TWO NEW ACTIVITIES AND A NEW INTERMEDIATE IN THE PURINE PATHWAY

by

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To my parents
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Abstract

Two new enzymatic activities for PurE and PurK and a new intermediate N5-carboxyaminoimidazole ribonucleotide (N5-CAIR) were discovered in the de novo purine biosynthetic pathway of E. coli. Three enzymes of the E. coli de novo purine biosynthetic pathway PurK, PurE, and PurC were purified and characterized. The first two enzymes performed the carboxylation of 5-aminoimidazole ribonucleotide (AIR) to 4-carboxyaminoimidazole ribonucleotide (CAIR). This activity differs from the pathway discovered in chicken liver, in which only PurE was involved with the carboxylation of AIR to CAIR in the presence of 200 mM bicarbonate. The third enzyme, PurC, converts CAIR to 5-aminoimidazole-4-N-succinylcarboxamide ribonucleotide (SAICAR). Enzymatic conversion of AIR to SAICAR is possible with PurE and PurC in the presence of high bicarbonate, questioning the purpose of PurK. At low concentrations of bicarbonate, we discovered that PurK utilizes ATP in a AIR dependent reaction, and that all three enzymes were required for SAICAR formation.

These observations suggested that an intermediate was formed during the conversion of AIR to CAIR by PurE and PurK. We proved this intermediate to be N5-CAIR, using NMR and kinetic analysis. Utilizing 1H NMR spectroscopy, PurK and its substrates (HCO3-, ATP, and AIR) produced N5-CAIR. With N5-CAIR, kinetic evidence revealed that PurE converts N5-CAIR to CAIR. In the reverse reaction of PurE, 1H NMR analysis confirmed the enzymatic carboxyl-rearrangement of CAIR to N5-CAIR followed by nonenzymatic decarboxylation to AIR. This unique carbon transfer, a decarboxylation/ recarboxylation of the substrate by PurE, was further investigated. The carbamate carbon (C7), by using [7-14C] N5-CAIR and [4,7-13C] N5-CAIR, was demonstrated to be the same carbon in the product, CAIR.

Thesis Supervisor: Prof. JoAnne Stubbe
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<tr>
<td>ADP</td>
<td>adenosine-5'-diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>L-asp</td>
<td>L-aspartate</td>
</tr>
<tr>
<td>CAIRs</td>
<td>carboxyaminoimidazole ribonucleoside</td>
</tr>
<tr>
<td>CHES</td>
<td>2[N-cyclohexylamino]ethanesulfonic acid</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DSS</td>
<td>sodium 2,2-dimethyl-2-silapentane-5-sulfonate</td>
</tr>
<tr>
<td>IPOH</td>
<td>isopropanol</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>NADH</td>
<td>β-nicotinamide adenine dinucleotide diphosphate, reduced</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-mercapto ethanol</td>
</tr>
<tr>
<td>MES</td>
<td>2-[N-morpholino]ethanesulfonic acid</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>PK</td>
<td>pyruvate kinase</td>
</tr>
<tr>
<td>RuBPC/O</td>
<td>ribulose-1,5-diphosphate carboxylase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>TAPS</td>
<td>[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloro acetic acid</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>TEAB</td>
<td>triethyl ammonium bicarbonate</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris(hydroxymethyl) aminomethane</td>
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Abbreviations for the purine enzymes and intermediates can be found in Scheme 1.1.
All experience is valuable, 
perhaps that's why it's so expensive.

Jean Stubbs
Chapter 1

Introduction to the *de novo* Purine Biosynthetic Pathway
**Introduction.** The *de novo* biosynthesis of purine nucleotides utilizes fifteen enzymes. Initiated with 5'-phosphoribosyl-α-1'-pyrophosphate (PRPP), eleven of these enzymes are involved in the biosynthesis of inosine monophosphate (IMP), a precursor for adenosine monophosphate (AMP) or guanosine monophosphate (GMP) (Scheme 1.1). Because the purine biosynthetic pathway is generally conserved in many organisms (human, chicken, *E. coli, B subtilis, S. cerevisiae*), they have all been extensively studied (Ebbole & Zalkin, 1987; Ebbole & Zalkin, 1989; Schild, 1990; Zalkin & Dixon, 1992) (Neuhard & Nygaard, 1987). Precise control of purine biosynthesis and the appropriate ratio to pyrimidine production is essential for the fidelity of DNA biosynthesis, and thus requires substrate/product regulation at multiple levels along the biosynthetic pathway.

In *E. coli*, regulation at the transcription level of *de novo* purine biosynthesis involves the PurR repressor, which has significant sequence homology with the LacI family of bacterial transcription repressors (Weicket & Adhya, 1992). The three dimensional structure of PurR was the first member of this family to be solved and facilitated the determination of the Lac repressor (Schumacher et al., 1994). The *E. coli* purR product is dependent on cooperative binding of hypoxanthine (Kd of 9.3 μM) or guanine (Kd of 1.5 μM), which results in a conformational change in PurR (Choi & Zalkin, 1992) (Schumacher et al., 1995). This conformational change allows the repressor to bind a specific 16-base pair region (the pur box) overlapping the promoter sequence of each gene (except purB) and to reduce transcription by 5-17 fold (He, 1990; Meng et al., 1990). The PurR-corepressors complex has an apparent Kd of 3.4 nM when bound to pur box of purF.

In addition to transcriptional control, the pathway is allosterically regulated at the enzymatic level. PRPP aminotransferase catalyzes the first committed step in
Scheme Legend 1.1:
The purine enzymes in avian and human systems contain multifunctional proteins: steps 2, 5, and 3 corresponds to the activity of glycineamide ribonucleotide synthetase, aminoimidazole ribonucleotide synthetase, glycineamide ribonucleotide transformylase (PurDMN) (Aimi et al., 1990b), steps 10 and 11 corresponds to the activity of 5-aminoimidazole-4-carboxamide-ribo nucleotide transformylase and inosine monophosphate cyclohydrolase (PurH) (Ni et al., 1991), steps 6, 7, and 8 corresponds to the activity of 5-aminoimidazolecarboxylase and SAICAR synthetase (PurEC) (Chen et al., 1990). The remaining steps have a single enzymatic activity: step 1 (5'-phosphoribosyl-1'-pyrophosphate amidotransferase) PurF (Cheng et al., 1990), step 4 (formylglycineamide ribonucleotide amidotransferase; PurL) (Barnes et al., 1992), and step 9 (Adenylosuccinate lyase; PurB) (Aimi et al., 1990).
Scheme 1.1: The *de novo* Purine Biosynthetic Pathway in *E. coli*.
the pathway (Scheme 1.1, step 1), and is the only enzyme within the pathway towards IMP that is negatively regulated by the pathways final products: AMP and GMP (Messenger & Zalkin, 1978). Subsequent to IMP formation, the enzymes initiating the branch towards AMP or GMP are cross regulated (product of one branch inhibits the first enzyme in the other branch). The purine pathway is an area of interest for cancer research, since cancer cells have high levels of purine biosynthesis (Daubner et al., 1985; Christopherson & Lyons, 1990). It has been shown that chemotherapeutic inhibitors to dihydrofolate reductase (methotrexate and aminopterin (Blaney et al., 1984)) were also inhibitors, subsequent to their glutamation, for GAR-TF and AICAR-TF (Allegra et al., 1985; Chabner et al., 1985), the two folate requiring enzymes of the purine pathway. Later, the discovery that 5,10-dideazatetrahydrofolate, an inhibitor of GAR-TF, was able to stop tumor group, renewing interest in drug design (Shih et al., 1987; Beardsley et al., 1989; Ray et al., 1993). With the recent structure of GARTF, more potential candidates are being studied (Chen et al., 1992; Klein et al., 1995).

Because of the biological importance of purines, several salvage enzymes exist to recycle purines, including: adenyl kinase, methylthioadenosine phosphorylase, xanthine oxidase, adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP), adenine phosphoribosyltransferase (APRT), hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (Weber et al., 1990). Deficiency in these enzymes and other imbalances in purine metabolism are related to several disorders: trisomy 21 Downs syndrome (Fuller, 1962; Patterson et al., 1981), Rheumatoid arthritis (Cornstein et al., 1995), gout (Sorenson, 1962), Lesch-Nyhan syndrom (Seegmiller 1967), urinary and bladder stones (de Vries, 1977), and ischemia (Grune et al., 1995). The immune system in particular is also affected by purine imbalances (Camici et al., 1995): ADA defects destroy lymphocytes, and
defective PNP destroys T lymphocytes. Superactive phosphoribosyl pyrophosphate synthetase causes elevated levels of PRPP, which in turn stimulates the pathway to produce more purines (Becker et al., 1987; Bory et al., 1994). A common feature of these diseases include an increase in uric acid levels (Camici et al., 1995).

Since its original discovery in pigeon liver by Buchanan and coworkers (Lukens & Buchanan, 1959), the de novo biosynthetic pathway has been shown to be conserved from bacteria to man. Enzyme mutants of all the steps in the purine biosynthetic pathway have been identified in E. coli (Bachmann & Low, 1980). Complementation of these mutants with human and avian DNA, with selection for removal of purine auxotrophy, has allowed cloning of all of the mammalian and avian genes in the pathway except for aminotransferase (Zalkin&Dixon, 1992). In higher organisms many of the enzymes in the pathway have been shown to contain multiple activities (see Figure legend 1.1). With genetic identification of all the genes in the E. coli pathway, several new observation were uncovered. An interesting discovery, and of great importance anticancer agent design, is that deletion of the purN product, GAR-TFase, did not lead to purine auxotrophy. This observation led to the discovery of PurT (Nygaard, 1993), which catalyzed the same reaction as PurN (Marolewski et al., 1994). Instead of using N-10-formyltetrahydrofolate (formyl-FH4) in the formylation of glycineamide ribonucleotide (GAR) (Scheme 1.1, step 3), PurT utilizes ATP and formate, generating formylphosphate which formylates GAR. However, this reaction is 100 fold slower (Marolewski et al., 1994). As a source of formate for PurT, another enzyme was discovered (PurU), which supplies this unique substrate for PurT (Nagy et al., 1993; Nagy et al., 1995).

The second discovery involves aminooimidazole ribonucleotide (AIR) carboxylase (Scheme 1.1, step 6, Scheme 1.2). From genetic studies by Gots, a point mutation in either purE or purK generated organisms auxotrophic for purines (Gots
et al., 1977). In the purK mutant case, growth of *E. coli* in an enriched CO₂ atmosphere also overcame the auxotrophic phenotype. This suggested that the function of PurK was to deliver CO₂ to PurE. The genes were isolated from

![Scheme 1.2: Proposed Catalysis of AIR carboxylase.](image)

*E. coli* (Tiedeman et al., 1989; Watanabe et al., 1989) and were determined to be on the same operon. The hypothesis was thus considered that PurE and PurK were subunits for AIR carboxylase and that PurK delivered CO₂ to the PurE enzyme.

From the original work by Buchanan (Lukens & Buchanan, 1959), and subsequent studies with purified chicken liver enzyme and yeast enzyme, revealed that the only substrate requirement for this reaction is bicarbonate. No additional cofactors, like ATP, were required for this process (Ahmad et al., 1965). The concentration of this substrate is exceedingly high ~200 mM. A single enzyme involved with the carboxylation of AIR carboxylase from *S. cerevisiae* has a Km for AIR of 10 μM and Km for bicarbonate of 130 mM (Nikolaeva et al., 1975), and therefore generated some suspicions. From avian, as discussed above, the enzyme is bifunctional with AIR carboxylase having a Km for AIR of 76 μM, and a Km for bicarbonate of 23 mM (Firestine & Davisson, 1994). In these two systems, the high concentrations of bicarbonate required for this reaction were quite disturbing.
As a substrate, carbon dioxide can be a problematic for an enzyme. Atmospheric composition of CO\textsubscript{2} is 0.033\%, and its concentration in solution is only 10 μM; however, the hydrated form is 20 fold greater at pH 7.4 (Inoue & Yamazaki, 1982). Within organisms, biosynthetic pathways can provide internal sources of CO\textsubscript{2} through oxidative or nonoxidative decarboxylations. However, single cell organisms cannot control diffusion of carbon dioxide effectively. This is an advantages in multicellular organisms which utilize a circulator systems to maintain homeostasis. In the human circulatory system, the level of bicarbonate in blood is 28 mM (1.4 mM CO\textsubscript{2}) (Guyton, 1986). In addition to the concentration difference between CO\textsubscript{2}/bicarbonate, the chemical properties also differ. Carbon dioxide has an electrophilic carbon facilitating nucleophilic attack, while CO\textsubscript{2} has enhanced reactivity relative to bicarbonate, its structure provides no binding energy. Bicarbonate, on the other hand, is negatively charged and has a handle to facilitate binding within the active site.

Interestingly, nature has taken several approaches to solve this problem. One approach is to activate bicarbonate chemically using ATP. It is proposed, that an enzyme forms carboxyphosphate, an unstable intermediate with an inferred half life of 70 msec (Caplow, 1968). Carboxyphosphate allows either for nucleophilic attack by the substrate or enzyme releasing phosphate, or allows for its decomposition to phosphate and carbon dioxide, generating a high local concentration of CO\textsubscript{2} for nucleophilic attack by the substrate or enzyme (Sauers et al., 1975). In all cases, the initial recipient of the carboxyphosphate carboxylation is an amine which can generate a carbamate. To facilitate this type of carbon transfer, a covalently bound biotin cofactor is employed by many enzymes (Table 1.1). However, as also revealed in Table 1.1 a number of enzymes can in fact use CO\textsubscript{2} directly and efficiently. The details of CO\textsubscript{2} binding remain to be established.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>RuBP carboxylase</td>
<td>CO2</td>
</tr>
<tr>
<td>PEP carboxykinase</td>
<td>CO2</td>
</tr>
<tr>
<td>PEP carboxylase (plants)</td>
<td>CO2</td>
</tr>
<tr>
<td>PEP carboxytransphosphorylase</td>
<td>CO2</td>
</tr>
<tr>
<td>Malic enzyme</td>
<td>CO2</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>CO2</td>
</tr>
<tr>
<td>6-Phosphogluconate dehydrogenase</td>
<td>CO2</td>
</tr>
<tr>
<td>CO2-reductase/ carbonate dehydratase</td>
<td>CO2</td>
</tr>
<tr>
<td>Prothrombin-precursor carboxylation enzyme</td>
<td>CO2</td>
</tr>
<tr>
<td>PEP carboxylase *</td>
<td>HCO$_3^-$</td>
</tr>
<tr>
<td>Acetyl CoA carboxylase *</td>
<td>HCO$_3^-$</td>
</tr>
<tr>
<td>Propionyl CoA carboxylase *</td>
<td>HCO$_3^-$</td>
</tr>
<tr>
<td>Pyruvate carboxylase*</td>
<td>HCO$_3^-$</td>
</tr>
<tr>
<td>β-Methylcrotonyl CoA carboxylase *</td>
<td>HCO$_3^-$</td>
</tr>
<tr>
<td>Geranoyl CoA carboxylase *</td>
<td>HCO$_3^-$</td>
</tr>
<tr>
<td>Urea carboxylase *</td>
<td>HCO$_3^-$</td>
</tr>
<tr>
<td>Carbamate kinase</td>
<td>HCO$_3^-$</td>
</tr>
<tr>
<td>Carbamoyl phosphate synthase</td>
<td>HCO$_3^-$</td>
</tr>
</tbody>
</table>

Table 1.1: Enzymes which Utilize Carbon Dioxide or Bicarbonate.
* - utilizes biotin

Of particular interest in this thesis is the only carbon-carbon bond formed in the purine biosynthetic pathway, through a carboxylation of AIR to CAIR (Scheme 1.2). The purE and purK gene products in E. coli catalyze this reaction. To undertake this study, the functions of the enzymes generated by purC, purE, and purK genes in E. coli have been purified to homogeneity. The PurC (Scheme 1.1, step 8) was very useful in assaying PurE and PurK. Studies were presented which demonstrate that PurK exhibited a previously unrecognized activity in the purine biosynthetic pathway, catalyzing the conversion of AIR to N$_5$-CAIR while requiring ATP and HCO$_3^-$ (Scheme 1.1, step 6). The enzyme, PurE, has been shown to catalyze a novel rearrangement in which N$_5$-CAIR is converted to CAIR.
Ironically, sequence comparison of PurT and PurK revealed a 55% conserved and a 27% exact identity, and both are involved with a single carbon addition. Thus the homology between PurT and PurK, two newly discovered enzymes in this well studied pathway may confirms a logical pattern which has now been elucidated chemically.

However, several key question remain unresolved. Do PurT and PurK have counterparts in mammalian systems? Recent studies of (Chen et al., 1990) shed some light on this question with respects to PurK, they used an E. coli purK auxotroph (TX209 - the codon for Tyr147 (TAC) was changed to a TAA termination codon), to screen for the mammalian AIR carboxylase. A gene was isolated and sequenced; however, it strikingly revealed no sequence relation to PurK, but had 22% identity to PurE. Mammalian PurE alone was able to make CAIR in the absence of N5-CAIR processing enzyme. These results, coupled to the recent studies of Firestein et al. (1994) suggest that the mammalian AIR carboxylase uses CO2, while the prokaryote system uses HCO3- and ATP. The lack of conservation of chemistry in a primary metabolic pathway is extremely unusual and underlines the relevance of these investigations. Similarly, PurT, has been sought and not yet found in mammalian systems. Thus, the discovery of two new enzymatic activities and a new intermediate in the purine biosynthetic pathway, without observable mammalian counterparts, suggests that these enzymes are unique to prokaryotes and lower eukaryotes.
References


Chapter 2

Purification and Initial Characterization of 5-aminomidazole-4-N-succinylcarboxamide ribonucleotide synthetase
Introduction: 4-[(N-succinylamino)carboxyl]-5-aminomidazole ribonucleotide (SAICAR) synthetase is the eighth enzyme in the purine biosynthetic pathway in E. coli and the seventh in eukaryotes. It catalyzes the conversion of 4-carboxy-5-aminomidazole ribonucleotide (CAIR) in the presence of Asp and ATP to SAICAR, ADP and Pi (Scheme 2.1). Since its discovery by Buchanan and coworkers (1959), the protein has been purified to homogeneity from chicken liver (Patey & Shaw, 1973; Firestine & Davisson, 1994) and Saccharomyces cerevisiae (Alenin et al., 1987). Furthermore, the genes coding for this protein from a number of sources have been cloned and sequenced (see Figure 2.15 at the end of this chapter).

Studies of (Patey & Shaw, 1973) revealed that the chicken liver enzyme is 52 kDa, which agrees with the molecular weight based on the gene sequence of 47 kDa (Firestine & Davisson, 1994), and contains in addition to SAICAR synthetase activity, the activity of the preceding enzyme in the pathway: 5-aminomidazole ribonucleotide carboxylase. The yeast enzyme on the other hand is a monomer of 36 kDa and contains only SAICAR synthetase activity. Recent studies have reported

![Scheme 2.1: SAICAR synthetase catalyzed reaction.](image-url)
the crystallization of this protein which should soon give rise to a high resolution structure (Grebenko et al., 1992).

The substrate specificity of the yeast enzyme has been examined in some detail and revealed, as shown in Table 2.1, that the 2 and 3 hydroxyls of the ribose and the amino group of the imidazole moiety are not essential for turnover (Ostanin et al., 1989). Studies with aspartate analogs revealed that alanosine was a substrate for this enzyme.

![Chemical Modification Sites on CAIR](image)

**Scheme 2.2:** Chemical Modification Sites on CAIR.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Vmax</th>
<th>Km (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAIR</td>
<td>substrate</td>
<td>76</td>
</tr>
<tr>
<td>R1 CH3-</td>
<td>&quot;</td>
<td>76</td>
</tr>
<tr>
<td>R1 C2H5-</td>
<td>&quot;</td>
<td>80</td>
</tr>
<tr>
<td>R2 H-</td>
<td>&quot;</td>
<td>54</td>
</tr>
<tr>
<td>R3 H-</td>
<td>&quot;</td>
<td>37</td>
</tr>
<tr>
<td>R4 H-</td>
<td>&quot;</td>
<td>40</td>
</tr>
<tr>
<td>R5 Br-</td>
<td>&quot;</td>
<td>4.8</td>
</tr>
<tr>
<td>R5 CH3-</td>
<td>&quot;</td>
<td>79</td>
</tr>
<tr>
<td>R6 N</td>
<td>inhibitor</td>
<td></td>
</tr>
<tr>
<td>Aspartate alanosine</td>
<td>substrate</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1: Substrate analogs for yeast PurC.
Our laboratory has been interested in studying the purine biosynthesis pathway in *E. coli*. To facilitate our analysis of the PurE and PurK catalyzed reaction, a coupled assay using SAICAR synthetase was developed. However, *E. coli* PurC had not been previously isolated and characterized. Therefore, Smith and his colleagues cloned the *purC* gene by complementation of *E. coli* *purC*-mutants (Tiedemann et al., 1990). The gene was sequenced and then sub-cloned into an overexpression system behind a λpL promoter. The plasmid carrying the gene has been designated pJS408.

This chapter reports the purification and characterization of SAICAR synthetase and the chemical synthesis of the substrate required for this enzyme, CAIR. These studies are both prerequisites for the studies reported in chapters 3 and 4 of this thesis.

**Materials.** Lactate dehydrogenase (LDH, 860 U/mg), pyruvate kinase (PK, 470 U/mg), malate dehydrogenase (MD, 100 U/mg), phosphoenolpyruvate (PEP), PEP carboxylase (PEPC, 3 U/mg), Glutamic-oxalacetic transaminase (GOT, 350 U/mg), L-aspartate, β-mercaptoethanol (β-ME), phenylmethanesulfonyl fluoride (PMSF), β-nicotinamide adenine dinucleotidediphosphate - reduced (NADH), diethylaminoethyl (DEAE)-Sepharose CL-6B, Dalton Mark VII molecular weight markers, bovine serum albumin (fraction V, BSA), 5-amino-1(β-D-ribfuranosyl) imidazole-4-carboxamide (AICARs) and adenosine-5 -triphosphate (ATP) were obtained from Sigma Chemical Co. Affi-Gel Blue and Dowex 50W-X8 were purchased from BioRad. Triethyl phosphate, phosphorus oxychloride, and triethylamine (TEA) were obtained from Aldrich. Amicon membranes were purchased from WR Grace. Sephadex G-25, and DEAE A-25 Sephadex was obtained from Pharmacia. Alkaline phosphatase was purchased from Boehringer Mannheim. Plasmid pJS408 encoding *purC* from *E. coli* was a gift from Dr. John Smith.
Methods. The concentration of aspartate was determined enzymatically with GOT and MD (Yagi, 1985). The phosphate concentration were determined by the method of Ames & Dubin (1960), with inorganic phosphate as a standard. The protein concentration was determined by the method of Lowry (1951), with BSA as a standard. TEAB solution was made by bubbling carbon dioxide (from dry ice) through the solution until saturated (pH 7.4).

Substrate Synthesis

Synthesis of CAIR

Phosphorylation of AICARs (1). Following the method of Yoshikawa (Yoshikawa et al., 1967; Srivastava et al., 1975), 1 (7.46 mmol) was dried with P$_2$O$_5$ overnight, then suspended in triethyl phosphate (70 mL, 540 mmol) and cooled to 4°C. Triethyl phosphate and POCl$_3$ were distilled prior to use. The POCl$_3$ (7 mL, 75 mmol) was added dropwise into the stirring solution (4°C) with a syringe over a

\[ \text{Scheme 2.3: Synthesis of CAIR with (A) phosphorylation and (B) saponification.} \]
period of three min, and the reaction was stirred under argon for an additional 2 h at 4°C. The ribonucleotide was precipitated with 2 L of ether, and the solution was centrifuged in 150 mL Corvex tubes at 2500 g for 20 min. The pellets were each washed with an additional 100 mL of ether and centrifuged again. The supernatant was decanted, and the pellets were dissolved in 100 mL water at 4°C. The pH was immediately adjusted to 8 by addition of 1 N NaOH, diluted to 500 mL with 10 mM TEAB (pH 7.4), and then loaded onto an DEAE A-25 column (10 x 2.5 cm, HCO₃⁻ form). The column was developed with a linear gradient from 0 to 500 mM TEAB (500 x 500 mL, pH 7.4), and 10 mL fractions were collected and monitored by A 268 nm. AICAR eluted at 150 mM TEAB, and fractions (23-54) were concentrated in vacuo. (Anion exchange chromatography was not required if the subsequent step was carried out immediately.) AICAR was produced in a 66% yield (4.96 mmol, ε₂₆₈=12,900 M⁻¹cm⁻¹)(Huang, 1965). A portion of this product was exchanged into D₂O for NMR analysis: ¹H NMR (300 MHz, D₂O, pD 7, TSP): δ 7.46 (s, 1, H2), 5.62 (d, J = 5.75, 1, H1 ), 4.63 (m, 1, H2 ), 4.38 (m, 1, H3 ), 4.26 (m, 1, H4 ), 3.95 (m, 2, H5 , H5") (Figure 2.1). ¹³C NMR (75 MHz, D₂O, pD 7, methanol at δ = 50 ppm): δ 169.3 (s, C6), 144.8 (s, C5), 132.3 (s, C2), 112.9 (s, C4), 89.1 (s, C1 ), 85.9 (s, C4 ), 73.3 (s, C3 ), 71.6 (s, C2 ), 64.7 (s, C5 ), (Figure 2.2).

**Saponification of AICAR(2) to CAIR(4).** Following the method of Srivastava et al. (1974), 5.09 mmol 2 (from ether precipitation of the previous phosphorylation) was dissolved in 6 N NaOH (5.1 mL, 30 mmol). Under argon, the solution was refluxed for 4 h and was then chilled on ice. The mixture was triturated in glass Corvex centrifuged tubes with 10 mLs ethanol, vortexed for one min, and then centrifuged for 2 min in a clinical centrifuge. The supernatant was decanted and the remaining solid was washed three times with 2.5 mL of ethanol, and three times with 1.3 mLs ethanol. (The methanol trituration step was avoided because of CAIR's solubility.) The resulting paste was stored at -80°C and was stable for at least 2
years. Before further use, a portion of the paste was purified on a DEAE A-25 column by dissolving 500 µmol of 4 in 500 mL 10 mM TEAB (pH 7.4) and loading it onto an A-25 column (15 x 2.5 cm, HCO₃⁻ form). The column was developed with a linear gradient from 50-650 mM TEAB (500 x 500 mL, pH 7.4, collected 10 mL fractions). Fractions were monitored for A250 nm. This chromatography separates 3, 2, 4, diphosphorylated 2, diphosphorylated 4; eluting with 100, 200, 300, 450, and 550 mM TEAB respectively. The ratios of these compounds relative to the starting material were: 3%, 6%, 50%, 1%, and 9%. Compounds 2 and 4 appear to be doubly phosphorylated, since they eluted after 4 (Figure 2.3). The appropriate fractions (41-79) were concentrated in vacuo, with repeated methanol and water dilution to remove the TEAB. A portion of this product (4) was characterized utilizing NMR spectroscopy: ¹H NMR (D₂O, acetone standard δ = 2.2 ppm) δ 7.47 (s, 1, H2), 5.62 (d, 1, J= 7 Hz, H1 ), 4.67 (m, 1, H2 ), 4.40 (m, 1, H3 ), 4.25 (s, 1, H4 ), 3.95 (s, 2, H5 , 5"), (Figure 2.4). ¹³C NMR (75 MHz, D₂O, pH 7, methanol at δ = 50 ppm): δ 172.9 (s, C6), 143.6 (s, C5), 132.4 (s, C2), 117.8 (s, C4), 89.1 (s, C1 ), 86.0 (s, C4 ), 73.8(s, C3 ), 71.7 (s, C2 ), 64.7 (s, C5 ).

