THE STRUCTURE AND MOLECULAR CHARACTERIZATION OF OF L-ASPARTATE-α-DECARBOXYLASE FROM *ESCHERICHIA COLI*

by

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The Structure and Molecular Characterization of L-Aspartate-α-decarboxylase from *Escherichia coli*

by

Roy Carl Smith

Submitted to the Department of Chemistry on the first of February, 1988 in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The *panD* locus encoding L-Aspartate-α-decarboxylase (ADC) of *Escherichia coli* was isolated from a lambda library prepared with restriction endonuclease fragments generated by digestion of total *E. coli* chromosomal DNA by EcoRI. The 12.5 kb fragment was obtained by screening the library with the *Pan* auxotroph AB355. The *panD* locus was further subcloned by ligation of the EcoRI fragment into the plasmid vector pBR325 resulting in the subclone pADC1. The *panD* locus was further localized to a 1079 basepair NruI fragment in pADC201 by the isolation of several deletion derivatives of the parent plasmid. Both strands of this NruI fragment were sequenced by the dideoxynucleotide chain termination method. All subclones satisfied the β-alanine auxotrophy of AB354. Localization of the *panD* locus within the 2.2 kilobase PvuII/EcoRI fragment of the subclone pADC200 by deletion of a 240 basepair HpaI fragment resulting in pADC2001 which was unable to provide for the growth of AB354 in the absence of exogenous β-alanine.

Analysis of both strands for open reading frames capable of encoding the ADC proteins revealed the presence of a complete open reading frame which exhibited a low occurrence of rare codon usage in a region defined by the deletion in pADC2001. The open reading frame encoded a single polypeptide with a molecular weight of 13,834 daltons obtained by translation of the open reading frame. The *panD* transcript was determined to be 470 nucleotides long by S1 nuclease mapping and originates from the region coinciding with the putative open reading frame. The termini of the transcript correlated with putative promoter and terminator structures. Analysis of restriction endonuclease digests of *E. coli* and *Salmonella typhimurium* chromosomal DNA by Southern analysis with a *panD* specific probe indicated that the single locus encodes the *panD* proteins of *E. coli*. An homologus locus or loci was found in *S. typhimurium* and was estimated to be from 5 to 15% divergent from the *E. coli* locus.

The molecular weights (M_r) of the proteins of ADC were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis to be 14,650 daltons for protein I, 10,580 daltons for protein II, and 2,230 daltons for protein III. Comparison of the individual protein components of ADC by limited proteolysis mapping with *Staphylococcus auereus* V8 protease indicated that both proteins II and III are related to protein I and may be derived from that peptide by proteolysis. Amino-terminal sequence analysis of the individual proteins of ADC indicated that they correspond to the single protein of the translated sequence from the putative *panD* ORF. The amino-terminal sequences determined for proteins I and III correspond exactly to the amino terminus of the translated sequence. The amino-terminal sequence of

protein II corresponds to an internal region of the translated sequence with the exception of an amino-terminal alanine residue which is predicted to be a serine in the translated sequence. The amino acid analyses of the ADC proteins correspond approximately to the predicted amino acid compositions obtained from the *panD* translated sequence.

Antiserum directed against ADC was obtained from rabbits inoculated with homogeneous ADC. The antiserum detected proteins I and II, but not protein III, that had been transferred to nitrocellulose. Treatment of maxicell extracts of pADC201, labeled with L-[^{35}S]methionine, with the antiserum immunoprecipitated a single prominent protein which had an electrophoretic mobility identical to that of protein I when subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Two minor labeled proteins were also immunoprecipitated from the extracts and correspond to proteins II and III of ADC. Analysis of extracts of the deletion plasmid pADC2001 indicated that none of the proteins immunoprecipitated from pADC201 are present in those extracts.

The correspondence of the proteins to the translated sequence of *panD* suggested that proteins II and III are probably derived from protein I, possibly by a single autocatalytic proteolytic cleavage. This proteolysis is anticipated to be involved in generation of the catalytically active proteins (II and III) of ADC from a proenzyme (protein I) by analogy to the activation of histidine decarboxylase of *Lactobacillus* 30a. A pyruvoyl group is known to be covalently attached to ADC and may be generated from the serine residue predicted as the amino terminus of protein II by the translated sequence. The alanine residue observed at the amino terminus of protein II is postulated to be derived from an amino-terminal pyruvoyl group via an anomalous decarboxylation-dependent transamination that results from the action of ADC on aspartate. Comparison of the translated *panD* sequence to the well characterized histidine decarboxylase of *Lactobacillus* 30a indicates the lack of any significant homology.

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I would like to thank Steve Wasserman for the initial isolation and characterization of the *panD* clone and for advice concerning the subsequent experiments. Steve Elledge proved an indispensable source of reagents, advice, interpretation, and encouragement in the subcloning of *panD* and characterization of the gene product by Maxicell anaysis. Andy Laudano provided considerable and extremely valuable expertise with respect to Cleveland mapping, electrophoresis and immunological methods. Steve Ludmerer kindly assisted with information and guidance in the DNA sequencing and S1 nuclease mapping of *panD*.

I owe considerable thanks to numerable members of several laboratories. First, I would like to thank all the members of the Gene Brown laboratory whom I have had the great pleasure of counting as both associates and close freinds. I would also like to thank the members of the Chris Walsh laboratory who were important to me in the early part of my graduate career. I have also had a third "home" and I owe a considerable thanks to the members of the Graham Walker laboratory for reagents, advice and a large quantity of frienship. I also owe much thanks to various members of both the Schimmel and Horvitz laboratories.

Finally, I would like to state that I have intentionally mentioned only a small number of individuals by name. In my time here at MIT, I have known and valued such a large number of people that I did not want to simply provide a long list of names from which I might inadvertantly omit someone. Rather, I would say that it has always been my considerable good fortune to associate with and obtain guidance from those whom I considered freinds and I wish to thank all of them for the very great pleasure of their acquaintance, advice and support.

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List of Abbreviations Used

AADC, Aromatic amino acid decarboxylase

ADC, L-aspartate-α-decarboxylase

bisacrylamide, N, N'-methyleneacrylamide

DTT, dithiothreitol

EDTA, ethylenediaminetetraacetic acid

HDC, L-histidine decarboxylase

kb, kilobase

kd, kilodalton

nt, nucleotide

ORF, open reading frame

PLP, pyridoxal 5'-phosphate

PSDC, phosphatidylserine decarboxylase

PAGE, polyacrylamide gel electrophoresis

SDC, S-adenosylmethionine decarboxylase

SDS, sodium dodecyl sulfate

Tris, tris(hydroxymethyl)aminomethane

XGal, 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside

Introduction

L-Aspartate-α-Decarboxylase (ADC) is an enzyme isolated from *Escherichia coli* in this laboratory by Joanne Williamson (Williamson, 1977) and is involved in the decarboxylation of L-aspartate to produce β-alanine, a precursor to pantothenate. The initial work in this laboratory on this enzyme grew out of the interest in the biosynthesis of pantothenate. The results of Joanne Williamson indicated that the enzyme was a pyruvoyl-dependent decarboxylase making it one of a small number of decarboxylases that utilize a covalently attached pyruvoyl prosthetic group instead of the ubiquitous pyridoxal 5'-phosphate.

My initial interest in this enzyme was to examine its mechanistic properties through the use of mechanism-based inhibitors (Smith, 1982). In the course of this work with a number of β-substituted amino acids, it became obvious that the small quantities of enzyme that could be obtained from wild type strains would hamper further mechanistic studies. In addition, the subunit structure and generation of the active form of the enzyme were subjects of interest since generation of the pyruvoyl group would require post-translational modification of some sort of proenzyme.

Under these circumstances, we felt the objectives would be best served by cloning the gene for ADC and placing it on a plasmid where, under control of its own promoter in *E. coli*, we could obtain increased quantities of the enzyme. In addition, with the gene localized to a relatively short stretch of DNA, we would be able to determine the nucleotide sequence of the gene encoding ADC and thereby obtain information regarding the proenzyme and its expression.

We have accomplished the cloning and sequencing of *panD*, the gene encoding ADC, and have obtained some information about its proenzyme form. We have correlated this information with the protein sequence of ADC and the results of that work have some implications as to the regulation of ADC in the pantothenate biosynthetic pathway. For that reason, I will present some background information on the pantothenate biosynthesis and its metabolism to 4'-phosphopantetheine and Coenzyme A. In addition, information regarding histidine decarboxylase from *Lactobacillus* 30a, by far the best characterized of the pyruvoyl-dependent decarboxylases, will also be presented.

1.1 The Biosynthesis of Panthothenic Acid and Coenzyme A

Panthothenic acid is a B vitamin which most microorganisms can synthesize but animals are unable to produce and must obtain from dietary sources. The biosynthesis of pantothenic acid and its conversion to 4'-phosphopantetheine has long been a subject of some interest in this laboratory (Brown, 1971). Both are precursors to Coenzyme A (Lipman *et al.*, 1947) which can subsequently serve as a source of 4'-phosphopantetheine for apo-acyl carrier protein (Sauer *et al.*, 1964). The biosynthetic pathway, from pantothenic acid to coenzyme A (CoA), indicated in Figure 1-1, has been shown to be operative in a number of micro-organisms as well as in rat liver and kidney (Brown, 1959). The pathway for the formation of pantothenic acid and Coenzyme A has been reviewed a number of times (Brown and Williamson, 1982; Abiko, 1975; Plaut, 1974; and Brown, 1971).

Figure 1-1. The Biosynthetic Pathway from Pantoic Acid to Coenzyme A.

Substrates and Products

A. Pantothenic Acid

B. 4'-Phosphopantothenate

C. 4'-Phosphopantothenylcysteine

D. 4'-Phosphopantetheine

E. Dephopho Coenzyme A

F. Coenzyme A

Enzymes

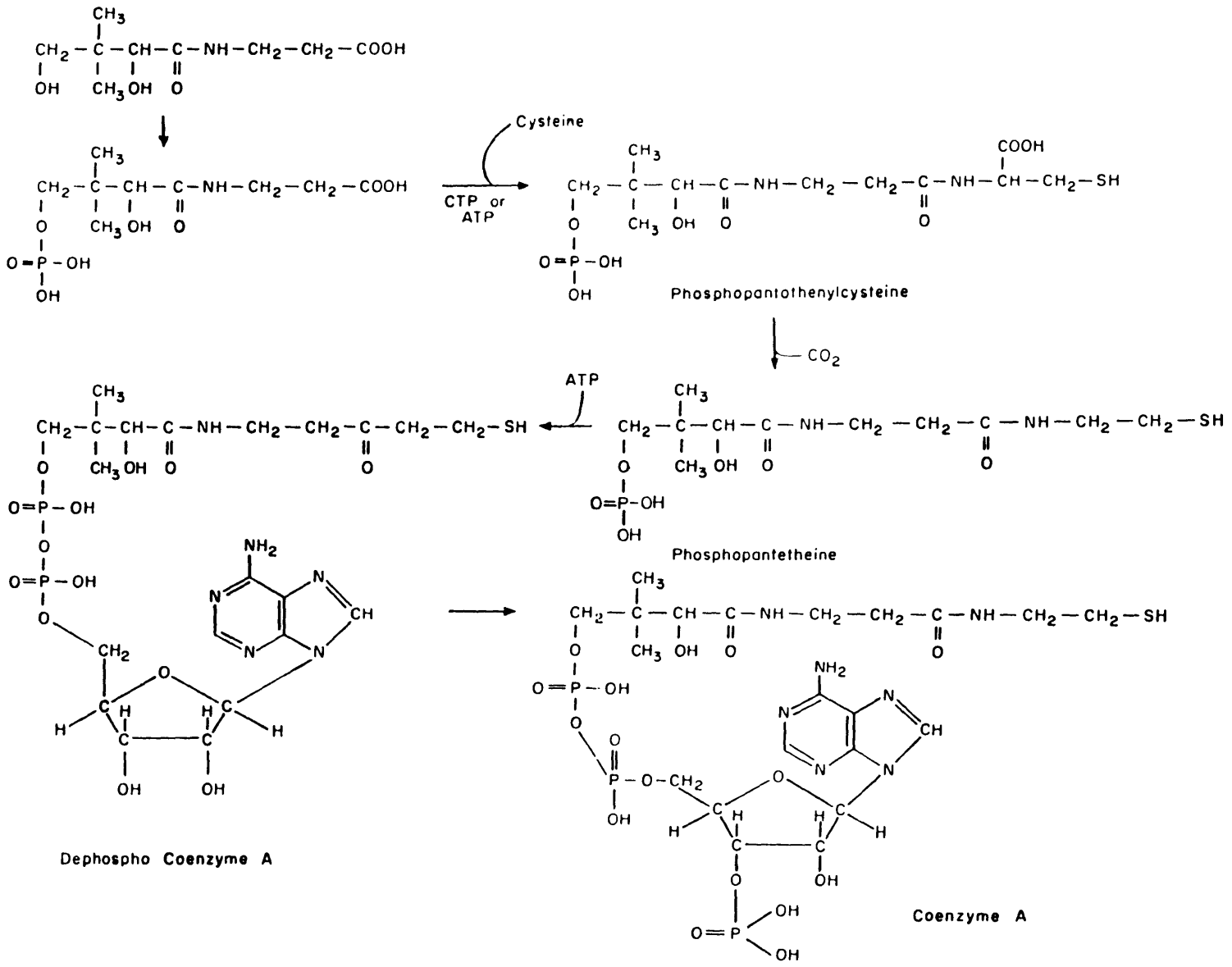
I. Pantothenate Kinase

II. Phosphopantothenylcysteine Synthetase

III. Phosphopantothenylcysteine Decarboxylase

IV. Dephospho-CoA Pyrophosporylase

V. Dephospho-CoA Kinase


Cysteine
CTP or ATP
Phosphopantothenylcysteine
CO_2
ATP
Phosphopantetheine
Dephospho Coenzyme A
Coenzyme A

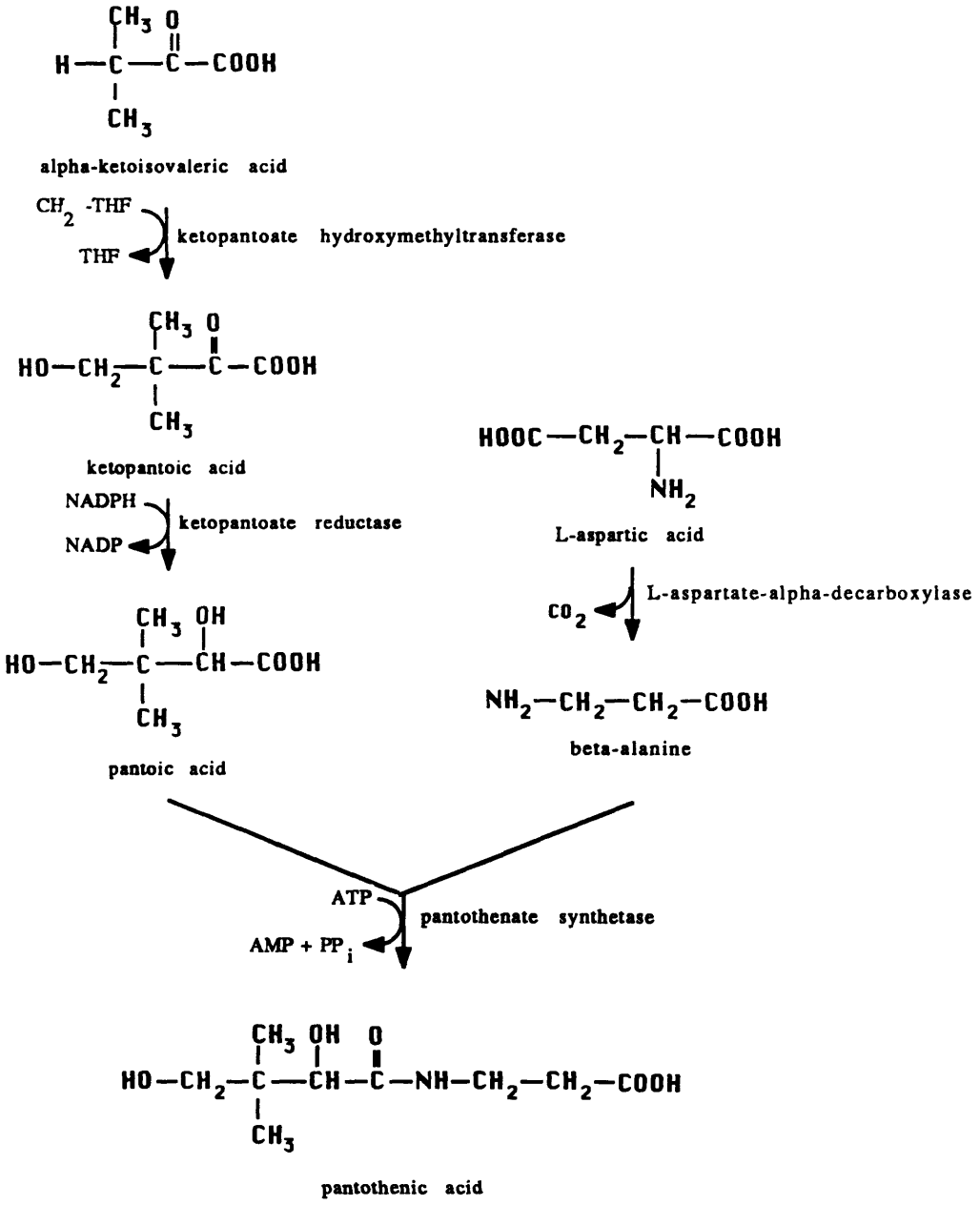


Figure 1-2. The Biosynthetic Pathway to Pantothenic Acid.

The first commited step in the biosynthesis of the pantothenic acid and Coenzyme A involves the conversion of α-keto-isovalerate to ketopantoate as depicted in Figure 1-2. The utilization of α-keto-isovalerate was first suggested by Maas and Vogel (1953) based on the observation that *E. coli* could synthesize ketopantoate from α-keto-isovalerate. The enzyme catalyzing the reaction, ketopantoate hydroxymethltransferase, has been purified to homogeneity from *E. coli* (Teller *et al.*, 1976). The enzyme utilizes methylenetetrahydrofolate as a cofactor and the reaction is readily reversible. The enzyme exhibits positive cooperativity towards substrate and this cooperative effect is enhanced by the inhibitors pantoate, pantothenate and coenzyme A (Powers and Snell, 1976). Powers and Snell (1976) view these inhibitory properties as having possible regulatory effects on pantothenate biosynthesis. In addition, α-ketobutyrate, an intermediate in isoleucine biosynthesis, has been shown to be nearly as efficacious a substrate as α-keto-isovalerate, although its effect *in vivo* has not been determined (Powers and Snell, 1976).

The reduction of ketopantoic acid to pantoate was first demonstrated by Kuhn and Wieland (1942) and they implicated this compound as a precursor to pantoic acid. This was confirmed by Lansford and Shive (1952) who isolated pantoate-requiring mutants of *E. coli* whose pantothenate auxotrophy could be satisified by α-ketopantoic acid. Enzymes catalyzing this reduction, α-ketopantoic acid reductases, have been purified from both yeast and *E. coli* and utilize NADPH as the source of reducing power (Wilken *et al.*, 1975). The *E. coli* enzyme has been purified to near homogeneity (Wilken *et al.*, 1975) and was separated from two distinct and highly similiar α-ketopantoyl lactone

reductases. The functions of the latter enzymes are currently unknown but the product of the reduction, α-ketopantoyl lactone, is a highly unstable ($t_{1/2}$ = 2.2 min.) pantoyl lactone which rapidly hydrolyzes to pantoic acid (Wilken *et al.*, 1975).

The coupling of β-alanine and pantoic acid to form pantothenic acid had been shown to occur in extracts of *Escherichia coli* by Maas (1952). Maas and Novelli (1953) have demonstrated that ATP is cleaved to AMP and PP_i by this activity. Aspartate was unable to serve as substrate for the formation of pantothenic acid when incubated with a more purified preparation of this pantothenate synthetase activity (Pfleiderer *et al.*, 1960).

The inability to directly utilize aspartate for the synthesis of pantothenate indicated the need for a source of β-alanine. Early studies had indicated the ability of aspartate, β-alanine and pantothenic acid to reverse growth inhibition caused by β-hydroxyaspartic acid and cysteic acid (Shive and Macow, 1946; Ravel and Shive, 1946), and D-serine (Durham *et al.*, 1964; Maas and Davis, 1950; Cosloy and McFall, 1973). Additional evidence indicated that whole cells of several microorganisms could produce β-alanine from aspartate (Virtanen and Laine, 1937; Billen and Lichstein, 1949; Grula and Grula, 1963). The α-decarboxylation of aspartate was the obvious means of producing β-alanine, although radioactive tracer studies had not confirmed this suggestion (Brown, 1971) previous to the work of Williamson and Brown (1979).

The direct synthesis of β-alanine from aspartate has been demonstrated in *E. coli* by the isolation of L-aspartate-α-decarboxylase (Williamson and Brown, 1979). Previously, Nakano and Kitaoka (1971) had

reported the partial isolation of an aspartate-α-decarboxylase activity from *E. coli* that was inducible during growth in acidic medium containing aspartate and glutamate as nitrogen sources. However, the activity reported by Williamson (Williamson and Brown, 1979) and purified to homogeneity in this laboratory, was not inducible under such conditions and was shown to contain a pyruvoyl prosthetic group. An aspartate-α-decarboxylase activity indistinguishable from that reported by Williamson and Brown had been independently discovered and partially purified from *E. coli* by Cronan *et al.* (1980). Attempts to purify an inducible PLP-dependent activity of Nakano and Kitaoka (1971) in this laboratory have been unsuccesful (Williamson, 1977) and casts doubt on the existence of such an activity.

The conversion of panthothenic acid to 4'-phosphopantothenic acid has been shown to precede the generation of the β-mercaptoethylamine moeity of pantetheine. Earlier work had indicated that the generation of the β-mercaptoethylamine moeity preceded the phosphorylation of the 4'-hydroxyl of the pantoyl residue of pantothenate (Brown, 1971 and references therein) based mainly on work in *Acetobacter suboxydans* and *Lactobacillus helveticus*. However, the inability of bacteria other than *A. suboxydans* and *L. helveticus* to decarboxylate pantothenylcysteine (Brown, 1957) and the inability of pantothenylcysteine to replace pantothenate as a nutritional factor in rats and chicks (Thompson and Bird, 1954) suggested that this pathway to 4'-phosphopantetheine was unique to *A. suboxydans* and *L. helveticus* (Brown, 1971).

A reinvestigation of bacterial and animal systems by Brown (1959) indicated that pantothenic acid was first converted to 4'-phosphopantothenic acid which is then condensed with cysteine to form

4'-phosphopantothenylcysteine with subsequent decarboxylation to 4'-phosphopantetheine. Brown was able to demonstrate the presence of these activities, as depicted in Figure 1-1, in cell-free extracts, leading to the conclusion that phosphorylation of panthothenate precedes the generation of the β-mercaptoethylamine moeity. The earlier pathway had been based on the ability of pantothenate kinases from bacteria (Ward *et al.*, 1955) and rat tissues (Hoagland and Novelli, 1954) to phosphorylate pantetheine. However, the ability of pantothenate kinase to phosphorylate both pantothenate and pantetheine only indicated a lack of strict specificity for this kinase (Hoagland and Novelli, 1954; Ward *et al.*, 1955) and, although Brown (1959) was unable to demonstrate this acitivity in rat liver, Abiko (1967a) confirmed the lack of specificity with pantothenate kinase purified 27-fold from rat liver which was able to phosphorylate pantothenylcysteine in addition to pantothenate.

Brown (1959) demonstrated direct condensation of 4'-phosphopantothenic acid with β-mercaptoethylamine to generate 4'-phosphopantetheine but dismissed its biological significance since the extracts were unable to generate β-mercaptoethylamine by decarboxylation of cysteine. The biological significance of this reaction was further discounted since this moeity was known to be derived from cysteine based on studies with *A. suboxydans* (Pierpont and Hughes, 1952) and *L. helveticus* (Brown and Snell, 1953).

A phosphopantothenylcysteine synthetase has been purified 26-fold from rat liver by Abiko *et al.* (1968) and they were able to show that the enzyme condensed cysteine with 4'-phosphopantothenate utilizing ATP which is cleaved to ADP and P_i. The enzyme was completely unaffected by the presence of pantothenate.

A phosphopantothenylcysteine decarboxylase activity was purified 112-fold by Abiko (1967b). The enzyme from horse liver has been purified to near homogeneity and Scandurra *et al.* (1974) have not been able to demonstrate the presence of pyridoxal 5'-phosphate although it is inhibited by a number of carbonyl reagents. The lack of PLP cofactor was not surprising given the fact that the amino group of the cysteine moiety is bound in amide linkage to the phosphopantothenate portion of the molecule, but the nature of its prosthetic group remains undecided. The horse liver enzyme is unaffected by either pantothenoylcysteine or 4'-phosphopantetheine and has an absolute specificity for 4-'phosphopantothenylcysteine (Scandurra *et al.*, 1974) while 4'-phosphopantetheine is a strong inhibitor of the rat liver enzyme (Abiko, 1967b).

The final two enzymes involved in the biosyntheis of coenzyme A, dephospho-CoA pyrophosphorylase and dephospho-CoA kinase, have been purified from rat liver extracts (Suzuki *et al.*, 1967) and may exist as a bifunctional enzyme complex.

1.2 The Genetic Organization of the Pantothenate (*pan*) Activities of *Escherichia coli*

The pantothenate (*pan*) activities are involved in the biosynthesis of panothenate in *E. coli* from α-ketoisovalerate and aspartate. Cronan *et al.* (1982) have assigned the *panB* allele to a deficiency in α-keto-pantoate hydroxymethyltransferase; *panC* to a deficiency in pantothenate synthetase and *panD* to a deficiency in L-asparatate-α-decarboxylase, in agreement with previous enzymatic analysis of *E. coli* W auxotrophs

(Teller *et al.*, 1976; Maas, 1952; and Williamson and Brown, 1979). In addition, Cronan (1980) has mapped the *panD* mutants AB353, AB354, and AB355 and shown that all three lesions represent a single complementation group that maps to min 1.5 of the *E. coli* K12 chromosome. P1 infections of CY257 (*panB*) to pan^+ with phage grown on AB354 (*panD*) gave 73 transductants whose auxotrophy were satisified by β-alanine and an analogous transduction into AB1371 (*panB*) gave greater than 95% cotransduction of *panB* with *panC*. Conjugational crosses of the *panD* mutants with HfrH and HfrC strains confirmed the results of the transductions which establish that the *panD* locus is closely linked to both the *panB* and *panC* loci.

On the basis of this tight linkage of the *pan* loci, Cronan (1980) proposed that *panB*, *panC* and *panD* may comprise an operon for the coordinate expression of the panothenate biosynthetic enzymes, although transcription of the *pan* region has not yet been examined. In addition, Cronan *et al.* (1982) have utilized a Tn10 insertion closely linked to the *pan* region (99.6% by P1 transduction) for three factor crosses to establish the order of the loci on the *E. coli* chromosome as *aceF-panB-panD-panC-tonA*.

This corrresponds to the organization of the *pan* activities of *Salmonella typhimurium* as established by Primerano and Burns (1983). The *panB* and *panC* loci are located at min 4.5 on the *S. typhimurium* LT2 linkage map (Sanderson, 1978). Recently, Primerano and Burns (1983) have isolated a number of β-alanine requiring auxotrophs and have shown that this *panD* allele is closely linked to the *panC* locus (99.1% and 97.4% by transduction with two genetically distinct *panD* mutant strains). This assignment differs from that obtained by Ortega *et al.*

(1975) which placed the *panD* locus at min 89 of the *S. typhimurium* LT2 map. In both cases the biochemical basis for the β-alanine auxotrophy was not addressed preventing determination of whether two distinct genetic lesion(s) result in a lack of aspartate-α-decarboxylase activity.

Primerano and Burns (1983) have isolated a number of *S. typhimurium* mutants that require either pantoate or pantothenate for growth. Two of these mutants, DU65463 and DU6563, had decreased levels of ketopantoic acid reductase; 12% and 3.2% of wild type levels, respectively. Mutants in the gene encoding this enzyme, which Primerano and Burns (1983) designated *panE*, had not previously been isolated. They attributed this discrepency to the observation that acetohydroxy acid isomeroreductase (*ilvC*), an enzyme involved in the biosynthesis of isoleucine and valine, is able to reduce α-ketopantoic acid (V_{max} = .136 μmoles/min) at 1\20 the rate of ketopantoic acid reductase (V_{max} = 2.45 μmoles/min). When acetohydroxy acid isomeroreductase is present in *panE* mutant strains it is able to supress the pantoic acid auxotrophy and permits growth of these strains on medium containing α-ketopantoic acid. This results in a phenotype identical to that seen with *panB* auxotrophs, which are deficient in α-keto-pantoate hydroxymethyltransferase. This necessitated obtaining the *panE* mutations in a *ilvC* mutant background in order to allow determination of the proper *panE* phenotype.

1.3 Regulation of Panthothenic Acid, 4'Phosphopantethenic Acid and Coenzyme A Levels in Animals and *Escherichia coli*

Control of CoA levels in *E. coli* has been postulated to show a dependence on the level of pantothenate kinase (Jackowski and Rock, 1981). Their studies on the growth of the β-alanine auxotroph SJ16 indicated that maximal growth was obtained at a concentration of 0.5 μM exogenous β-alanine indicating that this was the minimal level required to support uninhibited growth of *E. coli*. At this level of [^{14}C]-β-alanine, they were unable to detect the presence of either pantothenate or 4'-phosphopantetheine. The CoA intermediates 4'-phosphopantothenylcysteine, 4'-phosphopantothenate and dephospho-CoA were not detectable at any concentration of exogenous β-alanine. However, as the level of β-alanine was increased to 8 μm the CoA pool increased from 3.5 pmol to obtain a maximum of 38 pmol per 10^8 cells. The level of β-alanine incorporation into the 4'-phoshpopantetheine prosthetic group of acyl carrier protein doubled to a final value of 3.4 pmol per 10^8 cells as the β-alanine concentration was increased. As the level of exogenous [^{14}C]-β-alanine was increased from 4 to 32 μM the panthothenate and 4'-phosphopantotheine levels increased from initially undetectable levels to reach maximum concentrations of 8 and 7 pmol per 10^8 cells, respectively.

In the medium, Jackowski and Rock (1981) found that 4'-phosphopantetheine constituted 88% of the label while only trace amounts of 4'-phosphopantothenate, β-alanine, pantothenate and pantetheine were detected at 0.5 μM exogenous β-alanine. As they increased the concentration of the β-alanine to 4 μM the phosphorylated

components represented an increasing proportion of the label in the medium until they reached a maximum value of 22 pmol per 10^8 cells at 4 μM β-alanine at which point 4'-phosphopantothenate constituted 20% of this label found in the medium. From 4 to 16 μM β-alanine, the pantothenate levels in the medium increased until it became the major labeled component at a maximal level of 600 pmol per 10^8 cells (Jackowski and Rock, 1981).

Jackowski and Rock (1981) interpret this data as indicating that pantoic acid does not represent a limiting factor since pantothenate is able to support increasing CoA synthesis up to 8 μM β-alanine at which point considerable pantothenate is present in the medium. They also detected pantothenate excretion in wild-type cells, at levels corresponding to the amounts excreted by SJ16 in 16-32 μM β-alanine. This indicated to Jackowski and Rock (1981) that pantothenate production is 15-fold greater than the amount required to supply sufficient quantities of CoA to support metabolic processes and that β-alanine is not a limiting factor in CoA biosynthesis. β-alanine concentrations in excess of 32 μM resulted in substantial quantities of unmetabolized β-alanine remaining in the medium and pantothenate levels ceased to increase linearly, indicating that pantoic acid production had become limiting. This accumulation of pantothenate in the medium and the failure to detect appreciable levels of other β-alanine metabolites indicated to Jackowski and Rock (1981) that pantothenate kinase is the most plausable site for regulation of CoA biosynthesis in *E. coli*.

Holo-acyl carrier protein (holo-ACP) serves as an additional source of 4'-phosphopantetheine which can be monitored for turnover of its cofactor. Jackowski and Rock (1984a, 1984b) have utilized the

covalent attachment of this cofactor to acyl carrier protein as a means of monitoring the turnover of the intracellular CoA pool since the transfer of 4'-phosphopantetheine moiety from CoA to apo-ACP is the only known fate for CoA in *E. coli* (Powell *et al.*, 1969). In addition, incorporation of deuterium into non-exchangeable positions of ACP enhanced pH-induced hydrodynamic expansion allowing separation of pre-existing ACP (deuterated) from newly-synthesized ACP (nondeuterated) by conformationally sensitive gel electrophoresis (Jackowski and Rock, 1984a). They conducted pulse-chase experiments by labeling CoA in the β-alanine auxotroph SJ16 with β-[^{3}H]alanine in D_2O followed by labeling with a β-[^{14}C]alanine chase in H_2O allowing analysis of the turnover of the ACP prosthetic group by measuring the incoporation of ^{14}C. Measurment of deuterio-[*Pan*-^{3}H]ACP during growth indicated that the loss of tritium from the deuterio-ACP pool was very rapid during the first hour of growth, with a 2% turnover of the pool per minute, followed by a greatly decreased turnover, approximately 25% per generation (doubling time of 1 hour), after the culture entered the logarithmic growth phase.

Jackowski and Rock (1984a) interpreted this turnover of the ACP prosthetic group as representing a means of controlling CoA metabolism since it does not appear to be involved in the regulation of lipid metabolism. They based this conclusion on the observation that apo-ACP is absent even during β-alanine starvation (Jackowski and Rock, 1983) and the apparent futility of releasing the 4'-phosphopantetheine cofactor under conditions where holo-ACP would be rapidly regenerated by [ACP]synthase (Elovson and Vagelos, 1968) at the expense of an additional CoA molecule. This implicates the [ACP]phosphodiesterase

(Vagelos and Larrabee, 1967), that catalyzes the removal of 4'-phosphopantetheine from ACP, as the controlling factor in CoA turnover.

Previous experiments (Jackowski and Rock, 1984b) utilizing β-[^{3}H]alanine incorporation in strain SJ16 had indicated that intracellular CoA levels were not constant throughout the exponential growth phase of *E. coli*. During growth the CoA pool increased from early log phase to middle log phase and then dropped precipitously in late log phase. Following the cessation of growth, the pool increased to a maximum in stationary phase at which point both β-alanine and pantothenate were exhausted from the medium. The 4'-phosphopantetheine content of ACP showed analogous trends. Pantothenate appeared in the medium immediately after the start of exponential growth and increased slowly at first but then began increasing rapidly in correspondence with the drop in the CoA pool until it reached a maximum in late log phase where it remained until it was depleted from the medium in the stationary phase. 4'-phosphopantetheine began to appear in the medium in the late log phase corresponding to the drop in the CoA pool and then increased rapidly during stationary phase.

Double label experiments were conducted with SJ16 grown up overnight on β-[^{3}H]alanine, resulting in all of the ^{3}H being incorporated into CoA and holo-ACP as 4'-phosphopantetheine (Jackowski and Rock, 1984b). This culture was then diluted into medium containing β-[^{14}C]alanine and grown to late logarithmic stage. The result of this experiment indicated that all of the extracellular pantothenate was derived from β-[^{14}C]alanine while both the extra and intra-cellular

4'-phosphopantetheine had similiar ratios of $^{3}H/^{14}C$. Jackowski and Rock (1984b) interpreted this as indicating that the pantothenate was derived soley from *de novo* biosynthesis while the 4'-phosphopantetheine was derived from the turnover of the ACP prosthetic group. In additional experiments they observed that pantothenate but not 4'-phosphopantetheine could be taken up from the medium by SJ16.

The rapid decline in the CoA levels detected in the late log phase of growth and the concommitant rise in extracellular pantothenate was taken by Jackowski and Rock (1984b) to indicate the onset of inhibition of CoA biosynthesis via the inhibition of pantothenate kinase. This inhibition could conceivably be the result of competitive inhibition of the kinase by CoA or its thioester derivatives, an effect Vallari *et al.* (1987) have recently shown *in vitro*. This was anticipated to result in the accumulation of pantothenate and its excretion into the medium. The results of a double label experiment (Jackowski and Rock, 1984b) indicated that 4'-phosphopantetheine accumulation in the medium and its failure to be taken up by the cell was the result of ACP turnover and excretion. The labeling of the intracellular pool of 4'-phosphopantetheine by both ^{14}C and ^{3}H indicated that this prosthetic group can be either excreted or reutilized via its conversion into CoA. Vallari *et al.* (1987) interpret the inability of the cell to take up the extracellular 4'-phosphopantetheine and the turnover of ACP, via [ACP]phosphodiesterase (Vagelos and Larrabee, 1967), as providing a means of regulating CoA levels and fatty acid biosynthesis.

The recent studies by Vallari *et al.* (1987) indicated that pantothenate kinase from *E. coli* may be regulated by the energy state of the cell. They have demonstrated that the kinase was inhibited more

strongly by free CoA than its thioester derivatives, with acetyl CoA being five times less effective than free CoA. In addition, the inhibition of the kinase by CoA was competitive with respect to the ATP cosubstrate and this suggested to the authors that the activity of the kinase is linked to the energy state of the cell.

Vallari *et al.* (1987) demonstrated that a shift from glucose to acetate as a carbon source resulted in a change in the ratio of free CoA:acetyl-CoA from 0.7 to 4.3 during which they observed a selective inhibition of pantothenate phosporylation (Vallari *et al.*, 1987). They estimate, based on intracellular levels of CoA and ATP that the kinase exhibits only 20-25% of its maximal activity during logarithmic growth on glucose. Vallari *et al.* (1987) have also shown that the free-CoA:CoA-thioester ratio varies in correspondence with the ATP levels determined by Lowry *et al.* (1971) for E. coli grown on various carbon sources (glucose>succinate>glycerol>acetate>casein hydrosylate) although they have determined changes in pantothenate phosphorylation only in the shift from glucose to acetate.

Finally, the position that ketopantoate hydroxymethyltransferase occupies as the first commited step in the synthesis of pantothenate makes it a likely site for regulation. Powers and Snell (1976) noted that the level of ketopantoate hydroxymethyltransferase was unaffected by growth of *E. coli* K12 in nutrient broth or minimal medium containing pantothenate. In addition, the level of activity was not increased by growth in the presence of 2 mM D-serine, conditions where Cosloy and McFall (1973) had noted growth inhibition resulting from the inhibition of pantothenate biosynthesis. This suggested to Powers and Snell (1976) that the regulation of ketopantoate hydroxymethyltransferase activity

does not involve repression or induction of activity. However, Powers and Snell (1976) had noted inhibition of the purified ketopantoate hydroxymethyltransferase by pantoate, panthothenate and coenzyme A. Inhibition by pantoate occured at low enough levels (50 μM) compared to the K_M of the pantothenate synthetase (2 mM) for pantoate to allow the possibility that feedback inhibition of ketopantoate hydroxymethyltransferase may provide a physiologically significant control. This supposition was bolstered by the observed positive cooperativity exhibited by the enzyme for breakdown of α-ketopantoate via reversal of the biosyntheic activity (Powers and Snell, 1976). Unfortunately, the regulatory properties of ketopantoate hydroxymethyltransferase on pantothenate and CoA formation have not been addresed.

The regulation of Coenzyme A levels has been the subject of a number of studies in mammalian systems (see Plaut *et al.*, 1974 and Abiko, 1975 for reviews). Pantothenate kinase, as the first enzyme in the animal pathway for CoA biosynthesis, has been cited as the rate limiting step in the synthesis of CoA based on studies of CoA levels in perfused hearts (Abiko, 1975) and a number of studies have indicated that this control may be exerted through feedback inhibition by CoA. The partially-purified rat kidney enzyme (Karasawa *et al.*, 1972) was inhibited 30% by 10 μM CoA while the partially-purified enzyme from rat liver was inhibited more strongly by 4'-phosphopantetheine, 4'-phosphopantothenate and 4'-phosphopantothenylcysteine with CoA being much less effective in this case (Abiko *et al.*, 1972). Studies on the distribution of CoA precursors in rat liver have indicated that 4'-phosphopantetheine was present in much higher concentrations than

other precursors and may serve, along with CoA, as an inhibitor of pantothenate kinase activity *in vivo* (Plaut *et al.*, 1974 and references therein).

Halvorsen and Skrede (1982) have observed that short chain esters of CoA are more potent inhibitors of the partially purified rat liver pantothenate kinase than CoA, suggesting that the ratio of free to esterifed forms of CoA may be important in determining the level of feedback inhibition. A recent study by Fisher *et al.* (1985) with the partially purified pantothenate kinase from rat heart indicated that inhibition by CoA and its acyl esters is equally strong and that the inhibition by free CoA is uncompetitive in nature. The K_i for the inhibition was shown to be 0.2 μM for free CoA which is 70 fold lower than the cytostolic CoA concentration, suggesting that the enzyme would be largely inactive under such conditions (Fisher *et al.*, 1985). They postulate the need for a mechanism for reversal of inhibition since the level of pantothenate kinase activity is known to reach near maximum capacity in heart perfused in the absence of oxidizable substrate. One mechanism advanced for this relief from inhibition involves stimulation of the kinase by free carnitine, a molecule involved in the shuttling of fatty acids between the cytosol and the mitochondrial matrix. Inhibition of kinase activity by CoA was shown to be reversed by free carnitine (K_a = 0.27 mM) at concentrations well below the maximal level of free carnitine, estimated at 2.6 mM, under conditions in which pantothenate kinase is known to be at maximal activity in the perfused heart (Fisher *et al.*, 1985). However, even under conditions that generate maximal concentrations of free carnitine, the carnitine levels would not be sufficient to account for the level of deinhibition of

pantothenate kinase, as measured by CoA levels, observed in studies on perfused hearts. As a result, Fisher *et al.* (1985) postulated the need for another, as yet unidentified, deinhibitor of the kinase.

The results of the studies with both the *E. coli* and mammalian systems implicate pantothenate kinase (Jackowski and Rock, 1984b; Vallari *et al.*,1987; Karasawa *et al.*, 1972; Abiko *et al.*, 1972; Halvorsen and Skrede; 1982; Fisher *et. al.*, 1985) as being a major factor in determining the level of available 4'-phosphopantothenate and CoA. The *E. coli* system has also indicated the importance of the [ACP]phosphodiesterase (Jackowski and Rock, 1984a) in the turnover of ACP and CoA while the mammalian system appears to have modulators that increase the level of kinase acitivity (Fisher *et. al.*, 1985). However, the possibility that precursors to pantothenate may have an effect on levels of CoA and holo-ACP has not yet been fully addressed. Regulation of α-ketopantoic acid biosynthesis via the feedback inhibition of ketopantoate hydroxymethyltransferase by pantoate (Teller *et al.*, 1976) may be justified based on the finding of Jackowski and Rock (1981) that α-ketopantoic acid appears to be the limiting factor at high levels of β-alanine.

1.4 L-Aspartate-α-Decarboxylase of *Escherichia coli*

The activity reported by Williamson and Brown (1979) was a small enzyme of 58,000 daltons molecular weight (M_r) with a K_m of 0.16 mM for L-aspartate and a turnover number of 40 moles of CO_2 released/minute/mole cofactor. The enzyme had a pH optimum of 6.8-7.5, a temperature optimum of 55 °C and an isoelectric point of 4.67. The

enzyme appeared to be composed of three subunits with molecular weights (M_r), determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis, to be 11,800 (protein I), 9,800 (protein II), and 6,400 (protein III) daltons. The subunit stoichiometry was not determined. The 9,800 dalton subunit was shown to contain a covalently bound pyruvoyl group that appears to be essential for activity. Reduction of the enzyme with sodium borohydride or treatment with either hydroxylamine or phenylhydrazine abolished all activity. Inhibition of the enzyme by such carbonyl reagents indicated the presence of an essential carbonyl moeity but the absence of any detectable pyridoxal 5'-phosphate (PLP), as determined by bioassay and the lack of a characteristic PLP absorption spectrum, suggested the presence of the pyruvoyl prosthetic group. ^{3}H-lactate was isolated from acid hydrolysates of enzyme reduced with [^{3}H]-$NaBH_4$ and was identified by paper chromatography and its oxidation by lactate dehydrogenase.

The involvement of ADC in the biosynthesis of β-alanine and pantothenate biosynthesis was demonstrated by several criteria. The enzyme was shown by Williamson and Brown (1979) to be inhibited by L-cysteic acid, β-hydroxyaspartate and D-serine, known inhibitors of pantothenate biosynthesis (Ravel and Shive, 1946; Shive and Macow, 1946; Cosloy and McFall, 1973; and Maas and Davis, 1950). In addition, Williamson and Brown (1979) demonstrated the lack of an L-asparate-α-decarboxylase activity in a pantothenic acid requiring auxotroph of *E. coli* W (99-2) whose nutritional requirements could also be met by the addition of β-alanine to the growth medium. A prototrophic revertent of this strain contained an increased level of decarboxylase activity that was 2.5-fold greater than the activity found in wild type strains.