**Alternate Preparation of CAIR**

*Saponification of AICARs (1) to CAIRs (3).* AICARs (1) was saponified by a procedure previously described for saponification of 2. Following the ethanol trituration, the product was not purified, but taken onto the next step, phosphorylation. Quantitating the saponification reaction was difficult, since some starting material remains after the ethanol trituration. A portion of this product (3) was characterized utilizing NMR spectroscopy: ¹H NMR (D₂O, acetone standard δ = 2.2 ppm) δ 7.4 (s, 1, H2), 5.6 (d, J=6 Hz, 1, H1 ), 4.55 (m, 1, H2 ), 4.30 (m, 1, H3 ), 4.15 (m, 1, H4 ), 3.80 (m, 2, H5 , H5").

*Phosphorylation of CAIRs (3).* CAIRs was phosphorylated using the procedure described above for the conversion of (1) to (2). CAIRs (520 µmol) was
phosphorylated, placed on a DEAE A-25 Sephadex column (15 x 2.5 cm, HCO₃⁻ form) and eluted with a linear gradient of TEAB (500 x 500 mL, pH 8.0) at 350 mM TEAB. The appropriate fractions (45-60) were identified by monitoring A250 nm and were pooled and concentrated in vacuo to give 249 μmol of 4, (48% yield).

**Extinction coefficient of CAIR.** CAIR was purified by anion exchange chromatography and appeared to be homogeneous by NMR spectroscopic analysis. The quantity of CAIR was assayed using a PurC endpoint assay, with 100% conversion to SAICAR. In a final volume of 700 μL, the assay solution contained: 50 mM Hepes (pH 7.8), 20 mM KCl, 4.5 mM MgCl₂, 0.9 mM ATP, 0.2 mM NADH, 2.0 mM PEP, 6 mM aspartate, 10 U PK, 5 U LDH, and 0.6 U PurC. The reaction mixture was pre-incubated at 37°C, initiated with CAIR (16-75 nmol), and resulted in a stock solution of 3.73±.16 mM CAIR. In addition, the concentration of CAIR was also assayed using the phosphate assay of Ames and Dubin (3.64±.4 mM). Before addition of CAIR (13.6 μM - final concentration) to the cuvette, the spectrophotometer was blanked with buffer (500 μL, 100 mM): Ches (pH 10.0), Tris (pH 8.0), phosphate (pH 7.0), and Mes (pH 6.0). The extinction coefficient was determined from the average of 4 trials (Table 2.3).

**Absorption Spectrum of CAIR as a Function of pH.** In the following 100 mM buffers, the absorbance spectra of CAIR (45 μM) was monitored between 220 to 300 nm: Ches-KOH (pH 9.6), Tris-HCl (pH 8.1), potassium phosphate (pH 6.8), Mes (pH 6.1), NaOAc (pH 4.8), and HCl (pH 1.2) (Figure 2.5).

**Stability of CAIRs as a Function of pH.** CAIRs (0.76 mM) was incubated at room temperature, in the following buffers (100 mM buffer, pH, pKa): Ches, 9.5, 9.3; Tris, 8.0, 8.2; phosphate, 7.0, 6.8; Mes, 6.0, 6.1; and NaOAc, 4.8, 4.8. The spectrophotometer was blanked with buffer, and the absorption of CAIRs (76 μM) at 250 nm was monitored over time (0, 1, 5, 17, 28, 43 h) (Figure 2.10).
Enzyme Purification and Characterization

Growth of *E. coli* TX635/pJS408. *E. coli* TX635 containing the heat-inducible plasmid pJS408 (Tiedemann et al., 1990) was grown in 5 L of medium containing 10 g/L tryptone, 5 g/L yeast extract, 50 μg/mL ampicillin, and 10 g/L NaCl, all adjusted to pH 7.5. The cells were grown at 30°C (doubling time 45 min). At an *A*₆₀₀ nm of 1.3, the temperature was raised to 42°C with addition of an equal volume (5 L) of media at 54°C. The cells were allowed to grow at 42°C for 30 min and then at 37°C for an additional 5.5 h. The bacteria were harvested with a Sharples centrifuge to give 1.8 g cell paste per liter of medium, frozen in liquid nitrogen, and stored at -80°C.

Purification of purC Gene Product: SAICAR Synthetase. All purification steps were carried out at 4°C. The cells (15.8 g) of TX635/pJS408 were suspended at 5 mL/g in 50 mM Tris-HCl (pH 8.0), 15 mM MgCl₂, 6 mM β-ME, and 0.1% PMSF (Buffer A). The cells were ruptured using a French press pressure cell at 10,000 psi, and the cell debris was removed by centrifugation for 20 min at 10,000 g. A solution of protamine sulfate (3.7% in Buffer A) was added to the supernatant over 30 min to give a final concentration of 0.2%. At this point the absorbance spectrum of the supernatant should indicate an *A*₂₈₀/*A*₂₆₀ ratio of 0.75-0.8, otherwise more protamine sulfate was added until a ratio of 0.75-0.80 at *A*₂₈₀/*A*₂₆₀ ratio is achieved (Cooper, 1977). After an additional 20 min of stirring, the precipitate was removed by centrifugation. The supernatant (66 mL) was adjusted to 50% saturation in ammonium sulfate by addition of the solid (0.31 g/mL) over 30 min with stirring. The solution was stirred for an additional 20 min and then centrifuged at 10,000 g for 20 min. The pellet was re-dissolved in a minimal volume (~7 mL) of 25 mM Tris-HCl (pH 8.0) and 1 mM MgCl₂ (Buffer B), and desalted on a Sephadex G-25 column (42 x 2.5 cm) equilibrated in Buffer B. The fractions containing protein were diluted to 250 mL with Buffer B and loaded onto a DEAE-Sepharose CL-6B anion exchange column (13.5x1.5 cm) equilibrated with Buffer B. The activity was eluted
with 100 mM KCl using a linear gradient from 0 to 250 mM KCl (250 x 250 mL, Buffer B) (Figure 2.6). The fractions (43-60) with highest specific activity were pooled and concentrated to 30 mLs with an Amicon Diaflow Ultrafilter equipped with YM-30 membrane. The concentrated protein was then diluted to 200 mL with Buffer B, and reconcentrated to desalt the protein. This procedure was repeated twice, and then the protein (200 mL) was loaded onto an Affi-Gel Blue column (12 x 2.5 cm) equilibrated in Buffer B. (It should be noted that excessive exposure to potassium chloride reduces the Affi-Gel blue capacity, so the column needs to be washed with base (form OH-) prior to being equilibrated with Buffer B.) The enzyme eluted at 100 mM KCl using a 0 to 300 mM KCl linear gradient (300 x 300 mL, Buffer B) (Figure 2.7). The fractions containing protein of the highest specific activity (24-60) were pooled and concentrated to 30 mg/mL, using an Amicon Diaflow Ultrafilter equipped with a YM-30 membrane. The final protein solution contained 20% (v/v) with glycerol. Aliquots were frozen with liquid nitrogen and stored at -20°C.

Spectrophotometric assay for SAICAR Synthetase. In a final volume of 700 μL, the assay solution contained: 50 mM Hepes (pH 7.8), 20 mM KCl, 6.0 mM MgCl₂, 1.0 mM ATP, 0.2 mM NADH, 2.0 mM PEP, 0.5 mM CAIR, 10 U PK, 5 U LDH, and 6 x 10⁻³ U (0.2 μg, 11 nM) of PurC. The reaction mixture was pre-incubated at 37°C and initiated with 7 mM aspartate. The reaction was monitored by change in absorbance at 340 nm.

Radioactive Assay for [¹⁴C] SAICAR Synthetase. In a final volume of 250 μL at 37°C, the reaction mixture contained: 50 mM Hepes (pH 7.8), 20 mM KCl, 4.5 mM MgCl₂, 1.0 mM ATP, 2.0 mM PEP, 0.5 mM CAIR, 5 U PK, 0.015 U of PurC, and 6 mM [¹⁴C] aspartate (specific activity 1.2 x 10⁶ cpm/μmol). At various times, aliquots (45 μL) were withdrawn from the reaction mixture and quenched with 30% trichloroacetic acid (15 μL, room temperature). The quenched sample was centrifuged in an Eppendorf centrifuge for one min to pellet the denatured protein,
and the supernatant (55 µL) was loaded onto a Dowex 50W-X8 (1 mL, NH₄⁺ form), previously equilibrated with 50 mM ammonium formate (pH 3.3). The column consisted of a nine inch glass pasture pipette with a glass wool frit containing the resin. [¹⁴C] SAICAR was eluted with 5 mLs of 50 mM ammonium formate (pH 3.3); a portion of the elution (1 mL) was analyzed for radioactivity with scintillation counting.

**Determination of the Stoichiometry of the SAICAR Synthetase Reaction.** In a final volume of 500 µL (37°C), a typical assay contained: 50 mM Hepes(pH 7.8), 20 mM KCl, 1.0 mM ATP, 5.4 mM MgCl₂, 0.2 mM NADH, 4.3 mM [¹⁴C] aspartate (1.4 x 10⁶ cpm/µmol), 0.4 mM CAIR, 2 mU PurC, 10 U PK, and 5 U LDH. Using the coupled assay, the rate of ATP hydrolysis was monitored at 340 nm. During this continuous assay, aliquots (45 µL) were removed from the cuvette at fixed times (0.5, 1.5, 3, 5, 10 min), and the amount of [¹⁴C] SAICAR produced was quantitated as described above.

**Kinetic Analysis of SAICAR Synthetase.** All kinetic studies were conducted using the spectrophotometric assay described above. The Michaelis constants for aspartate, CAIR, and ATP were determined by varying the concentration of one substrate at saturating levels of the other substrates: (1) For the Km of CAIR, concentrations of CAIR (6-770 µM) were assayed with aspartate at 10 mM and ATP at 2.1 mM, (2) For the Km of aspartate, the concentrations of aspartate (0.29-12 mM) were assayed with ATP at 1.0 mM and CAIR at 0.45 mM, (3) For the Km of ATP, the concentrations of ATP (10-240 µM) were assayed with aspartate at 10 mM and CAIR at 0.45 mM (Figure 2.9). The Km determination involved patterns using five substrate concentrations evenly spaced between 0.2 to 5 Km. The data were fit to equation 1 (simplified for saturating >10 Km in other substrates) using non-linear regression analysis (Cleland, 1975) v= initial velocity, V= maximum velocity, S= substrate concentration, Kₘₐₜ= (k₋₁ + k₉₅)/k₁.
Product Analysis of the PurC Catalyzed Reaction:

Characterization of SAICAR. In a final volume of 1 mL, the reaction mixture contained: 2.0 mM ATP, 3.6 mM aspartate, 2.0 mM PEP, 6.0 mM MgCl_2, 2 mM NADH, 20 mM KCl, 1.65 mM CAIR, 100 mM Tris-HCl (pH 7.8), 10 U PK, and 5.0 U LDH. After incubation for 1.5 h at 37°C, the entire sample was loaded onto a semi-preparative HPLC reverse-phase Alltech C-18 column (25 x 1.5 cm), equilibrated in 50 mM ammonium formate (pH 3.0) and 7.5 mM tetrabutyrammonium bromide (Buffer A) at 2 mL/min. The products separated using the following program: an isocratic elution with Buffer A from 0 to 20 min, a linear gradient from 0 to 5% CH₃OH in Buffer A from 20 to 60 min, followed by a linear gradient from 5 to 10% CH₃OH in Buffer A from 60 to 80 min. The compounds and retention times were as follows: ADP, 21 min; NAD⁺, 33-39 min; SAICAR, 50-60 min; ATP and NADH, >90 min. The SAICAR was pooled and concentrated in vacuo, to give 85% recovery. It was then added to 30 mL of 10 mM TEAB (pH 7.8) and loaded onto a DEAE-Sephadex A-25 column (three mL, bicarbonate form). The product was eluted with a linear gradient from 0 to 600 mM TEAB (150 x 150 mL), and 3 mL fractions were collected, with SAICAR eluting at 250 mM. The appropriate fractions (34-46) were pooled, and the buffer removed in vacuo. Use of methanol to facilitate removal of TEAB yields SAICAR as a white solid. ¹H NMR (D₂O, pH 7, TSP at 0.0 ppm) δ 7.5 (s, 1, C2H), 5.65 (d, 1, H1 , J=7 Hz), 4.7 (m, 1, H₂ ), 4.55 (m, 1, H₃"), 4.4 (m, 1, H₃ ), 4.3 (m, 1, H₄ ), 4.0 (m, 2, H₅ , H₅"), 2.65 (m, 2, H₄"), (Figure 2.12). ¹³C NMR (D₂O, methanol at 50 ppm) δ 180.0 (s, C5"), 166.3 (s, C1"), 143.9 (s, C5), 132.3 (s, C2), 113.7 (s, C4), 89.1 (s, C1 ), 85.9 (s, C4 ), 73.2 (s, C3 ), 71.6 (s, C2 ), 64.8 (s, C5 ), 53.3 (s, C3"), 41.2 (s, C4"), (Figure 2.13, for numbering see Scheme 2.1).
**FAB mass spectrum of SAICAR.** SAICAR was analyzed in a 3-nitrobenzyl alcohol matrix and a glycerol/water matrix by negative ion FAB mass spectroscopy, using an 8200 Finnigan MAT with 8000 volts power and Xenon reaction gas: (M-1) 453 (Figure 2.14).

**Extinction Coefficient Determination for SAICAR.** In a final volume of 10 mLs (37°C), the reaction mixture contained: 100 mM Tris-HCl (pH 7.8), 20 mM KCl, 6 mM MgCl₂, 1.8 mM ATP, 5.5 mM PEP, 200 U PK, 2.53 mM [¹⁴C] Asp (9.44 x 10⁵ cpm/µmol), 9.17 mM CAIR, and 20 U PurC. The reaction was initiated with 10 U PurC, and the rest added 30 min later. After 1 h at 37°C, 2 mLs of 500 mM Tris-HCl (pH 8.5), and 1 mM EDTA were added, along with 200 U of alkaline phosphatase. After 4 h, the reaction was diluted with water to 500 mL. The pH was reduced with acetic acid to pH 4, and then loaded on to a column (DEAE A-25, 10 x 2.5 cm, formate form). The column was developed with a linear gradient from 0 to 500 mM TEA-Ac (pH 6.0, 500 x 500 mL). Collecting 10 mL fractions, an elution profile was generated with A₂₆₈ and scintillation counting. While loading the column, the flow through and the first four fractions of the gradient contained all the adenosine (previously ATP). Similarly, the radioactivity, [¹⁴C] Asp and [¹⁴C] SAICARs were recovered in fractions 10-25 and 60-80, respectively. The region containing [¹⁴C] SAICAR was pooled and concentrated in vacuo. The SAICARs was analyzed by UV spectroscopy in 100 mM Tris (pH 7.8), 6 mM MgCl₂, and 20 mM KCl. The concentration of SAICARs was based on the specific activity of aspartate.

**Results**

*Synthesis and Characterization of CAIR.* In order to assay SAICAR synthetase, a source of readily available substrate CAIR was sought. Two different approaches to synthesize CAIR were used (Scheme 2.3), based on previously published procedures.
Both methods started with AICARs, and involved hydrolysis of AICARs to the free acid and phosphorylation of the 5 hydroxyl group with POC13 in triethyl phosphate (Figure 2.1, Figure 2.2). These two procedures gave overall yields of 35%. The preferred sequence was phosphorylation followed with saponification, as the conditions for phosphorylation require anhydrous reagent which allowed for AICARs (a powder) to be dried over P2O5. CAIR was purified with anion exchange chromatography (Figure 2.3) and was characterized with NMR (Figure 2.4) and UV spectroscopy.

**Determination of the Extinction Coefficient for CAIR.** Previous studies of Srivastava (1974) and Litchfield (1971), reported conflicting numbers: \( \varepsilon_{249}=10,003 \) M\(^{-1}\)cm\(^{-1}\) in water and \( \varepsilon_{250}=8,700 \) M\(^{-1}\)cm\(^{-1}\) at pH 7.3; respectively, for the nucleoside CAIRs. Since the phosphorylated derivative was a major player in all of the studies reported in this thesis, its extinction coefficient needed to be determined. The amount of CAIR was quantitated using the enzymatic end point assay involving PurC (3.73±.16 mM), and was cooberated using the phosphate assay (3.64±.4 mM). The purity (±5%) was confirmed by \(^1\)H NMR spectroscopy. Using these methods, the extinction coefficients for CAIR was determined at 250 and 260 nm at various pHs as summarized in Table 2.3.

<table>
<thead>
<tr>
<th>pH</th>
<th>( \varepsilon_{250} ) (M(^{-1})cm(^{-1}))</th>
<th>( \varepsilon_{260} ) (M(^{-1})cm(^{-1}))</th>
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<tbody>
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<td>10500±100</td>
</tr>
<tr>
<td>pH 7.0</td>
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<td>10080±110</td>
</tr>
<tr>
<td>pH 6.0</td>
<td>9440±200</td>
<td>9080±200</td>
</tr>
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</table>

Table 2.3: Extinction Coefficients for CAIR.

The spectrum of CAIR, as revealed in Figure 2.5 changes as a function of pH. The \( \lambda_{\text{max}} \) 252 at pH 8 (\( \varepsilon_{252} = 10,980 \) M\(^{-1}\)cm\(^{-1}\)) shifts under acidic conditions of pH 4.8 to 244 nm(\( \varepsilon_{244} = 9,730 \) M\(^{-1}\)cm\(^{-1}\)). At pH 1.2, the \( \lambda_{\text{max}} \) shifts to 268 nm (\( \varepsilon_{268} \)
which is very similar to the absorbance AICAR ($\lambda_{\text{max}}$ 268 nm, 
$\varepsilon_{268}=12,900$ M$^{-1}$cm$^{-1}$) (Flaks et al., 1957). The stability of the nucleoside was
examined under a variety of conditions by monitoring $A_{250}$ nm changes (Figure
2.10). Under acidic conditions, CAIRs is unstable and is converted to AIRs via
decarboxylation, as determined by product analysis (Groziak et al., 1988). CAIR is
spectroscopically distinct from AIR, as shown in the Figure 3.5. This experiment
suggests alkaline storage conditions are appropriate for CAIR.

Purification of PurC. The *E. coli* gene for SAICAR synthetase was cloned into a
$\lambda$pL expression vector pJS408 and transformed into *E. coli* TX635 (Tiedemann et al.,
1990). The heat induction of *E. coli* strain TX635 containing this plasmid resulted in
over-expression of a 27-kDa protein. A DEAE CL-6B and an Affi-Gel Blue were used
to purify the enzyme to homogeneity (Figure 2.6, Figure 2.7). The purification was
monitored with activity (Table 2.2) and was observed by SDS gel electrophoresis
(Figure 2.8).

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein mg</th>
<th>Volume mL</th>
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<th>Specific Activity units/mg</th>
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<td>2156</td>
<td>70</td>
<td>1230</td>
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<td>80</td>
<td>2180</td>
<td>4.26</td>
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<tr>
<td>DEAE CL-6B</td>
<td>122</td>
<td>30</td>
<td>1477</td>
<td>12.1</td>
</tr>
<tr>
<td>Affi-Gel Blue</td>
<td>58</td>
<td>1.9</td>
<td>1800</td>
<td>31</td>
</tr>
</tbody>
</table>

Table 2.2: Purification of PurC. a 15.8 g of TX635 containing pJS408.

Incubation of SAICAR synthetase with ATP, CAIR, and aspartate resulted in
the stoichiometric production of SAICAR, ADP and Pi (Scheme 2.1). The ADP was
quantitated using the PK/LDH coupled enzyme assay by monitoring NADH
oxidation. Using $[^{14}\text{C}]$ Asp of known specific activity, $[^{14}\text{C}]$ SAICAR was isolated and
quantitated with scintillation counting. The Km values for aspartate, MgATP, and
Figure 2.1: $^1$H NMR of AICAR. The NMR spectrum of AICAR at pH 7 in D$_2$O.

Figure 2.2: $^{13}$C NMR of AICAR. The NMR spectrum of AICAR at pH 7 in D$_2$O, using methanol as the reference at 48 ppm.
AICAR (2)
Figure 2.3: A typical DEAE Sephadex A-25 column profile of CAIR. The compounds eluting from this column with TEAB are as follows (fractions): AICARs (10-20), CAIRs (27-35), CAIR (40-60), doubly phosphorylated AICARs (68-72), and doubly phosphorylated CAIRs (79-92).

Figure 2.4: 1H NMR of CAIR. The NMR spectrum of CAIR at pH 8.3 in D2O, using acetone as the reference at 2.2 ppm.
Figure 2.5: Absorption Spectrum of CAIR as a Function of pH. CAIR was diluted to 45 μM in the following buffers: Ches-KOH (pH 9.6), Tris-HCl (pH 8.1), Potassium Phosphate (pH 6.8), Mes (pH 6.1), NaOAc (pH 4.8), and HCl (pH 1.2).
Figure 2.6: A typical DEAE-Sepharose CL-6B column profile for PurC. From a CL-6B column, an elution profile was generated with protein absorbance at $A_{280}$ (○) and PurC activity (●). The column was loaded (fractions -80 to -40), washed with buffer (fractions -40 to 0), and then developed with a 0 to 250 mM KCl gradient. PurC activity was found in the first peak, eluting after 50 mM KCl. Fractions (43-60) were pooled.

Figure 2.7: A typical Affi-Gel Blue Column Profile for PurC. From an Affi-Gel Blue column, an elution profile was generated with protein absorbance at $A_{280}$ (○) and PurC activity (●). The column was loaded (fractions -30 to -10), washed with buffer (fractions -10 to 0), and then developed with a 0 to 300 mM KCl gradient. PurC activity was eluted after 50 mM KCl.
Figure 2.8: SDS-Page Gel of PurC Preparations. The gel depicted is 10% polyacrylamide with 2.7% cross linking. All lanes contain 50 μg total protein. The gel was loaded as follows: Lane (1) molecular weight standards, (2) crude *E. coli* TX635/pJS408, (3) Sephadex G-25 0-50% ammonium sulfate, (4) Sepharose CL-6B, (5) Sepharose CL-6B, (6) Affi-Gel Blue, (7) Affi-Gel Blue, 9 μg, (8) molecular weight standards (kDa): Bovine Serum Albumin, 66; Hen Egg Albumin, 45; Glyceraldehyde-3-Phosphate Dehydrogenase, 36; Carbonic Anhydrase, 29; Trypsinogen, 24; Trypsin Inhibitor, 20.1; and α-Lactalbumin, 14.2.
Figure 2.9: Kinetic data for PurC. (A) For the Km of ATP, the concentrations of ATP (10-240 μM) were assayed with aspartate at 10 mM and CAIR at 0.45 mM, (B) For the Km of aspartate, the concentrations of aspartate (0.29-12 mM) were assayed with ATP at 1.0 mM and CAIR at 0.45 mM, (C) For the Km of CAIR, concentrations of CAIR (6-770 μM) were assayed with aspartate at 10 mM and ATP at 2.1 mM.
Figure 2.10: Stability of CAIRs as a Function of pH. The absorbance at 250 nm of CAIRs was monitored in the following buffers (pH): ○ - HOAc at pH 4.8, × - Mes at pH 6.0, ◆ - Phos at pH 7.0, ◇ - Tris at pH 8.0, ● - Ches at 9.5, and Δ - NaOH at 1N.
CAIR were determined to be 1.04±0.08 mM, 39±1 μM, and 36±14 μM, respectively (Figure 2.9).

**Characterization of purC Product: SAICAR.** Enzymatically produced SAICAR was purified by HPLC and anion exchange chromatography (absorption spectrum, Figure 2.11). The NMR spectrum (Figure 2.12) is consistent with the structure; however, the carbon spectrum is missing one resonance (Figure 2.13). The two carbonyl groups originating from aspartic acid (C5", C6") have resonances which were superimposable at 180 ppm on both the 300 and 500 MHz NMR spectrometers. An FAB mass spectrum in the negative mode confirmed SAICAR (M-1, 453) (Figure 2.14).

The extinction coefficient for [\(^{14}\)C] SAICAR was determined by preparing [\(^{14}\)C] SAICAR using [\(^{14}\)C] aspartic acid and quantitating the amount of labeled nucleoside. To facilitate the separation of SAICAR, the reagents were dephosphorylated with alkaline phosphatase. Dephosphorylated SAICAR was purified using anion exchange chromatography which allowed separation of adenosine. The observed λmax is at 268 nm with an ε\(_{268}\) = 16,235±92 M\(^{-1}\)cm\(^{-1}\), in 100 mM Tris-HCl (pH 7.8), 20 mM KCl, 6 mM MgCl\(_{2}\). This value at 268 nm differs from previous reported values (pH 8.0): ε=13,300 M\(^{-1}\)cm\(^{-1}\) (Lukens & Buchanan, 1959), ε=14,500 M\(^{-1}\)cm\(^{-1}\) (Huang, 1965), ε=13,800 M\(^{-1}\)cm\(^{-1}\) (Shaw & Wilson, 1963) ε=14,895 M\(^{-1}\)cm\(^{-1}\) (Firestine&Davisson, 1994)). My extinction coefficient value is larger than those previously published for reasons that are unclear. SAICAR stability was not determined, and extended handling may result in a higher extinction coefficient species (similar to AIR).
Figure 2.11: Absorption of SAICAR. The absorption spectrum of SAICAR purified by HPLC and concentrated in vacuo, is shown in 80 mM Phosphate, 10 mM (Bu)$_4$N$^+$Br$^-$ (pH 3.0).
Figure 2.12: $^1$H NMR of SAICAR. The 88 mM natural abundance SAICAR (pH 7) was scanned 100 times, with TSP at 0 ppm.

Figure 2.13: $^{13}$C NMR of SAICAR. The 88 mM natural abundance SAICAR (pH 7) was scanned 4000 times, D1=0.5, with methanol reference at 50 ppm.
SAICAR (5)

180 120 60 0 ppm

ppm

O

=PO

HO

OH

CO₂⁻

CO₂⁻

N

N

H₂

H₂
Figure 2.14: Mass spectra of SAICAR. Negative ion FAB mass spectra revealed SAICAR (M/z = 453) in two different matrices: (A) glycerol matrix and (B) nitrobenzyl alcohol matrix. In Spectrum A, the glycerol ion was at 367. In spectrum B, the nitrobenzyl alcohol matrix ions were at 459.
Discussion

SAICAR synthetase was purified to homogeneity. Analysis of starting material and products revealed that CAIR in the presence of ATP and aspartate is quantitatively converted to ADP, SAICAR and Pi. Kinetic analysis revealed parameters similar to other SAICAR synthetases (Table 2.4). It seems likely that the discrepancy in Km values between our data with E. coli and that for yeast and chicken SAICAR synthetase may be related to their use of quantitating product at 282 nm, and our use of a PK/LDH coupled assay for ADP formation. Accurate quantitation of the rates below the Km value found for the yeast and chicken requires a special assay technique to measure only 10-20% CAIR turnover. In addition, the change in A282nm, due to the absorption of substrates (CAIR and ATP) in the same region, makes this assay unreliable.

The present studies have set the stage to study the enzymes PurE and PurK in subsequent chapters. PurC is readily available in large quantities to function in the coupled assays described subsequently.

<table>
<thead>
<tr>
<th>Species</th>
<th>CAIR (µM)</th>
<th>ATP (µM)</th>
<th>Asp (µM)</th>
<th>Reference</th>
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<td>39</td>
<td>1040</td>
<td></td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>3</td>
<td>10</td>
<td>1400</td>
<td>(Ostanin et al., 1989)</td>
</tr>
<tr>
<td>chicken</td>
<td>1.6</td>
<td>14</td>
<td>960</td>
<td>(Firestone&amp;Davisson, 1994).</td>
</tr>
</tbody>
</table>

Table 2.4: Km values for PurC from various species.

PurC has been isolated from many sources and the genes for these synthetase have been cloned and sequenced. Despite the observation that SAICAR synthetase in certain systems (human, rat, and chicken) exist as part of a bifunctional protein with PurE, the remaining examples are single proteins (Table 2.5, Figure 2.15). A comparison of these gene sequences revealed that out of 237 amino acids in E. coli, no distinct region is conserved, and only 35 amino acids are conserved.
Adenylosuccinate synthetase (PurA) is an enzyme in the purine pathway that utilizes aspartate. The enzyme catalyzes the addition of Asp with GTP to IMP and forms GDP, Pi and adenylosuccinate, the precursor to AMP. There is 50% similarity and 12% identity between PurA and PurC of *E. coli* sequences. Neither the region containing an active site amino acid, K140 in PurA, or the nucleotide binding site (G-X-X-G-X-G) aligned with PurC (Dong & Fromm, 1990). Another enzyme which also uses Asp and ATP is argininosuccinate synthetase in the urea cycle. This enzyme catalyzes the addition of Asp with ATP to citrulline, to form AMP, PPI and argininosuccinate with an intermediate of citrullyl-AMP intermediate (Ratners, 1973). There is 48% similarity and 9% identity between ArgG and PurC of *E. coli* sequences (Van Vliet et al., 1990). In all three case another enzyme is involved with the release of fumarate.

Table 2.5: Homology to *E. coli* PurC in other organisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>% homology for PurC</th>
<th>Similarity</th>
<th>Identity</th>
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<tr>
<td>Human <em>b</em></td>
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</tr>
<tr>
<td>Chicken <em>c</em></td>
<td>47</td>
<td>8</td>
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<td><em>B. subtilis</em> d</td>
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<td>38</td>
<td></td>
</tr>
<tr>
<td><em>S. pneumoniae</em> e</td>
<td>43</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td><em>A. thaliana</em> g</td>
<td>50</td>
<td>9</td>
<td></td>
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<tr>
<td><em>V. aconilifolia</em> h</td>
<td>48</td>
<td>6</td>
<td></td>
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<tr>
<td><em>C. maltose</em> i</td>
<td>48</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td><em>S. cerevisiae</em> j</td>
<td>46</td>
<td>9</td>
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</table>

*a* accession number D37979; (Iwahana et al., 1995)
*b* accession number P22234; (Minet & Lacroute, 1990)
*c* accession number P38024; (Chen et al., 1990)
*d* accession number D37979, (Ebbole & Zalkin, 1987)
*e* accession number Q07296; (Hui & Morrison, 1993)
*f* accession number P21155; (Tiedemann et al., 1990)
*gg* accession number P38025; submitted
*h* accession number S45524, (Chapman et al., 1994)
*i* accession number P27602; (Sasnauskas et al., 1991)
*j* accession number P27616; (Myasnikov et al., 1991)
Figure 2.15: Alignment of PurC from Different Sources. Alignment made using the program PILEUP (Feng & Doolittle, 1987) creates a multiple sequence alignment of between *E. coli*, rat, human, chicken, *B. subtilis*, *S. pneumoniae*, *Arabidopsis thaliana*, *Vigna aconilifolia* (moth bean), *Candida maltose* (yeast), *S. cerevisiae* (bakers yeast). Positions with identical residues for nine of the ten sequences are indicated with a capital letter, and are recorded on the consensus line. The literature source for each sequence is listed in Table 2.5. For the bifunctional enzymes, PurE begins after 411.
Plurality: 9.0  Threshold: 1.0  AveWeight 1.0  AveMatch 0.54  AvMisMatch -0.4

Rat
Human
Chicken
B. subtilis
S. pneumoniae
E. coli
A. thaliana
V. aconilifolia
C. maltosa
S. cerevisiae

Consensus

Rat
Human
Chicken
B. subtilis
S. pneumoniae
E. coli
A. thaliana
V. aconilifolia
C. maltosa
S. cerevisiae

Consensus
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Chapter 3

Purification and Characterization of PurE and PurK:
Discovery of a new intermediate in the Purine pathway.
Introduction. 5-Amino-imidazole ribonucleotide (AIR) carboxylase catalyzes the conversion of AIR in the presence of bicarbonate to 4-carboxy-5-aminoimidazole ribonucleotide (CAIR)(Scheme 3.1). This step in purine biosynthesis was originally elucidated by Buchanan and coworkers from chicken liver as a single enzymatic reaction requiring high concentrations of bicarbonate (0.2 M) as a substrate (Lukens&Buchanan, 1959). From genetic studies of Gots, a putative point mutation in either purE (purE1) or purK (purE2) created a mutant auxotrophic for purines. Unexpectedly, the auxotrophic phenotype for purines due to the mutation in purK could be overcome when grown in an enriched CO₂ atmosphere (Gots et al., 1977). The hypothesis was therefore put forth that the function of PurK was to deliver the CO₂ required for AIR carboxylation. A small molecule such as biotin has been known to function in this capacity, and thus it was curious why PurK, a 39 kDa protein, would be required as a CO₂ carrier.

Later, the E. coli genes for purE and purK were cloned and sequenced in the laboratories of Smith (Tiedeman et al., 1989) and Mizobuchi (Watanabe et al., 1989) and were discovered to be components of the same operon as expected for subunits of the same enzyme. DNA sequencing revealed that PurE was a protein of subunit molecular weight 17,649 Da, and PurK was a protein of subunit molecular weight
39,385 Da. Using an E. coli PurK mutant, Zalkin was successful in complimenting AIR carboxylase from avian systems (Chen et al., 1990). The sequence was homologous only with PurE. Lacking any evidence for PurK may suggest the mechanism for AIR carboxylation in eukaryotes might be different from prokaryotes.

AIR carboxylase has been isolated from both yeast and chicken (Nikolaeva et al., 1975; Firestine & Davisson, 1994). In S. cerevisiae, a 50 kDa protein catalyzes the carboxylation of AIR to CAIR in the presence of high bicarbonate. The Km values for AIR and bicarbonate are 10 μM and 130 mM respectively (Nikolaeva et al., 1975). Such a high Km for HCO₃⁻ might suggest that something is missing for enzyme catalysis. In chicken, the AIR carboxylase was shown under physiological conditions to be a bifunctional protein, containing PurC activity (Firestine & Davisson, 1994). Its Km values for AIR and bicarbonate are 76 μM and 23 mM, respectively. From sequence homology comparison, the yeast enzyme has homology to both PurE and PurK, while the chicken enzymes has homology only to PurE (see Appendix B).

This chapter presents the overexpression and purification of PurE and PurK, with efforts to characterize and understand how PurK functions as a CO₂ carrier in the AIR carboxylase reaction. Homogeneous PurE alone can catalyze the carboxylation of AIR to CAIR in the presence of high bicarbonate concentration (0.2 M), and the addition of purified PurK failed to reduce this high bicarbonate requirement. PurK was also purified to homogeneity and was found to possess an AIR dependent ATPase. This led to the hypothesis that carboxyphosphate was formed by PurK and utilized by PurE (Scheme 3.2).
Studies of Alenin et al. (1987) changed our ideas about the PurE/PurK catalyzed reaction. Their NMR studies suggested that in the presence of 1M bicarbonate, N₁-alkyl(R)-5-aminoimidazoles (R= CH₃ or ribose-5-phosphate - i.e. AIR!) could rapidly form a new carboxylated species which differed from N₁-alkyl(R) 4-carboxy-5-aminoimidazoles that forms on a much slower time scale (Scheme 3.3). Upon dilution of the high bicarbonate, the newly generated species decomposed back to starting material. The alternate carboxylation of a purine intermediate was postulated to be an N⁵-carbamate.

This chemical modification provided a unifying explanation for all of the unusual observations made while investigating PurE and PurK, reported in this chapter. It was postulated that PurK catalyzed the ATP and HCO₃⁻ dependent conversion of AIR to this new labile carboxylated imidazole (designated N⁵-CAIR), which is then utilized by PurE to catalyze the rearrangement to CAIR (Scheme 3.4). The synthesis
and characterization of \( N^5\text{-CAIR} \), along with evidence that this compound is actually a new intermediate in the purine biosynthetic pathway is present in this chapter.

\[
\text{AIR} + \text{ATP} + \text{HCO}_3^- \xrightleftharpoons{\text{PurK}} \left[ \begin{array}{c} \text{O} \\
\text{P} \\
\end{array} \right] \xrightarrow{\text{PurK}} \text{N}^5\text{-CAIR} + \text{ADP} + \text{Pi}
\]

\[
\text{N}^5\text{-CAIR} \xrightarrow{\text{PurE}} \text{CAIR}
\]

Scheme 3.4: Proposed Intermediate for Carboxylation of AIR by PurE and PurK.

**Materials.** Lactate dehydrogenase (LDH, 860 U/mg), pyruvate kinase (PK, 470 U/mg), malate dehydrogenase (MD, 100 U/mg), phosphoenolpyruvate (PEP), PEP carboxylase (PEPC, 3 U/mg), L-aspartate, \( \beta \)-mercaptoethanol (\( \beta \)-ME), phenylmethanesulfonyl fluoride (PMSF), \( \beta \)-nicotinamide adenine dinucleotide diphosphate, reduced (NADH), DEAE-Sepharose CL-6B, Dalton Mark VII molecular weight markers, and adenosine-5-triphosphate (ATP) were obtained from Sigma Chemical Co. \( \text{KH}^{13}\text{CO}_3 \) (WCS 171, batch 8,90%) and \( \text{H}_2^{18}\text{O} \) (WOW 420, batch 3, 95%) were purchased from Amersham. Isotope ratio mass spectrometry was performed by Geochron Laboratories, Cambridge, MA. Hydroxylapatite, Dowex 1-X8 were purchased from BioRad. Immobilon-P (a PVDF filter with a 0.45-\( \mu \text{m} \) pore size) was obtained from Millipore. Amicon membranes were purchased from WR Grace. DEAE A-25 Sephadex was obtained from Pharmacia. NMR spectra were recorded on either a Varian VXR-500 NMR or a Varian Unity 300 NMR spectrometer. \( ^{13}\text{C} \) NMR spectra were collected using broad-band decoupling. The plasmid, pJS355, containing PurE and PurK was a gift from Dr. John Smith.
Method. Bratton-Marshall assays were carried out as described (Schrimsher et al., 1986a). Fixed UV/visible wavelength assays were carried out using a Cary 210 spectrophotometer. Multiple wavelength assays were monitored on a Hewlett Packard 8452A diode array spectrophotometer. NMR spectra were recorded on a Varian 300 NMR spectrometer. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) samples were prepared and run as described by Laemmli (1970), as modified by Cooper (1977). Protein concentrations were determined according to the method of Lowry (1951) using BSA ($\varepsilon_{279} = 0.667 \text{mL mg}^{-1} \text{cm}^{-1}$) (Schachman, 1966) as a standard. Phosphate assays were accomplished using the method of Ames and Dubin (1960b) with inorganic phosphate as a standard. $N^5$-CAIR was stored at pH >12 due to its instability at neutral pH. Use of $N^5$-CAIR in enzymatic reactions therefore required monitoring the pH following its addition to the assay mixture, because of the alkaline storage condition.

Determination of the Bicarbonate Concentration. In a final volume of 400 µL, a typical assay contained 50 mM HEPES pH 7.8, 2 mM PEP, 2 mM MgCl$_2$, 0.2 mM NADH, 0.25 U PEP-carboxylase and 10 U of MDH (Peled, 1983). The assay solution was used immediately or stoppered for later use, since alkaline solutions absorb carbon dioxide. Bicarbonate concentrations were calculated using $\varepsilon_{340} = 6220 \text{M}^{-1} \text{cm}^{-1}$.

Enzyme Purification

Growth of TX635/pJS355. E. coli strain TX635 containing the heat-inducible plasmid pJS355 (Tiedeman et al., 1989) was grown in 5 L of medium containing 10 g/L tryptone, 5 g/L yeast extract, 50 µg/mL ampicillin, and 10 g/L NaCl, all adjusted to pH 7.5. The cells were grown at 30°C (doubling time 45 min) in a 10 L New Brunswick fermentor stirring at 300 rpm and aerating with 10 L per min (15.5 psi). At an A$_{600nm}$ of 1.3, the cells were heat induced by raising the temperature to 42°C with addition of an equal volume (5 L) of media at 54°C. The cells were incubated at
42°C for 30 min and then at 37°C for an additional 5.5 h. The bacteria were harvested with a Sharples centrifuge to give 2.6 g cell paste per liter of medium, frozen with liquid nitrogen, and stored at -80°C.

**Purification of the purE Gene Product:** At 4°C, cells of TX635/pJS355 (9.6g) were suspended in 49 mL (5 mL/g) of 100 mM Tris-HCl (pH 7.8), 30 mM MgCl₂, 6 mM β-ME, and 0.1% PMSF (Buffer A). The cells were ruptured in a French press pressure cell at 10,000 psi, and the cell debris was removed by centrifugation for 20 min at 10,000 g. The DNA was precipitated by the addition of 4.9 mL protamine sulfate (1.2% in Buffer A) to the supernatant over 30 min, to give a final concentration of 0.12%. At this point the absorbance spectrum of the supernatant should indicate an A₂₈₀/A₂₆₀ ratio of 0.75-0.8, otherwise more protamine sulfate was added (Cooper, 1977). After an additional 20 min of stirring, the precipitate was removed by centrifugation. The supernatant was adjusted to 50% saturation with the slow addition of solid ammonium sulfate (0.31 g/mL). After the ammonium sulfate had dissolved (30 min), the solution was stirred for 20 min and then centrifuged at 10,000 g for 20 min. The supernatant was then brought to 80% saturation with addition of solid ammonium sulfate (0.214g/mL over 30 min). The solution was stirred an additional 20 min and centrifuged at 10,000 g for 20 min. The pellet was dissolved in a minimal volume (~7 mL) of 100 mM Tris (pH 7.8, Buffer B) and desalted by passing it through a (42 x 2.5 cm) Sephadex G-25 column equilibrated in Buffer B. The fractions containing protein were pooled, diluted 1:1 with Buffer B and loaded onto a DEAE-Sepharose CL-6B column (16.5 x 1.5 cm, equilibrated with Buffer B). The column was washed with Buffer A until the absorbance at 280 nm was less than 0.05. The column was developed with a linear gradient from 0 to 200 mM KCl (200 x 200 mL, Buffer B) (Figure 3.6). The fractions (57-81, 100 mM KCl) containing activity were pooled and concentrated with an Amicon (YM-30) to 30 mLs. In order to desalt the protein, it was diluted with 250 mLs of 50 mM potassium
phosphate (pH 7.0, Buffer C), and reconcentrated twice. The sample was diluted to 200 mLs with Buffer C, checking the pH, and loaded onto an Hydroxylapatite column (7 x 2.5 cm) equilibrated in Buffer C. The column was washed with 60 mL of Buffer C and the developed with a 150 x 150 mL linear gradient from 50 mM to 500 mM potassium phosphate (pH 7.0), resulting in elution of AIR carboxylase after 300 mM potassium phosphate (Figure 3.7). The fractions containing AIR carboxylase were pooled (fractions 65-100), and then concentrated using an Amicon Diaflow Ultra filter equipped with a YM-30 membrane. The enzyme was stored at 20 mg/mL with at -20°C. The purity of the enzyme was observed on a SDS gel (Figure 3.8).

Purification of the purK Gene Product. All steps for the PurK purification were carried out at 4°C. TX635/pJS355 cells (14.9 g) were suspended in 5 mL/g of 100 mM Tris-HCl (pH 7.8), 30 mM MgCl₂ (Buffer D). The cells were ruptured using a French press pressure cell at 10,000 psi, and the cell debris was removed by centrifugation for 20 min at 10,000 g. The DNA was precipitated with addition of protamine sulfate (5 mLs of a 1.7% solution in Buffer D) to the supernatant over 30 min to give a final concentration of 0.12%. At this point the absorbance spectrum of the supernatant should indicate an A₂₈₀/A₂₆₀ ratio of 0.75-0.8, otherwise more protamine sulfate was added (Cooper, 1977). This solution was stirred for an additional 20 min and the precipitate was removed with centrifugation. The supernatant was then adjusted to 50% saturation by the slow addition of solid ammonium sulfate (0.31 g/mL). After the ammonium sulfate had dissolved (30 min), the solution was stirred for 20 min and then centrifuged at 10,000 g for 20 min. The PurK protein precipitated. The pellet was re-dissolved in minimal volume of 100 mM Tris-HCl (pH 7.8, Buffer E, ~7 mLs) and was desalted on a Sephadex G-25 column (48 x 2.5 cm) equilibrated with Buffer E. The pooled protein fractions were loaded onto a DEAE-Sepharose CL-6B column (16.5 x 1.5 cm) equilibrated in Buffer E. The column was washed with 250 mLs of Buffer E until the A₂₈₀ was <0.1. The protein was eluted with step gradients.
The first one used 100 mLs of 10 mM KCl in Buffer E, followed by 100 mLs of Buffer E containing 20 mM KCl. The protein eluted with 40 mM KCl in Buffer E (Figure 3.9). The fractions containing protein of high specific activity were pooled and concentrated with a YM-30 Amicon membrane to 42.3 mg/mL and stored in 20% glycerol at -20°C. The purity was determined with a 10% polyacrylamide gel (Laemmli, 1970) (Figure 3.8).

*N-Terminal Sequence Analysis of Purified Protein.* The purified proteins, PurK and PurE, were chromatographed on SDS 10% and 15% polyacrylamide gels, respectively (Laemmli, 1970). The protein was transferred to Immobilon-P (a PVDF filter with a 0.45-μm pore size) using a Semi-Phor TE-70 instrument from Hoefer Scientific following the procedure of (Matsudaira, 1987). The N-terminal sequence was determined using automated Edman degradation, performed at the Harvard Microchemistry Facility (Harvard University, Cambridge, MA) with an AB1 470A protein sequencer with a 120A on-line DRH-AA analyzer.

*Molecular Weight Determinations.* A Sephadex G-100 (83 x 1.5 cm) size exclusion column was equilibrated with 50 mM Tris-HCl (pH 7.5, 4°C) at 4 mL/h, and 1 mL fractions were collected. The column was calibrated with the following standards (Stokes radii, \(M_r\)): bovine serum albumin (35.5 Å, 66 kDa), horse heart cytochrome C (17 Å, 12.4 kDa), and yeast alcohol dehydrogenase (46 Å, 150 kDa) (Siegel, 1966). The column void volume was determined to be 40 mL using Blue Dextran (2 x 10^6 Da). The standards and the purine enzymes (PurE, PurK, and PurC) were loaded individually onto the column (200 μL, 10 mg/mL). From the column elution profile of \(A_{280}\) nm, the fraction number containing the maximum absorbance was used for the calculations. The data from these experiments were fit to Equation 3.1 to obtain the relative elution constant: \(V_e=\)elution volume, \(V_o=\)void volume, and \(V_t=\)total column volume.
\[ K_{ev} = \frac{(V_e - V_0)}{(V_t - V_0)} \quad \text{Equation 3.1} \]

A sucrose density ultracentrifugation experiment was performed according to the procedure of Martin & Ames (1961). Polyallomer centrifuge tubes (14 x 89 mm, Beckman) contained a 5-20% sucrose gradient (12 mL) in 100 mM Tris (pH 7.5). The gradient was poured at room temperature, and used immediately. The experimental samples included: PurE, PurK, PurC, a 1:1 molar mixture of PurE and PurK (0.054 mM) ± 30 mM MgCl₂, and freshly French press ruptured cells - the TX635/pJS355 lysate (100 μL of 15 mg/mL). The sedimentation coefficients were then determined with known standards (100 μL, 2 mg/mL): catalase (250 kDa, 11.3 S); bovine serum albumin (66 kDa, 4.3 S); lysozyme (17.2 kDa, 2.15 S); and yeast alcohol dehydrogenase (150 kDa, 7.65 S). The tubes were placed in a Beckman SW-40 rotor and centrifuged for 18 h at 39 000 rpm in a Beckman L7-5 ultracentrifuge. The sedimentation profile was made by removing 150 μL aliquots using a 200 μL pipetman from the top of the gradient. These fractions were then assayed for protein (Lowry, 1951) and for enzyme activity (PurE, PurC, and PurK).

The data from these experiments were fit to Equation 3.2 to obtain the molecular weight: \[ M = \frac{6\pi \eta N \beta r^2}{(1-\nu^2)} \quad \text{Equation 3.2} \]

**Substrate Synthesis: AIR**

*Enzymatic decarboxylation of CAIR.* CAIR (16.7 mM, 250 μmol, described in Chapter 2) was dissolved in 15 mL of 200 mM Tris (pH 7.8), 20 mM KCl, 16.7 mM EDTA at room temperature. The reaction was initiated by the addition of 15 U E. coli PurE (20°C). The reaction was monitored at A₂₄₀ and/or A₂₅₀, and reached
equilibrium after approximately 20 min. The reaction mixture was diluted to 400 mL, and then loaded onto a DEAE A-25 (15 x 2.5 cm, HCO₃⁻ form). The column was developed with a linear gradient from 0 to 400 mM TEAB (pH 7.4, 500 x 500 mL, 10 mL fractions). An elution profile was generated from A₂₄₀ and the Bratton-Marshall assay. Eluting at 200 mM TEAB, the fractions (40-55) containing AIR were pooled and concentrated in vacuo. With most of the TEAB gone, one mL aliquots were placed on the lyophilizer for final concentration and storage at -20°C, resulting in 128 µmol (51% yield). CAIR eluted after AIR in the profile, but only accounted for 5% of the starting material. With AIR(s), extensive purification and rotor evaporation decreases yields. ¹H NMR (D₂O, pH 6.5, DSS) δ 8.55 (s, 1, H2), 6.75 (s, 1, H4), 5.85 (d, 1, J=5 Hz, H1), 4.62 (m, 1, H2), 4.45 (m, 1, H3), 4.35 (m, 1, H4), 4.08 (m, 2, H5, H5”) (Figure 3.1a). The C-4 proton exchange rates were 0.09 min⁻¹ (pH 7.2) and 0.03 min⁻¹ (pH 5.1) (Schendel, 1986a). ¹³C NMR (125 MHz (D₂O, pD 7), ¹³CH₃OH standard δ = 48 ppm): δ 135.1 (s, C5), 131.5 (s, C2), 111.6 (s, C4), 86.4 (s, C1), 83.4 (s, C4), 72.5 (s, C3), 69.9 (s, C2), 62.8 (s, C5).

Determination of Extinction Coefficient for AIR. AIR (50 µmol) was rechromatographed on a Sephadex A-25 column (6 x 2.5 cm, HCO₃⁻ form), and eluted with a linear gradient of triethylammonium bicarbonate (0-400 mM TEAB, pH 8.0, 250 x 250 mL). Collecting 10 mL fractions, an elution profile was generated by monitoring A₂₅₀. AIR eluted at 150 mM TEAB (fractions 20 to 26). Concentrated in vacuo, the sample was used the same day. The concentration of AIR was determined by three independent methods: the Bratton-Marshall assay, the phosphate assay, and an enzymatic end-point assay described below. The Bratton-Marshall assay determined an AIR concentration of 35.0±0.6 mM. Using the phosphate assay, total amount of phosphate, inorganic and organic, were determined (110.6 mM, 74.1 mM, 36.5 mM respectively). The enzymatic end point assay used the following conditions: 50 mM HEPES (pH 7.8), 20 mM KCl, 4.5 mM
MgCl₂, 0.9 mM ATP, 2.0 mM PEP, 6.0 mM aspartate, 0.2 mM NADH, 10 U PK, 10 U LDH, and 1.4 U PurC, 0.01 U PurK, 0.095 U PurE. This end point assay determined an AIR concentration of 34.4±1.2 mM. The extinction coefficient for AIR was determined at several pHs. In the following buffers at 100 mM, AIR was diluted to 0.5 mM in 500 µL: Tris-HCl (pH 8.0), potassium phosphate (pH 7.0), Mes (pH 6.0), and Ches (pH 9.5) (Table 3.1). The average absorbance from ten separate experiments determined the extinction coefficient for each pH value. Further characterizing the spectral changes as a function of pH, for AIR (39 µM) were also monitored between 220 to 300 nm in 100 mM buffer: Ches (pH 9.5), Tris (pH 8.0), Phosphate (pH 7.0), Mes (pH 6.0), NaOAc (pH 4.8), and HCl (pH 1.0) (Figure 3.3).

Rate of non-enzymatic carboxylation of AIR. In a final volume of 750 µL at 37°C, AIR (0.067 to 1.33 mM) was added to a 1 mL cuvette containing: 100 mM Tris (pH 7.8), 180 mM KHCO₃, 1.0 mM ATP, 2.0 mM PEP, 6.0 mM MgCl₂, 10.0 mM aspartate, 0.2 mM NADH, 10 U PK, 10 U LDH, and 0.3 U of PurC. To maintain bicarbonate levels, the cuvette was sealed with a septum. The rate of CAIR formation was monitored using the coupling enzymes PurC, PK, LDH by trapping the ADP generated and observing the NADH oxidation.