The enzyme is not significantly inhibited by D-pantoate, β-alanine, D-pantothenate, Coenzyme A or acetyl-Coenzyme A at concentrations up to 250 μM (Williamson, 1977). This suggested that feedback inhibition by intermediates in the pantothenate pathway are not involved in controlling the activity of ADC *in vivo*. This is in accord with the findings of Jackowski and Rock (1981) that pantothenate kinase was the rate limiting step in this stage of coenzyme A biosynthesis.

ADC has been shown to be inactivated in a time dependent manner by the substrate L-aspartate (Smith, 1982). This inactivation, shown in Figure 1-3, exhibits first-order kinetics with respect to substrate as anticipated for a process that occurs from the enzyme-substrate complex. This suggested that the inactivation may occur as the result of an aberrant reaction that arises during the course of normal catalysis. D-aspartate had been shown to be a non-competitive inhibitor of substrate (K_I = 2.2 mM) and did not demonstrate a time-dependent inactivation of ADC. The proposed mechanism for ADC-catalyzed decarboxylation of L-aspartate is illustrated in Figure 1-4 along with a possible mechanism for the inactivation. This inactivation has been postulated to occur by a decarboxylation-dependent transamination of the pyruvoyl prosthetic group to yield an alanyl residue (Smith, 1982).

Initially our interest in the enzyme was to explore the mechanism of this puruvoyl-dependent decarboxylase through the inhibitory action of a number of β-substituted amino acids that were anticipated to serve as mechanism-based inhibitors. Earlier work (Smith, 1982) found that ADC is irreversibly inhibited by several β-substituted amino acids including β-chloro-D-alanine, O-carbamoyl-D-serine, β-trifluoro-DL-alanine, β-trifluoro-D-alanine, β-fluoro-D-alanine and

Figure 1-3 Inactivation of L-Aspartate-α-Decarboxylase by L-Aspartate.

20 μg of L-Aspartate-α-Decarboxylase was added to an assay mixture containing 50 mM potassium phosphate buffer pH 7.5, 5 mM EDTA, 50 μM dithiothreitol, 1 mM L-[$U^{14}C$]aspartate (3×10^5 dpm), in a final reaction mixture of 0.5 ml. Incubated at 42°C for the indicated length of time and the reaction mixture was forced onto minicolumns of Dowex 1-X8 (phosphate form) and the β-alanine was eluted with 4.0 ml of deionized distilled water. Radioactivity in the eluate was determined by scintillation counting.

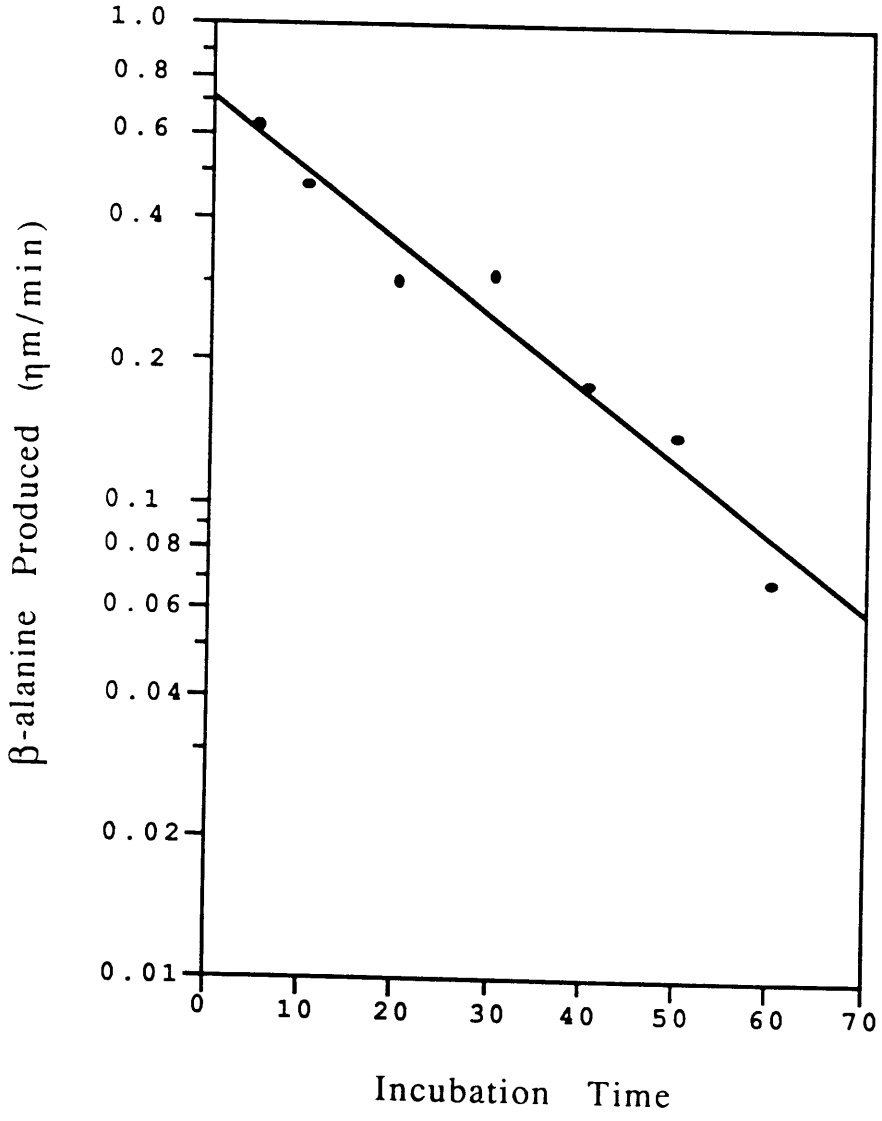
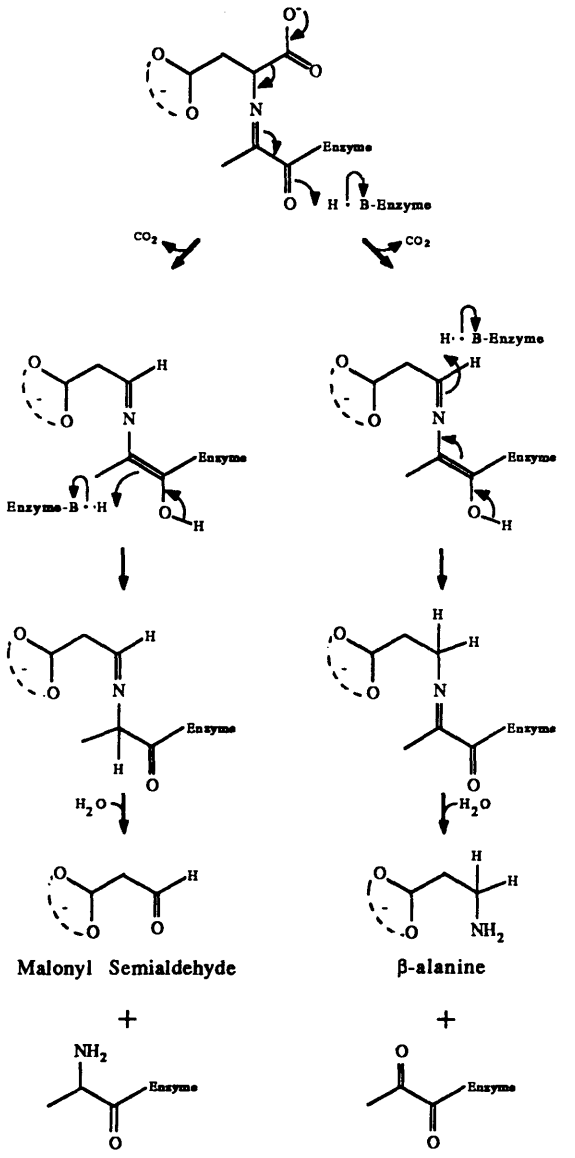

1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
0.01
β-alanine Produced (ηm/min)
0
10
20
30
40
50
60
70
Incubation Time

Figure 1-4. Postulated Mechanisms for L-Aspartate-α-Decarboxylase: Decarboxylation and Decarboxylation-Dependent Transamination.



Deamination Decarboxylation

O-acetyl-D-serine (Smith, 1982). The L isomers of chloro-alanine, O-carbamoyl-serine, β-fluoro-alanine and O-acetyl-serine were inactive. In addition, both the D and L isomers of serine demonstrate time dependent inactivation of the enzyme.

Initially, the inhibitors exhibit a time-dependent inactivation of ADC which reduces the activity to an intermediate level. At this point the inhibition enters a second phase where inactivation occurs at a much reduced rate or ceases altogether. The level of activity remaining after the initial rapid inactivation was shown to be concentration dependent for O-carbamoyl-D-serine and the initial rate appeared to exhibit a saturation effect (K_I = 3.6 mM). Removal of β-chloro-D-alanine via dialysis indicated that the inhibition was irreversbile. The addition of a second aliquot of inhibitor, either β-chloro-D-alanine or O-carbamoyl-D-serine, had no further effect on the level of residual activity indicating that the incomplete inactivation was not the result of their depletion from the incubation mixture (Smith, 1982).

The D isomer of serine exhibits a slow linear inactivation while the L isomer exhibits inactivation similiar to that seen with the β-substituted amino acids (Smith, 1982).

Inhibition by S-carbamoyl-D-cysteine was rapid and complete at considerably lower concentrations (90 μM) than was observed for β-chloro-D-alanine (15 μM) and O-carbamoyl-D-serine (15 μM) (Smith, 1982). However, at the time of the studies it was not known that the inhibitor, S-carbamoyl-D-cysteine, is unstable at the pH (7.5) used for the studies (Stark, 1964) and was hydrolized rapidly to cysteine and cyanate. Calculations based on the second order rate constant (Stark,

1964) for hydrolysis of the inhibitor suggested that the inhibitor was 93% hydrolyzed within the first two minutes of the incubation. Later studies indicated that cyanate alone was inactive against ADC, but D-cysteine was a powerful inhibitor (complete inhibition at 100 μM) while L-cysteine was 10-100 fold less effective, although this effect may have been the result of D-cysteine contamination (unpublished results). Active site involvement in the inactivation of ADC was demonstrated by protection of ADC from inactivation by S-carbamoyl-D-cysteine in the presence of saturating quantities of L- (15mM) or D- (30 mM) aspartate. Protection with L-aspartate resulted in the anticipated time-dependent inactivation (Smith, 1982).

The anticipated mechanisms for the inactivation of ADC by the inhibitors are illustrated in Figures 1-5 and 1-6. The pyruvoyl group is anticipated to function through the formation of a Schiff base with substrate, as demonstrated for histidine with histidine decarboxylase by Recsei and Snell (1970). In Figure 1-5, the inhibitor is assumed to be decarboxylated by ADC in a manner consistent with the conventional mechanism inactivation of PLP-dependent enzymes by β-substituted amino acids as described by Walsh (1984). The postulated eneamino-pyruvoyl intermediate may partition between alternate fates through turnover to acetaldehyde or by the addition of a nucleophile in a Michael sense. The alternate mechanism illustrated in Figure 1-6 assumes deprotonation of the inhibitor to generate an eneamino-intermediate that can either turnover to pyruvate or undergo Michael addition. The differing turnover products predicted, either acetaldehyde or pyruvate, allowed descrimination between the two mechanisms (Smith, 1982).

Figure 1.5. Postulated Mechanism for the Inactivation of L-Aspartate-α-Decarboxylase by β-substituted Amino Acids: Decarboxylation

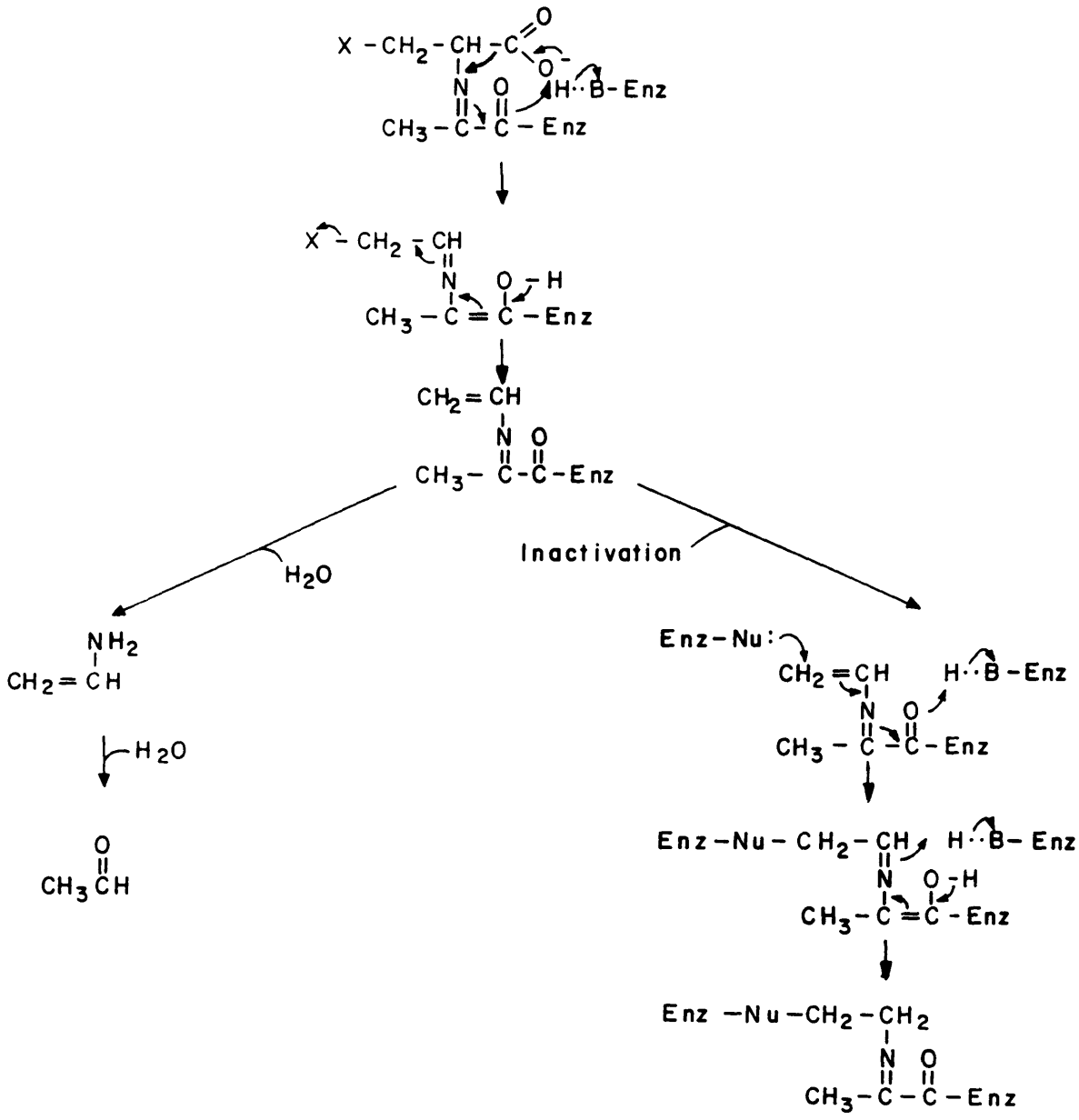
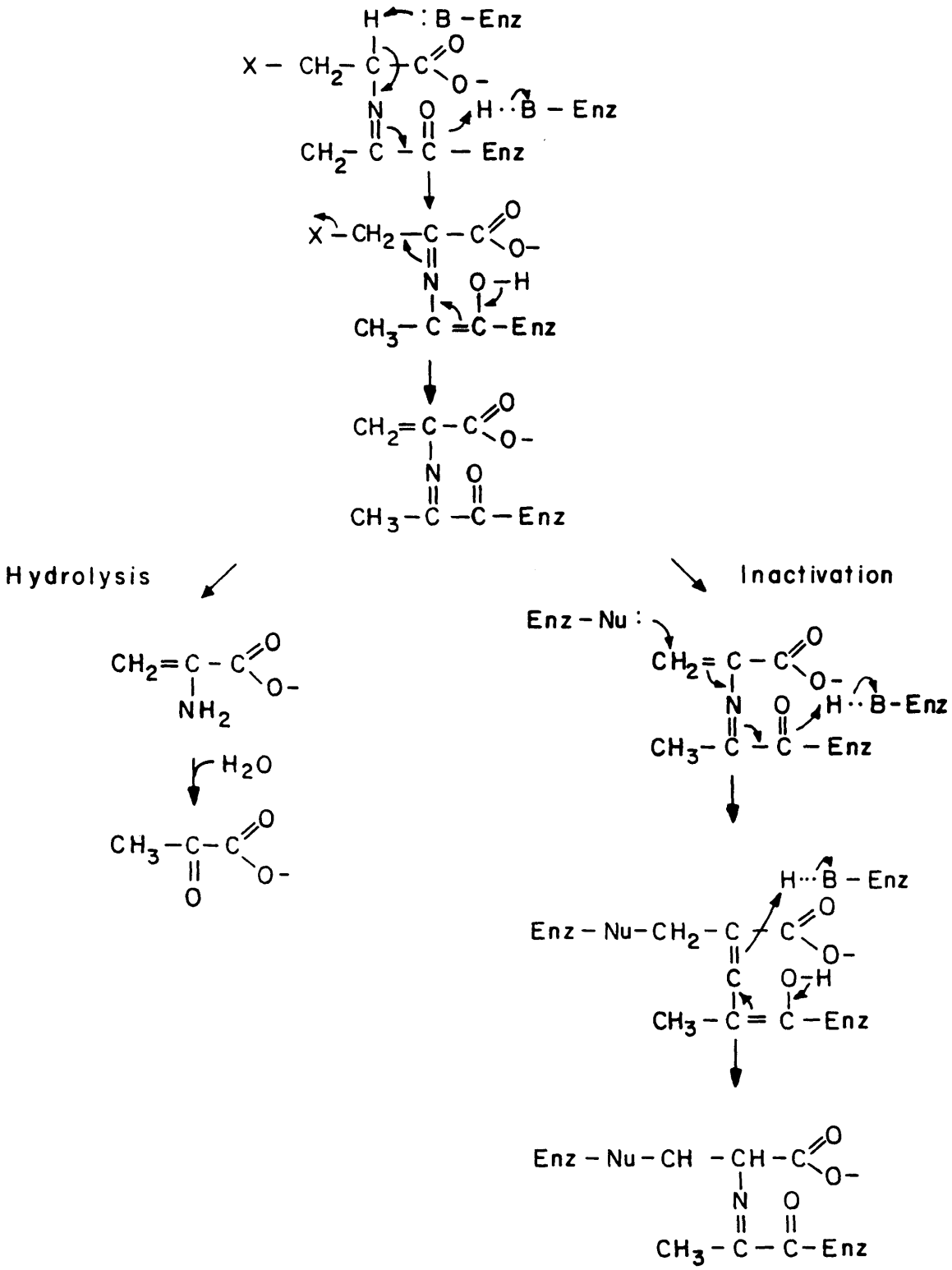

X - CH2 - CH - C
O
O-
N O
H··B-Enz
CH3 - C = C - Enz
X - CH2 - CH
N O - H
CH3 - C = C - Enz
CH2 = CH
N O
CH3 - C - C - Enz
H2O
Inactivation
NH2
CH2 = CH
H2O
O
CH3 CH
Enz-Nu:
CH2 = CH
H··B-Enz
N O
CH3 - C = C - Enz
Enz-Nu - CH2 - CH
H··B-Enz
N O - H
CH3 - C = C - Enz
Enz - Nu - CH2 - CH2
N O
CH3 - C - C - Enz

Figure 1.6. Postulated Mechanism for the Inactivation of L-Aspartate-α-Decarboxylase of β-substituted Amino Acids: Deprotonation.


Hydrolysis
Inactivation
Enz – Nu :
H_2O

Assay of the incubation mixtures with either lactate dehydrogenase (LDH) or alcohol dehydrogenase (ADH) indicated that β-chloro-DL-alanine, β-fluoro-D-alanine, O-acetyl-D-serine, D-serine and L-serine all turnover to pyruvate when assayed with lactate dehydrogenase. The rate of pyruvate production decreased in a time-dependent manner analogous to the inhibition of the ADC activity by these inhibitors. When the same incubations were examined with ADH there was no indication of turnover to acetaldehyde. The turnover of β-chloro-DL-alanine to pyruvate was inhibited by addition of saturating quantities of L-aspartate to the LDH assay mixture or by preincubation of ADC with either S-carbamoyl-D-cysteine or O-carbamoyl-D-serine. Assay of incubation mixtures from S-carbamoyl-D-cysteine or O-carbamoyl-D-serine with either LDH or ADH indicated that there was no significant turnover to either pyruvate or acetaldehyde (Smith, 1982).

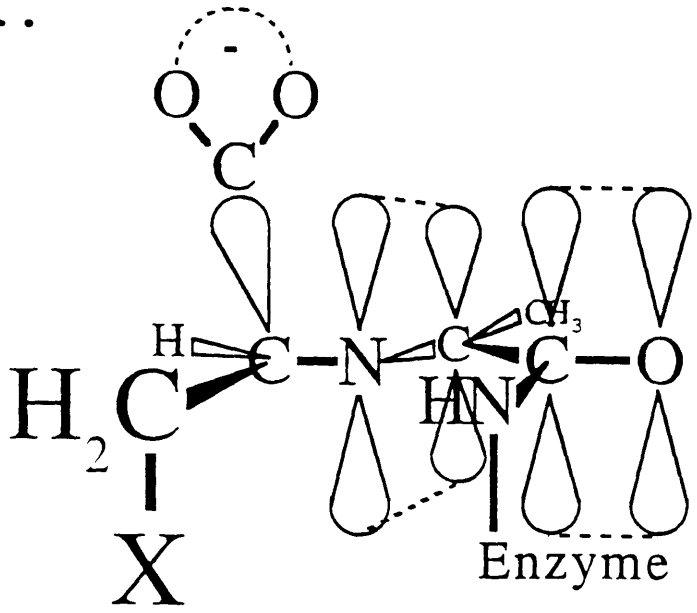
The results of the LDH assays suggested a deprotonation mechanism for the inhibition, as illustrated in Figure 1-6, and leads to some conjecture as to the active site configuration that would allow inhibition only by isomers of the opposite configuration from the substrate. The apparent labilization of the α-hydrogen suggested the orientation at the active site depicted in Figure 1-7. This figure indicates that the bond to be labilized is held in a position which allows the formation of an extended π-orbital system which would serve to stabilize the carbanion formed upon cleavage of the labile bond. In the analogous situation for PLP-dependent decarboxylases Dunathan (1966) has proposed that the bond to be labilized would serve as a second point of attachment to the enzyme. This mode of substrate attachment would prevent any alternate configuration at the active site that could result

Figure 1.7. Orientation of Inhibitor at the Active Site of L-Aspartate-α-Decarboxylase.

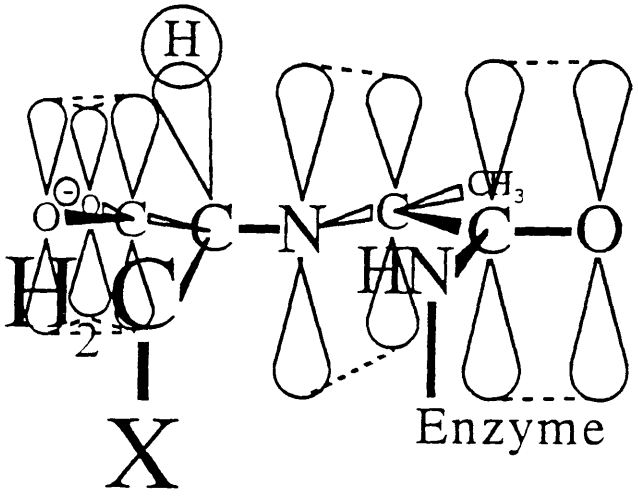
A. L-isomer

B. D-isomer

A.



B.



in differential labilization based on the stereochemistry of the substrate. An alternate means of attachment through binding of the amino acid side chain proposed by Snell and Di Mari (1970) and this means of attachment, as illustrated in Figure 1-7, that could result in alternate labilization of the C_α-H or C_α-carboxylate bonds in the D or L isomers (Smith, 1982).

1.5 Pyruvoyl Dependent Decarboxylases

Amino acid decarboxylases are present in animals, plants and microorganisms where they serve to produce amines that are known to serve serve a number of biological functions. Most amino acid decarboxylases utilize pyridoxal 5'-phosphate (PLP) as an essential coenzyme (Boeker and Snell, 1972). However, Riley and Snell (1968) demonstrated the existance of a histidine decarboxylase from *Lactobacillus* 30a that utilized a pyruvoyl prosthetic group at the active site. In the time since Riley and Snell demonstrated the presence of the pyruvoyl prosthetic group in histidine decarboxylase (HDC) of *Lactobacillus* 30a a small number of decarboxylases have been found that contain an essential pyruvoyl prosthetic group (reviewed recently by Recsei and Snell, 1984). The number of known pyruvoyl-dependent decarboxylases has not increased in recent times although several of the decarboxylases have been purified or identified from new sources. The known pyruvoyl-dependent decarboxylases and some of their physical properties are provided in Table 1-1.

Table: 1-1 Structures of known pyruvoyl-dependent decarboxylases

Enzyme	Subunit structure	Molecular weight (Kd)			Pyruvate containing subunit	Molecular weight proenzyme π (Kd)	References
		Native	Subunit α	Subunit β			
Histidine							
C. perfringens	$(\alpha\beta)_6$	210	27	9	α	* 8.32§	1,2
Lactobacillus 30a	$(\alpha\beta)_6$	208	28 24.9§	9 8.84§	α	37 33.7§	3-7
L. buchneri	$(\alpha\beta)_6$	210	27	9 8.81§	α	36.0	1.2
Micrococcus sp. n.	$(\alpha\beta)_3$	110	29	8.5	α	-	8-12
S-Adenosylmethionine							
E. coli	$(\alpha\beta)_4$	136	19	14	α	35	13,14
S. cerevisiae	$(\alpha)_2$	88	41		α	-	15,16
Murine							
Liver	$(\alpha)_2$	68	32		α	-	17
Mammary gland	$(\alpha)_2$	74	32			-	17
Rat liver and muscle	$(\alpha)_2$	68	32.5		α	37†	18-21
Bovine liver	$(\alpha)_x$	-	32.0		-	32^x	22
Rabbit liver	$(\alpha)_2$	70	34		ND		23
Phosphatidylserine							
E. coli	$(\alpha)_x$	-	36		α	-	24,25
Aspartate							
E. coli	$(\alpha\beta)_x(\pi)_y$	54.7	10.58 11.02φ	2.23 2.83φ	α	14.7 13.8φ	26,27

* = Precursor not detected with rabbit antiserum directed against Lactobacillus 30a histidine decarboxylase.

† = Obtained by immunoprecipitation of extracts obtained from the translation of rat prostate mRNA in a reticulocyte lysate system. Immunoprecipitations were conducted with rabbit anitserum raised against the purified S-adenosylmethionine decarboxylase (20).

§ = Calculated molecular weight determined from the protein sequence.

φ = Determined by translation of the DNA sequence described in this thesis.

References for Table: 1-1

1. Recsei *et al.* (1983).
2. Huynh and Snell (1985).
3. Riley and Snell (1970).
4. Hackert *et al.* (1981).
5. Recsei and Snell (1973).
6. Huynh *et al.* (1984).
7. Vaaler *et al.* (1982).
8. Semina and Mardashev (1965)
9. Alekseeva and Prozorovskii (1976).
10. Mardashev *et al.* (1974)
11. Alekseeva *et al.* (1976).
12. Prozorovski and Jornvall (1974).
13. Anton and Kutny (1987).
14. Tabor and Tabor (1985).
15. Cohn *et al.* (1977).
16. Andrews (1965).
17. Sakai *et al.* (1979).
18. Pegg (1977).
19. Poso and Pegg (1982).
20. Shirahata and Pegg (1986).
21. Shirahata *et al.* (1985).
22. Degen *et al.* (1981).
23. Balana-Fouce *et al.* (1986).
24. Satre and Kennedy (1978).
25. Dowhan *et al.* (1974).
26. Williamson and Brown (1979).
27. This work.

1.5.1 Histidine Decarboxylase

S-adenosylmethionine and phosphatidylserine decarboxylases have been less extensively studied than the histidine decarboxylase from *Lactobacillus* 30a (Snell, 1984) while the wealth of information available on HDC has made it the paradigm for the study of this small category of enzymes. As a result HDC will be described in greater detail in the following section.

The histidine decarboxylase (HDC) from *Lactobacillus* 30a was first reported by Rodwell (1953) in a study on bacteria, obtained from horse stomach and sheep rumen, which exhibited amino acid decarboxylase activities. The enzyme decarboxylates L-histidine to produce histamine which has been noted by Recsei and Snell (1984) to accumulate in the growth medium of *Lactobacillus* 30a. They postulate that the presence of histamine in the host's digestive track may affect the physiology of the host in a manner that provides a selective advantage for the microorganism or the host. In the time since Rodwell's initial report, the enzyme has been the subject of intensive study by E. E. Snell and associates (Recsei and Snell, 1984). HDC was purified to homogeneity and crystallized in large quantities (Chang and Snell, 1968), a process facilitated by the induction of the enzyme during growth in acidic medium containing large quantities of histidine (Guirard and Snell, 1964). The native enzyme was shown, based on the crystal structure, to have an $(\alpha\beta)_6$ structure (Hackert *et al.*, 1981) composed of two non-identical subunits of 28,000 (subunit α) and 9,000 (subunit β) daltons (Riley and Snell, 1970) consistent with the molecular weight established for the native enzyme as 208,000 daltons (Hackert *et al.*, 1981).

HDC was inhibited by a number of carbonyl reagents including cyanide, phenylhydrazine, and sodium borohydride (Riley and Snell, 1968; Rosenthaler, 1965) indicating the presence of a carbonyl group that was essential for activity. The [^{14}C]phenylhydrazine-inactivated enzyme was digested with pronase and chymotrypsin and following isolation the labeled fragment was shown to contain only phenylalanine in a a 1:1 ratio of phenylhydrazone:phenylalanine (Riley and Snell, 1968). This phenylhydrazone fragment was catalytically hydrogenated to give a 1:1 ratio of alanine:phenylalanine (Riley and Snell, 1968). This, in combination with the ability to identify the [^{3}H]lactate from the hydrolysate of [^{3}H]$NaBH_4$ reduced enzyme, indicated that the carbonyl group was a pyruvoyl residue bound via an amide linkage to an amino-terminal phenylalanine (Riley and Snell, 1968). The pyruvoyl group has been shown to be bound to the amino terminus of the larger α-subunit (Riley and Snell, 1970). The growth of Lactobacillus 30a on medium containing [^{14}C]serine resulted in the labeling of the serine and pyruvoyl residues indicating that the pyruvoyl group was derived from a serine residue by post-translational modification (Riley and Snell, 1970).

The HDC was shown to have a high degree of specificity for L-histidine but several structurally related compounds were able to serve as substrate at high concentrations (Chang and Snell, 1968a). The enzyme exhibited little variation in V_{max} over a pH range from 2.9 to 6.5 but groups that ionize at pH 4.2 (possibly a carboxyl group) and 6.6 (possibly an imidazole group) were shown to be involved in the binding of substrate (Rescsei and Snell, 1970). A K_M of 0.4 mM and a V_{max} of 80 μmoles of CO_2 min^{-1} mg^{-1}, were measured at pH 5 (Recsei and Snell, 1970).

HDC exhibited Michaelis-Menten kinetics, but above pH 7.0 sigmoidal effects become pronounced. When the enzyme was incubated in the presence of L-[^{14}C]histidine and $NaBH_4$ at 0 °C the label was covalently incorporated into the enzyme. Hydrolysis of the reduced inactive enzyme yielded N^2-(1-carboxyethyl)[^{14}C]histidine and N^1-(1-carboxyethyl) [^{14}C]histamine indicating the formation of a Schiff base between the substrate and the pyruvoyl prosthetic group during catalysis (Recsei and Snell, 1970).

The mechanism of HDC postulated by Recsei and Snell (1970) can be considered analogous to that postulated for ADC as illustrated in Figure 1-4. The decarboxylation and subsequent protonation has been shown to occur with retention of configuration (Chang and Snell, 1968a; Battersby *et al.*, 1980).

The presence of an active site pyruvoyl group indicated the need for some type of post-tranlational modification to generate the prosthetic group (Recsei and Snell, 1984). The existance of a proenzyme was postulated and the possibility existed that mutants could be obtained that were deficient in activation of the proenzyme. Seven such mutants were generated by nitrosoguanidine treatment of *Lactbacillus* 30a (Recsei and Snell, 1972). All 7 mutants were shown to contain material that cross-reacted with antibodies directed against HDC (Recsei and Snell, 1973) which had a molecular weight of 37,000 daltons. This protein was later shown to be the proenzyme form of HDC (designated by Snell as the π chain) which can also be detected in wild type strains of *Lactobacillus* 30a in cultures grown to stationary phase on low histidine medium and then induced for HDC production for 7 hours by the addition of L-histidine (Recsei and Snell, 1982).

The proenzymes from mutant strain 3 and wild type have been purified to homogeneity (Recsei and Snell, 1982). Both were maximally activated at pH 7.1-7.6 in the presence of monovalent cations at 37°C but the mutant proenzyme was activated at one-third the rate of the wild type proenzyme. The activation of both wild type and mutant proenzyme resulted in the formation of the pyruvoyl group and two subunits identical in size to those of the wild type enzyme (Recsei and Snell, 1973; Recsei and Snell, 1982). However, the mutant HDC exhibited a narrower optimal pH range, decreased strength of subunit interactions, a decreased V_{max} and an increased K_M (Recsei and Snell, 1982). Activation was shown to be first-order with respect to proenzyme or protein concentration indicating that activation of the proenzyme was either autocatalytic or nonenzymatic (Recsei and Snell, 1973).

The mechanism of activation of the mutant 3 proenzyme of HDC has been determined (Recsei *et al.*, 1983) and shown to be autocatalytic. A cleavage of the proenzyme occurred between two adjacent serine residues (residues 81 and 82 of the proenzyme) and generated a pyruvoyl residue at the amino terminus of the α-subunit from a serine at position 81 of the proenzyme while the serine at position 82 became the carboxy-terminus of the β-subunit (Recsei *et al.*, 1983). The cleavage of the proenzyme was shown to occur without the incorporation of O^{18} from solvent H_2O^{18} into the carboxyl group of the carboxy-terminal serine residue of the β-subunit. Activation of proenzyme labeled with L-[hydroxyl-O^{18}]serine resulted in the quantitative incorporation of the O^{18} into the serine carboxyl of the β-subunit. When the proenzyme was labeled with L-[carboxyl-O^{18}]serine, there was no change in the content or the distribution of the label upon activation indicating that the

incorporation of the hydroxyl-O^{18} from L-[hydroxyl-O^{18}]serine into the carboxyl group was specific.

This lead Recsei *et al.* (1983) to propose two alternate mechanisms for the activation of the proenzyme. The first requires the formation of an ester intermediate by attack of the hydroxyl of serine 81 on the peptide bond with subsequent elimination of the amide nitrogen to generate an ester intermediate between the adjacent serines at positions 81 and 82. This intermediate is cleaved by deprotonation of the α-carbon of serine 81 and subsequent elimination of the bridging hydroxyl oxygen. This oxygen is incorporated into the carboxyl of serine 82 generating a dehydroalanine residue at the amino terminus of the newly formed α-subunit. An alternate mechanism would require the initial dehydration of serine-81 to generate a dehydroalanine residue at that position in the proenzyme. The hydroxide ion generated from the dehydration of serine-81 would have to remain on the enzyme in a site sequestered from solvent. Hydrolysis of the peptide bond between serines 81 and 82 would occur by attack of this sequestered hydroxide ion on the amide bond, resulting in its incorporation into the newly formed carboxyl terminus of the β-subunit. In both cases the dehydroalanine residue generated at the amino terminus of the α-subunit undergoes hydrolysis to from the pyruvoyl prosthetic group.

Recently, the proenzyme of HDC from *Lactobacillus buchneri* has been analyzed in a manner identical to that used for the *Lactobacillus* 30a HDC proenzyme and has been shown to undergo a similiar cleavage to form the active enzyme (Recsei and Snell, 1985). In this case, degradation of the exogenous L-[hydroxyl-O^{18}]serine required the

omission of PLP from the growth medium to limit the catabolism of the precursor or its dilution by biosynthetically derived serine.

The amino acid sequence of the HDC from *Lactobacillus* 30a has been determined for both the α and β subunits (Vaaler *at al.*, 1982; Huynh *et al.*, 1984a). The amino acid sequence of the α-chain is 226 residues long and has a predicted molecular weight of 24,892 daltons. The amino acid sequence of the β-subunit is 81 residues long and has a predicted molecular weight of 8,840 daltons (Vaaler *at al.*, 1982). When the amino acid sequences of the α and β-chains are combined to generate the π-chain of the proenzyme (Huynh *et al.*, 1984a), the resulting protein is predicted by Chou-Fasman rules to have 35% helix, 22% β sheet and 26% reverse turn structures. The putative proenzyme cleavage site is predicted to be located within a sequence of 20 uncharged residues that would form a β sheet structure in the proenzyme.

As indicated in Table 1-1, HDC has also been purified from *Micrococcus* sp. n. (Semina and Mardashev, 1965), *Lactobacillus buchnerii*, and *Clostridium perfringens* (Recsei *et al.*, 1983). The complete sequences of the β-chains and the amino terminal regions of the α-chains of the *C. perfringens* and *L. buchneri* enzymes have been determined (Huynh and Snell, 1985). In addition, a substantial portion of the sequence of the β-chain (Alekseeva *et al.*, 1976) along with a portion of the α-chain (Prozorovski *et al.*, 1981) of the *Micrococcus* sp. n. HDC have been published. A comparison of the β-chains and the amino termini of the α-chains by Huynh and Snell (1985) indicated a region of considerable homology centered around the activation site of the *Lactobacillus* 30a proenzyme. Only 23% of the β-chain residues were identical in all four of the proteins but the majority of these residues

were found in the carboxy-terminal region which corresponded to the carboxy-terminal serine residue generated during activation of the proenzyme. In addition, the α-chains exhibited the highest degree of homology in the region corresponding to the pyruvoyl-terminal portion of the four enzymes (Huynh and Snell, 1985). The putative proenzymes for the three decarboxylases from *C. perfringens*, *L. buchneri*, and *Micrococcus* sp. n. share a 5 amino acid sequence spanning the activation site as defined by the proenzyme of *Lactobacillus* 30a. The high degree of homology exhibited by the four enzymes in the region corresponding to the activation site suggested to Huynh and Snell (1985) that all four enzymes were probably generated from a proenzyme in the manner determined for HDC and were derived from a common ancestral protein.

The sequence of the β-chain of histidine decarboxylase from the slow-activating mutant 3 of *Lactobacillus* 30a has been determined and compared with the wild type sequence to identify two amino acid substitutions within the mutant's sequence (Vaaler *et al.*, 1982). The two changes involved conversion of a serine and a glycine in the wild type to an alanine and an aspartate, respectively, in the mutant. These substitutions resulted in changes to the secondary structure predicted by the Chou-Fasman. The serine to alanine transition was predicted to increase the alpha-helical structure of the mutant β-chain with the concommitant elimination of a β-turn structure while the replacement of the glycine by an aspartate resulted in a change in the isoelectric point but only extended an alpha-helix by a single residue (Vaaler *et al.*, 1982). The resulting increase in the predicted alpha-helical

content from 35% to 43% of the β-chain was anticipated by Vaaler *et al.* (1982) to account for the observed changes in the physical and catalytic properties of the mutant enzyme.

The genes encoding the wild type and mutant histidine decarboxylases have been cloned and sequenced (Vanderslice *et al.*, 1986). The wild type gene contained an open reading frame that encoded a protein of 310 residues. This is longer than the 307 amino acids predicted by protein sequencing (Vaaler *et al.*, 1982; Huynh *et al.*, 1984) and Vanderslice *et al.* (1986) attribute this discrepency to a loss of a tripeptide cyanogen bromide fragment during protein sequencing. In addition, they report three additional minor differences with respect to the peptide sequence as determined by Snell and associates (Vaaler *et al.*, 1982; Huynh *et al.*, 1984).

The mutant 3 nucleotide sequence indicated only a single nucleotide change which resulted in the glycine to aspartate substitution noted by Vaaler *et al.* (1982). Vanderslice *et al.* (1986) were unable to confirm the serine to alanine substitution that Vaaler *et al.* (1982) had noted. The clear assignment of the serine to alanine transition at that position in the mutant protein sequence (Vaaler *et al.*, 1982) indicated to Vanderslice *et al.* (1986) that the mutant 3 strain (Guirard and Snell, 1964), from which they had obtained the HDC gene, might be of a different genotype. They were unable to note any difference in the rate of activation or pH dependence of their strain, which they designated as D58, and proenzyme obtained from mutant 3.

Examination of the 5' region of the open reading frame indicated the presence of a putative Shine-Dalgarno sequence as well as a plausable -35 region and with a Pribnow box sequence 89-94 nucleotides

upstream of the methionine initiation codon. Downstream of the open reading frame, they noted stop codons in all three reading frames within 20 bases of the putative transcriptional termination site which they note is a common feature in polycistronic messages. Consistent with this observation was the presence of a second open reading frame 80 nucleotides downstream of the HDC open reading frame. This second open reading frame also exhibited a plausable Shine-Dalgarno sequence and this led Vanderslice *et al.* (1986) to propose the possibility of a histidine decarboxylase operon.

Histidine decarboxylases have recently been purified from *Morganella morganii* AM-15 (Tanase *et al.*, 1985), fetal rat (Taguchi *et al.*, 1984), and mouse kidney (Martin and Bishop, 1986) and have been shown to require PLP for activity. In addition, the enzyme from *Klebsiella pneumoniae* has been reported to require the presence of exogenous PLP for activity (Tanase *et al.*, 1984). The gene for the *M. morganii* AM-15 enzyme has recently been cloned and sequenced (Vaaler *et al.*, 1986).

1.5.2 S-Adenosylmethionine Decarboxylase

S-Adenosylmethionine decarboxylase (SDC) is required for the synthesis of the two metabolically ambiguous polyamines, spermine and spermidine, and is believed to be the rate limiting step in their synthesis in mammals (Sakai *et al.*, 1979). Of particular interest is the increased levels of enzyme activity associated with cell proliferation (Tabor and Tabor, 1976). SDC has been purified from a number of sources, as indicated in Table 1-1, and in all cases where

cofactor requirements have been examined the SDC has been shown to lack PLP and contain a pyruvoyl group (Recsei and Snell, 1984).

The structural gene encoding the *E. coli* SDC (*speD*) has been mapped to min 2.7 on the *E. coli* chromosome (Tabor *et al.* 1978) and has been cloned as a 9 kilobase PstI fragment which has been inserted into the plasmid pBR322 (Markham *et al.*, 1982). A strain carrying this *speD* plasmid expressed the SDC activity 15 fold over wild type levels, allowing the enzyme to be purified rapidly to homogeneity using an affinity column technique (Markham *et al.*, 1982). The protein appeared to be composed of a single subunit of 17,000 daltons (M_r) (Markham *et al.*, 1982). More recently, the protein has been shown to contain two subunits of nearly identical molecular weights (M_r) of 19,000 and 17,000 daltons (Anton and Kutny, 1987). The recent molecular weights are in agreement with the report that the enzyme is synthesized as a 35,000 dalton (M_r) precursor that appears to be cleaved to subunits of (M_r) 17,000 and 19,000 daltons (Tabor and Tabor, 1985). Amino terminal sequence analysis indicated that the 19,000 dalton subunit has a blocked amino terminus which can be sequenced following reductive amination which yields an amino terminal alanine residue (Anton and Kutny, 1987). The partial amino terminal sequences of both subunits have been determined but the full sequence has not yet been published (Anton and Kutny, 1987). The pyruvoyl group forms a Schiff base intermediate with substrate which can be reduced with $NaCNBH_4$ (Anton and Kutny, 1987) to inactivate the enzyme and resulted in the incorporation of approximately 4 moles of substrate per mole of enzyme, consistent with the reported $(\alpha\beta)_4$ structure.