Equilibrium Constant [CAIR]/[HCO₃⁻][AIR]. In a final volume of 500 µL, a 0.5 mL Eppendorf tube contained the reaction mixture: 100 mM Tris-HCl (pH 7.8), variable amounts of KHCO₃, (20-180 mM), and either AIR (1.2 mM) or CAIR (1.1 mM). A rubber septum, fastened with copper wire, was placed on each Eppendorf. The reaction mixture was incubated at 37°C for 5 min, prior to addition of the nucleotide. At various times aliquots were removed with a syringe and the rate of approach to equilibrium was monitored by a reverse-phase HPLC chromatography on an Altech C-18 column (10 µm, 25 x 0.46 cm) equilibrated in 80 mM potassium phosphate and 10 mM tetrabutylammonium bromide (pH 7.0, 1 mL/min). For the endpoint of the reaction, 0.1 mg PurE was added to this reaction mixture. AIR eluted at 5 min and

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CAIR at 10 min as monitored at 250 nm. Standard curves were prepared using known quantities of AIR and CAIR (A$_{250}$ nm) and quantified using an HP 3396A integrator. Alternatively, the peaks were cut out and weighed.

$N^5$-CAIR:

Nonenzymatic Conversion of AIR to $N^5$-CAIR Monitored by NMR Spectroscopy. A solution, 600 µL, containing AIR (29 mM) and 200 mM potassium phosphate in D$_2$O (pD 6.0) was placed in an NMR tube. After an initial spectrum was recorded, solid KHCO$_3$ (56.1 mg, 0.63 mmol) was added to give a final volume of 700 µL and the NMR tube was sealed using a flame. The reaction was initiated by inverting the tube and mixing the solid and liquid phases. $^1$H NMR spectra were recorded at various intervals from 1 min to 600 h. Each spectrum was integrated to determine the amount of each species formed at a given time. At the end of the experiment the tube was opened and the pD was determined to be 8.0.

Enzymatic Synthesis of [amidino-$^5$N]-β-FGAM. The reaction mixture contained 40 mM Tris-HCl (pH 7.5), 20 mM ATP, 8.8 mM [γ-amido-$^{15}$N]-glutamine, 80 mM KCl, 30 mM MgCl$_2$, 11 mM PEP, 1.87 mM β-FGAR (formylglycineamid ribonucleotide), 20 U PK and 5 U FGAR-AT (FGAR-amino transferase) in a final volume of 4 mL. The mixture containing everything except the enzymes was pre-incubated at 37°C for 10 min, and the reaction was initiated by addition of FGAR-AT. After 25 min, the mixture was diluted to 100 mL with water at 4°C and applied to a DEAE A-25 column (2.5 x 10 cm, HCO$_3^-$ form). The column was washed with 100 mL water and the product eluted with a 500 x 500 mL linear gradient from 0 to 400 mM TEAB (pH 7.6). β-FGAM eluted at 75 mM TEAB, determined using the Orcinol assay. Fractions containing product were concentrated in vacuo and assayed by Bratton-Marshall endpoint assay indicated the recovery of 5.2 µmol β-FGAM (70% yield).
**Enzymatic Synthesis of [amino-5-15N]-β-AIR.** The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 20 mM ATP, 400 mM KCl, 3 mM MgCl₂, 14 mM PEP, 1.7 mM [amidino-15N]-β-FGAM, 10 U PK and 3 U AIR synthetase in a final volume of 2 mL. The mixture without enzymes was pre-incubated for 10 min at 37°C, and the reaction was then initiated by addition of PK and AIR synthetase. The reaction was allowed to proceed for 30 min, after which time the mixture was diluted to 100 mL with cold water and loaded onto a DEAE A-25 column (2.5 x 10 cm, HCO₃⁻ form). The column was developed with a 500 x 500 mL gradient of 0 to 500 mM TEAB, pH 7.6. Fractions were monitored using A₂₅₀ and the Bratton-Marshall and Orcinol assays. AIR eluted at 200 mM TEAB and the appropriate fractions were pooled and concentrated in vacuo to give 1.7 µmol AIR (51% yield). ¹³C NMR (125 MHz, D₂O, ¹³CH₃OH standard δ = 48 ppm): 135.1 (d, J=12 Hz, C5), 131.5 (s, C2), 111.6 (s, C4), 86.4 (s, C1), 83.8 (d, J=8.4 Hz, C4), 72.5 (s, C3), 69.9 (s, C2), 62.8 (d, J=3.8 Hz, C5) and a small amount of HCO₃⁻ (δ 167.5, s). Assignments are based on the work of Schendel and Stubbe (1986) and Alenin et al. (1987). The ¹³C-¹⁵N coupling observed at the C₅ resonance is in the range expected (Levy & Lichter, 1979).

**¹³C NMR Study of [amino-5-¹⁵N, carbamoyl-¹³C]-N⁵-CAIR.** [amino-5-¹⁵N ]-AIR (3.8 µmol) and NaH¹³CO₃ (375 µmol) were combined in 600 µL D₂O (final pD 7.4) and incubated on ice for 30 min. The sample was then adjusted to pD 12.5 using 1M KOD and ¹³CH₃OH was added as a standard (δ = 48 ppm). The final volume was 1.2 mL. The spectrum is shown in Figure 3.16.

**Synthesis of N⁵-CAIR**

i.) **Enzymatic Synthesis of N⁵-CAIR.** N⁵-CAIR was synthesized enzymatically with PurK from AIR, ATP and KHCO₃. In a final volume of 10 mL at 20°C, a 15 mL Falcon tube contained: 200 mM Tris (pH 8.0), 12 mM MgCl₂, 60 mM KCl, 4.5 mM ATP, 10 mM PEP, 50 µmol AIR, 156 U PurK, and 200 U PK. Solid potassium bicarbonate was added as a fine powder to the reaction mixture to give a final
concentration of 15 mM. After dissolving the HCO$_3^-$, the reaction was initiated with PurK. After 50 sec at 20°C, the reaction was quenched with 1 mL 10 M KOH (20°C). In all cases, the KOH solution was prepared immediately before use from solid KOH pellets. Basic solutions absorb carbon dioxide. The purification of N$^5$-CAIR is described subsequently.

ii.) Non-enzymatic Synthesis of N$^5$-CAIR: In a 2 mL screw top Eppendorf, 1.0 mL 100 mM Tris (pH 7.0) was added to 19 µmol of lyophilized AIR. KHCO$_3$ (0.1 g, 1 mmol, fine powder) was added to a final concentration of 1M (pH 8.0). The reaction was sealed with a septum and placed on ice (4°C). After 30 min, the solution was quenched with 1 mL of 4.2 M KOH. The sample was used in its impure state for crude assays or purified (described subsequently) with the yield depending on the reaction seal, 10-60%. A linear correlation exists between bicarbonate concentration and amount of N$^5$-CAIR produced (Mueller, 1994).

Isolation of N$^5$-CAIR. After the base quench of the N$^5$-CAIR synthetic reaction (described previously, i.), the sample was diluted with 300 mL of 10 mM KOH, and loaded onto a Dowex 1-X8 column (7 mL, -OH form). The N$^5$-CAIR was eluted with a linear gradient from 0 to 300 mM KCl (250 x 250 mL, 4.5 mL fractions) in 50 mM TEA, with N$^5$-CAIR eluting at 150 mM KCl. The prepared TEA solution (pH 11.4) was used immediately, without adjusting the pH. An elution profile was generated by monitoring A$_{240}$, A$_{250}$ and the Bratton-Marshall assay at A$_{500}$. When applicable, 0.5 mL from every fifth fraction was assayed for radio activity in the scintillation counter. Avoiding the fractions containing unreacted AIR (47-53), fractions containing N$^5$-CAIR (60-66) were pooled (Figure 3.14). To maintain alkaline conditions, 300 µL of 2 N KOH was added to the pooled fractions, and then the sample was concentrated in vacuo. An average yield from 8 attempts was 50%. The concentration of salt required for the elution prevents rechromatographing either
N⁵-CAIR or AIR on an anion exchange column; however, a resin from BioRad (Affi-Gel 601 gel) can be used. The sample was stored as a solution (pH >12) at -80°C.

**Salt Removal from N⁵-CAIR.** The concentrated salts can be removed from the sample of N⁵-CAIR using a methanol extraction procedure at 4°C. The N⁵-CAIR solution, described above was evaporated to dryness in a round bottom flask and placed in the cold room. The solid was triturated with 4 mL of HPLC grade methanol at 4°C. The salts were then allowed to settle and the supernatant was centrifuged in an Eppifuge for 20 s. The supernatant was then transferred (leaving 100 μL) to a new round bottom flask containing 25 μL of 1N KOH. The methanol extraction of the salts was repeated four times. The fractions were pooled and the methanol was then removed in vacuo. There was a 10% loss of N⁵-CAIR based on the Bratton-Marshall. The amount of KCl and KHCO₃ are greatly reduced. ¹H NMR (250 MHz, D₂O, TSP δ = 0.0 ppm, pD 12): δ 8.04 (s, 1H, C₂), 6.87 (s, 1H, C₄), 5.67 (d, J = 4.9, 1H, C₁), 4.52 (m, 1H, C₂), 4.35 (m, 1H, C₃), 4.24 (m, 1H, C₄), 3.10 (m, 2H, C₅), (Figure 3.15). ¹³C NMR (75 MHz, methanol standard at 48 ppm, D₂O, pD 14): δ 167.3 (KHCO₃), 161.6 (s, C₆), 133.5 (s, C₅), 127.7 (s, C₂), 122.7 (s, C₄), 86.5 (s, C₁), 82.5 (d, C₄, J = 8.0 Hz), 73.7 (s, C₃), 69.3 (s, C₂), 63.1 (d, C₅, J = 5.3 Hz), based on (Schendel, 1986b; Schrimsher et al., 1986; Alenin et al., 1987), (Figure 3.16).

**Quantitation of N⁵-CAIR.** The concentration of N⁵-CAIR was determined with the Bratton-Marshall assay. The initial step in this assay decarboxylates the carbamate to the diazotizable amine, thus any contaminating AIR in the assay was not distinguished from N⁵-CAIR.

**Absorbance Spectra.** To help with the decomposition studies to AIR, the absorption spectrum of N⁵-CAIR was determined under physiological conditions: 200 mM Tris (pH 7.8, 4°C) 20 mM KCl (Figure 3.4). Using a temperature cooled cuvette holder, a solution of 78.8 μM N⁵-CAIR was scanned (Table 3.7). The purity of the starting material was established with ¹H NMR spectroscopy, and the
concentration was determined with the Bratton-Marshall assay. To ensure complete decomposition, the cuvette was warmed to 37°C for 5 min, and then rescanned at 4°C.

**Stability of N5-CAIR.** Three different methods were used to monitor the decomposition of N5-CAIR.

i) *Direct monitoring of Decomposition by change in A_{250} nm.* The decomposition rate of N5-CAIR to AIR was followed at 250 nm in a temperature controlled cuvette (Figure 3.4). In a final volume of 1 mL, a typical assay contained: 200 mM Tris-HCl, 20 mM KCl and 125 μM N5-CAIR at 20°C. The increase in A_{250} nm was monitored spectrophotometrically under the following conditions: a) various temperatures (5, 10, 20, and 30 °C), b) various pH (7.6, 7.8, 8.0, 8.2, 8.4), and 8.6, c) two buffers (HEPES and Tris) at various pH, and d) various concentrations of Tris (100, 200, and 300 mM).

ii) *Indirect monitoring with a coupled Assay with PurC, PK, and LDH.* To establish the validity of the assay described in (i.) an enzymatic endpoint assay determined the remaining concentration of N5-CAIR as it decomposed under various buffer conditions, for example - 2.6 mM N5-CAIR in 500 μL of either 50 mM Phosphate (pH 12) or 50 mM Ches (pH 9.0). Over time, 10 μL aliquots were removed to be assayed for the remaining N5-CAIR by PurE. In a volume of 700 μL at 20°C, the reaction mixture contained: 50 mM HEPES (pH 7.8), 20 mM KCl, 4.5 mM MgCl2, 0.9 mM ATP, 2.0 mM PEP, 7.2 mM aspartate, 0.2 mM NADH, 10 U PK, 10 U LDH, 0.6 U PurE, and 0.7 U PurC. The decomposition was initiated by the addition of N5-CAIR, and the total Δ A_{340} nm was monitored.

iii) *[1^{14}C]-5-Aminoimidazole-4-N-succinylcarboxamide ribonucleotide (SAICAR) Formation.* The assay mixture was identical to that described above except [1-14C] aspartate (7.5 x 10^5 cpm/μmol) replaced aspartate, and NADH and LDH were omitted. The decomposition reactions were initiated with N5-CAIR, and 44 μL
aliquots were removed at various time points and placed in an Eppendorf tube containing 0.1 U PurE. Reactions were quenched 3 min later with 40 μL 33%(w/v) trichloroacetic acid. The [14C] SAICAR was quantitated as previously described (Chapter 2).

\[ \text{N}^5\text{-CAIR} \xrightarrow{\text{PurE}} \text{CAIR} \xrightarrow{\text{PurC}} \text{SAICAR} \]

PurE Enzyme Assays

i) Conversion of N\(^5\)-CAIR to CAIR. In a final volume of 700 μL at 20°C, the following reagents were present: 100 mM Tris (pH 7.8), 20 mM KCl, 5 mM MgCl\(_2\), 1.1 mM ATP, 2.0 mM PEP, 0.2 mM NADH, 7 mM aspartate, 1.1 U PurC, U PurE, 10 U PK, and 5 U LDH. Addition of N\(^5\)-CAIR initiated the reaction and the assay monitored the absorbance change at 340 nm. The pH was confirmed following the completed assay (Scheme 3.5).

ii) Reverse Reaction- Conversion of CAIR to AIR. The assay mixture in a total volume of 400 μL contained 100 mM Tris-HCl (pH 7.8) and 100 μM CAIR. The cuvette was stoppered with a septum and incubated at 37°C prior to initiation of the reaction with 0.2 mU PurE. The decrease in absorbance of CAIR to AIR was monitored as a function of time at 260 nm (\(\Delta \varepsilon_{260} = 8930 \text{ M}^{-1}\text{cm}^{-1}\)). For valid kinetic data, the rate limiting step needs to be the enzymatic decarboxylation of CAIR to \(N^5\)-CAIR rather than the nonenzymatic decarboxylation of \(N^5\)-CAIR to AIR (k=0.749 min\(^{-1}\) at 30°C, pH 7.8). Appropriate temperature and enzyme concentration avoid this potential problem.
iii) Conversion of AIR/N^5-CAIR to CAIR observed at 260 nm. To 100 mM Tris-HCl (pH 7.3) was added 180 mM KHCO₃, for a final pH of 7.8. The buffer was made fresh, stored at 4°C in an Erlenmeyer flask fitted with a rubber septum, and used immediately. A syringe transferred buffer to a rubber septum sealed 1 cm cuvette. The AIR concentrations varied between 60 µM and 1.5 mM. At millimolar concentrations of AIR, 0.2 cm path-length cells were required. The assay mixture was equilibrated at 37°C prior to its initiation by the addition of PurE. The reaction was monitored by decreased at 260 nm (Δε₂₆₀ = 8930 M⁻¹cm⁻¹) (Figure 3.5, Table 2.3, Table 3.1).

iv) Conversion of AIR/N^5-CAIR to CAIR Coupled with PurC. In a volume of 700 µL at 37°C, the reaction mixture contained: 100 mM Tris-HCl (pH 7.8), 180 mM KHCO₃, 0.84 mM AIR, 1.1 mM ATP, 2.0 mM PEP, 0.2 mM NADH, 6.0 mM MgCl₂, 10 mM KCl, 3.7 mM aspartate, 1.1 U PurC, U PurE, 10 U PK, and 5 U LDH. The assay was initiated by addition of PurE (3 mU, 0.062 µg).

Kinetics

Kinetic Analysis by NMR Spectroscopy of PurE Catalyzed Conversion of CAIR to N⁵-CAIR. CAIR (30 µmol) was carefully titrated to pH 7.0 using 1M HCl and brought to dryness in vacuo. The sample was exchanged into 500 µL D₂O, titrated to pD 8.0 at 20°C with 1M NaOD in D₂O and was assayed for CAIR (ε₂₅₀=10,980 M⁻¹ cm⁻¹) and bicarbonate using the PEP-C assay described above. PurE was exchanged into 300 mM Tris-HCl, pD 8.0, in D₂O using a Centricon-30 (dilution of original buffer salts was 1/100). Enzyme activity was assayed before and after exchange into deuterated buffer. In a final volume of 700 µL at 20°C, NMR samples contained: 300 mM Tris-HCl (pD 8.0), 7.5 mM CAIR, and PurE (1.5-15 U). After an initial spectrum of CAIR, the reactions were initiated with PurE, and successive 16 scan FIDs were collected over time. Spectra were acquired every 30 sec for the first 10
min, every min for the subsequent 15 mins, and finally every 5 min until the reaction had proceeded for 1 h.

The concentrations of substrates and products were determined by integration of the NMR spectra taken at each time point. The kinetic data were analyzed using an IBM compatible version of GIT [PC version 1.31, 12/87, © E. I. DuPont de Nemours by McKinney, R. and Weigert, F. J. (Stabler & Chesick, 1978; Weigert, 1987)] and plotted using a MacIntosh version of KINSIM (Barshop et al., 1983), 1.3, provided by Professor Robert Kuchta, U of Colorado, Boulder, CO.

Determination of Kinetic Constants. The Michaelis constants for AIR, CAIR, and KHCO₃ were determined by varying the concentration of one substrate while maintaining high levels of the second substrate. The kinetic parameters for the PurE reaction were determined using the PurC and PK/LDH coupled assay; while the reverse reaction monitored the decarboxylation of CAIR measured as a decrease in A₂₆₀. For determination of the Km for AIR, the concentration of AIR was varied from 0.1-1.0 mM with 180 mM KHCO₃. For the Km of KHCO₃, the concentration of KHCO₃ ranged from 16-390 mM in the presence of 1.0 mM AIR. (Note: This assay was used prior to our understanding of the function of PurE.) For the Km of N⁵-CAIR, the concentrations of N⁵-CAIR ranged from 60-750 μM in 200 mM Tris (pH 7.8) (Figure 3.19). For the Km of CAIR, the concentration of CAIR ranged from 8-83 μM with ambient bicarbonate concentration. The initial velocities were analyzed using the programs of Cleland (Cleland, 1979). The data (S= substrate concentration, v= initial velocity) was fit using non-linear regression analysis to Equation 3.3 to obtain the kinetic constants: \( V = \frac{v}{S/K_m + S} \)  

\[ v = \frac{VS}{K_m + S} \quad \text{Equation 3.3} \]
Inhibition of PurE by AIR. An effect of AIR on the $N^5$-CAIR initiated PurE activity was assayed with the following conditions at 20°C in 730 µL: 100 mM Tris (pH 7.8), 20 mM KCl, 2.0 mM PEP, 1.0 mM ATP, 0.2 mM NADH, 4.5 mM MgCl$_2$, 7.0 mM Asp, 10 U PK, 10 U LDH, 0.4 U purC, 0.6 µg purE, 115 µM $N^5$-CAIR, and 0 to 1.01 mM AIR. The enzyme was incubated with AIR prior to addition of $N^5$-CAIR to follow the decrease of NADH at 340 nm.

Inhibition Pattern of PurE by AIR. In a final volume of 730 µL at 20°C, the reaction mixture contained: 100 mM Tris (pH 7.8), 20 mM KCl, 2.0 mM PEP, 1.0 mM ATP, 0.2 mM NADH, 4.5 mM MgCl$_2$, 7.0 mM Asp, 10 U PK, 10 U LDH, 0.4 U purC, 0.6 µg purE, (37, 51, 74, 132, 678 µM) $N^5$-CAIR, and (0, 116, 500 µM) AIR. The cuvette was in a 20°C water bath for 3 min and the reaction was initiated with addition of $N^5$-CAIR. There was a 0.1 pH unit change between the low and high concentrations of $N^5$-CAIR due to the pH of the media in which $N^5$-CAIR was stored. The initial velocities were analyzed using the programs of Cleland (1979) (Figure 3.20).

\[
\text{ATP} + \text{HCO}_3^- + \text{AIR} \xrightarrow{\text{PurK}} \text{N}^5\text{-CAIR} + \text{Pi} + \text{ADP} \xrightarrow{\text{PK/LDH}} \text{ATP}
\]

Scheme 3.6: Nonenzymatic decomposition of $N^5$-CAIR monitored with PK/LDH Assay.

PurK

Spectrophotometric Coupled Assay for PurK. In final volume of 700 µL, the reaction mixture contained: 100 mM Tris (pH 7.8), 20 mM KCl, 6 mM MgCl$_2$, 1 mM ATP, 2.0 mM PEP, 0.2 mM NADH, 5 U PK, 5 U LDH, and AIR. The reaction was initiated by addition of 250 µM AIR, and the consumption of NADH monitored at 340 nm, at 37°C (Scheme 3.6).
*Heat Inactivation of PurK.* In a 1 mL Eppendorf tube, PurK (0.065 mg) was diluted in 50 mM HEPES (pH 7.9), 20 mM KCl; and it was placed in a 55°C water bath. At various times (0, 5, 10, 15, 20, 25, 30, 40, and 50 min), 100-μL aliquots were removed and cooled in an ice bath. Then, each aliquot was assayed for AIR-dependent ATPase activity and for [14C] SAICAR production.

*Requirement of both PurK and PurE for the production of CAIR under Conditions of Low Concentrations of Bicarbonate.* The reaction mixture contained in 250 μL: 50 mM Hepes (pH 7.8), 20 mM KCl, 7.0 mM MgCl₂, 1.2 mM ATP, 2.3 mM PEP, 5.0 mM [14C] aspartate (specific activity 1.4 x 10⁶ cpm/μmol), 0.8 mM AIR, <100 μM HCO₃⁻, and 0.3 U of PurC. Three control experiments in which the reaction mixture contained PurE, PurK, no PurE or PurK or both PurE and PurK were incubated at 37°C and analyzed for the product at 0, 2, 3, 5, and 10 min. Reactions were initiated by the addition of 0.05 U of PurE, and 0.09 U PurK. The final product [14C] SAICAR was isolated from an anion Dowex 50W-X8 column as described in chapter 2 (Figure 3.11).

*Stoichiometry of the PurK Reaction.* In a final volume of 400 μL at 37°C, the reaction mixture contained the following: 50 mM Hepes (pH 7.8), 20 mM NADH, 20 mM KCl, 0.9 mM ATP, 4.5 mM MgCl₂, 2.0 mM PEP, 20 mM NADH, 0.045-0.11 mM AIR, 6.0 mM [³H] aspartate (specific activity 2.1x10⁶ cpm/μmol), 10 U PK, 5 U LDH, 0.5 U PurE, and 1.1 U PurC. The reaction mixture was monitored spectrophotometrically at 340 nm prior to initiation by addition of 5 mU PurK. The total amount of ATP consumed was determined by the burst of NADH consumption. All of the AIR was consumed under these conditions. Once the NADH oxidation returned to steady state, a 100 μL aliquot was withdrawn from the cuvette and [³H] SAICAR isolated using a Dowex 50W-X8 column as described in Chapter 2. The ratios of AIR, ATP, and SAICAR were determined.
**PurK Catalyzed Transfer of $^{18}$O from NaH$^{13}$C$^{18}$O$_3$ to Pi.** NaH$^{13}$CO$_3$ (1.61 mg, 18.9 μmol) was placed in an oven dried Wheaton vial. H$_2^{18}$O (100 μL) was added through the septum using an oven dried syringe. The concentration of HCO$_3^-$ was confirmed to be 189 mM using the PEP-C assay. The bicarbonate was allowed to incubate at room temperature for 5 h to insure complete exchange. Isotope ratio mass spectroscopy of the resulting bicarbonate revealed that the $^{18}$O/$^{16}$O ratio was 89:11.

AIR (10 μmol) was acidified to pH 5.5 with 1M HCl, diluted to 1 mL and degassed using two freeze-pump-thaw cycles followed by bubbling argon through the solution at room temperature for 1 h. The pH of the sample was adjusted to 8.0, and then assayed for AIR (Bratton-Marshall assay), bicarbonate (PEP-C assay) and for total phosphate (Ames and Dubin, 1960).

Reaction samples contained in a final volume of 500 μL: 100 mM HEPES (pH 8.0), 15 mM KCl, 20 mM MgCl$_2$, 11.4 mM ATP, 1 mM AIR, 11.3 mM NaH$^{13}$C$^{18}$O$_3$ and 1.8 U PurK. A control reaction containing no AIR was also performed. Samples were pre-incubated for 2 min at 4°C in the absence of HCO$_3^-$ and PurK, and then the reactions were initiated with bicarbonate followed 5 sec later by PurK. Reactions were allowed to proceed for 2 min, then were quenched by addition of 300 μL of 600 mM CHES, 500 mM EDTA (pH 9.0). These samples were rapidly frozen using liquid nitrogen and stored at -80°C until NMR analysis was possible.

NMR samples were thawed in an Eppifuge at 4°C (spun for 10 min), $d_6$-acetone was added as a deuterium lock standard to each reaction, and the samples were forced through a 0.45 μm filter by centrifugation (2800 x g). $^{31}$P NMR spectra were collected for each sample on a VXR-500 NMR spectrometer ($^{31}$P 121 MHz) using a sweep width of 7272.7 Hz and an acquisition time of 2.002 seconds.

*Requirement for ATP in a PurK and PurE Reaction Monitored by HPLC.* In a final volume of 700 μL at 37°C, the reaction mixture contained: 50 mM Hepes (pH
7.8), 20 mM KCl, 0.9 mM ATP, 4.5 mM MgCl₂, 0.2 mM AIR, 0.4 U PurE, and 0.01 U PurK. The reaction mixture was incubated at 37°C for 5 min, and a 200 μL aliquot was removed and analyzed by HPLC chromatography using reversed-phase Altech C-18 column equilibrated with 80 mM Tris (pH 7.8) and 10 mM tetrabutylammonium bromide, flow rate 2 mL/min. AIR eluted with a retention time of 4 min, while CAIR eluted at 6 min. Control experiments lacking PurE, PurK, or ATP were carried out. In addition, standards of AIR and CAIR were also chromatographed (Figure 3.12).

PurK Catalyzed Conversion of AIR to N⁵-CAIR and Nonenzymatic Decomposition to AIR Monitored by NADH Consumption. In a final volume of 710 mL, the reaction mixture contained: 100 mM Tris-HCl (pH 7.8), 20 mM KCl, 4.5 mM MgCl₂, 1.0 mM ATP, 0.2 mM NADH, 2 mM PEP, 10 U PK, 10 U LDH, and 0.19 U PurK. The reaction was carried out at 20°C. The reactions were initiated with AIR (3-24 nmol, 4.2-34 μM). From the initial burst of product and the following steady state rate, the stoichiometry and decomposition rate were calculated using ε₂₄₀=6220 M⁻¹ cm⁻¹.

NMR Analysis of PurK Catalyzed Conversion of AIR to N⁵-CAIR. Reactions contained in a final volume of 560 μL: 50 mM Tris-HCl (pH 8.0), 15 mM KCl, 10 mM MgCl₂, 3 mM ATP, 3 mM KHCO₃, 3 mM AIR, 3 mM PEP, 8 U PK and 0.4 U PurK. Samples without enzymes were preincubated at 5°C for 2 min. To each sample was added PK, after which the reactions were initiated with PurK. Reactions were quenched after 2 or 5 min by adding 140 μL 1M NaOD in D₂O (final pH 12.5), and then were rapidly frozen in liquid nitrogen until work-up was possible, within 3 h. A control using no enzyme was also run.

Samples were thawed, brought to dryness in vacuo, and then exchanged with 2 x 1 mL aliquots of D₂O. After dissolving each sample in 700 μL D₂O, ¹H NMR spectra were collected.
Determination of the Reversibility of the PurK Reaction using Hexokinase and G-6-P dehydrogenase  In a final volume of 700μL at 37°C, the reaction mixture contained: 50 mM HEPES (pH 7.9) or 200 mM Tris-HCl (7.9), 20 mM KCl, 4.5 mM MgCl₂, 0.60 mM NADP⁺, 2 mM Glucose, 1.0 mM ADP, 1 mM Pi, 5 U HK, 5 U G-6-P dehydrogenase, and 0.075 U PurK. The reaction was monitored for increase in absorbance of NADPH. In addition, a variety of AIR analogs were used to see if one could effect the reverse reaction: 0.35 mM AIR, 1.5 mM CAIR or 0.04 mM N⁵-CAIR, with 0.8 U PurE, and a carboxyphosphate analog - 2.1 mM carbamyl phosphate.

Alternatively, the reversibility was monitored by ³²P incorporation into glucose-6-P. In a final volume of 300 μL at 20°C, the reaction mixture contained: 100 mM Tris (pH 7.8), 20 mM KCl, 9.0 mM MgCl₂, 1.0 mM ADP, 0.9 mM [³²P] Pi (1.35 x 10⁶ cpm/μmol), 2.0 mM glucose, 10 U hexokinase, and 1.2 mM CAIR. The PurE storage buffer (high phosphate) was diluted with 100 mM Tris (pH 7.8), 20 mM KCl, and then concentrated three times using a 30 kDa Centricon (1 to 1000 dilution of storage salts). The reaction was initiated with addition of PurE and PurK. The enzyme ratios were varied from 5:1 to 1:16 (purE:purK) to alter the rate of N⁵-CAIR formation by PurE in the presence of PurK. A 100 μL aliquot was removed and analyzed for G-6-³²P formation using a phosphate extraction technique previously described (Schrimsher et al., 1986). To a 100 μL sample in a 15 mL Falcon tube, 2 mL of (80% 1 N HClO₄ and 1% (NH₄)₆Mo₇O₄·4H₂O), and 2 mL of 1:1 benzene/isobutanol were added. The sample was vortexed, and centrifuged for 5 min in a clinical centrifuge. One mL of the organic (top) layer was counted for the presence inorganic phosphate [³²P], while the aqueous layer was counted to determine the amount of organic phosphate [³²P] G-6-P.
Results

Synthesis and Characterization of AIR. In order to develop an assay for the purification of PurE and PurK, a reproducible source of substrate was required. Two methods were utilized for synthesis of AIR. The first was decarboxylation of CAIRs (from chapter 2) under acidic conditions by the procedure of Groziak (Litchfield & Shaw, 1971; Groziak et al., 1988). The resulting AIRs was phosphorylated originally with the method described by Bhat et al. (1990) using m-cresol and pyrophosphoryl chloride. After anion exchange purification, this method typically gave 15-30% yields. Alternatively, a modified procedure of Yoshikawa et al. (1967) utilizing triethyl phosphate and phosphorous oxychloride was used. After anion exchange chromatography purification, this method typically gave 30-50% yields. Purity was based on $^1$H NMR.

The most reproducible procedure for AIR preparation was enzymatic decarboxylation of CAIR (from chapter 2) with PurE. This simple and rapid reaction generated CAIR that was purified with anion exchange chromatography, and resulted in yields of 50%. Purity was confirmed with NMR spectroscopy (Figure 3.1a, Figure 3.2a). The Bratton-Marshall assay was the most reliable method to quantify AIR. In order to determine the kinetic parameters for PurE, an accurate method to quantitate the change in AIR was required. Absorbance spectra of AIR are shown in Figure 3.3 as a function of pH (Levenberg & Buchanan, 1957), and the extinction coefficients (Table 3.1, Figure 3.4, Figure 3.5) were determined absorbance and the Bratton-Marshall assay. This method could be used to monitor the decarboxylation of CAIR catalyzed by PurE. As detailed subsequently in this chapter; however, AIR is not the product of the PurE catalyzed decarboxylation, but results from nonenzymatic decarboxylation of $N^5$-CAIR. Aminoimidazoles are know to be unstable, decomposing at room temperature to form black insoluble pigments (Hunter & Nelson, 1941; White & Rudolph, 1979). During storage, AIR decomposes.
to a darker colored material (Levenberg & Buchanan, 1957; Schendel, 1986a), either by oxidation or polymerization (see Appendix A). This impurity does not seem to effect the enzymatic assay or the Bratton-Marshall assay.

<table>
<thead>
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<th>pH</th>
<th>$\epsilon_{250}$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\epsilon_{260}$ (M$^{-1}$cm$^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td>9.5</td>
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<td>1340</td>
</tr>
<tr>
<td>8.0</td>
<td>3270±80</td>
<td>1570±120</td>
</tr>
<tr>
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<td>2410±90</td>
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<td>4170±170</td>
<td>2900±70</td>
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<tr>
<td>1.0</td>
<td>4230</td>
<td>3210</td>
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Table 3.1: Extinction Coefficient of AIR in various buffers.

Assay for PurE: AIR carboxylase has been previously purified to homogeneity from yeast and chicken (Nikolaeva et al., 1982), (Patey & Shaw, 1973). The reported assay for enzymatic activity monitored an increase in absorbance at 250 nm due to the carboxylation of AIR to produce CAIR. In all of these assays, bicarbonate was present at concentrations of 0.2 M. Initially we used this assay, and carefully analyzed the absorption spectra of both AIR and CAIR at various pHs in order to define a set of conditions that maximized the $\Delta \epsilon$ between these species. As revealed in Figure 3.5, at 260 nm the $\Delta \epsilon$ is 8930 M$^{-1}$cm$^{-1}$ (Table 2.3,3.2), and the increase in A260 nm can thus be used to monitor the PurE reaction. This assay allowed for accurate quantitation of CAIR production; however, it proved unreliable for kinetic
Figure 3.1a: $^1$H NMR of AIR. The NMR spectrum containing 19.5 mM AIR (pH 6.5) with TSP as the reference at 0.0 ppm. The C4 proton was absent due to exchange under these experimental conditions.
Figure 3.2a: The $^1$H NMR Resonances of C2H (A) and 1 (B) for CAIR and AIR at Various pHs. In a volume of 1 mL D$_2$O, the NMR sample contained both AIR (口 - 0.8 mM) and CAIR (■ - 5.4 mM) in either 100 mM Tris (pD 8.5, 8.0, 7.5) or Phosphate (pD 7.5 and 7.0). On a 300 MHz NMR spectrometer, each spectrum consisted of 100 scans with the TSP reference at 0.0 ppm. Afterwards, the pD of the NMR sample was confirmed with a pH meter.
Figure 3.2A

Figure 3.2B
Figure 3.3: Absorbance Spectra of AIR at Various pHs. The absorbance of 39 μM AIR was taken in the following 100 mM buffers: Ches (pH 9.5), Tris (pH 8.0), Phos (pH 7.0), Mes (pH 6.0), NaOAc (pH 4.8), and HCl (pH 1.0).
Have the length (nm)

Absorbance (AU)

Wavelength (nm)

pH 9.5
pH 8.0
pH 7.0
pH 6.0
pH 4.8
pH 1.0
Figure 3.4: Absorption of N⁵-CAIR and AIR. Absorbance spectrum of N⁵-CAIR (—) and its conversion to AIR (--). The cuvette contained a solution of 200 mM Tris (pH 7.8), 20 mM KCl, and 78.8 μM N⁵-CAIR at 4°C. The sample was scanned, heated to 37°C for 5 min to form AIR, cooled to 4°C, and re-scanned.

Figure 3.5: Absorption of CAIR and AIR. The superposition of AIR (—) and CAIR (---) absorption at 50 μM, in 100 mM Tris-HCl (pH 8.0). The concentrations were determined with the Bratton-Marshall assay and the phosphate assay, respectively.
studies since the equilibrium constant for the PurE catalyzed carboxylation in 0.2 M bicarbonate favored decarboxylation ($K_{eq}=1.8$) and thus the rate of approach to equilibrium is rapid and it is difficult to measure initial velocities. Addition of metals (Mg++, Ca++) to the assay mixture has allowed for longer linear rates by shifting the equilibrium to the right (Ahmad et al., 1965). CAIR apparently chelates these metals (Chipperfield et al., 1987) Alternatively, the next enzyme in the purine biosynthetic pathway converts CAIR into SAICAR with the conversion of ATP to ADP. Using a coupled enzyme assay, the presence of ADP can be coupled to PK an LDH, and thus monitor the amount of NADH oxidation. (The stoichiometry of CAIR/ATP to SAICAR/ADP by PurC was determined in the previous chapter.)

**Purification of PurE.** PurE was purified to homogeneity using a CL-6B column (Figure 3.6) and a hydroxylapatite column (Figure 3.7). The specific activity of the protein was 40 U/mg using the assay which contained 0.2 M bicarbonate (Figure 3.8, Table 3.2).

<table>
<thead>
<tr>
<th>step(^a)</th>
<th>protein (mg)</th>
<th>volume (mL)</th>
<th>total activity (units)</th>
<th>specific activity(^b) (units/mg)</th>
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<tr>
<td>crude</td>
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<td>56</td>
<td>6010</td>
<td>3.4</td>
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<td>G-25 (50-80% ammonium sulfate)</td>
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<td>80</td>
<td>3940</td>
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<tr>
<td>DEAE CL-6B</td>
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<td>3470</td>
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<td>Hydoxylapatite</td>
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<td>8</td>
<td>3610</td>
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</table>

Table 3.2: Purification of PurE. \(^a\)15.8 g of TX635 containing pJS408. \(^b\)All assays were carried out using 0.3 mM AIR, 180 mM KHCO₃, and the PurC coupled assay procedure.

SDS gel electrophoresis of PurE revealed a single polypeptide of $M_r = 17.4$ kDa, when compared with molecular weights standards (Figure 3.8). The native molecular weight was determined using Sephadex G-100 chromatography and sucrose density ultracentrifugation in comparison with known proteins. An
apparent native molecular weight of 137 kDa (Table 3.3) was obtained. Thus, PurE appears to be an octomer composed of eight equivalent subunits.

<table>
<thead>
<tr>
<th>Gene</th>
<th>SDS Da</th>
<th>Gel kDa</th>
<th>G-100 kDa</th>
<th>Stokes radii Å</th>
<th>Sucrose (kDa)</th>
<th>s20,w</th>
<th>Calculated</th>
<th>Eq. XX</th>
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<td></td>
<td>53.8±3.1</td>
<td>3.8</td>
<td></td>
<td>(n=2)</td>
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</tbody>
</table>

Table 3.3: Native Molecular Weight Determination.

The Michaelis constants for AIR and HCO$_3^-$ with PurE were 420±30 μM and 110±10 mM, respectively. In comparison to other substrates in the purine biosynthetic pathway, these values were very high, and the Km for HCO$_3^-$ is sufficiently high to warrant concern about its physiological relevance. As discussed subsequently, the Km value with the actual substrate for PurE was determined once PurE's biological function was established.

PurK

**Purification of PurK.** In order to study the function of PurK in the PurE catalyzed AIR carboxylation reaction, PurK needs to be purified. As observed on the SDS gel (Figure 3.8), both PurE (17 kDa) and PurK (39 kDa) were overexpressed on heat induction, but surprisingly, they separated from each other during the first ammonium fractionation! The PurK fractions had no effect on the AIR carboxylase assay, while monitoring CAIR formation at 260 nm. As a result, this assay was unavailable for purification of PurK. Without a spectrophotometric assay, purification was monitored by SDS PAGE. The overexpression was sufficiently high to monitor fractions eluting from a CL-6B column (Figure 3.9). This single chromatographic step was successful in purifying to >90% homogeneity.
Figure 3.6: A typical DEAE-Sepharose CL-6B column profile for purification of PurE. From a CL-6B column, an elution profile was generated with protein absorbance at A\textsubscript{280} (○) and PurE activity (●). The column was loaded (fractions -100 to -40), washed with buffer (fractions -40 to 0), and then developed with a 0 to 200 mM KCl gradient. PurE activity was found in the third peak, eluting after 100 mM KCl. Fractions (57-81) were pooled.

Figure 3.7: A typical Hydroxylapatite column profile for purification of PurE. From a hydroxylapatite column, an elution profile was generated with protein absorbance at A\textsubscript{280} (○) and PurE activity (●). The column was loaded (fractions -90 to -30), washed with buffer (fractions -20 to 0), and then developed with a 50 to 500 mM potassium phosphate (pH 7.0) gradient. PurE activity was found in the second major peak, eluting after 300 mM phosphate. Fractions (65-100) were pooled.
Figure 3.8: SDS-Page Gel of PurE and PurK Preparations. The gel depicted is 12% polyacrylamide with 2.7% cross linking. All lanes contain 50 μg total protein. The gel was loaded as follows: Lane (1) molecular weight standards, (2) crude E. coli TX635/pJS355, (3) Sephadex G-25 50-80% ammonium sulfate, (4) Sepharose CL-6B, (5) hydroxylapatite, (6) molecular weight standards, (7) crude E. coli TX635/pJS355, (8) Sepadex G-25 0-50% ammonium sulfate, (9) Sepharose CL-6B, (10) molecular weight standards (kDa): Bovine Serum Albumin, 66; Hen Egg Albumin, 45; Glyceraldehyde-3-Phosphate Dehydrogenase, 36; Carbonic Anhydrase, 29; Trypsinogen, 24; Trypsin Inhibitor, 20.1; and α-Lactalbumin, 14.2.
From a CL-6B column, an elution profile was generated with protein absorbance at $A_{280}$ (○) and PurE activity (●). The column was loaded (fractions 0 to 18), washed with buffer (fractions 19 to 66), and then developed with a (10 mM, 20 mM and 40 mM) KCl step gradient. The PurK eluted with 40 mM KCl. Fractions (110-160) were pooled.
SDS PAGE of PurK revealed a single polypeptide of $M_r = 38$ kDa when compared with molecular weights standards (Figure 3.8). The native molecular weight for PurK was calculated to be 84 kDa, in comparison with proteins of known molecular weight using Sephadex G-100 chromatography and sucrose gradient centrifugation (Table 3.3). Thus, PurK from appears to be a dimer composed of two equivalent subunits.

Numerous assays combining PurE and PurK at various stages of purification were attempted to decrease the bicarbonate requirement for CAIR formation. Addition and pre-incubation with various divalent metals (following the example of ribulose bisphosphate carboxylase)(Lorimer et al., 1976) and small molecules from crude extracts were also unsuccessful at detecting any effect of PurK on the PurE catalyzed carboxylation of AIR.

Characterization of Protein Products: N-Terminal Sequence Analysis. In order to establish that the 39 kDa and 17 kDa proteins were derived from the purK and purE genes, both proteins were blotted onto Immobilon and subjected to N-terminal sequencing with automated Edman degradation. A comparison of the protein sequence derived from the gene with that obtained by sequencing the proteins indicated that both proteins were as expected (Table 3.4).

<table>
<thead>
<tr>
<th>PurK</th>
<th>PurE</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKQVCVLGNQGLGRMLRQAG</td>
<td>MSSRNNPARVAV</td>
</tr>
</tbody>
</table>

Table 3.4: Comparison of Sequenced Protein and the Genetic Prediction.
**PurK Possesses an unusual AIR-dependent ATPase.** A breakthrough in identifying an assay for PurK came when it was noticed that PurK contained a Gly-X-Gly-X-X-Gly sequence, indicative of an ATP binding domains (Kikkawa et al., 1989). ATP consumption could be easily monitored using the pyruvate kinase and lactate dehydrogenase coupled assay, monitoring the consumption of NADH. This assay was effective only in the presence of AIR, and independent of PurE! The assay simplified the purification of PurK (Table 3.5, Figure 3.8). If PurK is in fact a "CO₂" carrier, it might be anticipated that its function would be to convert HCO₃⁻ in an ATP-dependent reaction to carboxy-phosphate, which could then be directly delivered to purE. Any attempts to examine the stoichiometry of ATP and AIR revealed that AIR did not appear to be chemically modified as consumption of ATP proceeded. Thus the catalytic function of PurK at this stage still remained a mystery. This uncoupled ATPase assay was used to determine a Km for ATP and AIR of 90 and 26 μM, respectively.

<table>
<thead>
<tr>
<th>Stepa</th>
<th>Protein (mgs)</th>
<th>Volume (mL)</th>
<th>Total activity (units)</th>
<th>specific activityb (U/mg)</th>
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<td>16629.</td>
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<tr>
<td>G-25</td>
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<td>[45% (NH₄)₂SO₄]</td>
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<td>2.</td>
<td>6836.</td>
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</tr>
<tr>
<td>CL-6B</td>
<td>84.6</td>
<td>2.</td>
<td>6836.</td>
<td>80.8</td>
</tr>
</tbody>
</table>

Table 3.5: Purification of the PurK. a 14.9 g of TX635/pJS355. b The coupled assay measuring AIR-dependent ATPase with pyruvate kinase and lactate dehydrogenase.

Unable to demonstrate any HCO₃⁻ concentration dependence on the ATPase activity, raised the possibility of a contaminating enzyme. While the ATPase may be due to another enzyme in the purK protein preparation, its strict dependence on the presence of AIR makes this possibility seem unlikely.
*Time Dependent Heat Inactivation of PurK.* To ensure that the observed ATPase with PurK was not a contaminating enzyme, a heat inactivation study was carried out on the purified protein. Following SAICAR formation and ATPase activity, the ability of PurK to support CAIR production was monitored in the presence of PurE/PurC (Figure 3.10). Both activities were lost simultaneously, suggesting that they were associated with the same protein. While the physiological significance of this AIR-dependent ATPase was questionable, these results suggest that PurK and ATP together might be required for the PurE-dependent conversion of AIR to CAIR.

*PurK is Required for AIR Carboxylase Activity under Low [HCO₃⁻].* The consumption of ATP and the low concentration of bicarbonate in solution, based on an analogy with biotin dependent systems, suggests that PurK might be generating carboxyphosphate. In some manner, carboxyphosphate then acts as the CO₂ source, and enables PurE to catalyze the conversion of AIR to CAIR. However, this hypothesis did not explain the requirement of AIR in the PurK catalyzed ATP consumption reaction. Thus, an assay containing PurK, PurE and PurC (to couple CAIR formation forward) under moderate bicarbonate conditions (10 mM) was monitored for [¹⁴C] SAICAR formation (Figure 3.11). In the absence of either PurE, PurK or both, no [¹⁴C] SAICAR was observed; however, in the presence of both enzymes, [¹⁴C] SAICAR was produced. This assay presented the first *in vitro* evidence that PurK served an enzymatic function in the purine pathway.

The previous experiment was simplified with HPLC analysis of CAIR. Even at ambient bicarbonate concentration (200 μM HCO₃⁻), PurE and PurK generated CAIR only in the presence of ATP, as monitored with HPLC (Figure 3.12).

*ATP Stoichiometry in the PurE/PurK Dependent Carboxylase Reaction.* An experiment was devised to minimize the PurK-AIR-dependent ATPase activity, so that the stoichiometry of ATP could be correlated to the amount of CAIR produced.
Figure 3.10: Heat-dependent inactivation of PurK. (□) loss of AIR dependent ATPase, (▲) loss of ability to produce SAICAR. Fig. D Heat Inactivation

Figure 3.11: [14C] SAICAR Synthesis with PurEKC. The formation of [14C] SAICAR was monitored using PurEKC in the presence of only <0.2 mM bicarbonate: (▲) no (purE, PurK); (■) only PurK, (○) only PurE, (△) PurE and PurK.
Figure 3.12: Requirement for ATP in the conversion of AIR to CAIR monitored by HPLC analysis. Each reaction mixture contained 50 mM Hepes (pH 7.8), 20 mM KCl, 4.5 mM MgCl₂, 0.2 mM AIR, and <100 μM HCO₃⁻, with the following variations: (A) PurE and 0.9 mM ATP were added, (B) PurE, PurK and 0.9 mM ATP were added, (C) PurE and PurK. Panel D in the standard CAIR in 50 mM Hepes (pH 7.8) and 20 mM KCl. The retention time for AIR was 4 min.
Using low concentrations of AIR and a ten fold excess of PurE and PurC relative to PurK, the consumption of AIR was monitored by ATP hydrolysis to ADP and SAICAR formation. The ADP analysis involved PEP, NADH and the coupling enzymes PK and LDH. A burst of NADH oxidation indicates ATP utilization and modification of AIR. The product can be quantitated as [14C] SAICAR. The initial amount of AIR, the stoichiometry of NADH oxidation and the final [14C] SAICAR produced are then compared (Table 3.6).

<table>
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<tr>
<th>Compound</th>
<th>AIR</th>
<th>ATP</th>
<th>SAICAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantity</td>
<td>33.7 nmol</td>
<td>62.7 nmol</td>
<td>33.1 nmol</td>
</tr>
<tr>
<td>Ratioa</td>
<td>1.0</td>
<td>2.0±0.1</td>
<td>1.0±0.1</td>
</tr>
</tbody>
</table>

Table 3.6: Amount of Substrate/Product in Enzymatic Conversion of AIR to SAICAR.; a - Average of six determinations

The observed stoichiometry reveals that for each mole of AIR consumed, 1 mole of SAICAR is produced and 2 moles of ATP are utilized. From the previous chapter, PurC was shown to utilize one mole ATP for each mole [14C] SAICAR produced. A control with PurE and PurC in the presence of 180 mM bicarbonate and AIR required only one ATP for every [14C] SAICAR. These results suggests the ability of PurK to use ATP is intimately linked to the carboxylation of AIR!

**PurK Catalyzed Transfer of [18O] from HC[18]O₃⁻ to Pi.** The model in scheme 3.2 and Scheme 3.7 predicts that, as in biotin dependent reactions (Knowles, 1989), the function of ATP and HCO₃⁻ is to generate the elusive compound carboxyphosphate. HC[18]O₃⁻ was prepared by incubating bicarbonate in H₂[18]O using the methods of Knoche (1980) and Faurholt (1925) and its [18O] content established (Material and Methods).
Scheme 3.7: Incorporation of $[^{18}\text{O}]$ bicarbonate in to Phosphate.

Incubation of HC$^{18}$O$_3^-$ with ATP and PurK in the presence of 1 mM AIR gave the $^{31}$P NMR spectra shown in Figure 3.13. This experiment was carried out in collaboration with E. Mueller. Integration of the $\gamma$-phosphate of ATP (~4.30 ppm) and $\beta$-phosphate of ADP (~4.81 ppm) in the $^{31}$P NMR spectrum revealed that 8% of the ATP has been hydrolyzed during the reaction (Figure 3.13.B). The amount of ADP is stoichiometric with respect to the amount of AIR consumed. Figure 3.13.C displays an expanded spectral region for inorganic phosphate which contains a resonance at 3.44 ppm for [HP$^{16}$O$_4$]$^{2-}$, this was added as carrier and as an internal reference; and a resonance at 3.42 ppm for [H$^{18}$OP$^{16}$O$_3$]$^{2-}$, the product of the reaction. Quantitation of [H$^{18}$OP$^{16}$O$_3$]$^{2-}$ generated revealed that ~one atom of $^{18}$O was transferred from bicarbonate to phosphate for every ADP generated. Control reactions containing without 0.1 mM AIR or PurK revealed no feature at 3.42 ppm in the $^{31}$P NMR spectrum. These results are consistent with the intermediacy of carboxyphosphate (Scheme 3.4).
Figure 3.13: $^{31}$P NMR spectrum of the products when AIR is incubated with ATP, HC$^{18}$O$_3^-$, and PurK. (A) Spectrum on completion of the reaction of 1 mM AIR, HC$^{18}$O$_3^-$, and 11.4 mM ATP with 1.8 units of PurK. The sample also contained 2.3 mM carrier Pi. δ 4.54 (s, AIR), 3.44 (Pi), -4.30 (d, $J = 31$ Hz, γ-P of ATP), -4.81 (d, $J = 36$ Hz, α-P of ATP), -19.64 (t, $J = 31$ Hz, β-P of ATP). (B) expanded region at -3 to -5 ppm reveals that ~8% of the ATP has been hydrolyzed to ADP. (C) Expanded region at 3.4 ppm reveals 71% [HP$^{16}$O$_4$]$^{2-}$ (3.44 ppm) and 29% [HP$^{18}$O$^{16}$O$_3$]$^{2-}$ (3.42 ppm).
Studies of Alenin et al. allowed us to formulate a hypothesis for the PurE/PurK catalyzed reaction that accommodated all available information. These investigators carried out NMR studies which suggested that N^1-alkyl(R)-5-aminoimidazoles (R=ribose-5-phosphate or CH_3, in includes AIR!) in the presence of 1M bicarbonate could rapidly form a carboxylated species (Scheme 3.3). This new species differed from N^1-alkyl(R) 4-carboxy-5-aminoimidazoles (CAIR) which is eventually formed on a much slower time scale. This newly generated species decomposed upon dilution of the bicarbonate concentration regenerating starting material. Thus the following model was put forth (Scheme 3.4) to account for our data. N^5-CAIR was synthesized and used to test this model.

Synthesis and Purification of N^5-CAIR. A method had to be developed to synthesize and isolate N^5-CAIR so that it could be characterized structurally and for use to determine the kinetic parameters of PurE and PurK. The putative carbamate can be generated chemically in the presence of 1 M bicarbonate by the published procedure (Alenin et al., 1987). Alternatively, N^5-CAIR can be enzymatically synthesized with PurK. Carbamates are known to decompose at neutral pH and have increased stability at pH >12 requiring alkaline purification conditions (Caplow, 1968; Ewing et al., 1980). The N^5-CAIR generated enzymatically was purified on a Dowex 1W-X8 and the appropriate fractions were concentrated in vacuo, and extracted with methanol to reduce the KCl and bicarbonate concentration (40-55% yields)(Figure 3.14). N^5-CAIR was stored at pH >12. In the elution profile, N^5-CAIR elutes prior to another absorbing species which does not have a positive reaction with the Bratton-Marshall assay but does have end absorption similar to AIR and N^5-CAIR. This impurity decreases if AIR is purified immediately prior to synthesis of N^5-CAIR. Since this unidentified species is not detected with NMR analysis, its extinction coefficient must be large. The resulting N^5-CAIR made chemically or enzymatically has been characterized by ^13C and ^1H
Figure 3.14: A typical Dowex 1W-X8 column profile of an $N^5$-CAIR purification. From a Dowex column, an elution profile was generated with compound end absorption at $A_{250}$ (○) and Bratton-Marshall activity (✧). The eluted compounds were as follows: AIR (fractions 47-53), $N^5$-CAIR (fractions 60-66), and decomposed AIR (fractions 68-75).
Figure 3.15: $^1{H}$ NMR of N$^5$-CAIR. The NMR spectrum containing 41 mM N$^5$-CAIR (pH 12), with TSP as the reference at 0.0 ppm.
Figure 3.16: $^{13}$C NMR of [amino-$^{15}$N] AIR with $[^{13}$C$]$ HCO$_3^-$. A) Natural abundance $^{13}$C NMR spectrum of $N^5$-CAIR prepared by incubating HCO$_3^-$ with AIR.
Assignments: $\delta$ 167.5 (s, HCO$_3^-$), 161.6 (s, carbamate C), 135.1 (d, $J = 12$ Hz, C5), 131.5 (s, C2), 111.6 (s, C4), 86.4 (s, C1), 83.8 (d, $J = 3.8$ Hz, C5). (B) An expansion of the spectrum of $N^5$-CAIR (Figure 3.16) in the region of the carbon of the putative carbamate of $N^5$-CAIR. (C) The spectrum of the carbamate carbon produced from [amino-$^{15}$N]AIR incubate with $[^{13}$C$]$HCO$_3^-$ ($J_{C,N} = 19.7$ Hz).
NMR spectroscopy (Figure 3.15). Except for the salts, the sample appears to be >95 homogeneous.