A cDNA clone for the gene encoding the bovine liver S-adenosylmethionine decarboxylase has recently been isolated by Mach *et al.* (1986). The decarboxylase had been purified to homogeneity and contained a blocked amino terminus consistent with an amino terminal pyruvoyl group. Probes were derived from the sequences of proteolytic fragments and were utilized for screening a bovine cDNA library prepared from bovine lymphocyte poly $(A)^+$ RNA in phage λgt11. The clone isolated from the library contained a 1350-nucleotide insert and open reading frames coding for two proteins. The reading frame established by the partial amino acid sequences was terminated at the vector boundary indicating that the clone was lacking the 5′ region of the bovine gene. When the cDNA clone was used for Northern analysis two bands, 2.4 and 3.6 kb in length, hybridized to the probe and exhibited apparent equal complementarity. Unfortunately, the sequence of the bovine SDC coding region has not yet been published.

Total poly(A) mRNA from rat ventral prostate was translated in a reticulocyte system and two proteins corresponding to SDC were immunoprecipitated by antiserum prepared against SDC (Shirahata and Pegg, 1986). The proteins exhibited molecular weights (M_r) of 37,000 and 32,000 daltons. The 32,000 dalton protein corresponded to the molecular weight of the subunit of SDC (Shirahata *et al.*, 1985) while the 37,000 dalton protein was a proenzyme that is cleaved on continued incubation, following the addition of cycloheximide to inhibit protein synthesis, to yield the 32,000 dalton protein (Shirahata and Pegg, 1986). A similiar experiment with antibodies prepared against the bovine liver SDC revealed a single 32,000 dalton (M_r) protein, corresponding to the mature subunit, as the only immunoprecipitable

material obtained from a reticulocyte translation system indicating that a precursor, if any exists, is no more than 10 amino acids longer than the mature subunit (Degen *et al.*, 1981).

1.5.3 Phosphatidylserine Decarboxylase

Phosphatidylserine decarboxylase catalyzes the decarboxylation of phosphatidylserine (Dowhan *et al.*, 1974) in the final step of the biosynthesis of phosphatidylethanolamine, the major phospholipid of the *E. coli* membrane. The gene encoding the enzyme (*psd*) has been obtained on a Clarke-Carbon plasmid (Tyhach *et al.*, 1979) and the plasmid bearing strain is able to overproduce the enzyme 40-fold. The *E. coli* enzyme has been purified to homogeneity from the plasmid bearing strain (Dowhan *et al.*, 1974) and shown to be susceptable to inactivation by carbonyl reagents. The pyruvoyl group has been identified by reduction of the enzyme with [^{3}H] $NaBH_4$ and identification of the [^{3}H]lactate from acid hydrosylates (Satre and Kennedy, 1978). SDC can be inactivated by $NaCNBH_4$ in the presence of substrate indicating that phosphatidylserine forms a Schiff base intermediate with the pyruvoyl group (Satre and Kennedy, 1978).

Experimental Methods

2.1 Materials

Restriction endonucleases were obtained from either New England Biolabs, Bethesda Research Laboratories,or Boehringer Mannheim Biochemical. λ DNA-HindIII digest, ϕX174 RF DNA-HaeIII digest, M13mp18 RF I DNA, T4 DNA ligase, and polynucleotide kinase were also obtained from New England Biolabs. Sodium ampicillin, tetracycline hydrochloride, chloramphenicol, D-cycloserine, isopropyl-β-D-thiogalactopyranoside, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) were obtained from Sigma. Dithiothreitol (DTT), ultra-pure agarose for gel electrophoresis, and the Nick Translation Reagent Kit were obtained from Bethesda Research Laboratories. Components of culture media were obtained from Difco. S1 exonuclease, and the Large fragment of DNA polymerase I were obtained from Boehrigner Mannheim Biochemicals. The deoxy-nucleotide triphosphates, and dideoxy-nucleotoide triphosphates used for DNA sequencing were obtained from Pharmacia PL Biochemicals. The 17 base -20 universal primer for M13 sequencing was obtained from IBI. Electrophoresis grade acrylamide, ammonium persulfate, bis-acrylamide, and SDS were obtained from BioRad. Ultra-pure urea was obtained from Accugen. 5'-[α-^{32}P]dATP, 5'-(α[^{35}S]thio)dATP, L-[^{35}S]methionine, L-[4-^{14}C]aspartic acid, and affinity-purified [^{125}I]Protein A were obtained from Amersham. DL-[4-^{14}C]aspartic acid was obtained from Research Products International. DL-[1^{14}C]aspartic acid was obtained from ICN Pharmaceuticals, Inc. Complete Freunds adjuvant, Trizma base, Dowex 1X8-400 chloride form and DEAE-Sephadex A-50 were

obtained from Sigma. Ultrogel AcA44 was obtained from LKB. Hydroxylapatite was obtained from BioRad. Ultra-pure ammonium sulfate was obtained from Schwartz/Mann Inc. PM10 Diaflo ultrafiltration membranes were obtained from Amicon. Enhance and hyamine hydroxide were obtained from New England Nuclear. Nitrocellulose (0.10 μm, PH79) was obtained from Schleicher and Schuell. *Staphylococcus auereus* V8 protease was obtained from Miles Scientific. Protein-A Sepharose CL-4B was obtained from Pharmacia. Phenol was obtained from Mallinckrodt.

2.2 Media for Bacterial Growth

Liquid media used for cultures included LB broth, M9 minimal medium, NZC medium and λ broth (Maniatis *et al.*, 1982). M9 minimal plates contained glucose (0.2%) as a carbon source and were supplemented with 20 μg/ml DL-threonine, 20 μg/ml L-leucine and 1 μg/ml thiamine when used to select for complementation of the *panD*$^-$ phenotype of the β-alanine requiring auxotrophs. When necessary minimal plates were supplemented with 100 μg/ml β-alanine. In addition, LB plates, λ plates and X-gal indicator plates were used (Maniatis *et al.*, 1982). Plates generally contained 1.5% agar while top agar, when used, contained 0.65% agar. Antibiotic concentrations used were 15-25 μg/ml tetracycline hydrochloride, 25-50 μg/ml ampicillin and 10 μg/ml chloramphenicol.

When large cultures of bacteria were required then the M63 minimal medium of Pardee *et al.* (1959) was used: KH_2PO_4 (13.6 g/l), $(NH_4)_2SO_4$ (2.0 g/l), $MgSO_4.7H_2O$ (0.2 g/l) and $FeSO_4.7H_2O$ (0.5 mg/l). The pH was adjusted to 7.0. This was routinely supplemented with .5% glucose (5 g/l), yeast extract (3 g/l) and Bacto-peptone (5 g/l).

Table 2.1: *Escherichia coli* K12 Strains Used in this Work.

Strain	Synonym	Genotype	Reference or Source
AB354		Hfr *lacZ4 leuB6 pan2 rpsL8 supE44 thi1 thr1* (λ-)	B. Bachmann (ECGSC)
AB355		Hfr *lacZ4 leuB6 pan3 rpsL8 supE44 thi1 thr1* (λ-)	B. Bachmann (ECGSC)
DB4548	BNN45	*hsdR supE44 supF met*$^-$ *thi*$^-$	D. Botstein and S. Wasserman
RB901	Δ21	F$^-$ Δ(*recA srl*)21 *lacY? leu6 lexA3*(Ind$^-$) *lexA51*(Def) *mtlA rpsL31 sulA11 thr1*	G. Walker and S. Elledge
JM101		Δ(*lac-pro*) *thi*$^-$ *supE44* / F' *traD36 proAB lacI*^{q} *lacZ*ΔM15	P. Schimmel and S. Ludmerer
W3110		λ$^-$	G. Walker and S. Winans

ECGSC - *E. coli* Genetic Stock Center at Yale University.

2.3 Bacterial Strains

The bacterial strains used in this work are listed in Table 2.1. The plasmids used are listed in Table 2.2. λ_{SW1} is a clone of *dadB* of *Salmonella typhimurium* (S. Wasserman *et al.*, 1983).

2.4 Growth of AB355/pADC1 for ADC Preparation

The routine source for large preparations of ADC was the subclone AB355/pADC 1 maintained under drug pressure (12.5 μg/ml tetracycline). Large cultures of *E. coli* (24 liters) were grown in a New Brunswick Scientific Microferm fermentor Model MF-128S. Salts were sterilized *in situ* for 1 hour as per the manufacturers instructions. Glucose, yeast extract, and Bacto-peptone were sterilized separately and added before inoculation. Foaming was controlled by the use of Sigma antifoam C. The inoculum for the fermentor was a one liter culture grown overnight in the same medium on a laboratory shaker at 37° C. Cells were grown at 37° C with maximum aeration with the stirring speed set at 200 rpm. Cells were harvested in late log phase when the turbidity had reached a reading of >250 Klett units.

Cells were harvested in 1 liter bottles by centrifugation at 4,000 rpm for 20 minutes in a Beckman J-6B centrifuge. Aeration and stirring were continued while harvesting proceeded at a rate of 6 liters per centrifugation. Cells were then resuspended in 1 liter of 0.89% saline and removed from suspension by centrifugation for 10 minutes at 16,000 x g on a Sorvall RC2-B refrigerated centrifuge. The resulting wet cell paste was separated into 45 g quantities and stored frozen at -20° C. The cells were used for preparation of ADC, generally, within 1-2 weeks after harvesting.

2.5 Preparation of ADC from AB355/pADC1

ADC was prepared from the over-producing strain AB355/pADC1 according to the method of Williamson and Brown (1979) with the ommisions indicated. The dialyzed, concentrated eluate from the DEAE-Sephadex A-50 column was added directly to the Ultrogel AcA44 column without previous purification on QAE-Sephadex Q-50. In addition, the dialyzed concentrated eluate from the hydroxylapatite column was submitted directly to preparative gel electrophoresis without previous purification by preparative isoelectric focusing.

Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin for preparation of standard protein solutions.

The buffer commonly used throughout the protein purification is referred to as standard buffer and contains 50 mM potassium phosphate pH 7.0, 5 mM EDTA and 50 μM DTT.

2.6 Preparative Gel Electrophoresis of ADC

When preparative native polyacrylamide gels were required for ADC preparation, 0.5 cm wide slab gels were used, the stacking gel was eliminated, and the buffer used in the Davis system was replaced with the Tris-borate buffer system of Peacock *et al.* (1965) as described by Williamson and Brown (1979). 4X preparative sample buffer contained salts at 4 times the running buffer concentrations in addition to 40% glycerol, 280 mM β-mercaptoethanol, and 84 μM bromophenol blue.

Following preparative native slab gel electrophoresis, the sides of the slab were cut off and stained for protein. Once the bands had been localized the pertinent area of the gel was cut into sections horizontally and placed in dialysis bags with electrode buffer. The bags were placed in a horizontal flat bed apparatus containing the running buffer used for the preparative gel, and the protein was electroeluted overnight at 50 volts constant voltage. Following electroelution, the dialyzed eluate was removed and concentrated in an Amicon pressure cell equipped with a PM-10 membrane. The resulting concentrates were analyzed by native disc gel electrophoresis using the Davis system. The homogeneous fractions were pooled and dialyzed against two changes of standard buffer (1000 volumes) for 8 hours each. Homogeneous ADC was stored at 4° C.

2.7 Assay for ADC Activity

L-asparate-α-decarboxylase activity was assayed by the producton of either CO_2 or β-alanine from aspartate. $^{14}CO_2$ production from DL-[1-^{14}C]-aspartate was measured by capture in the form of a carbonate salt using hyamine hydroxide as previously described (Williamson and Brown, 1979). β-[1-^{14}C]-alanine production from DL-[4-^{14}C]-aspartate or L-[4-^{14}C]-aspartate was measured using the Dowex-1X8 minicolumn procedure as previously described (Smith, 1982). A unit of ADC is defined as the amount of enzyme required to catalyze the formation of one nanomole of CO_2 from L-aspartate per minute at 42° C.

2.8 Analytical Gel Electrophoresis

2.8.1 Non-denaturing Gel Electrophoresis. Analytical non-denaturing protein polyacrylamide gel electrophoresis was carried out by the method of Davis (1964) with 0.15 cm wide slab gels and an acrylamide concentration of 10%. 5X Sample buffer contained 300 mM Tris-HCl pH 6.8, 50% glycerol, 350 mM β-mercaptoethanol, and 105 mM bromophenol blue.

2.8.2 Denaturing Gel Electrophoresis. Analytical denaturing slab gels were 10-18% linear gradient polyacrylamide (60:1.5% acrylamide:bisacrylamide ratio) gels containing 0.1% SDS, 7 M urea, and a 0-10% sucrose linear gradient utilizing a discontinuous buffer system as described by Hashimoto *et al.* (1983). This gel system gave an effective separation range of from 1.5-43 kd molecular weight (M_R) with excellent resolution in the low molecular weight range. On occasion, a 10-18% polyacrylamide gradient gel containing 0.1% SDS was prepared using the buffer system of Laemmli (1970). 5X Sample buffer contained 300 mM Tris.HCl pH 6.8, 10% SDS, 50% glycerol, 350 mM β-mercaptoethanol, and 105 mM bromophenol blue. Samples were denatured in sample buffer by incubation for 10 minutes in a boiling water bath.

Polyacrylamide protein gels were stained with a solution containing 0.17% Coomassie Brilliant Blue, 50% methanol, and 10% glacial acetic acid. Hashimoto gels were photographed while still wet using Polaroid Type 55 film due to their tendency to crack upon drying. Polyacrylamide gels containing peptides from L-[^{35}S]-methionine labeled maxicells preparations were fixed and stained as above then treated with

Enhance cocktail (New England Nuclear) as described by the manufacturer. The gels are then dried for 1 hour on a BioRad gel dryer under vacuum and exposed at -70° C using Kodak X-Omat AR film.

Molecular weight standards for determination of subunit size are generated by cyanogen bromide cleavage of horse heart cytochrome C (Chu *et al.*, 1964) and equine myoglobin (Edmundson, 1963) as described by Gross (1967). The resulting fragments are treated with SDS sample buffer and used as standards with the Hashimoto gel system.

2.9 Maxicell Analysis of Plasmid Encoded Proteins

Preparation of cells for the labeling of plasmid encoded proteins with L-[^{35}S]-methionine was accomplished essentially by the method of Sancar *et al.* (1981). Plasmids of interest were transformed into a *recA*$^-$ *lexA*$^-$ background (RB901) as described. The cells were grown to mid-log phase (turbidity reading of 40 Klett units, 2 x 10^8 cells/ml) under the appropriate drug pressure in K Media of Rupp et al. (1971). Cells were exposed to a General Electric germicidal lamp calibrated at a UV dose of 1 Joule/m^2/sec (determined with a calibrated Dosimetre Latarjet number 221) for 30 seconds to provide a dose of 30 J/m^2. The cells were then allowed to incubate for 1 hour at 37°C and then D-cycloserine was added to a final concentration of 100 μg/ml. The cultures were then incubated for 8-12 hours at 37°C in the dark. The cells were then concentrated by cenrifugation and washed several times with modified Hershey sulfate free salts (Worcel and Burgi, 1974). The cells are then resuspended in modified Hershey medium (0.4% glucose) and the proteins were labeled with 25 μCi of L-[^{35}S]-methionine (specific

activity >1,000 Ci/mmol) at 25 μCi/ml with aeration at 37° C for 15 minutes and were then concentrated by centrifugation for 10 minutes at 12,000 x g at 4° C. Cells were then treated in one of two ways. Maxicell preparations not required for immunoprecipitations were placed in SDS sample buffer and lysed for 10 minutes in a boiling water bath. Preparations to be treated with antisera were resuspended in ice cold TE buffer and treated with 1.25 μg/ml of lysozyme for 10 minutes on ice and frozen. Frozen aliquots were thawed on ice and the resulting cellular debris was removed by centrifugation for 15 minutes in an Eppendorf microfuge.

2.10 Preparation of Antiserum Directed Against ADC

Preimmune sera was obtained from blood collected from the ear vein of each of four male New Zealand white rabbits weighing approximately 3 kilograms each. The blood was allowed to clot overnight at 4° C and the sera was cleared by two succesive centrifugations for 15 minutes at 1,500 x g at 4° C. The cleared supernatant was stored frozen in aliquots at -20° C.

ADC (390 μg of gel purified homogeneous enzyme) was prepared for injection by mixing equal volumes of ADC in standard buffer with .15 M NaCl and complete Freund's adjuvant and homogenizing the mixture to obtain a thick emulsion. Each rabbit was then injected with 2 ml of the homogenate (97.5 μg of ADC) by the administration of injections of the homogenate in equal volumes subcutaneously on both sides of the neck, 10 intradermal injections down the length of the back on each side of the spinal column and intramuscular injections into both femoral regions.

At six weeks after the first inoculation the rabbits were injected in an identical manner with a preparation containing 65 μg of ADC per rabbit. At 7, 8, and 9 weeks after the first inoculation the rabbits were each bled from an ear vein and the blood was treated to obtain antisera as described for the preimmune sera.

2.11 Immunoprecipitations with Antiserum Directed Against ADC

Protein-A Sepharose CL-4B was suspended at 40 mg/ml in the same buffer and allowed to swell for 1 hour. The gel was then removed from suspension by centrifugation for 5 minutes in an Eppendorf microfuge and washed in two changes of buffer. The swollen gel was then resuspended in RIPA buffer to a final concentration of 10% (w/v). Immunoprecipitations were prepared in RIPA buffer (10 mM sodium phosphate pH 7.4, 150 mM NaCl, 1% Triton X100, 1% sodium desoxycholate and 1% SDS) containing 1% aprotinin (1 mg/ml with a specific activity of 15 TIU/mg in sterile 0.89% saline), and 1% of 100 mM PMSF (prepared fresh in ethanol).

Immunoprecipitations of maxicell extracts were prepared in RIPA buffer in a volume of 200 μl to which was added 10 μl of antiserum (diluted 1:100 in the same buffer). Precipitations were allowed to occur for 2 hours on ice after which the immunoprecipitations are treated with 50 μl of the Protein-A Sepharose suspension. Each sample was vortexed once a minute for 30 minutes and the gel is removed from suspension by centrifugation for 5 minutes in an Eppendorf microfuge. The gel was then subjected to 5 washes in RIPA buffer. After the final wash the gel is resuspended in SDS sample buffer and subjected to

denaturation for 10 minutes in a boiling water bath to release the bound protein. Samples were routinely analyzed on the denaturing gel system of Hashimoto as previously described.

2.12 Transfer of Proteins to Nitrocellulose and Detection with anti-ADC Antiserum

Preparations of ADC separated by denaturing gel electrophoresis were transfered from 1.5 mm thick 10-18% polyacrylamide gradient gels to nitrocellulose (Schleicher and Schuell PH79, 0.10 μm) via the method of vacuum-blotting as described by Peforoen *et al.* (1982). Analysis of the proteins transfered to the nitrocellulose was accomplished with the Bovine Lacto Transfer Technique Optimizer (BLOTTO) cocktail (Johnson *et al.*, 1984). The nitrocellulose strips were blocked in BLOTTO cocktail [5% w/v nonfat dry milk, 0.01% Sigma Antifoam A and 0.0001% merthiolate in phosphate buffered saline (PBS; 0.8% NaCl, 0.2% KCl, 8 mM $Na_2HPO_4.7H_2O$, and 1.5 mM KH_2PO_4)] at room temperature for 18 hours.

The blocked filters were then incubated with BLOTTO cocktail containing a 1:250 dilution of either preimmune or anti-ADC serum at 37° C for 2 hours on a rocker platform. Following incubation with serum, the filters were washed with 5 changes of BLOTTO cocktail (100 ml) for 10 minutes each at room temperature with agitation.

The filters were then placed in 11.25 ml of BLOTTO buffer containing 1 μCi of [^{125}I]Protein A for 1 hour at room temperature. At the end of the incubation, the unbound Protein A was removed by 5 washes in BLOTTO cocktail (100 ml each) at room temperature. After the final wash the filter was blotted dry, wrapped in Saran Wrap and exposed at

-70° C to Kodak X-Omat AR film utilizing a Dupont Cronex Lightning-Plus intensifying screen.

The location of protein transfered to nitrocellulose was visualized by the direct staining of a duplicate portion of the filter with the amido black method of Towbin *et al.* (1979). Filters were stained in 0.1% amido black 10B, 45% methanol, and 10% glacial acetic acid for 15 minutes. The filter is then destained in 3 changes of 90% methanol, and 2% glacial acetic acid.

2.13 Isolation of ADC Peptides by Preparative Gel Electrophoresis

ADC peptides submitted for amino terminal sequence analysis and determination of amino acid composition were prepared from homogeneous ADC (300 μg) separated by preparative SDS polyacrylamide disc gel electrophoresis. The sample was denatured by incubation in a boiling water bath for 10 minutes in the reducing sample buffer previously described for the analytical gels. Separation of the resulting peptides was obtained on preparative 10-18% polyacrylamide gradient gels (16 cm x 17 cm x 0.5 cm) prepared according to the Hashimoto system with fresh electrophoresis grade urea to prevent the formation of cyanates. The peptides of interest were electroeluted by David Andrews of the Harvard Microchemistry Facility using the methods of Hunkapiller *et al.* (1983).

2.14 Peptide Mapping by Limited Proteolysis

Peptide mapping by limited proteolysis was performed as described by Cleveland *et al.* (1977). ADC was separated into its component

peptides by analytical denaturing polyacrylamide disc gel electrophoresis (16 cm x 17 cm x 0.15 cm) using the Hashimoto gel system as previously described. The separated peptides were located by staining with Coomassie Brilliant Blue, and the band cut out with a scalpel. The gel slices were stored at -20° C until needed. When needed, the gel slices were thawed at room temperature and trimmed to 5 mm in width to fit in a standard gel lane. The gel slices were then placed in the well of a Hashimoto gel prepared with a stacking gel at least 2 cm in length and overlayed with 20% glycerol, 25 mM Tris-HCl pH 6.8, 1 mM EDTA, and 0.1% SDS. This was followed by an overlay with 10 μl of the same buffer containing 10% glycerol and protease. Voltage was applied until the dye from the gel slices was 1 cm from the bottom of the stacking gel and electrophoresis was interrupted. Proteolysis was allowed to occur for 30 minutes in the stacking gel and then electrophoresis was continued to completion. Following electrophoresis, the gel was stained and destained as usual to visualize the separated proteolytic fragments.

2.15 Amino-terminal Sequence Analysis

Amino terminal sequence analysis was performed by William Lane of the Harvard Microchemistry Facility using an Applied Biosystems 470A protein gas-phase sequencer. The amino acid phenylthiohydantoin derivatives were identified by determination of their retention time on a Hewlett-Packard 1090 HPLC equipped with a Zorbax ODS column and a diode array detector.

2.16 Amino Acid Composition Analysis

Amino acid compositions were obtained for each of the three peptides obtained by separation of the gel purified ADC as described above. The amino acid composition was determined by David Andrews of the Harvard Microchemistry Facility. Samples were sealed under vacuum in Pyrex tubes and hydrolyzed in 6N HCl at 110° C for 24 hours. Upon completion of the hydrolysis, the solvent is removed by lyophylization and the residue is dissolved in 20 μl of a mixture of ethanol:H_2O :triethylamine:phenylisothiocyanate (7:1:1:1). This reaction mixture is allowed to remain at room temperature for 20 minutes, after which it is lyophilized and the residue is stored at -20 °C.

The resulting mixture of PTC-amino acids is resolved on a Hewlett-Packard 1084B HPLC equipped with an Altex-ODS 3μ column using a gradient program.

2.17 Growth of Lambda Phage

The strain (DB4548) was grown in k broth supplemented with 0.2% maltose and 0.01% yeast extract and routinely used for the growth of kgt7 derivatives. Small quantities of λ phage lysate were prepared by means of plate lysates (Miller, 1972). Large quantities of phage were prepared when needed by means of the liquid NZC culture method (Blattner, 1977). Plating culture for preparation of a lawn was prepared with DB4548 grown to early log phase and concentrated by centrifugation followed by resusupension in one half of the original

volume in 10 mM $MgSO_4$. λ phage transfections were conducted by preincubating the phage with 100 μl of plating culture for 20 minutes at room temperature followed by the addition of 2.5 ml of λ top agar and overlaying of the mixture onto a λ plate. Plates were incubated for 8-12 hours at 37° C.

2.18 Preparation of Lambda Phage DNA

The method used for the large scale purification of λ phage is a variation of that used by Yamamoto *et. al.* (1970). 5 x 10^6 plaque forming units (pfu) of λgt7 recombinant phage were adsorbed for 25 minutes at 37° C to 1 ml of an overnight culture of DB4548 that had been resuspended in 10 mM $MgCl_2$ and 10 mM $CaCl_2$. This was added to 500 ml of NZC broth in a 2 l ehrlenmeyer flask and grown on a reciprocating shaker at 37° C until lysis was apparent by the appearance of cellular debris. The culture was then brought to 0.5 M NaCl by the addition of 14.6 gm of solid NaCl, and 1 ml of chloroform was added after solution of the salt was obtained. The culture was then replaced on the shaker for 10 minutes after which it was chilled on ice. The cellular debris was removed from suspension by centrifugation at 43,000 x g for 10 minutes at 4° C. Polyethylene glycol (6,000-8,000 MW) was added to the supernatant to a final concentration of 10%, and the mixture was chilled overnight at 0° C to precipitate the phage.

The precipitate was removed from suspension by centrifugation for 20 minutes at 19,700 x g at 4° C. The fine precipitate was then redissolved in 7 ml of 10 mM $MgCl_2$, 50 mM Tris.HCl pH 7.5. The solution was then brought to 50 μg/ml RNase I and 5 μg/ml DNase I with 10 mg/ml

RNase I and 10 mg/ml DNase I solutions. This was incubated for 1 hour at 37° C. At the end of incubation 0.75 gm CsCl per ml was added, and the resulting solution was subjected to centrifugation at 57,400 x g for 20 hours at 15° C in a Beckmann L2-65B ultracentrifuge equipped with a type 65 rotor.

The visible phage band was removed in a volume of 1 ml by syringe via side puncture. Proteinase K was added to the purified phage to a final concentration of 1 mg/ml along with solid Na_2EDTA to 25 mM. This mixture was then loaded into dialysis bags and incubated for 2 hours at 37° C in a constant temperature bath during dialysis against 100 volumes of 20 mM Tris.HCl, 0.25 M NaCl, 2 mM EDTA and 0.002% Triton X-100. The diasylate was then extracted twice with equilibrated phenol followed by an extraction with chloroform. The aqueous layer was ethanol precipitated, and the precipitate removed from suspension by centrifugation for 30 minutes at 16,500 x g in an HB-4 rotor. The dried pellet was resuspended in STE (10 mM Tris.HCl pH 8.0, 5 mM NaCl, 1 mM EDTA).

2.19 Isolation of the *panD* Clones

panD clones were isolated from a λgt7 (*nin*5, *cI*$^-$, *att*$^-$, *int*$^-$) hybrid pool created by ligation of fragments generated by digestion of *E. coli* W3110 DNA with EcoRI into the EcoRI site of λgt7 (Williams and Blattner, 1980) as described by Thomas *et al.* (1974). The library was screened by Steven Wasserman, and clones were selected for complementation of the β-alanine auxotrophy of the *panD*$^-$ *lacZ4* strain AB354 via double-lysogen selection by coinfection with λgt4-lac5 (*nin5*, *c*I857, *att*$^+$, *int*$^+$) helper phage and plated in soft agar on M9 minimal

plates (supplemented with 20 μg/ml leucine and threonine and 1 μg/ml thiamine). Single colonies were picked and grown in λ broth at 30° C followed by induction of lytic growth at 42° C for 20 minutes. The resulting lysates were streaked on lawns of DB4548 plated in soft agar on λ plates containing XGal. Stocks prepared from nonblue plaques were retested for complementation.

2.20 Assay for Expression of ADC during Lytic Induction of the Lambda Clones

Induction of lytic growth by recombinant phage derived from λgt7 was obtained by transfection of a 1l culture (contained in a 4l Ehrlenmeyer flask) of AB354 (λ^-) grown to mid-log phase (3×10^8 cells/ml) on λ_{ym} with a multiplicity of infection of 2. Phage were allowed to preadsorb for 15 minutes at 37° C followed by growth at 37° C on a reciprocating shaker. Samples (500 ml) were taken at 30 and 50 minutes after initiation of growth. Growth was arrested by the addition of ice to lower the temperature to 0° C. The cells were removed from suspension by centrifugation for 20 minutes at 4000 rpm in a Beckmann J-6B centrifuge and then resuspended in 25 ml of ice cold standard buffer.

The samples were assayed for the production of ADC activity using toluene permeablized cells. This involved treating the cells by the addition of 0.5 ml of ice cold toluene (20 μl/ml) and vortexing for 30 seconds followed by incubation on ice for 20 minutes. Samples (490 μl) were taken and controls were denatured in a boiling water bath before determination of ADC activity. Activity was measured by the evolution of $^{14}CO_2$ from DL-[1-^{14}C]-aspartate.

2.21 *E. coli* Transformation and Selection

Competent cells were prepared fresh the day they were required from a 1:200 dilution of a stationary phase, overnight culture into fresh LB broth (or YT broth in the case of JM101) and grown to early log phase (1×10^8 cells/ml) at 37° C in a shaking water bath. The cells were then removed from suspension by centrifugation for 8-10 minutes at 7,600 x g in a Sorvall refrigerated centrifuge. The cells were then resuspended in 1/2 volume of ice-cold 50 mM $CaCl_2$, 10 mM Tris.HCl pH 8.0 and were stored on ice for 15-20 minutes. At the end of the incubation the cells were once again concentrated by centrifugation and then resuspended in 1/15 of the original volume of fresh buffer. The cells were retained on ice for up to 8 hours until needed.

Transformations were carried out in culture tubes with the addition of DNA in buffer volumes of 1-10 μl with 200 μl of competent cells. The transformation mixture was incubated on ice for 30 minutes and then was placed in a 42° C water bath for 2 minutes. At the end of the incubation 2 ml of LB broth is added and the culture was incubated at 37° C for 30 or 60 minutes to allow establishment of drug resistance to tetracycline or ampicillin respectively.

When selecting for complementation of the *panD*$^-$ phenotype, the transformed cells were removed from suspension in LB broth by centrifugation for 8 minutes at 7,600 x g. The cells were then resuspended in 2 ml of 0.89% saline and concentrated a second time by centrifugation. The saline wash was repeated twice, and the cells were finally resuspended in 1 ml of saline before being spread onto minimal

plates. Typically, 100 μl of this resuspension was used for plating on minimal medium minus β-alanine.

Clones answering the selection for β-alanine independent growth were routinely purified on LB plates by isolation of single colonies. Plasmid DNA was obtained from the transformants by the methods to be described, and the ability of the plasmid to satisfy the β-alanine auxotrophy was tested by transformation into AB354 or AB355 followed by selection for drug resistance and complementation.

2.22 Subcloning of panD

Incubation mixtures for restriction digests were prepared as per the manufacturers conditions for optimal activity and incubated at the recommended temperature. Ligations reactions involving complementary ends were prepared in 50 mM Tris.HCl pH 7.8, 7 mM $MgCl_2$, 1 mM DTT in 100 μl and were heated to 55° C for 10 minutes and then cooled on ice before the addition of 400 units of T4 DNA ligase (New England Biolabs) and ATP to 1 mM. Ligations were incubated for 1 hour to overnight at 16° C. When the ligations involved blunt-ended DNA fragments the 55° C treatment was eliminated and the volume of the ligation mixture was reduced to 10-20 μl while the duration of the incubation at 16° C was extended to overnight. When necessary, the fragments were treated with T4 polynucleotide kinase in the ligation buffer at 37° C with 1-2 units of enzyme for 1 hour.

Electroelution of DNA fragments from gels was accomplished by either of two methods. In the case of agarose gels the band of interest was located via long wave UV transillumination and a trough was cut in

the gel immediately in front of the band. A piece of dialysis tubing cut to the width of the trough was used to line the bottom and far end of the trough, and the desired band was electroeluted onto the dialysis membrane. The current was momentarily reversed and once the band was visibly separated from the membrane the buffer was removed from the trough with a pipettor. In the case of polyacrylamide gels and occasionally with agarose gels the band was cut out and electroeluted into a dialysis bag by the method of McDonnell *et al.* (1977) as described by Maniatis *et al.* (1982). Following electroelution, the diasylate was routinely phenol extracted followed by a single chloroform extraction and concentration by ethanol precipitation.

2.23 Construction of the *panD* Subclones

pADC1. 4 μg of the phage λgt4b and 1 μg of pBR325 was restricted with EcoRI for 1 hour at 37° and protein removed by successive phenol and chloroform extractions followed by ethanol precipitation. 1.3 μg of restricted λgt7-4b DNA and 1 μg of restricted pBR325 were resuspended in ligation buffer containing 1 mM ATP to and 800 units of T4 DNA Ligase in a final volume of 100 μl. The resulting ligation mixture was incubated at 16° C for 16 hours. 5 μl of the ligation mixture was transformed into 200 μl of competent AB355 and plated on M9 minimal plates (25 μg/ml of tetracycline).

pADC101. 1.5 μg of pADC1 was restricted with 10 units of SalI and 20 units of EcoRI and purified by the serial of phenol extraction and chloroform extractions followed by ethanol precipitation. Half of the restricted pADC1 and 7.5 μg of the gel purified 3.7 Kb Ap^r EcoRI/SalI

fragment of pBR322 were ligated in a volume of 100 μl. 5 μl of the ligation mixture was transformed into 200 μl competent AB354 and 5 μl of the transformation mixture was plated on M9 minimal media (50 μg/ml ampicillin).

pADC102. 5 μg of pADC101 was restricted with 20 units of XhoI for 2 hours at 37° C. Protein was removed by the serial phenol and chloroform extractions followed by ethanol precipitation. 4 μg of the restricted pADC101 was resuspended in ligation medium including 1 mM ATP and 400 units of T4 DNA Ligase in a final volume of 100 μl for 2 hours at 16° C. 5 μl of the ligation mixture was used to transform 200 μl of competent AB354. The transformation mixture was then plated on M9 minimal media (50 μg/ml ampicillin).

pADC103. 10 μg of pADC102 was restricted with 16 units of SalI and 50 units of XhoI at 37° C for 2 hours. Protein was again removed by serial phenol and chloroform extractions followed by ethanol precipitation. The restricted pADC101 was resuspsended in ligation mixture with 1 mM ATP and 400 units of T4 DNA Ligase in a final volume of 100 μl. The ligation was incubated for 2 hours at 16°C. 5 μl of the ligation mixture was plated on M9 Minimal media (50 μg/ml ampicillin).

pADC 200. 2.5 μg of pADC103 and 2.5 μg of pBR322 were submitted to restriction with 20 units of PvuII for 1 hour at 37°C. Protein was removed by serial phenol and chloroform extractions followed by ethanol precipitation. The restricted plasmids were then resuspended in ligation mixture with 1 mM ATP and 400 units of T4 DNA Ligase to a final volume of 100 μl and incubated for 2 hours at 16°C. 5 μl of the ligation mixture was then transformed into 200 μl of competent AB354.

The transformation mixture was then plated on M9 minimal media (25 lg/ml ampicillin).

pADC201 4 μg of pADC200 was restricted with 4 units of NruI for 2 hours at 37°C. The restricted DNA was fractionated by electrophoresis through 0.8% agarose and a 1.1 Kb fragment was eluted from the gel and purified by the serial phenol and chloroform extractions followed by ethanol precipitation. The NruI fragment was resuspended in ligation buffer with 1 μg of pBR322 that had been restricted with NruI. The ligation mixture was incubated at 16°C for 16 hours and 10 μl of the ligation mixture was used to transform 200 μl of competent AB354. The transformation mixture was plated on M9 minimal media (25 μg/ml tetracycline).

2.24 Rapid Small-Scale Preparation of Plasmid DNA

A rapid small scale plasmid preparation was performed essentially according to the method of Holmes and Quigley (1981) as described by Maniatis *et al.* (1982). The RNase I treatment was eliminated and phenol and chloroform extractions added as described for cleared lysates.

Cleared lysates of plasmid DNA were routinely prepared from 20 ml overnight cultures grown on LB under drug pressure. Cells were removed from suspension by centrifugation for 10 minutes at 10,000 rpm. The resulting pellet is resuspended in 0.6 ml of 25% sucrose, 50 mM Tris.HCl pH 8.0, 40 mM EDTA, and 100 μl of a solution of 25 mg/ml lysozyme in sucrose buffer is added. The mixture is incubated on ice for 5 minutes, and then 125 μl of 0.5 M EDTA pH 8.0 is added followed by 460 μl of 0.2% Triton X-100, 62.5 mM EDTA, 50 mM Tris.HCl pH 8.0. The mixture is

incubated on ice for 10 minutes or until lysis is evident by the increased viscosity of the suspension. The suspension is cleared of cellular debris by centrifugation in an Eppendorf microfuge for 15 minutes at 4° C. The resulting pellet is removed and the supernatant is incubated in a constant temperature water bath set at 65° C for 15 minutes. The denatured protein is removed by centrifugation for 15 minutes in an Eppendorf centrifuge. The supernatant is then brought to 250 mM NaCl and 10% polyethylene glycol (PEG 6,000) with solutions of 5 M NaCl and 40% PEG, respectively. The PEG coprecipitation of plasmid DNA is allowed to proceed for 2 hours to overnight at 4° C.

The precipitate is removed from suspension by centrifugation for 15 minutes in an Eppendorf microfuge and the supernatant drained off the pellet. The pellet is resuspended in 300 μl of TE buffer (10 mM Tris.HCl pH 8.0, 1 mM EDTA). The solution is extracted once with an equal volume of liquified phenol (brought to a pH > 7.0 with 200 mM Tris.HCl pH 8.0) and the phases separated by centrifugation. The aqueous phase is then extracted once with chloroform and the phases separated by centrifugation. The aqueous phase is brought to 300 mM NaCl with 5 M NaCl followed by addition of 2 volumes of anhydrous ethanol. The DNA was precipitated from solution by placing the mixture at -70° C for 1 hour or at -20° C overnight. The precipitate was obtained by centrifugation for 30 minutes in an Eppendorf microfuge after which the pellet was dried under vacuum and resuspended in 100 μl TE.

2.25 Preparation of Plasmid DNA by Centrifugation to Equilibration in Cesium Chloride Gradients

Large quantities of plasmid DNA were prepared using a cleared lysate method. An overnight culture of the plasmid-bearing strain grown on LB under the appropriate drug pressure was used to inoculate a 1 liter culture which was allowed to grow overnight at 37° C on a reciprocating shaker. The cells were removed from suspension by centrifugfation at 4,000 rpm in a Beckmann J-6B centrifuge for 15 minutes. The pellet was then reuspended in 5 ml of ice cold 25% sucrose, 50 mM Tris.HCl pH 8.0 and allowed to remain 10 minutes on ice. To this suspension was added 1 ml of a cold solution of lysozyme (10 mg/ml) and the mixture was allowed to incubate for 10 minutes on ice. At the end of this incubation 2 ml of cold 250 mM EDTA pH 8.0 and 8 ml of 10% Triton X-100 in 50 mM Tris.HCl pH 8.0 was added. The tube containing the lysate was gently inverted and immediately subjected to centrifugation for 30 minutes at 43,000 x g at 4° C.

The supernatant was removed from the pellet and its volume measured. The supernatant was then brought to 10% CsCl (w/v) with solid CsCl. After obtaining solubilization of the CsCl, a 10 mg/ml solution of ethidium bromide was added to 8% (v/v). The mixture was subjected to centrifugation at 100,000 x g for 40 hours at 20° in a Beckmann L2-65B ultracentrifuge equipped with a type 65 rotor.

Following centrifugation, the DNA bands were visualized by long wave UV irradiation and the lower band (containing covalently-closed circular forms) was removed in a 1 ml volume by syringe via side

puncture. This fraction was extracted five times with isopropanol equilibrated with a saturated solution of CsCl. The plasmid preparation was then dialyzed against three changes of 100 ml of TE buffer (4 hours each). The resulting dialyzate was ethanol precipitated as described above, and the DNA resuspended in TE buffer. This solution was then phenol extracted followed by a chloroform extraction and a final ethanol precipitation. The DNA concentration was determined by measurement of the absorbance of an aliquot at a wavelength of 260 nm (OD_{260} = 1.00 for [DNA] = 50 μg/ml) (Maniatis *et al.*, 1982).

2.26 Restriction Endonuclease Mapping

Restriction digests for the purpose of restriction mapping were carried out using the three buffer-system described by Davis *et al.* (1980). The resulting fragments were separated by means of either horizontal agarose gel flat bed or vertical polyacrylamide slab gel electrophoresis and the bands obtained by staining with ethidium bromide were sized by comparison to standard λ DNA-HindIII or ϕX174 RF DNA-HaeIII digests. Agarose electrophoresis was routinely performed with 0.8% agarose containing TBE buffer (89 mM Tris-borate pH 8.0, 89 mM boric acid, 2 mM EDTA) with either 19.5 cm x 22.5 cm gels or 6.6 cm x 6.6 cm minigels as described by Maniatis *et al.* (1982). When separation of restriction fragments smaller than 1,000 bp was required, 5% polyacrylamide gels were employed using the TBE buffer system. Sample buffer contained 15% Ficoll type 400, 0.25% bromophenol blue, 0.25% xylene cyanole. Gels were stained with a 0.5 μg/ml solution of ethidium

bromide and photographed using a 256 nm UV transilluminator as a light source and Polaroid Type 57 film.

2.27 Preparation of *E. coli and S. typhimurium* Chromosomal DNA

E. coli chromosomal DNA was prepared from a 50 ml culture of W3110 grown to stationary phase in LB broth at 37° C on a reciprocating shaker. The DNA was prepared by the Marmur (1961) procedure. Following isolation, the DNA was resuspended in 2 ml of TE buffer at a concentration of 0.6 mg/ml.

2.28 Preparation of Radiolabeled Probes by Nick Translation

^{32}P-labeled probes were prepared from plasmid DNA by incorporation of deoxyadenosine 5'-[α-^{32}P]triphosphate (Amersham 410 Ci/mmol) with the Nick Translation Reagent kit obtained from BRL. Probe was prepared with 1 μg of a gel-purified restriction fragment as per the manufacturers instructions and the probe was separated from unincorporated label by two sequential precipitations through the addition of 0.5 volumes of 7.5 M ammonium acetate and 2 volumes of absolute ethanol.

2.29 Analysis of Restiction Digests by Southern Transfer

DNA fragments obtained by restriction digest and separated by agarose gel electrophoresis were tansfered to nitrocellulose (Schleicher and Schuell PH79, 0.10 μm) by the method of Southern (1975) as described by Maniatis *et al.* (1982).

2.30 Construction of the *panD* Deletion

A deletion of *panD* encompassing the -26 to +213 region was obtained by removal of the 239 bp HpaI fragment from pADC200 and insertion of an 8 bp SalI linker (New England Biolabs #1012) according to methods described by Maniatis *et al.* (1982). The successful insertion and ligation of the linker was determined by selection for ampicillin resistance and screening for the inability to complement the *panD*$^-$ phenotype. The resulting plasmid, pADC2001, contained a single SalI site and remained uncut when subjected to restriction with HpaI.

2.31 DNA Sequencing of the *panD* Gene

Sequencing of the *panD* gene was accomplished using the NruI fragment from pADC201 as a source of DNA for the isolation of subclones in the M13mp18 vector. The NruI fragment was restricted with PstI, FnuDII, RsaI, EcoRV and HpaII. The NruI-PstI fragments were ligated into M13mp8 and mp9 that had been restricted with PstI and HincII. The fragments generated from restriction with FnuDII, RsaI and EcoRV were ligated into M13mp18 that had been restricted with either SmaI or HincII. Fragments generated by HpaII were ligated into AccI restricted M13mp18. Subcloning into the polycloning sites of M13 mp18w was performed as described by Messing *et al.* (1981).

DNA sequencing of the M13 subclones was performed by the dideoxynucleotide chain termination method of Sanger *et al.* (1977) using 5'-(α[^{35}S]thio)dATP and the 17 base universal M13 primer. Sequencing

reactions were fractionated on 8 M urea denaturing gels containing either 6% or 8% polyacrylamide [acrylamide:bisacrylamide (19:1% w/v)] and run at 40 mamps/gel constant current.

2.32 RNA preparation from *E. coli*

RNA was prepared from the ADC overproducer AB355/pADC1. All solutions are prepared in acid washed glassware and sterilized by autoclaving. RNA was prepared from a culture of AB355/pADC1 grown on 125 ml LB broth with ampicillin at 25 μg/ml to an OD_{420} of 0.380 (1:10 dilution) corresponding to one quarter of the maximum stationary phase. Chloramphenicol was added to 100 μg/ml and sodium azide was added to obtain a final concentration of 1 mg/ml. The culture was then poured over 100 gm of sterile ice and the ice was allowed to melt at room temperature.