While the studies of Alenin et al. (1987) strongly suggested that N⁵-CAIR was a carbamate, and our ¹H and ¹³C-NMR spectra (Figure 3.15) are consistent with this proposal, further evidence was sought to define its structure. [Amino-5-¹⁵N]-AIR was biosynthetically prepared by Dr. E. Mueller, which he then incubated in H¹³CO₃⁻, and analyzed the resulting product with ¹³C NMR spectroscopy (Figure 3.16). A signal is observed at 161.6 ppm with a ¹³C-¹⁵N coupling constant of 19.7 Hz. These results are consistent with one-bond coupling and unambiguously define the structure as the predicted carbamate (Levy & Lichter, 1979).

**Stability and Decomposition of N⁵-CAIR.** The UV spectrum of N⁵-CAIR is sufficiently different from AIR (Figure 3.4, Table 3.7) that UV can be used to monitor its decomposition to AIR. The stability of N⁵-CAIR was thus examined as a function of ionic strength, pH, temperature, and buffer (Table 3.8A-D).

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Table 3.7: Extinction Coefficients of N⁵-CAIR and AIR.

a) 100 mM Tris (pH 7.8), 4°C, b) 100 mM Tris (pH 7.8), 4°C,
   c) 100 mM Tris (pH 8.0)(Meyer et al., 1992), d) 50 mM phosphate (pH 12.5), 20°C.

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Table 3.8.A

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<td>7.8</td>
<td>0.228</td>
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<tr>
<td>8.0</td>
<td>0.173</td>
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<tr>
<td>8.2</td>
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</tr>
<tr>
<td>8.4</td>
<td>0.0845</td>
</tr>
<tr>
<td>8.6</td>
<td>0.0479</td>
</tr>
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</table>

A) Variation of temperature in 200 mM Tris (pH 7.8), 20 mM KCl.
B) Variation of pH in 200 mM Tris (pH 7.8), 20 mM KCl, 20°C.
Table 3.8.C

<table>
<thead>
<tr>
<th>pH</th>
<th>a</th>
<th>b</th>
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<tr>
<td>8.1</td>
<td>0.221</td>
<td>0.072</td>
</tr>
<tr>
<td>7.9</td>
<td>0.279</td>
<td>0.124</td>
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<tr>
<td>7.6</td>
<td>0.524</td>
<td>0.206</td>
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<td>7.3</td>
<td>0.997</td>
<td>0.383</td>
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Table 3.8.D

<table>
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<tr>
<th>buffer</th>
<th>pH</th>
<th>k (min(^{-1}))</th>
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</thead>
<tbody>
<tr>
<td>100</td>
<td>7.82</td>
<td>0.344</td>
</tr>
<tr>
<td>200</td>
<td>7.85</td>
<td>0.227</td>
</tr>
<tr>
<td>300</td>
<td>7.83</td>
<td>0.176</td>
</tr>
</tbody>
</table>

Table 3.8: Rate of Decomposition of \(N^5\)-CAIR as a Function of Temperature (A), pH (B), buffer (C), buffer concentration (D).

C) Variation of pH in Two Buffers
   a.) 200 mM Hepes, 200 mM KCl, 10 mM MgCl\(_2\) at 20°C,
   b.) 200 mM Tris, 200 mM KCl, 10 mM, MgCl\(_2\) at 20°C.

D) Variation of buffer concentrations with 20 mM KCl at 20°C.

The decomposition rate can also be monitored with an enzymatic end point assay using PurE/PurC, in which the remaining \(N^5\)-CAIR is converted to SAICAR. At pH 12 in 50 mM phosphate buffer, its half life is 8 h, while at pH 9.0 in 50 mM Ches, its half life is 42 min. These decomposition rates confirm that this carbamates is stable under basic conditions; however, the high salt and pH required to isolate and stabilize \(N^5\)-CAIR has been problematic in controlling the ionic strength and pH in the kinetic assays.

**PurE Catalyzed Conversion of CAIR to \(N^5\)-CAIR.** With homogeneous PurE available and knowledge of \(N^5\)-CAIR stability, we examined the PurE catalyzed conversion of CAIR to \(N^5\)-CAIR with NMR spectroscopy (Scheme 3.8). The hypothesis predicts that the \(N^5\)-CAIR formed by PurE would rapidly decompose to AIR in a non-enzyme dependent fashion. To test this hypothesis, CAIR was incubated with PurE and the reaction monitored with NMR spectroscopy. This reaction was carried by E. Mueller. The ability to differentiate between the C1 proton resonances of CAIR, \(N^5\)-CAIR and AIR (δ 5.6 to 5.8 ppm), and the C2 protons (7.5-8.1 ppm) have allowed the reaction to easily monitored (Figure 3.17). The number of units of PurE and the temperature of the reaction were chosen to facilitate this kinetic analysis with NMR spectroscopy. Typical NMR spectra are shown in Figure
3.17 and the kinetics of CAIR decomposition, N⁵-CAIR formation and
decomposition, and AIR production are shown in Figure 3.18. The data have been
fit by computer to equation (Scheme 3.8) giving values of $k_1 = 0.18 \text{ min}^{-1}$, $k_{-1} = 0.12 \text{ min}^{-1}$, $k_2 = 0.26 \text{ min}^{-1}$, $k_{-2} = 2.8 \times 10^{-4} \text{ min}^{-1}$ in 300 mM Tris-HCl (pD 8.0). The rate of

\[
\text{CAIR} \xrightleftharpoons{k_1}{k_{-1}} \text{N}^5\text{-CAIR} \xrightleftharpoons{k_2}{k_{-2}} \text{AIR} + \text{HCO}_3^-
\]

Scheme 3.8: Rates of CAIR to N-CAIR to AIR equilibrium.

conversion of CAIR to N⁵-CAIR was shown to be dependent on the concentration of
PurE, while the rate of conversion of N⁵-CAIR to AIR was found to be independent
of PurE (data not shown). The rate constant for the decomposition of N⁵-CAIR ($k_2$),
under these conditions, is virtually identical to that determined as described above,
when pH differences, temperature, and ionic strength are taken into account. Thus
PurE catalyzes the conversion of CAIR to N⁵-CAIR as predicted by our model
(Scheme 3.4).

*Kinetics of N⁵-CAIR with PurE.* The studies reveal that N⁵-CAIR is the actual
substrate for PurE and thus the kinetic studies described above have been
reexamined. As outlined above, the high pH and ionic strength required for
isolation and storage of N⁵-CAIR has made reproducibility of the kinetic difficult.
The methanol extraction reduced the salt concentrations in the N⁵-CAIR sample,
and allowed for reproducible assays. Concentrations of 5 times its Km value
resulted in a pH changes of less than 0.1 pH. The Km N⁵-CAIR was determined to
be 178±30 µM with a Vmax of 70 U/mg. These numbers are the average of two
determinations and a typical set of kinetic data is shown in Figure 3.19.
Figure 3.17: PurE-dependent generation of $N^5$-CAIR from CAIR at 25°C. (A) A zero time point in which only CAIR (and a small amount of contaminating AIR) is present. (B) A 1.5-min time point after addition of PurE reveals that $N^5$-CAIR has formed along with a small amount of breakdown product, AIR. (C) A 20-min time point after addition of PurE reveals a nearly completed reaction, with AIR as the major species.
Figure 3.18: Kinetics of PurE-dependent conversion of CAIR to $N^5$-CAIR at 20°C followed by nonenzymatic conversion of $N^5$-CAIR to AIR. NMR spectroscopy (Figure 3.17) allows monitoring of the reaction species: CAIR (×), $N^5$-CAIR (●), AIR (□). The solid lines are a nonlinear least-square fit to equation (Scheme 3.8) where $k_1 = 0.18 \text{ min}^{-1}$, $k_{-1} = 0.12 \text{ min}^{-1}$, $k_2 = 0.26 \text{ min}^{-1}$, and $k_{-2} = 2.8 \times 10^{-4} \text{ min}^{-1}$. Error bars reflect integration errors.
**Inhibition by AIR.** The structural similarities between AIR and N⁵-CAIR and the observation that N⁵-CAIR decomposes to AIR, it was important to determine if AIR is an inhibitor of the PurE catalyzed reaction since it is always present in the assay solution. A kinetic study was carried out with 150 M N⁵-CAIR and concentrations of AIR varying from 10 µM to 1 mM. Data analysis using the equations 3.6.a,b,c revealed that the data fit best to a competitive pattern (Figure 3.20). The kinetic constants are summarized in Table 3.9.

\[
v = \frac{V_{\text{max}}[S]}{K_m(1+[I]/K_{iS})+[S]} \quad \text{competitive} \quad \text{Eq. 3.6a}
\]
\[
v = \frac{V_{\text{max}}[S]}{(K_m(1+[I]/K_{iS})+[S](1+[I]/K_{ii}))} \quad \text{noncompetitive} \quad \text{Eq. 3.6b}
\]
\[
v = \frac{V_{\text{max}}[S]}{(K_m+[S](1+[I]/K_{ii}))} \quad \text{uncompetitive} \quad \text{Eq. 3.6c}
\]

where \(K_{iS} = [E][I]/[EI], K_{ii} = [ES][I]/[ESI], S = \text{substrate concentration}, v = \text{initial velocity}\) Michaelis constants: \(V = \text{maximum velocity and } K_m = (k_{-1} + k_{\text{cat}})/k_1.\)

The inhibition of PurE with AIR was interesting, since the original enzymatic assay only contained the substrates AIR and bicarbonate (180 mM). Under these conditions, 16% of the AIR was converted to N⁵-CAIR as observed with NMR spectroscopy. This implies that N⁵-CAIR was always present for PurE to use as a substrate; however, an inhibitor was also present. From the inhibition pattern, the calculated N⁵-CAIR \(K_m\) of 210 µM is in moderate agreement with the \(K_m\) value of 178 µM described above.

<table>
<thead>
<tr>
<th></th>
<th>Comp</th>
<th>Noncomp</th>
<th>Uncomp</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_{\text{max}})</td>
<td>96±2 U/mg</td>
<td>97±3 U/mg</td>
<td>113±9 U/mg</td>
</tr>
<tr>
<td>(K_m) (N⁵-CAIR)</td>
<td>207±9 µM</td>
<td>216±12 µM</td>
<td>348±52 µM</td>
</tr>
<tr>
<td>(K_{iS}) (AIR)</td>
<td>236±15 µM</td>
<td>261±34 µM</td>
<td>5386±5078 µM</td>
</tr>
<tr>
<td>(K_{ii}) (AIR)</td>
<td>5386±5078 µM</td>
<td></td>
<td>483±117 µM</td>
</tr>
</tbody>
</table>

Table 3.9: Competitive Inhibition By AIR on PurE.
Figure 3.19: PurE $K_m$ determination with $N^5$-CAIR. The concentrations of $N^5$-CAIR ranged from 60 to 750 μM, with the assay monitoring CAIR formation via PurC at 20°C.

Figure 3.20: PurE Inhibition Pattern with AIR. Initiating the reaction with PurE, a 3 by 5 inhibition pattern was designed with varying concentrations of AIR (♦ - 0 μM AIR, ■ - 116 μM AIR, ▲ - 500 μM AIR) and $N^5$-CAIR (37, 51, 74, 132, 678 μM). The y-axis is 1/(μmol/min/mg).
PurK Catalyzed Conversion of AIR to N⁵-CAIR in the Presence of ATP.

While the above studies established that PurE can catalyze conversion of CAIR to N⁵-CAIR, the model also requires that PurK can generate N⁵-CAIR. The following experiment was designed to show the formation of N⁵-CAIR catalyzed by PurK (Scheme 3.6). Our hypothesis predicts that incubation of a small amount of AIR with an excess of ATP, HCO₃⁻ and PurK should produce a burst of N⁵-CAIR, Pi and ADP. The latter can be monitored using a PK/LDH coupled assay which follows NADH consumption. If sufficient PurK is present, then the rate determining step in the reaction will be the non-enzymatic decomposition of N⁵-CAIR to AIR. The initial burst will be followed by a slower rate of NADH consumption and can be compared to the rate of nonenzymatic decomposition of N⁵-CAIR. The results of a variety of experiments in which the amount of AIR and PurK were varied are summarized in Table 3.10. The observed bursts are equivalent to the amount of AIR present,

<table>
<thead>
<tr>
<th>AIR added (nmol)</th>
<th>NADH Rapidly Consumed (nmol)</th>
<th>Steady State NADH Consumption (min⁻¹)</th>
</tr>
</thead>
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<tr>
<td>3.2</td>
<td>3.5</td>
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<td>11.9</td>
<td>12.9</td>
<td>0.39</td>
</tr>
<tr>
<td>23.9</td>
<td>23.6</td>
<td>0.40</td>
</tr>
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</table>

Table 3.10: Evidence for N⁵-CAIR Formation by PurK Using A Coupled Assay. a 100 mM Tris-HCl (pH 7.8, 20°C)

while the slow rates of NADH consumption are independent of the concentration of PurK and are equivalent to the non-enzymatic breakdown of N⁵-CAIR (0.40 sec⁻¹) under identical conditions. These results support our hypothesis that PurK catalyzes the formation of N⁵-CAIR.
Detection of PurK Catalyzed Formation of $N^5$-CAIR by NMR Spectroscopy. The experiments described in the previous sections all corroborate the model proposed in scheme 3.4. It remained to be shown; however, that the compound whose formation is responsible for the burst of NADH consumption is in fact $N^5$-CAIR. Thus, an experiment was set up under conditions identical to the "burst" experiments described, so that $N^5$-CAIR could be detected by NMR spectroscopy. The reaction was quenched with base to a final pH of 13 to decrease the rate of $N^5$-CAIR decomposition, with the help of Dr. E. J. Mueller. The results of this experiment are shown in Figure 3.21. The observation of new resonances at 7.96, 6.82, and 5.58 ppm (Figure 3.21.B, arrow), identical to those associated with $N^5$-CAIR at 7.98, 6.83, and 5.63 ppm (Figure 3.21.C) provides direct evidence that PurK catalyzes formation of $N^5$-CAIR.\footnote{The chemical shifts are very sensitive to both changes in the pH and the ionic strength of the solutions. The pH of the solution, the spectrum of which is shown in Fig. 3.21.B is ~ 13, while that in Fig. 3.21.C is ~ 14. The C4 proton of AIR (starting material) had been partially exchanged in D$_2$O during its characterization. Consequently, the appearance of less than one equivalent of a proton at C4 in $N^5$-CAIR is a consequence of the exchanged starting material and not additional exchange during the experiment or workup.}
Figure 3.21: PurK-catalyzed conversion of AIR to $N^5$-CAIR monitored with NMR spectroscopy subsequent to quenching at pH 12.5. (A) The reaction mixture with everything present prior to the addition of enzyme: $\delta$ 8.56 (C8, ATP), 8.28 (C2, ATP), 7.69 (C2, AIR), 6.45 (C4, AIR), 6.06 (d, $J = 6$ Hz, C1 of ATP), 5.56 (d, $J = 6.9$ Hz, C1 of AIR), 5.39 (s, vinyl protons of PEP), 5.21 (s, vinyl protons of PEP). (B) PurK was added to initiate the reaction, which was quenched after 2 min by adjusting the pH to 13. New resonances (indicated by arrows) are observed at $\delta$ 7.96 (s, C2 of $N^5$-CAIR), 6.8 (s, C4 of $N^5$-CAIR), 6.02 (d, $J = 6$ Hz, C1 of ADP), 5.58 (d, $J = 5.0$ Hz, C1 of $N^5$-CAIR). (C) Spectrum of $N^5$-CAIR at pH 14, $\delta$ 7.98 (s, C2 of $N^5$-CAIR), 6.83 (C4, $N^5$-CAIR), 5.63 (d, $J = 4.8$ Hz, C1 of $N^5$-CAIR). Differences in the new resonances and the $N^5$-CAIR standard are attributed to the pH differences in the two samples. In addition, the C4 proton of AIR and consequently $N^5$-CAIR has undergone partial exchange.
Reverse reaction of PurK with Hexokinase and G-6-P dehydrogenase. The reversibility of PurK reaction was examined using the coupled assay shown in Scheme 3.9.

Scheme 3.9: Assay for PurK reverse reaction. (HK - hexokinase, G-6-P - glucose-6-phosphate, G-6-P DH - glucose-6-phosphate dehydrogenase, 6-PGL - 6-Phosphoglucono-δ-lactone), and ambient bicarbonate concentrations.

$N^5$-CAIR synthesized chemically or generated enzymatically from PurE gave no detectable formation of ATP as monitored by NADP$^+$ reduction or as organic phosphate starting with radiolabeled $^{32}$Pi. In addition, use of carbamoyl phosphate and ADP also failed to generate ATP. Thus no evidence for the reversibility of PurK was obtained.
Discussion

Genetic studies by Gots suggested that two proteins were involved in the carboxylation of AIR to CAIR in *E. coli de novo* purine biosynthesis. Mutation studies suggested that PurK was a "CO₂" carrier, an unusual function for a 39 kDa protein. *PurK* and *purE* genes were constituents of the same operon as discovered by Smith (Tiedeman et al., 1989) and Mizobuchi (Watanabe et al., 1989) and thus it seemed likely that they were subunits of AIR carboxylase. Both proteins were placed in a vector behind a heat inducible promoter and were overexpressed as observed by SDS PAGE (Figure 3.8). Initial attempts to purify PurK revealed unexpectedly that it separated from PurE during the first ammonium sulfate fractionation. Previously described assays conditions for AIR carboxylase, observing CAIR formation at 250 nm in the presence of 200 mM bicarbonate and AIR, were successful with PurE to allow purification to homogeneity. Unfortunately, addition of PurK to this same assay had no effect on the rate of AIR carboxylation.

Without an assay for PurK, sufficiently high overexpression (15% of cellular protein) allowed purification of PurK by monitoring protein with SDS PAGE. In hopes of determining a function for PurK, purified fractions of PurK and PurE were combined in anticipation of decreasing the bicarbonate requirement by PurE in carboxylating AIR. Using the assay which monitors CAIR formation at A260nm, PurK had no effect on its rate of formation. Previous characterization of purified AIR carboxylases from other sources, all assays required non-physiological concentrations of bicarbonate, >100 mM KHCO₃ (Patey&Shaw, 1973); (Lukens&Buchanan, 1959), (Nikolaeva et al., 1975), (Nikolaeva et al., 1982), (Ahmad et al., 1965), (Yanulaitis et al., 1975). In addition, no requirement for ATP was mentioned, even though it was looked for. With my observation that PurK catalyzed the consumption of ATP in the presence of AIR, a new activity was
monitored with the PK/LDH coupled assay, which facilitated purification of the protein to homogeneity, but raised questions about the role and function of PurK.

One of the observations made with PurK, which required an explanation, was the absolute dependence on AIR for ATPase activity without AIR being consumed (as monitored with HPLC). This uncoupled ATP hydrolysis was disturbing. Several experiments suggested that the observed ATPase was actually an integral part of PurK's role in purine biosynthesis. First, the enzyme contains an AIR (but not CAIR) dependent ATPase. Second, an HPLC analysis containing low concentrations of HCO₃⁻ and ATP revealed that in the presence of PurE and PurK and AIR, that "CAIR" was produced; however, in the absence of ATP, no "CAIR" was produced. Third, in coupling the PurE/PurK product with PurC, it was shown that all three enzymes were required for the AIR to SAICAR conversion with low bicarbonate. Finally, during the inactivation of PurK, both the ATPase activity and SAICAR formation were lost simultaneously. The kinetic constants for PurK were determined (Km of 26 µM for AIR, Km of 90 µM for ATP), and the values were reasonable with respect to PurK's postulated role. These methods have shown for the first time that ATP is stoichiometrically consumed in the conversion of AIR to CAIR and that PurK is involved in lowering the concentration of HCO₃⁻ for CAIR.

The strict dependence on AIR to observe the ATP hydrolysis catalyzed by PurK still remained to be unexplained. However, the ATPase activity itself suggests a role for PurK, functioning as the missing factor required to make PurE function under physiological conditions. Specifically, PurK could catalyze the reaction of ATP and HCO₃⁻ to produce carbonyl phosphate, which could then be delivered to PurE as a source of CO₂ required to carboxylate AIR (Scheme 3.2).

A classic "CO₂" carrier in biological systems has been biotin. The mechanism for biotin carboxylation have been studied extensively (Knowles, 1989). Failure in
locating the common biotin binding region (AMKM) in the PurK sequence, and PurKs continuous ATPase activity even in the presence of avidin, suggests that biotin is not involved in this carboxylation reaction. With evidence proposing that PurK is involved with "CO₂" delivery of the substrate for PurE, whether CO₂ or HCO₃⁻ is the substrate needs to be established. Since the enzyme utilizes ATP, the actual substrate for PurK was believed to be HCO₃⁻. This was confirmed by Dr. Ernie Mueller, by using ¹⁸O labeled bicarbonate. The ATP hydrolysis products generated in the presence of [¹⁸O] HCO₃⁻ by PurK were analyzed with ³¹P NMR spectroscopy. As shown in Scheme 3.7, if bicarbonate is labeled, one atom of ¹⁸O should be found in Pi. The observed results are consistent with a carboxyphosphate intermediate, confirming that bicarbonate and not carbon dioxide is the actual substrate for PurK. (A control experiment revealed that ¹⁸O was not washed out of the HCO₃⁻ during the course of the reaction.)

Efforts to analyze the kinetics of bicarbonate dependence were all unsuccessful, due to our inability to remove the endogenous HCO₃⁻ from our solutions to sufficiently low levels. However, Dr. Steven Oh in our lab has recently succeeded in determining a Km for of 42 μM. This constant was well below the concentrations of HCO₃⁻ (200 μM) routinely fund in buffers, allowing for a continual supply of this substrate for PurK without any addition to assay mixtures.

A change in the approach in thinking about this enzyme came with closer analysis of the experiments reported by Alenin et al. in 1987. Their studies suggested that N-substituted carbamates could be rapidly generated in a chemical process from 5-aminoimidazole derivatives in 1 M HCO₃⁻ solutions. Furthermore, their studies revealed that with dilution of the high HCO₃⁻ solution, the putative carbamates decomposed back to starting aminoimidazole derivatives.
Initial efforts focused on isolation and structural characterization of the compound produced when 1M HCO$_3^-$ was incubated with AIR. Studies of Caplow (1968) on model carbamates for biotin-dependent decarboxylations provided the insights needed about carbamate stability to successfully complete these tasks. This new species was designated N$^5$-carboxyaminoimidazole ribonucleotide (N$^5$-CAIR). $^1$H, $^{15}$N, and $^{13}$C-NMR experiments indicate that this species is the N$^5$-carbamate of AIR (Mueller et al., 1994). Kinetic analyses have shown that its half life in solution at 30 °C, pH 7.8, was 0.9 min, and dependent on pH, temperature, and ionic strength.

Identification of this novel modified purine pathway compound, N$^5$-CAIR, raised hopes that this might be a new intermediate in purine biosynthesis: a product of PurK and a substrate for PurE. Using NMR spectroscopic methods, Dr. E. Mueller, showed first that PurK catalyzes conversion of AIR, ATP, and HCO$_3^-$ to N$^5$-CAIR, ADP, and Pi and that PurE catalyzes the reversible interconversion of N$^5$-CAIR and AIR.

In quenching the PurK catalyzed reaction with base, a new species is characterized in the $^1$H-NMR spectrum, which is identical to N$^5$-CAIR. PurK catalyzed formation of N$^5$-CAIR can now explain the observed AIR-dependent ATPase observed with PurK. Since the compound rapidly decarboxylates to AIR non-enzymatically, a small amount of AIR can continuously be recycled and account for the millimolar quantities of ATP consumed during a standard assay. Interestingly, reduction in temperature during the ATPase assay resulted in the observation of two distinct rates, the initial rate of NADH oxidation (equivalent to AIR) was followed by a slower rate. This slower rate corresponds to nonenzymatic decomposition of N$^5$-CAIR back to AIR, which was observed in this assay with its recarboxylation by purK. The instability of N$^5$-CAIR precluded its identification by HPLC.
The instability of $N^5$-CAIR also complicated the process for determining if $N^5$-CAIR is the real substrate for PurE generating CAIR. This process was analyzed with NMR spectroscopy and by kinetic experiments. In using NMR spectroscopy, the reverse reaction of PurE was monitored, by following the specific resonances associated with C1 and C2 of CAIR, $N^5$-CAIR and AIR. Fortunately, the starting material is stable and appropriate conditions of temperature and pH were chosen to accommodate $N^5$-CAIR detection. These results unambiguously established that PurE catalyzes the conversion of CAIR to $N^5$-CAIR, which non-enzymatically and irreversibly decomposes to AIR.

For the biosynthetic direction, kinetic parameters for PurE with $N^5$-CAIR were determined. Assay conditions required appropriate pH and ionic strength control, as well as selection of a temperature that reduced decomposition of $N^5$-CAIR. The assays were carried out in 200 mM Tris buffer (pH 7.8) and 20°C. The half-life for decomposition of $N^5$-CAIR in the assay mixture under these conditions was ~3.0 min. Under these conditions the $N^5$-CAIR $K_m$ was 174 μM with a Vmax of 70 μmol/min/mg. In the reverse direction in 100 mM Tris (pH 7.8, 37°C), the CAIR $K_m$ was 36 μM with a Vmax of 20 U/mg.

These experiments established that $N^5$-CAIR is a newly discovered intermediate in the purine pathway, and a novel method to generate a carboxylated species. Using ATP and bicarbonate, PurK is proposed to generate a carboxyphosphate intermediate that carboxylates AIR to generate $N^5$-CAIR. The resulting carbamate is utilized by PurE to place the carbon in its final location on the purine base. This proposed rearrangement now requires that "AIR carboxylase" describing the AIR to CAIR conversion be renamed, such that PurK is designated as $N^5$-CAIR synthetase and PurE as $N^5$-CAIR mutase (established in Chapter 4 of this thesis).
An interesting comparison was made between the assay conditions for PurE before and after the discovery of N5-CAIR as the actual substrate. Originally, PurE was assayed in 180 mM HCO3- and AIR. Under these conditions through NMR analysis, it was revealed that 16% of the AIR was nonenzymatically converted to N5-CAIR at 37°C. Thus, PurE was actually utilizing N5-CAIR as a substrate in the presence of the potential inhibitor AIR. Taking into consideration the Ks and the ratio of AIR to N5-CAIR, the turnover numbers of the high bicarbonate assay (37°C) agreed with the N5-CAIR assay (20°C). This observation may also explain why the yeast enzyme activity was detected without use of ATP (Nikolaeva et al., 1975), even though a portion of the yeast enzyme contained 26% sequence identity to the *E. coli* PurK.

The purine biosynthetic pathway is found in all organisms and thus it might be of interest to compare the available "AIR carboxylase" amino acid sequences based on the gene sequence and their organization for PurE and PurK. The following organisms contain separate proteins for either PurE and/or PurK: *Synechococcus sp.* (purK) (Schwarz et al., 1992), *M. thermoautotrophicum* (purE) (Hamilton & Reeves, 1985b) *M. smithii* (purE) (Hamilton & Reeves, 1985a) and *E. coli*. (purE and purK) (Tiedeman et al., 1989). The second group of organisms contain a single protein with homology with both PurE and PurK: *B. subtilis* (Ebbole & Zalkin, 1987), *S. pombe* (Szankasi et al., 1988), and *S. cerevisiae* (Sasnaukas & Janulaitis, 1992). The third group also possess a gene coding for a bifunctional protein with activity for PurC and PurE: human (Minet & Lacroute, 1990) and chicken (Chen et al., 1990). No sequence homology with PurK was found in the third group, and they contain 22% identity and 60% similarity with *E. coli* PurE. The question can be raised as to whether PurE from these organism possesses an intrinsic PurK activity or whether a different mechanism of carboxylation is employed by these organisms. This
development may suggest something about the environment in which these enzymes function in their respective organisms. The first and second groups are single cellular organelles which are dependent on their environment for the level of carbon dioxide and bicarbonate, 200 μM HCO₃⁻ at pH 7.4. Fortunately for E. coli, the characterized PurK has a bicarbonate Km of only 42 μM, an abundance of substrate for this step in the pathway.

In organisms in which no PurK has been thus far detected, the common feature include bifunctional PurC-PurE activity, and a circulatory system for homeostasis. This allows for a constant supply of substrate since venous blood is 28 mM bicarbonate, suggesting that tissue levels are comparable. In characterization of the avian PurE, the Km for bicarbonate was 23 mM; however, the exact substrate has yet to be determined (i.e. CO₂ or HCO₃⁻). Since ATP was not utilized by the avian PurE, CO₂ may be the substrate. The mechanistic implications of this in comparison with studies on the E. coli enzyme are discussed in next chapter.

Regardless of the mechanism of CAIR biosynthesis in eukaryotes, this new twist in the E. coli pathway returns us to a long standing unresolved problem in carboxylation chemistry: the mechanism by which carboxybiotin and now N⁵-CAIR delivers CO₂ to its substrate(s). There are striking similarities and a few differences in these two CO₂ delivery systems (Knowles, 1989). Both are chemically unstable: carboxybiotin has a half-life of 103 minutes (Tipton, 1988), while N⁵-CAIR has a half-life of 0.9 minutes at 30°C. Carboxybiotin and N⁵-CAIR are both generated in ATP dependent processes which presumably generates carboxyphosphate. In both systems, the existence of carboxyphosphate was inferred by incorporation of ¹⁸O from HC¹⁸O₃⁻ into Pi. Both cases consist of two half reaction. In the first reaction, biotin is carboxylated with ATP and bicarbonate (AIR is carboxylated to N⁵-CAIR). This is followed by a transfer in space to the final carboxylation site: the 14 Å arm of biotin reaches the active site of the substrate to be carboxylated, while PurK releases
$N^5$-CAIR as a substrate for PurE which performs the carboxylation in the active site. An interesting difference between these two systems is that $N^5$-CAIR can form nonenzymatically, while biotin is not nucleophilic enough, and needs activation, which may explain why its half life is so much longer.
References


White, R.H. & Rudolph, F.B. (1979) 18, 2632-2636.


Chapter 4

Mechanistic Study of the Reaction Catalyzed by PurE:
Conversion of $N^5$-CAIR to CAIR
Introduction. We have shown in chapter 3 that PurE catalyzes the reversible conversion of $N^5$-CAIR to CAIR. The mechanism for decarboxylation-recarboxylation is chemically unprecedented and is the major focus of the present chapter (Scheme 4.1). Several mechanistic options for this carboxylate rearrangement have been considered and are outlined in Scheme 4.2. The first possibility shows a concerted transfer of $\text{CO}_2$ from $N^5$-CAIR to CAIR. The second involves decarboxylation of $N^5$-CAIR, releasing $\text{CO}_2$ in the active site, followed by nucleophilic attack with C4 of the resulting AIR. In the third case, covalent catalysis via a lysine intermediate could be involved in the transfer. The fourth mechanism involves two substrates, $N^5$-CAIR and AIR, to generate AIR and CAIR.

![Scheme 4.1: Label transfer by PurE.](image)
\[ 1) \quad N^5-CAIR \quad \rightarrow \quad \quad \quad \quad \quad \quad \rightarrow \quad CAIR \]

\[ 2) \quad N^5-CAIR \quad \rightarrow \quad \quad \quad \quad \quad \quad \rightarrow \quad CAIR \]
Scheme 4.2: Possible mechanistic intermediates of PurE.
Two experimental approaches have been used to examine the mechanism of this decarboxylation/recarboxylation catalyzed by PurE. The first one utilizes NMR spectroscopic analysis of the [4,7-\textsuperscript{13}C] N\textsuperscript{5}-CAIR to determine the fate of the label in the product (Scheme 4.1, Scheme 4.4). The second involves use of [7-\textsuperscript{14}C] N\textsuperscript{5}-CAIR to followed its conversion to CAIR and determination of the final specific activity.

\[ R = \text{ribose 5'-phosphate} \]

Scheme 4.3: SAICAR synthetase reaction.

To facilitate product analysis and prevent the reverse reaction catalyzed by PurE, CAIR was converted to SAICAR using SAICAR synthetase (Scheme 4.3). This chapter describes the enzymatic synthesis of N\textsuperscript{5}-CAIR with \textsuperscript{13}C and \textsuperscript{14}C labels and analysis of the resulting SAICAR.

**Materials.** Lactate dehydrogenase (LDH, 860 U/mg), pyruvate kinase (PK, 470 U/mg), phosphoenolpyruvate (PEP), phosphoenolpyruvate carboxylase (PEP-C, 3.6 U/mg), malate dehydrogenase (MDH, 3000 U/mg), and adenosine-5-triphosphate (ATP) were obtained from Sigma. [2-\textsuperscript{13}C] Gly 99%, 83-12204, and [\textsuperscript{13}C] NaHCO\textsubscript{3} 99.0%, 83-70006 was purchased from Isotec. Dowex 1-X8 was purchased from BioRad. DEAE Sephadex A-25 was obtained from Pharmacia. [\textsuperscript{14}C] NaHCO\textsubscript{3} (6.8 mCi/mmol) was purchased from New England Nuclear. Scint\textsuperscript{A} scintillation fluid was purchased from Packard. 5-Phosphoribosyl-1-pyrophosphate amidotransferase (PRPP-
AT) (6.8 U/mg), (Rudolph, 1993)), glycineamide ribonucleotide synthetase (GAR syn) (30 U/mg), (Cheng et al., 1990)), glycineamide ribonucleotide amidotransferase (GAR-TF, PurT) (14.8 U/mg), (Marolewski et al., 1994)), formylglycineamide ribonucleotide amidotransferase (FGAR-AT) (1.0 U/mg), (Schendel et al., 1989)), aminomimidazole ribonucleotide synthetase (AIR syn) (1.9 U/mg), (Schrimsher et al., 1986)) were isolated as described previously and then assayed at 20°C. The synthesis of AIR, N5-CAIR, and CAIR, the purification of enzymes and the assays of PurE, PurK, and PurC were carried out as previously described(Meyer et al., 1992; Mueller et al., 1994). Unless otherwise stated, in all these experiments described below, the KOH solution was prepared immediately before use by dissolving solid KOH into distilled water.

Methods Orcinol assay for quantitation of reducing pentoses was performed according to the procedure described by Dische[1962]. The concentration of β-FGAR, AIR, N5-CAIR were determined using the Bratton-Marshall assay, modified by Schendel (1986). The concentration of bicarbonate was determined enzymatically, utilizing PEP carboxylase and malate dehydrogenase (Peled, 1983)

Synthesis

Enzymatic Synthesis of [3- 13C] FGAR. [3- 13C] FGAR was synthesized from PRPP using the first three enzymes in the purine pathway: PRPP-AT (PurF), GAR syn (PurD), and GAR-TF (PurT)(Scheme 4.4), following the procedure of Dr. E. J. Mueller (Thesis p.54). In a final volume of 50 mL at 20°C, the reaction mixture contained: 100 mM Tris (pH 7.8), 10 mM MgCl2, 135 mM KCl, 4.86 mM α-PRPP (243 µmol), 35 mM Gln, 12.1 mM [2- 13C] Gly (99%, Isotec, 83-12204), 2.22 mM ATP, 8.0 mM formate, 20 mM PEP, 37 U
Scheme 4.4: The de novo Purine Biosynthetic Pathway following two labeled carbons.
PRPP-AT, 52 U GAR syn, 700 U GAR-TF, and 250 U PK. Prior to enzyme addition, the reaction mixture was adjusted to pH 7.8 and incubated at 20°C for five min. When the enzymes were added, PRPP-AT was added last to initiate the reaction. After 1 h, the reaction mixture was diluted to 500 mL (pH 7.4) with de-ionized water at 4°C and purified in the cold room using the following procedure. The sample was loaded onto a DEAE Sephadex A-25 column (10 x 2.5 cm, HCO$_3^-$ form), and the column was developed with a linear gradient from 0 to 500 mM TEAB (500 x 500 mL, pH 7.4). Fractions (12 mL) were collected and 100 μL of every third fraction was analyzed using Orcinol assay (Figure 4.1). The fractions containing FGAR (fractions 34-44, at 200 mM TEAB) were pooled. To facilitate removal of TEAB, the sample was repeatedly dissolved in water and methanol, and concentrated in vacuo. To prevent anomerization during the concentration process, it was essential that the pH remained slightly basic. This was accomplished through dropwise addition of freshly prepared 1 N KOH. A yield of 50% was determined using the Bratton-Marshall assay. A portion of this product was exchanged into D$_2$O for NMR analysis: $^1$H NMR (300 MHz, D$_2$O, TSP at 0.0 ppm, pH 7.0) δ 8.13 (d, 1, J=5.7 Hz, formyl), 5.43 (d, 1, J=5.4 Hz, 1), 4.20 (m, 1, H2), 4.12 (m, 2, H3), 4.06 (m, 1, H4), 3.97 (m, 2, J=137.1 Hz, H3), 3.89 (m, 2, H5) (Figure 4.2a). $^{13}$C NMR (75 MHz, D$_2$O, MeOH at 50 ppm): δ 56.1 (TEAB), 53.4 (s, C3), 17.3 (TEAB) (Figure 4.2b).

Enzymatic Synthesis of [4- $^{13}$C] AIR. In a final volume of 50 mL at 20°C, the reaction mixture contained: 100 mM Tris (pH 7.9), 10 mM MgCl$_2$, 200 mM KCl, 30 mM Gln, 3.16 mM [3- $^{13}$C] FGAR (159 μmol), 1.8 mM ATP, 10.5 mM PEP, 16 U FGAR-AT, 15 U AIR syn, and 200 U PK. The reaction was allowed to proceed for 20 min and the mixture was diluted to 500 mL with de-ionized water at 4°C, and then loaded onto a DEAE Sephadex A-25 column (10 x 2.5
cm, HCO$_3^-$ form). The column was developed with a linear gradient from 0 to 500 mM TEAB (pH 7.6, 500 x 500 mL). An elution profile of the fractions (11 mLs) was generated by monitoring at A250 nm and by the Bratton-Marshall assay (Figure 4.3). The appropriate fractions containing AIR (fractions 43-50, at 250 mM TEAB) were pooled. To facilitate removal of TEAB, the sample was repeatedly dissolved in water and methanol and concentrated in vacuo. The solution was kept basic with addition of 1N KOH. In the later stages of TEAB removal, the sample was not concentrated completely to dryness and was enzymatically monitored for decrease in bicarbonate. The upper limit for $^{12}$C bicarbonate ranged from 12-73 µmol. An average yield (from eight preparations) for this reaction was 30%, and it was characterized with $^1$H and $^{13}$C NMR spectroscopy: $^1$H NMR of [4- $^{13}$C] AIR (D$_2$O, pH 8.0, TSP at 0.0 ppm): δ 7.9 (d, J=7.6 Hz, H2), 6.5 (d, J=195 Hz, H4), 5.75 (d, J=4.9, H1), 4.65 (t, H2), 4.53 (t, H3), 4.42 (m, H4), 3.97 (m, H5). $^{13}$C NMR (75 MHz, D$_2$O, MeOH at 50 ppm): δ 113.6 (s, C4).

**Enzymatic Synthesis and Isolation of [4- $^{13}$C] N$^5$-CAIR Synthesis.** In a final volume of 10 mL, a 15 mL Falcon tube contained: 200 mM Tris (pH 8.0), 12 mM MgCl$_2$, 60 mM KCl, 4.5 mM ATP, 10 mM PEP, 50 µmol [4- $^{13}$C]AIR, 156 U PurK, and 200 U PK. The solution was equilibrated for 5 min in a 20°C water bath. Solid bicarbonate, [$^{13}$C] NaHCO$_3$ (99.0%), was added as a fine powder to the reaction mixture to produce a final concentration of 120 mM. An excess of labeled bicarbonate was added to dilute the unlabeled bicarbonate (upper limit of 10% $^{12}$C) originating from AIR isolation. After dissolving the labeled bicarbonate, the reaction was initiated by the addition of PurK. After 50 sec at 20°C, the reaction was quenched with 1 mL of 10 M KOH (20°C). The reaction mixture was then diluted with 300 mL of 10 mM KOH. This was loaded onto a Dowex 1-X8 column (7 mL, OH form), and the column was
developed with a linear gradient from 0 to 300 m KCl (250 x 250 mL in 50 mM TEA, 4.5 mL fractions). The TEA solution (pH 11.4) was prepared and used immediately without adjusting the pH. An elution profile was generated by monitoring A240, A250 and for Bratton-Marshall positive material. When applicable, 0.5 mL from every fifth fraction was assayed for radioactivity in the scintillation counter. In a typical reaction, AIR eluted in fractions 47-53, while N5-CAIR eluted in fractions 60-66 (Figure 4.4). To maintain alkaline conditions, 300 μL of 2 N KOH was added to the pooled fractions, and the sample was concentrated *in vacuo* to obtain a 50% yield. The presence of high concentrations of salt prevents rechromatographing either N5-CAIR or AIR on an anion exchange column (can use a BioRad affinity column - Affi-Gel 601). The N5-CAIR was typically stored as a solution (pH >12) at -80°C. The purity of the sample was determined with NMR spectroscopy: 1H NMR (300 MHz, TSP, D2O, pD 14): δ 7.93 (d, 1, J=8.5 Hz, C2H), 6.84 (d, 1, J=191.6 Hz, C4H), 5.49 (d, 1, J=4.92 Hz, H1), 4.4 (m, 1, H2), 4.3 (m, 1, H3), 4.21 (m, 1, H4), 3.91 (m, 2, H5) (Figure 4.6). 13C NMR (75 MHz, MeOH at 50 ppm, D2O, pD 14): δ 169.6 (CO3=), 163.8 (s, C7), 124.6 (s, C4) (Figure 4.7).

**Methanol Extraction of N5-CAIR.** The KCl and KHCO3 in the N5-CAIR sample can be removed using the methanol extraction procedure described below. The N5-CAIR and salt solution was evaporated to dryness in a round bottom flask and placed in the cold room. The resulting solid was triturated with 4 mL of HPLC grade methanol at 4°C. The salts were then allowed to settle and the sample was centrifuged in an Eppifuge for 20 s in 1.5 mL Eppendorf tubes. The supernatant was then transferred into a new round bottom flask. The methanol extraction of the salts was repeated four times. To insure basic conditions, 25 μL of 1N KOH was added to the MeOH/N5-CAIR mixture. The combined methanol fractions were pooled and the
solvent was then removed *in vacuo*. A 10% loss of N⁵-CAIR during this desalting process was based on the Bratton-Marshall; while, the concentrations of salts was greatly reduced (final concentrations of KHCO₃⁻ was 50-100 mM).

**Enzymatic Conversion of [4,7⁻¹³C] N⁵-CAIR to [4,1⁻¹³C] SAICAR.** In a final volume of 5 mL at 20°C, the reaction mixture contained: 200 mM Tris (pH 8), 10 mM KCl, 10 mM MgCl₂, 14 mM Asp, 1.62 mM ATP, 4.0 mM PEP, 250 U PK, 132 U PurC, 11.3 U PurE (0.07 µmol), and 0.92 mM [4,7⁻¹³C]N⁵-CAIR. A sample of the reaction mixture without enzyme (45 µL) was mixed with N⁵-CAIR (5 µL) to determine the final reaction pH with pH paper. The buffer was then adjusted accordingly, so that the final reaction mixture had a pH of 8. The reaction mixture with enzymes was initiated by addition of N⁵-CAIR, and was quenched after 141 sec with 1 mL of 13 N KOH. The mixture was diluted with 300 mLs of 10 mM KOH at 4°C, and then loaded onto a Dowex 1-X8 column (8 mL, OH⁻ form). The column was developed with a linear gradient from 0 to 500 mM KCl (300 x 300 mL, 50 mM TEA, pH 11). An elution profile was generated by monitoring A₂₆₈ and A₂₆₀ (Figure 4.12). Fractions (79-96) containing SAICAR were pooled so as to minimize the amount of ATP. The sample was concentrated *in vacuo* without methanol, and exchanged with D₂O for analysis with NMR spectroscopy (yield 60%): ¹H NMR (300 MHz, 0.8 mM, TSP, D₂O, pD 6): δ 7.56 (d, 1, J=10.5 Hz, C2H), 5.72 (d, 1, J=6.5 Hz, H1). (The other resonances were obscured by ATP and TEAB.) ¹³C NMR (75 MHz, MeOH at 50 ppm, D₂O, pD 14): δ 166.26 (d, J=84.19 Hz, C1"), 161.2 (CO₃⁻), 113.54 (d, J=84.19 Hz, C4), (Figure 4.8).

**Carbon T₁ Relaxation Determinations.** Using the inversion-recovery method and a Varian 300 MHz NMR equipped with a broad band probe at 75.429 MHz, the resonance intensities were fit to Eq. 1 for an exponential least-
squares analysis to determine the longitudinal relaxation values (T1) for N5-CAIR (Varian, 1987).

\[ M_z = M_o \frac{1 - e^{-(d_2/T_1)}}{1 - e^{-(d_2/T_1)} \cos \alpha} \]  
Equation 1

\( M_z \) - steady state z magnetization, \( M_o \) - equilibrium magnetization, \( T_1 \) - longest longitudinal relaxation value, \( \alpha \) - flip angle, \( d_2 \) - interval between pulses

First, all nuclei were allowed to relax to equilibrium (stage: \( d_1 \)), second, the nuclei were inverted with a 180° pulse (\( p_1 \)); third, the nuclei were allowed to equilibrate for variable times (\( d_2 \)); and finally, the nuclei were monitored with a 90° pulse (\( p_w \)) to measure their peak height as a function of \( d_2 \). (Scheme 4.5). (Under most circumstances, the resonance peak height varies exponentially with \( T_1 \), Eq. 1). The parameters were as follows: 6.8 mM \([4,7-^{13}C] N^5\text{-CAIR} \) (pH 12), decoupler sequence = nny (the sample was proton decoupled only during acquisition), number of transients = 168, temperature = 17-23°C, pulse width \( p_w \) = 12 µsec, first pulse width = 24 µsec, first delay \( d_1 \) = 60 sec, second delay \( d_2 = \tau \) (array - 0.469, 0.938, 1.875, 3.750, 7.50, 15, 30, and 60 sec), (Figure 4.10).

Scheme 4.5: Two-Pulse Sequence Diagram for Inversion Recovery
\( d_1 \)- equilibration time, \( p_1 \)- 180° pulse width, \( d_2 \)- recovery time, \( p_w \)- - 90° pulse width, \( AT \)- signal accumulation, \( NT \)- number of transients.
For SAICAR, the same method was utilized to determine the T1 values as described above for N5-CAIR. A solution of SAICAR (181 mM) at pH 7.0 was analyzed by NMR using the following parameters: decoupler sequence = nny, number of transients = 144, temperature = 17-23°C, pulse width pw = 12.2 μ sec, first pulse width = 24.4 μ sec, first delay d1 = 32 sec, and second delay d2 = τ (array - 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 sec) (Figure 4.11).

**Enzymatic Synthesis of [7- 14C] N5-CAIR with 14C bicarbonate by PurK.** In a final volume of 5 mL at 20°C, a 15 mL Falcon tube contained: 200 mM Tris (pH 8.0), 6 mM MgCl2, 20 mM KCl, 5 mM ATP, 10 mM PEP, 1.35 μmol AIR, 92 U PurK, and 125 U PK. In the hood, a granule of [14C] NaHCO3 (NEC-086, 0.25 GBq/mmol, 6.8 mCi/mmol from NEN) was transferred with a glass micro cap into the reaction mixture. The reaction was initiated with PurK and allowed to proceed for 30 sec at 20°C. The reaction was quenched with 1 mL of 10 M KOH. The previously described isolation procedure for [4,7-13C] N5-CAIR with anion exchange chromatography and methanol extraction were used to purify [7-14C] N5-CAIR (Figure 4.5). To prevent the loss of the label during scintillation counting, [14C] N5-CAIR was diluted into 1 mL of 140 mM KOH before addition of the scintillation fluid. The N5-CAIR was quantitated using the Bratton-Marshall assay. (Note: the first step of the Bratton-Marshall assay decomposes N5-CAIR to AIR). Typical specific activities were from 1.1x10^6 to 1.67x10^6 cpm/μmol. With success in collecting pure [4,7-13C]-N5-CAIR, an NMR was not taken of the 14C compound.

**Synthesis of [14C] SAICAR from [14C] N5-CAIR.** In a final volume of 5 mL at 20°C, the reagents were added in the following order: 200 mM Tris HCl (pH 8, from an initial pH 7.3), 20 mM KCl, 5.2 mM MgCl2, 1 mM ATP, 3 mM PEP, 7 mM Asp, 11.6 U PurE, 123 U PurC, 250 U PK, and 0.27 mM [14C] N5-CAIR
(1.17x10^6 cpm/μmol, impurities: 5.0 mM HCO_3^-, 260 mM KCl). The effect of \textit{N}^5-CAIR on the final pH was taken into consideration prior to the actual experiment (see previously described experiment). The reaction was quenched after 35 seconds with 1 mL of 13.5 M KOH. The mixture was diluted to 300 mL with 10 mM KOH at 4°C, and loaded onto a Dowex 1-X8 column (7 mL, OH^- form). The compounds were eluted with a linear gradient from 0 to 500 mM KCl (320 x 320 mL, 50 mM TEA, pH 11.4) (Figure 4.13). An elution profile was generated by monitoring \textit{A}_{268}, \textit{A}_{260}, and by scintillation counting of every fifth fraction (0.5 mL). Based on relative specific activities, the [^{14}C] SAICAR was pooled, and then rechromatographed on a DEAE A-25 column (6 mL, HCO_3^- form) to separate ATP. SAICAR was eluted with a linear gradient from 0 to 750 mM TEAB (pH 7.5, 200 x 200 mL), pooled and then concentrated \textit{in vacuo} (Figure 4.14). The specific activity was determined with repeated absorbance spectra of SAICAR in 100 mM Tris HCl (pH 8.0) and with scintillation counting.

Results and Discussion

\textit{Synthesis of Labeled \textit{[4-}{^{13}C}\textit{]} AIR}. The first three enzymes in the purine pathway (PRPP-AT, GAR syn, and GAR-TF) were used to incorporate [2-^{13}C] glycine into [3-^{13}C] FGAR (Scheme 4.4), that was purified using anion exchange chromatography (Figure 4.1). The fractions containing [3-^{13}C] FGAR were pooled and concentrated \textit{in vacuo} (50% yield, see NMR Figure 4.2). The next two enzymes in the purine biosynthetic pathway (FGAR-AT and AIR syn) were then used to convert [3-^{13}C] FGAR to [4-^{13}C] AIR (Scheme 4.4). The resulting [4-^{13}C] AIR was purified using anion exchange chromatography, and the appropriate fractions were pooled and concentrated \textit{in vacuo} (30% yield). The \textit{1H} and \textit{13C} NMR spectra were used to establish the
position of the $^{13}$C label at C4 of AIR as well as its purity (Figure 4.3). Longer reaction times in the second series of enzymatic transformations resulted in a compound which elutes subsequent to AIR during the purification. The compound had an absorption spectrum similar to AIR, and a Bratton-Marshall assay absorption spectrum similar to CAIR ($\lambda_{\text{max}}$ at 520 nm, while AIR is at 502 nm). Its identity remains to be established.