The cells are removed from suspension by centrifugation at 5900 x g for 10 minutes and resuspended in 10 ml of 10 mM Tris.HCl pH 7.3, 10 mM KCl, 5 mM $MgCl_2$ to which 4.5 mg of lysozyme was added. The suspension was then frozen briefly at -70° C and thawed by brief immersion in a 65° C water bath. 0.7 ml of 10% SDS was added and the mixture is incubated 2 minutes at 65° C with gentle agitation. 1.1 ml of 1 M sodium acetate pH 5.2 was added followed by 12 ml of redistilled liquified phenol and the mixture was placed in a 65° C water bath with maximum agitation for 4 minutes. The phenol layer was seperated from the aqueous by centrifugation at 12,000 x g for 3-4 minutes. The hot phenol extraction was repeated and the resulting aqueous layer was brought to 0.3 M sodium acetate with 3 M sodium acetate pH 8.0 and 1 mM

EDTA. 2.5 volumes of absolute ethanol was added and the RNA was precipitated at -70° C for 18 hours.

The RNA was removed from suspension by centrifugation at 16,500 x g for 20 minutes and the pellet dried briefly under vacuum. The pellet was immediately resuspended in sterile deionized water and divided into 150 μl aliquots which were stored as ethanol precipitates at -70° C until immediately before use. The OD_{260}/OD_{280} of the RNA preparation from AB355/pADC1 was 2.02 and two distinct rRNA bands were obtained on electrophorsis of 0.29 μg of RNA in a 0.8% agarose gel.

2.33 Preparation of Radiolabeled Probes by Primer Extension

^{32}P-labeled probes were obtained by primer extension of the M13mp18 subclones by preparation of the sequencing reaction for adenosine termination, but ommitting the dideoxyadenosine triphosphate. The reaction contained 2.5 ng of the 17 base -20 universal M13 primer (IBI), 14 mM Tris.HCl pH 7.5, 14 mM $MgCl_2$, 1 μg of M13mp18 template and deionized water to 10 μl. The primer was allowed to anneal for 30 minutes at 37° C after which the polymerization reaction was initiated by the addition of 10 μl of a mixture containing 20 μCi (α-^{32}P)deoxyadenosine triphosphate (specific activity >400 Ci/mmole), 10 mM DTT, 7 mM Tris.HCl pH 7.5, 7 mM $MgCl_2$, 2.5 units of the Klenow fragment of DNA polymerase I and 50 μM concentrations of each of the deoxynucleotide triphosphates of guanine, cytosine and thymine. The incubation was continued at 37° C for 15 minutes at which time an additional 2.5 units of the Klenow fragment was added. The incubation was continued an additional 15 minutes and then 2.5 μl of 0.5 mM dATP

was added to ensure completion of the polymerization reaction. Upon completion of the incubations, 10 mM Tris.HCl pH 8.0 and 1 mM EDTA was added to a final volume of 100 μl and the mixture was extracted with an equal volume of equilibrated phenol. The aqueous phase of the phenol extraction was extracted twice with equal volumes of chloroform and ethanol precipitated for 1 hour at -70° C.

The resulting precipitate was removed from suspension by centrifugation for 15 minutes in an Eppendorf microfuge. The pellet was resuspended, after being dried under vacuum, in 8 μl of deionized water and was brought to 100 mM NaCl, 10 mM Tris.HCl pH 7.5, and 10 mM $MgCl_2$. 10 units of EcoRI are added and the restriction digest was incubated 1 hour at 37° C. At the end of the incubation period, 20 μl of formamide dye mixture (95% deionized formamide, 0.1% (w/v) xylene cyanol FF, 0.1% (w/v) bromophenol blue and 10 mM disodium EDTA) was added and the mixture placed in a boiling water bath for 3 minutes. The denatured single-stranded probe was then purified by electrophoresis in a 6% polyacrylamide sequencing gel (33 x 40 x .04 cm) which was run until the bromophenol blue dye had eluted off the bottom of the gel. The gel was then exposed to Kodak X-Omat AR film for 10 minutes to determine the location of the probe.

The gel section corresponding to a band on the exposed film was cut out and the probe was eluted in 600 μl of 0.5 M sodium acetate pH 8.0 for 18 hours with agitation. The gel was removed by centrifugation and the supernatant was extracted with phenol followed by two chloroform extractions. The extracted aqueous phase was ethanol precipitated at -80° C for 1 hour in the presence of 10 μg of carrier yeast tRNA. The precipitated DNA was removed from suspension by centrifugation for 15

minutes in an Eppendorf microfuge. The pellet was dried under vacuum and resuspended in 100 μl of deionized water. The quantity of label incorporated was determined by Cherenkov counting on a Packard Tri-Carb 460 liquid scintillation counter.

2.34 S1 Nuclease Mapping of the 5′ and 3′ Termini of the *panD* Transcript

S1 nuclease mapping of the 5′ and 3′ termini of the mRNA encoding the ADC activity was performed by a variation of the method of Berk and Sharp (1977) where the probes were obtained by primer extension of the M13mp18 subclones as described previously.

mRNA for S1 nuclease mapping that had been stored as an ethanol precipitate was removed from suspension by centrifugation in an Eppendorf microfuge for 15 minutes. The pellet was dried under vacuum and resuspended in sterile deionized water to give a final concentration of 2.9 mg/ml. 58 μg of mRNA per S1 digest was ethanol precipitated in the presence of 50,000 cpm of probe labeled with 5′-[α-^{32}P]dATP. After centrifugation and drying under vacuum the pellet is resuspended in 25 μl of 80% formamide containing 40 mM PIPES pH 6.4, 400 mM NaCl and 1 mM EDTA. The hybridization mixture was heated to 70° C for 10 minutes and then transferred rapidly to a 42° C water bath and allowed to hybridize 18 hours. At the end of the hybridization period, 250 μl of ice cold S1 reagent (1 mM $ZnSO_4$, 30 mM sodium acetate pH 4.6, 250 mM NaCl, 5% glycerol and 2,000 units/ml S1 endonuclease) was added and the tube was transferred to a 37° C water bath. Digeston with S1 nuclease was carried out for 30 minutes. At the end of the incubation 14 μg of yeast

tRNA was added to each digest and nucleic acid was ethanol precipitated 1 hour at -70° C.

Following centrifugation and drying under vacuum, the pellet is resuspended in 5 μl 0.1 M NaOH, 5 mM EDTA and 5 μl of formamide dye mixture. Reactions were denatured for 2 minutes at 90° C then quick chilled on ice. Samples of each reaction (4 μl) were run on 6% and 8% polyacrylamide denaturing sequencing gels. The length of the fragments obtained by S1 endonuclease mapping was determined by comparison to a sequencing ladder run alongside. The results were evaluated by autoradiography by exposure of the dried gel to Kodak X-Omat AR film aided by a Dupont Cronex Lightning-Plus intensifying screen.

2.35 Computer-Aided Analysis of DNA Sequence Data

Analysis of the DNA sequence obtained for the *panD* region was aided by the use of the sequence analysis programs available at the Whitaker College Computer Facility and published by the University of Wisconson Genetic Computer Group as described by Devereux et. al. (1984). The 6 possible open reading frames on both strands were determined using Codonpreference program of Gribskov *et al.* (1984) which also provides a codon preference plot based on the GenDataBase E_coli.CDF codon frequency table. Coding probabilities were determined based on the frequency of codon usage for 61,028 codons from 235 coding regions in 118 entries from the National Biomedical Research Foundation NUCLEIC database, release 26 that were designated as "*coli*" and exclusive of those designated as being plasmid related.

Determination of the sequence with optimal complementarity to the 3′ region of 16S rRNA, as well as the best segments of similarity to the consensus sequences for the putative -35 and -10 promoter regions was determined with the BESTFIT program utilizing the alogarithm of Smith and Waterman (1981). The presence of possible stem-loop secondary structures and determination of their order of stability was achieved by use of the STEMLOOP program. Determination of the translated sequence of the open reading frame and the sites of type II restriction endonucleases was acheived with the MAP program. Comparison of the translated sequence of ADC with the known peptide sequence of the proenzyme of L-histidine decarboxylase was achieved through the use of the COMPARE program based on the method of Maizel and Lenk (1981) with graphic matrix analysis of the data utilizing the DOTPLOT program.

RESULTS

3.1 Molecular Cloning of the *panD* Gene

The low level of L-aspartate-α-decarboxylase activity present in the wild type strains of *Escherichia coli* K12 and B indicated that obtaining sufficient quantities of the enzyme for study would require amplification of the level of expression of the *PanD* gene product. The existance of several *PanD* auxotrophs with depressed levels of ADC activity indicated that molecular cloning of the *PanD* gene was feasible. An initial attempt to screen a K12 library with the *panD* auxotroph M99-2 was unsuccessful, possibly due to residual levels of ADC activity sufficient to allow slow growth on M9 minimal media. As a result, we obtained the three *panD* auxotrophs (AB353, AB354, and AB355) described by Cronan (1980) which proved to be sufficiently "tight" to preclude growth on minimal media. In addition, careful removal of growth media via extensive washing, in conjunction with subsequent starvation on β-alanine deficient minimal media, provided conditions whereby dependency of the bacteria on supplemental pantothenate could be increased to sufficiently stringent levels.

Under such conditions, a λgt7 library containing EcoRI DNA from *E. coli* W3110 was screened utilizing a lysogenic selection for recombinant phage by infection of the *panD* auxotroph AB354 in the presence of helper phage. Two orientations of a single 12.5 kb EcoRI fragment were obtained entitled 4b and 5c by the means described in Experimental Methods.

Table 3-1. L-Aspartate-α-Decarboxylase Activity Following Induction of Lytic Growth of the Recombinant Phage.*

Phage	Specific activity at 30 min. after infection	Specific activity at 50 min. after infection
λgt7-1†	.010 U/mg	.037 U/mg
λgt7-4b	.023 U/mg	.088 U/mg
λgt7-5c	.013 U/mg	.070 U/mg

* Lytic assay performed as described in Experimental Methods by infection of AB354 with the indicated recombinant phage followed by growth with aeration at 37°C. Growth was arrested by rapidly cooling cells to 0°C and ADC activity was measured as the release of $^{14}CO_2$ from DL-[1-^{14}C]asparatate with toluene permeabilized cells.
† Recombinant phage containing the *dadB* gene from *S. typhimurium* (Wasserman *et al.*, 1983).

The two orientations in the λ clones provided a system for measurement of the expression of ADC activity after induction of lytic growth. When transcription of the *trp* operon occurs in the absence of endogenous promoter under control of the P_L promoter in a *cI At2 cro1 Qam73 Sam7* background, the result is an accumulation of *trp* proteins to levels where they represent 16% of the total soluble protein (Moir and Brammar *et al.*, 1976). Similiarly high levels of activity have been seen with anthranilate synthetase in λ*trpEDC Nam7am53* under control of its endogenous promoter, where over-expression has been attributed to gene dosage effects as well as relaxation of repression through titration of the *trp* repressor (Hopkins *et al.*, 1976). In order to determine if similiar results could be obtained for ADC, a lytic assay was performed by infection of AB354 with the chimeric phage in the absence of helper phage and determination of levels of ADC activity

expressed during early and late phases of the lytic cycle. The results of the assay are presented in Table 3-1. Neither orientation was able to provide levels of ADC activity considerably greater than that seen with the control phage containing the *dadB* gene.

3.2 Construction of the *panD* Subclone pADC1

The results of the lytic infection with the λ clones indicated that overexpression of the ADC activity would require further subcloning of *panD*. To this end, the EcoRI insert of λgt7-4b was ligated into the single EcoRI site of pBR325. The resulting ligation mixture was transformed into competent AB355 cells and the presence of the 12.5 kb EcoRI fragment was selected for tetracycline resistance (Tc^r) and complementation of the *PanD* phenotype as described in Experimental Methods. The plasmid, designated pADC1, contained the 12.5 kb EcoRI fragment inserted into the chloramphenicol acetyl-transferase gene and the transformant was sensitive to growth on M9 minimal plates containing 10 μg/ml chloramphenicol. A restriction map of the plasmid is given in Figure 3-1.

3.3 Further Subcloning of the *panD* Gene

Localization of the *panD* gene within the EcoRI fragment was possible via a series of subclones as indicated in Figure 3-2. The orientations of the cloned fragments in the subclones pADC101 and pADC201 are shown in Figure 3-3. The orientation of the cloned segment in subclones pADC101 through pADC200 remains constant with respect to

Figure 3-1

Restriction Map of the *panD* Subclone pADC1.

pADC1 was constructed as described in Experimental Methods. The presence of open reading frames are represented by arrows indicating the direction of transcription. The double-headed arrow indicates the sequences derived from pBR325. Restriction sites of the 12.5 kb EcoRI insert were determined by restriction digests and analysis of the resulting fragments by agarose gel electrophoresis. The NruI sites of the EcoRI fragment of pADC1, indicated by bold type, were not determined for this plasmid and are provided only as a reference. They define the fragment used in the construction of pADC201 and sequenced in Section 3.4.

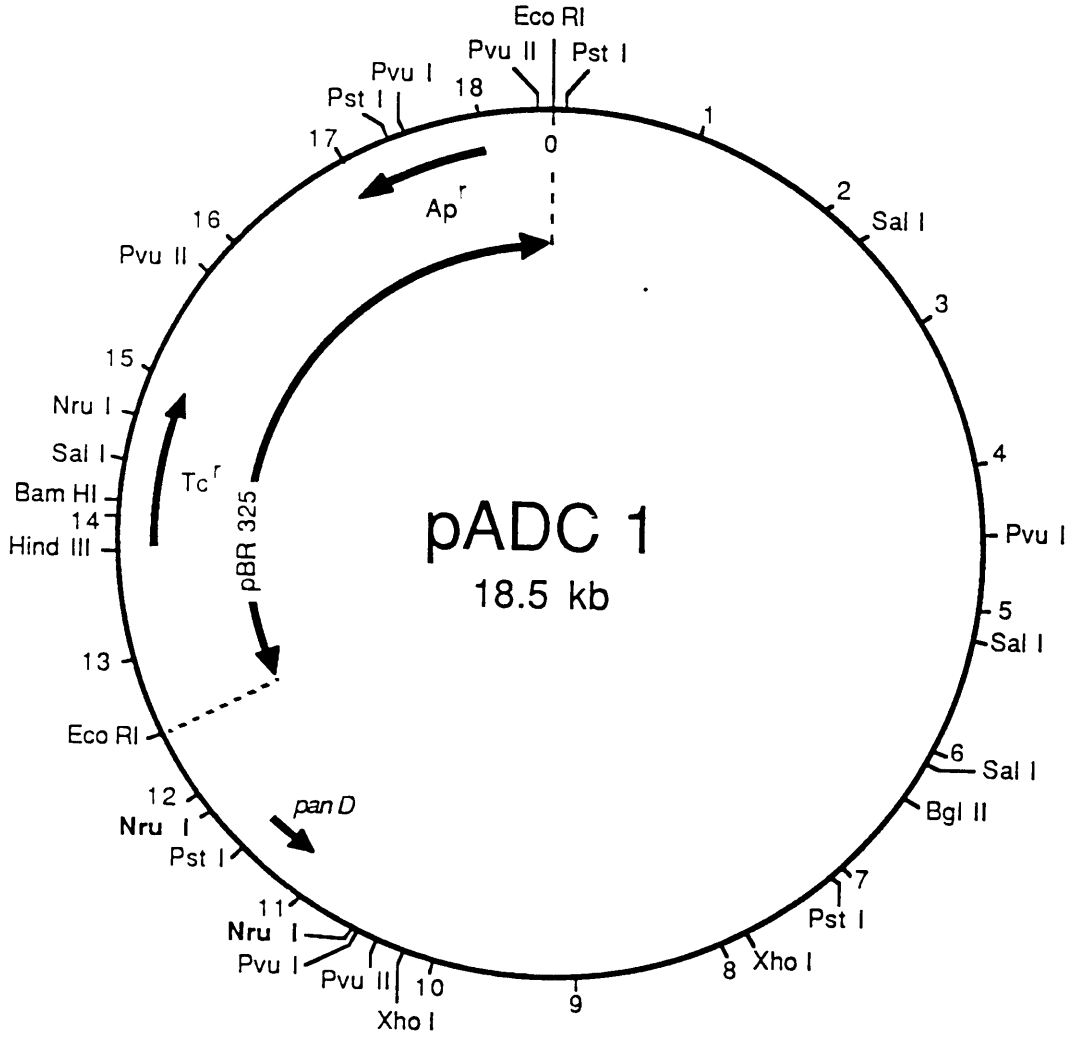
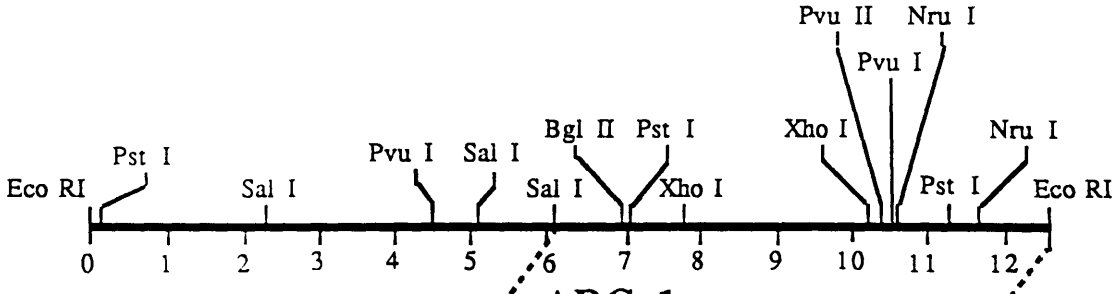

Eco RI
Pvu II
Pst I
Pvu I
Pst I
18
17
0
1
2
Sal I
3
4
Pvu I
5
Sal I
6
Sal I
Bgl II
7
Pst I
8
Xho I
9
10
Xho I
Pvu II
Pvu I
11
Nru I
12
Nru I
Pst I
13
Eco RI
14
Hind III
Bam HI
Sal I
15
Nru I
16
Pvu II
Ap r
Tc r
pBR 325
pan D
pADC 1
18.5 kb

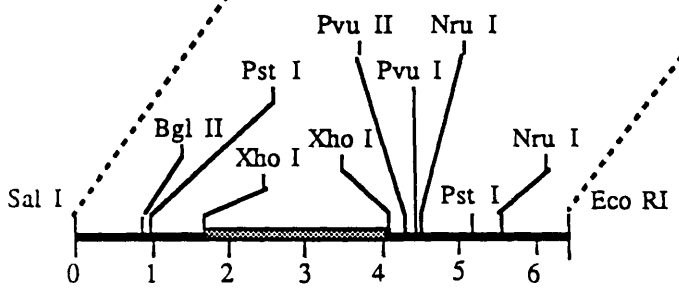
Figure 3-2

Intermediates Constructed in the Subcloning of *panD*

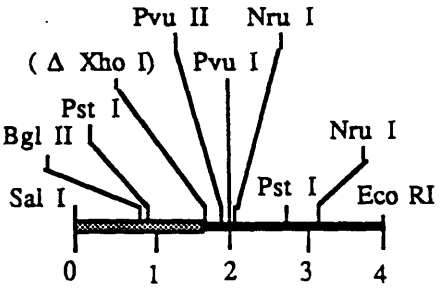
The *panD* region of the subclones involved in the isolation of the gene are shown in the order derived, from pADC1 to the final subclone pADC201. The dotted lines indicate the relationship of deletions to the parent fragment. Regions that are deleted in pADC101 to obtain pADC102 and in pADC102 to obtain pADC103 are indicated by the shaded regions. The details of the constructions are given in Experimental Methods. All subclones (except pADC 1) were derived from pBR322. Restriction maps are drawn to a scale of 1 kb to the centimeter.



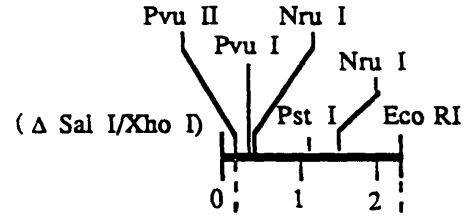
pADC 1



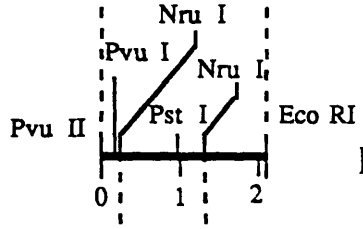
pADC 101



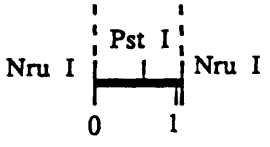
pADC 102



pADC 103



pADC 200



pADC 201

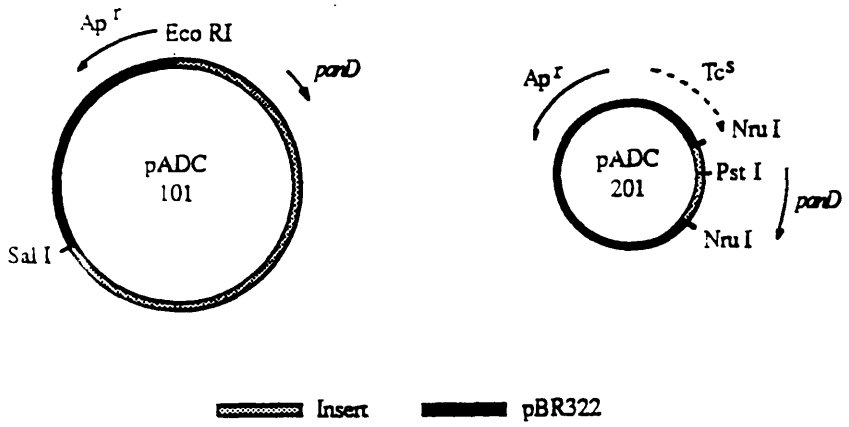


Figure 3-3 The orientation of the *panD* subclones with respect to vector sequences as determined by restriction endonuclease mapping. The arrows indicates the direction of transcription of the indicated ORF. The *panD* ORF is determined in this work.

pBR322 sequences. The 6.5 kb SalI-EcoRI fragment containing *panD* was obtained from pADCI and ligated to the SalI-EcoRI fragment of pBR322 containing the ampicillin resistance gene. The resulting plasmid was selected for as above and was designated pADC101.

The *panD* gene was subcloned further by isolation of deletion derivatives of pADC101. The plasmid was digested to completion with XhoI. After purification by phenol-chloroform extractions and ethanol precipitation, the resulting precipitate was resuspended at low plasmid concentration and ligated as described in Experimental Methods. The ligation mixture was transformed into competent AB355 and selected for the Ap^r and *panD* phenotypes to yield pADC102 from which the single XhoI fragment of pADC101 had been deleted. An additional deletion was obtained by digestion of this plasmid with SalI and XhoI followed by ligation of the purified plasmid at low concentrations as above. After transformation and selection for Ap^r and the *panD* phenotypes, the resulting plasmid, pADC103, lacks the 2.1 kb SalI-XhoI fragment adjacent

to the remainder of the tetracycline resistance gene of pADC102 and retains the 2.3 kb XhoI-EcoRI fragment. *panD* was further subcloned via ligation of the 2.2 kb PvuII-EcoRI fragment from pADC103 to the PvuII-EcoRI Ap^r fragment of pBR322 to yield pADC200 following transformation and selection as above.

This plasmid was used as the source of the 1.1 kb NruI fragment which was ligated into a unique NruI site located within the tetracycline resistance region of pBR322. The resulting plasmid, pADC201, was obtained by selection for Ap^r and *panD* phenotypes and localized *panD* to a 1.1 kb NruI fragment. The very low occurence of succesful ligation of NruI fragments resulted in only a single orientation being obtained.

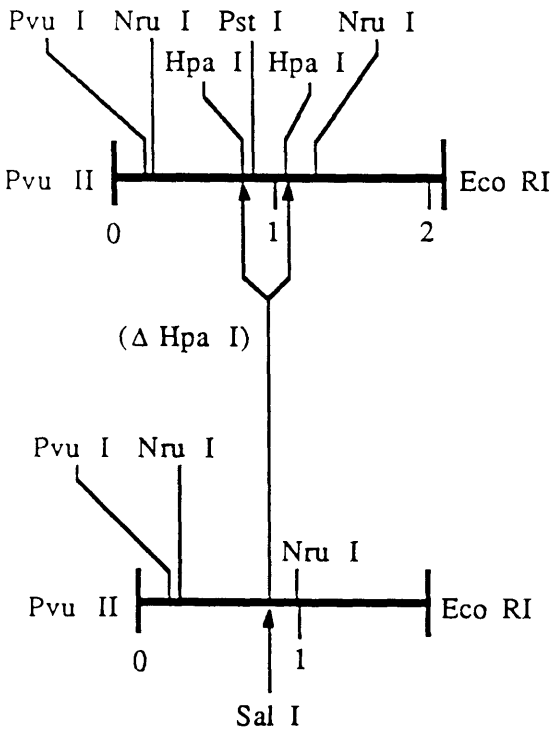
Attempts at further subcloning of the NruI fragment utilizing the unique PstI site results in fragments that are unable to complement the *PanD* auxotrophs AB353 or AB354. The inability to further subclone *panD* via the PstI site suggested that deletion of the 240 bp HpaI fragment containing the PstI site from pADC201 would verify the location of *panD* by the inability of the resulting plasmid to relieve the β-alanine auxotrophy of AB354. A deletion of this small region was obtained by digestion with HpaI followed by insertion of a SalI linker as shown in Figure 3-4. The plasmid was selected for Ap^r and screened for the presence of the inserted SalI sequence and loss of the HpaI sites. The resulting construction, pADC-2001, was unable to satisfy the β-alanine requirement of AB354. Crude lysates of strains carrying each plasmid in the series of *panD* subclones were assayed for ADC activity by production of [1-^{14}C]β-alanine from DL-[4-^{14}C]aspartate and the results are

Figure 3-4

Construction of the *panD* Deletion pADC2001

Details of the construction of the plasmid are given in Experimental Methods. The HpaI fragment was removed by digestion with HpaI and a SalI restriction site was created by ligation of the 8 bp SalI linkers to the HpaI ends. The ligated fragments were digested with SalI to generate cohesive ends and ligated to obtain a covalently-closed circle. The restriction maps are drawn to a scale of 1 Kb to the centimeter.

pADC 200



pADC 2001

Table 3-2. Levels of ADC Activity in *panD* the Subclones.

Plasmid[§]	Specific Activity[†]
pADC1	3.0 units/mg
pADC101	2.1
pADC102	2.4
pADC200	5.9
pADC201	3.0
pADC2001	ND*
pBR322	ND*
None	0.06∫

§ Activity measured in an AB354 background.
† ADC activity was measured by formation of β-[1-^{14}C]alanine from DL-[4-^{14}C]aspartate.
* None detected.
∫ Activity measured from E. coli K12 by the formation of $^{14}CO_2$ released from DL-[1-^{14}C]aspartate

presented in Table 3-2. As expected, the deletion possesses only background levels of ADC activity while all previous subclones gave high levels of activity that are 35-100 fold greater than obtained with wild type.

3.4 DNA Sequencing of the *panD* Region

The ability to subclone the *panD* region to a 1.1 kb fragment indicated that information regarding the protein sequence could be rapidly obtained through translation of the *panD* DNA sequence. V8 protease analysis (see section 3.15) of the individual subunits

indicated that such information would be important to the understanding of subunit composition and post-translational processing of the proenzyme when verified by amino-terminus sequence analysis.

The strategy used to sequence both strands of the 1.1 kb NruI *panD* fragment of pADC-201 is depicted in Figure 3-5. Sequencing was performed by the dideoxynucleotide method of Sanger *et al.* (1977) using the M13 vector system of Messing *et al.* (1981). The resulting complete double-stranded sequence of the NruI fragment of pADC201 is given in Figure 3-6 and a partial restriction endonuclease map is provided in Figure 3-7. Both strands were sequenced for the entire length of the fragment with the indicated exceptions. In all cases where both strands of a given region could not be sequenced, the sequence on the single strand was determined in an unambiguous manner.

3.5 Analysis of the Open Reading Frames of the *panD* Region

The DNA sequence was examined for the presence of open reading frames (ORF) and the frequency of rare codon usage with the Codonpreference program of Gribskov *et al.* (1984) utilizing the GenDataBase E_coli.CDF codon preference table. The results of that analysis are provided in Figures 3-8 and 3-9 with the presence of rare codons being indicated by hashmarks below the graph. The frequency of rare codon usage for the major open reading frames (larger than 50 bp in length) is given in Table 3-3.

Figure 3-5

Sequencing Strategy for the NruI Fragment of pADC201

The restriction map was derived by analysis of the DNA sequence for restriction sites. The arrows beneath the restriction map indicates the direction and the extent of the DNA sequence determined from each M13mp18 derived clone.The box labeled *panD* indicates the *panD* ORF determined is Section 3-5. The restriction map is drawn to a scale of 100 bp to the centimeter.

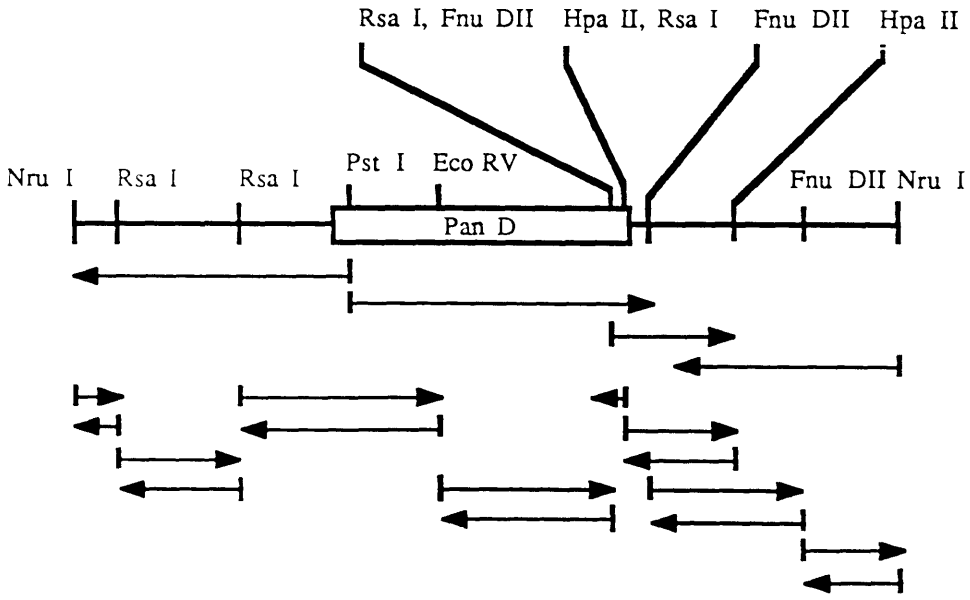

Rsa I, Fnu DII
Hpa II, Rsa I
Fnu DII
Hpa II
Nru I
Rsa I
Rsa I
Pst I
Eco RV
Fnu DII
Nru I
Pan D

Figure 3-6

Nucleotide Sequence of the NruI Fragment of pADC201

The nucleotide sequence of the *panD* NruI fragment of pADC201 was determined by the dideoxynuleotide method as described in Experimental Methods. The nucleotide sequence of 5'→3' strand is given in the upper register and the 3'→5' strand is given in the lower register. The position of the final nucleotide in a row is given at the end of each row and refers to its position with respect to the 5' end of the upper register. Nucleotides which were not determined on a given strand are indicated by the placement of an asterick (*) next to the unconfirmed nucleotide.

```
5'
CGAGCCGTCTCCGCATGCATTAAAAATTGTTTAAATACCGCGTCATGCGT
1--------+---------+---------+---------+---------+    50
GCTCGGCAGAGGCGTACGTAATTTTTAACAAATTTATGGCGCAGTACGCA
3'

GTGGTACTCGGTGCATCCATCTTCCCTGTCTTCCGTGTGGCTTTGCGTGA
---------+---------+---------+---------+---------+   100
CACCATGAGCCACGTAGGTAGAAGGGACAGAAGGCACACCGAAACGCACT

AGGCAGTATCCAGTCAAGGCGCGCTATCGTCAGCCACCTCTTTATTGTTT
---------+---------+---------+---------+---------+   150
TCCGTCATAGGTCAGTTCCGCGCGATAGCAGTCGGTGGAGAAATAACAAA

ACCGAGCAGCGTTCAGAGAAATATCAATTCGTTACAGGCGATACATGGCA
---------+---------+---------+---------+---------+   200
TGGCTCGTCGCAAGTCTCTTTATAGTTAAGCAATGTCCGCTATGTACCGT

CGCTTCGGCGCGGTACGCAAACTGCTGACGCAAATGCCGTCAGATGAGTA
---------+---------+---------+---------+---------+   250
GCGAAGCCGCGCCATGCGTTTGACGACTGCGTTTACGGCAGTCTACTCAT

GACACTAAACAAAAATCGGGCAATACTGCGTGAGAATTTCCTCAAAGCAG
---------+---------+---------+---------+---------+   300
CTGTGATTTGTTTTTAGCCCGTTATGACGCACTCTTAAAGGAGTTTCGTC

GCATCGCCTCGTTCGTTAACGACAGGGTAGAAAGGTAGAAGTTATGATTC
---------+---------+---------+---------+---------+   350
CGTAGCGGAGCAAGCAATTGCTGTCCCATCTTTCCATCTTCAATACTAAG

GCACGATGCTGCAGGGCAAACTCCACCGCGTGAAAGTGACTCATGCGGAC
---------+---------+---------+---------+---------+   400
CGTGCTACGACGTCCCGTTTGAGGTGGCGCACTTTCACTGAGTACGCCTG

CTGCACTATGAAGGTTCTTGCGCCATTGACCAGGATTTTCTTGACGCAGC
---------+---------+---------+---------+---------+   450
GACGTGATACTTCCAAGAACGCGGTAACTGGTCCTAAAAGAACTGCGTCG

CGGTATTCTCGAAAACGAAGCCATTGATATCTGGAATGTCACCAACGGCA
---------+---------+---------+---------+---------+   500
GCCATAAGAGCTTTTGCTTCGGTAACTATAGACCTTACAGTGGTTGCCGT
```

```
AGCGTTTCTCCACTTATGCCATCGCGGCAGAACGCGGTTCGAGAATTATT
---------+---------+---------+---------+---------+  550
TCGCAAAGAGGTGAATACGGTAGCGCCGTCTTGCGCCAAGCTCTTAATAA

TCTGTTAACGGTGCGGCGGCCCACTGCGCCAGTGTCGGCGATATTGTCAT
---------+---------+---------+---------+---------+  600
AGACAATTGCCACGCCGCCGGGTGACGCGGTCACAGCCGCTATAACAGTA

CATCGCCAGCTTCGTTACCATGCCAGATGAAGAAGCTCGCACCTGGCGAC
---------+---------+---------+---------+---------+  650
GTAGCGGTCGAAGCAATGGTACGGTCTACTTCTTCGAGCGTGGACCGCTG

                                         *********
CCAACGTCGCCTATTTTGAAGGCGACAATGAAATGAAACGTACCGCGAAA
---------+---------+---------+---------+---------+  700
GGTTGCAGCGGATAAAACTTCCGCTGTTACTTTACTTTGCATGGCGCTTT

*******
GCGATTCCGGTACAGGTTGCTTGATTGTTATCCCTGCGGCTGGTTACTCA
---------+---------+---------+---------+---------+  750
CGCTAAGGCCATGTCCAACGAACTAACAATAGGGACGCCGACCAATGAGT
                                      **

     ***
CCAGCCGCGACATCGTCTCCAGCGAATCCGTTCTTAAGATATAAAGTCGT
---------+---------+---------+---------+---------+  800
GGTCGGCGCTGTAGCAGAGGTCGCTTAGGCAAGAATTCTATATTTCAGCA

TTTAGTAACAACGGATTATCCCCCGGTTTTACTTTGCCTTTCATGGTTGT
---------+---------+---------+---------+---------+  850
AAATCATTGTTGCCTAATAGGGGGCCAAAATGAAACGGAAAGTACCAACA

CACCGCCAGGTGAAATCCGGCATCGTTTGCTGCCTTCACGGCGTTGTCAT
---------+---------+---------+---------+---------+  900
GTGGCGGTCCACTTTAGGCCGTAGCAAACGACGGAAGTGCCGCAACAGTA

TAAATCCGCCAAACGGATACGAAAGATACCAGACATGCGGATTAAATTGC
---------+---------+---------+---------+---------+  950
ATTTAGGCGGTTTGCCTATGCTTTCTATGGTCTGTACGCCTAATTTAACG

GCCAGAGCGCGGCGTGAACGTGCAAAATCAAACAGAATATTGTGCTCACT
---------+---------+---------+---------+---------+ 1000
CGGTCTCGCGCCGCACTTGCACGTTTTAGTTTGTCTTATAACACGAGTGA
```

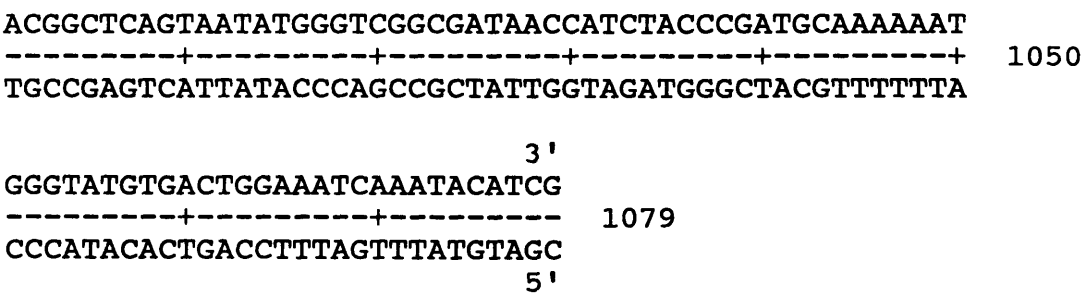

ACGGCTCAGTAATATGGGTCGGCGATAACCATCTACCCGATGCAAAAAAT
1050
TGCCGAGTCATTATACCCAGCCGCTATTGGTAGATGGGCTACGTTTTTTA
3'
GGGTATGTGACTGGAAATCAAATACATCG
1079
CCCATACACTGACCTTTAGTTTATGTAGC
5'

Figure 3-7

Restriction Map of the NruI Fragment of pADC201

This partial restriction map of the Nru I insert of the *panD* subclone pADC201 is derived by analysis of the DNA sequence of Figure 3-6. The fragment was inserted into the Nru I site of pBR322 within the Tc^r region. The arrow shown below the map indicates the orientation of the *panD* ORF as determined in Section 3-5 of Section 3-5. The restriction map is drawn to a scale of 100 nucleotides to the centimeter.

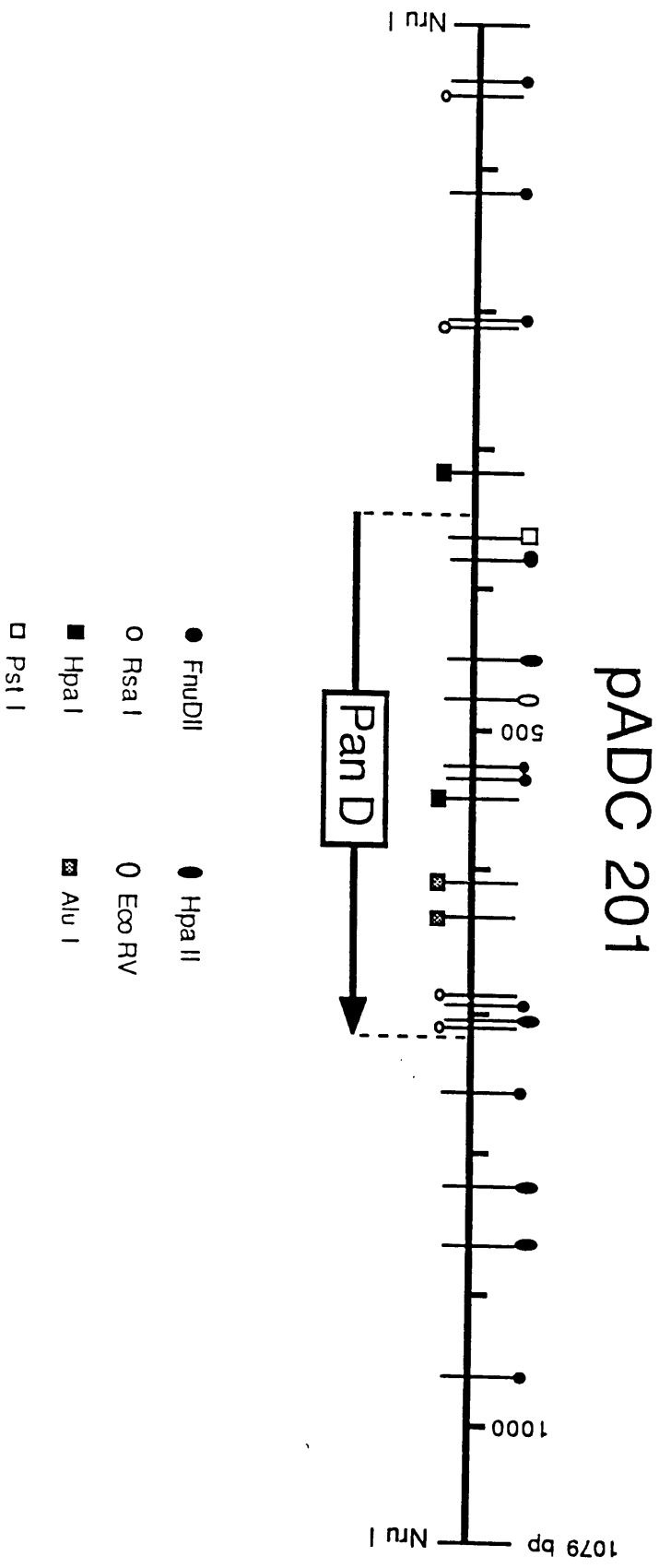

pADC 201
Nru I
500
1000
1079 bp
Nru I
Pan D
FnuDII
Hpa II
Rsa I
Eco RV
Hpa I
Alu I
Pst I

Figure 3-8

Open Reading Frame and Codon Usage Analysis of the 5'→3' Strand of the NruI Fragment of pADC201

The Codon preference plot is given in three parts for each of the three possible reading frames starting at the first, second, and third nucleotides for Parts A, B, and C, respectively. The dashed line in each section indicates the calculated codon preference preference statistic for a theoretical random sequence of the same composition as the E_coli.CDF codon usage table. Open reading frames are indicated by boxed regions at the bottom of each plot of codon preferences. Methionine codons (AUG) are represented by vertical lines that do not cross the lower horizontal frame. Stop codons are represented by vertical lines that cross the horizontal frame. Rare codons that represent less than 5% of a given synonomous family are represented by the vertical bars below each plot.

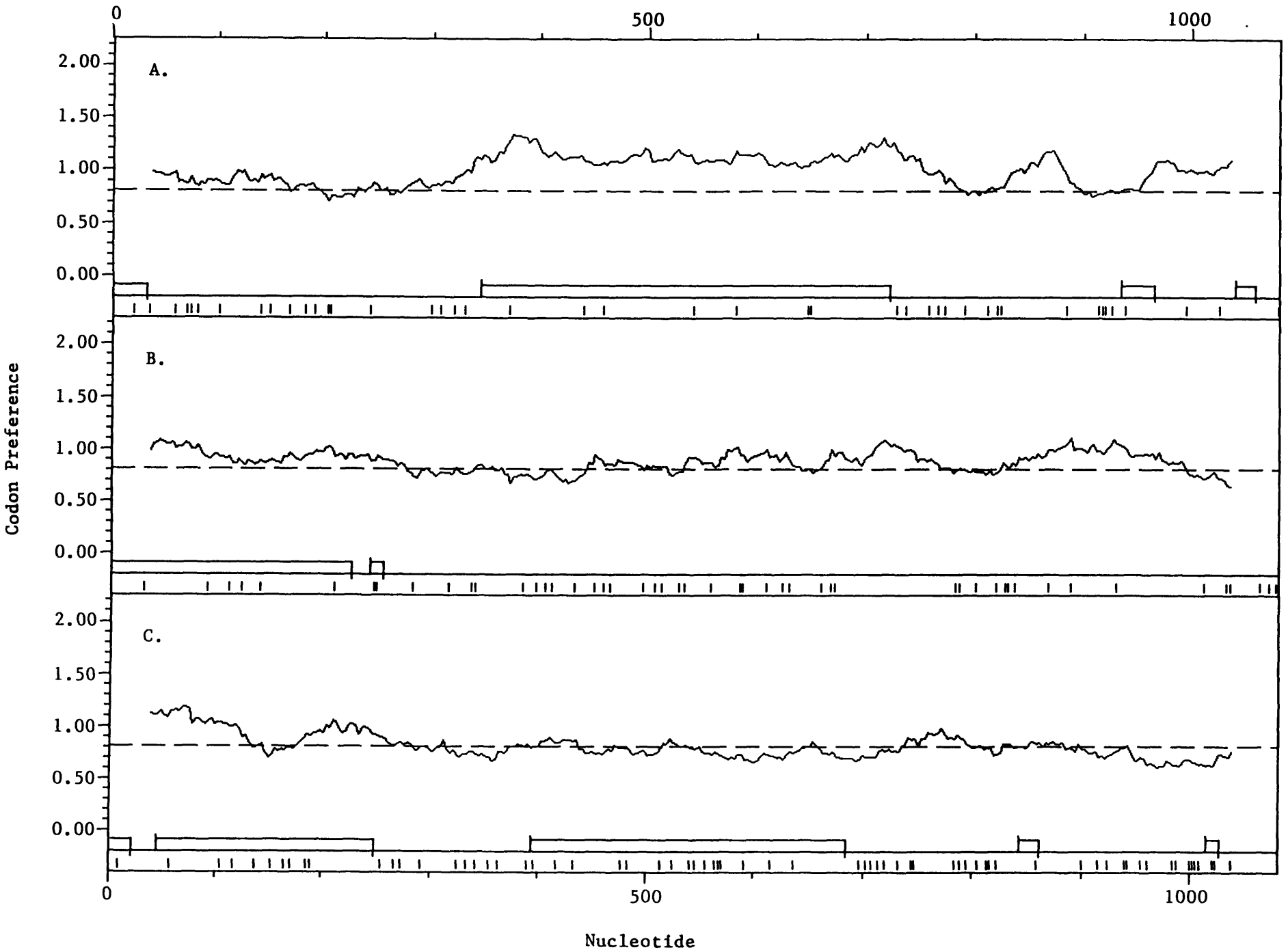

0
500
1000
A.
B.
C.
2.00
1.50
1.00
0.50
0.00
Codon Preference
Nucleotide

Figure 3-9

Open Reading Frame and Codon Usage Analysis of the 3'→5' Strand of the NruI Fragment of pADC201

The Codon preference plot is given in three parts for each of the three possible reading frames starting at the first, second and third nucleotides for A, B and C, respectively. The dashed line in each section indicates the calculated codon preference preference statistic for a theoretical random sequence of the same composition as the E_coli.CDF codon usage table. Open reading frames are indicated by boxed regions at the bottom of each plot of codon preferences. Methionine codons (AUG) are represented by vertical lines that do not cross the lower horizontal frame. Stop codons are represented by vertical lines that cross the horizontal frame. Rare codons that represent less than 5% of a given synonomous family are represented by the vertical bars below each plot.

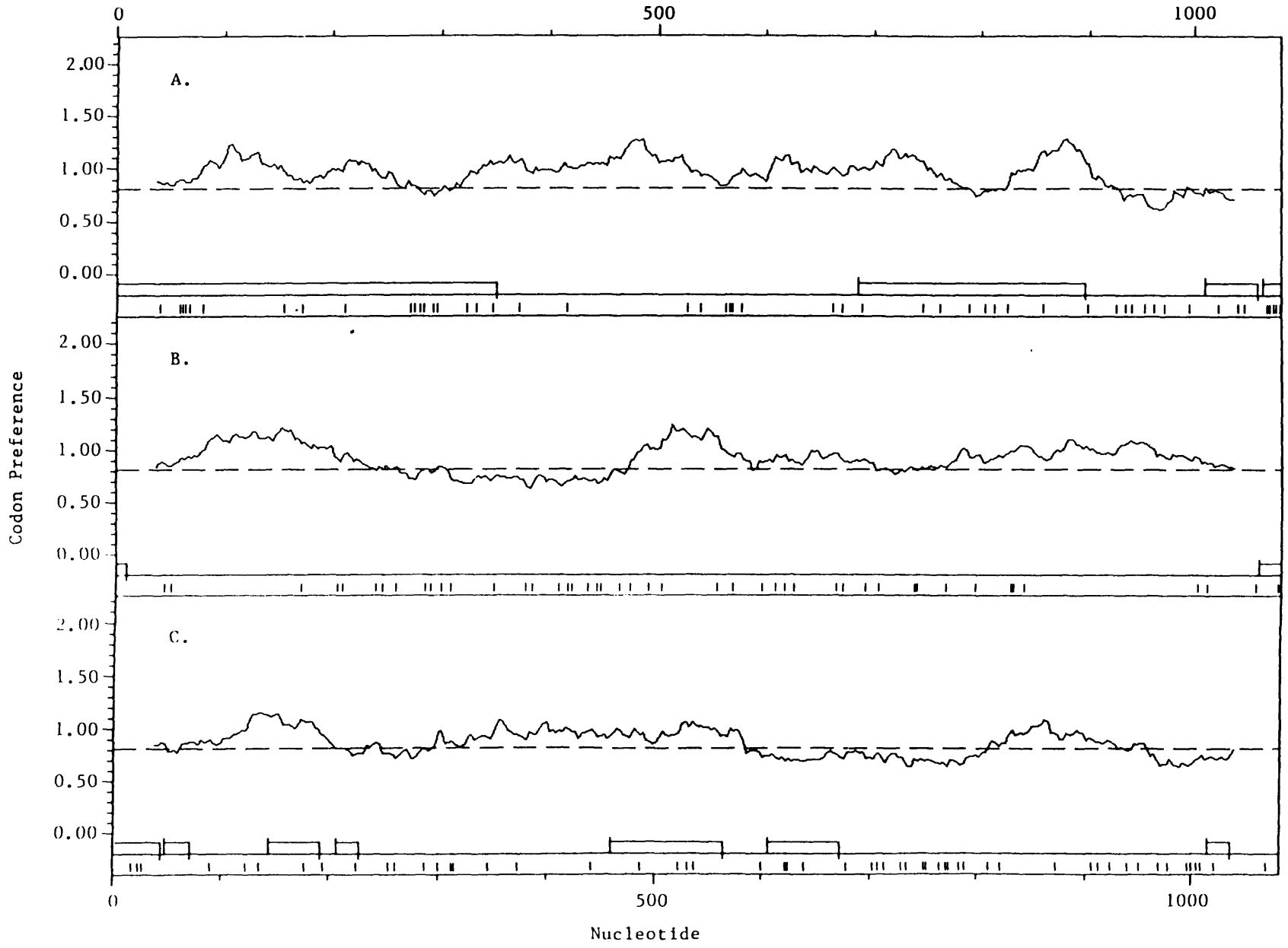

0
500
1000
A.
B.
C.
2.00
1.50
1.00
0.50
0.00
Codon Preference
Nucleotide

Table 3-3. The Frequency of Rare Codon Usage in the Open Reading Frame of the Sequence of Figure 3-6.§

Reading Frame	Starting Position of ORF†	Total Number of Codons	Number of Rare Codons	Percentage of Rare Codons
5'→3' Strand				
A	344	126	7	5.6
B	1	75	6	8.0
C	45	68	9	13.2
	393	97	16	16.5
3'→5' Strand				
A	1078	116	18	15.5
	394	70	8	11.4
C	621	4	35	11.4
	474	3	22	13.6

§ = Only Open Reading Frames larger than 50 nucleotides are included
† = Position as determined from 5' terminus as indicated in Figure 3-6

The utility of this form of analysis is based on the observation by Ikemura (1982) that a direct correlation exists between the frequency of a synonomous codon's usage in *E. coli* genes and the abundance of its cognate tRNA in the cell. Gribskov *et al.* (1984) have demonstrated a low occurrence of rare codons in moderately and highly expressed proteins providing the basis for the Codonpreference program's facility for identifying genuine transcriptional units.

The result of the Codonpreference analysis of the NruI fragment reveals that the rare codon usage is particularly low for two large open

Table 3-4. The Frequency of Codon Usage in the Coincident Open Reading Frames of the A and C Frames of Figure 3-7.

	Frame A	Frame C		Frame A	Frame C		Frame A	Frame C		Frame A	Frame C
AAA (lys):	4	2	AAC (asn):	4	1	AAG (lys):	1	5	AAT* (asn):	2	0
ACA (thr):	0	1	ACC (thr):	4	2	ACG* (thr):	1	5	ACT (thr):	2	2
AGA (arg):	1	0	AGC (ser):	1	1	AGG* (arg):	0	1	AGT (ser):	1	0
ATA* (ile):	0	2	ATC (ile):	4	0	ATG (met):	4	6	ATT (ile):	8	2
CAA* (gln):	0	0	CAC (his):	3	0	CAG (gln):	3	3	CAT (his):	1	0
CCA (pro):	1	9	CCC* (pro):	1	1	CCG (pro):	1	1	CCT (pro):	0	2
CGA (arg):	1	1	CGC (arg):	4	0	CGG (arg):	0	4	CGT* (arg):	2	0
CTA (leu):	0	0	CTC (leu):	2	1	CTG (leu):	2	1	CTT (leu):	1	2
GAA (glu):	8	1	GAC (asp):	4	1	GAG (glu):	0	0	GAT (asp):	4	0
GCA (ala):	2	2	GCC (ala):	8	0	GCG (ala):	6	5	GCT (ala):	2	1
GGA (gly):	0	1	GGC (gly):	4	1	GGG (gly):	0	0	GGT (gly):	4	0
GTA (val):	1	1	GTC (val):	4	0	GTG (val):	2	2	GTT (val):	3	3
TAA (Stop):	0	0	TAC (tyr):	0	0	TAG (Stop):	0	0	TAT (tyr):	3	0
TCA (ser):	0	3	TCC (ser):	1	0	TCG* (ser):	1	6	TCT (ser):	2	2
TGA (Stop):	1	1	TGC (cys):	2	2	TGG (trp):	2	0	TGT (cys):	0	0
TTA (leu):	0	3	TTC (phe):	2	2	TTG (leu):	0	5	TTT (phe):	2	1

* = Rare Codons

reading frames in frames A (5.6%) and B (8.0%) of Table 3-3, respectively. The two reading frames both are of sufficient size to encode one of the ADC proteins. However, the ORF in frame B of the 5'→3' strand, which encodes a protein fragment of 8,156 daltons, is truncated by the 5' end of the insert and would require upstream plasmid encoded promoter functions for expression, possibly resulting in a fused transcript. The ORF in frame A encodes for a protein of 13,818 daltons and appears to have a high probability of serving as a *bona fide* transcriptional unit based on the low usage of rare codons. Unfortunately, the situation is complicated by the presence of an ORF in reading frame C, encoding a protein with a molecular weight of 10,424 daltons, in the same region and fully contained within the ORF of frame

A. However, some distinctions can be made on the basis of the codon usage as compiled in Table 3-4 for the ORF's of frames A and C.

G. Nigel Godson and colleagues had previously encountered the utilization of codons corresponding to rare tRNA species as a form of translational control during studies of the macromolecular synthesis operon (Konigsberg and Godson, 1981; Smiley *et al.*, 1982; Peacock *et al.*, 1985). This operon is composed of the *rpsU* (ribosomal protein S21), *dnaG* (primase), and *rpoD* (sigma subunit of RNA polymerase). The three proteins are present in widely differing quantities in E. coli with 50,000 copies (Kjeldgaard and Gausing, 1974), 50 copies (Rowen and Kornberg, 1974), and 3,000 copies (Iwakura *et al.*, 1974; Engbaek *et al.*, 1976) per cell for *rpsU*, *dnaG*, and *rpoD*, respectively. S1 nuclease mapping by Burton *et al.* (1983) revealed that the operon is transcribed either coordinately (the order of transcription is *rpsU-dnaG-rpoD*) or separately with a strong terminator positioned between *rpsU* and *dnaG* with additional transcription of *rpoD* initiating from promoters internal to the *dnaG* gene (Peacock *et al.*, 1985). However, S1 mapping indicated that a considerable amount of transcription was proceeding through the *rpsU* terminator and that additional control is involved in maintaining the low level of the *dnaG* gene product.

Analysis of the *dnaG* gene suggested two possible means of post-transcriptional regulation. First, the putative Shine-Dalgarno sequence (Smiley *et al.*, 1982) was a poor match to the consensus sequence (GGGG as opposed to the canonical AGGA). In addition, they observed an unusually high occurrence of rare codons (9.1%) which they postulated may serve as a form of control via "translational modulation" (Konigsberg and Godson, 1981). In this form of control, the level of

modulation would be determined by the availability of the various isoaccepting tRNA molecules since ribosomal transit time would be lengthened by the increasing occurrence of rare codons requiring low abundance tRNA species. In addition, they postulate that the clustering of rare codons seen with *dnaG* would provide a highly effective means of inducing pauses in ribosomal transit. This rate limiting effect of scarce tRNA species on the rate of protein synthesis has been observed *in vitro* by Anderson (1969) and had previously been proposed as a means of controlling expression (see references 1-5 in Post *et al.*, 1979).

Konigsberg and Godson (1981) have calculated the frequency of usage of rare codons for non-regulatory proteins in *E. coli*. The frequency ranges from 0% for highly expressed proteins such as *rpsU* and *lpp* (murein lipoprotein) to 5.6% for *polA* (DNA Polymerase) to 8.46% for *uncB* (membrane-bound ATP synthase). This in contrast to the 10.0%, 9.2% and 6.74% for *lacI*, *araC*, and *trpR* regulatory proteins, respectively. In light of these values, the very high frequency of rare codon usage (16.5%) for the ORF of frame C indicates that if this does represent a valid transcriptional unit, it probably encodes a protein with a low level of expression.

Finally, the large ORF present in the A frame contains the unique PstI site which had been anticipated to be internal to the *panD* gene. In addition, the 5′ end of the ORF is spanned by the 240 bp HpaI fragment that was deleted in the construction of in the PanD plasmid pADC2001.

3.6 Fidelity of the Sequencing of the *panD* Region

The Codonpreference analysis serves an additional purpose besides its obvious facility for the identification of open reading frames. Sequencing errors that result in frameshifts can be identified by the change in codon usage. Following the occurrence of a frameshift error, a sudden increase in the usage of rare codons (decrease in the codon preference statistic) in the current open reading frame is accompanied by a concurrent decrease in rare codon usage (increase in the codon preference statistic) in an alternate reading frame (Gribskov *et al.*, 1984).

Examination of the results obtained for the analysis of the NruI fragment indicates that the ORF of frame A in Figure 3-8 has the highest overall codon preference statistic (lowest usage of rare codons) of any frame on both strands. The codon preference statistic is elevated from shortly before the start of the open reading frame, remaining at aproximately 1.1 throughout the reading frame and dropping to a minimum, after the termination of the ORF, at position 800. This decrease in reading frame A is the result of a cluster of rare codons following terminuation of the ORF. Beyond 800 the codon preference statistic increases again briefly and then descends to random levels and finally increasing a third time to 1.1 at the end of the NruI fragment. Frame B contains an area between 675 and 775 free of rare codons which spans the end point of the ORF of frame A (postion 720) followed by a decrease in the codon preference stastic with a cluster of rare codons at 800 after

which it increases above the random level between 825 and 1000. Frame C shows a consistently low codon preference stastic beyond position 250.

The short region free of rare codons in frame B suggests that the possibility of a frame shift error in the 3' terminal region of the ORF of frame A that results in shifting of the reading frame to B. However, the large number of rare codons in both alternate reading frames B and C shortly downstream of this region indicates that the possibility of such a sequencing error is low.

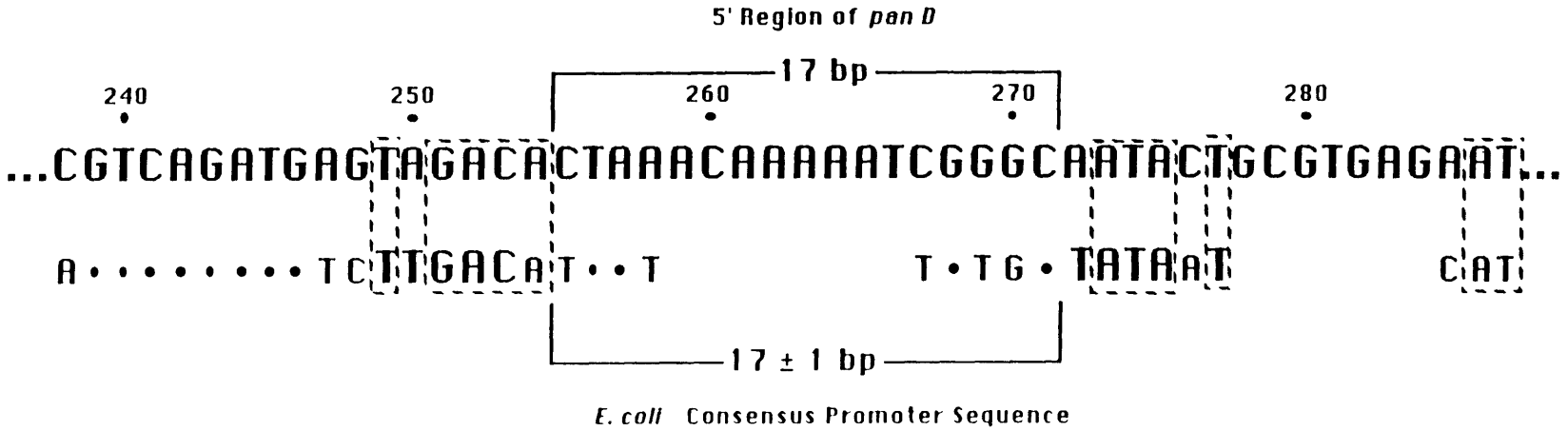
3.7 Analysis of the 5'and 3' Regions of the Putative *panD* ORF for Control Elements

Expression of the gene product of *panD* from the ORF in frame A would require a correctly positioned promoter in the 5' upstream region. Examination of this region revealed a match to the *E. coli* consensus promoter sequence (Hawley and McClure, 1983) was obtained at the positions 249-278 nucleotides (-65 to -95 from the ORF) as shown in Figure 3-10. The putative -10 region gives a 6 out of 7 match with a 5 out of 6 match for the -35 region. The adenosine residue at position 250 in the putative -35 region represents a severe departure from the consensus sequence with only 18 of the 112 promoters examined by Hawley and McClure (1983) having a nucleotide other than thymidine at that position. Regarding the -10 region, the adenosine residue at position 272 is seen in only 2 out of 112 promoters. Substitutions at both of these positions in the P_{ant} promoter controlling the antirepressor gene of bacteriophage P22 lead to severe depression of promoter activity as

Figure 3-10

Correspondence of the Putative *panD* Promoter to the *E. coli* Concensus Promoter Sequence

The DNA sequence of a portion of the 5' region of the putative *panD* ORF is shown in the top register while the E. coli concensus promoter sequence of Hawley and McClure (1983) is shown in the bottom register. Regions of homology are enclosed in the boxes with dashed lines. The distance between the -10 and -35 regions are shown. The larger characters in the consensus sequence are highly conserved (conserved in more than 54% of sequences compiled, greater than 6 standard deviations above random occurence) while smaller characters are conserved (greater than 3 standard deviations) in greater than 39% but less than 54% of the sequences compiled for the consensus sequence. The numbers above the *panD* sequence indicate the position within the panD region of the NruI fragment of pADC201 whose sequence is provided in Figure 3-6.


5' Region of pan D
17 bp
240
250
260
270
280
...CGTCAGATGAGTAGACACTAAACAAAAATCGGGCAATACTGCGTGAGAAT...
A·····TCTTGACAT··T T·TG·TATAAT CAT
17 ± 1 bp
E. coli Consensus Promoter Sequence

measured by the production of transcript by an *in vitro* assay (Youderian *et al.*, 1982). The 18 bp spacing between the -35 and -10 region falls in the optimal range of 17-19 nucleotides (Rosenberg and Court, 1979).

In addition to the promoter given in Figure 3-10, a second match to the consensus sequence exists located at positions 319-325 (-35 region) and at positions 323-328 (-10 region). This putative promoter contains a poor -35 region with two substitutions at positions 319 and 320 and one in the -10 region at position 32 that represent severe departures from the consensus sequence. The -10 region overlaps the start codon of the ORF of frame A of Figure 3-8 eliminating it as a possibility in that instance, but this sequence could serve as a promoter for the ORF in frame C which begins further downstream.

The involvement of a Shine-Dalgarno sequence in the binding of mRNA to the ribosome has been postulated as an essential element for efficient translation (Shine and Dalgarno, 1975; Shephard *et al.*, 1982) and would be expected to be present in the upstream region of any transcriptionally active ORF. An upstream region of the putative *panD* ORF complimentary to the *E. coli* 16S rRNA is capable of serving as a Shine-Dalgarno region as shown in Figure 3-11. This region, from 331-342 nucleotides (-12 to -2 upstream of the ORF), has 6 possible basepairing matches to the 3' terminus of the 16S rRNA with a cluster of 4 consecutive complementary bases 8 nt upstream of the ORF.

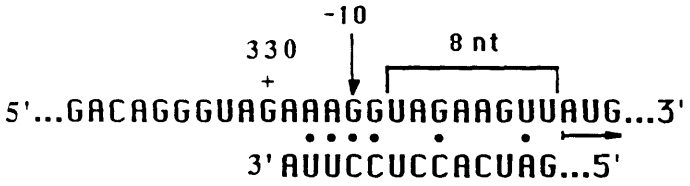
We hoped to obtain an indication of the size of the transcript of the putative panD transcript through location of a plausible termination site. The predominant features of prokaryotic rho-independent termination sites have been described (Platt, 1986). The features commonly seen with this type of termination include a

Figure 3-11

Possible Basepairing of the Putative Shine-Dalgarno Sequence with the 3′ Terminus of the *E. coli* 16S rRNA

The sequence shown is from the putative 5′ region of the *panD* transcript upstream of the large ORF of Frame A of Figure 3-8. The lower sequence is the 3′ terminus of the E. coli 16S rRNA (Baan *et al.*, 1977). Possible basepairings are indicated by closed circles and the distance between the start of the ORF and the tetranucleotide of consecutive basepairings anticipated to serve as the Shine-Dalgarno region is provided. The position of this region within the sequence of Figure 3-6 is shown by the cross.

5' region of pan D



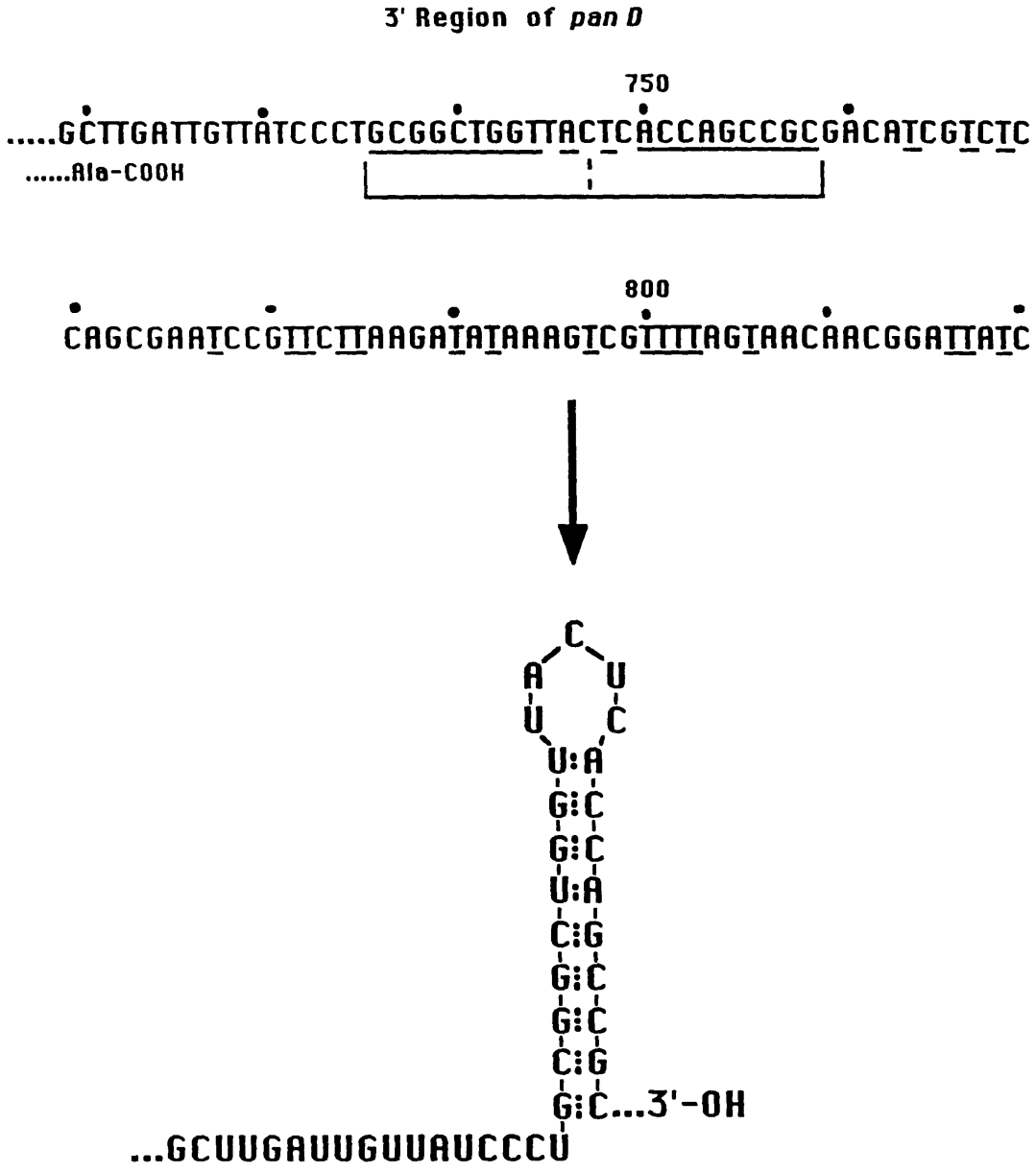
3' Terminus of the E. coli 16S rRNA

G/C rich (3-11 contiguous G/C base pairs) inverted repeat region followed by a run of consecutive thymidine residues located in the non-template strand. Analysis of the 3' region downstream of the ORF for possible termination sites located the postulated termination structure shown in Figure 3-12. The predominate feature of this analysis is the 9 bp long perfect inverted repeat (from 736-744 and 750-758 nucleotides) that is capable of forming a stable RNA stem-loop structure common to several rho-independent termination regions (Rosenberg and Court, 1979). This inverted repeat region is capable of forming 8 G-C basepairs out of the 10 bp possible within the postulated stem-forming region. This provides a highly stable structure with a calculated ΔG (25°C) = -20.9 kcal/mole, calculated according to Tinoco *et al.* (1973). In the vicinity of such structures, rho-independent termination of transcription generally occurs within 20 ($\pm$4) nucleotides downstream from the center of the dyad symmetry, generally with a terminus of 4 to 8 consecutive uridine residues (Rosenberg and Court, 1979). The expected series of thymidine residues on the non-template strand is not observed in the vicinity of the inverted repeat region at end of the putative *panD* ORF. Single thymidine residues are located at positions 763, 766, 768, and 777 which are 18, 21, 23, and 32 nucleotides from the center of dyad symmetry, respectively. The first pair of consecutive thymidine residues are at positions 781-782 bp located 34 bp from the center, after which several runs of consecutive thymidine residues exist and within an A-T rich region (75% A-T) at positions 780-820.

Figure 3-12

Analysis of the 3' Region of the *panD* ORF for Putative Termination Structures

The upper portion of the figure is the sequence of the sense strand downstream of the putative open reading frame encoding the *panD* gene product. The underlined nucleotides enclosed by the bracket are part of a dyad symmetry that is capable, in the form of mRNA, of forming the postulated stem-loop structure depicted below. Thymidine residues distal to the region of dyad symmetry, that may serve as rho-independent termination sites, are underlined.



Possible secondary structure at the
3' terminus of *pan D* mRNA

3.8 S1 Nuclease Mapping of the 3' and 5' Termini of *panD* mRNA

We decided to map the termini of the mRNA transcribed from this region in order to confirm the full extent of the ORF. This was accomplished by the method of S1 mapping as described in Experimental Methods through annealing of mRNA to single stranded probes and then determining the portion protected from digestion by S1 nuclease (under conditions providing specificity for digestion of only single-stranded nucleic acid). The radiolabeled probes were prepared by primer extension of the M13mp18 subclones containing complementary strands of the restriction fragments shown in Figure 3-13. Two probes were prepared for the putative 5' region from pADC201 derived fragments; 10119 containing the NruI-PstI fragment (positions 1→363 in Figure 3-4), and; 10113, containing the NruI-EcoRV fragment (positions 1→478). The two probes for the 3' region are: 101112 containing the AluI-NruI fragment (positions 635→1079) and; 10116 containing the EcoRV-NruI fragment (positions 478→1079).

The fragments obtained as a result of the S1 nuclease mapping were sized by comparison to a sequencing ladder obtained from a known M13mp18 subclone on sequencing gel. Autoradiograms obtained from such analyses are shown in Figure 3-14, 3-15, 3-16, and 3-17. From the standard, the lengths of the S1 nuclease generated fragments were determined and the results are shown in Table 3-5. The fragment lengths allow correlation of the termini of the *panD* transcript to the DNA sequence by virtue of their distance from the internal restriction sites as shown in Figure 3-18.

Figure 3-13

Origins of the S1 Nuclease Probes for Mapping of the *panD* Transcript

The termini of the probes obtained by single stranded M13mp18 subclones used for S1 nuclease mapping of the termini of the *panD* mRNA are shown with respect to the *panD* open reading frame. The bars at the end of the arrows indicate the restriction endonuclease sites that mark the start of *panD* sequences contained within the probes. Radioactively labeled probes were obtained by primer extension of m13mp18 subclones containing the sense strand of the Nru I insert of pADC1 as described in Experimental Methods. The numbers provided within parentheses indicate the positions of restriction sites and the open reading frame within the Nru I insert. The position of the putative *panD* ORF (the large ORF of the A frame of Figure 3-8) is shown by the box and the first and last nucleotide of the ORF is provided in parentheses.

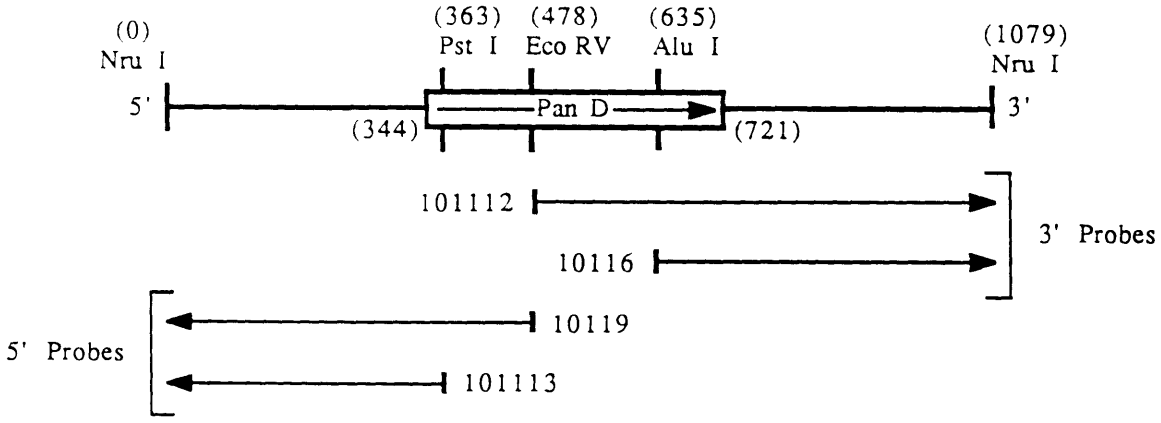

(0)
Nru I
5'
(363)
Pst I
(478)
Eco RV
(635)
Alu I
(344)
Pan D
(721)
(1079)
Nru I
3'
101112
10116
3' Probes
10119
101113
5' Probes

Figure 3-14

S1 Nuclease Mapping of the *panD* Transcript with the 5' Probe 10119

58 μg of mRNA isolated from AB355/pADC1 was hybridized with 50,000 cpm of the gel-purified probe 10119 (labeled by primer extension with 5'-[^{32}P]dATP) in 80% formamide containing buffer at 42°C for 18 hours. The hybridization was then treated with 500 units of S1 nuclease for 30 minutes at 37°C. The reaction was quenched with 14 μg of yeast tRNA and nucleic acid was precipitated with ethanol. The resuspended nucleic acid was resuspended in a basic formamide dye mixture and denatured for 2 minutes at 90°C and then quick chilled on ice. 4 μl (40%) of the sample was subjected to electrophoresis on a 6% sequencing gel. Reactions without mRNA contain 58 μg of tRNA. Full experimental details are provided in Experimental Methods. The positions of the S1 nuclease generated fragments are indicated by the arrows and the lengths provided were determined by comparison to the standard sequence shown.

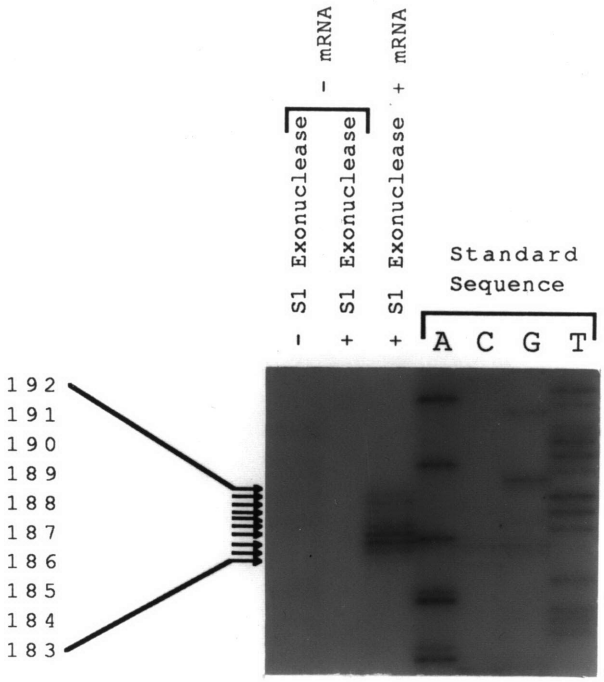

- mRNA
+ mRNA
- S1 Exonuclease
+ S1 Exonuclease
+ S1 Exonuclease
Standard Sequence
A C G T
192
191
190
189
188
187
186
185
184
183

Figure 3-15

S1 Nuclease Mapping of the *panD* Transcript with the 5' Probe 10113

58 μg of mRNA isolated from AB355/pADC1 was hybridized with 50,000 cpm of the gel-purified probe 10113 (labeled by primer extension with 5'-[^{32}P]dATP) in 80% formamide containing buffer at 42°C for 18 hours. The hybridization was then treated with 500 units of S1 nuclease for 30 minutes at 37°C. The reaction was quenched with 14 μg of yeast tRNA and nucleic acid was precipitated with ethanol. The resuspended nucleic acid was resuspended in a basic formamide dye mixture and denatured for 2 minutes at 90°C and then quick chilled on ice. 4 μl (40%) of the sample was subjected to electrophoresis on a 8% sequencing gel. Reactions without mRNA contain 58 μg of tRNA. Full experimental details are provided in Experimental Methods. The positions of the S1 nuclease generated fragments are indicated by the arrows and the lengths provided were determined by comparison to the standard sequence shown.

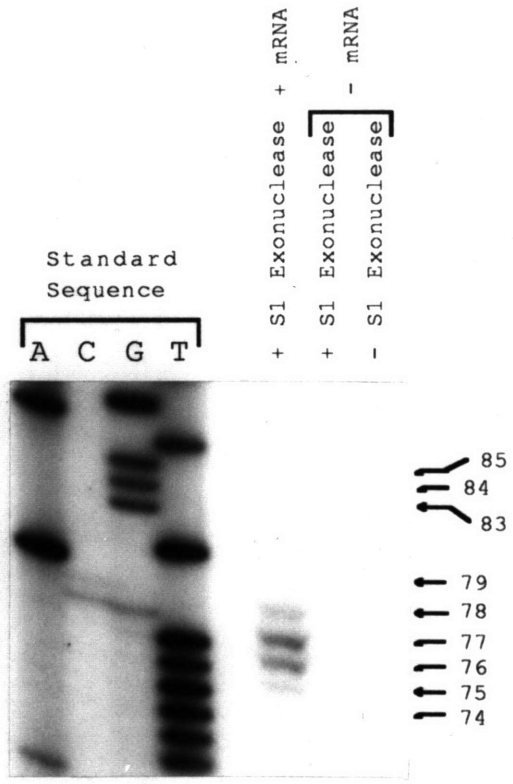

Standard
Sequence
A C G T
+ S1 Exonuclease + mRNA
+ S1 Exonuclease - mRNA
- S1 Exonuclease
85
84
83
79
78
77
76
75
74

Figure 3-16

S1 Nuclease Mapping of the *panD* Transcript with the 3' Probe 10116

58 μg of mRNA isolated from AB355/pADC1 was hybridized with 50,000 cpm of the gel-purified probe 10116 (labeled by primer extension with 5'-[^{32}P]dATP) in 80% formamide containing buffer at 42°C for 18 hours. The hybridization was then treated with 500 units of S1 nuclease for 30 minutes at 37°C. The reaction was quenched with 14 μg of yeast tRNA and nucleic acid was precipitated with ethanol. The resuspended nucleic acid was resuspended in a basic formamide dye mixture and denatured for 2 minutes at 90°C and then quick chilled on ice. 4 μl (40%) of the sample was subjected to electrophoresis on a 6% sequencing gel. Reactions without mRNA contain 58 μg of tRNA. Full experimental details are provided in Experimental Methods. The positions of the S1 nuclease generated fragments are indicated by the arrows and the lengths provided were determined by comparison to the standard sequence shown.

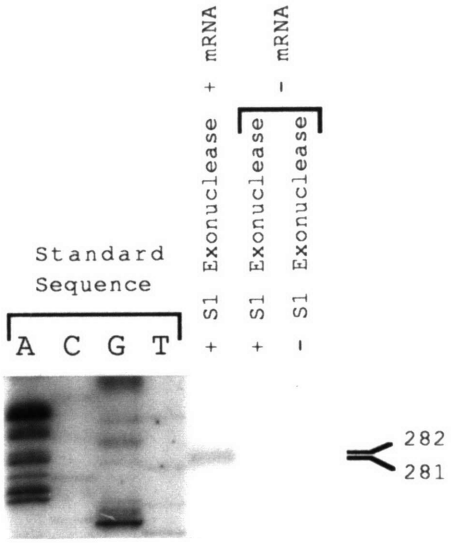

Standard
Sequence
A C G T
+ S1 Exonuclease + mRNA
+ S1 Exonuclease - mRNA
- S1 Exonuclease
282
281

Figure 3-17

S1 Nuclease Mapping of the *panD* Transcript with the 3' Probe 101112

58 μg of mRNA isolated from AB355/pADC1 was hybridized with 50,000 cpm of the gel-purified probe 101112 (labeled by primer extension with 5'-[^{32}P]dATP) in 80% formamide containing buffer at 42°C for 18 hours. The hybridization was then treated with 500 units of S1 nuclease for 30 minutes at 37°C. The reaction was quenched with 14 μg of yeast tRNA and nucleic acid was precipitated with ethanol. The resuspended nucleic acid was resuspended in a basic formamide dye mixture and denatured for 2 minutes at 90°C and then quick chilled on ice. 4 μl (40%) of the sample was subjected to electrophoresis on an 8% sequencing gel. Reactions without mRNA contain 58 μg of tRNA. Full experimental details are provided in Experimental Methods. The positions of the S1 nuclease generated fragments are indicated by the arrows and the lengths provided were determined by comparison to the standard sequence shown.

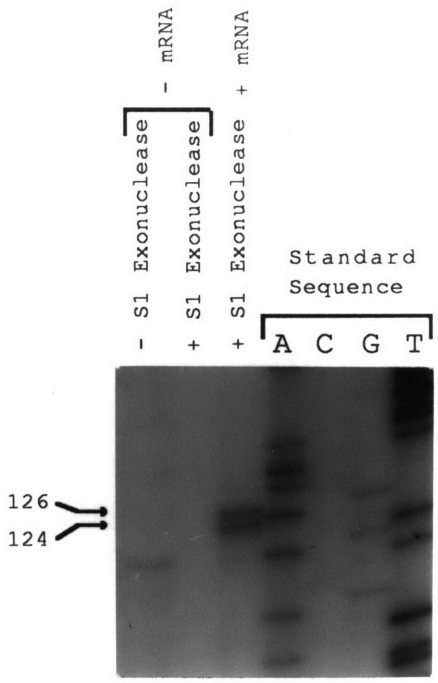

- mRNA
+ mRNA
- S1 Exonuclease
+ S1 Exonuclease
+ S1 Exonuclease
Standard Sequence
A C G T
126
124

Table 3-5. Length of the Fragments Generated by S1 Nuclease Mapping of mRNA Termini

Probe	Length of Fragments (Nucleotides)	Strength of Signal
5' Terminus		
10119		
	183	2
	184	4
	185	4
	186	4
	187	3
	188	1
	189	1
	190	3
	191	3
	192	1
10113		
	74	1
	75	2
	76	4
	77	4
	78	3
	79	1
	83	2
	84	2
	85	1
3' Terminus		
10116		
	281	4
	282	4
101112		
	124	4
	126	4

4 = strongest
1 = weakest

The almost identical results obtained with the two distinct probes for both the 5' and 3' termini indicates that the method has provided consistent results. The stuttering obtained at the 5' terminus of the transcript is unusual since initiation usually occurs at a single predominant nucleotide (Rosenberg and Court, 1979), but the effect is

Figure 3-18

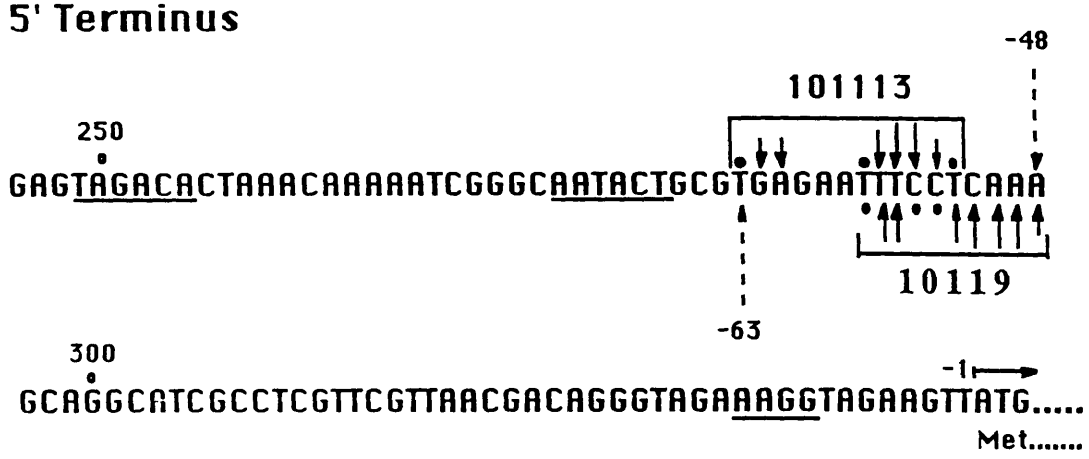
Termini of the *panD* mRNA as Determined by S1 Nuclease Mapping

The position of the *panD* mRNA termini as determined by S1 nuclease mapping with respect to the DNA sequence of the Nru I insert of pADC201. The lengths of the fragments were determined against a standard DNA sequence and are given in Table 3-5. The termini for a given probe are bracketed and the strength of the signal obtained for each species is represented. The weakest signals are designated by a closed circle. The arrows represent stronger signals with the strength of the signal increasing with the length of the shaft. The positions of each region within the DNA sequence of the Nru I fragment are shown by the open circles.

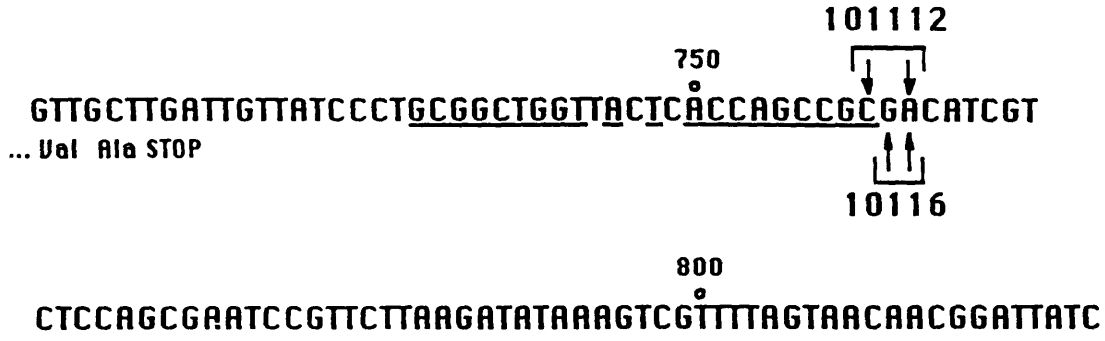
The underlined nucleotides for the 5' terminus represent the position of the putative -35 and -10 promoter regions and Shine-Dalgarno sequence. The position of the 5' termini with respect to the ORF of Frame A in Figure 3-8 is given by the dotted arrows.

The underlined sequences for the 3' region comprise the region of dyad symmetry that may function as a transcriptional termination signal.

5' Terminus



3' Terminus



reproducible since similiar patterns were obtained with both probes and may represent an artifact of the S1 mapping. The quality of the mRNA preparation is not a factor since the results obtained with the 3′ probes do not exhibit this pattern. Possibly, the multiple 5′ termini may be resulting from the instability of dA-rU basepairing (Martin and Tinoco, 1980) where the high A-T content in the region of the 5′ terminus allows some "nibbling" of frayed ends by S1.

The results of the S1 nuclease mapping indicate that the assumptions made about the size and positioning of the open reading frame were correct with the trancript being transcribed from the 3′→5′ strand depicted in Figure 3-6. Hence, the 5′→3′ strand represents a sense reading of the ORF. The total length of the transcript can be determined to be approximately 470 nucleotides from the results obtained with probes 101119 and 10116.

The 5′ end of the transcript is located at the position anticipated for the promoter region of the ORF in frame A (Figure 3-8) as predicted from homology to the *E. coli* promoter consensus sequence. Figure 3-18 indicates that the 5′ termini of the transcript, as specified by probe 10113, is from 3-5 nt and 8-13 nt from the -10 region. The strongest signals correspond to deoxythymidine and deoxycytidine residues 10 and 11 nt from the -10 region. However, additional strong signals correspond to deoxyguanosine (5 nt from the -10 region) and deoxyadenosine (6 nt) residues upstream of the former residues while a faint signal corresponded to a deoxythymidine residue (3 nt). The latter signals place the start of the transcript at purine residues within the 4-8 nucleotides spacing specified by Hawley and McClure (1983). This indicates that the terminus of the transcript is

located from -52→-63 nt upstream of the putative ORF. The absence of other signals for the S1 mapping indicates that if the ORF of frame C in Figure 3-5 is being transcribed from an alternate promoter then the level of transcription must be considerably lower than that seen for the putative promoter of Figure 3-10.

The results obtained at the 3′ terminus, also shown in Figure 3-18, again bears out the predictions for the ORF. Both probes give nearly identical results placing the termini at positions 758-760 of the sequence of Figure 3-6. This places cytidine, guanosine, and adenosine residues at the 3′ terminus of the treanscript immediately following the putative stem-loop termination structure. The predicted lack of 3′ terminal uridine residues in the transcript is highly unusual for an apparent rho-independent termination structure although a similiar situation has been seen previously with the transcript of the *serB* gene which encodes phosphoserine phosphatase (Neuwald and Stauffer, 1985).

In the absence of such a highly charateristic feature of rho-independent termination sites, the possibility of rho involvement is the obvious alternative. Unfortunately, the presence of the highly stable stem-loop structure at the immediate terminus of the transcript does not appear to be characteristic of that form of termination although the specific sequence determinants involved in signaling termination by the rho protein are not well understood. A large number of rho-dependent termination sites have been compiled but can only be noted for their lack of sequence specificity (Platt, 1986). Mechanistic studies of rho-dependent termination *in vitro* indicate that rho exhibits a lack of sequence specificity except for a requirement for several cytidine residues and prefers a binding site near the end of the transcript with

little or no secondary structure of sufficient length (72-84 nt) to activate the rho ATPase activity necessary for termination (Bear *et al.*, 1985 and references therein). While also elucidating the features provided by Bear *et al.*, Platt (1986) states that an unstructured region alone is not sufficient to cause termination and implicates the involvement of sequences considerably upstream of the termination point with conveying the signal for termination. In light of such information, the presence of strong secondary structure near the terminus of the transcript is likely to preclude any involvement of rho protein in the termination of transcription and leaves the mechanism of termination of the *panD* transcript rather nebulous.

3.9 Translated Protein Sequence of the *panD* ORF

The translated sequence of the putative *panD* ORF is shown in Figure 3-19. It encodes a single protein of 13,834 daltons molecular weight which appears to correspond to the molecular weight of the ADC protein I which has a M_r of 14,650 daltons (see section 3.14). We anticipated, at this point, the possibility that only a single protein would be encoded by the *panD* gene based on the extensive studies by Snell and coworkers on the structure and post-translational modification of the π-chain proenzyme of histidine decarboxylase from *Lactobacillus* (Recesi and Snell, 1984). However, the possibility of an additional proteins being encoded at unlinked loci could not be wholly disregarded even though the likelihood appears low since all known mutants displaying the *panD*$^-$ phenotype have been shown to map to a single *panD* locus (Cronan *et al.*, 1982).

Figure 3-19

Translation of the Open Reading Frame Encoding the *panD* Gene Product

The DNA sequence of the 5'→3' strand of the Nru I insert of pADC201 is shown. Translation of the ORF encoding for the *panD* gene product is given beneath the DNA sequence. The standard three letter abbreviation for the amino acid is given below its respective codon. The position of the nucleotides with respect to the full Nru I fragment of pADC 201 is given above the nucleotide sequence. The position of an amino acid from the amino terminus is given below the underlined amino acid. The underlined DNA sequences indicate the position of putative regulatory sequences. (a) -35 promoter region (b) -10 promoter region (c) Shine-Dalgarno sequence (d) Inverted repeat termination sequence.

```
                                        250
                                         ^
5'
ACGCAAACTGCTGACGCAAATGCCGTCAGATGAGTAGACACTAAACAAAAA
                                          (a)

                                        300
                                         ^
TCGGGCAATACTGCGTGAGAATTTCCTCAAAGCAGGCATCGCCTCGTTCGT
       (b)

                                          350
                                           ^
TAACGACAGGGTAGAAAGGTAGAAGTT ATG ATT CGC ACG ATG CTG
                (c)          Met Ile Arg Thr Met Leu
                              1

                                                    400
                                                     ^
CAG GGC AAA CTC CAC CGC GTG AAA GTG ACT CAT GCG GAC
Gln Gly Lys Leu His Arg Val Lys Val Thr His Ala Asp
           10

CTG CAC TAT GAA GGT TCT TGC GCC ATT GAC CAG GAT TTT
Leu His Tyr Glu Gly Ser Cys Ala Ile Asp Gln Asp Phe
20                                       30

            450
             ^
CTT GAC GCA GCC GGT ATT CTC GAA AAC GAA GCC ATT GAT
Leu Asp Ala Ala Gly Ile Leu Glu Asn Glu Ala Ile Asp
                           40

                                500
                                 ^
ATC TGG AAT GTC ACC AAC GGC AAG CGT TTC TCC ACT TAT
Ile Trp Asn Val Thr Asn Gly Lys Arg Phe Ser Thr Tyr
                 50

                                                550
                                                 ^
GCC ATC GCG GCA GAA CGC GGT TCG AGA ATT ATT TCT GTT
Ala Ile Ala Ala Glu Arg Gly Ser Arg Ile Ile Ser Val
    60                                          70
```

```
AAC GGT GCG GCG GCC CAC TGC GCC AGT GTC GGC GAT ATT
Asn Gly Ala Ala Ala His Cys Ala Ser Val Gly Asp Ile
                                  80

    600
     ^
GTC ATC ATC GCC AGC TTC GTT ACC ATG CCA GAT GAA GAA
Val Ile Ile Ala Ser Phe Val Thr Met Pro Asp Glu Glu
                    90

                   650
                    ^
GCT CGC ACC TGG CGA CCC AAC GTC GCC TAT TTT GAA GGC
Ala Arg Thr Trp Arg Pro Asn Val Ala Tyr Phe Glu Gly
        100                                     110

                                  700
                                   ^
GAC AAT GAA ATG AAA CGT ACC GCG AAA GCG ATT CCG GTA
Asp Asn Glu Met Lys Arg Thr Ala Lys Ala Ile Pro Val
                                    120

                                        750
                                         ^
CAG GTT GCT TGA TTGTTATCCCTGCGGCTGGTTACTCACCAGCCGCG
Gln Val Ala                          (d)
        126

                                        800
                                         ^
ACATCGTCTCCAGCGAATCCGTTCTTAAGATATAAAGTCGTTTTAGTAACA
                                                    3'
```

3.10 Analysis of *E. coli* and *S. typhimurium* Chromosomal DNA following Southern Transfer with a *panD* Probe

Analysis of *Escherichia coli* chromosomal DNA by Southern transfer (Southern, 1975) and probing with radioactively labeled NruI fragment of pADC201 was conducted to determine if the *panD* locus isolated in this work was solely responsible for production of the ADC proteins. In addition, *S. typhimurium* chromosomal DNA was analyzed for the presence of any homologous loci. A *panD* strain of this bacterium had been mapped to minute 128 of the *Salmonella typhimurium* chromosome prior to the mapping of *panD* locus of *E. coli*. However, the *panD* mutant of *S. typhimurium* has not been further characterized and the possibility that it encodes for an homologous ADC activity has not been explored. The result of the analysis is provided in Figure 3-20 which includes the results of both high and low stringency wash conditions. The analysis of *E. coli* chromosomal DNA indicated fragments that correspond exactly to the standard fragments obtained from identical digests of pADC 201 or pADC1 (for the EcorRI digest) at both levels of stringency. The low stringency conditions were calculated to be capable of detecting a minimum of 15% divergence from the DNA sequence based on the 53% G-C content of the *panD* ORF with a predicted T_m = 92.2°C calculated based on the methods of Marmur and Doty (1962) corrected for the wash conditions of 1xSSC as described by Maniatis *et al.* (Dove and Davidson, 1962; as described in Maniatis *et al.*, 1982) and assuming a 1°C decrease in T_m for each increase of 1% in

Figure 3-20

Analysis of *E. coli* and *S. typhimurium* Chromosomal DNA following Southern Transfer and Probing for *panD* Homology with the NruI Fragment of pADC201

3.6 μg of either *E. coli* or *S. Typhimurium* Chromosomal DNA was digested with the indicated restriction endonucleases. The resulting restrictions fragments were separated by electrophoresis in a 1.4% agarose gel and transfered to nitrocellulose. The resulting blots were prehybridized for 2 hours at 68°C and then hybridized with 2.5 x 10^5 cpm/ml (1 μg; 5 x 10^6 cpm total) of ^{32}P-labeled NruI fragment of pADC201 prepared by nick translation. The hybridization was allowed to occur for 16 hours at 68°C. Following hybridization, the filters were washed once in 2xSSC for 5 minutes at room temperature and a second at room temperature for 15 minutes in 2xSSC. At the end of the second wash the filters were treated separately at low and high stringency conditions: (A) high stringency: one wash in 0.1xSSC, 0.1% SDS for 2 hours at 68#C followed by a second wash under identical wash conditions for 30 minutes (B) low stringency: one wash in 2xSSC, 0.1% SDS for 2 hours at 68#C

followed by a second wash under identical conditions for 30 minutes. Dried filters were exposed overnight to Kodak X-OMAT film.

The arrows indicate the position of the *panD* specific restriction fragments generated by the following digests:

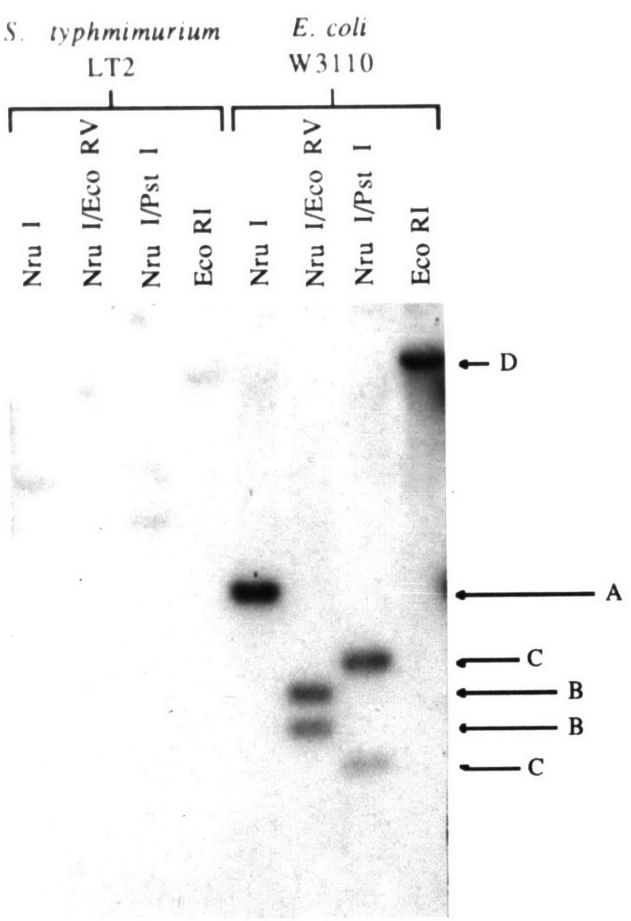
(A) NruI digest of pADC201

(B) NruI/EcoRV digest of pADC201

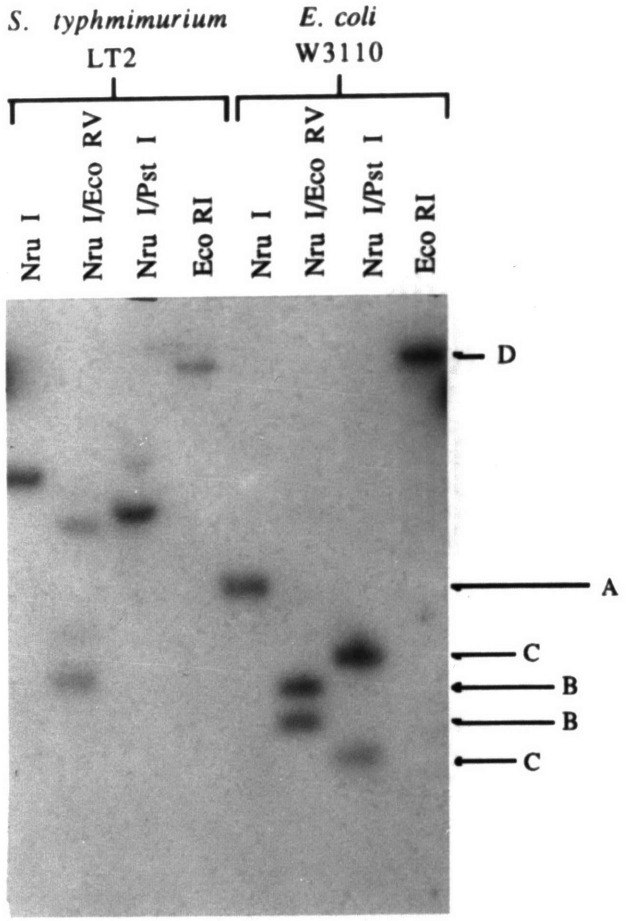
(C) NruI/PstI digest of pADC201

(D) EcoRI digest of pADC201

A.


S. typhmimurium
LT2
E. coli
W3110
Nru I
Nru I/Eco RV
Nru I/Pst I
Eco RI
Nru I
Nru I/Eco RV
Nru I/Pst I
Eco RI
D
A
C
B
B
C

B.


S. typhmimurium
LT2
E. coli
W3110
Nru I
Nru I/Eco RV
Nru I/Pst I
Eco RI
Nru I
Nru I/Eco RV
Nru I/Pst I
Eco RI
D
A
C
B
B
C

the number of mismatched basepairs (Bonner *et al.*, 1973). The lack of additional bands, when combined with the results of Cleveland mapping and the amino-terminal sequencing to follow, indicated that ADC is encoded by a single locus in *E. coli*.

The analysis of *S. typhimurium* DNA indicated the presence of a homologous locus which may encode a *panD* protein similiar to that from *E. coli*. The fragments are barely detectable in the high stringency wash which was anticipated to detect sequences with no greater than 5% divergence. This suggests that *S. typhimurium* has a locus with 85-95% homology to the *panD* locus of *E. coli*. The failure to detect fragments comparable in size to those obtained from *E. coli* is understandable since only the EcoRV and the PstI sites are internal to ORF and the other restriction sites lie outside the ORF in regions where conservation would not be anticipated.

3.11 Purification of ADC from AB355/pADC1

The initial objective of cloning the *panD* gene was to allow overproduction of ADC, present at low concentrations in the extracts from wild type *E. coli* K12, allowing further studies of inactivation of the mechanism-based inhibition of ADC by beta-substituted alanine derivatives. Eventually, we had anticipated being able to probe the active site of the enzyme through isolation of peptides that had been alkylated by action of the inhibitors. However, the structure of the enzyme had been in doubt since the three peptides composing ADC that had been identified as subunits by Williamson and Brown (1979) did not appear to be present in stoichiometric quantities (Williamson, 1977).

Observations by Joanne Williamson had suggested that the subunit stoichiometry appeared variable when analyzed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) suggesting the possibility of degradation of the protein, following denaturation, by an SDS-tolerant protease (Williamson, 1977).

The initial construct, pADC1, provided 50 fold greater levels of ADC over that found in wild type K12 and it was this subclone that was the source of the enzyme for all subsequent work. The purification of the enzyme was essentially that of Williamson and Brown (1979). However, in the course of this study it became apparent that the preparative isoelectric electrofocusing column was prohibitively costly in terms of lost enzymatic activity and was eliminated. This appeared an acceptable ommission since the single protein contaminant removed by that procedure could also be removed by the non-denaturing preparative gel electrophoresis fractionation that provided homogeneous ADC. The results of a typical preparation are provided in Table 3-6.

The results of electrophoresis of gel-purified ADC under non-denaturing conditions is shown in Part A of Figure 3-21. ADC migrates in the Davis buffer (1964) system as a large diffuse band regardless of the quantity of ADC loaded onto the gel. This is consistent with the results obtained by Joanne Williamson (1977) with this system. Part B of Figure 3-21 shows the results of SDS-PAGE of the ADC containing fraction resulting from each stage in the isolation of the homogeneous enzyme. The gel-purified ADC exhibits the three bands that are characteristic of the enzyme and are referred to hereafter as proteins I, II, and III with respect to their increasing order of electrophoretic mobility during SDS-PAGE.

Table: 3-6 Summary of the Purification of ADC from the Overproducing Strain AB354/pADC 1.

Procedure or fraction	Volume (ml)	Activity (units)	Protein (mg)	Specific Activity* (U/mg)	Purification (fold)	Yield (%)
Lysate	328	35,750	8730	4.1	-	-
Heat treated extract	285	15,110	6240	2.4	-	42
40-60% ammonium sulfate precipitate	68	12,850	1904	6.8	1.65	35
DEAE Sephadex	25	10,880	193	56.5	13.8	30
ACA 44	3.3	9,110	57	159	38.8	25
Hydroxylapatite	1.37	6,850	15.9	427	104	19
Preparative Gel Electrophoresis†	-	562	1.06	530	129	6.7

† = Data not available for total preparation

* = Units defined as nanomoles of CO_2 or β-alainine produced per minute

Figure 3-21

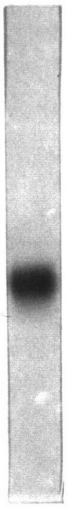
Gel Electrophoresis of the Fractions Obtained during the Isolation of ADC from AB355/pADC1

Part A. Non-denaturing gel electrophoresis of 3.3 μg gel-purified ADC in a 10% polyacrylamide gel utilizing the Davis (1964) discontinuous buffer system. Electrophoresis was conducted at 4°C at a constant current of 10 milliamps until the bromophenol blue in the sample buffer had migrated off the bottom of the gel. The gel was then stained for protein with Coomassie Brilliant Blue and destained as described for denaturing gel electrophoresis in Experimental Methods.

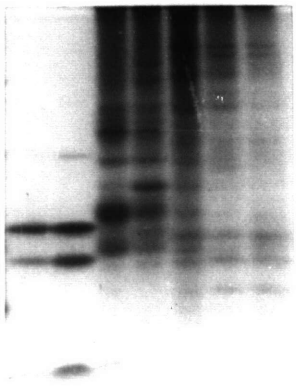
Part B. SDS polyacrylamide gel electrophoresis of the fractions obtained in the isolation of ADC. Samples were subjected to electroporesis in 10 cm x 1.5 mm 10-18% polyacrylamide gradient gels using the Hashimoto *et al.* (1983) system. Electrophoresis was conducted at 10 milliamps until the bromophenol blue in the sample buffer had migated off the end of the gel. The gel was then stained and destained as described in Experimental Methods. Samples were placed in SDS sample buffer and denatures 10 minutes in a boiling water bath.

Samples: 106 μg Crude lysate, 88 μg Heat Treated Lysate, 84 μg 40-60% Ammonium Sulfate Fraction, 31 μg DEAE Fraction, 52 μg AcA44 Fraction, 4.7 μg Hydroxylapatite Fraction and 2.4 μg gel-purified ADC.

A.


Gel Purified ADC

B.


Gel Purified ADC
Hydroxylapatite Fraction
AcA44 Fraction
DEAE Fraction
40-60% NH_4SO_4 Fraction
Heat Treated Lysate
Crude Lysate

3.12 Preparation of Antiserum Directed Against ADC

Previous work by Joanne Williamson in this laboratory had indicated the presence of a covalently attached pyruvoyl group associated with protein II was essential for decarboxylase activity (Williamson and Brown, 1979). It is obvious that the production of active enzyme requires post-translational attachment or generation of a pyruvoyl prosthetic group. Previous to completion of the subcloning of *panD*, we had decided to obtain antibodies directed against the native form of the enzyme. We hoped to use the antiserum to detect the presence of a proenzyme form by immunoprecipitation of the initial translational product.

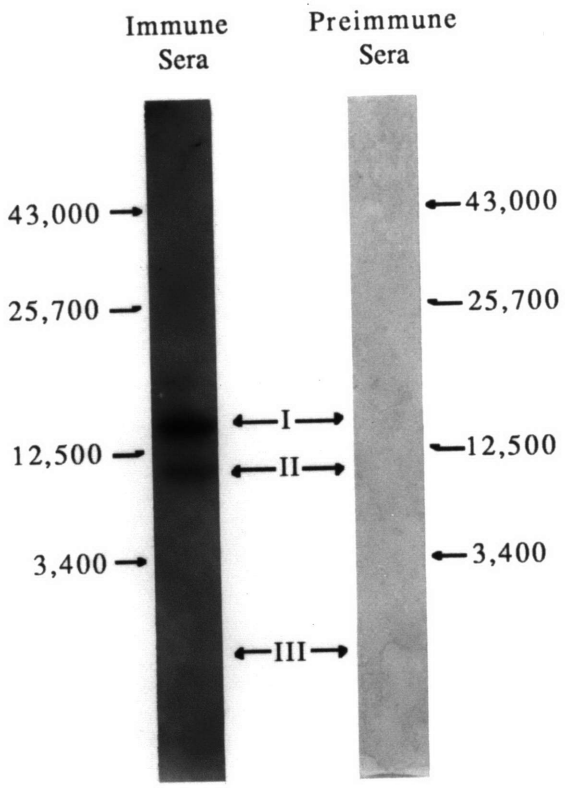
This was accomplished by inoculation of 4 male New Zealand white rabbits with gel-purified ADC as described in Experimental Methods. Preimmune serum was collected several weeks previous to inoculation and immune serum was collected at 7, 8, and 9 weeks after the second inoculation with the enzyme. The resulting antiserum was able to bind the ADC protein components that had been separated by SDS-PAGE and transferred to nitrocellulose as shown in Figure 3-22.

This figure indicates that proteins I and II both bind immunoglobulin present only in the immune serum, but protein III fails to provide any indication of bound antisera regardless of the length of exposure time. This failure to detect protein III with the antiserum was not due to poor transfer to the filter since staining of the filter

Figure 3-22

Analysis of the Protein Components of ADC with Antiserum following Transfer to Nitrocellulose

Details of the procedures may be found in Experimental Methods. Triplicate samples of ADC (13 μg) were subjected to SDS-PAGE as described in Figure 3-21. Molecular weight standards were also run in triplicate in lanes adjacent to the ADC. Following completion of electrophoresis, the proteins were transferred to nitrocellulose by vacuum blotting. The gel, following transfer, was stained for protein to determine electrophoretic mobility of ADC components and the molecular weight standards. The nitrocellulose was divided into three strips each containing both the ADC proteins and molecular weight standards. One strip was stained for protein to ascertain that the proteins had been transferred to the nitrocellulose. The remaining strips were then separately hybridized with 1:250 dilutions of either preimmune or 8th week immune serum from rabbit 3266 in BLOTTO buffer. Bound immunoglobulin was detected by the binding of [^{125}I]protein A. Detection of the [^{125}I]protein A was accomplished by autoradiography for 85 hours. The results are shown for both the preimmune and immune sera. The molecular weights provided are in daltons and were obtained form the co-electrophoresis of the following standards: ovalbumin (43,000 daltons), α-chymotrypsin (25,700 daltons), cytochrome C (12,500 daltons), and insulin β-chain (3,400 daltons). The central markers provide the location of the protein components I, II, and III of ADC.


Immune Sera
Preimmune Sera
43,000
25,700
12,500
3,400
I
II
III
43,000
25,700
12,500
3,400

for protein indicated that the protein had bound to the nitrocellulose. In addition, visualization of the bound immunoglobulin was also accomplished with horseradish peroxidase conjugated donkey anti-rabbit immunoglobulin with identical results (results not shown). This consistent failure of protein III to bind antiserum immunoglobulins perhaps indicates the loss of antigenic determinant(s) following SDS-PAGE and subsequent transference to nitrocellulose.

3.13 Analysis of the Initial Translational Product of *panD* Gene Product by Immunoprecipitation

We hoped to detect the presence of the proenzyme by immunoprecipitation from Maxicell extracts. The Maxicell procedure developed by Sancar *et al.* (1981) allows labeling of plasmid-encoded proteins with [^{35}S]methionine. Destruction of genomic DNA occurs by degradation via DNA excision and repair functions in RB901 (*recA*$^-$ *lexA*$^-$) strain following UV-induced damage provided by limited doses of UV radiation (Sancar *et al.*, 1979). Destruction of the genomic DNA allows labeling of only plasmid-encoded genes and short periods of labeling were expected to produce levels of proenzyme that could be detected by immunoprecipitation. This *in vivo* system was expected to allow us to detect an initial translational product prior to cleavage or modification that would generate the active form of the enzyme. This would possibly clarify the subunit structure by allowing us to follow maturation of the proenzyme to active ADC by pulse/chase experiments utilizing labeled methionine.

Extracts of pADC200 were immunoprecipitated with antiserum obtained at 7, 8, or 9 weeks after the second inoculation with ADC. The immunoprecipitations were then denatured with SDS and the extracts subjected to SDS-PAGE. The results are shown in Figure 3-23. A single protein was immunoprecipitated by all three of the immune sera and is absent from the preimmune serum lane. Identical results were obtained with all four rabbits. The immunoprecipitated protein displays an electrophoretic mobility identical to protein I of ADC. This suggested that the enzyme, as isolated, may contain a proenzyme form that produces the catalytic form of ADC by an autocatalytic cleavage step similiar to that seen with histidine decarboxylase (Recsei *et al.*, 1983b).

3.14 Determination of the Molecular Weight of the Protein Components of ADC

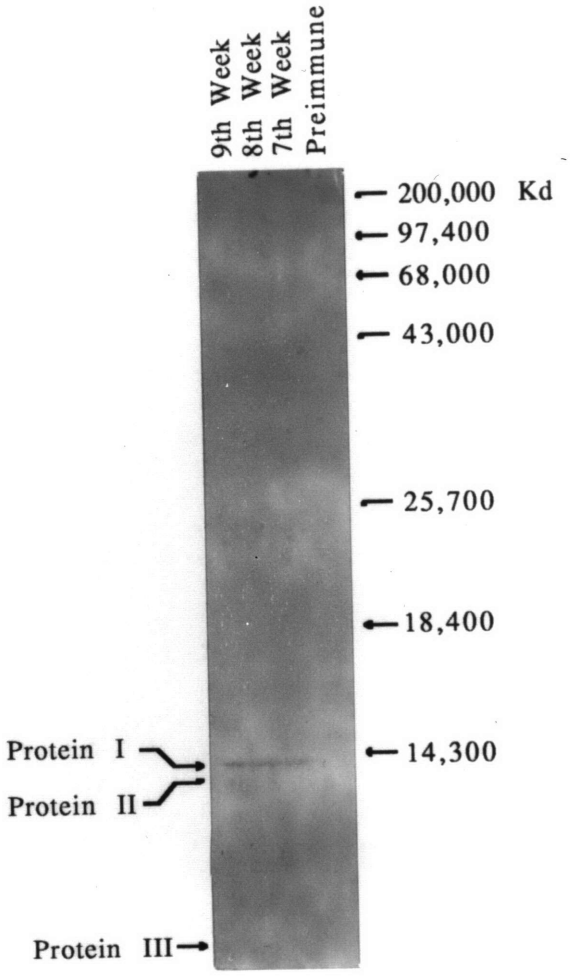
The molecular weight of the native form of the enzyme was determined by Joanne Williamson to be 58,000 daltons by gel exclusion chromatography (Williamson and Brown, 1979). Williamson had also determined the molecular weights (M_r) by SDS-PAGE of the protein components of ADC to be 11,800, 9,800 and 6,400 daltons which correspond to proteins I, II, and III, respectively. This indicated an $\alpha_2\beta_2\gamma_2$ subunit structure. However, the results obtained by Williamson (1977) provided only very poor resolution of proteins I and II and required extrapolation of the standard curve in order to provide molecular weights for proteins II and III. In addition, the result of staining for protein with Coomassie Blue suggested that the proteins were not present in stoichiometric quantities (Williamson, 1977). This lack

Figure 3-23

The Initial Translational Product of the *panD* Gene

Plasmid encoded proteins were labeled by the Maxicell technique as described in Experimental Methods. A 10 ml culture of RB901/pADC200 was labeled with 25 μCi of L-[^{35}S]methionine for 15 minutes. The cells were lysed with lysozyme in preparation for immunoprecipitations as described. Each immunoprecipitation contained lysate from the equivalent of 0.5 ml of the original culture and was incubated with 10 μl of a 1:250 dilution of the indicated serum from rabbit 3266. The immunoglobulins were precipitated with protein-A Sepharose CL-4B. The resulting precipitates were resuspended in SDS sample buffer and analyzed by SDS-PAGE in the 10-18% polyacrylamide gradient gels of the Hashimoto *et al.* (1983) gel system. Gels were prepared and analyzed as described in Experimental Methods.

7th, 8th, and 9th week refers to serum collected from rabbit 3266 at 7, 8, and 9 weeks after the second inoculation with ADC in Complete Freunds Adjuvant. Preimmune serum was collected 1 week before the first inoculation with ADC in Complete Freunds Adjuvant.


9th Week
8th Week
7th Week
Preimmune
200,000 Kd
97,400
68,000
43,000
25,700
18,400
14,300
Protein I
Protein II
Protein III

of an obvious stoichiometry and accurate molecular weights had initially indicated that new estimations of the molecular weights would be a prerequisite for ascertaining the correct subunit structure.

Initially, long polyacrylamide gradient gels (>20 cm in length) had provided the highest resolution, however, they still resulted in only poor resolution of proteins I and II and diffuse bands after staining with Coomassie Brilliant Blue which precluded the determination of accurate molecular weights. The discovery of the SDS-PAGE system of Hashimoto *et al.* (1983) provided a method with resolving power far superior to other systems available for the electrophoresis of the small proteins. This system utilizes a 10-18% polyacrylamide gradient containing 7 M urea and a 1-10% sucrose gradient which stabilizes the acrylamide gradient during preparation of the gel. Another important aspect of this system was a change in the acrylamide:bis ratio from the 29.2:.8% (weight/volume) ratio used by Davis (1964) to 20:1.

Denatured ADC was subjected to electrophoresis in the Hashimoto system using the cyanogen bromide cleavage products of cytochrome C and myoglobin as standards for the gel shown in Figure 3-24. The molecular weights for proteins I, II, and III were obtained from a semi-logarithmic plot of the molecular weight versus the electrophoretic mobility as shown in Figure 3-25. The average of four separate determinations provided molecular weights of 14,650, 10,580, and 2,230 daltons for proteins I, II, and III, respectively. The molecular weight for protein I was determined from the high molecular weight standards while those for proteins II and III were obtained from the low molecular weight standards. The biphasic nature of the regression analysis was highly reproducible. Protein I lies at this discontinuity of

Figure 3-24

Molecular Weight Determination of the Protein Components of ADC: SDS Polyacrylamide Gel Electrophoresis

2.4 μg of gel-purified ADC was subjected to SDS-PAGE utilizing the Hashimoto *et al.* (1983) gel system in a 1.5 mm x 10 cm gel. Electrophoresis was conducted as for Part B of Figure 3-21. Molecular weights are given in daltons.

Molecular weight standards: 20 μg cytochrome C cyanogen bromide fragments, 20 μg myoglobin cyanogen bromide fragments, 3 μg ovalbumin, 2 μg α-chymotrypsin, 2 μg cytochrome C, and 2 μg insulin β-chain.

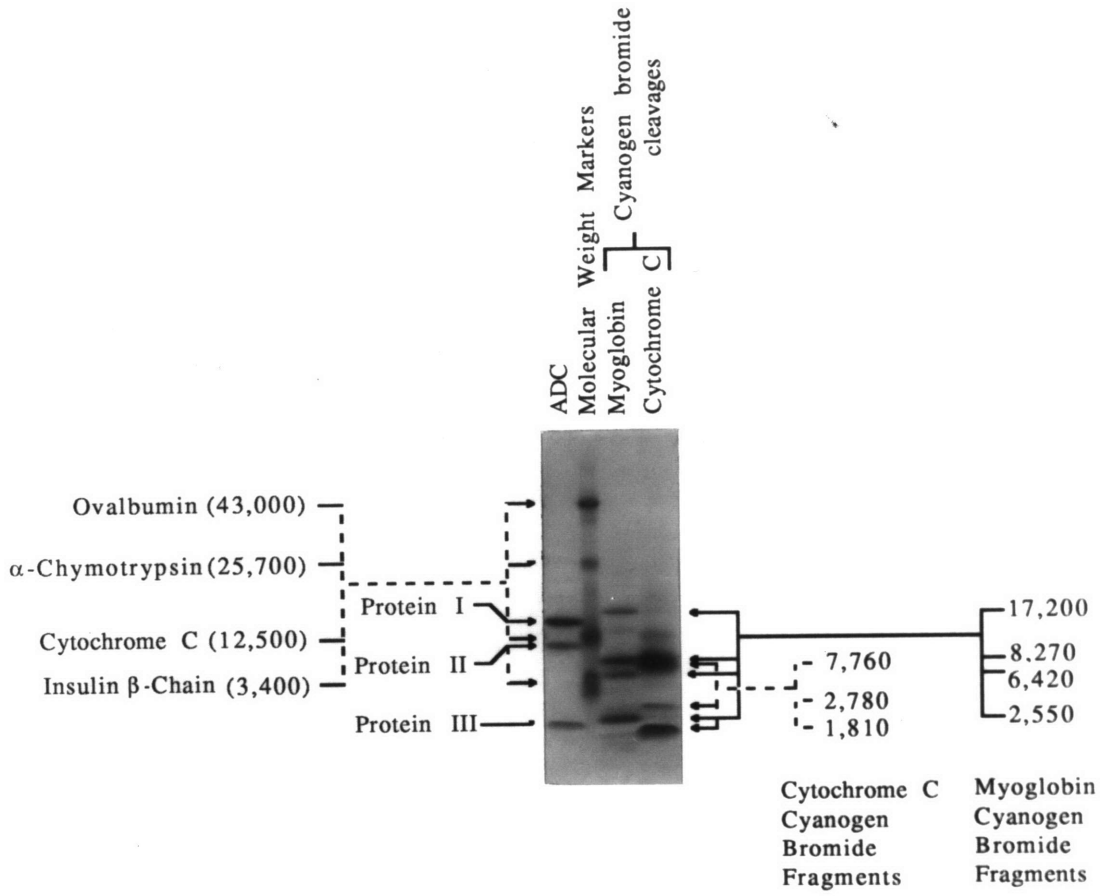
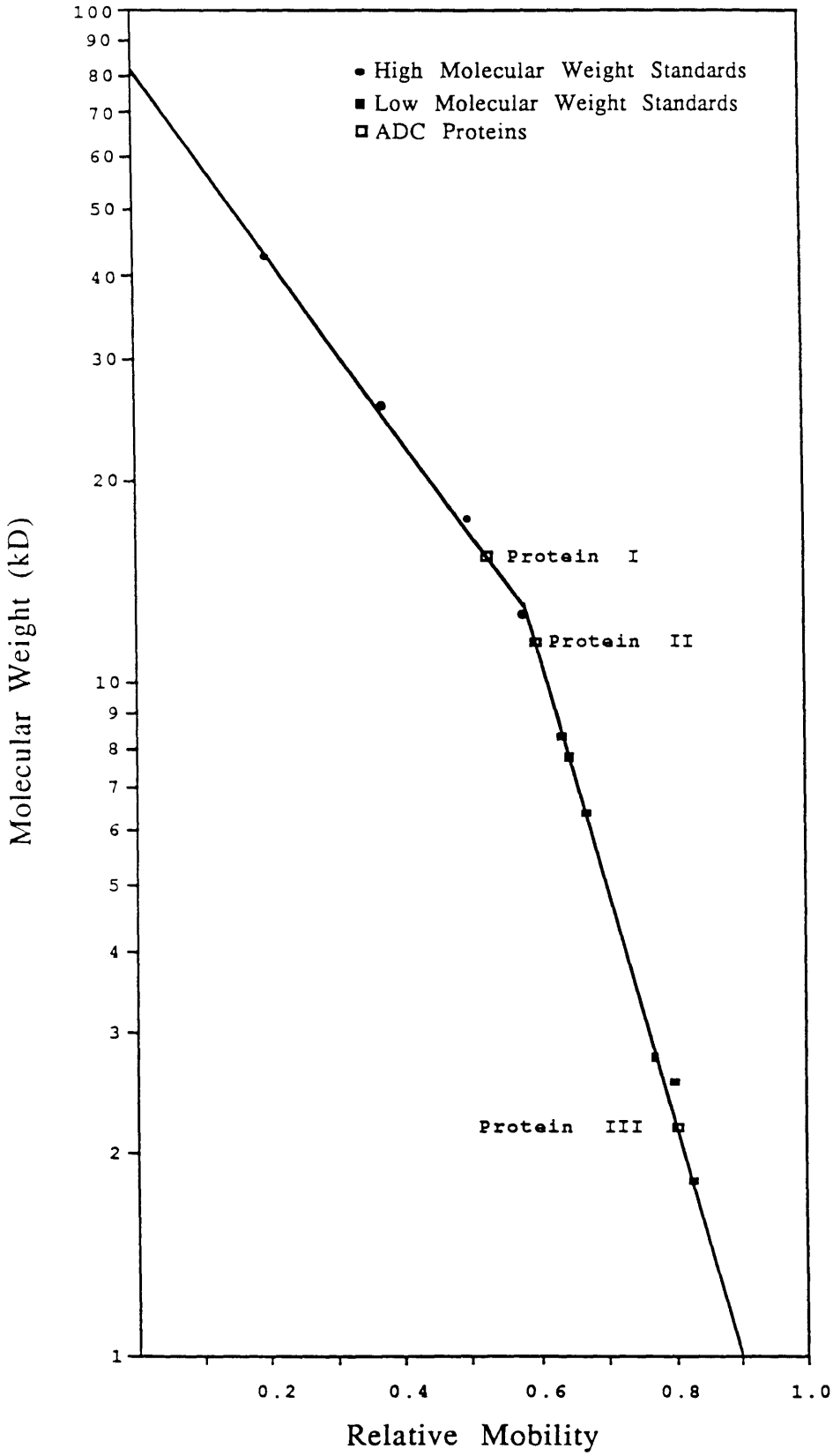

ADC
Molecular Weight Markers
Myoglobin
Cytochrome C
Cyanogen bromide cleavages
Ovalbumin (43,000)
α-Chymotrypsin (25,700)
Cytochrome C (12,500)
Insulin β-Chain (3,400)
Protein I
Protein II
Protein III
17,200
8,270
6,420
2,550
7,760
2,780
1,810
Cytochrome C Cyanogen Bromide Fragments
Myoglobin Cyanogen Bromide Fragments

Figure 3-25

Molecular Weight Determination of the Protein Components of ADC

The Molecular weights (M_r) obtained from Figure 25 were plotted versus their relative mobility with respect to the dye Bromophenol Blue. The molecular weight standards are divided into High and Low Molecular weight regions and with the curves derived by linear regression. The molecular weight of Protein I was determined from the high molecular weight standard curve while the molecular weights for Proteins II and III were determined from the low molecular weight curve.


High Molecular Weight Standards
Low Molecular Weight Standards
ADC Proteins
Protein I
Protein II
Protein III
Molecular Weight (kD)
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
0.2
0.4
0.6
0.8
1.0
Relative Mobility

the standard curves and as a result assignment of a molecular weight required deciding which curve provided an accurate estimation. The M_r reported here was determined from the curve generated by the high molecular weight standards based on the observation that protein I comigrates with lysozyme (results not shown) and in this system, the molecular weight of lysozyme (14,300 daltons) was best approximated from the high molecular weight standards.

The activation of the protein I by a mechanism analogous to that proposed for the activation of HDC (Recsei *et al.*, 1983) requires the cleavage of protein I into two smaller proteins. The mechanism indicates only a single cleavage which occurs without any loss of protein from the enzyme molecule. Under such circumstances, the sum of the molecular weights of proteins II and III would be anticipated to equal that of protein I. However, there is a discrepancy of 1840 daltons between the sum of the molecular weights of proteins II and III and the reported molecular weight of protein I. This can be viewed as a loss of protein upon multiple cleavages of the precursor, but is more likely an indication of the inaccuracy of determining the molecular weights of small proteins in urea containing gels.

Large anomolies in the molecular weights determined for several proteins, with molecular weights greater than 10,000 daltons, in the presence of urea and SDS (36% for ribonuclease A) have been noted previously (Dunker and Rueckert, 1969; Swank and Munkres, 1971) and errors of 5% to 6% from the actual molecular weight were common (Dunker and Rueckert, 1969). The discrepencies appear to be more pronounced for small peptides (below 10,000 daltons) and Swank and Munkres (1971) view the molecular weights determined for such proteins as only a "first

approximation". They attribute anomalous behavior in such systems to conformational effects of the protein in response to the denaturants and the poor masking by SDS of the intrinsic charges in small proteins. The small size of the proteins would be anticipated to enhance the magnitude of these effects and result in greater inaccuracies within the low molecular weight range.

The results of the molecular weight determinations reported here provide more accurate molecular weights for proteins I and II than those previously reported by Joanne Williamson (Williamson and Brown, 1979). The subunit structure of $\alpha_2\beta_2\gamma_2$ suggested by Joanne Williamson's data remains consistent. If the discrepancy between the sum of the molecular weights of proteins II and III compared to that of protein I is attributed to the inaccuracy of the gel system, then the current molecular weights strengthen the proposition that proteins II and III were obtained in stoichiometric quantities by a proteolytic cleavage of protein I.

3.15 Analysis of the Protein Components of ADC by Limited Proteolysis with V8 Protease

This putative relationship between the ADC proteins could be readily demonstrated by the limited proteolytic mapping method of Cleveland *et al.*, (1977). If the lower molecular weight proteins are derived from protein I then limited proteolysis of the separated subunits should reveal proteolytic fragments in common for the related peptides when the digests are subjected to SDS-PAGE. Initial studies indicated that ADC is highly resistant to proteolytic digestion.

Figure 3-26. Predicted Proteolytic Cleavage Sites within the Translated Peptide Sequence of the *panD* ORF. Crosses indicate the distance from the amino terminal methionine residue.

```
  P  P PPP P P        CVP↓       CP   P PV V    C
MIRTMLQGKLHRVKVTHADLHYEGSCAIDQDFLDAAGILENEAIDIWNVT
         +         +         +         +         + 50

 PPPC  C    VPP P     P        P       C     VV P
NGKRFSTYAIAAERGDRIISVNGAAAHCASVGDIVIIASFVTMPDEEART
         +         +         +         +         + 100

CP    CCVP  V PP  P
WRPNVAYFEGDNEMKRTAKAIPVQVA
         +         +
```

↓ = Putative Proenzyme Cleavage Site
C = Chymotrypsin
P = Papain
V = V8 Protease

Both papain and chymotrypsin failed to cleave the enzyme at all levels tested. At the highest concentrations, 161 milliunits (2.4 ug) of chymotrypsin or 65 milliunits (2.4 ug) of papain, fragments resulting from the auto-degradation of the proteases are apparent. This lack of cleavage occured despite the presence of cleavage sites predicted from the translated sequence as shown by the proteolytic map given in Figure 3-26.

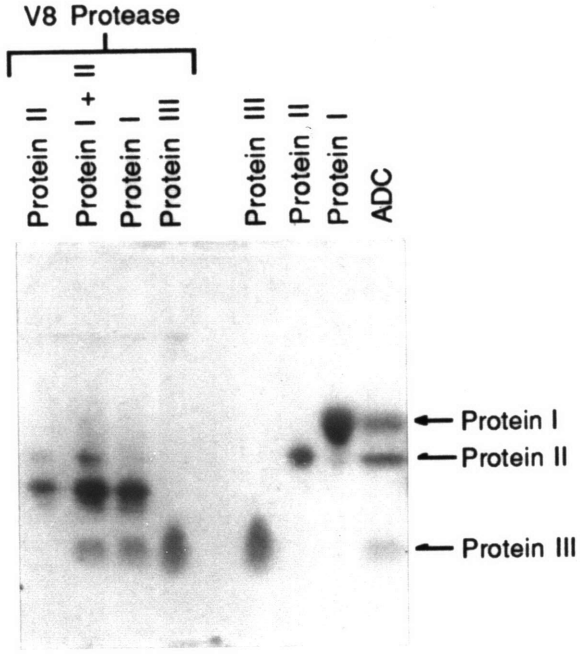
Initial experiments with *Staphylococcus aureus* V8 protease indicated that ADC was digested rapidly to a limit digest that was independent of the concentration of protease or the length of the incubation period. The results of the proteolysis of the purified protein components of ADC can be seen in Figure 3-27. Protein I is cleaved into a minimum of 6 bands of which 4 are coincident with

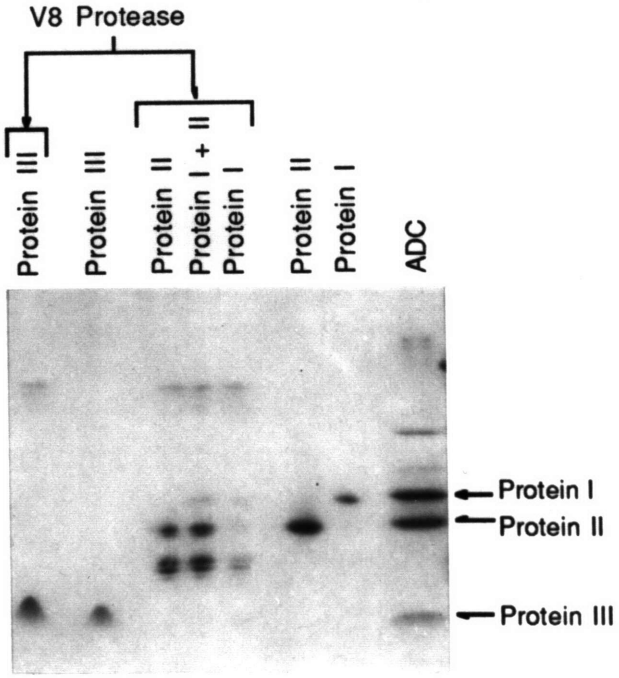
Figure 3-27

Analysis of the Protein Components of ADC by *S. auereus* V8 Protease Mapping

Upper Gel. The individual protein components of ADC were prepared by the electrophoresis of 14.5 μg per lane of gel-purified ADC for the isolation of proteins I and III by denaturing SDS-PAGE with the Hashimoto *et al.* (1983) gel system as described in Experimental Methods Section 2-14. Protein II was isolated by the electrophoresis of 4.8 μg of gel-purified ADC. Analysis of each individual protein was determined with a gel slice containing a protein from a single lane of the preparative gel and conducted as described in Experimental Methods with 0.5 μg of *S. auereus* V8 protease (0.27 units, 542 units/mg) in a 10% glycerol overlay and an incubation period of 30 minutes in the stacking gel. The mixed digests utilize a single gel slice of both proteins I and II. Analysis of the resulting proteolytic fragments was performed by SDS-PAGE as used for preparation of the proteins.

Lower Gel. Analysis was conducted as above with the protein components of ADC for the peptide mapping being prepared by the denaturing gel electrophoresis of 4.8 μg per lane of gel-purified ADC.


V8 Protease
Protein II
Protein I + II
Protein I
Protein III
Protein III
Protein II
Protein I
ADC
Protein I
Protein II
Protein III


V8 Protease
Protein III
Protein III
Protein II
Protein I + II
Protein I
Protein II
Protein I
ADC
Protein I
Protein II
Protein III

fragments from protein II as can be seen from in the mixed proteolytic digests of proteins I and II. Analysis of the fragments predicted from the proteolytic map indicates that the patterns obtained with proteins I and II may have resulted from incomplete digests. The largest fragment predicted for protein I, 3,357 daltons, is less than molecular weights of the three largest fragments obtained from the digests of both protein I and II (M_r = 5.3, and 4,6 and 3.9 kd).

In addition, the largest and smallest of the six fragments obtained in the digestion of protein I appear identical in size to proteins II and III, respectively. It should be noted from Figure 3-26, that a cleavage site exists between residues 23 and 24 for the V8 protease of *Staphylococcus aureus*. This would generate an amino-terminal fragment with a molecular weight of 2.22 kd, very close to the molecular weight of protein III (M_r = 2.23 kd), which would be resistant to further proteolysis by V8. A fragment exhibiting such properties is seen in the digest of protein I in Part A of Figure 3-27 and is missing from the digest of protein II. The molecular weight of the carboxy-terminal protein would be 11.3 kd which is similiar to protein II (M_r = 10.6 kd) and the fragment seen in a digest of protein I in part A.

The most important aspect of the experiment are the mixed digests of proteins I and II that indicate that the fragments generated from each protein were sufficiently similiar that the patterns generated during the individual digests were preserved in the mixed digests. The individul patterns are most clearly seen in the gel in Part B of this figure and the mixed digest provides a clear indication of this pattern. This suggests that proteins II and III are generated from protein I by a single cleavage, in the vicinity of the predicted V8 protease site, at

the serine residue (position 23 of the translated sequence) as indicated in Figure 3-26.

3.16 Amino Terminal Sequence Analysis of the Protein Components of ADC

Under such circumstances, we felt that correlation of the protein sequence to that obtained by translation would provide considerable evidence that all the ADC proteins where encoded by the single *panD* locus. ADC was prepared by purification from lysates of AB355/pADC1 as described in Experimental Methods and summarized in Table 3-6. Gel purified ADC was then separated into its polypeptide components by reducing preparative SDS-PAGE without further derivitization of the protein. Following identification and excision of the individual protein components from the preparative gel, the gel slices were submitted to the Harvard Microchemistry Facility. Amino acid composition and amino terminal sequence analyses were conducted on the electroeluted proteins. In addition, a sample of protein II was submitted for analysis that had been obtained from enzyme that had been subjected to reductive amination prior to preparative SDS-PAGE. This procedure produces an alanine residue from the pyruvoyl prosthetic group allowing subsequent amino-terminus sequencing when this prosthetic group is attached to the amino terminus of a peptide. This procedure had been developed by Snell and coworkers to allow sequencing of the α-subunit of histidine decarboxylase which contains the pyruvoyl prosthetic group (Huynh *et al.*, 1984b).

The relationship of the three proteins was obvious when the results of the amino acid sequencing analyses were compared to the

translated sequence of the *panD* ORF as shown in Figure 3-28. Proteins I and III share the first six amino acids in common and correspond to the amino terminus of the translated sequence. Protein II corresponds, with the exception of its amino terminus, with residues 26→30 of the translated sequence.

However, the amino terminus of the protein contains an alanine residue in the place of the serine at position 25 of the translated sequence. This was an unusual, albeit anticipated, finding since it was known that Protein II contains the pyruvoyl moiety that is essential for the decarboxylase activity (Williamson and Brown, 1979). This prosthetic group usually occupies an amino terminal position (Huynh et al., 1984; Prozorovski et al., 1975; Huynh and Snell, 1985; Anton and Kutny, 1987) and requires reductive amination of the pyruvoyl group to alanine before sequencing of the peptide is possible. We postulate that this alanine residue is derived from the pyruvoyl prosthetic group by means of a decarboxylative transamination of the aspartate substrate (see Discussion). This assumes that the pyruvoyl group is generated from the amino terminus from the serine residue encoded by the DNA sequence via a proteolytic activation process similiar to that seen with histidine decarboxylase of *Lactobacillus* 30a (Recsei *et al.*, 1983).

As had been mentioned previously, ADC had been subjected to reductive amination by the procedure of Huynh *et al.* (1984b) in anticipation of the inability to sequence the amino terminus of protein II. Nonetheless, the amino acid composition and amino terminal sequence analyses (results not shown) of the modified protein II were identical to the results obtained with protein II from the unmodified enzyme. The amino terminus appeared to be completely accesible to modification by

Figure 3-28

The Amino Terminal Sequences of the Proteins of ADC and their Alignment to the Translated Sequence of the *panD* ORF

The partial sequences of the three protein components of ADC were determined by amino-terminal sequence analysis by William Lane of the Harvard Microchemistry Facility through identification of phenylthiohydantoin amino acids obtained from amino terminal derivitization and subsequent cleavage. The purified proteins were obtained by electroelution of proteins separated by SDS-PAGE. The partial amino acid sequences are aligned with the matching sequences obtained from the tranlsated sequences of the *panD* ORF as given in Figure 3-19. The sequences are shown starting at the amino terminus. The distance of the underlined amino acid from the amino terminus of the translated *panD* sequence is provided.

10
Met Ile Arg Thr Met Leu Gln Gly Lys Leu His Arg Val Lys Val

Met Ile Arg Thr Met Leu Gln Gly Lys Leu... **Protein I**
Met Ile Arg Thr Met Leu... **Protein III**

20 30
Thr His Ala Asp Leu His Tyr Glu Gly Ser Cys Ala Ile Asp Gln

Protein II Ala Cys Ala Ile Asp Gln...

40
Asp Phe Leu Asp Ala Ala Gly Ile Leu Glu Asn Glu Ala Ile Asp

50 60
Ile Trp Asn Val Thr Asn Gly Lys Arg Phe Ser Thr Tyr Ala Ile

70
Ala Ala Glu Arg Gly Ser Arg Ile Ile Ser Val Asn Gly Ala Ala

80 90
Ala His Cys Ala Ser Val Gly Asp Ile Val Ile Ile Ala Ser Phe

100
Val Thr Met Pro Asp Glu Glu Ala Arg Thr Trp Arg Pro Asn Val

110 120
Ala Tyr Phe Glu Gly Asp Asn Glu Met Lys Arg Thr Ala Lys Ala

Ile Pro Val Gln Val Ala
126

phenylisothiocyanate in spite of the attachment of the pyruvoyl prosthetic group to this protein (Williamson and Brown, 1979).

The ability to sequence the amino terminus of the unmodified protein II suggests that the enzyme as isolated has a considerable quantity of peptide I bearing an amino terminal alanine residue. Based on the indicated assumptions, the enzyme as isolated may be largely inactive and this appears to correlate with the single pyruvoyl group found per enzyme molecule (Williamson and Brown, 1979).

3.17 Analysis of the Amino Acid Composition of the Protein Components of ADC Proteins

The results of the amino acid composition analyses are given in Tables 3-7, 3-8, and 3-9 for proteins I, II, and III, respectively. The expected results for proteins II and III are based on an assumed cleavage between the glycine and serine at positions 24 and 25 of the translated sequence of Figure 3-14. Protein I was taken to be dervied from residues 1-24 and Protein II from residues 25-126. For protein II, the serine at position 25 was taken to be an alanine residue.

The results of the amino acid analyses were rather disappointing with high percent errors obtained between the expected and obtained values as can be observed in the tabulations. This problem appears to be somewhat exaggerated by the small size of the proteins involved (14.7, 10.6, and 2.2 kd) and is particularly apparent in the results for protein III. Nonetheless, despite two separate analyses, the errors obtained were similiar and appear to be consistently high for several specific residues.

Table: 3-7 Predicted and Observed ADC Protein I Amino Acid Compositions.

Amino Acid Residue	mol/mol enzyme predicted	mol/mol enzyme observed	Error (%)
Ala	18	19	6
Arg	8	5	38
Asx	14	16	14
Cys	2	ND	-
Glx	11	14	27
Gly	8	19	138
His	4	3	25
Ile	12	9	25
Leu	5	6	20
Lys	5	4	20
Met	4	1	75
Phe	4	1	75
Pro	3	2	33
Ser	6	1	83
Thr	7	2	71
Trp	2	ND	-
Tyr	3	1	67
Val	10	9	10
Total residues = 126		Average error = 34%	

ND = Not determined

Table: 3-8 Predicted and Observed ADC Protein II Amino Acid Compositions.

Amino Acid Residue	mol/mol enzyme predicted	mol/mol enzyme observed	Error (%)
Ala	17	25	47
Arg	6	5	17
Asx	13	8	39
Cys	2	ND	-
Glx	9	6	33
Gly	6	12	100
His	1	0	*
Ile	11	10	9
Leu	2	4	100
Lys	3	3	0
Met	2	1	50
Phe	4	3	25
Pro	3	2	33
Ser	6	2	67
Thr	5	2	60
Trp	2	ND	-
Tyr	2	0	*
Val	8	9	13
Total residues = 102		Average error = 44%	

ND = Not determined
* = Not meaningful

Table: 3-9 Predicted and Observed ADC Protein III Amino Acid Compositions.

Amino Acid Residue	mol/mol Enzyme Predicted	mol/mol Enzyme Observed	Error (%)
Ala	1	2	100
Arg	2	1	100
Asx	1	2	100
Cys	0	ND	-
Glx	2	3	50
Gly	2	3	50
His	3	1	67
Ile	1	1	0
Leu	3	3	0
Lys	2	1	50
Met	2	1	50
Phe	0	1	*
Pro	0	1	*
Ser	0	1	*
Thr	2	1	50
Trp	0	ND	-
Tyr	1	1	0
Val	2	2	0
Total residues = 24		Average Error = 41%	

ND = Not determined
* = Not meaningful

The observed value for glycine is considerably higher than the predicted value for both proteins I and II which may be the result of contamination by the glycine component of the discontinuous buffer system used to isolate the proteins. This residue frequently presents a problem with proteins obtained from such systems (David Andrews, personal communication). The spuriously high values obtained for alanine for protein II is also a common contaminant of proteins isolated by SDS-PAGE (David Andrews, personal communication). Additionally, methionine, proline, serine, and threonine are consistently low for proteins I and II. The low values for threonine and serine were not particularly surprising in light of the known instability of these residues to the conditions used for the hydrolysis of peptides (Chang, 1979). The anomolously high error for results obtained for leucine and methionine with protein II serves as an indication of the unusually large errors obtained when small number of residues are present as in the peptides of ADC. A small difference (1) between the number of observed methionine (1) residues and predicted (2) results in a large error. The large error obtained for proline is not readily explained.

The results with protein III are particularly illustrative of the difficulties inherent in these comparisons. In several cases, for phenylalanine, proline, and serine where no residues had been predicted, the analysis indicates the presence of single residues possibly reflecting a high background of amino acid contamination with this protein.

Graphical representations of the results provide a better sense of the level of correspondence obtained between the predicted and the

observed number of residues. The results are presented in Figures 3-29, 3-30, and 3-31 for proteins I, II, and III, respectively. In each case, and particularly for proteins I and II, the general trend indicates that the greatest anomaly is obtained with glycine. For the other residues, the general trend of obtained to predicted can be seen to correspond fairly well. The amino acids predicted to be abundant are generally found in larger quantities and those predicted to be in low abundance are generally found in lesser quantities.

3.18 Analysis of the Initial Translational Product of the *panD* Gene in Maxicells and Evidence for its Post-translational Processing

The initial results obtained with immunoprecipitations of Maxicell extracts presented in Section 3.13 suggested that a single polypeptide was the product of the *panD* locus. However, the expression of this protein had not yet been fully linked to the expression of the putative *panD* ORF. The initial results were repeated using the Hashimoto gel system. Re-examination of the plasmid-encoded proteins provided some indications that processing of the putative proenzyme of protein I to proteins II and III in the UV-treated cells, albeit at barely discernable levels.

The production of the the protein seen in the Maxicell extracts by expression of *panD* was also confirmed by their absence in extracts of the *panD* deletion plasmid pADC2001 as shown in Figure 3-32. The intense band obtained in the *panD* subclone pADC200/RB901 corresponds to protein I of ADC and is absent from the extracts of both pBR322 and pADC2001 bearing strains. This protein is readily precipitated with antiserum

Figure 3-29

Amino Acid Composition of the ADC Protein I

The amino acid composition of protein I was determined by David Andrews of the Harvard Microchemistry Facility. Protein I was obtained by preparative denaturing gel electrophoresis using the 10-18% polyacrylamide gradient gels of the Hashimoto *et al.* (1983) gel system as described in Experimental Methods. The results represented graphically here are also provided in Table 3-7.

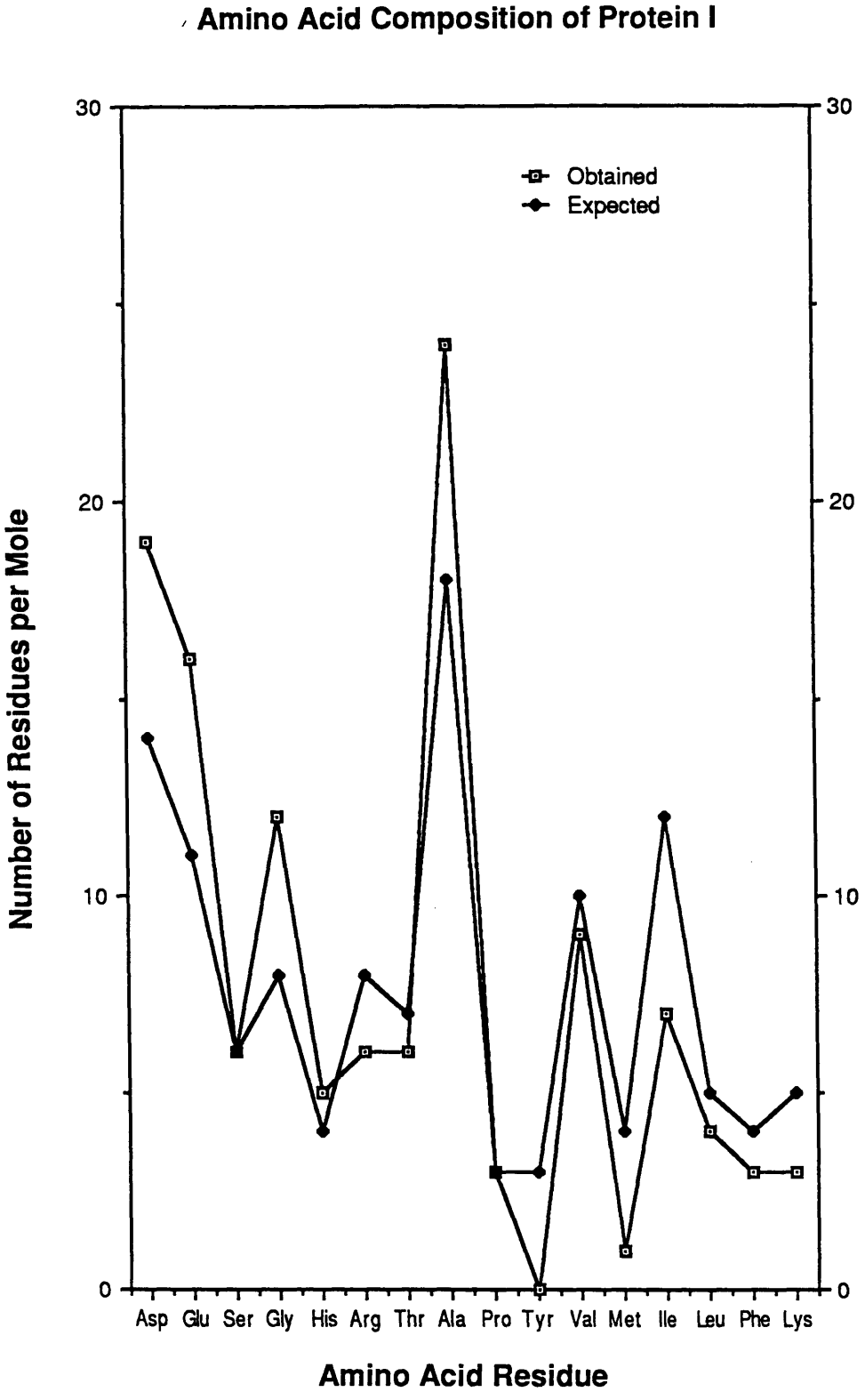

Amino Acid Composition of Protein I
Obtained
Expected
Number of Residues per Mole
30
20
10
0
Asp Glu Ser Gly His Arg Thr Ala Pro Tyr Val Met Ile Leu Phe Lys
Amino Acid Residue

Figure 3-30

Amino Acid Composition of the ADC Protein II

The amino acid composition of protein II was determined by David Andrews of the Harvard Microchemistry Facility. Protein II was obtained by preparative denaturing gel electrophoresis using the 10-18% polyacrylamide gradient gels of the Hashimoto *et al.* (1983) gel system as described in Experimental Methods. The results represented graphically here are also provided in Table 3-8.

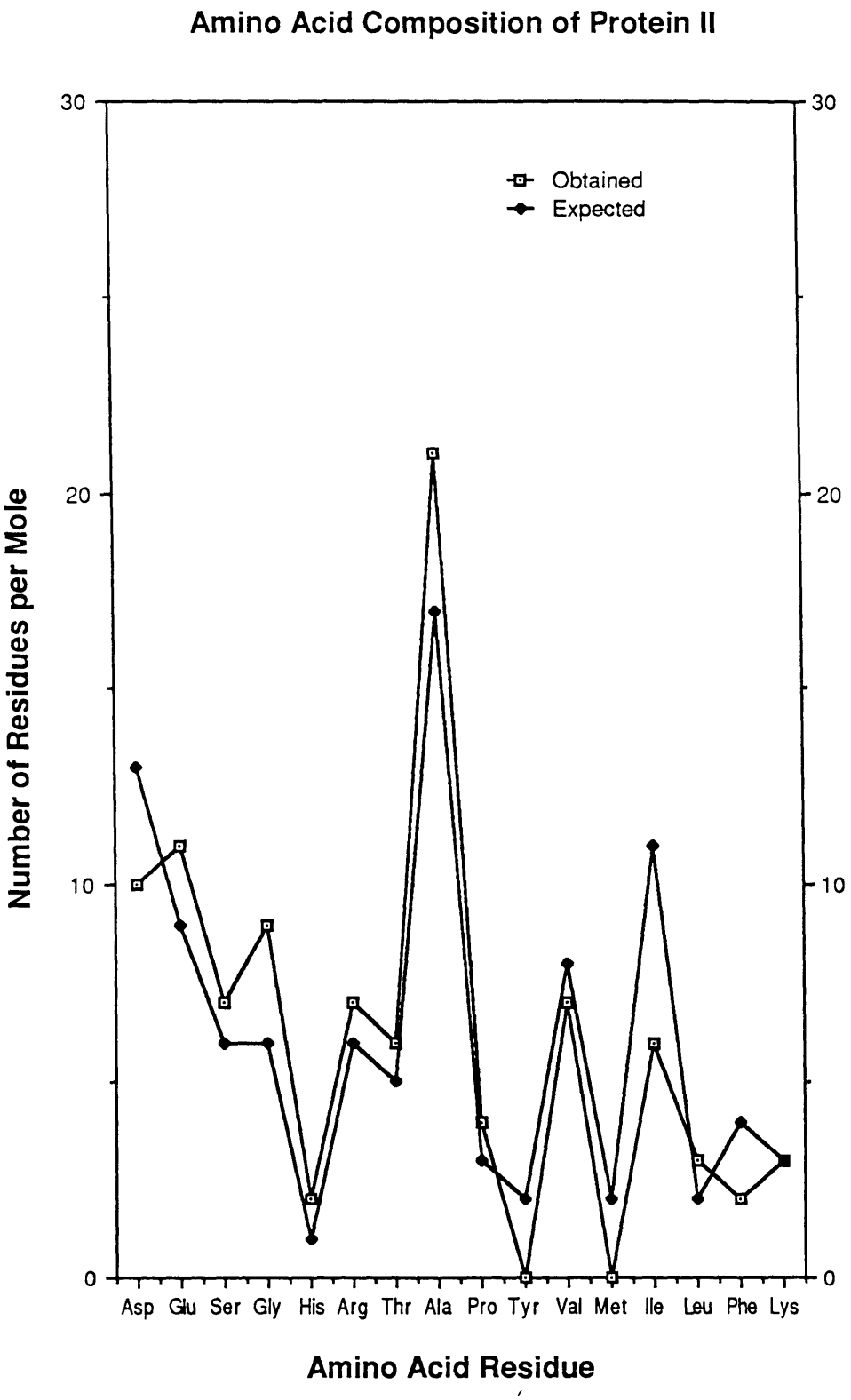

Amino Acid Composition of Protein II
Obtained
Expected
Number of Residues per Mole
30
20
10
0
Asp Glu Ser Gly His Arg Thr Ala Pro Tyr Val Met Ile Leu Phe Lys
Amino Acid Residue

Figure 3-31

Amino Acid Composition of the ADC Protein III

The amino acid composition of protein III was determined by David Andrews of the Harvard Microchemistry Facility. Protein II was obtained by preparative denaturing gel electrophoresis using the 10-18% polyacrylamide gradient gels of the Hashimoto *et al.* (1983) gel system as described in Experimental Methods. The results represented graphically here are also provided in Table 3-9.

Amino Acid Composition of Protein III

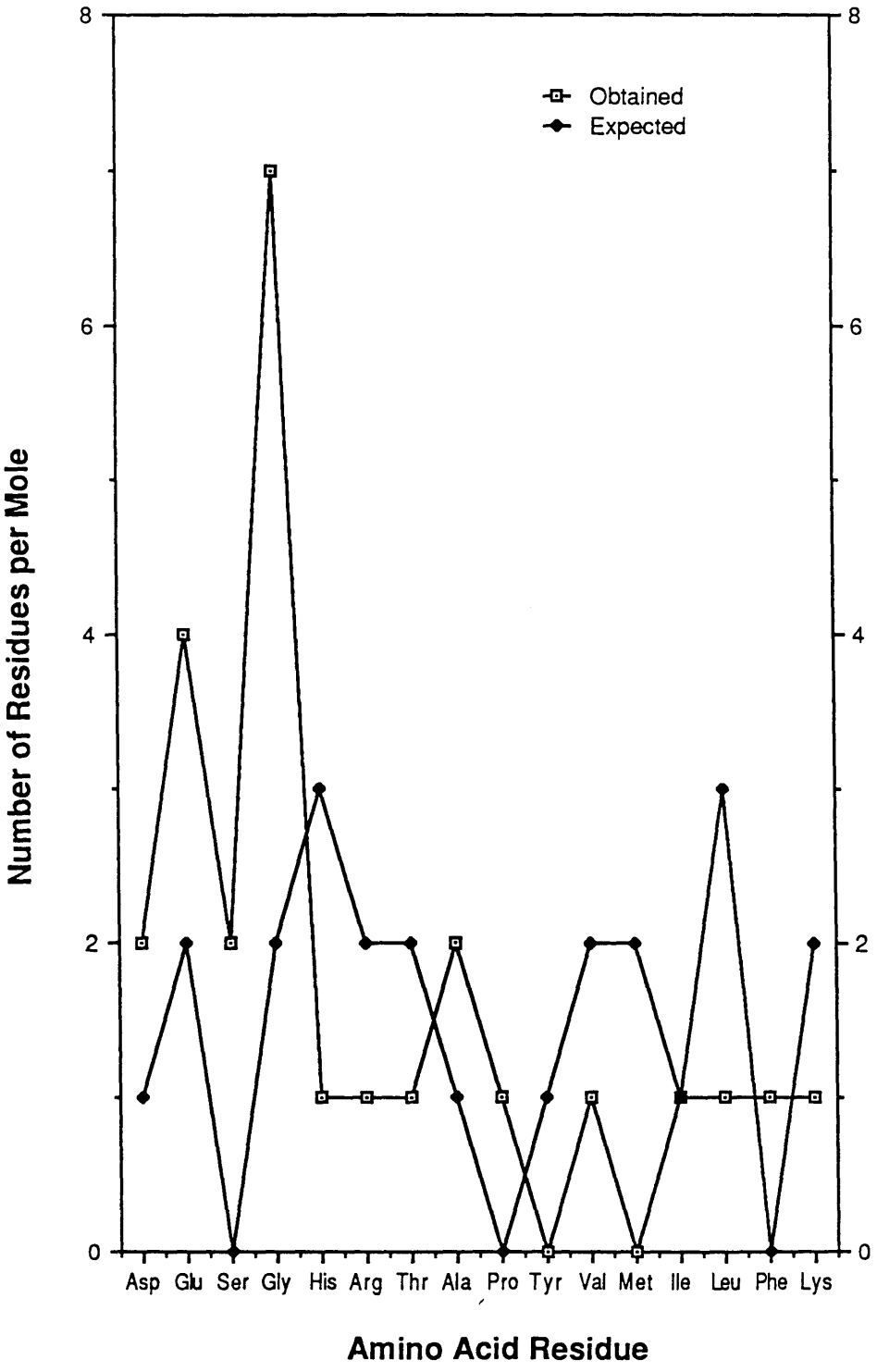


Figure 3-32

Analysis of the Gene Product of *panD* by Immunoprecipitations from the *panD* Subclone pADC200 and the Deletion Plasmid pADC2001

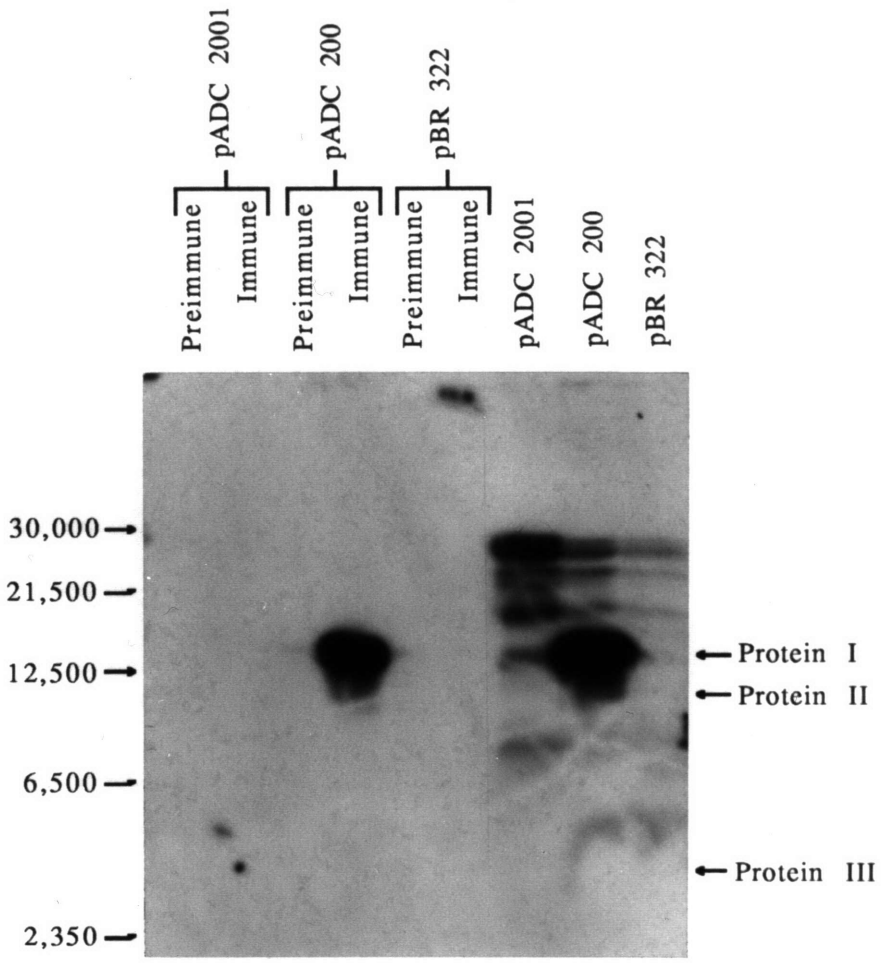
Plasmid encoded proteins were labeled by the Maxicell technique as described in Experimental Methods. 10 ml cultures of the strains RB901/pBr322, RB901/pADC200, and RB901/pADC2001 were labeled with 25 μCi each of L-[^{35}S]methionine for 15 minutes. The cells were lysed with lysozyme in preparation for immunoprecipitations as described for Maxicells. Analysis of the Maxicell extracts and immunoprecipitations was conducted by SDS 10-18% polyacrylamide gradient gel electrophoresis using the Hashimoto *et al.* (1983) gel system. Upon completion of electrophoresis, the gel was stained for protein and prepared for fluorography as described in the Experimental Methods section 2.8.

Maxicell Extracts. Direct analysis of denatured Maxicell extracts are designated by the plasmid harbored by the strain. Each analysis is conducted on cells from the equivalent of 2 ml of the original culture resuspended in SDS sample buffer and denatured for 10 minutes in a boiling water bath.

Immunoprecipitations. Immunoprecipitations were conducted with lysate, from the strain bearing the plasmid indicated in the figure, that represented the equivalent of 2 ml of the original culture. Immunoprecipitations were conducted by incubation of the Maxicell extracts with 10 μl of a 1:100 dilution of either the preimmune or the

8th week immune serum of rabbit 3266. Immunoglobulins were then precipitated with Protein-A Sepharose CL-4B as described in Experimental Methods. The resulting precipitates were resuspended in SDS sample buffer and denatured for 10 minutes in a boiling water bath.

The positions of the protein components I, II, and III are indicated by the arrows and were determined by the co-electrophoresis of gel-purified ADC with the Maxicell extracts. The position of molecular weight markers are indicated for: carbonic anhydrase (30,000 daltons); soybean trypsin inhibitor (21,500 daltons); cytochrome C (12,500 daltons); aprotinin (6,500 daltons); and insulin α-chain (2,350 daltons).


pADC 2001
pADC 200
pBR 322
Preimmune
Immune
Preimmune
Immune
Preimmune
Immune
pADC 2001
pADC 200
pBR 322
30,000
21,500
12,500
6,500
2,350
Protein I
Protein II
Protein III

directed against ADC from extracts of pADC200/RB901 while the preimmune lane appears devoid of any antigenic material. Immunoprecipitations of extracts from pBR322 and pADC2001 bearing strains also appear devoid of any antigenic material. This result confirms that the material seen in the maxicell analysis is the product of the panD gene and probably represents a proenzyme form of ADC prior to cleavage and activation.

Extracts from pBR322, pADC200, and pADC2001 bearing strains were also submitted to electrophoresis under non-denaturing conditions. The results are shown in Figure 3-33. The pADC200 extracts indicate the presence of an intense diffuse band with an electrophoretic mobility identical to gel-purified ADC. This corresponds to the diffuse band seen upon electrophoresis of homogeneous ADC in this system as depicted in Part A of Figure 3-21. Extracts from both pBR322 and pADC2001 exhibit a much less intense and focused band at this same position. Immunoprecipitations depleted the intense band from the pADC200 extracts while the corresponding bands in the pBR322 and pADC201 extracts are unaffected by immune serum (results not shown).

Unfortunately, consistent results for pBR322 have been difficult to obtain in the maxicell experiments. Figure 3-34 presents the results obtained by SDS-PAGE of maxicell extracts from strains bearing the plasmids pBR322, pADC200, and pADC2001. In this gel, the regions corresponding to the peptides of ADC are free of any discernable bands in the lane for pBR322 in either exposure. The lane for pADC200 in the 20 hour exposure of Part A shows, in addition to the intense band corresponding to protein I, a very faint band in the region corresponding to protein II. The 72 hour exposure in Part B shows a protein with an electrophoretic mobility approximately corresponding to

Figure 3-33

Examination of the *panD* Gene Product by Maxicell Analysis of the *panD* Subclone pADC201 and the Deletion Plasmid pADC2001 with Non-denaturing Gel Electrophoresis

Plasmid encoded proteins were labeled by the Maxicell technique as described in Experimental Methods. 10 ml cultures of the strain RB901 bearing the indicated plasmid were labeled with 25 μCi each of L-[^{35}S]methionine for 15 minutes. Cells were lysed by incubation with 10 μl of 25 mg/ml lysozyme and frozen. The thawed lysates were then mixed with sample buffer analysis of the Maxicell extracts was conducted by non-denaturing gel electrophoresis with 10% polyacrylamide gels using the Davis (1964) buffer system. Upon completion of electrophoresis, the gel was stained for protein and prepared for fluorography as described in the Experimental Methods section 2.8. Each lane contains the equivalent of 2.0 ml of the original culture volume of the plasmid bearing strain as indicated by the plasmid in the figure.

The position of gel-purified ADC is indicated by the arrow and was determined by co-electrophoresis of the enzyme with the Maxicell extracts.

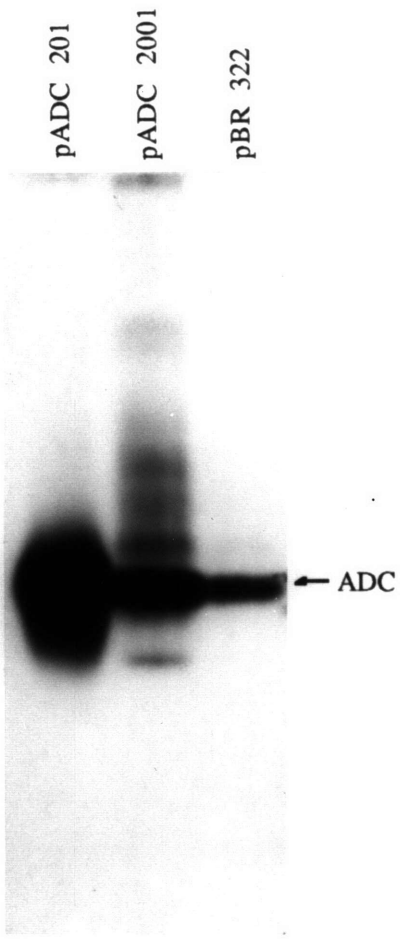

pADC 201
pADC 2001
pBR 322
ADC

Figure 3-34

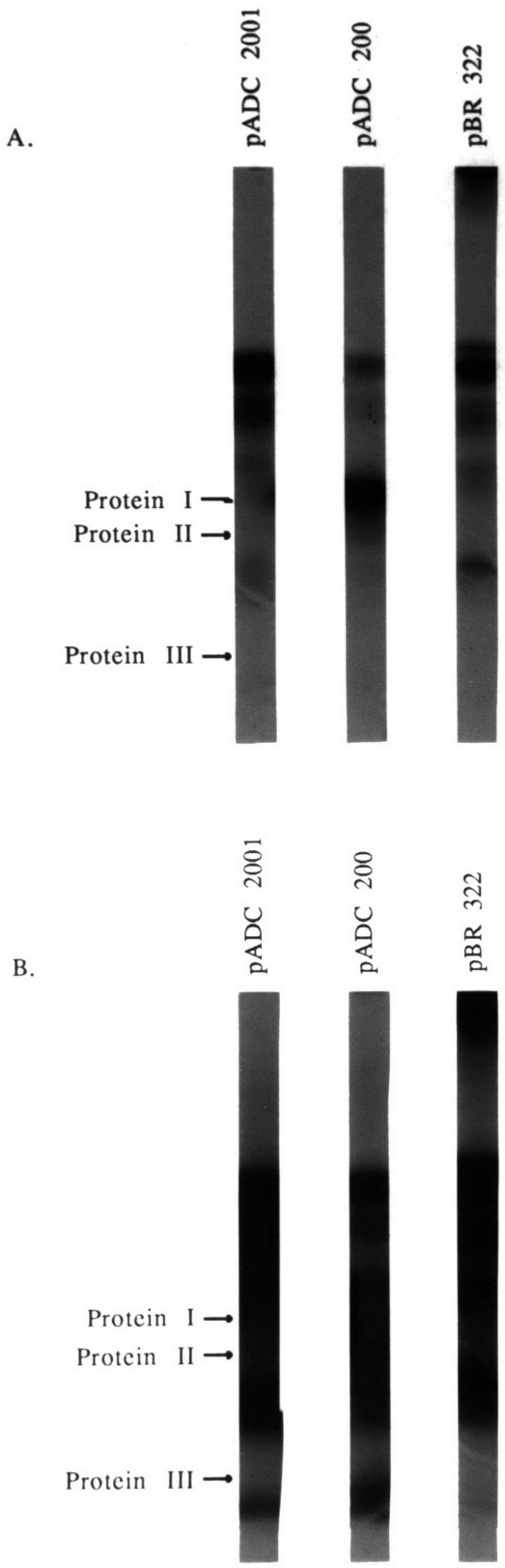
Comparison of the Plasmid Encoded Proteins of the *panD* Subclone pADC200 and the Deletion Plasmid pADC2001

Plasmid encoded proteins were labeled by the Maxicell technique as described in Experimental Methods. 10 ml cultures of the strain RB901 bearing the indicated plasmid were labeled with 25 μCi each of L-[^{35}S]methionine for 15 minutes. Cells are resuspended in SDS sample buffer and denatured for 10 minutes in a boiling water bath. Analysis of the Maxicell extracts was conducted by SDS 10-18% polyacrylamide gradient gel electrophoresis using the Hashimoto *et al.* (1983) gel system. Upon completion of electrophoresis, the gel was stained for protein and prepared for fluorography as described in the Experimental Methods Section 2.8. Each lane contains the equivalent of 2.5 ml of the original culture volume of the plasmid bearing strain indicated by the plasmid in the figure.

Part A is the result of a 20 hour exposure of the gel to Kodak X-OMAT film.

Part B is the result of a 72 hour exposure of the same gel.

The positions of the protein components I, II, and III are indicated by the arrows and were obtained by co-electrophoresis of gel-purified ADC.


A.
pADC 2001
pADC 200
pBR 322
Protein I
Protein II
Protein III
B.
pADC 2001
pADC 200
pBR 322
Protein I
Protein II
Protein III

that obtained for Protein III. This is in contrast to the results for the deletion plasmid, pADC2001, that was free of distinct bands in the region corresponding to both proteins I and II. The results are less clear cut for protein III; the 72 exposure contains a protein from extracts of pADC2001 with an electrophoretic mobility similiar, but not identical, to the band seen with the pADC200 extracts.

Figure 3-35 shows the results of the labeling of plasmid encoded proteins by the Maxicell technique with each of the plasmids generated during isolation of the 1.1 kb NruI fragment. Three bands corresponding in size to the three peptides of ADC are readily visible in the lane for pADC201. The most prominently labeled protein, corresponding to protein I, was again easily precipitated by immune serum from extracts of pADC201 and was absent from the pre-immune lane. This protein was readily observable in the overnight exposure, but proteins corresponding to proteins I and II were not discernable. Longer exposures times provided some indication that the low moleular weight proteins were indeed present in the maxicell extracts. Part C of Figure 3-35 was the result of a 48 hour exposure. The immune lane of pADC201 now indicates the presence of a pale band migrating with an electrophoretic mobility corresponding to Protein II. Although this band was nearly obfuscated by the predominant band, it does not represent an artifact of poor resolution since excellent resolution was obtained with the gel-purified ADC (results not shown). The 120 hour exposure shown in Part D indicates the presence of a third barely discernable band corresponding to the position of protein III of ADC in the immune lane of pADC201.

The sum total of the results obtained with the maxicell extracts indicates that ADC is indeed transcribed form the putative *panD* ORF in

Figure 3-35

Immunoprecipitation of the Plasmid Encoded *panD* Proteins by Antiserum Directed Against ADC

Plasmid encoded proteins were labeled by the Maxicell technique as described in Experimental Methods. 10 ml cultures of the strain RB901 bearing the indicated plasmid were labeled with 25 μCi each of L-[^{35}S]methionine for 15 minutes. The cells were lysed with lysozyme in preparation for immunoprecipitations as described for Maxicells. Analysis of the Maxicell extracts and immunoprecipitations was conducted by SDS 10-18% polyacrylamide gradient gel electrophoresis using the Hashimoto *et al.* (1983) gel system. Upon completion of electrophoresis, the gel was stained for protein and prepared for fluorography as described in the Experimental Methods Section 2.8.

Immunoprecipitations. Immunoprecipitations were conducted with lysate from the RB901/pADC201 that represented the equivalent of 2 ml of the original culture. Immunoprecipitations were conducted by incubation of the Maxicell extracts with 10 μl of a 1:100 dilution of either the preimmune or the 8th week immune serum of rabbit 3266. Immunoglobulins were then precipitated with Protein-A Sepharose CL-4B as described in Experimental Methods. The resulting precipitates were resuspended in SDS sample buffer and denatured for 10 minutes in a boiling water bath.

Maxicell extracts. Lysates, from the strain bearing the indicated plasmid, representing 2 ml of the original culture volume were

resuspended in SDS sample buffer and denatured immediately for 10 minutes in a boiling water bath.

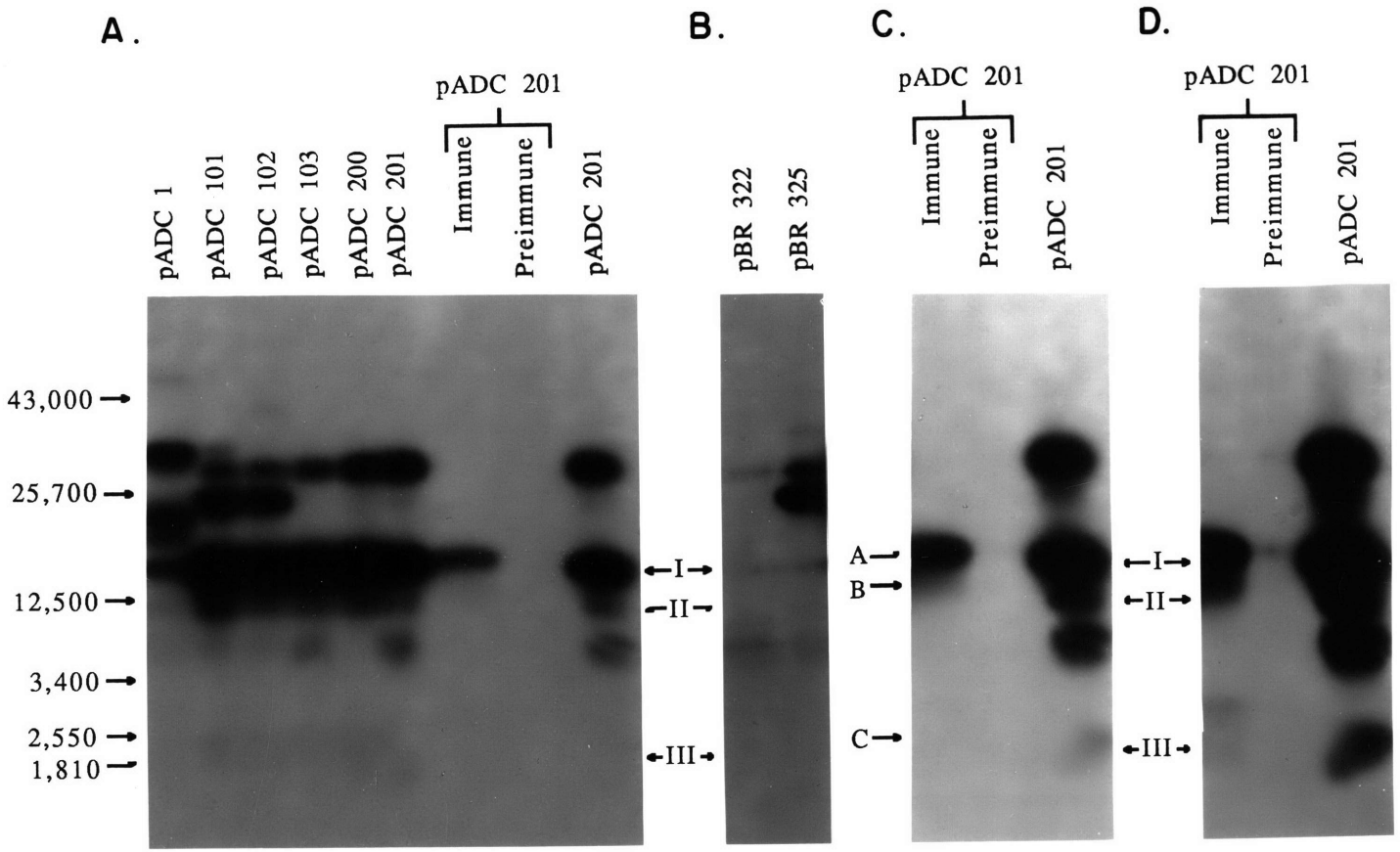
Part A. The results of Maxicell analysis of all the *panD* subclones obtained upon isolation of the gene. Also shown is the result of a immunoprecipitations with antiserum directed against ADC of pADC201 Maxicell extracts. The result shown is a 20 hour exposure of the gel to Kodak X-OMAT film.

Part B. Maxicell extracts of the parent vectors, pBR322 and pBR325, of the subclones are shown from a 220 hour exposure of the gel to film.

Part C. The exposure resulting from a 72 hours exposure to film of the gel from Part A.

Part D. The exposure resulting from a 220 hours exposure to film of the gel from Part A.

The position of the protein components I, II, and III are indicated by the arrows and were determined by the co-electrophoresis of gel-purified ADC with the Maxicell extracts. The position of the proteins precipitated by the antiserum directed against ADC are indicated by A, B, and C. The position of molecular weight markers are indicated for: ovalbumin (43,000 daltons); α-chymotrypsinlgen (25,700 daltons); cytochrome C (12,500 daltons); insulin β-chain (3,400 daltons); myoglobin cyanogen bromide cleavage fragment III (2,550 daltons); cytochrome C cyanogen bromide cleavage fragment III (1,8100 daltons).


A.
B.
C.
D.
pADC 1
pADC 101
pADC 102
pADC 103
pADC 200
pADC 201
pADC 201
Immune
Preimmune
pADC 201
pBR 322
pBR 325
pADC 201
Immune
Preimmune
pADC 201
pADC 201
Immune
Preimmune
pADC 201
43,000
25,700
12,500
3,400
2,550
1,810
I
II
III
A
B
C
I
II
III

the from of a single polypeptide that is precipitable by the antiserum directed against ADC. Processing of this peptide to proteins II and III also seems to be occurring in the UV-irradiated cells albeit at low rates and the presence of the three peptides was confirmed by their precipitation with antiserum. However, direct evidence for the production of these peptides from protein I is still lacking.

3.19 Comparison of the *panD* Protein Sequence with that of the Histidine Decarboxylase of *Lactobacillus* 30a

The complete primary sequence of histidine decarboxylase (HDC) from *Lactobacillus* 30a has been determined (Huynh *et al.*, 1984; Vaaler *et al.*, 1982; Vanderslice *et al.*, 1986) and has been compared to the partial sequences of several pyruvoyl-dependent histidine decarboxylases (Huynh and Snell, 1985). The enzymes have been shown to have 23% identity in the β-chain with both the α and β chains showing a high degree of homology within the vicinity of the pyruvoyl prosthetic group (Huynh and Snell, 1985).

The possibility that the conserved sequences may be important in the activation of the proenzyme prompted us to compare the translated *panD* sequence with that of the corrected sequence of HDC (Vanderslice *et al.*, 1986). We wanted to ascertain whether the possibility of a degree of homology existed between the pyruvoyl generating region of HDC (amino acid 82 of the proenzyme) and the apparent cleavage site of the *panD* sequence (amino acid 25 of Figure 3-26). The result of the comparison by the enhanced graphic matrix analysis using the COMPARE and DOTPLOT programs with the standard comparison parameters (window is 30 residues

wide and a stringency of 8 matches or greater) is shown in Figure 3-36. The almost total lack of points indicates the extremely low degree of homologous sequence shared by the two proteins. In addition, when the stringency is increased to 9 matches per window or the window decreased to 20 residues the result is a total lack of homology. This suggests that the local homology seen in Figure 3-36 is due to the minimum number of matches spread over the breadth of window. One alignment of the homologous regions indicated in Figure 3-36 is shown in Figure 3-37 indicating this situation.

Figure 3-37 shows that this alignment provides 8 matches within a 30 residue region and occurs when the amino terminus of the HDC sequence is aligned with residue 12 of the *panD* translated sequence. The homologous regions of ADC and HDC lie outside the region of optimal homology (residues 60-94 of *Lactobacillus* 30a HDC) obtained from the comparison of HDC from various sources (Huynh and Snell, 1985), but the putative cleavage site of the *panD* sequence falls within the sampling region of the window. This alignment provides only two instances of two consecutive homologous residues occurring between the two proteins. This nearly total lack of homology indicates that if any functional similarity between the proteins exists then it is not apparent from comparison of the primary sequence and may only be obtained from a knowledge of the tertiary structure of both enzymes.

Figure 3-36

Comparison of the Histidine Decarboxylase Protein Sequence with the Translated Protein Sequence of the *panD* Gene

Diagonal Dot Matrix comparison was based on the corrected protein sequence of HDC (Vanderslice *et al.*, 1986) and the translated protein sequence of *panD* as determined in Section 3-9. The comparison is based on the enhanced graphic analysis method of Maizel and Lenk (1981) as determined by the COMPARE and DOTPLOT programs. The comparison was conducted with a window setting of 30 residues and a stringency of 8 residues. The abcissa represents the primary sequence of HDC and the ordinate represents the primary sequence of ADC.

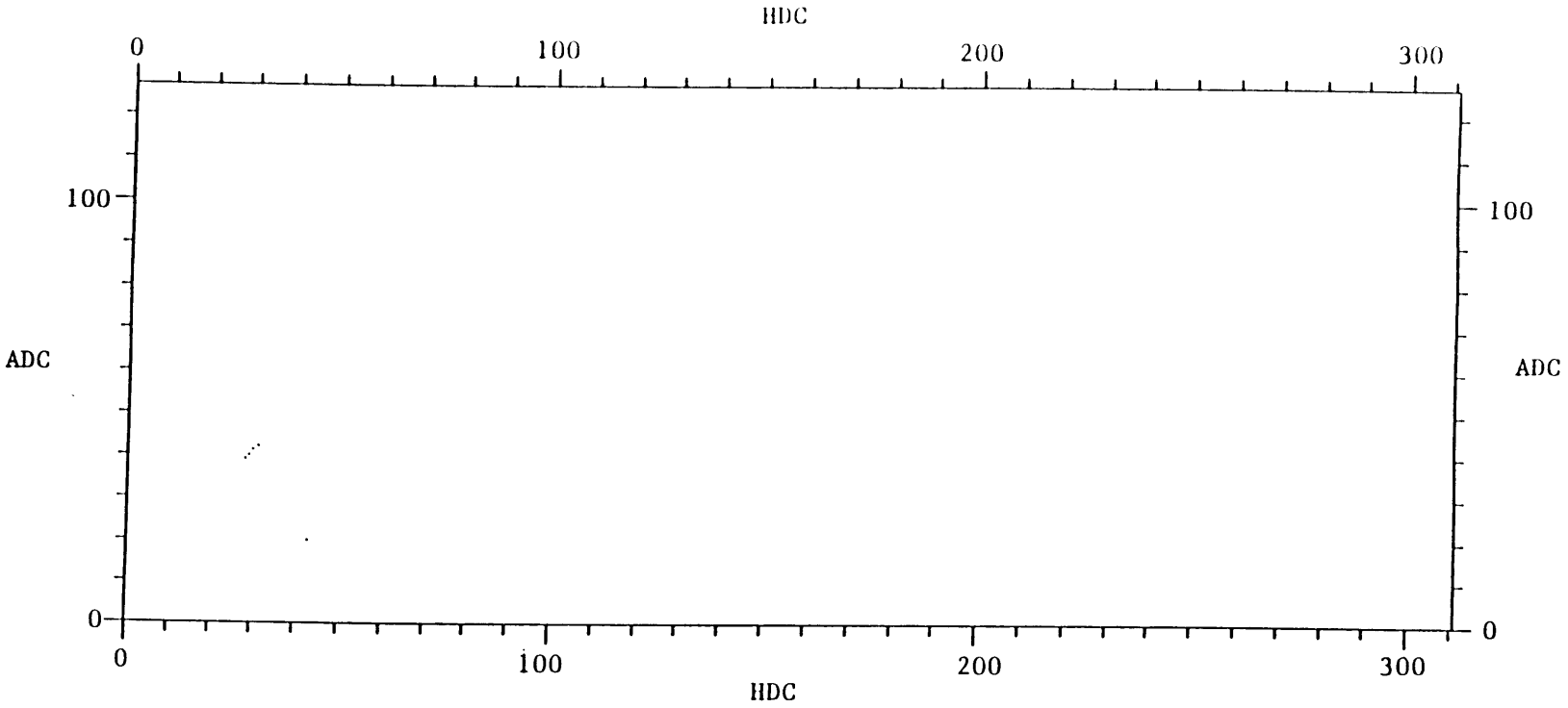

HDC
0
100
200
300
100
ADC
0
0
100
200
300
HDC
100
ADC
0

Figure 3-37 The optimal alignment of the *panD* sequence of *E. coli* and HDC from *Lactobacillus* 30a as determined by the BESTFIT program. The upper sequence is of histidine decarboxylase from *Lactobacillus* 30a and the lower underlined sequence is of L-aspartate-α-decarboxylase from *E. coli*. Crosses (+) indicate the presence of a match. Arrows indicate the actual (HDC) or putative (ADC) sites of cleavage of the proenzymes.

```
            SELDAKLNKLGVDRIAISPYKQWTRGYMEPGNIGNGYVT 39
                              ++        + +  +   ++
MIRTMLQGKLHRVKVTHADLHYEGSCAIDQDFLDAAGILENEAIDIWNVT 50
                             ↑

                                                  ↓
GLKVDAGVRDKSDDDVLDGIVSYDRAETKNAYIGQINMTTASSFTGVQGR 89
  +                    +    +         +
NGKRFSTYAIAAERGSRIISVNGAAAHCASVGDIVIIASFVTMPDEEART 100


VIGYDILRSPEVDKAKPLFTETQWDGSELPIYDAKPLQDALVEYFGTEQD 139

WRPNVAYFEGDNEMKRTAKAIPVQVA 126


IRRHYPAPGSFVCANKGVTAERPKNDADMKPGQGYGVWAIAISFAKDPTK 189


DSSMFVEDAGVWETPNEDELLEYLEGRRKAMAKSIAECGQDAHASFESSW 239


IGFAYTMMEPGQIGNAITVAPYVSIPIDSLPGGSILTPDKDMEIMENLTM 289


PEWLEKMGYKSLSANNALKY 310
```

Discussion

4.1 Transcription of the *panD* Locus and the Possible Coordinate Expression of the *pan* Loci

The order of the *pan* loci on the *E. coli* chromosome is *aceF-panB-panD-panC-tonA* (Cronan *et al.*, 1982) indicating that transcription originating at either *panB* or *panC* could encode all the *pan* loci. It is conceivable that the ORF of Frame B in Figure 3-8 terminating 120 nt 5' to the *panD* ORF could represent either the *panB* or *panC* gene. However, a lack of information concerning either the nucleotide sequences or the proteins encoded by the *panB* and *panC* genes prevents assignment of this ORF to either locus. Downstream of the *panD* gene, an ORF of noteworthy size is not observed within the remaining 320 nucleotides of the NruI fragment.

While the considerable distance separating the *panD* ORF from its nearest downstream neighbor may be an indication of the lack of any coordinate expression with downstream regions, but distance is not a definitive test of genetic organization. Typically, proteins that are dependent on coordinate expression (*i.e.* the subunits of an enzyme) are encoded by closely spaced open reading frames. The *trpE* and *trpD* structural genes, which encode the subunits of anthranilate synthetase, actually overlap by a single nucleotide (Nichols *et al.*, 1980) and exhibit co-ordinate translation in the process known as "translational coupling" (Oppenheim and Yanofsky, 1980). In the case of the *lac* operon, where translational coupling is not observed (Reznikoff, 1984), the intercistronic distances are larger; *lac Y* is 54 nt downstream of *lac Z* and 70 nt upstream of the *lac A* gene (Buchel *et al.*, 1980).

Greater intercistronic distances have been observed in several bacterial operons (Higgins *et al.*, 1982) and the *rplJL-rpoBC* operon (Post *et al.*, 1979) is an interesting example with 324 nt between *rplL* and *rpoB*.

The nonattenuated transcript arising from the *rplJL-rpoBC* operon contains a long intercistronic region between *rplL* and *rpoB* which contains an inverted repeat (Post *et al.*, 1979) that is cleaved by an RNase III type of activity (Barry *et al.*, 1980). The two ribosomal proteins *rplJ* and *rplL* (L10 and L7/L12, respectively) are encoded by closely spaced open reading frames (68 nt separation) while *rpoB* and *rpoC* (β and β' subunits of RNA polymerase, respectively) are located downstream of the large intercistronic region. The cleavage of the transcript generates separate transcripts for the ribosomal proteins and RNA polymerase subunits and may provide a basis for the observed differential expression of these two sets of proteins (Lindahl *et al.*, 1977).

The lack of terminal uridine residues in what otherwise appears to be a rho-independent termination structure for the *panD* could result from cleavage of a longer polycistronic transcript distal to the putative 3′ stem-loop structure of Figure 3-12. The susceptability of polycistronic transcripts to degradation in a stepwise manner has been postulated to occur by a process that involves initial cleavage within intercistronic regions (Stern *et al.*, 1984 and references therein) with the lifetime of the resulting single cistronic transcripts determined by their resistance to degradation (*i.e.* by RNase II), presumably by the presence of stable internal secondary structure. The existance of a stable stem-loop structure (ΔG = -20.9 kcal/mole) found at the immediate 3′ terminus of the *panD* transcript may serve an analogous function to

the *trp t* structure. Under those circumstances, it would function as a barrier to attack by a 3'→5' exonuclease activity thereby preventing degradation of the upstream *panD* structural gene.

Alternatively, the unusual 3' terminus of the *panD* transcript may be the result of another form of processing similiar to that which is operative at the *trp t'* termination region of the trp operon (Platt *et al.*, 1985). In this operon, there exists what appears to be a rho-independent termination region at *trp t* which includes an inverted repeat region postulated to form a stem-loop structure. However, the result of cloning experiments indicated that the *trp t'* site, which is distal to and downstream of the observed 3' terminus of the transcript which occurs at *trp t*, was involved in termination (Wu *et al.*, 1980). The level of termination at this site was found to be dependent on the presence of rho factor (Wu *et al.*, 1981). The observed 3' terminus of the mature *trp* transcript is then generated by RNase acitivity (Mott *et al.*, 1985) acting on the 3' terminus of the susceptable initial transcript. The *trp* transcript terminated at *trp t'* was shown to be resistant to *in vitro* degradation by RNase III past the *trp t* stem-loop structure, thus generating the *in vivo* 3' terminus (Mott *et al.*, 1985).

However, the mechanism of termination of the *panD* transcript still remains in question. An A/T rich (75%) region located from 20 to 60 nucleotides downstream of the stem-loop structure, the observed 3' terminus, may represent a possible rho-independent termination site unusually distant from the inverted repeat region which is degraded to yield the observed terminus. Alternatively, the possibility of rho involvement may be anticipated in light of the precedent provided by *trp t'*. Analysis of the 100 nucleotides downstream of the 3' terminus of

the *panD* transcript indicates the possibility of only weak secondary structure, which is the most salient feature of rho-dependent termination sites (Platt, 1986; Bear *et al.*, 1985). In such a case, the observed 3' terminus of the transcript could be generated, following rho-induced termination, by degradation of a longer transcript and the lack of secondary structure would facilitate this degradation by a 3'→5' exonuclease activity.

S1 nuclease mapping indicated the presence of only a single transcript from this region though the probes extended well beyond the region of the *panD* ORF. This observation was confirmed by hybridization of RNA, fractionated by agarose gel electrophoresis and transfered to nitrocellulose, to probes derived from either EcoRV/RsaI (positions 214-478 of Figure 3-7) or NlaIII (positions 197-395) fragments of the *panD* region. The results indicated the presence of only a single transcript approximately 700 nt in size (results not provided). In addition, the S1 nuclease mapping also correlated the termini of the *panD* transcript with both putative promoter and terminator regions indicating that the transcript contains only a single cistron. The combination of these results suggests that longer polycistronic transcripts are not originating from this region.

However, S1 nuclease mapping is not sufficiently accurate to allow the exact location of control regions in the absence of more detailed genetic or biochemical anaylsis. Hawley and McClure (1983) require either direct analysis of the 5' terminus of the transcript or genetic analysis of a promoter region to qualify for inclusion in their compilation of *E. coli* promoter regions. This indicates the need for further analysis to elucidate the regions affecting the initiation and

termination of transcription at the *panD* locus. Analysis of both the promoter and termination regions could be achieved by *in vitro* mutagenesis, using the single stranded M13mp18 subclones constructed for DNA sequencing, by synthetic oligodeoxyribonucleotide heteroduplex mutagenesis methods (M. Smith, 1984) to generate point mutations in the putative control regions. For the termination region in particular, the effect of mutations downstream of the observed 3' terminus would provide direct evidence for the involvement of this region in termination of the *panD* transcript.

Biochemical analysis of the initiation site may be confirmed, *in vitro*, by examination of run-off transcripts (Haldenwang and Losick, 1979) originating at the putative promoter region contained in the NruI/EcoRV (positions 1-478 of Figure 3-6) restiction fragment of pADC201. Alternatively, the *panD* transcript produced by *in vitro* transcription can help to determine the termini precisely, either by sequencing the RNA with reverse transcriptase (Zimmern and Kaesberg, 1978) or by direct anaylsis of the transcript by fingerprinting methods (Maxam and Gilbert, 1978).

Several methods exist for investigating the involvement of additional factors in the termination of transcription or post-translational processing. *In vivo* analysis of the transcript obtained from the temperature sensitive *rho*$^-$ strains of Das *et al.* (1976) while the direct effect of rho factor can be assesed by inclusion of the purified factor (Wu *et al.*, 1981) during *in vitro* transcription. Similiarly, RNase III (*rnc*) involvement may be investigated by analysis of the *panD* transcript obtained from the *rnc*$^-$ and *rnc*$^+$ transformed

isogenic strains N2076 and N2077, respectively, of Apirion and Watson (1975) and by examining the effect of RNase III on the transcript produced by *in vitro* transcription.

The involvement of independent promoter and terminator functions specific for the functioning of the *panD* locus would provide considerable evidence for independent expression, although the expression of a single locus from multiple promoters is known for the *int* gene of λ (Court *et al.*, 1983) and *dnaG* of the macromolecular synthesis operon (Lupski *et al.*, 1983). In both cases, the level of expression of one or more of the activities within the operon is required at levels that are anticipated to be highly divergent from that obtained from the polycistronic transcript. However, in the case of the *pan* activities, the efficacy of the differential expression of the various activities is not obvious and the *pan* loci are not known to exhibit pleiotropic phenotypes (Cronan *et al.*, 1982).

Finally, the ability of pADC1, containing the full 12.5 kb EcoRI fragment, to complement the auxotrophy of either or both *panB* and *panC* would allow us to determine the proximity of these loci to *panD* (Cronan *et al.*, 1982). If they are located within this fragment, it would then be quite simple to ascertain their location as a preliminary exercise to determining their nucleotide sequences.

4.2 Generation of the Active Form of ADC

The lack of homology exhibited between histidine decarboxylase and ADC was not unexpected. Although both are pyruvoyl-dependent decarboxylases and exhibit what we believe to be similiar patterns with

respect to the mechanism of activation, they are functionally different enzymes. HDC is an inducible catabolic enzyme in both *E. coli* (Gale, 1946) and *Lactobacillus* 30a (Guirard and Snell, 1964) while ADC is a constitutive biosynthetic enzyme. A comparison of the β chains of HDC isolated from a number of organisms indicates that they share only 23% of the residues in common to all four enzymes (Huynh and Snell, 1985) and virtually all are clustered near the carboxyl terminus generated by the cleavage of the proenzyme. Conserved residues in the α chain are also clustered near the pyruvoyl residue at the amino terminus. The conserved residues may function by providing either the proteolytic activity necessary for activation of the proenzyme or determine the active site for the decarboxylase activity or both.

With respect to these possibilities, the lack of homology of HDC to any region of ADC indicates that either the determinants of the proteolytic activity are located in regions distal to the cleavage site or that cleavage site is specified by more subtle features of secondary structure. However, the cleavage site of HDC proenzyme is located within a region of β sheet, as determined by x-ray crytallography (Parks *et al.*, 1985), while the putative cleavage site of ADC is located, as determined by the Chou-Fasman method for prediction of protein conformation (Chou and Fasman, 1974; 1977; and 1978), in a 4 amino acid region, possibly as β sheet, flanked on both sides by beta turns. This apparent lack of similiarity in protein conformation suggests that the homologous regions in HDC are involved mainly in determining the active site for histidine decarboxylation.

In spite of this lack of homology, we have utilized the extensively studied pyruvoyl-dependent histidine decarboxylase of

Lactobacillus 30a (Recsei and Snell, 1984) as a paradigm. The results obtained by DNA sequencing, examination of the initial translational product, and protein sequencing of ADC are all consistent with the concept of production of active enzyme from a single polypeptide precursor by cleavage of a peptide bond to generate two fragments. Unfortunately, we have not yet shown direct activation of the precursor either from the isolated protein I or the major product of maxicell analysis. This activation has not been observed even when maxicells are subjected to a chase of unlabeled methionine for up to 120 minutes after labeling is terminated (results not shown). This appears consistent with the results of the lytic assay performed on the λ clones (see Table 3-1) and suggests that activation of the proenzyme may be a slow process which does not occur to a significant extent in the time period examined in these experiments. By virtue of this slow activation, we may be able to obtain large quantities of unactivated enzyme by the rapid production of proenzyme from a strong inducible promoter.

Repeated attempts have been made to fuse the *panD* ORF to the isopropyl-thiogalactoside (IPTG) inducible *tac* promoter (de Boer *et al.*, 1983) which contains the -35 region of the *trp* operon fused to the -10 region of the *lac* UV5 promoter with a Shine-Dalgarno sequence upstream of a BamHI restriction site. However, we have been unable to ligate BamHI linkers to a properly positioned upstream MnlI restriction site (position 318 of Figure 3-6) which appears to reside within a region that had been identified as exhibiting secondary structure during sequencing of the M13mp18 subclones. As a result, construction of the *tac* promoter fusion will require the synthesis of a pair of oligonucleotides that will allow fusion of a BamHI restriction site to

an SfaNI restriction site (position 345) that had previously been used for cloning purposes and is just downstream of the *panD* initiation codon.

Based on the activation of HDC (Rescei *et al.*, 1983b), the generation of the active form of ADC may be postulated to occur by a cleavage between residues 24 and 25 of the *panD* translated sequence (see Figure 3-19). Our choice of the cleavage site would place the glycine (position 24) at the carboxyl terminus of protein III and the serine at position 25 would be located at the amino terminus of protein II. We anticipated the aforementioned cleavage at this position since it would place a serine residue at a position analogous to that seen in the proenzyme of HDC. If the mechanism for activation of HDC may be applied to ADC, then the serine residue at position 25 of the translated sequence is converted, through activation of the proenzyme, to a pyruvoyl residue at the amino terminus of protein II. For all pyruvoyl-dependent decarboxylases where the location has been determined (as indicated in Table 1-1), the pyruvoyl group was bound via a peptide linkage to the amino terminus (Recsei and Snell, 1984). ADC has been shown to contain a pyruvoyl group covalently attached to protein II (Williamson and Brown, 1979), but the precise site of attachment has not been determined. In other cases, the location of the pyruvoyl residue has been demonstrated by the inability to sequence the amino terminus of the protein prior to the reductive amination of the pyruvoyl residue (Hyunh, *et al.*, 1984b). The choice of the cleavage site for ADC is supported, with a single qualification, by the protein sequencing data (see Figure 3-28). In this case, the presence of the alanine residue at this position in protein II, as determined by peptide sequencing,

indicates the need for alternative methods of determining the site of attachment of the pyruvoyl residue.

In the absence of definitive proof for the involvement of the serine residue at position 25, one could argue that the alanine at the amino terminus is indeed encoded by *panD* and that the serine indicated in the translated sequence is the result of a sequencing error at this position. However, the amino-terminal sequences obtained for proteins I and II indicate that the first 30 nucleotides of the ORF are aligned in frame with the segment coding for protein II which is 42 nucleotides downstream. An alanine residue is not present in either of the alternate reading frames at this region eliminating the probability of a frame shift error and, in addition, the autoradiograms of the sequencing gels for this region lacked any indication of the compression that can result in the ommission of nucleotides thereby providing an unambiguous determination of the sequence of both strands.

The alanine residue may also be the result of a post-translational modification either via the addition of an alanine residue to an amino terminal cysteine residue following cleavage or by reduction of a dehydro-alanine prosthetic group. As to the former case, it can only be noted that the post-translational addition of an an amino acid residue would appear to be without precedent, although we have not attempted to address this possibility impirically. With respect to the latter case, the dehydro-alanine residue is generally found as a [^{3}H]alanine upon reduction of the enzyme with [^{3}H]$NaBH_4$ (Snell, 1977) while [^{3}H]lactate was the only labeled compound isolated upon reduction of ADC with [^{3}H]$NaBH_4$ (Williamson and Brown, 1979). Suffice it to say that while such means of generating the alanine residue may be proposed, the

propitious correspondence of ADC to several of the precedents provided by HDC (Recsei and Snell, 1984) suggests that a less radical mechanism for generation of the alanine residue is likely to be involved.

Previous studies had shown that the enzyme is highly susceptable to inactivation in the presence of aspartate (Smith, 1982) and this inactivation results in the time-dependent first order loss of activity as depicted in Figure 1-3. This indicates that inactivation occurs from an enzyme-substrate complex as would be anticipated for an enzyme catalyzed event. This leads to what we feel may be a more feasable explanation of the origin of the alanine residue based on a process known to occur in pyridoxal-phosphate dependent decarboxylases. Kalyankar and Snell (1962) observed decarboxylation-dependent transamination occuring when several α-alkyl amino acids are heated in dilute solution with pyridoxal. Since that time this reaction has been shown to occur with 3,4-dihydroxyphenylalanine (DOPA) decarboxylase (O'Leary and Baughn, 1975 and 1977; Minelli *et al.*, 1979), brain glutamate decarboxylase (Porter *et al.*, 1985), and aspartate β-decarboxylase (Novogrodsky and Meister, 1964). This results in the formation of pyridoxamine 5'-phosphate and the concommitant inactivation of the enzyme which can be restored to the active apoenzyme by the addition of endogenous pyridoxal 5'-phosphate. The irreversible inhibition of the pyruvoyl-dependent mammalian S-adenosyl-L-methionine decarboxylase by substrate has been reported (Pankaskie and Abdel-Monem, 1980) and it has been postulated to be the result of such a decarboxylation-dependent transamination although evidence supporting this supposition has not been published.

The results of the protein sequencing indicate the need to demonstrate the origin of the pyruvoyl residue, its involvement at the active site, and its subsequent conversion by decarboxylation-dependent transamination into the alanine residue observed at that position. As to the origin of the pyruvoyl group, the ability to separate the active and inactive forms of protein II would provide the location of the pyruvoyl residue in a straight forward manner by reductive amination and protein sequencing according to the methods of Huynh *et al.* (1984b). In the absence of this capability, demonstration of its amino terminal position should be possible in a similiar manner by reduction of the pyruvoyl residue with [^{3}H]$NaCNBH_4$ in the presence of NH_4Cl (Huynh *et al.*, 1984). Sequence analysis should yield a labeled alanine residue at the amino-terminus of protein II and the complete release of radioactivity after the first round of sequencing would demonstrate the location of the pyruvoyl residue at the amino terminus.

The origin of the pyruvoyl group may be demonstrated by analysis of protein II isolated from one of the plasmid bearing strains grown in the presence of [^{14}C]serine. This should produce a labeled alanine residue at the amino terminus of protein II and a labeled pyruvoyl group, determined as described above, while growth in the presence of [^{14}C]alanine should result in the absence of label at the amino terminus.

The alanine residue at this position as determined by amino-terminal sequencing is anticipated to be the product of a side reaction occuring during the decarboxylation of L-aspartate resulting from the anomalous protonation of the imine formed following decarboxylation of aspartate, as depicted in Figure 1-3. This scheme requires utilization

of the pyruvoyl group in the formation of a Schiff base with the substrate which then results in decarboxylation. The existance of the Schiff base intermediate has not been investigated in the case of ADC but could presumably be demonstrated, in the manner utilized for HDC (Rescei and Snell, 1970), by reduction of the enzyme with $NaCNBH_4$ followed by acid hydrolysis in the presence of the L-[2,3-^{3}H]aspartate which should produce both N^2-(1-carboxyethyl)[2,3-^{3}H]aspartate and N^3-(1-carboxyethyl)[2,3-^{3}H]β-alanine.

The transamination of aspartate should produce malonyl semi-aldehyde and convert the pyruvoyl prosthetic group to an alanine residue. We have not yet attempted to obtain direct proof for the formation of the alanine at the amino terminus as a result of this reaction. Isolation of the malonyl semi-aldehyde would require the utilization of substrate quantities of active enzyme (> 100 picomoles) to detect the production of malonyl semi-aldehyde from L-[^{14}C] aspartate. The instability of the malonlyl semi-aldehyde (Hayaiyshi *et al.* 1961; Den *et al.*, 1959) complicates its analysis, although it should be possible to identify it as the 2,4-dinitrophenylhydrazone derivative via paper chromatography (Hayashi *et al.*, 1961). However quantitation may prove difficult as the 2,4-dinitrophenylhydrazone provides a result that differs from other forms of analysis (Yamada and Jakoby, 1960). Hayaiyshi *et al.* (1961) found that malonyl semi-aldehyde appeared to polymerize nonenzymatically and also condensed with β-alanine above pH 9.0, a process also observed on prolonged incubation in the presence of β-alanine at pH 8.0 or when amine buffers were present. As a result, an alternate method which requires much smaller quantities of protein may prove more effective. Prolonged incubation of the enzyme in the

presence of substrate should yield a peptide that lacks any pyruvoyl residue susceptable to reduction with sodium borohydride. Quantitative amino-terminal analysis of the peptides should demonstrate a corresponding increase in the quantity of amino-terminal alanine for protein II. The DABITC/PITC double-coupling micro-sequencing method of Chang (1981) has been shown to be sensitive enough to allow quantitative determinations on 100 picomoles of protein (1.1 μg of protein II).

The seryl-seryl bond of HDC cleaved by the proenzyme to generate the active enzyme is highly susceptable to cleavage by hydroxylamine (Huynh and Snell, 1986a) under mild conditions. This susceptability is an exception to the known specificity of hydroxyl amine cleavage at Asn-Gly bonds (Bornstein and Balian, 1977) and appears to indicate that the seryl-seryl bond has rearranged to an active ester intermediate prior to cleavage of the proenzyme (Huynh and Snell, 1986a). This ester intermediate is a postulated intermediate in the autocatalytic activation of HDC (see section 1.5.1). This increase in susceptability of peptide bond at the activation site bears examination in ADC, although the basis for this increased susceptability in HDC has not been determined.

The possibility that the activation of the proenzyme is catalyzed by a trans-acting factor would appear unlikely based on the precedent of the autocatalytic activation HDC from *Lactobacillus* 30a (Rescei *et al.*, 1983) and *Lactobacillus buchneri* (Recsei and Snell, 1985). However, one possible explanation for the apparent slow activation of ADC may be the requirement for an activating factor whose activity is rate limiting. In the case of histidine ammonia-lyase from *Psuedomonas putida*, the dehydro-alanine residue appears to be attached to the side chain of a

backbone residue via a post-translational modification requiring an activating factor which maps to a different locus from the histidine ammonia-lyase structural gene (Phillips *et al.*, 1986) This suggests the possibility of the involvement of such a factor in the activation of ADC and demonstration of such a factor should be possible by conducting mutagenesis in a plasmid-bearing strain (i.e. pADC1/AB355). Under these circumstances, the high copy number of the pBR322 derived subclones would allow isolation of genomic mutations independent of the ADC structural gene.

4.3 The Structure of ADC

The results of both the limited proteolysis by V8 protease and the protein sequencing data indicate that the observed structure of ADC includes the presence of the proenzyme form which we postulated to be protein I. While the combined molecular weights determined in Section 3.14 for proteins II and III do not provide a neat stoichiometry for ADC, an apparent stoichiometry is probable may be postulated as being composed of three possibilities: a tetramer of protein I (π_4, the π proenzyme, {4(14,650) = 58,600 daltons}); an $(\alpha\beta)_4$ structure composed of proteins II (α) and III (β) {4(10,580 + 2,230) = 51,240 daltons}; or some mixture of the two structures, $\pi_{4-n}(\alpha\beta)_n$. A similiar analysis for these tetramers of the protein derived from the translated sequence yields a molecular weight of 55,330 daltons. These values are close to the 58,000 daltons determined for native ADC by gel filtration on AcA44 and the 54,000 daltons obtained for ADC, under mild denaturing

conditions, by SDS-PAGE (Williamson, 1977). A single determination by the author provided a value of 54,900 daltons from gel filtration on AcA44.

A few clues pertaining to the protein structure of ADC have been obtained by the separation of what may be distinct species during non-denaturing gel electrophoresis. When ADC is subjected to electrophoresis using the Davis (1964) buffer system and stained for protein with coomassie blue, the result is a diffuse band as seen in part A of Figure 3-21. This band can be divided into fractions which, after electroelution and evaluation by electrophoresis in the same system, again result in diffuse bands but with slightly differing mobilities which were directly related to the position of the gel slice from which it had been obtained. Enzyme isolated from the gel slice nearest the cathode was seen to migrate at a slightly lower mobility than enzyme isolated from gel slices nearer the anode. However, the fractions are not mutually exclusive and largely overlap in their individual mobilities. Hence, the separate fractions do not appear to represent isolates of distinct species, but may indicate enrichment of a mixed enzyme structure composed of proteins that vary slightly in their electrophoretic mobility.

This would be the anticipated result if one were dealing with a mixed subunit structure composed of either protein I or the nearly identical products of a single proteolytic cleavage of this protein to generate proteins II and III. One would anticipate that the result of such a cleavage would be two components which as a complex exhibited a slightly altered overall charge compared to that of the parent protein I as a result of generating an additional carboxyl terminus (and possibly

a new amino terminus). Assuming that the overall structure of the complex composed of proteins II and III is not drastically altered from that of protein I by such a cleavage, a change may be anticipated in the electrophoretic mobility between protein I and the associated proteins II and III. Hence, a tetramer composed of varying ratios of protein I and its putative cleavage products would be a series of enzyme molecules of slightly differing electrophoretic mobilities.

The diffuse bands seen on non-denaturing gels would be anticipated on the basis of this model for the structure of ADC. A number of closely related species, differing in the net charge of the total enzyme molecule, would likely result in a diffuse band during electrophoresis on non-denaturing gels while providing essentially identical results on denaturing gels where electrostatic properties of the native enzyme are generally discounted (Swank and Munkres, 1971). The complexity of the electrophoretic behavior would be further increased if equilibration between various species occurs during the course of electrophoresis resulting in diffuse bands lacking distinct entities.

An additional complexity is provided by the presence of the alanine residue at the amino terminus of protein II. We had anticipated a pyruvoyl group attached to the amino terminus of this protein by precedence to HDC (see section 1.5.1). This was bolstered by the prediction of a serine residue at this position in the translated sequence, the same residue utilized by HDC for the generation of the pyruvoyl residue (Hyunh *et al.*, 1983). The alanine residue obtained at this position from the amino-terminus sequencing of protein II indicates the possibilility of an additional species in the mixture of proteins anticipated to comprise ADC. Generation of this residue by

decarboxylative transamination would generate an additional amino terminus at a position that would contain an uncharged pyruvoyl residue in the putative active enzyme.

Under such circumstances, one would predict that, based on the charge differences, the various species could, theoretically, be separated by isoelectric focusing in a system of sufficiently high resolution. In fact, preparation of ADC by Joanne Williamson (1977) had utilized a preparative isoelectric focusing column which resulted in two overlapping peaks of activity (pI = 4.63 and 4.66). Examintation of the proteins contained in the overlapping peaks of ADC activity on Davis (1964) gels indicated that both species were identical. This may indicate, as Williamson (1977) had postulated, that two forms of the enzyme existed which she ascribed to active and inactive forms. The putative inactive form could contain the alanine-terminal protein II while the active form would contain a pyruvoyl-terminal protein II.

Regardless of the exact species resulting in the various forms of the enzyme, it may be possible to separate these species by a high resolution isoelectric focusing system, possibly using the immobiline system of LKB (Bjellqvist *et al.*, 1982). In addition, examination of the results obtained by Joanne Williamson during gel electrophoresis under various denaturing conditions (Williamson, 1977) indicates that it may be possible to separate protein I from proteins II and III by treatment of the enzyme with SDS under mild conditions or possibly by treatment with non-ionic detergents. The results of treating ADC with SDS at 37°C in the presence of DTT appears to yield only protein I from the homogeneous enzyme (Williamson, 1977) suggesting the possibility that protein I is loosely bound to the enzyme complex or that species of

ADC exist which exhibit a greater susceptability to solubilization. In either case, the ability to separate protein I from the two smaller components would allow us to study the putative activation of protein I and would provide definitive proof for our current suppositions regarding activation of the enzyme.

Alternatively, dissocation of the enzyme by treatment with acid (Cook and Koshland, 1969) may also prove useful since this method is known, in the case of several proteins, to result in only minor perturbations of structure (Cook and Koshland, 1969) and may allow isolation of the proteins in their native form.

4.4 Regulation of L-Aspartate-α-Decarboxylase Activity

The slow activation of the ADC precursor appears to be unique among the pyruvoyl-dependent decarboxylases. Of the decarboxylases for which the proenzyme has been detected (see Table 1-1), none has been found which exhibits the stability of the ADC proenzyme which is the only pyruvoyl-dependent decarboxylase for which the proenzyme can be readily isolated. The proenzyme for the HDC of *Lactobacillus* 30a can be detected when induced in stationary phase with L-histidine (Recsei and Snell, 1982) but the proenzyme is not a major component when the enzyme is obtained from *Lactobacillus* 30a grown under normal culture conditions (Chang and Snell, 1968b). This suggests that proenzyme activation may have implications as regulation of β-alanine production.

Jackowski and Rock (1981) have shown that wild type strains of *E. coli* produce copious quantities of pantothenate that is excreted into the medium indicating that ADC is not a limiting factor in the

panthothenate pathway. They estimate that wild type *E. coli* has 15-fold higher levels of CoA than is necessary to support metabolism. This suggests that regulation of ADC levels may only function by limiting production of β-alanine in order to avoid the unnecessary squandering of metabolic resources. ADC levels are not affected in *E. coli* grown on minimal medium containing β-alanine, pantoic acid or panthothenate indicating that repression of ADC does not occur (Williamson, 1977). The ADC has been shown to be insensitive to inhibition by β-alanine, D-pantoic acid, D-panthothenic acid, coenzyme A and acetyl-coenzyme A indicating that the activity is also not subject to feedback inhibition by any of the intermediates in the pantothenate and CoA biosynthetic pathway (Williamson and Brown, 1979).

Thus, ADC activity of *E. coli* appears to lack regulation beyond transcriptional controls. However, a number of inherent properties of the enzyme function in maintaining low basal levels of ADC actiivity. The high K_M (160 μM) for L-aspartate, low turnover number (40 moles CO_2/min/mole cofactor), and low copy number [estimated to be 30 copies per cell by Williamson (1977)] have obvious implications with respect to the rate of β-alanine production. However, the stoichiometry of one pyruvoyl residue per enzyme molecule (Williamson and Brown, 1979) indicates that the enzyme, as isolated, is at only 25% of full activity assuming that the subunit structure of fully activated ADC is $(\alpha\beta)_4$. Hence, regulation of the level of ADC activity may be postulated as occurring in two forms; by activation of the proenzyme and inactivation of the enzyme by substrate. Whether this is a dynamic control is a matter that requires further study.

One could envision a model whereby the slow activation of ADC coupled with its inactivation by aspartate would serve as a dynamic control of the steady state level of the biosynthesis of β-alanine, possibly as an interactive system exhibiting negative cooperativity effects. Hybrid molecules composed of the activated and proenzyme forms would act in concert to modulate the amount of proenzyme that undergoes cleavage. The presence of the activated peptides would exert a negative effect on the further activation of the proenzyme in a given molecule. Conversion of the active form of ADC by transamination would remove this negative influence and allow activation of the proenzyme. The appearance of this active form would then prevent further processing of the proenzyme and thereby maintain a steady level of active enzyme. Anecdotal evidence for this regulatory model is provided by the observation that, in spite of the presence of the proenzyme form in the homogeneous purified ADC, the enzyme can be stored for long periods of time at 4° C without any considerable increase in the activity of the preparation, although a slow increase is indeed observed. During this period, the quantity of the protein I proenzyme form appears to decrease while proteins II and III appear to increase as determined by SDS polyacrylamide gel electrophoresis. This model is also consistent with the slow activation of the enzyme seen in the Maxicell experiments.

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