**Enzymatic Synthesis of [$^{13}$C or $^{14}$C] Labeled N5-CAIR.** Utilizing [$^{13}$C or $^{14}$C] bicarbonate, PurK can be used to enzymatically label the carbamate of N5-CAIR (Scheme 4.1). The reaction variables (50 sec with 156 Units of PurK) were chosen to maximize formation of N5-CAIR and minimize its decomposition ($k=.23 \text{ min}^{-1}$, $t_{1/2}=3 \text{ min}$, Chapter 3). Alkaline conditions (pH 12) must be insured during its purification and storage to reduce its decarboxylation to AIR ($k=0.00144 \text{ min}^{-1}$, $t_{1/2}=8 \text{ h}$, pH 12, 20 °C). Typically for isolation of N5-CAIR, the reaction mixture was loaded onto a Dowex 1-X8 exchange column, and then eluted with a KCl gradient in 50 mM TEA (Figure 4.4, Figure 4.5). Under these conditions, the bicarbonate utilized during synthesis elutes prior to N5-CAIR. The presence of bicarbonate in the purified materials results from the well known fact that basic solutions rapidly absorb CO$_2$ from the atmosphere. Even with many precautions, some contaminating bicarbonate is always present in the product at the end of the purification sequences (Figure 4.7). After the appropriate fractions were identified, pooled and concentrated in vacuo, N5-CAIR was extracted with methanol to reduce the contaminating salts, KCl and KHCO$_3$. The methanol extraction takes advantage of the higher solubility of N5-CAIR in methanol, which allows removal of a substantial amount of the salt from the N5-CAIR sample. Overall yields for N5-CAIR were typically 30% (NMR - Figure 4.6, Figure 4.7). The low yields were from AIR decomposition prior to
Figure 4.1: Isolation of [3-^{13}C]FGAR Using DEAE Sephadex A-25 Chromatography.
The column was developed with a TEAB gradient, and the elution profile (♦) was determined using the Orcinol assay by the method of Dische (1962)
Figure 4.2a: $^1$H NMR Spectrum of [3-$^{13}$C] $\beta$-FGAR. $^1$H NMR (300 MHz, D$_2$O, TSP at 0.0 ppm, pH 8.0). The coupling constants were as follows: C3H (137.1 Hz), formyl (5.7 Hz).
[3-\textsuperscript{13}C] FGAR
Figure 4.2b: $^{13}$C NMR Spectrum of [3-$^{13}$C] $\beta$-FGAR. $^{13}$C NMR (300 MHz, D$_2$O, MeOH at 50 ppm, pH 8.0).
[3-\textsuperscript{13}C] FGAR
Figure 4.3: Isolation of [4-^{13}C] AIR with a DEAE Sephadex A-25 Chromatography. The column was developed with a TEAB gradient, and the profile was determined using the Bratton-Marshall assay (♦) and absorption at 250 nm (○). AIR eluted in fractions 43-50.
Figure 4.4: Isolation of [4,7-^{13}C]N^5-CAIR by Dowex 1-X8 Chromatography. The column was developed with a KCl gradient, and the elution profile was determined using the Bratton-Marshall assay (♦) and absorption at 250 nm (●). AIR eluted in fractions 47-53 and N^5-CAIR in fractions 61-69.
Figure 4.5: Isolation of $[^{14}C]$-$N^5$-CAIR by Dowex 1-X8 Chromatography. The column was developed with a KCl gradient, and the elution profile was determined using the Bratton-Marshall assay ($\mathbf{\star}$), absorption at 250 nm (○) and radio activity (○). Labeled bicarbonate eluted in fractions 20-40, AIR eluted in fractions 41-50, and $[^{14}C]$-$N^5$-CAIR eluted in fractions 51-63.
Figure 4.6: $^{1}$H NMR Spectrum of [4,7-$^{13}$C]$^{N5}$-CAIR. (300 MHz, TSP, D$_2$O, pD 14, 11 mM). The coupling constants were as follows: C2H (8.5 Hz), C4H (191.6 Hz).
Figure 4.7: $^{13}$C NMR Spectrum of [4,7-$^{13}$C]N$^5$-CAIR (75 MHz, MeOH at 50 ppm, D$_2$O, pD 14).
Figure 4.8: $^{13}$C NMR Spectrum of [4,1"-$^{13}$C] SAICAR. The coupling constant was 84.4 Hz. $^{13}$C NMR (D$_2$O, MeOH at 50 ppm) $\delta$ 166.3 (s, C6), 161.1 (s, HCO$_3^-$), 113.7 (s, C4).
synthesis. The purity of the sample was established with $^1$H NMR spectroscopy, since the aromatic and C1 protons of AIR and CAIR have different chemical shifts from those of $N^5$-CAIR (Table 3.2, Figure 4.6). Of some consideration in analysis of [4,7- $^{13}$C] $N^5$-CAIR was utilization of glycine and HCO$_3^-$ which was 99% [$^{13}$C] enriched. This results in a distribution of labels: 98% ($^{13}$C:$^{13}$C), 1% ($^{13}$C:$^{12}$C) and 1% ($^{12}$C:$^{13}$C).

Following the synthesis of [7- $^{14}$C ] $N^5$-CAIR, the specific activity was determined with the Bratton-Marshall assay in conjunction with scintillation counting (1-1.6 x 10$^6$ cpm/μmol). Quantitation with the Bratton-Marshall assay is problematic since the first step in this procedure decarboxylates $N^5$-CAIR to AIR before diazotization. Thus, contaminating AIR over estimates the concentration of $N^5$-CAIR and decreases the measured specific activity. Careful attention to pH during the isolation of $N^5$-CAIR can reduce contaminating AIR to less than 5% (by NMR analysis) of the total sample.

*Summary of $^{13}$C-NMR.* The $^{13}$C-chemical shifts and coupling constants of intermediates isolated during the synthesis of $N^5$-CAIR and SAICAR are summarized in Table 4.1.

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<th>Ref</th>
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<td>p. 228$^a$</td>
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Table 4.1: Coupling Constants Observed from the Two Labeled Carbons

$^a$(Hunsen, 1981), $^b$(Marshall, 1983)
Integration Parameters for $^{13}$C Spectra of [4,7-$^{13}$C] N$^5$-CAIR and [4,1"-$^{13}$C] SAICAR. Accurate NMR integration of the appropriate $^{13}$C-resonance of starting material and product requires an acquisition delay three to five fold greater than the longest longitudinal relaxation time (T1) (Derome, 1987). Therefore, to obtain meaningful integration of $^{13}$C spectrum required to determine the stoichiometry during the transfer of the C7 carbamate of [4,7-$^{13}$C] N$^5$-CAIR to [4,6-$^{13}$C] CAIR, the T1 of each of these carbons was determined. Using the inversion recovery method, the T1 of C7 of N$^5$-CAIR was determined to be 12.6 sec (Table 4.2, Figure 4.10). The T1s for C1" and C4 of SAICAR were 3.2 and 4.6 sec, respectively (Table 4.3, Figure 4.11). For integration of N$^5$-CAIR, an acquisition delay of 60 sec was used (Figure 4.7), while SAICAR a delay of 23 sec (Figure 4.8) was used. The methanol reference was effected by the conditions used in the experiment, and resulted in different T1 values.

<table>
<thead>
<tr>
<th>Carbon</th>
<th>T1 (sec)</th>
<th>ppm</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.53±0.04</td>
<td>124.6</td>
<td>71</td>
</tr>
<tr>
<td>7</td>
<td>12.58±0.139</td>
<td>163.8</td>
<td>150</td>
</tr>
<tr>
<td>CO$_3^-$</td>
<td>51.2±5.23</td>
<td>169.6</td>
<td>26.3</td>
</tr>
<tr>
<td>MeOH</td>
<td>13.52±0.30</td>
<td>50.0</td>
<td>70</td>
</tr>
</tbody>
</table>

Table 4.2: Carbon T1s for N$^5$-CAIR. (Least Square Analysis of Figure 4.10)

<table>
<thead>
<tr>
<th>Carbon</th>
<th>T1 (sec)</th>
<th>Published</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.317±0.018</td>
<td>0.16</td>
</tr>
<tr>
<td>4</td>
<td>4.623±0.127</td>
<td>5.3</td>
</tr>
<tr>
<td>5</td>
<td>3.243±0.163</td>
<td>4.7</td>
</tr>
<tr>
<td>1&quot;</td>
<td>3.246±0.238</td>
<td></td>
</tr>
<tr>
<td>3&quot;</td>
<td>0.418±0.065</td>
<td></td>
</tr>
<tr>
<td>4&quot;</td>
<td>0.240±0.038</td>
<td></td>
</tr>
<tr>
<td>5&quot;</td>
<td>3.070±0.118</td>
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</tr>
<tr>
<td>6&quot;</td>
<td>3.070±0.118</td>
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</tr>
<tr>
<td>MeOH</td>
<td>9.99±0.58</td>
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</table>

Table 4.3: Carbon T1s for SAICAR. (Least Square Analysis of Figure 4.11)
Published T1s are from adenosine-5-monophosphate (Levy et al., 1980).

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This data thus set the stage for analysis of the PurE catalyzed conversion of \( N^5\)-CAIR to CAIR as described subsequently.

*Enzymatic Catalyzed Conversion of \([4, 7-^{13}C]\) \( N^5\)-CAIR to \([4, 1''-^{13}C]\) SAICAR.* To address the question of whether the "CO\(_2"\) from the carbamate of \( N^5\)-CAIR is transferred directly to C4 to produce \([4,6-^{13}C]\) CAIR, without exchanging with the CO\(_2\) in solution, \([4,7-^{13}C]\) \( N^5\)-CAIR was incubated with PurE. The reaction was carried out in the presence of PurC, ATP and aspartate to trap the CAIR as SAICAR. This is an essential procedure since the PurE catalyzed reaction is reversible (Scheme 4.1 and 4.3). The number of units of PurE and PurC were chosen to minimize the non-enzymatic decomposition of \( N^5\)-CAIR. With 4.6 \( \mu \text{mol} \) \([4,7-^{13}C]\) \( N^5\)-CAIR and 11.3 units (\( \mu \text{mol/min} \)) PurE, the conversion was complete after 24 sec, limiting the amount of non-enzymatic decarboxylation of \( N^5\)-CAIR to 13% (0.6 \( \mu \text{mol} \)) (\( k=0.227 \) min\(^{-1}\), \( t_{1/2}=3 \) min).

The \([4,1''-^{13}C]\) SAICAR generated was purified by anion exchange chromatography (Figure 4.12). The acquisition delay of 23 sec allowed for integration of the \( ^{13}C \) spectrum and analysis of the amount of \( ^{13}C \) transferred. If \( ^{12}C \) from CO\(_2\) in solution is incorporated during this conversion, then the \( ^{13}C \)-resonance appears as a singlet at 113.5 ppm. On the other hand if \( ^{13}C \) from the carbamate is transferred to C4, the C4 carbon appears as a doublet with resonance of 113 and 114.1 ppm (\( J_{c-c} = 84.2 \) Hz). By integration of the relative intensities of these lines, the amount of direct transfer can be quantitated. From two separate reactions, the integration analysis reveal that 97% retention of the label during this enzymatic process (Table 4.4). This result establishes that the 4-carbonyl of CAIR originated from \( N^5\)-CAIR. Loss of this label during the trans-carboxylation reaction would require greater than 3% \( ^{12}C \) at C1 of SAICAR to be observed above the NMR spectrum noise. Thus no exchange with \( ^{12}C \) bicarbonate/carbon dioxide from solution was not detected during the PurE catalyzed reaction.
Quantitation of the [4,1"-14C] SAICAR. As a control for the previously described $^{13}$C experiment using NMR spectroscopy, a similar experiment was designed with [7-14C] N⁵-CAIR. The specific activity of [7-14C] N⁵-CAIR was determined using the Bratton-Marshall assay and scintillation counting. The conversion of N⁵-CAIR to SAICAR was carried out to minimize its nonenzymatic decomposition to CAIR. With 1.35 µmol [7-14C] N⁵-CAIR and 11.6 units (µmol/min) PurE, the reaction was complete after 7 sec, allowing for only 4% (0.05 µmol) of the starting material to decarboxylate non-enzymatically ($k=0.227\ \text{min}^{-1}, t_{1/2}=3\ \text{min}$). As in the case of the $^{13}$C experiment, the specific activity of [7-14C] N⁵-CAIR was estimated to be inaccurate by about 10%, due to nonenzymatic decomposition to AIR.

Anion exchange chromatography was problematic since the elution profile of SAICAR and ATP overlapped (Figure 4.13), and their separation is essential for accurate quantitation. The two species have similar UV spectra and extinction coefficient (ATP - $\varepsilon_{260} = 15,400\ \text{M}^{-1}\text{cm}^{-1}$, SAICAR - $\varepsilon_{268} = 13,300\ \text{M}^{-1}\text{cm}^{-1}$), making quantitation of SAICAR difficult (chapter 2).

The [14C] SAICAR was identified by scintillation counts of appropriate fractions and by its UV absorption spectrum. A spectrum of isolated [14C] SAICAR is shown in Figure 4.15, and has more end-absorption then the previously isolated SAICAR (Figure 2.11). With contaminating ATP, the specific activity of SAICAR was

<table>
<thead>
<tr>
<th>[4,7-13C] N⁵-CAIR</th>
<th>C4</th>
<th>C7</th>
<th>[4,1&quot;-13C] SAICAR</th>
<th>C4</th>
<th>C1&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100</td>
<td>98</td>
<td>A</td>
<td>99</td>
<td>100</td>
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<tr>
<td>B&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
<td>97.2</td>
<td>B&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
<td>98.6</td>
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</tbody>
</table>

Table 4.4: $^{13}$C NMR Integration of Starting Material ([4,7-13C] N⁵-CAIR) and Product ([4,1"-13C] SAICAR from the Carbamate Transfer Experiment.

<sup>a</sup> - calculations from Figure 4.7 and Figure 4.8.
Figure 4.10: Inversion Recovery for [4,7-\textsuperscript{13}C] N\textsuperscript{5}-CAIR. Using the double labeled sample of [4,7-\textsuperscript{13}C] N\textsuperscript{5}-CAIR, the longitudinal relaxation times were determined with the inversion recovery method. The array of plots correspond to the delay \(d2\) in acquiring the spectra following the 180\(^\circ\) pulse. The relative intensity of each resonance was subsequently fit to Eq. 1, for a least squares analysis, to determine the T\text{I} value. From the spectrum \textsuperscript{13}C NMR (75 MHz, MeOH at 50 ppm, D\textsubscript{2}O, pD 14) the resonances of interest were: \(\delta\) (A) 163.8 ppm (C7), (B) 124.6 (C4), with methanol at 50 ppm and HCO\textsubscript{3}\textsuperscript{-} at 169 ppm.
Figure 4.11: Inversion Recovery for SAICAR. Using natural abundance sample of SAICAR, the longitudinal relaxation times were determined with the of inversion recovery method. The array of plots correspond to the delay ($d_2$) in acquiring the spectra following the $180^\circ$ inversion pulse. The relative intensity of each resonance was subsequently fit to Eq. 1, for a least squares analysis, to determine the $T_1$ value. From the spectrum $^{13}$C NMR (75 MHz, MeOH at 50 ppm, D$_2$O, pD 7) the resonances of interest were: δ (A) 166.26 ppm (C1"), (B) 113.54 (C4), with methanol at 50 ppm.
determined (Table 4.5) and compared with that of the starting material. The results of two separate studies revealed that 50% of the label was lost during the PurE catalyzed reaction. This result is not consistent with the $^{13}$C-experiment described above. The basis for the discrepancy, the difficulties in quantitating $N^5$-CAIR and SAICAR, have been discussed (chapter 2 and 3), and errors in their quantitation are the most reasonable source of discrepancy between these methods. In the case of $N^5$-CAIR, the specific activity could be higher than reported, as it decarboxylates to AIR which is also detected in the Bratton-Marshall assay. Thus this error would make the discrepancy between the starting material and product larger than that reported on Table 4.5.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Sample</th>
<th>cpm/μmol</th>
<th>% S.A.</th>
<th>μmol (recovered)</th>
<th>Method</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>$N^5$-CAIR</td>
<td>1.17x10^6</td>
<td>1.31</td>
<td>1.31</td>
<td>B-M</td>
</tr>
<tr>
<td></td>
<td>SAICAR</td>
<td>0.75x10^6</td>
<td>64</td>
<td>0.62</td>
<td>$\varepsilon=13300$</td>
</tr>
<tr>
<td>2</td>
<td>$N^5$-CAIR</td>
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<td>1.33</td>
<td>1.33</td>
<td>B-M</td>
</tr>
<tr>
<td></td>
<td>SAICAR</td>
<td>0.64x10^6</td>
<td>50</td>
<td>1.12</td>
<td>$\varepsilon=13300$</td>
</tr>
</tbody>
</table>

Table 4.5: Transfer of the $^{14}$C label from $N^5$-CAIR to SAICAR. % S.A. - specific activity of product relative to starting material.

<table>
<thead>
<tr>
<th>Column</th>
<th>Total cpm</th>
<th>recovered μmol</th>
<th>cpm/μmol</th>
<th>% recovery from previous step</th>
</tr>
</thead>
<tbody>
<tr>
<td>Load $a$</td>
<td>1,405,118</td>
<td>1.33 $c$</td>
<td>1.11</td>
<td>-</td>
</tr>
<tr>
<td>Dowex 1-X8 $b$</td>
<td>905,856</td>
<td>1.32 $d$</td>
<td>0.69</td>
<td>70%</td>
</tr>
<tr>
<td>A-25 $b$</td>
<td>732,000</td>
<td>1.12 $d$</td>
<td>0.65</td>
<td>81%</td>
</tr>
</tbody>
</table>

Table 4.6: Recovery of $[^{14}$C] SAICAR through two columns.

Thus the most likely source of error results from our inability to obtain a good specific activity measurement for SAICAR. As described above, SAICAR and ATP overlap or purification with a Dowex 1-X8. A variety of conditions were tried to remove the ATP (Figure 4.11). Repurification of
SAICAR through a Sephadex DEAE-A25 column allowed better resolution of ATP and SAICAR (Figure 4.13). The specific activity of the SAICAR; however, instead of increasing as expected due to the removal of contaminating ATP remained the same (Table 4.6). While it was not anticipated that SAICAR would decompose during this repurification, another contaminant must copurify (for example the \( N^5 \)-CAIR/AIR decomposition compound - Figure 4.5, fraction 70).

The best way to separate SAICAR from ATP would be to remove their phosphates using alkaline phosphatase and then effect their separation via an anion exchange column. This experiment needs to be repeated.

An additional source of error during the \(^{14}\text{C}\) experiment was in the activity of the PurE. Originally, PurE activity monitoring CAIR formation via PurC in 180 mM bicarbonate and AIR. However, later observation revealed that AIR inhibits the reaction of PurE. This results in an incorrect amount of PurC coupling enzyme was calculated, and thus the possibility for the reverse reaction. The assay for PurE activity was determined with \( N^5 \)-CAIR for the \(^{13}\text{C}\) experiments.
Figure 4.12: Isolation of $[4,1^\text{t}. \ ^{13}\text{C}]$ SAICAR by Dowex 1-X8 Chromatography. The column was developed with a KCl gradient, and the elution profile was determined using absorption at 260 nm (◊) and 268 nm (♦). SAICAR eluted in fractions 80-96.
Figure 4.13: Isolation of $^{14}$C SAICAR with Dowex 1-X8 chromatography. The column was developed with a KCl gradient, and the elution profile was determined using absorption at 268 nm (◊) and cpm (♦). Fractions 78 to 105 were pooled. The radioactive peak at fraction 15 correlated to 10% decomposed $[^{14}$C]$N^5$-CAIR, and the peak at fraction 125 is ATP.

Figure 4.14: Isolation of $^{14}$C SAICAR with DEAE Sephadex A-25 Chromatography. The column was developed with a TEAB gradient, and the profile was determined using absorption at 260 nm (◊) and 268 nm (♦). Fractions 50 to 70 were pooled based on SAICAR to ATP ratio.
Figure 4.15 Absorption of $[^{14}\text{C}]$ SAICAR. A spectrum of the isolated SAICAR was taken in 50 mM HEPES (pH 7.9).
Conclusion

In trying to elucidate the mechanism of PurE, specific labels were incorporated in the substrate to follow their fate in the conversion to product. Using PurK, N5-CAIR was synthesized with either [13C or 14C] bicarbonate, AIR and ATP to place the label at the C7 carbamate. Because of the reversibility of PurE and non-enzymatic decarboxylation of CAIR, the product of the PurE catalyzed reaction was coupled with PurC to SAICAR which was then quantitated and characterized.

The question being addressed was to determine if the carbon of the N5-CAIR carbamate was transferred directly to the C4 of the same molecule of N5-CAIR or did the carbon exchange with CO2 in the media during the conversion? A double labeled 13C experiment was carried out to address this question. [4- 13C] AIR was synthesized using enzymes from the purine biosynthetic pathway and [2- 13C] glycine. N5-CAIR was then also made biosynthetically with PurK and integration of the carbon resonances revealed equivalent label incorporation into [4,7- 13 C] N5-CAIR: 100% in C4 and 97% C7. Using [4,7- 13 C] N5-CAIR, PurE catalyzed reaction was analyzed by trapping the CAIR produced with PurC. NMR analysis of SAICAR revealed a ratio of 100% C4 and 98% C1". This label was completely retained in N5-CAIRs conversion to CAIR.

These results are consistent with several mechanistic possibilities for PurEs conversion of N5-CAIR to CAIR, as outlined in the introduction (Scheme 4.2). The first proposal involves a concerted reaction involving a four member ring transition state with double bond character. The ring strain would make this transformation highly unlikely. In the second proposal, the substrate decarboxylates in the active site to form AIR and carbon dioxide. This creates a high, local concentration of carbon dioxide in the active site, which can then react at C4. As
measured by (Schendel Thesis), the C4 proton has an exchange rate at pH 7.2 of 0.09 min\(^{-1}\), so instead of exchanging a proton, nucleophilic attack could occur on an electrophilic carbon dioxide. This mechanism also has experimental support. In previous studies of Alenin et al. (1987) and Mueller et al (1994), NMR analysis of non-enzymatic AIR carboxylation in high concentrations of bicarbonate, it was observed that \(N^5\)-CAIR forms very rapidly (the kinetic product) while CAIR forms at a much slower rate (the thermodynamic product). The extent of the reaction was dependent on the concentration of bicarbonate or CO\(_2\). The commonality of the proposed enzymatic mechanism and this chemical example is the concentration of CO\(_2\); however, this mechanisms would also suggest that the free CO\(_2\) could dissociate from the active site, equilibrate with the carbon dioxide pool in solution and possibly dilute the label in both \(^{13}\)C, \(^{14}\)C experiments. No loss of label was detected and thus if this reaction occurs, the trapping of active site CO\(_2\) is very efficient. This is a very appealing model given the results of Firestein et al (1994) with avian PurE. In the case of eukaryotic systems (chapter 3) no PurK is apparent and CO\(_2\) appears to be a substrate for PurE with AIR. Given the easy of decarboxylation of \(N^5\)-CAIR, this is at present the most appealing model.

The third proposed mechanism involves covalent catalysis and has precedence in the reaction catalyzed by ribulose bisphosphate carboxylase, and hemoglobin which form protein bound carbamates. In the case of hemoglobin, the N-terminal valine forms a carbamate as a method to transport carbon dioxide, resulting in decreased affinity \(O_2\) binding (Roughton, 1970). The carbamate of valine has a half life of 2.5 min at 25\(^\circ\)C carbamate (Chipperfield, 1965). In the case of ribulose bisphosphate carboxylase, the enzyme requires activation of a lysine with CO\(_2\) to form a carbamate that is then stabilized with a Mg\(^{2+}\)(Lorimer & Mizioroko, 1980). The half life of this carbamate was 2 days (Donnelly, 1983). Formation of an enzyme bound intermediate is advantageous, since the enzyme stabilizes the carbon dioxide
to prevent diffusion, which then undergoes a nucleophilic attack by C4 of AIR. The last proposed mechanism (4), would require AIR to be present (decomposition of $N^5$-CAIR) and thus a lag in the synthesis of CAIR and loss of the C4 label. A single turnover experiment would be required to eliminate this possibility, or an experiment in which a 1:1 mixture of $^{13}$C:$^{13}$C $N^5$-CAIR and $^{12}$C:$^{12}$C $N^5$-CAIR were converted to SAICAR. This mechanism is unlikely given that the $K_i$ for AIR and the $K_m$ for $N^5$-CAIR are similar. However, PurE could catalyze rapidly the decomposition of $N^5$-CAIR to generate its required AIR. This possibility cannot be ruled out at this time. The most interesting analogy is biotin. Biotin carbamylates a second substrate. Is it possible that $N^5$-CAIR carboxylates a second subunit of AIR. This would be consistent with mechanism 4.

To further elucidate the mechanism of PurE, numerous experiments are possible: trapping the enzyme carbamate with diazomethane, or design a competition experiment between labeled and unlabeled $N^5$-CAIR and $N^5$-CAIR. In scenario 3, a carboxylated enzyme might be generated by incubation with high concentrations of bicarbonate in the reaction mixture, allowing carbon dioxide to carboxylate the putative lysine in the active site, and in turn use AIR as a alternative substrate to form CAIR. The $^{13}$C transfer experiment does not eliminate any of the proposed mechanisms; however, it confirms that the transferred carbon originates from $N^5$-CAIR and does not exchange with CO$_2$ in solution. As for the relative specific activity [$^{14}$C] between $N^5$-CAIR and SAICAR, there were several possible site for improvement: 1) separation of ATP and SAICAR, 2) exact extinction coefficient of SAICAR, 3) stability of SAICAR, and 4) correct enzyme ratios.
References


Appendix A

Does PurE catalyze an Oxygenase reaction or does AIR? An oxygen electrode was used to determine if PurE catalyzed O₂ consumption. This was performed by Dr. Lynn Abell (Schloss Laboratory, Du Pont Experimental Station, Wilmington, DE). In 100 mM Tricine buffer at pH 7.8, 25°C, and 2 mM AIR, the rate of oxygen consumption was 0.7 μM/min (at 0.25 mM O₂). This rate was independent of the amount of PurE (up to 0.6 mg) and PurK. Addition of 10 mM MgCl₂ and/or 2 mM bicarbonate with either PurE and/or PurK did not change the rate of AIR oxidation or modification. In the presence of 200 mM bicarbonate, the rate of oxygen consumption was reduced to 0.4 μM/min, an observation which is consistent with O₂ and CO₂ competing for the same enolate. PurE under a variety of conditions (similar to those used to investigate ribulose bisphosphate carboxylase (Lorimer et al., 1976) (Lorimer & Miziorko, 1980)does not appear to catalyze the oxidation of AIR any faster than the substrate oxidizes in the absence of enzyme, less than 0.015% of the normal reaction.
Appendix B

Alignment of PurEK from Different Sources. Alignment made using the program PILEUP (Feng & Doolittle, 1987) creates a multiple sequence alignment of between the organisms listed below. Positions with identical residues for ten of the sixteen sequences are indicated with a capital letter, and are recorded on the consensus line. The literature source for each sequence is listed below. For the bifunctional enzymes, PurE begins after 430.

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<th>Reference</th>
</tr>
</thead>
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Myco. leprae 151
Synec. sp. 200
Haem. influenzae
Bruc. melitensis
Human
Chicken
Meth. therm.
Meth. smithii
Bruc. melitensis
E. coli
S. pombe
S. cerevisiae
Myco. leprae
Vigna acon.
Bac. subtilis
Haem. influenzae
Consensus

Myco. leprae 201
Synec. sp.
Haem. influenzae
Bruc. melitensis
Human
Chicken
Meth. therm.
Meth. smithii
Bruc. melitensis
E. coli
S. pombe
S. cerevisiae
Myco. leprae
Vigna acon.
Bac. subtilis
Haem. influenzae
Consensus

Myco. leprae 251
Synec. sp.
Haem. influenzae
Bruc. melitensis
Human
Chicken
Meth. therm.
Meth. smithii
Bruc. melitensis
E. coli
S. pombe
S. cerevisiae
Myco. leprae
Vigna acon.
Bac. subtilis
Haem. influenzae
Consensus

Myco. leprae 300
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Haem. influenzae
Bruc. melitensis
Human
Chicken
Meth. therm.
Meth. smithii
Bruc. melitensis
E. coli
S. pombe
S. cerevisiae
Myco. leprae
Vigna acon.
Bac. subtilis
Haem. influenzae
Consensus

Consensus
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<td>Houng, H.S. (1994)</td>
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Appendix C

Construction of plasmid pJS335

Plasmid name: pJS335
Plasmid size: 4741 bp
Constructed by: Tiedeman, A. A. and Smith, J. M.
Construction date: