Studies of the Inactivation of Thymine Hydroxylase by 5-Ethynyluracil

by

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B.S., Chemistry (1994)

Yale College

Submitted to the Department of Chemistry
in Partial Fulfillment of the Requirements for the Degree of
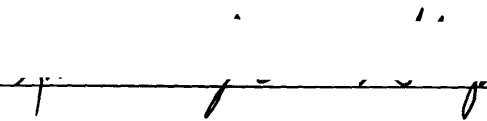Master of Science in Biological Chemistry

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ABSTRACT

The α-ketoglutarate-dependent dioxygenases comprise a large sub-class of the mononuclear non-heme iron metalloenzymes and require ferrous ion, dioxygen, and an α-keto acid for activity. Despite extensive studies undertaken on several enzymes from this class, little is known about either the detailed mechanism or the role of the iron center in catalysis. Investigation of the mechanism of the α-ketoglutarate-dependent dioxygenases in the Stubbe laboratory has focused on the enzyme thymine hydroxylase (TH) purified from *Rhodotorula glutinis*. TH catalyzes the conversion of thymine to its corresponding alcohol, aldehyde, and carboxylic acid in three steps, each accompanied by the conversion of α-ketoglutarate (αKG) to succinate and CO_2. Extensive studies of the enzyme substrate specificity and the reaction kinetics have demonstrated similarities in the chemistry of this system to that of Cytochrome P-450. Spectroscopic characterization of the iron center and its protein environment during turnover will be necessary for the unambiguous identification of the active iron species and elucidation of the detailed mechanism. Since large quantities of homogeneous protein are needed for such studies, the initial focus of this project was the cloning of the TH gene from *R. glutinis* for overexpression of the protein in *Escherichia coli*. Two different approaches have been taken in this work. First, peptide information from TH has been used to design synthetic oligonucleotide primers and probes. These oligomers were used to run PCR reactions and to screen Southern blots of restriction digests of genomic DNA. Second, isolation of mRNA was undertaken for the synthesis of single-stranded cDNA to be used as a template in PCR reactions and for screening in Northern blots.

In addition to isolating TH for peptide sequencing as part of the cloning project, protein was isolated for some preliminary mechanistic studies. It has been demonstrated that reaction of thymine hydroxylase with the mechanism-based inhibitor 5-ethynyluracil results in the inactivation of the protein and the production of two different covalent enzyme-inhibitor adducts in a 3:1 ratio. Characterization of the major adduct was the initial focus of investigation in the laboratory. Characterization of the minor adduct formed during the inactivation has been more difficult because of the smaller quantities of this product available for analysis. Data obtained from ^{1}H- and ^{13}C-NMR spectroscopy, FAB-MS

analysis, and UV spectroscopy of the minor modified adduct suggest that this adduct contains a benzoyluracil moiety. The synthesis of a model compound which mimics the postulated minor adduct structure is presented and a comparison of its structural data with that of the minor enzyme adduct is also presented. A mechanism for the formation of the minor adduct has been proposed. This mechanism predicts that formate should be released during the course of the inactivation. Preliminary work undertaken to test this proposal is presented.

Thesis Supervisor: Professor JoAnne Stubbe
Title: Novartis Professor of Chemistry and Biology

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ABBREVIATIONS

αKG	α-ketoglutarate
β-ME	β-mercaptoethanol
bp	base pair
5-BU	5-benzoyluracil
cDNA	complementary DNA
DEAE	diethylaminoethyl
DEPC	diethyl pyrocarbonate
DMSO	dimethylsulfoxide
dT	deoxythymidine
E. Coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
gDNA	genomic DNA
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HPLC	high pressure liquid chromatography
mRNA	messenger RNA
NMR	nuclear magnetic resonance
OD	optical density
PCIA	phenol: chloroform: isoamyl alcohol [25:24:1]
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonylfluoride
R. glutinis	*Rhodotorul glutinis*
SDS	sodium dodecylsulfate
TAE	tris-acetate/EDTA
TH	thymine 7-hydroxylase (E.C. 1.14.11.6)
TLC	thin-layer chromatography
Tris	tris(hydroxymethyl)aminomethane

Chapter 1

Introduction

The α-ketoglutarate-dependent dioxygenases comprise a large sub-class of the mononuclear non-heme iron metalloenzymes that require ferrous ion, dioxygen, and α-ketoglutarate (αKG) for activity (Figure 1-1). Members of this class have been

$$S_{RED} + HO_2C\overset{O}{\underset{\alpha KG}{\wedge}}\overset{O}{\wedge}OH + O_2 \xrightarrow[Fe^{2+}]{Enzyme} S_{OX} + HO_2C\overset{O}{\wedge}OH + CO_2$$
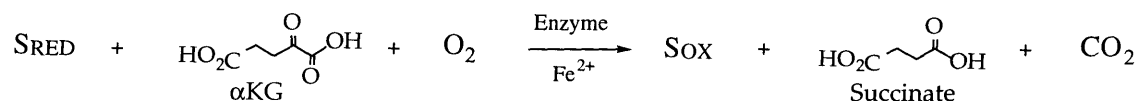
Figure 1-1. General reaction scheme for αKG dependent dioxygenases
(S_{RED} = substrate, S_{OX} = hydroxylated product)

isolated from bacteria, fungi, plants and mammals and are involved in many important biological processes. For example, the αKG dependent dioxygenases play a key role in both collagen biosynthesis (prolyl-4-hydroxylase) and the blood clotting cascade (aspartyl hydroxylase) in mammals, antibiotic biosynthesis in bacteria (deacetoxycephalosporin C synthase), and hormone biosynthesis in plants (gibberellin 20-oxidase) (Figure 1-2). Most of the αKG-dependent dioxygenases catalyze the hydroxylation of unactivated aliphatic substrates, although there also exist examples of desaturation, epoxidation, and rearrangement reactions catalyzed by this class. Dioxygenases incorporate both atoms of O_2 into substrate; in the case of the αKG-dependent dioxygenases one of the atoms ends up in succinate and one in the oxygenated product (or in water in the case of the desaturases).

Despite extensive studies undertaken on several enzymes from this class, little is known about either the details of their mechanisms or the role of the iron center in catalysis. Progress toward understanding the αKG-dependent dioxygenases has been slow due to the difficulties inherent in monitoring the spectroscopically-inaccessible ferrous reaction center. In contrast, the Fe^{3+} heme-

Figure 1-2. Hydroxylation reactions catalyzed by αKG dependent dioxygenases

Prolyl 4-hydroxylase

Aspartyl 3-hydroxylase

Deacetoxycephalosporin C synthase

Gibberellin 20-oxidase
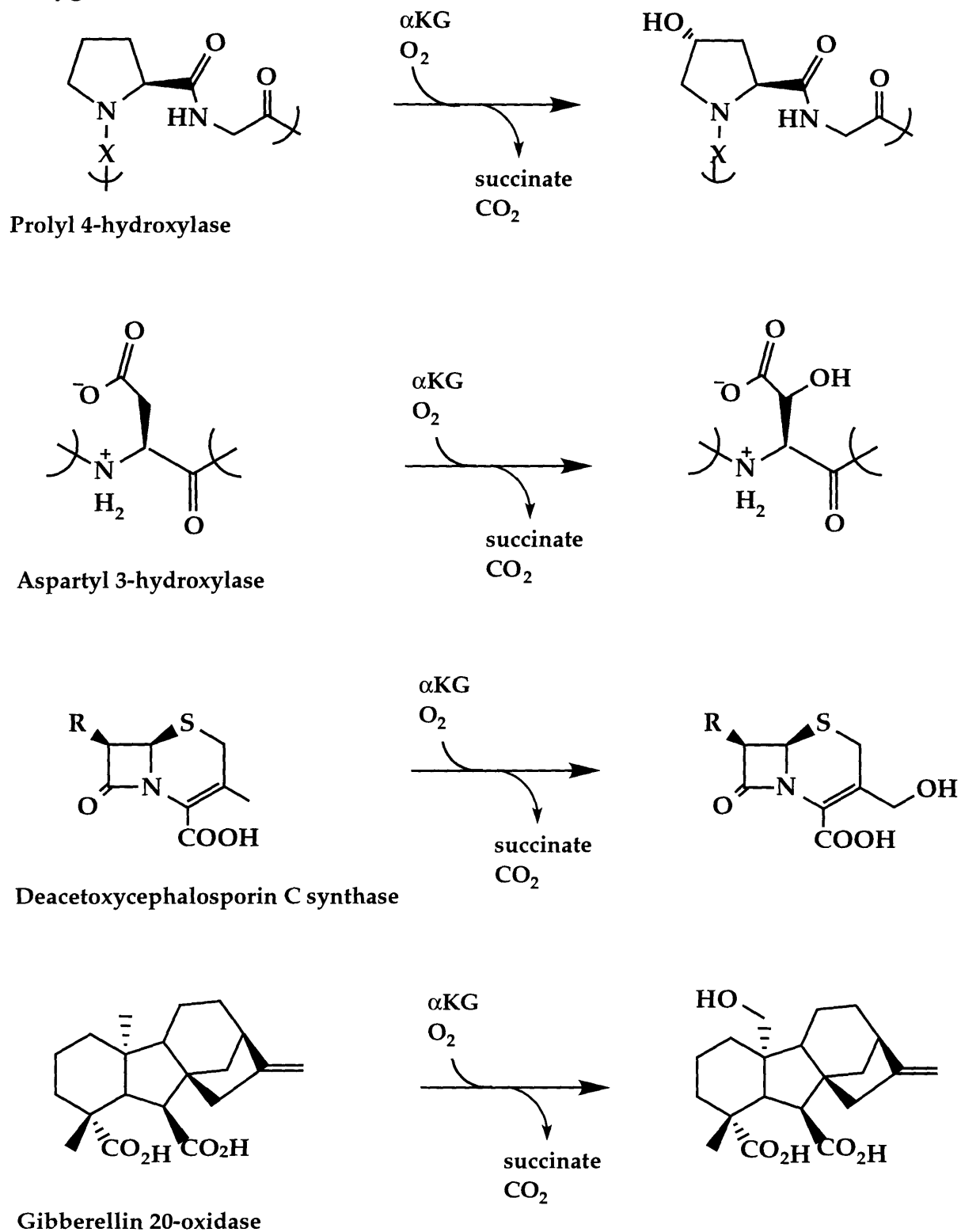
Figure 1-3. Proposed reaction mechanism for the αKG dependent dioxygenases[1, 2]
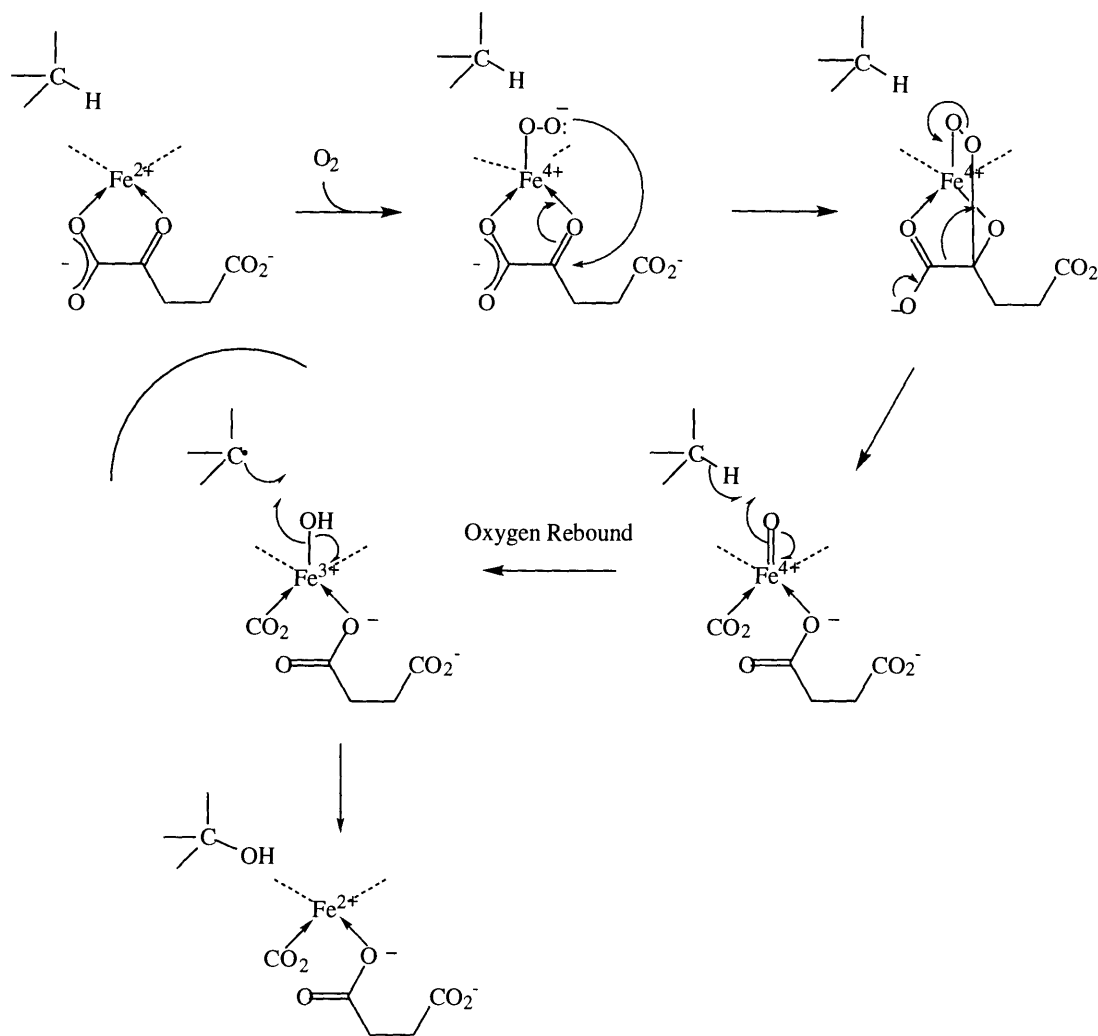
iron dependent cytochromes P-450 (cyt P-450), have been studied extensively and are relatively well understood. Studies of enzymatic systems as well as of synthetic models have limited the mechanisms plausibly used by the heme-iron dependent enzymes.[3] A general reaction mechanism has been proposed for the αKG-dependent dioxygenases involving a high valent iron-oxo intermediate similar to the proposed oxidizing species of the heme iron-dependent cyt P-450.[3] In the proposed scheme, attack of iron-bound O_2 on the C-2 carbonyl of the αKG is followed by decarboxylation of the αKG, which drives the heterolytic O-O

bond cleavage to yield a high valent iron-oxo species (Figure 1-3).[1, 2] The iron-oxo species then abstracts a hydrogen atom from the substrate producing a substrate radical which recombines to generate hydroxylated product by the oxygen rebound mechanism.[4]

Investigation of the mechanism of the αKG-dependent dioxygenases in the Stubbe laboratory has focused on the enzyme thymine 7-hydroxylase (TH, EC 1.14.11.6) purified from *Rhodotorula glutinis*. TH catalyzes the conversion of thymine to its corresponding alcohol, aldehyde, and carboxylic acid in three separate steps, each accompanied by the conversion of αKG to succinate and CO_2 (Figure 1-4).

$R_1 = CH_3$
$R_2 = CH_2OH$
$R_3 = CHO$

$R'_1 = CH_2OH$
$R'_2 = CHO$
$R'_3 = CO_2^-$
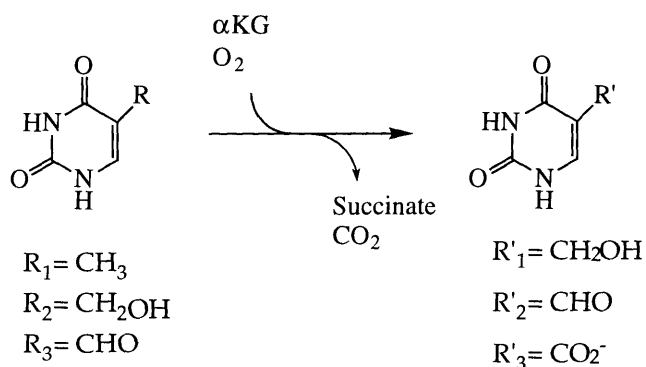
Figure 1-4. Reactions catalyzed by thymine hydroxylase

TH is particularly well-suited for mechanistic studies because of its relatively small size (39 kD), the simplicity of its substrate, and its ability to act on a wide range of modified substrates. This enzyme is also found in *Neurospora crassa* and *Aspergillus nidulans*. The highest levels of activity are attainable from *R. glutinis* grown in a defined medium with thymine as the sole nitrogen source from which the enzyme is obtained using a straightforward purification procedure. This procedure, developed by Lora Thornburg in the Stubbe laboratory as an improvement on the procedure of Warn-Cramer, can be used to

isolate 34 mg of 85% pure enzyme with a specific activity of 27 μmol min^{-1} mg^{-1} from a kg of cells.[5, 6]

Extensive studies of TH's substrate specificity and reaction kinetics have revealed similarities in the catalytic capabilities of this system to that of cyt P-450. Using alternate substrates, it was demonstrated that TH is able to catalyze epoxidations, sulfur oxidation and N-demethylation reactions in addition to the hydroxylation of unactivated C-H bonds (Figure 1-5).[6]

The inactivation of TH by the mechanism-based inhibitor 5-ethynyluracil (EU) has also been studied extensively.[7] It has been demonstrated that reaction of TH with EU results in the formation of carboxymethyluracil and uracil 5-acetylglycine in addition to inactivating the protein by covalent modification. Tryptic digests of the inactivated protein and analysis of modified protein fragments revealed the formation of two different covalent enzyme-inhibitor adducts in a 3:1 ratio, both modified at the same residue. Characterization of the major adduct revealed a novel norcaradiene moiety.[8] Formation of this adduct is proposed to occur via the insertion of a carbene intermediate into an active-site phenylalanine residue, followed by a second hydroxylation reaction (Figure 1-6). Amazingly, the second hydroxylation occurs despite the covalent modification of a residue in the active-site!

Characterization of the minor fragment has been more difficult due to the small quantities available for analysis. Based on data obtained by ^{13}C- and ^{1}H-NMR, FAB-MS analysis and UV spectroscopy, the structure of the minor adduct has been assigned as a benzoyluracil moiety. A mechanism for the formation of the minor adduct has also been proposed. To obtain additional support for the adduct structure, the synthesis of 5-benzoyluracil (BU) was accomplished for the purpose of comparing the ^{1}H- and ^{13}C-NMR data of BU with the adduct. Work has also been undertaken to examine the plausibility of the mechanism favored

for minor adduct formation. Both the synthesis of BU and the mechanistic work are discussed in Chapter 4.

The ultimate and as of yet unrealized goal of this research is the identification of the reactive iron species and the elucidation of the detailed enzyme mechanism by spectroscopic characterization of the iron center and its protein environment during turnover. Recent investigations in the Stubbe laboratory of the assembly of the dinuclear non-heme iron center of ribonucleotide reductase using stopped-flow absorption and rapid freeze-quench EPR, ENDOR, EXAFS, Resonance Raman, and Mössbauer spectroscopies have demonstrated the power of these techniques in the investigation of reactive intermediates.[9-13] Extension of these types of biophysical studies to TH should provide valuable insight into the identity of the iron species during turnover and into the detailed mechanism of the αKG-dependent dioxygenases.

Nitric oxide (NO) has been shown to bind to the EPR-silent active site ferrous ion in many mononuclear non-heme metalloenzymes to form EPR-active complexes.[14-16] Perturbations of the iron-nitrosyl EPR spectrum of TH upon addition of αKG and thymine, as well as a wide range of alternative substrates, might provide information about alterations in the ligand environment caused by binding of these molecules at or near the iron. Comparison of the spectra obtained from TH with those of related systems and model complexes could be used to identify species and potentially

Figure 1-5. Catalytic Capabilities of Thymine Hydroxylase

EPOXIDATION
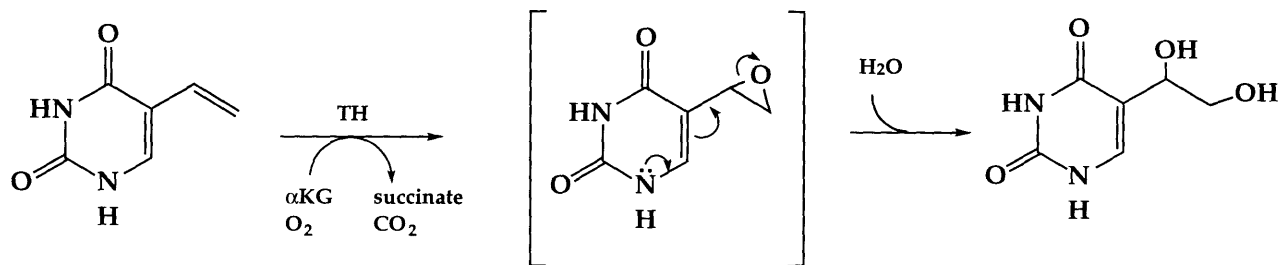
SULFUR OXIDATION
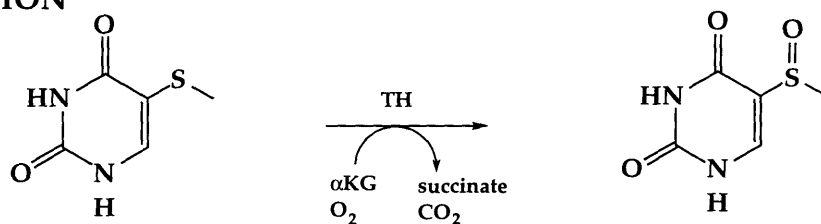
N-DEMETHYLATION

HYDROXYLATION OF UNACTIVATED C-H BONDS

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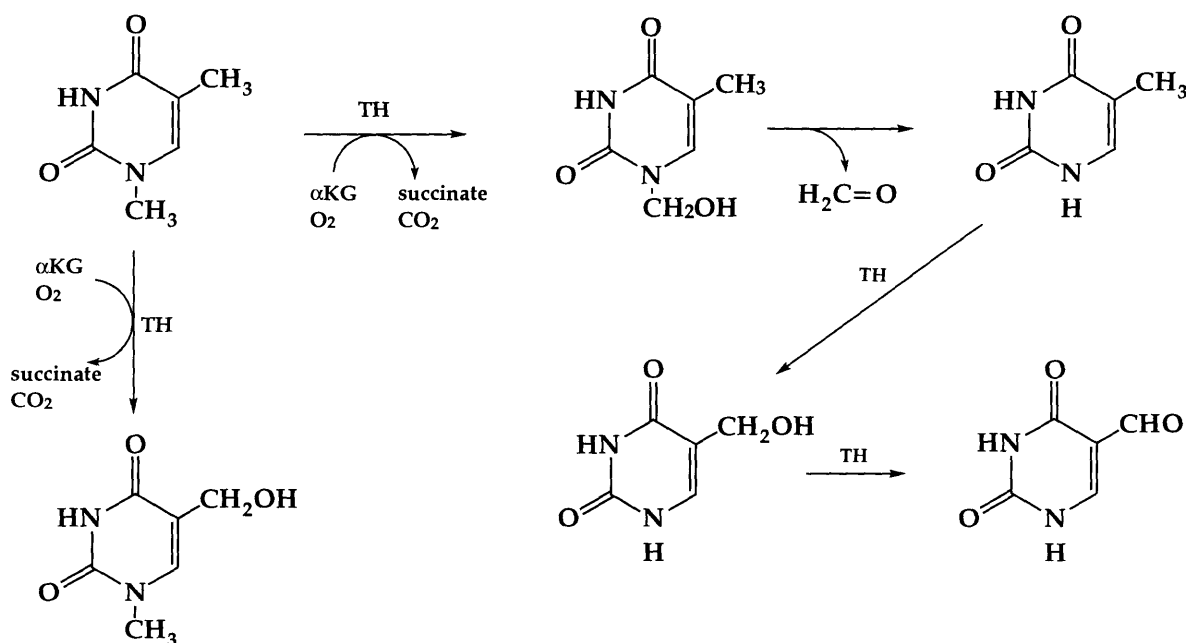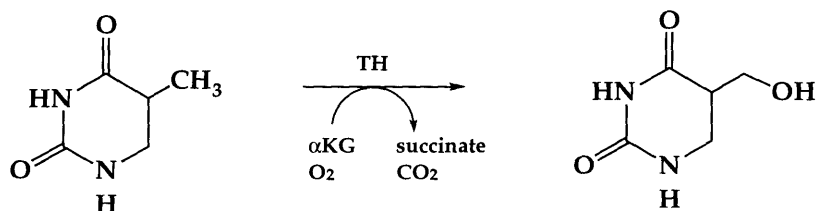
Figure 1-6. Inactivation of Thymine Hydroxylase by 5-Ethynyluracil
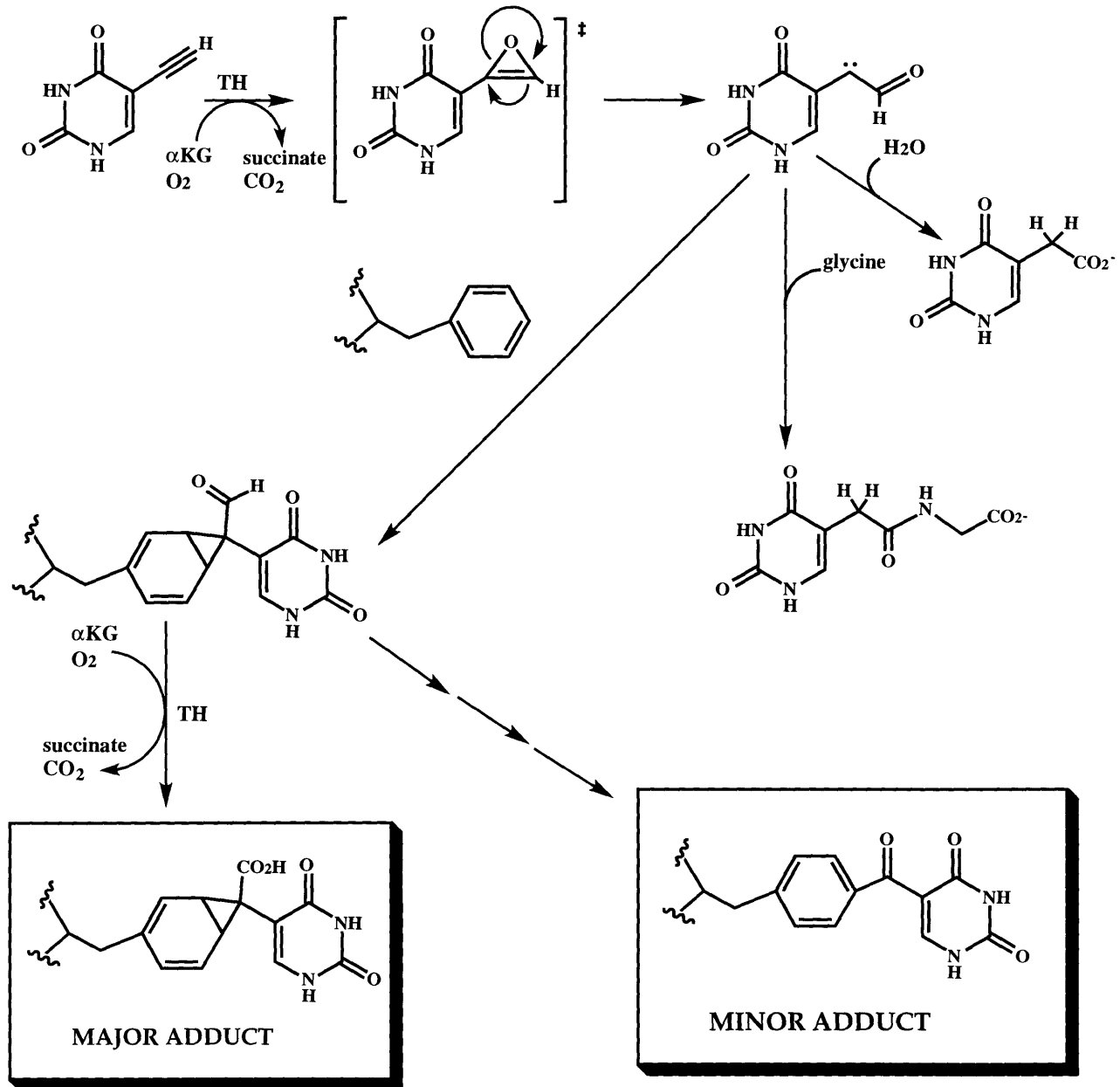
provide direct evidence for the reaction mechanism.[17, 18] UV-vis stopped flow

spectroscopy of the reaction could also be used to examine the interactions of O_2

with TH. The utility of this technique depends on the spectral properties of the

intermediates as well as the kinetics of the oxidation reaction. Standard reaction

conditions may have to be altered to retard the reaction and stabilize intermediates along the reaction pathway.

Unfortunately we have been limited in our mechanistic investigation of TH not only by the small quantities of protein isolated from *R. glutinis*, but by the limited stability of the purified enzyme from the last purification step as well. Since large quantities of homogeneous protein are needed for the spectroscopic studies mentioned above, the initial focus of this project was the cloning of the TH gene from *R. glutinis* for overexpression of the protein in *Escherichia coli*. Two different approaches have been taken in this work. First, peptide information from TH has been used to design synthetic oligonucleotide primers and probes. These oligomers were used to run PCR reactions and to screen Southern blots of restriction digests of genomic DNA. Second, isolation of mRNA was undertaken for the synthesis of single-stranded cDNA to be used as a template in PCR reactions and for screening in Northern blots. A detailed description of methods used and results obtained in the cloning work is found in Chapter 2. During the course of that work, more peptide information was obtained for the design of additional primers and probes. The protein submitted for sequencing was purified using a improved procedure which utilizes a BioCAD Sprint perfusion chromatography system. The new peptide information and a description of the improved purification method are found in Chapter 3.

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Chapter 2

Efforts Toward Cloning the Gene for
Thymine hydroxylase from
Rhodotorula glutinis

Since large quantities of homogeneous protein are needed for spectroscopic studies, we have focused on cloning the thymine hydroxylase (TH) gene from the yeast *Rhodotorula glutinis* for over-expression of the protein in *Escherichia coli.* Two different approaches have been taken in this work. First, we have used peptide information from TH to design synthetic oligonucleotide primers and probes. Peptide sequence data for TH has been derived from Edman degradation of the N-terminus, from peptide fragments generated from a trypsin digest, and from labeling of the active site by a mechanism-based inhibitor (Figure 2-1).[1,2]

1) N-terminus--V S S G I V P P I N F E P F L S G
2) V T Q E T D P E A I A K
3) T Q Q F F D L P M E V K
4) L S E T Y T K
5) W S N D V L K
6) Active-site--N S I A F F S N P S L R
7) L E V V L P A D G D P L E M T P R

Figure 2-1. TH Peptide Fragments Isolated from *R. glutinis*

The oligomers designed by back-translating the peptide information into the corresponding genetic code were used to run PCR reactions and to screen Southern blots of restriction digests of genomic DNA. Second, isolation of RNA was undertaken for Northern blotting as well as for the synthesis of single-stranded cDNA to be used as a template in PCR reactions.

This chapter provides a detailed description of the methods used and results obtained in the attempt to isolate the TH gene from *R glutinis*. This work has presented many challenges. Unlike the yeast *Saccharomyces cerevisiae, R. glutinis* has not been genetically characterized. There are no reports in the

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literature of genes cloned from this particular organism, and only very few genes have been cloned from related *Rhodotorula* strains.[3-7]

MATERIALS AND METHODS

Materials

Freeze-dried *R. glutinis* cultures were obtained from the American Type Culture Collection (ATCC #2527). Powdered YM broth and agar were supplied by Difco. Tris, Proteinase K, RNase A, and 10X TAE (0.4 M Tris-acetate pH 8.4, 0.1 M EDTA, 0.2 M glacial acetic acid) were purchased from Boehringer Mannheim. NaCl, EDTA, sucrose, glycerol, KCl, NaOAc, sodium citrate, formaldehyde solution, isopropanol and sodium phosphate were obtained from Mallinckrodt. Amresco supplied all phenol and chloroform reagents. Sorbitol and spermidine were products of Aldrich. Pharmco Products supplied absolute ethanol. Novozym 234, PEG-8000, denatured salmon sperm DNA, polyvinylpyrrolidone, glass beads, and diethylpyrocarbonate (DEPC) were ordered from Sigma. Triton X-100 was provided by Fluka. Bromophenol blue and β-mercaptoethanol were purchased from BioRad. Gibco BRL Life Technologies, Inc. supplied sodium dodecyl sulfate (SDS), hybridization bags, agarose, restriction enzymes, oligo-dT cellulose columns, and the mRNA Isolation System (cat# 18351-015). Probe grade dextran sulfate was purchased from Anchor, Inc. Ficoll 400, Taq polymerase, NAP-5 columns and Ultrapure dNTP's were obtained from Pharmacia. Oligonucleotides were synthesized by the MIT biopolymers lab, Research Genetics, or Integrated DNA Technology. Formamide was supplied by EM Science. Hybond membranes and MOPS were purchased from Amersham Life Sciences. GeneScreen Plus membranes, [γ-^{32}P]-

ATP (6000 Ci/mmol), and Nensorb columns are products of DuPont, New England Nuclear. New England Biolabs supplied restriction enzymes and T4 polynucleotide kinase. Gel blotting paper was manufactured by Schleicher and Schuell.

GROWTH OF *Rhodotorula glutinis*

Preparation of Media

YM agar was prepared by suspending agar powder in H_2O (41 g/L) and adjusting pH to 4.5 with 6 M HCl. YM broth powder was dissolved in H_2O (21 g/L) and the pH adjusted to 4.5 with 6 M HCl. All YM media was sterilized by autoclaving 30 minutes at 20 psi.

Recipes for carbon-based growth medium stock solutions are shown in Tables 2-1 and 2-2. Stock solutions of amino acids, trace elements and vitamins were prepared and filtered through 0.2 μm filters into autoclaved bottles for storage. Solution A was autoclaved 30 minutes at 20 psi. Solution B was prepared and sterile filtered. Prior to innoculation, solutions A and B were combined.

Starter and Storage Cultures

Freeze-dried *R. glutinis* cultures were rehydrated with 400 μL sterile H_2O. YM agar plates were innoculated with 100 μL aliquots and incubated at room temperature until growth was visible, then moved to 4°C for storage. Master plates were constructed by plating serial dilutions of the rehydrated culture to obtain single colonies. Single colonies were picked and spotted in rows on an agar plate. These plates were respotted every 2-4 months.

Storage cultures were prepared by innoculating YM broth (1 mL) with a loop of cells from the YM plates and incubating with shaking (250 rpm) at 26°C for 30 h (OD = 33). Aliquots were then tansferred into an equal volume sterile 50% glycerol and stored at -80°C. Storage cultures were re-plated on YM agar before using to innoculate YM broth.

Cell Density Measurements

R. glutinis growths were monitored spectrophotometrically by the absorbance at 600 nm. Samples were prepared by mixing 50 μL culture with 450 μL PEG-8000 (76 mg/mL) to prevent the rapid settling of cells. Absorbances were multipled by the dilution factor to obtain the OD.

Growth of *R. glutinis* in YM Broth for DNA Isolation

YM broth (1.5 mL) was innoculated with a loop of cells from actively growing YM agar plates and incubated at 26°C with shaking. After 24 h, cultures had reached late log phase (OD=30) and were transferred to 15 mL YM broth in 125 mL flasks. These cultures reached late log phase after 29 h and were transferred to 150 mL YM broth in 1 L flasks. Cells were harvested after reaching late log phase (OD=30) by centrifuging at 6000xg for 20 minutes. Cells were frozen in liquid nitrogen and stored at -80°C.

Growth of *R. glutinis* in Carbon-base Medium for RNA Isolation

YM broth (1.5 mL) was innoculated with a loop of cells from actively growing YM agar plates and incubated at 26°C with shaking. After 24 h, cultures had reached late log phase (OD=30) and were transferred to 15 mL carbon-base medium in 125 mL flasks. After 30 h at 26°C the C-base cultures had reached late log phase (OD=20) and were transferred to 150 mL C-base medium in 1 L flasks.

Table 2-1. Recipes for Stock Solutions for C-base Medium

AMINO ACIDS SOLUTION prepare by dissolving amino acids in 400 mL H_2O	40 80 80	mg mg mg	L-histidine·HCl·H_2O DL-methionine tryptophan
TRACE ELEMENTS SOLUTION prepare by dissolving trace elements in 400 mL H_2O plus 16 mL conc. HCl	200 1.6 40 80 160 80 160	mg mg mg mg mg mg mg	H_3BO_3 $CuSO_4·5H_2O$ KI $FeCl_3·6H_2O$ $MnSO_4·1H_2O$ $Na_2MoO_4·2H_2O$ $ZnSO_4·7H_2O$
VITAMINS SOLUTION prepare by dissolving vitamins in 350 mL H_2O plus 400 mL ethanol	16 0.8 160 80 160 80 160 800 80	mg mg mg mg mg mg mg mg mg	calcium pantothenate folic acid niacin p-aminobenzoic acid pyridoxine·HCl riboflavin thiamin·HCl inositol biotin (50 mL of a 1.6mg/mL solution)

Table 2-2. Solutions for 1 L C-base Medium with Thymine

SOLUTION A dissolve thymine and salts in 700 mL H_2O	1 1 0.5 0.1 0.1	g g g g g	thymine KH_2PO_4 $MgSO_4·7H_2O$ NaCl $CaCl_2·2H_2O$
SOLUTION B dissolve glucose in 300 mL H_2O and add remaining ingredients	10 1 1 2	g mL mL mL	glucose amino acids solution trace elements vitamins

After an additional 30 h the 150 mL cultures (OD=20) were transferred to 1.5 L C-base medium in 6 L flasks. The 1.5 L cultures were harvested after another 30 h.

ISOLATION OF GENOMIC DNA FROM *Rhodotorula glutinis*

Six different protocols were used in isolating genomic DNA from *R.glutinis*. The cells used in the isolations were grown in YM to saturation (OD=30) as described above.

Protocol 1

Protocol 1 used the procedure of Lai.[8] Frozen *R. glutinis* cells (8 g) were ground with liquid nitrogen in a mortar. The cell powder was suspended in 20 mL extraction buffer (0.2 M Tris pH 8.5, 0.25 M NaCl, 25 mM EDTA, 0.5% SDS) and then centrifuged at 3000xg for 20 min to pellet cell debris. Proteins were extracted from the supernatant first with 1 volume phenol, then with 1 volume phenol: chloroform: isoamyl alcohol (25:24:1), and finally, with 1 volume chloroform. After each extraction the sample was centrifuged at 3000xg for 30 min to separate the aqueous and non-aqueous layers. Nucleic acids were precipitated from the aqueous layer with the addition of 0.1 volume of a 2.5 M NaOAc solution and either 0.54 volume isopropanol or 2 volumes ethanol. The precipitate was collected by centrifugation and dissolved in 2 mL TE (10 mM Tris pH 8, 1 mM EDTA). Extractions were performed a second time as described above. To remove RNA in the DNA preparation, the solution was incubated with RNase A (final concentration 80 μg/mL) for 3 h at 37°C. A third series of extractions preceeded the final DNA precipitation. The DNA was either lifted out of solution with a pipette tip or centrifuged to pellet, dried under a stream of argon and dissolved in TE. Protocol 1 yielded 0.3 mg DNA per gram yeast cells with an A_{260}/A_{280} ratio ranging from 1.8-2.0.

Protocol 2

Protocol 2 used the procedure of Raeder and Broda.[9] *R. glutinis* cells (0.5 g) were ground in a mortar with liquid nitrogen. Cell powder was divided among 10 Eppendorf tubes and suspended in 500 μL extraction buffer (0.2 M Tris pH 8.5, 0.25 M NaCl, 25 mM EDTA, 0.5% SDS). Phenol (350 μL) and chloroform (150 μL) were added to each Eppendorf tube and mixed thoroughly before the suspension was centrifuged for 1 h in a microfuge at maximum speed (14000 rpm). The aqueous phase was removed and transferred to a clean Eppendorf containing 25 μL RNase A (20 mg/mL) and incubated 5-10 min at 37°C. Next, the solution was extracted with 1 volume chloroform and centrifuged 10 min at maximum speed. The aqueous phase was removed and transferred to an Eppendorf tube and mixed with 0.54 volume isopropanol. The DNA precipitate was allowed to settle in the tube and the supernatant removed. Tubes were spun for 5 sec and the remaining supernatant removed. Pelleted DNA was washed with 70% EtOH, dried under a stream of argon, and resuspended in TE. The yield was 0.8 mg per gram yeast cells. The DNA obtained by protocol 2 had an A_{260}/A_{280} ratio of 1.97.

Protocol 3

Protocol 3 used the procedure of de O. Azevedo, et al.[10] *R. glutinis* cells (4 g) were ground with liquid nitrogen and then suspended in 30 mL TTE (10 mM Tris pH 8.5, 10 mM EDTA, 4 mM spermidine, 10 mM β-mercaptoethanol (β-ME), 0.5 M sucrose, 36 mM KCl, 0.25% Triton X-100). The suspension was centrifuged at 7500xg for 10 min and the pelleted cells resuspended in 20 mL lysis buffer (40 mM Tris pH 8.0, 10 mM EDTA, 0.2 M NaCl, 1.5% w/v SDS). This suspension was extracted twice with 1 volume phenol and then once with 1 volume chloroform. 3 M NaOAc (3 M, pH 5.3, 0.1 vol) was added to the aqueous layer

and the DNA precipitated with the addition of 0.54 volume isopropanol at 4°C overnight. Visible DNA fibers were removed with a pipette, rinsed with 70% EtOH and allowed to air-dry. Purified DNA was dissolved in 2 mL TE to yield 0.14 mg per gram yeast cells with an A_{260}/A_{280} ratio of 1.83.

Protocol 4

Protocol 4 followed the procedure described in Current Protocols in Molecular Biology.[11] *R. glutinis* cells (1 g) were suspended in 5 mL sorbitol solution (0.9 M sorbitol, 0.1 M Tris pH 8.0, 0.1 M EDTA) by vortexing. Zymolase (500 μL, 0.3 mg/ml in sorbitol solution) and 500 μL 0.28 M β-ME were added to the suspension. After incubating 1 h at 37°C on a rolling shaker the mixture was centrifuged at 12000xg for 5 min to pellet the sphereoplasts. The supernatant was discarded and the sphereoplasts resuspended in 5 mL Tris/EDTA solution (50 mM Tris pH 8.0, 20 mM EDTA). The suspension was transferred to 1.5-mL Eppendorf tubes (500 μL each) and 50 μL 10% SDS added to each sample. The SDS mixture was incubated at 65°C for 20 min. Potassium acetate (5M, 200μL) was added and the samples were then placed on ice for 40 min. After centrifuging 3 min at room temperature, the supernatant was transferred to clean tubes and 1 mL EtOH added. No DNA was recovered using this protocol.

Protocol 5

Protocol 5 followed the procedure of Gilbert, et al.[5] *R. glutinis* cells (1.5 g) were suspended in 10 mL EDTA/β-ME solution (45 mM EDTA, 300 mM β-ME) by vortexing and then incubated at room temperature 30 min. The cells were centrifuged 5 min at 12000xg to pellet, resuspended in sorbitol solution (65 mM sodium phosphate, 1 M sorbitol, 25 mM EDTA pH 8.5) containing 8 mg/mL Novozym and incubated 19 h at 37°C on a rolling shaker to form protoplasts.

Protoplasts were centrifuged at 12000xg for 5 min and resuspended in 10 mL NaCl/EDTA solution (0.15 M NaCl, 0.1 M EDTA pH 8.0). Proteinase K (50 µg/mL) and SDS (1%) were added and the cells incubated overnight. Next, the suspension was extracted with an equal volume of chloroform: isoamyl alcohol (24:1). Two volumes ethanol were layered over the aqueous layer and the tube inverted to mix. The solution turned cloudy and after 30 min of centrifuging at 6000xg a pellet was visible. The pellet was dissolved in 2 mL 50 mM Tris, pH 7.4. RNase A was added to a final concentration of 80 µg/mL and the solution incubated 2 h at 37°C. This solution was extracted twice with 1 volume phenol and once with 1 volume chloroform. The aqueous phase was made 0.1 M NaCl by the addition of a 5 M NaCl solution and 0.54 volume isopropanol added to precipitate the DNA. No DNA was obtained.

Protocol 6

Protocol 6 followed the procedure of Levin, et al.[12] *R. glutinis* cells (0.5 g) were suspended in 0.4 mL extraction buffer (0.5 M NaCl, 0.2 M Tris pH 7.6, 10 mM EDTA, 1% SDS). The suspension was divided between 2 Eppendorf tubes and 0.4 g glass beads (0.45-0.55 mm) added to each. A 0.2 mL solution of phenol: chloroform: isoamyl alcohol [25:24:1] (PCIA) was added to the samples which were next vortexed at top speed for 2.5 min. An additional 0.3 mL extraction buffer and 0.3 mL PCIA were added and the tubes vortexed another min. Samples were centrifuged 5 min at maximum speed in a microfuge. The aqueous phase was removed and extracted twice with 1 volume PCIA. Two volumes EtOH were added to precipitate nucleic acids. The precipitate was pelleted and then washed with 0.2 mL 70% EtOH. Nucleic acids were resuspended in 50 µL TE and RNase A (2 µL of a 10 mg/mL solution) was added. Solutions were then incubated at 37°C for 1 h before extracting with 1 volume chloroform and

precipitating the DNA with 2 volumes EtOH. The yield from 0.5 g cells was 244 µg DNA with an A_{260}/A_{280} ratio of 1.7.

SOUTHERN BLOTTING

Restriction Digests

In a typical digest, 10 µL *R. glutinis* genomic DNA (5-24 µg) was mixed with 8 µL H$_2$O, 2 µL 10X buffer (supplied by New England Biolabs (NEB) with the restriction enzyme) and 2 µL of a restriction enzyme (EcoR I, Pst I, Hind III, Xba I, Bgl II, Sma I, Nco I and Nde I; 8-40 units). Double digests were run with 2 µL both enzymes and the buffer recommended by NEB for that particular pair of enzymes. Reaction mixtures were incubated at 37°C for 4-6 h. If reactions ran more than 6 h, additional enzyme was added over the course of the digest. After the digest was complete, 4 µL 6X loading dye (20 mL contains 3 g ficoll 400, 40 mg bromophenol blue, 40 mg xylene cyanol, and 50 mM EDTA) was added to each reaction mixture. DNA fragments were electrophoresed in ethidium bromide-containing agarose gels (1-1.5% agarose, 0.5 µg/mL ethidium bromide). Gels were run in 1X TAE until the bromophenol blue ran at least halfway down the gel and were photographed before continuing.

Southern Transfers

DNA fragments were prepared for transfer from agarose gels to GeneScreen*Plus* nylon membranes (Dupont, New England Nuclear) following the manufacturer's instructions. Gels were first soaked in denaturation solution (0.6 M NaCl, 0.4 M NaOH) 30 min with agitation and then in neutralization solution (1.5 M NaCl, 0.5 M Tris pH 7.5) 30 min. The membrane was prepared by wetting with deionized water and then soaking 15 min in 10X SSC (10X= 0.15 M sodium citrate, 1.5 M NaCl).

DNA fragments were transferred from the agarose gel to the membrane using standard molecular biological techniques according to the original procedure of Southern.[13] Capillary blots were set up with 10X SSC. Gel blotting paper was used to make the wick which was placed on a support over a dish of 10X SSC (Figure 2-2). The gel was placed on the wick with the bottom of the wells facing up. On top of the gel was placed the pre-wet GeneScreen*Plus* nylon membrane, 3 sheets of blotting paper pre-wet with 10X SSC, 5-8 cm of paper towels, and finally, a glass plate with a 0.5 kg weight on top. Transfer was allowed to proceed overnight. After transfer, the membrane was immersed in 0.4 M NaOH for 30-60 sec to ensure complete denaturation of DNA, then rinsed in 0.2 M Tris pH 7.5, 2X SSC (0.03 M sodium citrate, 0.3 M NaCl). Membranes were air-dried before proceeding with hybridization.
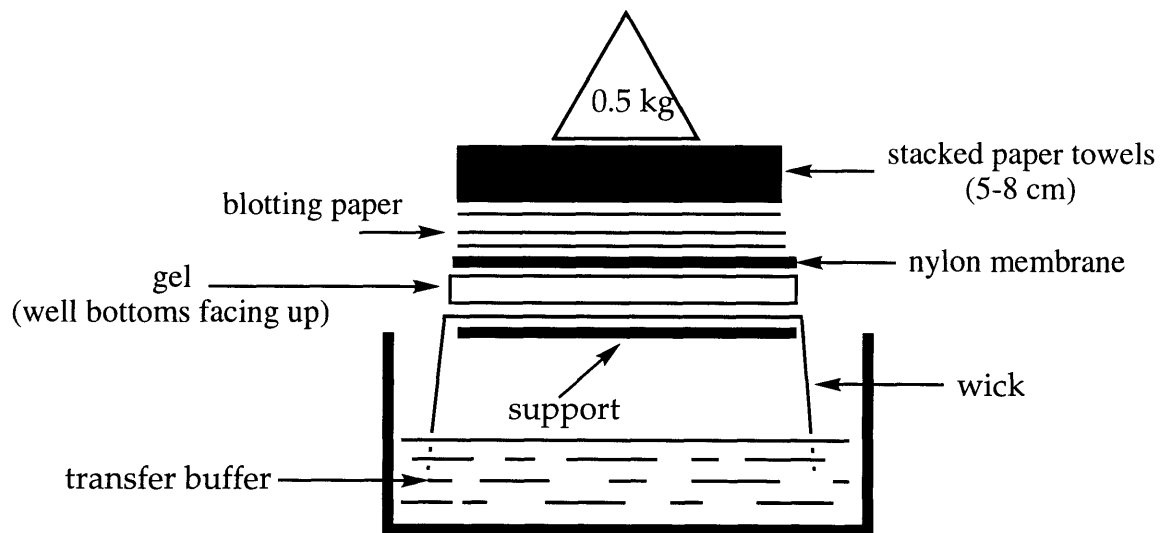
Figure 2-2. Capillary Blot Set-up for Transfer of DNA Fragments from Agarose Gel to Nylon Hybridization Membrane

Amersham Hybond-N nylon membranes were also used for Southern blots. Gels and membranes were prepared according to manufacturer's specifications. Agarose gels were soaked 30 min in denaturing solution (1.5 M

NaCl, 0.5 M NaOH), rinsed in water and then soaked in neutralizing solution (1.5 M NaCl, 0.5 M Tris pH 7.2, 1 mM EDTA) 30 min. Membranes did not require soaking prior to use. The transfer buffer used with the Hybond membranes was 20X SSC (0.3 M sodium citrate, 3 M NaCl). Following transfer, membranes were rinsed in 2X SSC and then baked 2 h at 80°C to fix the DNA to the membrane.

Radioactive Probe Labeling and Detection System

Radiolabelled oligonucleotide probes used for hybridization were 5' end-labeled with ^{32}P using $[\gamma-^{32}P]$-ATP and T4 polynucleotide kinase (PNK). The reaction consisted of 50 pmol oligo, 250 µCi $[\gamma-^{32}P]$-ATP, 5 µL 10X buffer [0.5 M Tris pH 7.6; 0.1 M $MgCl_2$; 50 mM dithiothreitol; 1 mM spermidine; 1 mM EDTA pH 8] and 20 units PNK. Total volume was adjusted to 50 µL with H_2O and the reaction mixture incubated at 37°C for 60 min. Probes were purified from unreacted $[\gamma-^{32}P]$-ATP with NENSORB columns following manufacturer's directions.

Blots were prehybridized in sealed bags containing 10 mL hybridization buffer [6X SSC (0.9 M NaCl, 0.09 M trisodium citrate); 5X Denhardt's reagent (50X = 5 g Ficoll, 5 g BSA, 5 g polyvinylpyrrolidone in 500 mL H_2O); 0.5% SDS; 100 µg/mL denatured salmon sperm DNA] for 3 h at 42°C. Blots were then hybridized 16 h in fresh hybridization solution containing the labeled probe (typically 1 x 10^7 Cerenkov counts per µg oligomer).

Following incubation with the labelled probe, blots were washed, wrapped in Saran wrap and exposed to a Phosphorimage plate at least 12 h. A typical series of washes began with 2- 30 min washes in 2X SSC at room temperature. These were followed by a 45 min wash at 42°C with 2X SSC/1%SDS and then a final 60 min wash in 2X SSC at room temperature.

Additional washes were often necessary. These washes were run in 0.1-1X SSC/0-1%SDS solutions at temperatures up to 45°C.

Blots could be stripped of the radiolabelled probe by washing in 2X SSC/1%SDS for 1 h at 50°C and then in 2X SSC at 50°C for another h. Complete removal of the probe was verified by exposing the stripped blot to a Phosphorimage plate at least 12 h.

PCR

PCR reactions were run in a total volume of 100 µL consisting of 1X PCR buffer (10 mM Tris pH 9.0, 1.5 mM $MgCl_2$, and 50 mM KCl) with 0.2 mM dNTP's, 100 pmol of each forward and reverse primer and 0.5-1.5 µg of the reaction template. Reactions were overlayed with a drop of mineral oil to prevent sample evaporation during the temperature cycling and then heated 4 min at 95°C before adding 2.5 units Taq polymerase. For each reaction that was set up, four controls were run: forward and reverse primers minus template; forward primer plus template; reverse primer plus template; template without primers. Typical thermocycler parameters were as follows: 10 cycles of 94°C for 1 min (denaturation); 37°C for 1 min (annealing); and 72°C for 2 minutes (synthesis). This was followed by 25 cycles of 94°C for 1 min, 40-50°C for 1 min, and 72°C for 2 min.

Reaction products were separated on ethidium bromide containing agarose gels (1-1.5% agarose, 0.5 µg/mL ethidium bromide) run with 1X TAE. Samples were prepared for loading onto gels by mixing 20-40 µL reaction mixture with 6X loading dye (20 mL contains 3 g ficoll 400, 40 mg bromophenol blue, 40 mg xylene cyanol, and 50 mM EDTA). If the entire reaction mixture was to be loaded, mineral oil was first extracted with chloroform and then the sample

concentrated either by precipitating with ethanol or by ultrafiltration with a Centricon-10 concentrator (YM membrane).

Southern Blots of PCR Products

Gel and membrane preparations were identical to those discussed above for genomic digests.

ISOLATION OF mRNA FROM *R. glutinis*

mRNA was isolated from *R. glutinis* cells grown in carbon-base medium. All solutions and equipment were properly treated to ensure the inactivation of RNases. Glassware was baked overnight at 180°C, and all plastics and nalgene centrifuge bottles soaked in 0.5 M NaOH for 10 min, rinsed thoroughly with distilled water and then autoclaved for 25 min at 18 psi. Water was prepared in RNase-free glassware by treating with 0.01% diethyl pyrocarbonate (DEPC) overnight and then autoclaving. All solutions were prepared from previously un-opened reagents in treated glassware.

Isolation of total RNA

Protocol 1

Protocol 1 used the Gibco BRL Life Technologies mRNA isolation system. *R. glutinis* cells (6 g) were ground in liquid nitrogen to a fine powder and suspended in 90 mL ice-chilled guanidine isothiocyanate (GuSCN)/β-ME solution (4 M guanidine isothiocyanate, 1.14 M β-ME, 50 mM Tris-HCl, pH 7.5, 25 mM EDTA). Cold EtOH (0.3 vol) was added to the cell suspension and the sample mixed by inversion. After centrifuging for 5 min at 16000xg, the supernatant containing denatured RNases was carefully removed. The pellet

and interior of the centrifuge tube were rinsed with several milliliters GuSCN/β-ME solution before resuspending the RNA-containing pellet in 45 mL GuSCN/β-ME solution. This suspension was then centrifuged at 16000xg for 3 min to pellet insoluble debris and denatured proteins.

The RNA-containing supernatant was removed to a clean tube (45 mL). A 1M acetic acid solution (0.025 vol, 1.125 mL) and EtOH (0.75 vol, 33.75 mL) were added. After mixing tube contents by inversion, the solution was chilled at -20°C for 20 min and then centrifuged 10 min at 7000xg. The RNA pellet was resuspended in 60 mL GuHCl/β-ME solution (6 M guanidine hydrochloride pH 7.5, 11 mM β-ME, 25 mM EDTA) before adding 1M acetic acid (0.05 vol, 3 mL) and EtOH (0.5 vol, 30 mL). The mixture was placed at -20°C for 20 min before centrifuging 10 min at 7000xg. Two more cycles of suspending, precipitating and pelleting the RNA followed with decreasing volumes of the GuHCl/β-ME solution used to resuspend the RNA pellet (42 mL and 30 mL, respectively). After the last precipitation step, the RNA pellet was suspended in 4 mL binding buffer (10 mM Tris pH 7.5, 1 mM EDTA, 0.3 M NaCl, 0.1% SDS). Cold EtOH (2 volumes) was added and the sample chilled at -20°C for 20 min. RNA was pelleted by centrifugation at 7000xg for 10 min and the pellet dissolved in 3 mL binding buffer to yield 0.197 mg total RNA per gram cells with an A_{260}/A_{280} ratio of 1.45.

Protocol 2

All centrifugations were done at 4°C in an Eppendorf centrifuge at maximum speed.

R. glutinis cells (0.8 g) were divided among 4 Eppendorf tubes. Cells were suspended in 1 mL H_2O and then quickly pelleted by centrifuging. The pellet was resuspended in 400 µL TES (10 mM Tris pH 7.5, 10 mM EDTA, 0.5% SDS).

Acid phenol (pH 4.3, 400 µL) was added and the mixtures vortexed vigorously for 10 sec. The mixtures were then incubated for 60 min at 65°C with occasional vortexing. Samples were removed from the heated bath and placed on ice for 5 min before centrifuging for 6 min. The aqueous phase was transferred to clean tubes and 400 µL acid phenol added. Samples were vortexed vigorously and then spun 6 minutes. Next, 400 µL of chloroform was added to the aqueous layer, the samples vortexed, and then spun 5 min. The aqueous phase was brought to 0.3 M sodium acetate by addition of a 3 M solution after which 2.5 volumes cold EtOH was added to precipitate the RNA. A white precipitate was immediately visible. Samples were placed at -20°C for 30 min before centrifuging 5 min. The RNA pellet was washed once with 70% EtOH and then air-dried. DEPC-treated H_2O (50 µL) was added to each dry pellet and the RNA dissolved by heating at 65°C for 15 min and then pipetting up and down repeatedly. Samples prepared for mRNA isolation were dissolved in binding buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.3 M NaCl, 0.1% (w/v) SDS) instead of water. Protocol 2 yielded 1 mg total RNA per gram cells with an A_{260}/A_{280} ratio of 1.79.

Isolation of mRNA by Oligo-dT Cellulose Chromatography

Total RNA samples were dissolved in binding buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.3 M NaCl, 0.1% (w/v) SDS), heated at 70°C for 5 min, and then chilled on ice for 5 min. Total RNA was loaded onto an oligo-dT cellulose column (100 mg resin per column) which had been pre-equilibrated with binding buffer. The column was washed with 4 mL binding buffer to elute non-mRNA and the mRNA then eluted with 1.5 mL elution buffer (10 mM Tris pH 7.5, 1 mM EDTA, 0.1% (w/v) SDS). While the column was being re-equilibrated with binding buffer, the mRNA solution was heated 5 min in a 70°C water bath and

chilled 5 min on ice. The RNA solution was then allowed to sit 20 min at room temperature. After the addition of 90 μL 5 M NaCl, the RNA sample was loaded for a second run through the oligo-dT cellulose column. The column was washed with 4 mL of binding buffer and the mRNA then eluted with 1.5 mL elution buffer. 5 M NaCl (90 μL) and EtOH (3 mL) were added to the mRNA fraction which was placed at -20°C until needed. This protocol yielded 1.8 μg mRNA per mg total RNA isolated using protocol 1 and 2.6 μg mRNA per mg total RNA isolated using protocol 2.

Northern Blots

RNA samples were electrophoresed on denaturing agarose gels which were prepared by mixing 1.5 g agarose with 10 mL 10X MOPS (0.2 M 3-[N-Morpholino] propane-sulphonic acid), 73 mL H_2O and 3 μL ethidium bromide (10 mg/mL). The mixture was heated in a microwave to dissolve the agarose. After cooling to 50°C, 17 mL formaldehyde (37%) was added and the gel poured immediately.

Samples were prepared for electrophoresis by dissolving RNA in a minimal amount of water and adding 4 vol loading buffer (250 μL formamide, 50 μL 10X MOPS, 80 μL formaldehyde [37%]). Samples were incubated at 65°C for five min and chilled on ice before adding 0.3 vol dye solution (50% glycerol, 0.1 mg/mL bromophenol blue). Gels were run with 1X MOPS until the dye had run halfway down the gel.

RNA was transferred from the denaturing gel to Amersham Hybond-C Extra supported nitrocellulose membranes as described above for Southern transfers. 20X SSC was the transfer buffer. Membranes were baked at 80°C under vacuum on a gel dryer to fix the RNA.

Northern blots were prehybridized in a solution of 7 mL H_2O, 2 mL 50% dextran sulfate, and 1 mL 10% SDS. Blots were sealed in hybridization bags and

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prehybridized 90 min at 60°C. Radiolabeled probe (approximately 40 µg, typically 2 x 10^5 Cerenkov counts per µg oligomer) plus 130 µL of 10.8 mg/mL salmon sperm DNA solution were incubated at 100°C for 10 minutes and then added to the hybridization bag. Blots were hybridized overnight at 65°C with constant agitation.

Following hybridization, blots were washed twice in 100 mL 2X SSC for 5 min at room temperature. Next, blots were washed in 200 mL 2X SSC/1%SDS for 25 min at 60°C. Blots were then rinsed 3 min at room temperature in 100 mL 2X SSC before wrapping in Saran Wrap and exposing to a Phosphorimage plate overnight.

RESULTS AND DISCUSSION

Genomic DNA Isolation

Six different protocols were tested in the isolation of R. *glutinis* genomic DNA. Dr. Lai, a post-doctoral fellow in the laboratory who initiated the cloning work, had observed that DNA isolated by Protocol 1 (see experimental methods) was resistant to digestion by restriction enzymes, including those enzymes that cut frequently such as EcoR I and Hind III. It is not unusual to have problems such as contaminating pigments and polysaccharides in DNA preparations from fungi; it was possible that impurities in the DNA samples were inhibiting the restriction enzymes. To find a procedure that yielded high-quality DNA, isolation procedures ranging from the standard *Saccharomyces cerevisiae* DNA preparation to fungal DNA isolation protocols were tested. The quality of DNA obtained in each case was evaluated by agarose gel, by the ratio of the absorbance at 260 and 280 nm, and by restriction digests.

The standard protocol for isolating S. *cerevisiae* DNA employs Zymolyase, a β-glucanase that hydrolyzes the yeast cell wall to form sphereoplasts.[11]

Sphereoplasts are lysed with the addition of an SDS solution and the SDS, proteins, and cell debris are precipitated with potassium acetate. Nucleic acids remain in solution and are precipitated with ethanol. The combination of Zymolyase and SDS failed to rupture the *R. glutinis* cell wall and no DNA was obtained with Protocol 4. This observation that the *R. glutinis* cell wall resisted hydrolysis was substantiated by a report in the literature comparing the different compostitions of the *S. cerevisiae* and *R. glutinis* cell walls.[14] The *S. cerevisiae* cell wall is composed mainly of β-glucan and α-mannan in contrast to *R. glutinis* whose cell wall is composed of a glucomannon whose structure is not characterized because of its resistance to common glucanases. Another protocol (Protocol 5) employed Novozym 234, a mixture of lysing enzymes containing cellulase, protease and chitinase activities. This protocol had been used to isolate DNA from *Rhodosporidium toruloides*, a strain of yeast closely related to *R. glutinis*.[5] Unfortunately, this protocol also failed to lyse *R. glutinis* cells and no DNA was obtained.

In all other protocols mechanical force was used to rupture the cells. Cells were either ground in liquid nitrogen and then suspended in lysing buffer, or suspended in lysing buffer and vortexed with glass beads. Cell homogenates were then extracted several times with phenol and chloroform/isoamyl alcohol (24:1 [vol/vol]) to remove proteins. Nucleic acids were precipitated with ethanol or isopropyl alcohol, resuspended, and incubated with RNase A. Yields varied from 0.3-0.6 mg DNA per gram cells.

Genomic DNA isolated by Protocols 1, 2, 3, and 6 ran near the 23 kb marker on 1% agarose gels as expected. A_{260}/A_{280} ratios were within the normal range (1.8-2.0) with the exception of Protocol 6 which yielded DNA with a lower ratio (1.7). This DNA appeared to be contaminated with an impurity which rendered the DNA resistant to digestion by restriction enzymes. Protocol

3 provided the highest yield of DNA and was the primary source of DNA used in the experiments discussed below.

POLYMERASE CHAIN REACTION

One of the main focuses of the cloning projects has been use of the polymerase chain reaction (PCR) with degenerate primers based on the peptide information to amplify a region of the thymine hydroxylase gene from *R. glutinis* genomic DNA. This amplified region of the gene could in turn be used as an exact match probe for Southern blots, or used to design additional primers based on the codon usage to amplify other regions of the gene. The typical haploid yeast genome is only 3-4 times larger than a bacterial genome and introns, if present at all, tend to be small and range from 50 bases to several hundred bases in length. Assuming that the *R. glutinis* genome is approximately 14,000 kilobases, PCR primers must be at least 12 bases to correspond to a unique sequence (see Figure 2-3). Based on this calculation, we had sufficient peptide information for the design of multiple primers.

Figure 2-3. Calculation showing the minimum length of an oligonucleotide necessary to specify a unique sequence in a yeast genome.

$$4^x = 14 \times 10^6$$

$$x(\log 4) = \log 14 \times 10^6$$

$$x(0.6021) = 7.146$$

$$x = 11.8$$

Primer Design

Oligonucleotide primers for PCR were designed by back-translation of peptide sequence and were synthesized with either mixed bases or inosines in

positions of codon degeneracy (see Figure 2-4). Forward primers were designed with the anti-sense strand sequence, and reverse primers with the sense strand sequence.

Unfortunately many of the amino acids in the sequenced peptide fragments have highly degenerate codons (i.e. serine-- 6 codons, leucine-- 6 codons) so, in designing the primers the information yielding the least-degenerate primers was used. PCR primers were designed without including the translated information at the 3'-end. Any PCR product could easily be identified as either the correct product or a product resulting from non-specific binding by reading the 9-12 bases following the primer sequence.
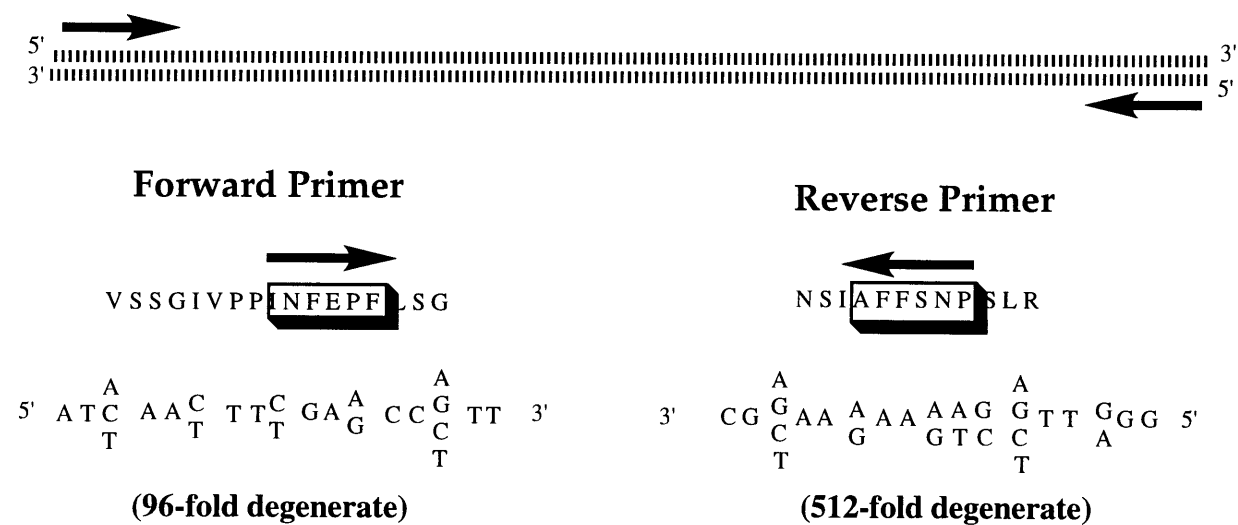
Figure 2-4. PCR Primer Design

Shown below are the primers designed by Dr. Lai which were used in the earliest PCR reactions (Table 2-3). He designed a single forward primer from the N-terminus information and 6 reverse primers from the remaining peptide fragments.

Table 2-3. PCR Primers Designed by Dr. Lai ('*' indicates mixture of 4 bases)

Primer ID	Sequence (5'-->3')	length/degeneracy
NTERM#1 **INFEPF**		
	AT (T/A/C) AA (T/C) TT (T/C) GA (A/G) CC*TT	17/96
MRP#1 **ETYTK**		
	(T/C) TT*GT (A/G) TA*GT (T/C) TC	
	15/128	
MRP#2 **QETDPEA**		
	(T/C) TC*GG (A/G) TC*GT (T/C) TC (T/C) TG	18/256
MRP#3 **LPADGDP**		
	CAT (T/C) TC*A (A/G) *GG (A/G) TC*CC (A/G) TC	21/1024
MRP#4 **TQQFFDL**		
	A (A/G) (A/G) TC (A/G) AA (A/G) AA (T/C) TG (T/C) TG	17/64
MRP#5 **PMEVK**		
	(T/C) TT*AC (T/C) TCCAT*GG	15/64
MRP#6 **DEFAQL**		
	A (A/G) (T/C) TG*GC (A/G) AA (T/C) TC (A/G) TC	17/128

Three additional forward primers and a single reverse primer were designed to incorporate either a singly degenerate codon or a G/C clamp on the 3' end of the oligonucleotide (Table 2-4). This region of the primer is the most critical for

Table 2-4. Additional Primers

Primer ID	Sequence (5'-->3')	length/degeneracy
FP#1 **DPLEM**		
	GA (T/C) CC* (T/C) T*GA (A/G) ATG	15/96
FP#2 **FDLPM**		
	TT (T/C) GA (T/C) (T/C) T*CC*ATG	15/96
FP#3 **ETDPEA**		
	GA (A/G) AC*GA (T/C) CC*GA (A/T) GC	17/128
RP#1 **WSNDV**		
	AC (A/G) TC (A/G) TT* (G/C) (A/T) CCA	14/24

controlling specificity in the annealing step and aiding binding of the primer to its target sequence. Two forward primers (FP#1 and FP#2) were designed to contain the singly degenerate codon for methionine at the 3' end and a third (FP#3) chosen for it's high G/C content. The reverse primer (RP#1) was designed to use the singly degenerate tryptophan codon.

Once the sequence homology had been discovered between the TH peptide fragments and gibberellin 20-oxidase (see discussion below), the N-terminus primer was redesigned (NTERM#2) from sequence information with a higher G/C content and an additional reverse primer was ordered (KRP#4). The new reverse primer was designed from the active site peptide information. The sequence alignment suggested that the active site was located close to the C-terminus of the protein and that these primers could potentially lead to the amplification of a large part of the gene.

Table 2-5. N-Terminus and Active Site Primers

Primer ID	Sequence (5'-->3')	length/degeneracy
NTERM#2 **GIVPP** GG*AT(T/C/A)GT*CC*CC		14/192
KRP#4 **PNSFFA**		
GG(A/G)TT*(G/C)(A/T)(A/G)AA(A/G)AA*GC		17/512

Another set of primers was designed with inosines in the third position of each codon and mixtures of bases in positions with double degeneracy. Even with the base mixtures in some positions, the primers were only 16 and 64-fold degenerate. To compensate for the lack of specificity in the third position, these primers were longer than all other primers used in this project.

Table 2-6. Inosine Primers

Primer ID	Sequence (5'-->3')	length/degeneracy
IFP **SSGIVPP**	(T/A)(G/C)I(T/A)(G/C)IGGIATIGTICCICC	20/16
IRP **RLSPNSFFA**	IC(G/T)IA(A/G)I(G/C)(A/T)IGGITTI(G/C)(A/T)IAAIAAIGC	27/64

A set of singly degenerate primers was designed for this cloning work by Dr. Loredano Pollegioni. He recently cloned the gene for D-amino acid oxidase (DAAO) from *Rhodotorula gracilis*, a close relative of *R. glutinis*. Dr. Pollegioni claimed that the use of singly degenerate primers had been key to his success. These primers were based on the codon preferences of *R. gracilis* found in the DAAO gene.

Table 2-7. Singly Degenerate Primers Based on *R. gracilis* Codon Preferences

Primer ID	Sequence (5'-->3')	length/degeneracy
GFP#1 **VSSGIV**	GTCTCGTCGGGCATCGTC	18/1
GFP#2 **VTQETD**	GTCACGCAGGAGACGGAC	18/1
GRP#1 **KLVDN**	CTTGAGGACGTCGTTCG	17/1
GRP#2 **SFFAIS**	CGAGAAGAACGCGATCG	17/1

The last set of primers were designed to amplify a portion of the gene corresponding to a single peptide fragment. Forward and reverse primers were designed from the ends of the 2 longest peptide fragments (Figure 2-5, Table 2-8). These primers were designed such that the size of the expected product would be known, making identification of the correct product simple. In addition, short stretches of DNA sequence would be obtained and could be used to identify codon preferences and to design exact-match primers.

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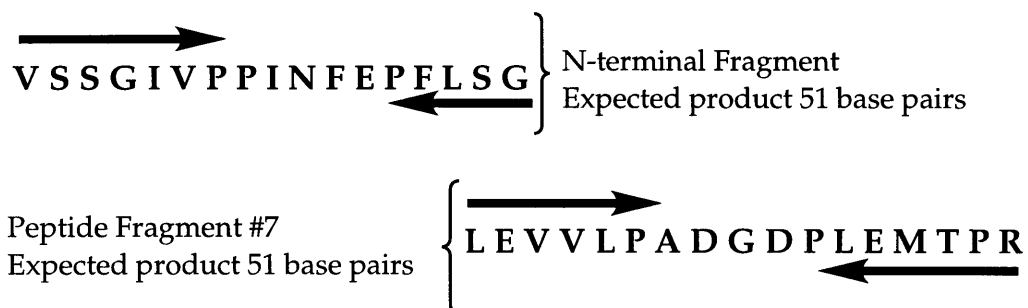
Figure 2-5. Peptide Fragment PCR

Table 2-8. Peptide Fragment Primers

Primer ID length/degeneracy	Sequence (5'-->3')	
FPC#1 **VSSGIVP**		
20/8	GTCTC (C/G) TC (C/G) GG (T/C) ATCGTCCC	
RPC#1 **GSLFP**		
15/8	(A/G) CC (G/C) GAGAGGAA (C/G) GG	
FPC#7 **LEVVLPA**		
20/8	CTCGAGGT (C/G) GT (G/C) CTCCC (C/G) GC	
RPC#7 **RPTMELP**		
	GCG (G/C) GG (C/G/A) GTCATCTCGAG (C/G) GG	17/12

Reaction Conditions and Analysis

Early PCR reactions were run with a single forward primer designed from the N-terminus sequence and 6 different reverse primers designed from the remaining peptide information (Table 2-1). Conditions such as salt concentration ($[MgCl_2]$=1.5-50 mM), annealing temperatures (25-50°C), and the ratio of forward to reverse primers in the reaction mixture were varied to find optimal reaction conditions. Salt concentration appeared to have negligible effect on the reactions.

Annealing temperatures were critical and in all reactions it was necessary to use a low annealing temperature in the first 10 reaction cycles to detect any products. The annealing temperature was then increased in the last 25 reaction cycles to encourage amplification of the correct product out of the mixtures produced in the first 10 cycles. Varying the ratios of forward to reverse primers is a technique used to account for the varying binding efficiencies of the forward and reverse primers and resulted in the appearance of different products in varying quantities.

Analysis of these reactions was difficult for two reasons. First, there were multiple products in all reactions. Second, the sequential ordering of the peptide fragments was unknown, and therefore the sizes of desired products was also unknown. To determine which of the multiple products were primer artifacts, 4 controls were run for each reaction; (-) template control, (-) forward primer control, (-) reverse primer control and (-) both primers control. By comparing the bands in the reaction and controls, primer artifacts were easily identified and could be eliminated from consideration as possible gene fragments. Of the bands which were both primer and template dependent it was impossible to know which product was the desired one without sequencing the DNA. A number of products from these early reactions were sequenced and turned out to be products resulting from non-specific binding events as established by the bases following the primer sequence.

Nested PCR and PCR Southerns

A search for proteins with sequence homology to the TH peptides identified a non-heme iron-containing dioxygenase, gibberellin 20-oxidase (GA20OX) which is involved in gibberellin biosynthesis.[15] The significant homology between these proteins throughout the entire GA20OX sequence is quite remarkable (Figure 2-6) and has been useful for designing PCR experiments. Based on the alignment we have been able to tentatively assign the order of the peptide fragments. This information allowed us to design new primers, choose new primer combinations for reactions, and estimate relative sizes of expected products.

Figure 2-6. Sequence alignment of TH peptide fragments with Gibberellin 20-oxidase

(GA20OX sequence in capital letters, TH fragments shown underneath)

```
1   MALNGKVATE  SAPSNLNEEM  KGEYRPPFGG  SDESKVPEDF  IWSEKFEASE

51 .LLPVLDVPTI  DLEKFMSGDK  SYVEEATRLV  DEACRQHGIF  FVVNHGVDIE
    :.    ||.|  ::|.|:||                          |.::.| |
    vssgivppi   nfepflsg                           vtqetdpe

101 MMGRVHDCMN  EFFTMPLDVK  QRAKRKVGES  YGYTNSFFGR  FASNLPWKET
    :::          .  :||.:|::||
    aiak        q   qffdlpmevk

151 FSLRCVAAQN  SSAAHDYVLD  TLGPSFSHHG  KAYQECGIAL  NELGTKIVEL

201 LGLSLGISRE  YFKNFFEDND  SILRLNYYPT  CDKPEVVLGT  GPHTDPTSVT

251 ILHQDPVSGL  QVCSNDQWYS  IPPNPEAFVI  NIGDTFTSLT  NGIYKGCIHR
                                       :::|:|.:.   |::.|
                                       lsetytkws   ndvlk

301 AVVNSMNARK  SLAFFLCPSH  DKVVRAPEEL  VEKSPPRKYP  DYKWPMLLEM
       .        |:|||  ||   : |:.|.::   :|..|.
       n        siaffsnps     evvlpadgdp lemtpr

351 TQKRYRPDCN  TLEAFKTWVQ  EGKALDTGST  ITAPSA
```

Figure 2-7. Primer Alignment with GA20OX Sequence and TH Peptide Fragments

```
  1  MALNGKVATE   SAPSNLNEEM   KGEYRPPFGG   SDESKVPEDF   IWSEKFEASE

 51  LLPVLDVPTI   DLEKFMSGDK   SYVEEATRLV   DEACRQHGIF   FVVNHGVDIE
```

GFP#2 ➞

IFP ➞

vssgivppi   nfepflsg   vtqetdpe FP#3

NTERM#2   NTERM#1   ⬅ MRP#2

GFP#1 ➞

```
101  MMGRVHDCMN   EFFTMPLDVK   QRAKRKVGES   YGYTNSFFGR   FASNLPWKET
```

FP#2 ➞

aiak   q   qffdlpmevk

⬅ MRP#4   ⬅ MRP#5

```
151  FSLRCVAAQN   SSAAHDYVLD   TLGPSFSHHG   KAYQECGIAL   NELGTKIVEL

201  LGLSLGISRE   YFKNFFEDND   SILRLNYYPT   CDKPEVVLGT   GPHTDPTSVT

251  ILHQDPVSGL   QVCSNDQWYS   IPPNPEAFVI   NIGDTFTSLT   NGIYKGCIHR
```

⬅ RP#1

lsetytkws ndvlk

⬅ MRP#1   ⬅ GRP#1

```
301  AVVNSMNARK   SLAFFLCPSH   DKVVRAPEEL   VEKSPPRKYP   DYKWPMLLEM
```

IRP ⬅   FP#1 ➞

n   siaffsnpslr evvlpadgdp   lemtpr

KRP#4 ⬅

⬅ GRP#2   MRP#3

```
351  TQKRYRPDCN   TLEAFKTWVQ   EGKALDTGST   ITAPSA
```

In addition, techniques such as nested PCR reactions and Southern blots were available for the analysis of reaction mixtures. In a nested PCR reaction, 1-3 μL of a reaction mixture containing multiple products or a single product

(isolated from an agarose gel slice) is used as the template for a second PCR reaction. Primers located within the original product are chosen for the nested reaction so that in a mixture of products only the desired product is amplified. Product mixtures separated on a gel for Southern blotting can be probed with an oligomer which is complementary to an internal sequence and used to identify single products from mixtures (Figure 2-8).

NESTED PCR

Template --
PCR reaction mixture
containing multiple products

2nd PCR reaction

Primers --
Internal to desired sequence amplified in first PCR reaction

Amplify correct product

Mixture of products from PCR reaction

SOUTHERN BLOT

1) Blot
2) Hybridize with Internal Probe(s)

Separate mixture on agarose gel

Identify correct product from mixture
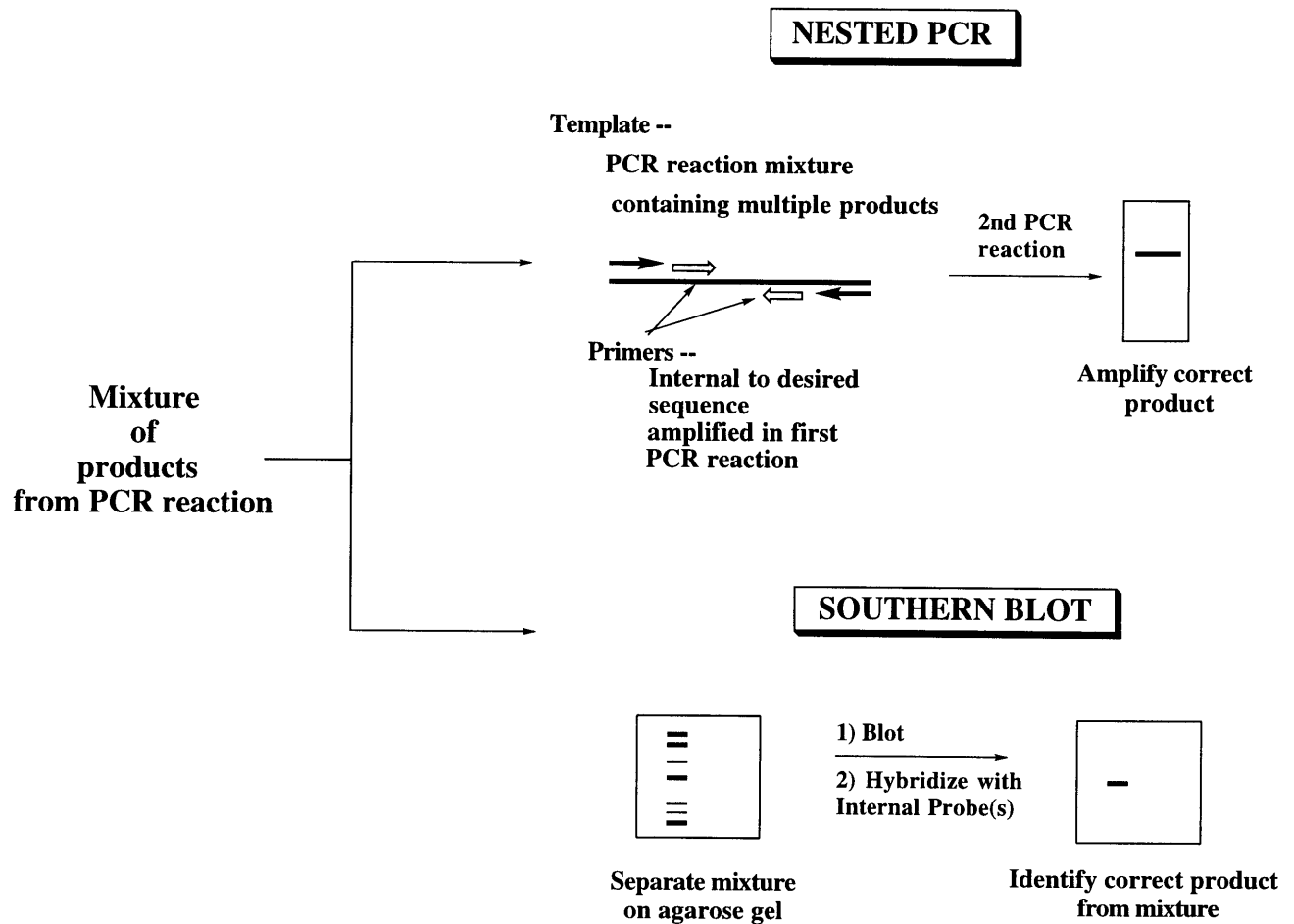
Figure 2-8. Methods of Analysis for PCR Product Mixtures

With the nested PCR and Southern blot techniques there seemed to be an increased chance of identifying the correct product. Unfortunately, even with the

alignment information the analysis of reaction products was not straightforward as had been anticipated. Many times a nested PCR reaction gave smaller products as expected, yet no hybridization was seen on the Southern blot. Other times no products were seen in the nested reactions. In one instance both nested reactions and Southern blots appeared to give positive results, however, sequencing revealed that the DNA was a product resulting from non-specific hybridization. PCR reactions with FP#3, KPR#4 and genomic DNA yielded two products at 2.4 kb and 0.9 kb in reactions that were absent from the controls (Figure 2-9).
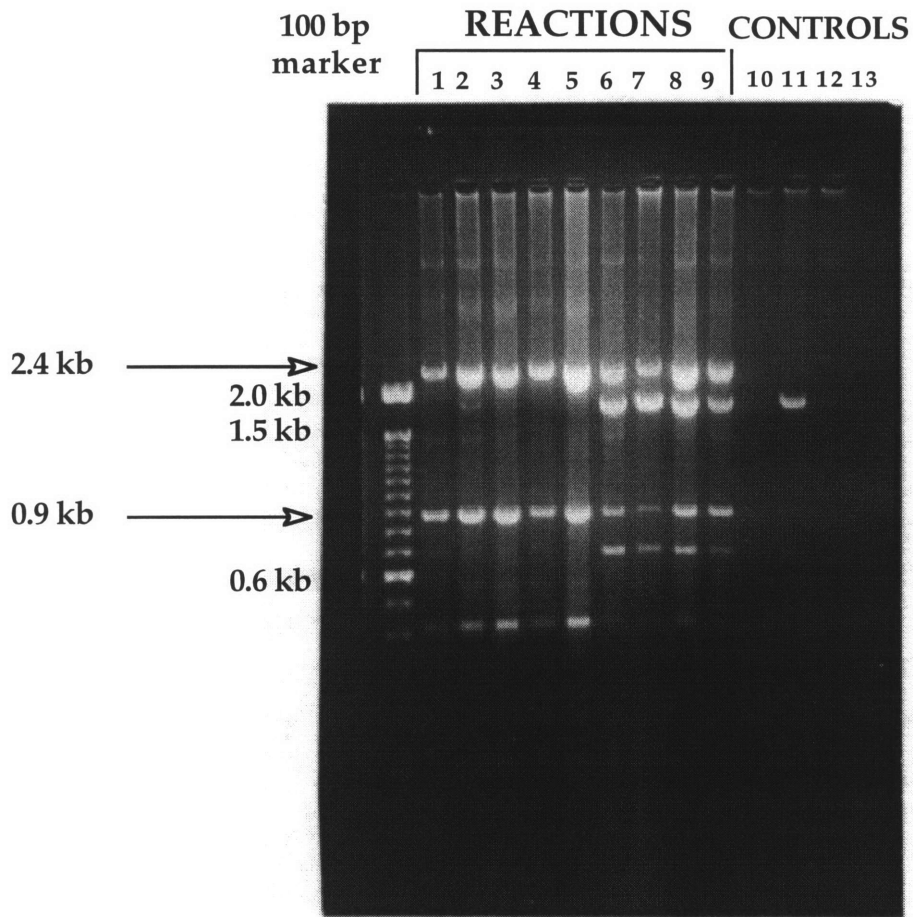
Figure 2-9. Agarose Gel Analysis of PCR (Genomic DNA Template, FP#3 and KPR#4) Lanes 1-9 show reactions with varying ratios of forward primer (FP#3) to reverse primer (KRP#4). Products at 2.4 and 0.9 kb looked promising. Another band between the 1.5 and 2 kb markers was seen in reaction lanes 2 and 6-9, however control lane 11 showed it was a single-primer dependent product.

REACTIONS		CONTROLS	
Lane	FP#3:KRP#4	Lane	Control
1	1:1	10	(-) primers
2	1:2	11	(-) KRP#4
3	1:3	12	(-) FP#3
4	1:4	13	(-)genomic DNA
5	1:5		
6	5:1		
7	4:1		
8	3:1		
9	2:1		

The PCR products were transferred to a membrane for Southern blotting with inosine probe WSNDVLK (Table 2-9) whose sequence is internal to the two primers. The 2.4 kb piece lit up with probe WSNDVLK and the membrane was stripped and re-probed with a second probe, QQFFDLPMEVK (Table 2-9) that should have also been included in the correct product. This probe showed hybridization to the 2.4 kb band, as well. Next, the 2.4 kb DNA was isolated from a gel slice for use as a template in nested reactions. In the nested reactions 11 different primer combinations were used of which 9 gave products that seemed reasonable sizes and 2 gave no products (Figure 2-10).
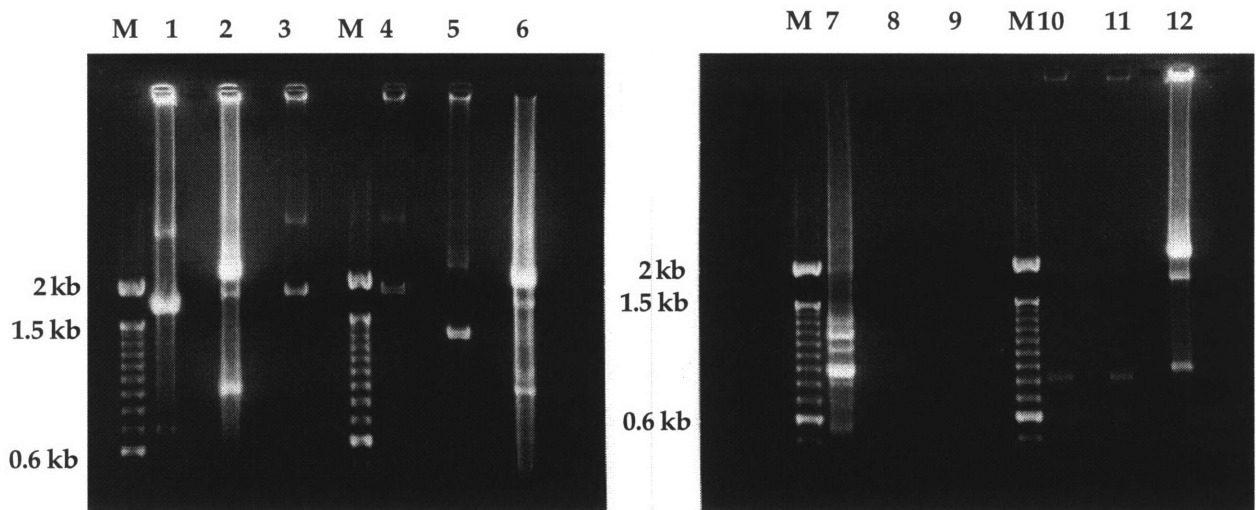
Figure 2-10. Agarose Gel Analysis of Nested PCR Reactions on 2.4 kb Product Generated from Reactions with FP#3, KRP#4, and genomic DNA

M= 100 bp DNA marker

Lane	FP	RP	Lane	FP	RP
1	KFP#3	KRP#1	7	GENEFP	KRP#4
2	KFP#3	IFP	8	KFP#	KRP#1
3	KFP#3	GRP#1	9	KFP#2	GRP#1
4	KFP#3	GRP#1	10	GENEFP	KRP#1
5	KFP#2	KRP#4	11	GENEFP	GRP#1
6	GFP#2	KRP#4	12	KFP#3	KRP#4[a]

[a] Original reaction primers

These results were encouraging and the original PCR product was sub-cloned into a vector for sequencing. Sequencing showed that although the primers had been incorporated into the 2.4 kb product, the rest of the sequence was not related to any of the TH peptide information including the 2 probe sequences. This was one of the first indications that the hybridization seen in all Southern blots was a non-specific, concentration-dependent phenomenon (see discussion under Southern Blots). Blots were rinsed until most of the radioactivity was washed off. What remained on the membrane was bound to the species present in the highest concentration and not necessarily the desired one.

Reactions run with some of the specially designed primers such as the inosine containing primers, singly degenerate R. gracilis-based primers, and internal peptide fragment primers did not result in amplification of any portion of the TH gene. Reactions with inosine primers generated no products. With the singly degenerate primers there were very few products (Figure 2-11) and then in nested reactions smears of products (Figure 2-12). Changing reaction conditions such as the $MgCl_2$ concentration and raising the primer annealing temperature in the thermocycler program did nothing to reduce the smears to discernable products (Figure 2-13). When these PCR gels were blotted with internal inosine probes no hybridization was seen. In reactions run with primers designed from the ends of a single peptide fragment results were uninterpretable. Reaction and (-) template controls contained identical products. Single primer + template controls showed many more products than seen in the reaction (Figure 2-14).

Figure 2-11. Agarose Gel Analysis of PCR Reactions Using Singly-Degenerate Primers
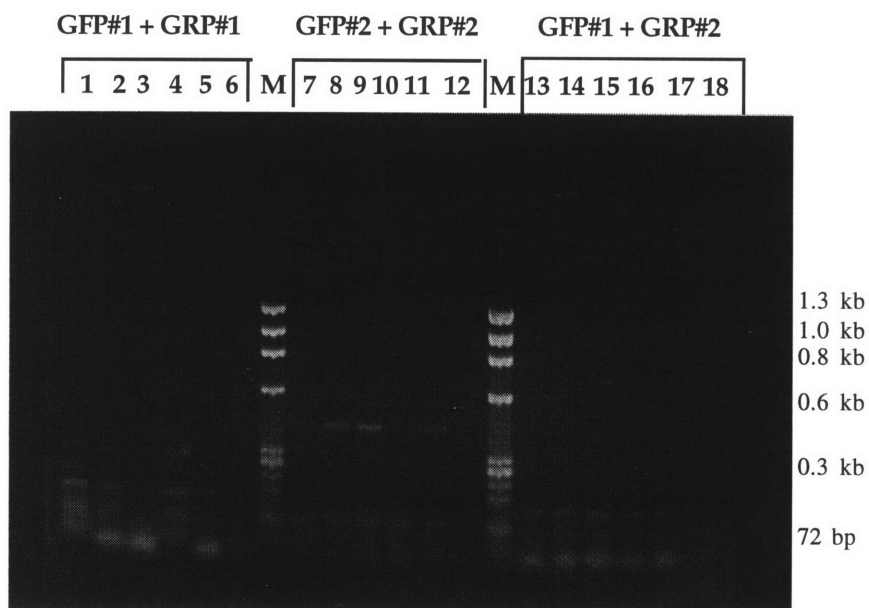
Lanes 1-5:   Reactions with GFP#1, GRP#1 and gDNA template.
Lane 6:   Control with GFP#1 and GRP#1, no DNA.

M: ΦX174 RF DNA/Hae III marker

Lanes 7-11:   Reactions with GFP#2, GRP#2 and gDNA template.
Lane 12:   Control with GFP#2 and GRP#2, no DNA.

M: λ DNA-BstE II Digest marker

Lanes 13-17: Reactions with GFP#1, GRP#2 and gDNA template.
Lane 18:   Control with GFP#1 and GRP#2, no DNA.

Figure 2-12. Nested PCR Reactions Using Singly-Degenerate Primers and Reaction Mixture Templates
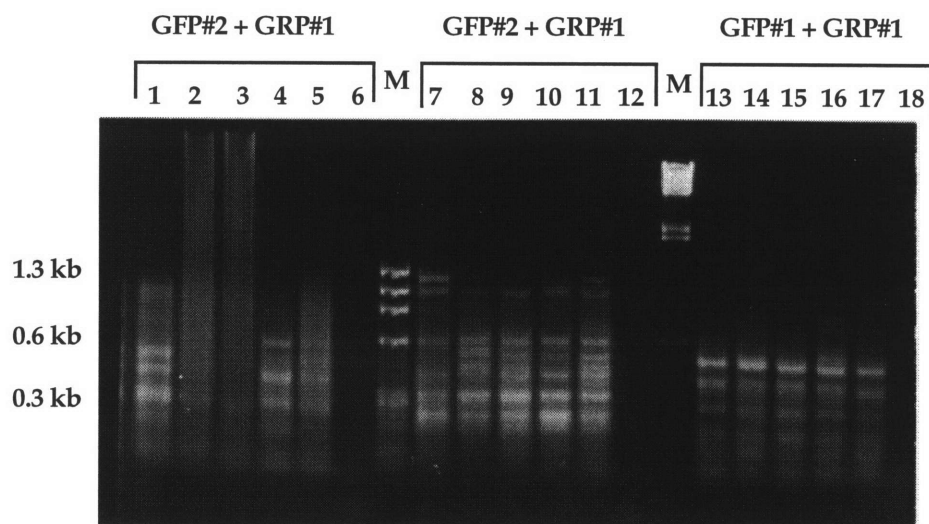
Lanes 1-6: Reactions with GFP#2, GRP#1 and 2 µL reaction mixtures 1-6 shown in figure 2-11 as the template.

M: ΦX174 RF DNA/Hae III marker

Lanes 7-12: Reactions with GFP#2, GRP#1 and 2 µL reactions 7-12 shown in figure 2-11 as the template.

M: λ DNA-BstE II Digest marker

Lanes 13-18: Reactions with GFP#1, GRP#1 and 2 µL reactions 13-18 shown in figure 2-11 as the template.

Figure 2-13. Affects of Changing Annealing Temperature and MgCl$_2$ Concentrations on Reaction Products Seen in Nested PCR Reactions with Singly-Degenerate Primers

Raising the primer annealing temperature from 40°C to 50°C did not result in a reduction of the number of products seen in these reactions. Reactions shown in the gel below are set up as those described in figure 2-12.
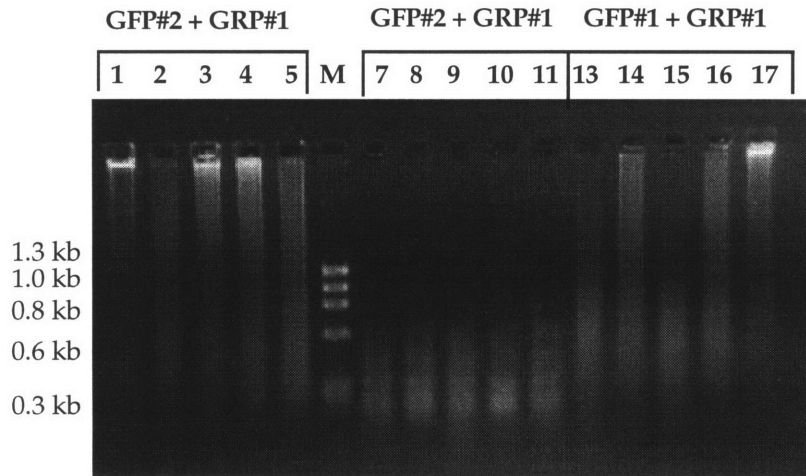
Raising the MgCl$_2$ concentration from 1.5 mM to 3-12 mM also did nothing to reduce the number of products seen in the nested reactions.
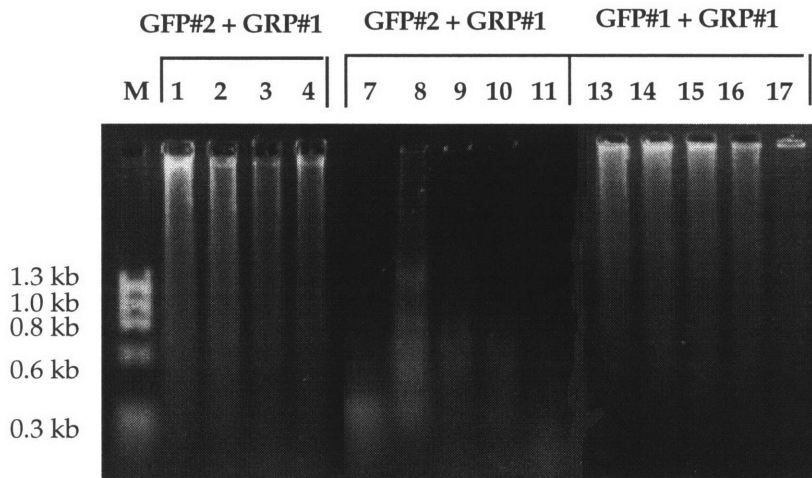
Lane	[MgCl$_2$]	Lane	[MgCl$_2$]	Lane	[MgCl$_2$]
1	3 mM	7	3 mM	13	3 mM
2	6	8	6	14	6
3	9	9	9	15	9
4	12	10	12	16	12

Figure 2-14. Agarose Gel Analysis of Internal Peptide Fragment PCR reactions with Genomic DNA, FPC#1 and RPC#1
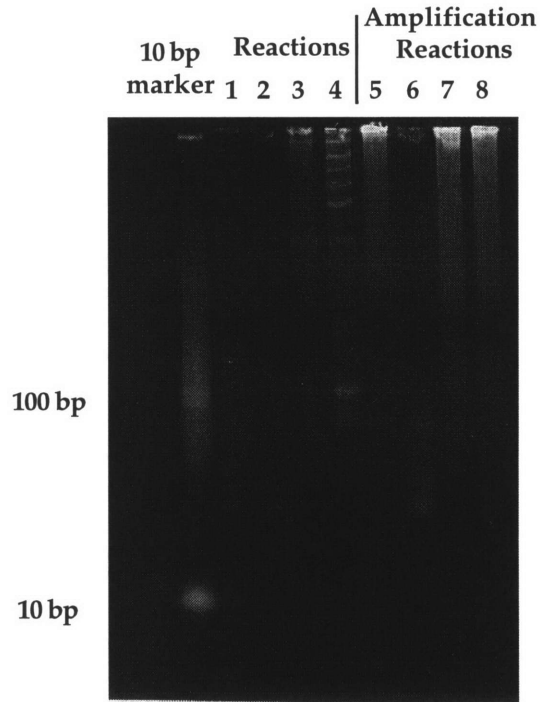
Lane	FP	RP	template
1	FPC#1	RPC#1	gDNA[a]
2	FPC#1	RPC#1	--
3	FPC#1	--	gDNA
4	--	RPC#1	gDNA
5	FPC#1	RPC#1	2 µL rxn mixture 1[b]
6	FPC#1	RPC#1	--
7	FPC#1	--	2 µL rxn mixture 1
8	--	RPC#1	2 µL rxn mixture 1

[a] gDNA = genomic DNA

[b] Lanes 5-8 show results from the amplification reactions using the reaction mixture generated with FPC#1, RPC#1 and gDNA (shown in lane 1) as the template.

SOUTHERN BLOTS OF RESTRICTION DIGESTS

Restriction Digests

Unlike other eukaryotic genomic DNA digests which appear as smears on ethidium-bromide stained gels, typical yeast restriction digests show discrete bands with the most intense bands corresponding to 2μm plasmid and tandemly repeated ribosomal RNA genes.[16] Restriction digests of R. glutinis DNA showed little digestion in many cases (EcoR I, Hind III, Xba I, Nde I) or gave smears containing some descrete bands (Pst I, Bgl I, Sma I, Nco I, Sau3A I, Hae III). Inefficient digestion was problematic in early Southern blots. Reaction conditions were varied to find appropriate conditions for digestion. Variables such as the isolation protocol for the DNA, the units of enzyme added to the reaction, the total reaction volume, and the time the reaction ran were examined. The DNA isolation protocol had the most dramatic effect on digests and suggested that an impurity in the DNA was at least partially inhibiting the restriction enzymes. Other factors that may also contribute to the inefficient digestion include a low number of the common restriction sites in R. glutinis DNA or DNA modifications preventing enzyme recognition of cut sites.

Southern Probes and Detection Systems

Southern probes were designed with both mixed bases and inosine in positions of redundancy. Probes were designed using the longest possible sequences with the lowest possible codon usage. Shown below (Table 2-9) are the probe sequences with the length, degeneracy and the number of potential hybridization sequences (taking into account that inosine base-pairs with all 4 bases).

In early experiments, probes were labeled with a non-radioactive oligolabeling method and detection system developed by Amersham. This

detection system uses the enhanced chemiluminescence associated with the horseradish peroxidase catalyzed oxidation of luminol to detect the presence of oligos labeled with fluorescein-11-dUTP. Detection solution 1 decays to hydrogen peroxide, the substrate for HRP. Reduction of hydrogen peroxide by the enzyme is coupled to a light producing reaction by detection reagent 2 which contains luminol (Figure 2-15).

Figure 2-15. Enhanced Chemiluminescence Reaction

luminol   3-aminophthalate
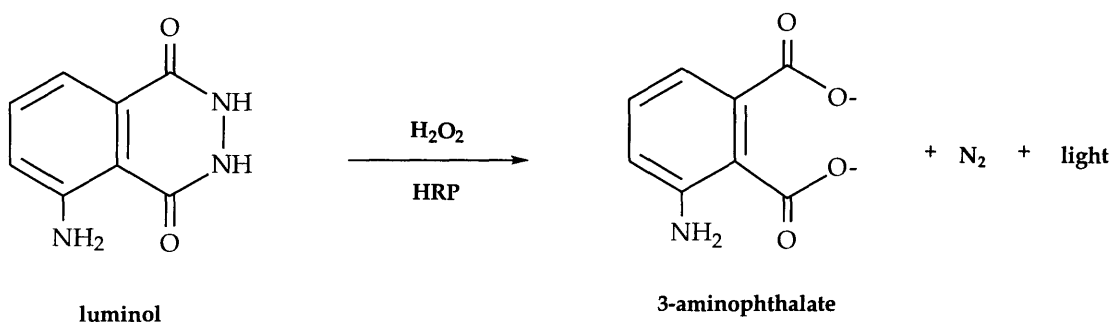
Oxidation of the luminol produces the blue light that is detected by blue-light sensitive film. Positives were never seen on Southern blots of restriction digests probed with this labeling and detection system. Wash conditions recommended by the maufacturer were probably too stringent for the short probes being used.

With the switch from the Amersham labeling and detection system to radiolabeled probes, hybridization of probes to Southern blots of restriction digests was finally observed. One advantage of using radiolabeled probes was that the extent of washing could be closely monitored by a Ludlum Measurements radioactivity survey meter. Stringency of the blot washes could therefore be easily adjusted for each blot and probe.

Table 2-9. Inosine Probes for Southern Blots

Probe Sequence (5'-->3') sequences	length/degeneracy # of complementary
QQFFDLPMEVK	
CAICAITTITTIGAI(C/T)TICCIATGGAIGTIAA	32/2
	524,288
NSIAFFSNPSLR	
AAI(A/T)(G/C)IATIGCITTITTI(A/T)(G/C)IAAICCI(A/T)(G/C)I (T/C)TI(A/C)GI	33/256
	4.3×10^9
PADGDPLEM	
CCIGCIGAIGGIGAICCIITIGAIATG	27/1
	262,144
SSGIVPP	
(T/A)(G/C)I(T/A)(G/C)IGGIATIGTICCICC	20/16
	65,536
QQFFDLPMEV	
CA(A/G)CA(A/G)TT(C/T)TT(C/T)GA(C/T)(C/T)TICCIATGGA(A/G)G T	29/128
	2048
WSNDVLK	20/32
TGG(T/A)(G/C)IAA(C/T)GA(C/T)GTI(C/T)TIAA	2048

Southern Blots

Due to the lack of digestion, the only hybridization seen on the first blots was to uncut DNA at the 23kb marker (Figure 2-16). Faint bands at lower molecular weights looked promising and prompted the optimization of digest conditions (see Restriction Digest discussion). After altering reaction conditions

to get more thorough digestion, hybridization was observed in regions from 5-9 kb (Figure 2-17).

Figure 2-16. Example of Inefficient Restriction Digests of Genomic DNA and a Southern Blot of the Digests Probed With Probe QQFFDLPMEVK

LANE #	RESTRICTION DIGEST
1	none- uncut gDNA
2	Pst I
3	Bgl I
4	Hind III
5	Nde I
6	EcoR I
7	Sau3A I
8	Xba I
9	Hae III
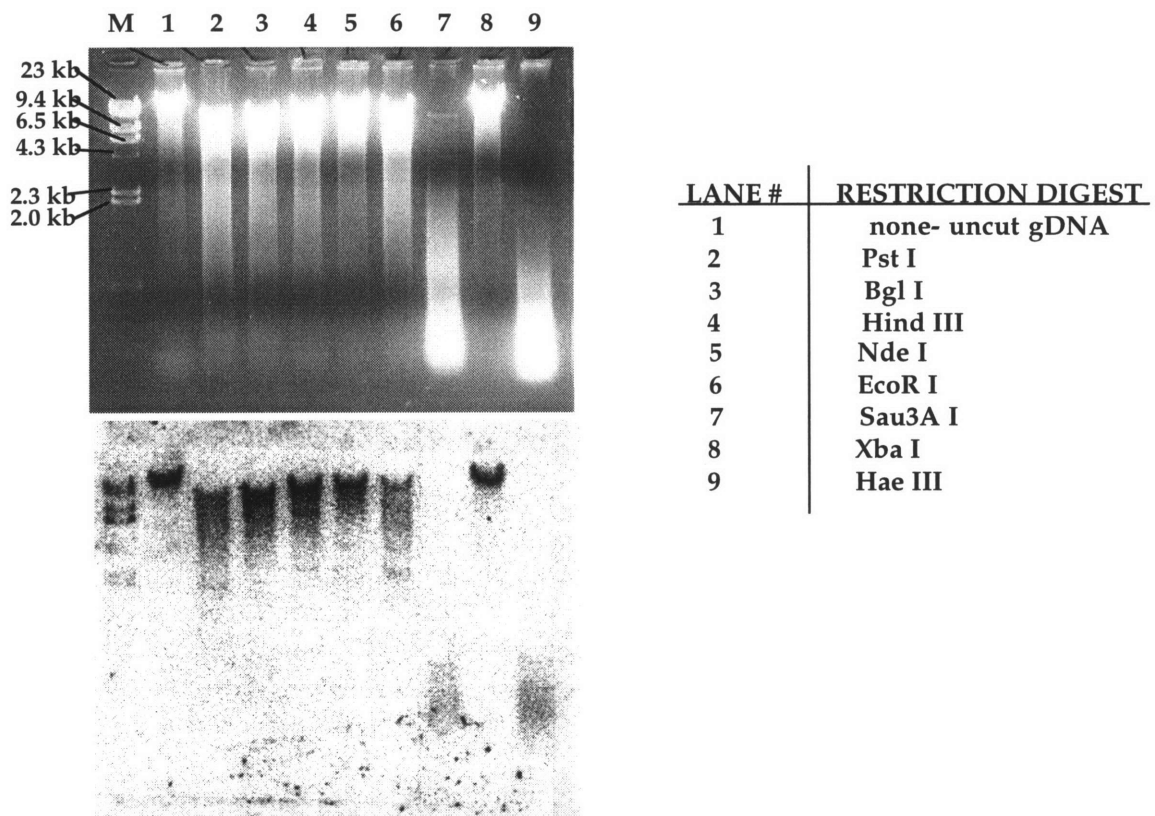
Figure 2-17. Example of Improved Restriction Digests of Genomic DNA and a Southern Blot of the Digests Probed With Probe QQFFDLPMEVK

With improved restriction digestion of the DNA there was an increased concentration of DNA fragments at smaller sizes. Hybridization bands were finally seen on Southern Blots below the 6.5 kb marker. Arrows point to some of the bands seen in this Southern Blot.

LANE #	RESTRICTION DIGEST
1	EcoR I
2	Pst I
3	Bgl II
4	Sma I
5	Nco I
6	Nde I
7	uncut gDNA
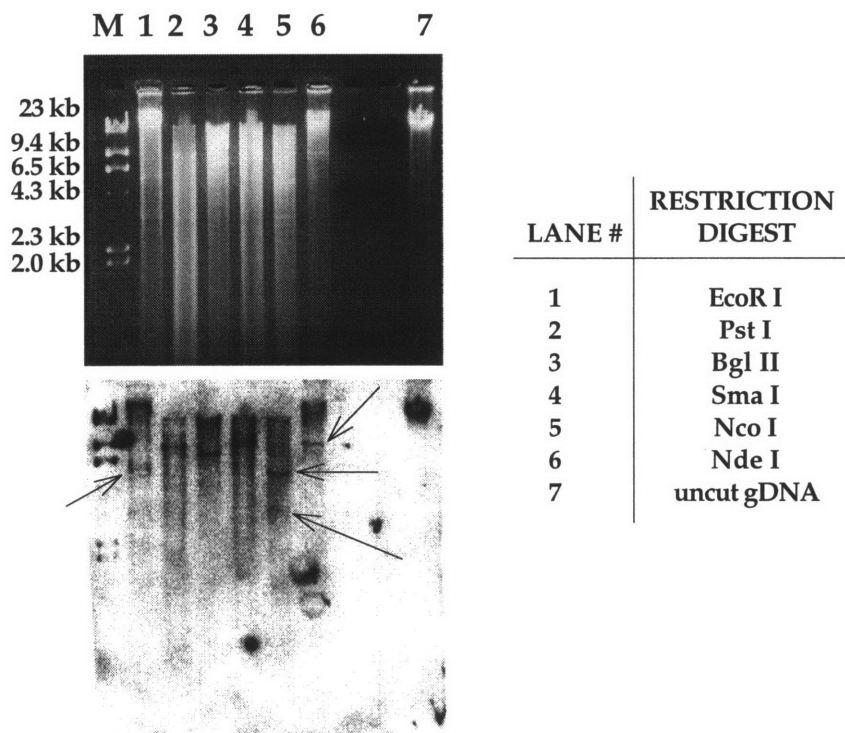
Nco I, Bgl I, and Pst I digests looked especially promising and Nco I digests and Pst I + Bgl I double digests were run for another Southern blot. The agarose gel for this Southern blot was loaded with 2 identical sets of lanes so that after the DNA fragments were transferred to the membrane, the membrane could be cut into two identical halves. One half was then probed with probe QQFFDLPMEVK and the other probed with probe PADGDPLEM (Table 2-9). Unfortunately, a careful comparison of the hybridization bands seen in these two blots and the bands seen on the ethidium bromide-visualized agarose gel of the digests revealed that the hybridization was non-specific (Figure 2-18). Wherever there was a discrete band in the restriction digest, there was a corresponding band on the membrane.

Figure 2-18. Southern Blot of Restriction Digests Probed Simultaneously with Two Different Probes

Agarose Gel of Restriction Digests
for Southern Blot

Lanes 1: Pst I + Bgl II Double Digests
Lanes 2: Nco I Digests
M: Lambda DNA Hind III Digest

Gel half probed with
probe QQFFDLPMEV

Gel half probed with
probe PADGDPLEMT
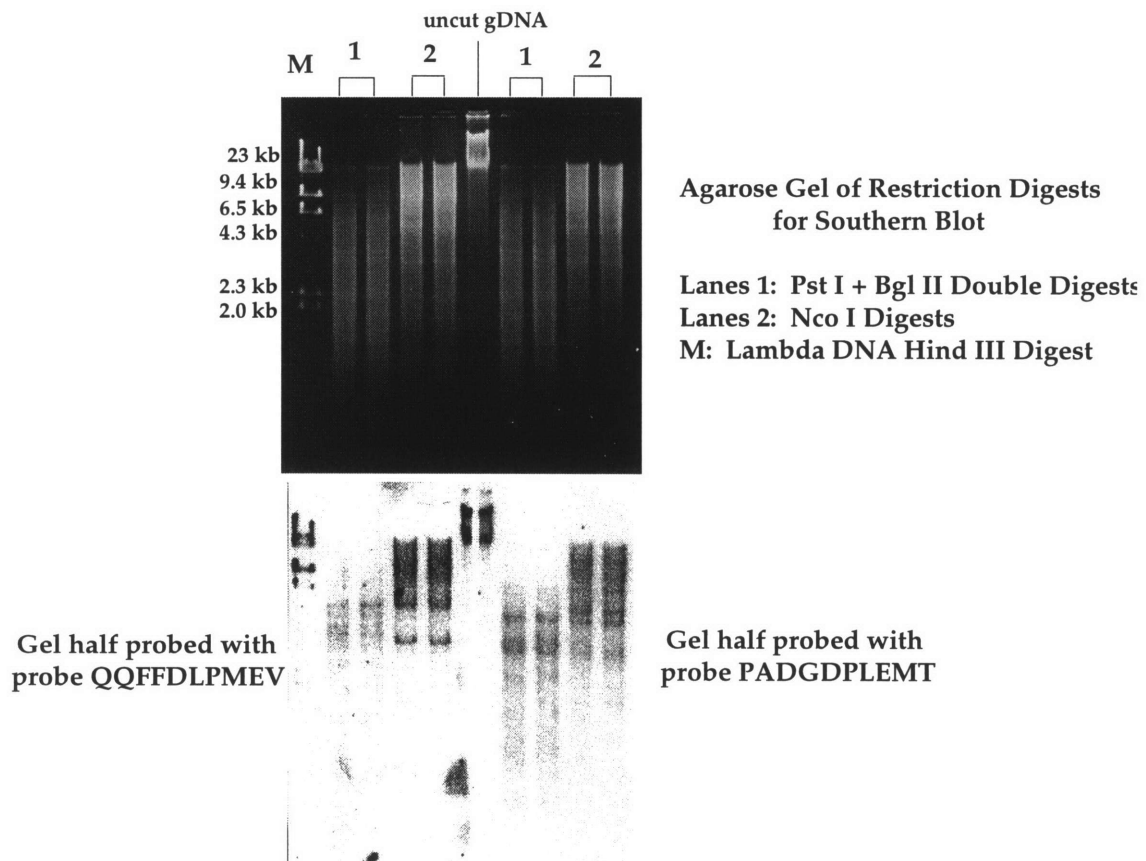
RNA ISOLATION

During the course of this work, we discovered that Dr. Pilone and co-workers at the University of Milan had recently cloned the gene for D-amino acid oxidase (DAAO) from the yeast *Rhodotorula gracilis*.[17] These researchers had amplified the sequence by PCR using primers designed from peptide sequence data. Instead of using genomic DNA as the template in amplification reactions, however, they used single-stranded cDNA. We decided to try this approach and began isolating mRNA from *R. glutinis* for single-stranded cDNA synthesis. We were also interested in the possibility of constructing a cDNA library. It is not unusual for fungal genes to have several introns ranging from 50 to several hundred base pairs each and all *Rhodotorula* genomic DNA sequences reported in the literature contain introns.[3-7] Working with cDNA would ensure that introns were not complicating the cloning work.
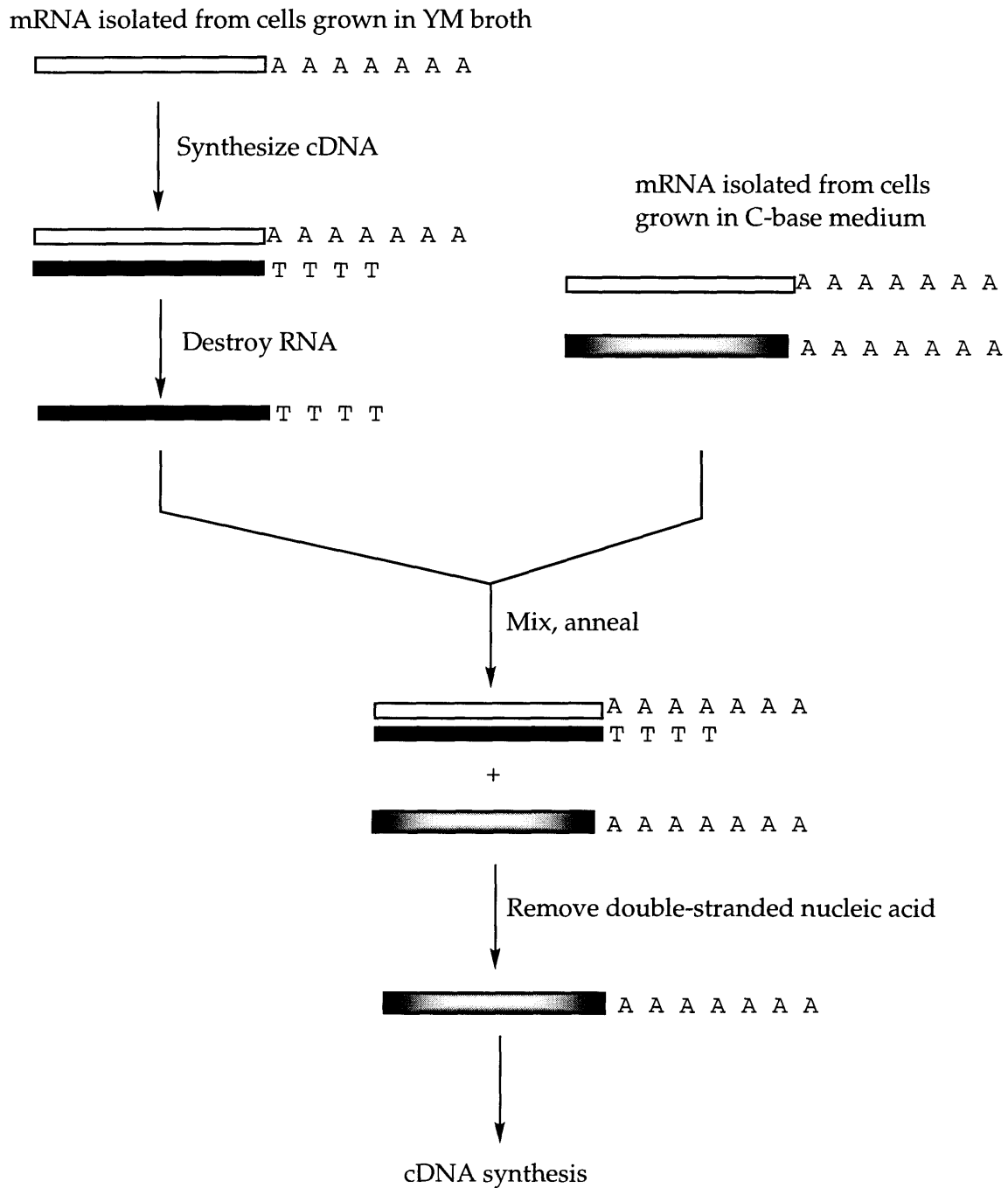
A standard guanidine isothiocyanate protocol (Gibco BRL) and an acid phenol/SDS extraction protocol were both tested for RNA isolation. The acid phenol/SDS protocol was more efficient of the two and increased the yield from 0.2 to 1.0 mg total RNA per gram cells. Yields from separation of mRNA from total RNA by oligo-dT cellulose chormatography were poor. To check whether the desired mRNA was present in the isolated samples, a Northern blot was probed with the reverse complement of Southern probe QQFFDLPMEVK. No hybridization was detected on the Northern blot and synthesis of cDNA was not pursued.

At this point the focus of the cloning work was directed toward obtaining additional peptide information (Chapter 3). The work with RNA, however, is worth pursuing in the future. The methods used for isolating total RNA and separating mRNA from total RNA need to be optimized to increase yields and improve stability of samples. In addition to the benefit of getting rid of introns

within the gene sequence, working with mRNA is attractive because of the controls that can be run to verify the presence of the desired sequence in the samples isolated. For example the presence of the TH gene in a RNA preparation can be verified by *in vitro* translation of the sample followed by the enzyme assay or by probing a Northern blot. Once this information is verified, the possibilities of working with mRNA and cDNA are numerous.

One method worth investigating is the construction of a subtraction library (Figure 2-19). Subtractive hybridization techniques result in the enrichment of RNA species that are present in one type of cell (type A) but absent in another (type B).[18] The difference between types A and B could be caused by a mutation in one of the cell types or by the different treatment of the two cell types to induce mRNA synthesis in one but not the other. To construct a subtraction library mRNA is isolated from both types of cells and single-stranded cDNA is synthesized with an oligo-dT primer on the type A cells. The RNA of these duplexes is destroyed leaving the single-stranded cDNA. This cDNA is mixed with the mRNA

mRNA isolated from cells grown in YM broth

```
[============]A  A  A  A  A  A  A
```

Synthesize cDNA

```
[============]A  A  A  A  A  A  A
[████████████]T  T  T  T
```

mRNA isolated from cells
grown in C-base medium

```
[============]A  A  A  A  A  A  A
[████████████] A  A  A  A  A  A  A
```

Destroy RNA

```
[████████████]T  T  T  T
```

Mix, anneal

```
[============]A  A  A  A  A  A  A
[████████████]T  T  T  T
```

+

```
[████████████] A  A  A  A  A  A  A
```

Remove double-stranded nucleic acid

```
[████████████] A  A  A  A  A  A  A
```

cDNA synthesis

```
[======]A  A  A  = mRNA present in cells grown in both YM broth and C-base medium

[██████]A  A  A  = mRNA's enriched in cells grown in C-base medium

[██████]T  T  T  = cDNA synthesized from mRNA
```

Figure 2-19. Subtractive Hybridization

from type B cells and the two are allowed to anneal. The mRNA unique to or present in higher quantities in type B cells will not be able to anneal to the cDNA from the type A cells and will remain single-stranded. After the annealing step the single- and double-stranded species are separated on a hydroxyapatite column which binds double stranded nucleic acids more tightly than single-stranded nucleic acids. The single-stranded mRNA is recovered and used for cloning work. Of course, the usefulness of this technique depends on whether our gene is regulated at the level of transcription or translation. This could be verified by comparing Northern blots of mRNA isolated from YM growths and C-base medium growths.

CONCLUSION

All experimental analysis revealed that the hybridization seen in Southern blots and products in PCR reactions were the result of non-specific hybridization events. It is possible that the peptide information we had for the design of primers for PCR reactions and probes for Southern blots was not sufficient for this cloning project so at this point the cloning work was put on hold until more peptide information could be obtained. With additional peptide information there is the potential of designing new PCR primers and hybridization probes from longer and less degenerate sequences. There might also be additional opportunities to try PCR within a single peptide fragment. Synthesis of the gene is yet another option if the entire protein is sequenced. In addition to obtaining additional peptide information, there are many possibilities for work with mRNA and cDNA that have not yet been examined and may prove to be the key to success in the cloning of the TH gene.

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Chapter 3

Purification of Thymine Hydroxylase for Additional Peptide Sequencing:

Improved Purification with Perfusion Chromatography

In addition to isolating TH for peptide sequencing as part of the cloning project, we were interested in isolating protein for some preliminary spectroscopic studies on the iron center of TH using EPR and stopped-flow UV-vis spectroscopy. We had not anticipated the amount of time and effort the cloning project would require and wanted to start experiments to help determine whether cloning the gene for over-expression would be time well spent.

Recently our lab obtained a BioCAD system from PerSeptive Biosystems. Used in conjunction with the POROS Perfusion Chromatography resins, this system can be used to develop highly optimized purifications quickly. Traditionally, the final step of the enzyme isolation has involved chromatography on a MonoQ FPLC anion exchange column. This step results in 85% pure protein; however, recoveries are poor and the enzyme is not stable to storage. Development of a purification that minimizes the time the enzyme spends dilute while increasing both its stability and purity is absolutely necessary for future experiments.

This chapter provides an overview of the TH enzyme isolation procedure and activity assay. In addition, a detailed description of the methods used and results obtained in the protein purification with Biocad POROS 20HQ resin is included. A sample of Biocad-purified TH was submitted to the MIT biopolymers sequencing facility and the results from this peptide sequencing are reported.

MATERIALS AND METHODS
Materials

PEG-8000, protamine sulfate (grade X, salmon), Sephadex G-25 and G-100 resins, molecular weight standards for SDS-PAGE, hyamin hydroxide (1M

solution in methanol), monosodium α-ketoglutarate, thymine, and sodium ascorbate were purchased from Sigma. Ammonium sulfate and ferrous sulfate were purchased from Aldrich. Protein assay dye reagent was supplied by Bio-Rad. DEAE 52 resin was supplied by Whatman. Center wells and red rubber septa for assay vials were purchased from Kontes (cat# 882320-0000) and Aldrich (cat# Z12744-2), respectively. Radiolabelled α-ketoglutarate ([1-^{14}C]-αKG, 59 μCi/μmol) was supplied by New England Nuclear. Scintillation fluid was purched from Packard (Scint-A XF and Poly Fluor). PerSeptive Biosystems supplied all perfusion resins.

Large-scale Growth of *R. glutinis* in C-base Medium

All OD's were taken by diluting cultures in a solution of PEG-8000 (76 mg/mL) and multiplying the absorbance at 600 nm by the dilution factor.

Large-scale C-base growths were started in YM broth. YM broth (1.5 mL) was innoculated with a loop of cells from actively growing YM agar plates and incubated at 26°C with shaking (detailed description of start-up growths in Chapter 2 Methods). After 24 h the saturated YM cultures (OD_{600}=30) were used to innoculate 15 mL C-base medium in 125 mL flasks. At 29 h the cells had reached late log phase (OD_{600}=23) and were transfered to 1 L flasks containing 150 mL C-base medium. After 30 h the cells (OD_{600}=18) were transferred to 1.5 L medium in 6 L shake-flasks. At 27 h (OD_{600}=12) the 1.5 L cultures were either harvested or used to innoculate the 10 L fermentor. Fermentor growth continued for 33 h until OD_{600}=12. Cells were harvested using a Sharples continuous-flow centrifuge and then washed twice in 50 mM Tris, 0.1 mM EDTA solution and centrifuged to pellet. Washed cells were frozen in liquid nitrogen and stored at -70°C until needed.

BUFFERS AND SOLUTIONS

0.1 M EDTA solution, pH 8

$Na_2 \cdot EDTA \cdot 2H_2O$ (3.7 g) was mixed with 70 mL H_2O. NaOH (5 M) was added

until EDTA dissolved and the solution was diluted to 100 mL with H_2O.

0.1 M glycine, 0.1 mM EDTA solution

Glycine (60 g) was dissolved in 8 L H_2O containing 8 mL 0.1 M EDTA solution.

1 M potassium phosphate

1 M KH_2PO_4-- 136 g dissolved in 1 L H_2O

1 M K_2HPO_4-- 212 g dissolved in 1 L H_2O

Column Buffer (20 mM potassium phosphate, 0.1 M glycine, 0.1 mM EDTA, pH 7.5)

A mixture of glycine/EDTA solution (5 L) and 1 M K_2HPO_4 (100 mL) was titrated to pH 7.5 with a mixture of glycine/EDTA solution (2 L) and 1 M KH_2PO_4 (40 mL).

0.5 M potassium phosphate, pH 7.5

K_2HPO_4 (261 g) was dissolved in 3 L H_2O and titrated to pH 7.5 with a 0.5 M solution KH_2PO_4 (68 g/L).

50 mM Tris, 0.1 mM EDTA, pH 8

Tris (12.11 g) was dissolved in 2 L H_2O plus 2 mL 0.1 M EDTA solution, pH 8.0 and the pH was adjusted with 1 M HCl.

COLUMNS (sizes given for 130 g preparation):

Sephadex G-25 (3 x 48 cm, 340 mL)

The Sephadex G-25 column was poured and packed with water, then equilibrated with column buffer. Conductivity and pH were checked at column inlet and outlet to verify that equilibration was complete. Total volume loaded onto this column should be less than 5% total column volume for good separation.

Column resin is cleaned after the isolation in a fritted funnel with 0.2 M NaOH until eluent is basic and then re-equilibrated with H_2O. Resin is stored at 4°C in 20% ethanol.

DE-52 (5.5 x 10 cm, 240 mL)

Following manufacturer's instructions, 90 g pre-swollen Whatman DE-52 resin was suspended in 225 mL 0.5 M potassium phosphate (pH 7.5). The suspension was allowed to settle 15 min and the fines were decanted. An additional 0.2 vol 0.5 M potassium phosphate was added to the settled resin before pouring the column. The column was equilibrated first with 0.5 M potassium phosphate then with column buffer.

To clean the resin at the end of the isolation the resin is washed on a fritted funnel with 5-6 volumes 50 mM NaOH and then with water until pH 7-8. Resin is stored at 4°C in 20% ethanol.

Sephadex G-100 (1.5 x 63 cm, 111 mL)

Column was poured and equilibrated with column buffer. This column is poured once and used several times before cleaning.

The resin is cleaned in a fritted funnel with 5-6 volumes 100 mM NaOH and then with water until pH 7-8, then stored at 4°C in 20% ethanol

ENZYME ASSAY

Enzyme activity was measured by quantitating the amount of $^{14}CO_2$ produced from the decarboxylation of [1-^{14}C]-α-ketoglutarate. The standard enzyme mixture consisted of 10-25 μL enzyme in column buffer, 0.9 mM thymine, 0.45 mM αKG (specific activity typically 1 x 10^5 cpm/μmol), 2.3 mM sodium ascorbate, 11 μM ferrous sulfate and 45 mM HEPES, pH 7.5 in 220 μL total volume.

Assay Solutions

50 mM HEPES, pH 7.5

HEPES (1.43 g) was dissolved in 120 mL H_2O and the pH adjusted with KOH.

10 mM thymine

A 1 M thymine solution was prepared by dissolving thymine (2.46 g) in 50 mM HEPES, pH 7.5 (21 mL). A 10 μL aliquot of the 1 M thymine solution was diluted in 990 μL H_2O. Thymine solution (10 mM) was stored in 1 mL aliquots at -20°C.

20 mM α-ketoglutarate, pH 2

The α-ketoglutarate solution was prepared by dissolving 29.2 mg in 10 mL H_2O and was stored in 1 mL aliquots at -20°C. [1-^{14}C]-αKG (20 μL, 0.05 μCi/μmol) was added to the unlabeled solution before using in the assay. Final specific activity was typically 1 x 10^5 cpm/μmol.

20 mM sodium ascorbate (prepared fresh)

Sodium ascorbate (3.97 mg) was dissolved in 1 mL HEPES.

10 mM ferrous sulfate (prepared fresh)

Ferrous sulfate (3.0 mg) was dissolved in 1.08 mL H_2O.

Reaction Mixture Stock Solution

    1260 µL HEPES

    200 µL ascorbate

    20 µL $FeSO_4$

    40 µL ^{14}C-αKG (1×10^5 cpm/µmol)

[NOTE: When preparing the assay solution, $FeSO_4$ must be added after ascorbate to prevent the precipitation of ferric hydroxide.]

Reaction mixture stock (190 µL) and thymine solution (20 µL) were pipetted into 15 x 45 mm glass vials. Vials were sealed with septa equipped with center wells containing accordian-folded filter paper soaked with a 1 M hyamin hydroxide solution (20 µL). Assays were conducted at 30°C. Reactions were initiated by injecting enzyme and terminated by injecting 100 µL 20% (w/w) TCA. Reaction vials were incubated at 37°C for at least 1 hour before the wells and filter paper were removed for liquid scintillation counting. Wells and filters were placed in 20 mL scintillation vials with 9 mL Scint-A XF or Poly Fluor and counted by a Beckman LS 6500 multi-purpose liquid scintillation counter. Controls lacking thymine were run in parallel and background counts subtracted from reaction values before calculating specific activities. Protein concentrations of assay solutions were determined by the Bradford method using BioRad assay dye reagent. BSA was used as the protein standard (1 mg/mL solution-- $A_{280}=0.667$).

ENZYME ISOLATION (130 g cells)

Frozen, washed cells (130 g from 20 L culture) were thawed in Tris buffer (300 mL) containing PMSF (approximately 1 mM). [Note: PMSF solutions were made fresh directly before use. PMSF was dissolved in minimal amount of ethanol and added to vigorously stirred buffer.] Cells were ruptured in 30 mL

aliquots with a French pressure cell at 18,000 psi. The cell was chilled on ice prior to use, and the ruptured cells collected in flasks cooled on ice. Cell debris was pelleted by centrifugation at 11,000xg for 45 min. The resulting supernatant was filtered through cheesecloth to remove lipids (350 mL). All remaining steps were done at 4°C.

Nucleic acids were removed by the addition of a protamine sulfate solution (10 mg/mL Tris buffer). This solution was prepared the night before it was needed since protamine sulfate becomes very sticky once buffer is added and it is difficult to dissolve. The protamine solution was added by pipet to the pink cell extract until solution turned a milky-pink color. A final concentration of 0.1-0.23% (w/v) protamine sulfate is required for complete precipitation of nucleic acids. [NOTE: The exact amount of protamine sulfate needed should be determined before beginning the prep on a small batch of cells since the amount can vary between different *R. glutinis* growths.] After stirring 30 min, the mixture was centrifuged at 15,000xg 30 min. The yellow supernatant was decanted from the pink pellet and filtered through cheesecloth (375 mL).

Solid ammonium sulfate was added to the stirring protamine sulfate solution over 45 min to yield a 45% saturated solution (277 g/L). The solution was stirred an additional 30 min before centrifuging (15,000xg, 30 min). The supernatant was decanted and the pellet discarded. Additional ammonium sulfate (210 g/L) was added to the supernatant (425 mL) to bring it to a 75% saturated solution. After stirring for 30 min, the solution was centrifuged at 15000xg for 45 min.

The large protein pellet which had precipitated with the 75% solution was dissolved in a minimal volume of Tris buffer (20 mL). This orange colored solution was loaded onto a Sephadex G-25 column and eluted with column buffer. Fractions containing protein were located in the pinkish fractions and

were pooled together (80 mL). The desalted protein was loaded onto the DE-52 column and left on the column overnight.

The DE-52 column was first washed with 2 column volumes buffer before eluting the protein with a 7.3 column volume linear KCl gradient (0 to 0.17 M in column buffer). A column profile was plotted from the absorbance at 280 nm for even-numbered fractions. Fractions 61-99 were assayed to locate thymine hydroxylase activity (50 μL of each fraction assayed at room temperature for 3-4 min). Fractions 75-89 were pooled and concentrated by Amicon to 20 mL with a PM-30 membrane. The concentrated sample was divided into 1.5 mL aliquots and stored at -20°C.

The DE-52 sample (5 mL) was thawed on ice and then centrifuged in a microfuge at maximum speed (14000 rpm) before loading onto the Sephadex G-100 column and eluting with column buffer. A column profile was plotted from the absorbance at 280 nm for even-numbered fractions. Odd numbered fractions 1-15 were assayed to locate enzyme activity (10 μL of each fraction assayed 3 min at 30°C). Activity appeared in fraction 13 and fractions 12-16 were assayed to decide which fractions to pool. Fractions 13-14 were pooled and concentrated by Amicon with a PM-30 membrane. Fraction 12 was concentrated separately. Protein samples were frozen and stored at -20°C.

BIOCAD PURIFICATION OF THYMINE HYDROXYLASE

pH Mapping Study

Column: 20HQ POROS, 4.6 x 100 mm, 1.5 mL

Buffers: 50 mM Tris + 50 mM BisTris, pH 6

                50 mM Tris + 50 mM BisTris, pH 9

                3 M NaCl

                H_2O

(Buffers were filtered and chilled before adjusting pH.)

Samples: 200 µg G-100 protein loaded per run. Protein samples were from the 7/96 preparation starting with 50 g cells or G-100 samples that had been prepared by Lora Thornburg (dated 1988).

The pH mapping study covered a range of pHs from 6-9. The method (shown below) used the appropriate mixture of the 2 different Tris buffers to achieve the desired pH for each run. The pHs examined were: 6, 6.6, 7.2, 7.8, 8.4 and 9. Runs were analyzed by assaying column fractions for TH activity and by concentrating fractions with Centricon-30 concentrators and running the concentrated samples on 10% SDS-PAGE mini-gels.[1]

Method: Equilibration-- 10 column volumes 50 mM Tris buffer

Elution Gradient--0-1 M NaCl in 50 mM Tris buffer

over 50 column volumes

Column Wash-- 10 column volumes 1 M NaCl in 50 mM Tris buffer

Development of Biocad Purification to Replace MonoQ FPLC Column

Column: 10HQ POROS, 4.6 x 100 mm, 1.5 mL

Buffers: Column buffer (20 mM KPO$_4$, 0.1 M glycine, 0.1 mM EDTA, pH 7.5)

Column buffer + 0.5 M KCl

(Buffers were filtered and chilled prior to use.)

Samples: 100 µg G-100 samples

Method: Equilibration-- 10 column volumes column buffer

Elution-- linear gradient, 0-0.5 M KCl in column buffer

Wash-- 10 column volumes column buffer + 0.5 M KCl

A wide range of gradient slopes (0-0.5 M KCl over 10-200 column volumes) were examined to determine which gave optimal separation. Protein

peaks were analyzed by 10% SDS-PAGE mini-gel and TH activity assays to assess purification.

Preparation of Thymine Hydroxylase Sample Submitted to the MIT Biopolymers Lab for Lys C Digest and Peptide Sequencing

Column: 10HQ POROS, 4.6 x 100 mm, 1.5 mL

Buffers: Column buffer

                   Column buffer + 0.5 M KCl

Method: Equilibration-- 10 column volumes column buffer

                   Elution-- 0-0.5 M KCl linear gradient over 150 column volumes

                   Wash-- 10 column volumes column buffer + 0.5 M KCl

Sample: 100 μg of 7/96 G-100 sample

The peak containing TH was concentrated with a Centricon-30 concentrator from 2 mL to 45 μL. The concentrated sample was divided for analysis; 5 μL was diluted 50-fold to assay for activity, 10 μL was loaded onto a 10% SDS-PAGE mini-gel and the remaining 30 μL prepared for sequencing (9 μg, 229 pmol). The sequencing sample was loaded in one lane of a 18 x 16 x 0.15 cm 10% SDS-PAGE gel. [NOTE: Sequencing facility suggested using 15% gel in the future since protein is relatively small.] Gel was run overnight at 22 V and then 4 h at 130 V until the dye front reached the bottom of the gel (voltage for gel to run to completion overnight was underestimated and the voltage was increased to finish the electrophoresis in a timely manner). The gel was stained for 15 min in fresh stain (0.1% Comassie Brilliant Blue R-250, 10% acetic acid, 50% MeOH) and then destained in slow destain overnight (10% acetic acid, 50% MeOH in H_2O). Before excising the protein band, the gel was rinsed in H_2O for 15 min to remove MeOH and acetic acid. The slice containing TH was removed (2 x 1 x 10 mm),

placed in an Eppendorf tube and stored at -20°C. In addition, a gel slice of identical dimensions was excised for the control. Samples were submitted to the sequencing lab for in-gel Lys-C digestion and peptide sequencing.

RESULTS AND DISCUSSION

R. glutinis growths and TH Activity

Problems detecting TH activity in multiple *R. glutinis* growths under activity inducing conditions (C-base medium) necessitated a careful look at both the yeast's growth and enzyme assay. Three 1.5 L C-base growths started from 3 separate agar cultures were monitored for growth and assayed for enzyme activity. Culture #1 was harvested at 24 h, culture #2 at 33 h, and culture #3 at 36 h. Cell extracts were measured for activity under standard assay conditions. Results from these growths demonstrated that the doubling time for the cells was normal (approximately 13 h) and that the enzyme specific activity (0.1 units/mg) in the crude extract was also normal (Table 3-1). In addition, the reproducibility of the growth and enzyme activity from colony to colony was established.

Several problems were encountered in measuring enzyme activity. High levels of chemiluminescence obscurred detection of labelled carbon dioxide from the enzyme assay. This problem is caused by residual methanol on the filter paper from the hyamin hydroxide solution. Incubating the reaction vials at 37°C prior to scintillation counting of the wells and filter is crucial for the thorough drying of the filters and eliminated the problem. There appeared to be no loss of counts from incubations up to 2 hours. Scintillation readings of the incubated samples were reproducible over several hours. On the other hand, vials incubated less than 1 h showed elevated levels of chemiluminescence and gave

less stable scintillation readings. It was also observed that using a fresh source of ferrous sulfate in the assay solution was important. In the future, it may be useful to degas assay solutions or make up the ferrous sulfate solution in weak acid.

Table 3-1. TH activity in 3 small-scale *R. glutinis* growths

Culture #	μg protein assayed	CPM (rxn- control)[a]	nmol CO_2 produced	Specific Act. (Units/mg)
1	8 20 40	173 455 713	2.16 5.68 8.9	0.09 0.09 0.07
2	8.5 21.25 42.5	272 511 813	3.4 6.38 10.16	0.13 0.10 0.08
3	8.7 21.75 43.5	258 571 950	3.2 7.13 11.8	0.12 0.10 0.09

[a] In a typical control, background counts ranged from 40-80 cpm.

Enzyme Isolation

Once growth and assay conditions had been worked out, large-scale growths of *R. glutinis* were set up in preparation for TH isolation. A total of 180 g *R. glutinis* was obtained from three 10 L fermentations. Each growth was assayed to verify that normal levels of TH activity were present in the cells. TH was isolated first on a small scale (50 g cells) to get acquainted with the isolation

procedure before isolating the enzyme from the remaining 130 g cells. The enzyme preparation was developed by Lora Thornburg and a detailed discussion of the methods can be found in her thesis (University of Wisconson, Madison 1992).

Before beginning the isolation, cells must be washed twice in Tris buffer to ensure complete removal of the *R. glutinis* cell coating. This is most easily done directly after harvesting. Lora Thornburg had noticed that incompletely washed cells led not only to lower protein yields, but also lower or no activity and attributed this to an extracellular protease or inhibitor that had not been removed. Insufficient cell washes may have been a contributing factor in failure to detect TH activity in early growths. Washed *R. glutinis* cells were thawed in Tris buffer containing PMSF and cracked using a French pressure cell. Cell debris was pelletted by centrifugation.

The next step in the enzyme isolation is the removal of nucleic acids by protamine sulfate. The amount of protamine sulfate added to the cells varies between growths and since a portion of the protein is also precipitated in this step, it is necessary to determine how much to add before beginning the enzyme isolation by testing a small batch of cells. [NOTE: One of the first preps attempted had to be abandoned after most protein was precipitated in this step.] Lora Thornburg reported that 25% protein was typically precipitated, resulting in a 1.5-fold purification of TH activity. After centrifuging the protamine sulfate solution to pellet nucleic acids the solution should lose most of the pink color.

Ammonium sulfate fractionations follow the nucleic acid precipitation. Proteins precipitated in the first cut (0-45%) are discarded (30% of total protein). The second cut (45-75%) contains TH activity. The pelleted protein is redissolved in minimal amounts of column buffer and desalted on a Sephadex G-25 column. The protein solution is orange-pink and the protein-containing fractions are

easily located since they are colored. Protein fractions are pooled and loaded onto the DEAE-52 column. This is a good place to stop if a break is necessary.

Through the next two columns, DE-52 followed by Sephadex G-100, the isolation is highly reproducible and straightforward. The column profiles for the 130 g preparation are shown in Figures 3-1 and 3-2, respectively. The enzyme isolation summary for the 130 g TH prep is shown below in Table 3-2.
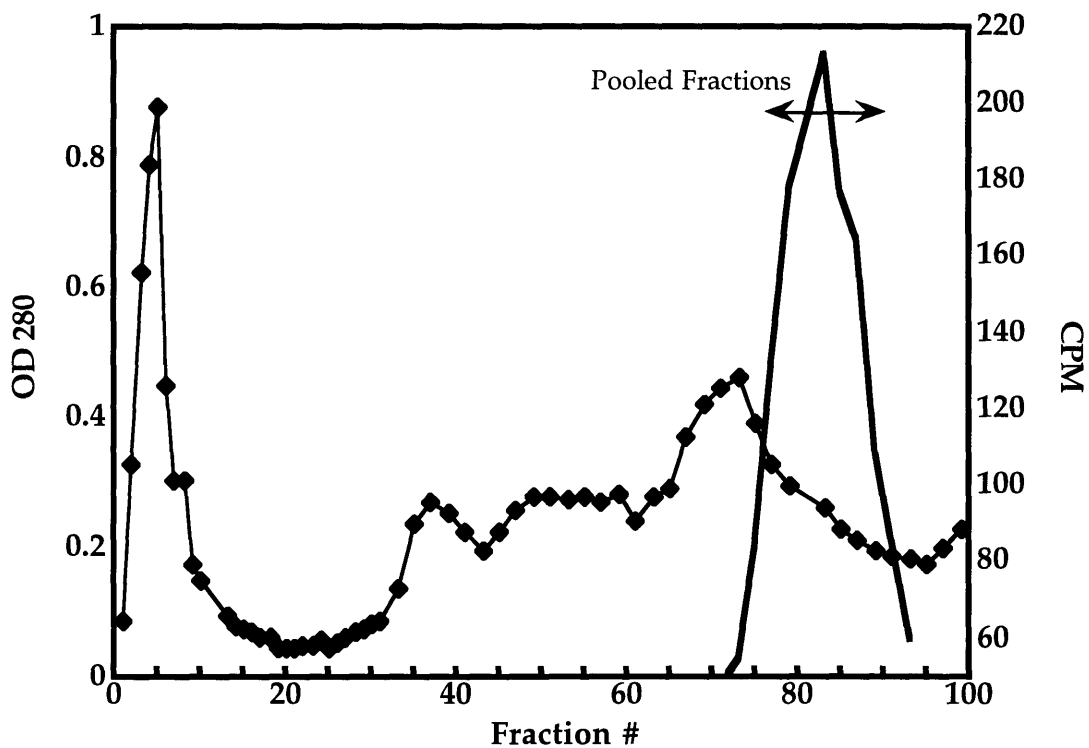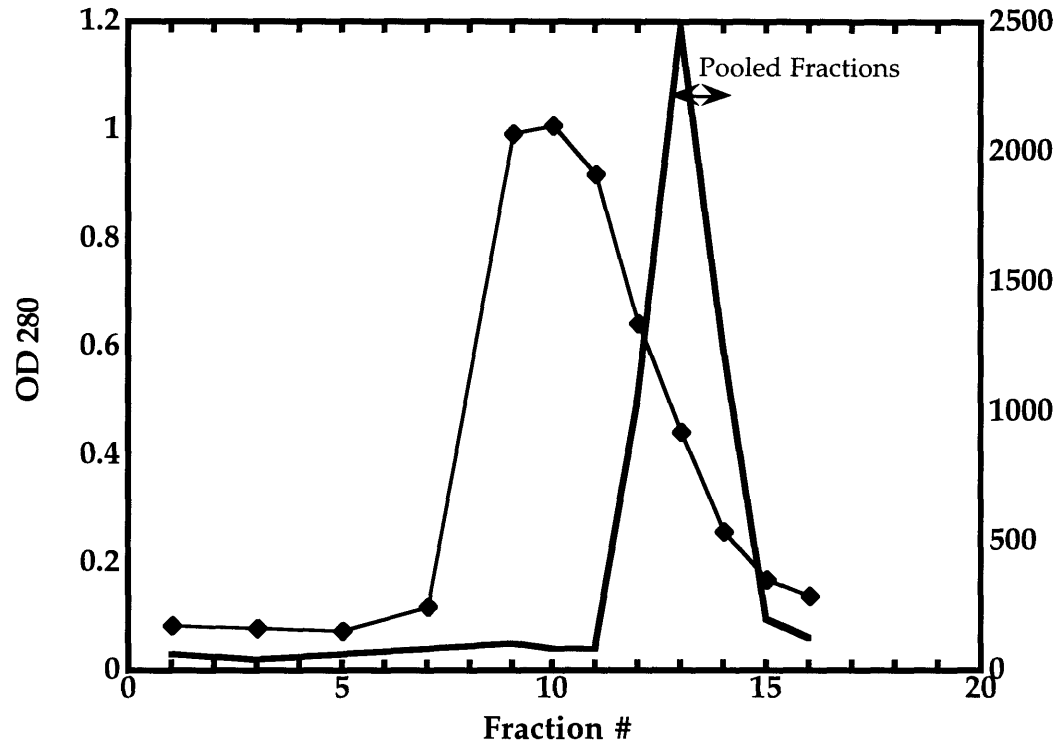
Figure 3-1. DE-52 Elution Profile

Figure 3-2. Sephadex G-100 Elution Profile

The 130 g TH preparation began with a total of 100 units of activity and a specific activity of 0.124 in the crude extract. The protamine sulfate precipitation resulted in 36% decrease in total protein with no increase in specific activity and a purification of 1.16. The amount of protein precipitated was high compared to Lora Thornburg's reported value (25%). The first ammonium sulfate fractionation failed to precipitate any protein in contrast to the 30% decrease in total protein reported by Lora Thornburg. This could be explained by either the excess protein precipitated in the protamine sulfate purification or an error in protein quantitation.

Following the anion exchange column Lora Thornburg typically saw specific activities of 1-1.5 units/mg with a $\leq$ 2-fold increase in total units most likely due to removal of a reaction inhibitor. In this preparation a 2-fold increase in units was seen, however activity was lower than expected (0.75 units/mg).

Table 3-2. Enzyme Isolation Summary (130g *R. glutinis* cells)

Prep Stage	Volume (mL)	Total Activity (Units)	Specific Activity	Total Protein (mg)	Protein Conc. (mg/mL)	Recovery (%)	Purification
CRUDE	350	97.6	0.124	787	2.25	---	---
PROT. SULF.	375	61.5	0.144	427	1.14	63	1.16
0-45% Amm. Sulf.	425	60.8	0.149	408	0.96	62	1.20
G-25 45-75% desalted	80	65.3	0.23	284	3.55	67	1.85
DE-52	20	120	0.75	160	8	123	6.05
G-100 [a]	2	11.76	6.0	1.96	0.98	60	48

[a] Only 20% of the DE-52 sample was loaded in this step. Recovery is corrected for the fact that only a portion of the sample was carried through at this point.

Failure to concentrate the column fractions quickly enough could be one explaination for the low specific activity since TH is unstable under dilute conditions.

Recoveries from the size exclusion chromatography step were also low. Lora reported that the size exclusion chromatography step yielded 11% recovery of total protein and 100% recovery of total units with an increase in specific activity to 10-14 units/mg. In this preparation only 2.5% of total protein and 60% of total units were recovered and the specific activity was measured at 6 units/mg. Low protein recovery resulted from Amicon problems. The Amicon apparatus should always be checked for leaks with buffer before concentrating protein solutions. In addition, if the apparatus is assembled as room temperature it should be retightened after chilling.

A comparison of the 130 g TH prep with a 226 g TH prep by Dr. Thornburg (Notebook 12-- 2/23/90) is outlined in Table 3-3. Values were scaled for comparison.

Table 3-3. Comparison of TH Isolations from 2/90 and 12/96

Prep Stage	LORA protein (mg)	12/96 protein (mg)	LORA Units	12/96 Units	LORA Specific Activity	12/96 Specific Activity	LORA recovery (%)	12/96 recovery (%)
CRUDE	1700	787	139	98	0.082	0.124	---	---
PROT. SULF.	782	427	180	61.5	0.23	0.144	129	63
0-45% Amm. Sulf.	600	408	126	60.8	0.21	0.149	91	62
G-25 45-75% desalted	564	284	141	65	0.25	0.23	101	65
DE-52	118	160	160	120	1.35	0.75	115	123
G-100	24	10	140	59	9.2	6.0	100	60

Biocad Purification

Recently our lab obtained a BioCAD Sprint perfusion chromatography system from PerSeptive Biosystems. Used in conjunction with the POROS Perfusion Chromatography resins, this system can be used to develop highly

optimized purifications quickly. Unlike traditional resins, POROS beads have 2 types of pores-- throughpores that cross through the bead, and diffusive pores that branch off from the throughpores and greatly increase the internal surface area. Sample particles are carried to the diffusive pores by the throughpore network where they quickly diffuse to and from the internal binding sites in the diffusive pores. These two factors allow for much quicker separations than those possible with traditional resins which rely solely on diffusion. POROS columns are small (1.5 mL total volume), can be run at flow rates up to 15 mL/min, and separate up to milligram amounts (depending on particular separation and sample purity) in minutes.

In the past, the final purification step in the TH prep has been accomplished on a MonoQ FPLC anion exchange column. The enzyme activity from this column is unstable in the dilute solutions that are recovered. With this new chromatography system we hoped to replace the last step in the TH purification with a quicker purification step that would increase the stability of the enzyme.

On recommendation of the manufacturer, pH was the first variable systematically examined for optimization of the TH purification on anion exchange 10HQ resin. Unfortunately, TH was not stable in the Tris buffering system used for the pH mapping study. A range of pHs from 6-9 were tested and in all cases no activity was detected at the end of the separation. Gel analysis of the fractions collected from the column revealed that little separation had occurred under any of the pH conditions tested.

Based on these results, a method was develped using conditions similar to those used with the MonoQ FPLC column. The column was equilibrated with column buffer (20 mM potassium phosphate, 0.1 M glycine, 0.1 mM EDTA, pH 7.5) and protein was eluted with a 0-0.5 M KCl linear gradient. Starting with

conditions identical to those used by Lora for the MonoQ column, a method was worked out that afforded good separation with 10HQ. Since we were interested in purifying large amounts of protein, we switched to 20HQ resin which has a higher loading volume. Separation was not affected. Shown in Figure 3-3 is the column profile for purification by 20HQ (0-0.5M KCl gradient over 150 column volumes). Figure 3-4 displays the gels showing the purity of the fractions through the major peaks.
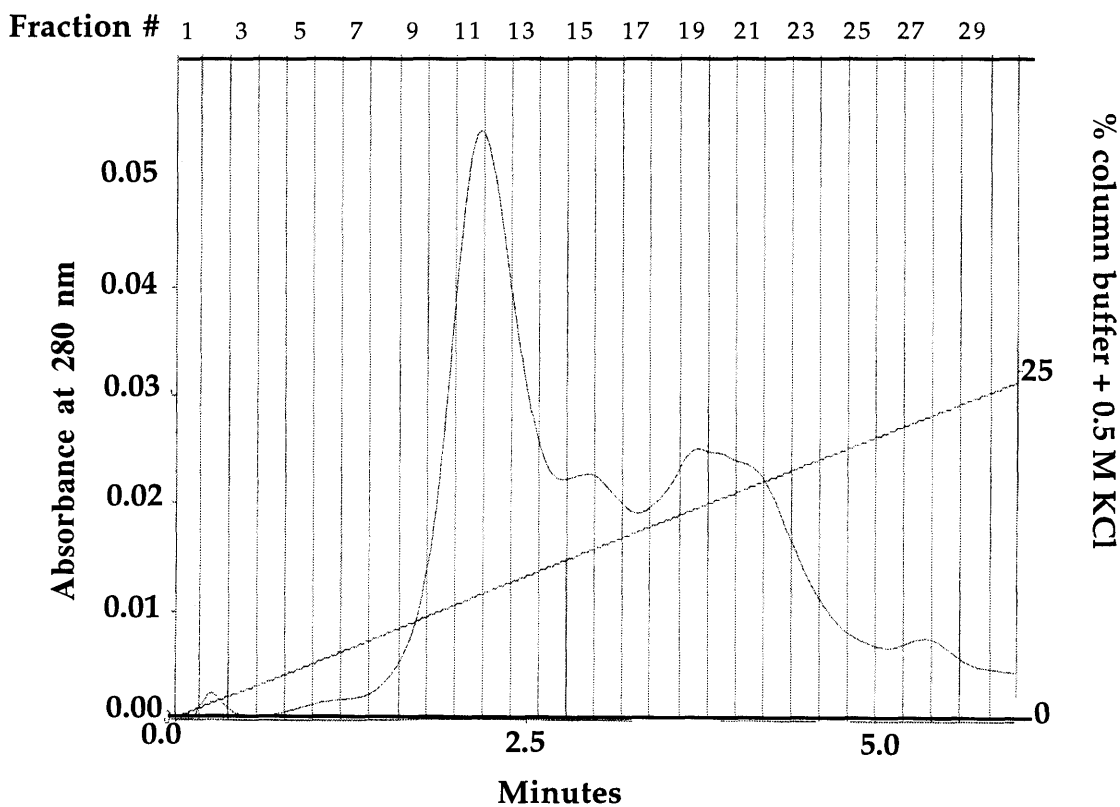

Figure 3-3. POROS 20HQ Elution Profile
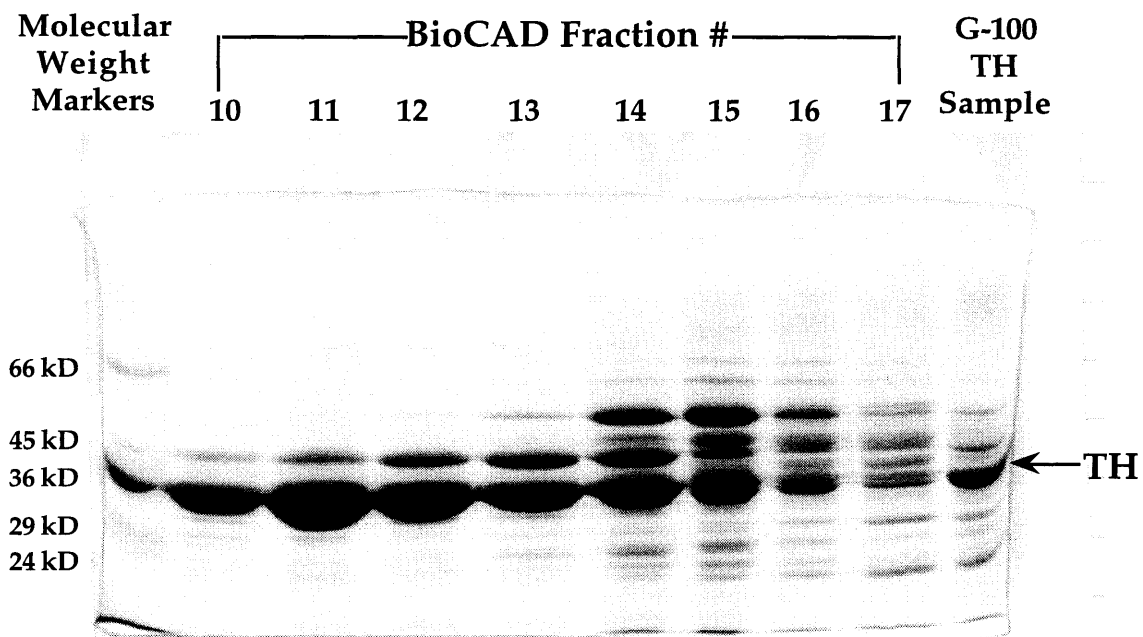
Figure 3-4. SDS-PAGE Analysis of the POROS 20HQ Purification of TH
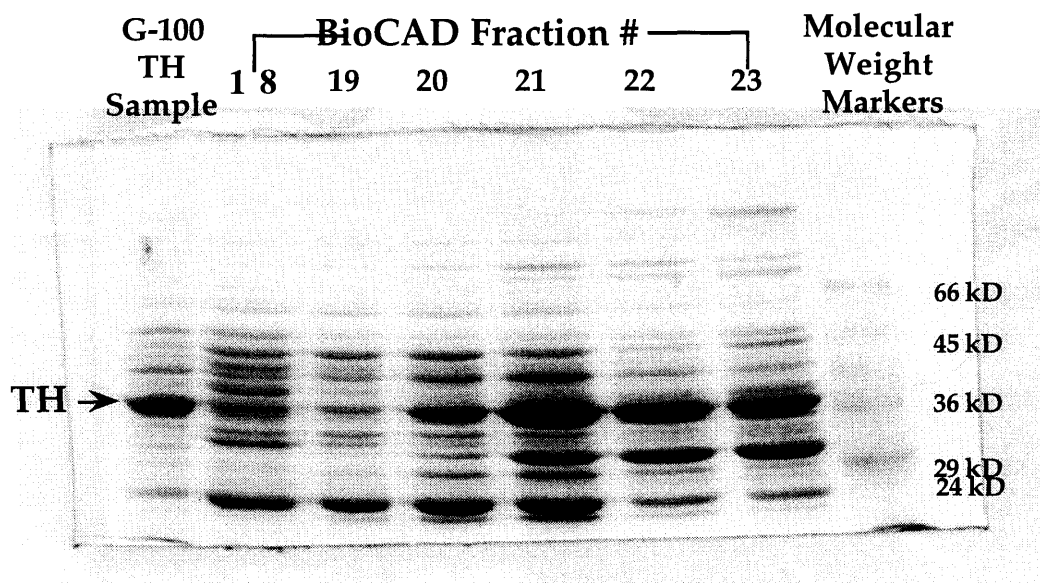
Figure 3-4. SDS-PAGE Analysis of the POROS 20HQ Purification of TH

Shown below is a purification summary table for the BioCAD purification

step (Table 3-4). A sample of G-100 protein (1 mg loaded in 1 mL) was loaded

onto the POROS 20HQ column (4.6 x 100 mm, 1.5 mL). Of the sample loaded

60% of the total protein was recovered, and 72% of units. Specific activity

increased dramatically from 6 to 30 units/mg.

Table 3-4. Summary for BioCAD Purification of TH (1/14/97)

Prep Stage	Volume (mL)	Total Activity (Units)	Specific Activity	Total Protein (mg)	Protein Conc. (mg/mL)	Recovery (%)	Purification
G-100	1	6	6	1	1	---	---
BioCAD	0.5	4.33	30	0.61	1.22	72	5

Comparison of the MonoQ and BioCAD purifications is shown below in Table 3-

5. The BioCAD purification compares favorably with MonoQ for both protein

recovery and purification. Additional benefits that the BioCAD purification

**Table 3-5. Comparison of MonoQ and BioCAD 20HQ Purification Steps
(3/22/90 MonoQ purification values scaled for 1 mg protein loaded)**

Method	Volume (mL)	Total Activity (Units)	Specific Activity	Total Protein (mg)	Protein Conc. (mg/mL)	Recovery (%)	Purification
MonoQ	1.5	7.3	26	0.28	0.19	63	2.2
BioCAD	0.5	4.33	30	0.61	1.22	72	5

offers are shorter purification times, less dilution of the enzyme on the column, and increased stablity of the enzyme. BioCAD purification takes 2.5 minutes compared to the 40 minute MonoQ column. Volumes coming off the Biocad column are smaller and are quickly and easily concentrated by centricon. The BioCAD purified enzyme appears to be stable to storage unlike the MonoQ purified samples and has been shown to retain all activity through one freeze-thaw cycle. This result needs to be repeated for verification. It may be advantageous in the future to add a cryoprotectant to the BioCAD sample before storing at -20°C.

Protein Sequencing

One motive for working out the Biocad purification was to obtain clean protein for sequencing. A protocol has been developed for digesting proteins directly in a SDS-PAGE gel slice.[2] It produces yields that are similar or better to those obtained from membrane digests. A Biocad purified sample of TH was sent to the Biopolymers lab for an in-gel digest by Lys-C and sequencing of the fragments generated. From this digest 6 new peptide fragments were sequenced (Figure 3-5).

Figure 3-5. Peptide Fragments Sequenced from a Lys-C Digest of TH

```
(T) V I S A L P G T
E R L E W E [C] A E S N (R) (R)
E R L E W E [-] A E - - R   - Y V
A F M N D F F E K
D Y R E G P D D S P F E N R
V L A L I M P I G N S F P D L K
D G V F V P A ? P V A D T V V V N V G D L L Q
```

() = somewhat confident, reasonable
[] = low confidence, possible
- = no detection

Unfortunately none of the new information included portions of previously sequenced peptides. We had hoped to obtain some overlapping information both to validate the Lys-C sequencing results and for the synthesis of longer probes. The new peptide sequence information was aligned with the GA20OX sequence using the BCM Search Launcher.

Figure 3-6. Sequence alignment of TH peptide fragments with Gibberellin 20-oxidase

(GA20OX sequence in capital letters, TH fragments underneath GA20OX= old sequence, TH fragments above GA20OX= new sequence)

```
1   MALNGKVATE   SAPSNLNEEM   KGEYRPPFGG   SDESKVPEDF   IWSEKFEASE

51. LLPVLDVPTI   DLEKFMSGDK   SYVEEATRLV   DEACRQHGIF   FVVNHGVDIE
    :.     ||.|  ::|.|:||                              |.::.| |
    vssgivppi    nfepflsg                              vtqetdpe


101 MMGRVHDCMN   EFFTMPLDVK   QRAKRKVGES   YGYTNSFFGR   FASNLPWKET
    :::       .  :||.:|::||
    aiak      q  qffdlpmevk


151 FSLRCVAAQN   SSAAHDYVLD   TLGPSFSHHG   KAYQECGIAL   NELGTKIVEL


            A    FMNDFFEK                    VISA   LPGT
                 |||                         |      | |
201 LGLSLGISRE   YFKNFFEDND   SILRLNYYPT   CDKPEVVLGT   GPHTDPTSVT


                              VLALIM   PIGNSFPDLK
                                       || | |
251 ILHQDPVSGL   QVCSNDQWYS   IPPNPEAFVI   NIGDTFTSLT   NGIYKGCIHR
                                          :::|:|.:.  |::.|
                                          lsetytkws   ndvlk


            D    GVFVPACPVA   DTVVVNVGDL   LQ
                 ||          | ||
301 AVVNSMNARK   SLAFFLCPSH   DKVVRAPEEL   VEKSPPRKYP   DYKWPMLLEM
    .            |:|||  ||    : |:.|.::   :|..|.
            n    siaffsnps   evvlpadgdp   lemtpr


351 TQKRYRPDCN   TLEAFKTWVQ   EGKALDTGST   ITAPSA
              |            |
    DVREGPDDS   PFENR
```

At this stage it is difficult to interpret the results of the peptide sequencing and alignment. Levels of identity between the new peptide sequences and the GA-20OX sequence is lower than those previously aligned. Also, some of the peptides overlap with the older peptide information. Lys-C digests should be repeated to verify this information.

BIBLIOGRAPHY

(1)   Laemmli, U. K. *Nature* **1970**, *227*, 681-685.

(2)   *Techniques in Protein Chemistry*; Vol. VI, pp 143.

Chapter 4

Inactivation of Thymine Hydroxylase by 5-Ethynyluracil:
Examination of the Structure and Formation of a
Minor Enzyme-Inhibitor Adduct

As mentioned briefly in Chapter 1, the reaction of thymine hydroxylase with the mechanism-based inhibitor 5-ethynyluracil results in the inactivation of the protein and the production of two different covalent enzyme-inhibitor adducts in a 3:1 ratio.[1] Tryptic digests of the inactivated protein, identification of the modified fragments, and peptide sequencing of the fragments showed that both adducts were derived from the same sequence, NSIAFFSNPSLR. In both cases the second phenylalanine in the sequence is the site of modification.

Characterization of the major adduct was the initial focus of investigation in the laboratory. Large-scale inactivation reactions with isotopically labeled EU ([2-^{14}C]-5-[1', 2'-^{13}C$_2$]-EU) made it possible to use 1D and 2D NMR spectroscopic methods in the structural identification of the major adduct. A novel norcaradiene structure is proposed to be the major species formed in the inactivation. A mechanism has been proposed for the formation of this unusual species and involves the insertion of a carbene intermediate into an active-site phenylalanine residue followed by a second hydroxylation reaction (Figure 4-1). Characterization of the minor adduct formed during the inactivation has been more difficult because of the smaller quantities of this product available for analysis. Data obtained from ^{1}H- and ^{13}C-NMR spectroscopy, FAB-MS analysis, and UV spectroscopy of the minor modified adduct suggest that this adduct contains a benzoyluracil moiety (see discussion section). Prior to this work, there were no suitable chemical models available to confirm this structural model.

In this chapter the synthesis of a model compound which mimics the postulated minor adduct structure is presented and a comparison of its structural data with that of the minor enzyme adduct is also presented. A mechanism for the formation of the minor adduct has been proposed. This mechanism predicts that formate should be released during the course of the inactivation. Preliminary work undertaken to test this proposal is presented.
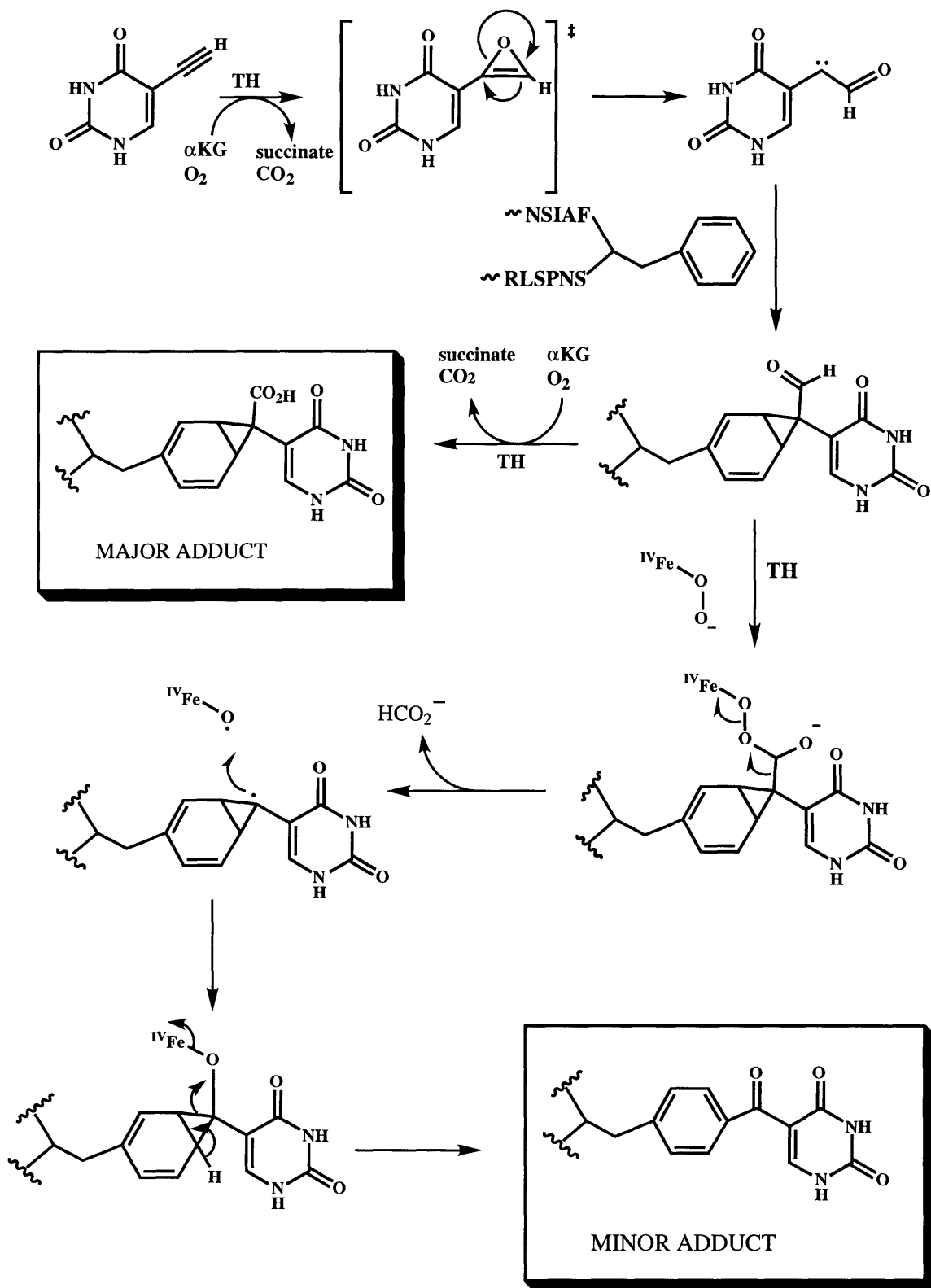
Figure 4-1. Proposed Mechanism for the Formation of the Major and Minor Adducts

MATERIALS AND METHODS

Materials

Ethyl benzoylacetate, triethylorthoformate, 2,6-Di-*tert*-butyl-4-methylphenol (BHT), *N*-benzyl-*N*-nitroso-*p*-toluenesulfonamide, di(ethylene glycol) ethyl ether, and silica gel (60Å, 230-400 mesh) were purchased from Aldrich. Acetic anhydride, HCl, and KOH were obtained from Mallinckrodt. Formate dehydrogenase from yeast (cat. # F8649) and NAD were supplied by Sigma. Isotopically labeled sodium formate (^{14}C, 0.1 mCi/mL) was purchased from Dupont NEN. An authentic sample of benzyl formate was purchased from Lancaster Synthesis, Inc. Sequencing grade urea was purchased from American Bioanalytical. EM Science supplied Silica Gel 60 F_{254} TLC plates. All solvents used were of the highest quality available.

All HPLC separations were performed on a Waters system (consisting of Tunable Absorbance Detector 486, two 510 pumps and Automated Gradient Controller 680). Column profiles were recorded by an OmniScribe Series D5000 Recorder. An Alltech Econosil C18 10U semipreparative column (250 x 10 mm) was used for all separations. ^1H and ^{13}C NMR spectra were recorded on a Varian VXR 500 MHz instrument. Chemical shifts were referenced to either trimethylsilane (TMS) or sodium 3-(trimethylsilyl)-1-propanesulfonate (TSP). A Varian Cary 3 spectrophotometer equipped with a temperature-controlled cell holder was used for the formate dehydrogenase assays.

SYNTHESIS OF 5-BENZOYLURACIL (Figure 4-2)

Alkylidene 1 [mixture of (*E*)- and (*Z*)- isomers]:

Ethyl benzoylacetate (5 g, 26 mmol) was combined with triethylorthoformate (3.85 g, 26 mmol) in acetic anhydride (5 mL). The reaction mixture was refluxed at 120°C. After 2 h the solution had turned from yellow to red. TLC analysis (20% ethylacetate in hexanes) indicated that the starting material (R_f= 0.55) had been

partially converted to product (R_f= 0.25). Heating at reflux for additional time did not result in increased conversion of starting material to product, so reactions were typically run 2 h. The product was separated from unreacted starting material by flash chromatography (silica gel column 20 x 4.5 cm, 20% ethylacetate in hexanes). The solvent was removed under reduced pressure to give 0.82 g (3.3 mmol) of a red oil in 13% yield. ^{1}H-NMR showed the presence of both the (*E*)- and (*Z*)- isomers with a ratio of major and minor isomer of 7:1 (Figure 4-3).

^{1}H-NMR of the major isomer **1** (300 MHz, CDCl$_3$) δ: 7.38-7.88 (m, 5H ArH̲, 1H vinyl-H̲), 4.13 (q, J= 7.2 Hz, 2H, -OCH̲$_2$CH$_3$), 4.05(q, J= 7.2 Hz, 2H, -OCH̲$_2$CH$_3$), 1.22 (t, J= 6.9 Hz, 3H, -OCH$_2$CH̲$_3$), 1.11 (t, J= 6.9 Hz, 3H, -OCH$_2$CH̲$_3$). [NOTE: Additional resonances seen at 2.06 and 2.01 ppm are acetone (used to clean all NMR tubes) and ethyl acetate, respectively.]

Urea derivative 2 [mixture of (*E*)- and (*Z*)- isomers]:

Alkylidene **1** (0.22 g, 0.88 mmol) was dissolved in ethanol (2 mL). Urea (0.053 g, 0.88 mmol) and 2,6-Di-*tert*-butyl-4-methylphenol, BHT (2 mg, 8.8 μmol) were added and the mixture heated at reflux. The reaction was removed from the heat once TLC analysis (50% ethyl acetate in hexanes) indicated that starting material (R_f= 0.87) was no longer being converted to product (R_f= 0.24). Reactions were typically run 12 h. Urea derivative **2** was separated from unreacted starting material and BHT by flash chromatography (silica gel column 18 x 3 cm, 50% ethyl acetate in hexanes). The solvent was removed under reduced pressure and the compound dried under vacuum. Weight of the dried compound was 40 mg (0.15 mmol) corresponding to a 20% isolated yield. ^{1}H-NMR shows the presence of equal amounts of the (*E*)- and (*Z*)-isomers of the product (Figure 4-4).

^{1}H-NMR (Figure 4-4) [300 MHz, DMSO] δ: 10.43 (d, J= 13 Hz, 1H, -NH̲-CO-NH$_2$), 10.25 (d, J= 13 Hz, 1H, -NH̲-CO-NH$_2$), 8.37 (d, J= 13 Hz, 1H, vinyl-H̲), 8.06 (d, J= 13 Hz,

1H, vinyl-$\underline{H}$), 7.61-7.426 (m, 5H, Ar$\underline{H}$), 7.31 (b, 4H, -NH-CO-N$\underline{H}_2$), 4.02 (q, J= 7.1 Hz, 2H, -OC$\underline{H}_2$CH$_3$), 3.97 (q, J= 7 Hz, 2H, -OC$\underline{H}_2$CH$_3$), 0.97 (t, J= 7 Hz, 3H, -OCH$_2$C$\underline{H}_3$), 0.92 (t, J= 7.1 Hz, 3H, -OCH$_2$C$\underline{H}_3$). The resonances seen at 2.5 and 3.35 ppm are DMSO and H$_2$O, respectively.

^{13}C-NMR (Figure 4-5) [500MHz, DMSO] δ: 194.38 & 192.71, Ar-$\underline{C}$O; 166.68 & 166.406, -NH-$\underline{C}$O-NH$_2$; 153.183 & 153.08, -$\underline{C}$O-OCH$_2$CH$_3$; 147.98 & 147.04, Ar-CO-$\underline{C}$=; 139.88, 139.58, 131.85, 131.77, 128.37, 128.18, 128.09, & 127.63, Ar$\underline{C}$; 105.7 & 105.19, =$\underline{C}$-NH-CO-NH$_2$; 59.88 & 59.79, -O$\underline{C}$H$_2$CH$_3$; 13.79 & 13.65 -OCH$_2$$\underline{C}H_3$.

5-Benzoyluracil (3):

Urea derivative **2** (0.05 g, 0.19 mmol) was added to 5% aqueous KOH (500 µL) which had been heated to 75°C in a 5 mL round bottom flask equipped with a micro-stir bar. After the compound had dissolved (2 min), one equivalent of HCl (50 µL, 4 M) was added to the reaction and the mixture left heating and stirring approximately 5 min. The reaction was removed from the heat and allowed to cool to room temperature on the bench. A white precipitate was visible upon removal of trace amounts of EtOH (which is formed during the reaction) from the reaction mixture under reduced pressure. The reaction slurry was pipetted from the flask, placed in an Eppendorf tube, and the white precipitate was pelleted in a microfuge. [NOTE: The reaction flask was rinsed with several aliquots of H$_2$O (300-500 µL) to maximize recovery of the precipitate. The rinse fractions were added to the reaction mixture in the Eppendorf tubes.] The supernatant was discarded. The pellet was dissolved in 30% MeOH in H$_2$O. Compound **3** was purified by HPLC on an Econosil-C18 10U semi-preparative column (250 x 10 mm). Samples were injected onto the column equilibrated with 30% MeOH in H$_2$O. The HPLC detector was set to monitor absorbance at 284 nm. The compound was eluted with a linear gradient

Figure 4-2. Synthesis of 5-Benzoyluracil (3)

Figure 4-3. ^{1}H-NMR of Alkylidene 1 in CDCl$_3$

109

Figure 4-4. ^{1}H-NMR of Urea Derivative 2 in DMSO

110

Figure 4-5. ^{13}C-NMR of Urea Derivative 2 in DMSO

of MeOH (30-100% MeOH over 20 min at a flow rate of 2 mL/min). The compound eluted with 72% MeOH. The retention time of **3** under these conditions is 12 min. Reaction yield is undetermined.

UV (Figure 4-6): λ_{max} = 280 nm

^{1}H-NMR (Figure 4-7) [500 MHz, D_2O] δ: 8.28 (s, 1H, C-6 H̲), 7.68-7.52 (m, 5H, ArH̲) [NOTE: Water suppression pulse sequence used to obtain spectrum.] Additional peaks at 1.91, 1.33, 1.32 and 1.29 ppm were not identified, however, integration suggests that these impurities are present in insignificant amounts.

^{13}C-NMR (Figure 4-8) [500 MHz, DMSO] δ: 191.67, 165.30, 163.80, 160.36, 141.33, 130.03, 128.46, 127.25, 107.48

Figure 4-7. UV Spectrum of 5-Benzoyluracil (3)
λ max = 280 nm

112

Figure 4-7. ^{1}H-NMR of 5-Benzoyluracil in D_2O Water suppression pulse sequence was used in obtaining spectrum.

Figure 4-8. ^{13}C-NMR of 5-Benzoyluracil in DMSO

INACTIVATION OF THYMINE HYDROXYLASE BY 5-ETHYNYLURACIL

Synthesis of Phenyldiazomethane (PDM):

N-benzyl-N-nitroso-p-toluenesulfonamide (7.9 g, 27 mmol) was added slowly with stirring to a mixture of KOH (3.4 g, mol), di(ethylene glycol) ethyl ether (20 g, 0.15 mol), H_2O (5.6 mL) and ether (5.6 mL).[2] The reaction mixture changed from yellow to a red-orange over the course of the addition (approximately 45 min). Next the reaction mixture was poured into a separatory funnel containing ice-cold H_2O and extracted with four-70 mL portions of hexanes. The combined hexane extracts were washed with three-50 mL portions of a saturated aqueous $NaHCO_3$ solution. After drying the organic layer over anhydrous $MgSO_4$ for 30 min, the solution was filtered to remove $MgSO_4$ and hexanes were removed under reduced pressure. The product was purified by vacuum distillation (26°C, 1.5 Torr). The reaction yield was not determined.

^{1}H-NMR (Figure 4-10) [300 MHz, CD_2Cl_2] δ: 7.29-6.90 (m, 5H, Ar$\underline{H}$), 4.97 (s, 1H, Ar-C$\underline{H}$-N$_2$)

Figure 4-9. Synthesis of Phenyldiazomethane (PDM)

Figure 4-10. ^{1}H-NMR of Phenyldiazomethane in CD_2Cl_2

Derivatization of Formate[3]

The reported procedure for the derivatization of formate with PDM was tested for use in the formate release experiment. In a typical reaction sodium formate (0.9 μmol, 30μL of 0.03 M methanolic solution) was acidified to pH 1 with the addition of HCl (15 μL, 1 M solution in THF). PDM was added in 10 μL aliquots to the acidified solution until the red color of the solution (due to unreacted PDM) persisted (40 μL).

GC-MS analysis of all derivatized samples was performed by Pete Wishnok on a HP-5890 GC equipped with a Hewlett Packard 100% dimethylpolysiloxane gum column (HP-1, 12 m x 0.22 mm, film thickness 0.33 μm). Samples were injected in 1 μL total volume. The temperature program was as follows: 50°C for 0.5 min followed by a ramp of 10°C/min to 85°C and finally a temperature ramp of 20°C/min to 180°C. Under these conditions benzyl formate has a retention time of 3.65 min. A standard curve for the response of the GC-MS detector for benzyl formate was constructed using authentic samples (Lancaster cat # 13405). The response of the detector was linear for concentrations ranging from 0.002 - 2 nmol/μL.

Formate Dehydrogenase End-Point Assay for Formate Quantitation[4]

The formate dehydrogenase (FDH) assay for the quantitation of formate is based on the measurement of the increase in absorbance at 340 nm due to the reduction of NAD^+ in the presence of formate:

$$\text{formate} + NAD^+ + H_2O \longleftrightarrow HCO_3^- + NADH + H^+$$
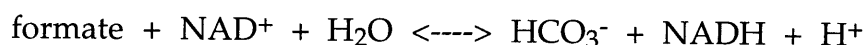
The assay mixture contained in a final volume of 255 μL: 100 μL H_2O, 100 μL of 0.2 M sodium phosphate buffer pH 7.5 (78 mM), 25 μL of 0.02 M NAD (2 mM), 10 μL FDH from yeast (Sigma F8649) in sodium phosphate buffer (10-20 units; 1 unit will

oxidize 1 µmol formate to CO_2 per minute at pH 7.6, 37°C), and 20 µL of the formate solution to be analyzed (2-8 nmol was added to yield formate concentrations in the assay mixture ranging from 0.01 to 0.04 mM). Reactions were run at 37°C. All reaction components except for the formate solution were mixed in a cuvette and the absorbance at 340 nm was recorded. The reaction was started with the addition of the formate solution and the absorbance at 340 nm monitored. The amount of formate in the sample was then calculated from the final absorbance change at 340 nm using the extinction coefficient of NAD^+ (6.3 mM^{-1} cm^{-1}).

Bulb-to-Bulb Distillations of ^{14}C-Formate to Test Distillation Efficiency

Bulb-to-bulb distillation efficiencies were determined under mock reaction conditions substituting BSA for TH and adding ^{14}C-labeled formate to the reaction mixture. A typical distillation mixture contained BSA (250 µg, 4 nmol), EU (30 nmol, 100 µM), and ^{14}C-formate (20 µg, 0.3 µmol, 4.4 x 10^4 CPM/µmol) in 295 µL 45 mM HEPES (pH 7.5) containing 0.45 mM α-KG, 0.11 mM $FeSO_4$, 2.3 mM ascorbate, and 0.4% (v/v) DMSO. The components were mixed, incubated at 30°C for 15 min, and then acidified to pH 1 with HCl (20 µL, 1 M) [NOTE: A final pH of 2 should be sufficient since the pK of formate is 3.75]. Following acidification, volatile components in the reaction mixture were distilled. The pH of the collected distillate was adjusted to pH 13 with NaOH (20 µL, 1 M) immediately following distillation [NOTE: Neutralization should be sufficient, however this was not tested in this set of experiments.] This was lyophilized to dryness and the residue redissolved in 500 µL H_2O. Other distillations were set up under identical conditions with 0.14, 0.29, 0.44 and 0.58 µmol formate. Samples were counted with a Beckman LS 6500 multi-purpose scintillation counter in 9 mL Scint-A XF. Distillation efficiencies of 80-90% were measured based on recovery of counts in collection flask following lyophilization.

Thymine Hydroxylase Inactivation Reactions

Enzyme used in the inactivation reactions was purified through the Poros 20HQ perfusion chromatography step by the procedure described in Chapter 3. The $^{14}CO_2$ assay described in Chapter 3 was used to measure TH activity.

Control Reaction to Verify Inactivation of TH Under Conditions to be used for Formate-Release Experiment

To test the conditions to be used for the formate-release experiment, TH (460 µg, 12 nmol, 17 units/mg) was inactivated with EU (5.8 µg, 43 nmol) in 220 µL 45 mM HEPES (pH 7.5) containing 0.45 mM α-KG, 0.11 mM FeSO$_4$, 2.3 mM ascorbate, and 0.4% (v/v) DMSO. The reaction was run for 13 min at 30°C. Aliquots (10 µL) of the reaction mixture were withdrawn from the reaction flask approximately every 2 min and were assayed for TH activity. A parallel reaction lacking only the inhibitor served as a control. The amount of TH activity in the reaction vial had decreased to less than 1% of the starting activity after 11 min and the formate release experiment was set up to run at least 13 min to ensure complete inactivation.

Formate Release Experiment

The formate-release experiment was set up as follows. TH (440 µg, 11 nmol, 8.7 units/mg) was inactivated with EU (6.6 µg, 49 nmol) in 220 µL 45 mM HEPES (pH 7.5) containing 0.45 mM α-KG, 0.11 mM FeSO$_4$, 2.3 mM ascorbate, and 0.4% DMSO. After incubating at 30°C for 13 min, a [^{13}C]-formate internal standard (2.4 µg, 35 nmol) was added to the reaction. [NOTE: Concentration of the [^{13}C]-formate standard was determined using the FDH assay.] Following acidification of the reaction mixture to pH 1 with HCl (60 µL, 1 M), the volatile fraction was collected by bulb-to-bulb distillation. The volatile fraction was adjusted to pH 13 with NaOH (60

μL, 1 M) and the solution lyophilized to dryness. The resulting residue was dissolved in methanol and the methanol solution was adjusted to pH 2 by the addition of HCl (1 M solution in THF). Neat PDM was added dropwise to the methanolic solution until the red color of the solution (due to unreacted PDM) persisted.

GC-MS analysis of the derivitized samples was performed by Pete Wishnok on a HP-5890 GC equipped with an HP-1 column (12 m x 0.22 mm, film thickness 0.33 μm) as described the the section discussing the derivatization of formate.

RESULTS AND DISCUSSION

The structure of the minor adduct formed during the inactivation of TH by EU has been assigned as a benzoyluracil moiety based on analysis of the minor peptide fragment by methods including ^{13}C- and ^{1}H-NMR, FAB-MS analysis and UV spectroscopy (Figure 4-11). Characterization of the adduct by 1D ^{1}H-and ^{13}C-NMR spectroscopies has been especially revealing.

MINOR ADDUCT

MODEL COMPOUND
5-Benzoyluracil
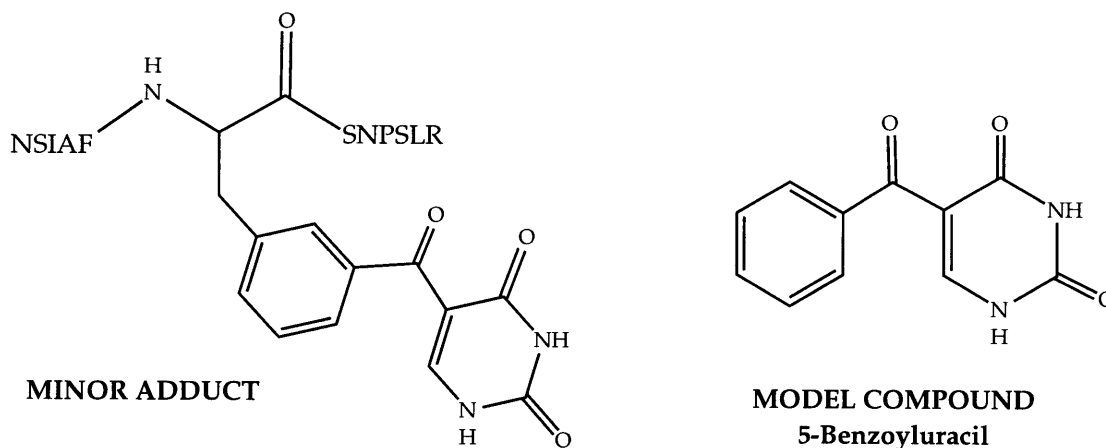
Figure 4-11. Proposed Structure of the Minor Adduct Formed During the Inactivation of Thymine Hydroxylase by 5-Ethynyluracil and the Model Compound, 5-Benzoyluracil

120

From the ^{1}H-NMR spectrum it is evident from the resonances between 7.15 and 7.63 ppm that the aromaticity of both phenylalanines in the sequence is maintained (in the major adduct this is not the case) (Figure 4-12). The doublet splitting of the aromatic protons at 7.65 ppm is indicative of the presence of an electron withdrawing group at the *para* position of the aromatic ring of the phenylalanine. The resonance at 8.24 ppm has been assigned as the C-6 proton of a uracil moiety. The C-6 chemical shift of uracil is normally 7.20 ppm; this downfield shift is consistent with examples in the literature of conjugated uracils (Figure 4-13) and supports the proposal that the uracil is conjugated with a carbonyl group.

The ^{13}C-NMR spectrum of a sample obtained from the inactivation of TH with EU that had been 100% isotopically enriched (^{13}C) at the acetylene carbons shows a downfield shifted carbon resonance at 196.8 ppm which appears to belong to a carbonyl carbon bridged between two aromatic groups (Figure 4-14). This chemical shift is comparable to the ^{13}C chemical shift of the carbonyl carbon of benzophenone (196.6 ppm). The ^{13}C-NMR data also provided evidence that one of the acetylenic carbons is lost during formation of the minor adduct.

FAB-MS analysis revealed a molecular weight of 1490.2 for the minor peptide which is consistent with a structure containing the unmodified peptide (1352.4), EU, and two additional mass units. This mass value corresponds to the mass of the major adduct (1520.3) minus a molecule of formate (30).

The UV spectrum of the minor adduct shows significant absorbance above 265 nm unlike that of the major peptide (Figure 4-15). This observation is consistent with the proposed structure where the uracil and phenylalanine are bridged by an unsaturated group which is responsible for the extended conjugation. The UV difference spectrum obtained from the subtraction of the standard peptide (NSIAFFSNPSLR) from the minor modified peptide shows an absorbance at 284
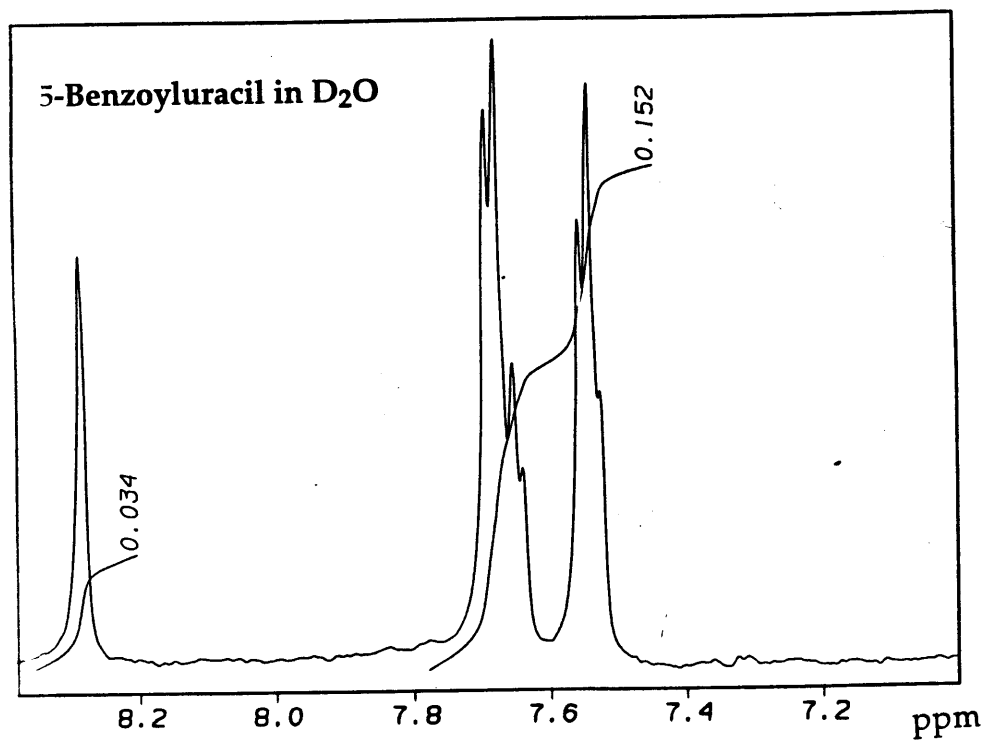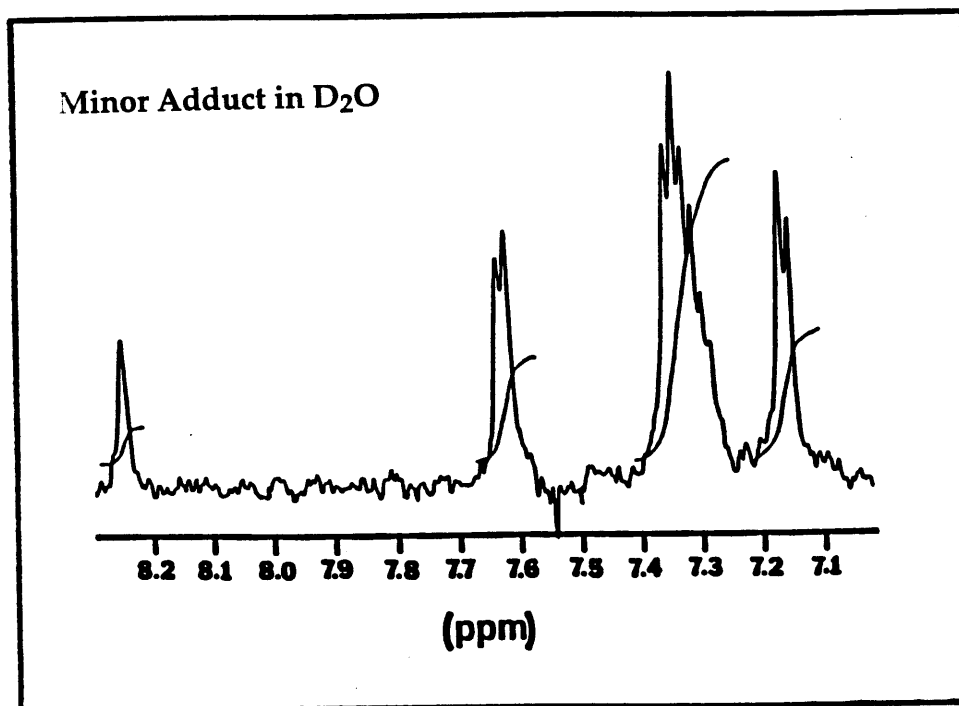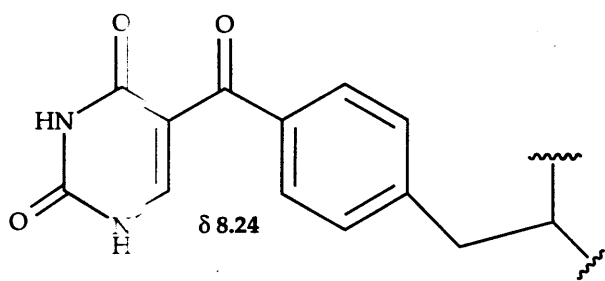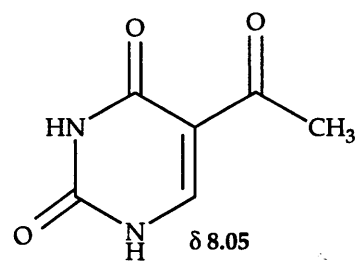
121

Minor Adduct in D2O

5-Benzoyluracil in D2O

0.152

0.034

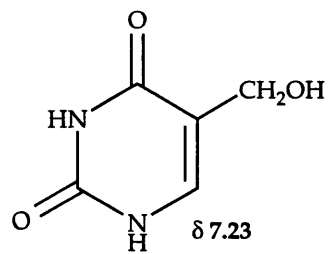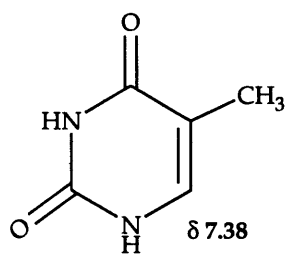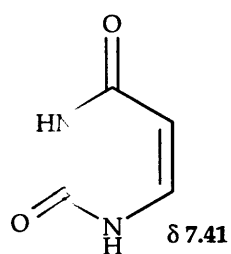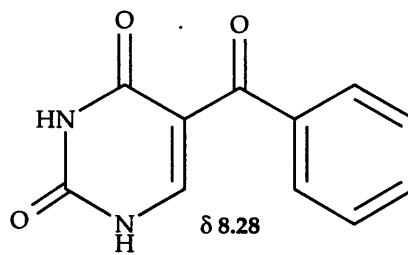Figure 4-12. Comparison of the Aromatic Regions of the 1H-NMR Spectra of the Minor Adduct and the Model Compound

δ 7.41

δ 7.38

δ 7.23

δ 8.41

δ 8.27

δ 8.05

δ 8.24

MINOR ADDUCT

δ 8.28
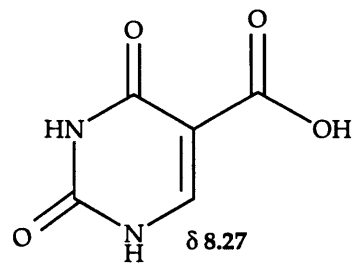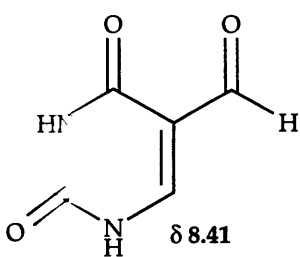
5-Benzoyluracil

Figure 4-13. Proton Chemical Shifts of C-6 Protons of Uracil Analogs Compared to the Minor Adduct and BU
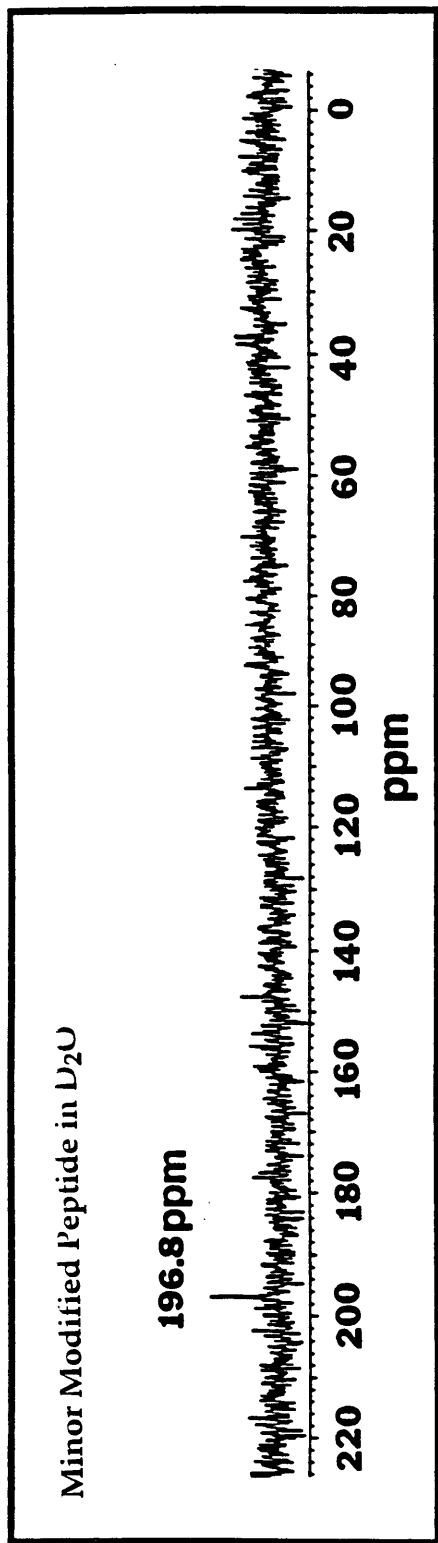
123

Figure 4-14. Comparison of the 13C-NMR Spectra of the Minor Modified Peptide with that of 5-Benzoyluracil

nm, also supporting a conjugated system (Figure 4-16).

To provide additional support for the proposed structure of the minor adduct the synthesis of 5-benzoyluracil, BU, has been undertaken. A number of synthetic approaches were tried in the synthesis of BU before the desired product was obtained. The first synthetic method was based on recent work from Bruce Eaton's laboratory.[5] His co-workers have developed methods for the synthesis of uridine derivatives with appended carbonyls using the palladium-mediated cabonylative carbon-carbon bond forming reactions known as Stille carbonylative couplings.

Outlined below is the general reaction scheme for the Stille carbonylative coupling showing the transformation of 5-iodouracil to 5-benzoyl uracil (Figure 4-17). In this reaction the palladium first inserts into the uracil-iodine bond. CO insertion occurs after this oxidative addition and prior to the transmetallation step with tributylphenyltin. Isomerization of the palladium complex then places the uracil and phenyl *cis* so that reductive elimination yields the desired product. Unfortunately, this procedure proved unsuitable for derivatizing 5-iodouracil most likely because of the electronic differences between the protected 5-iodouridine used by Eaton and our starting material, 5-iodouracil. The procedure would perhaps be successful used in conjunction with protected or modified uracils.

A : major modified peptide
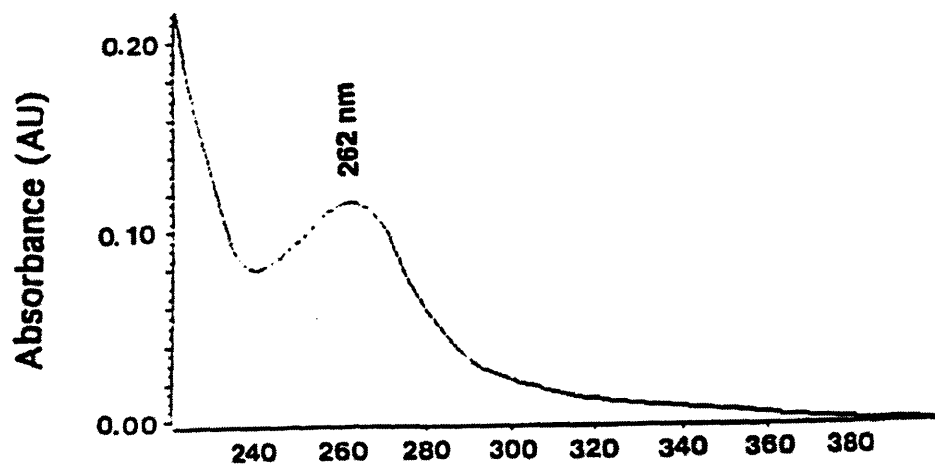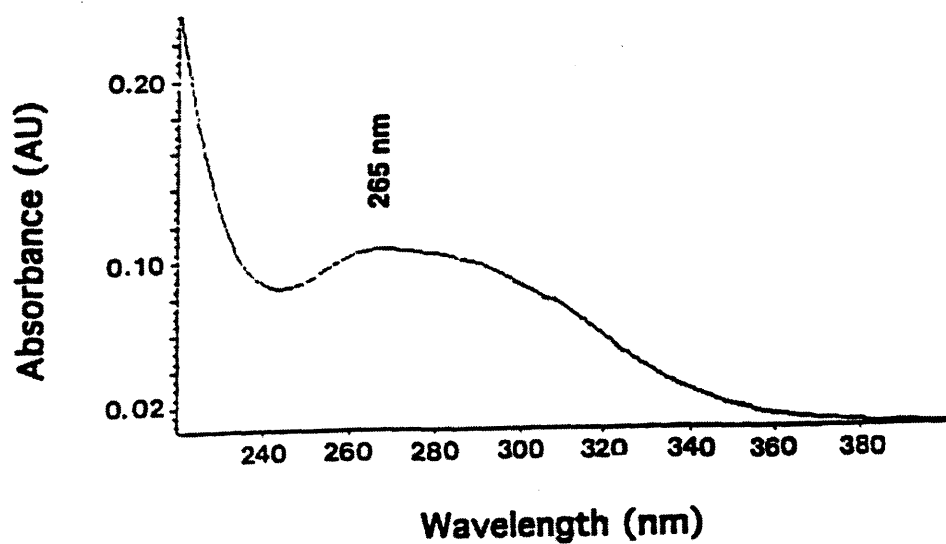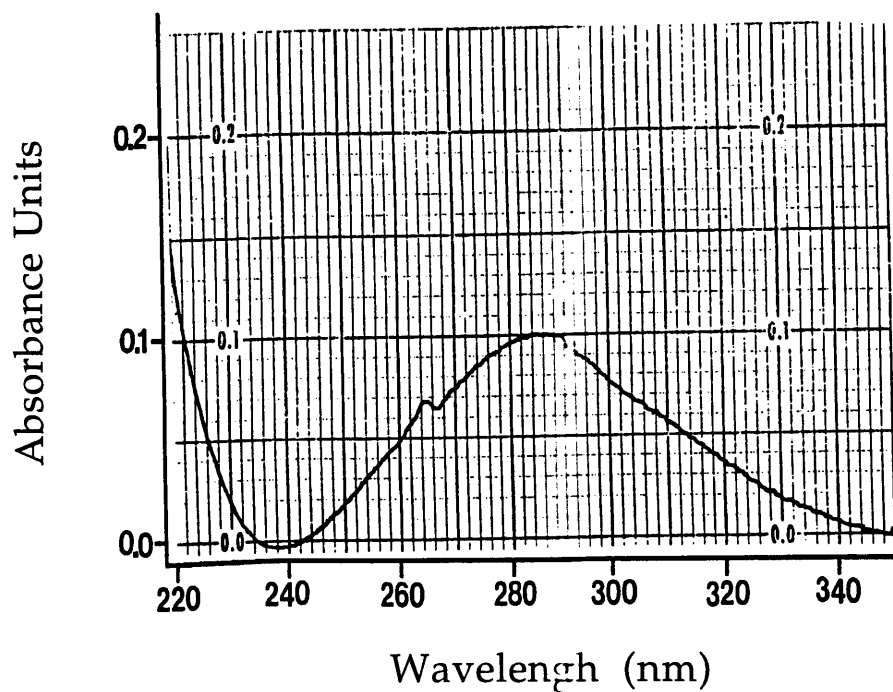

B : minor modified peptide

Figure 4-15. UV-Vis Spectra of Major and Minor Adducts from the Inactivation of Thymine Hydroxylase by 5-Ethynyluracil
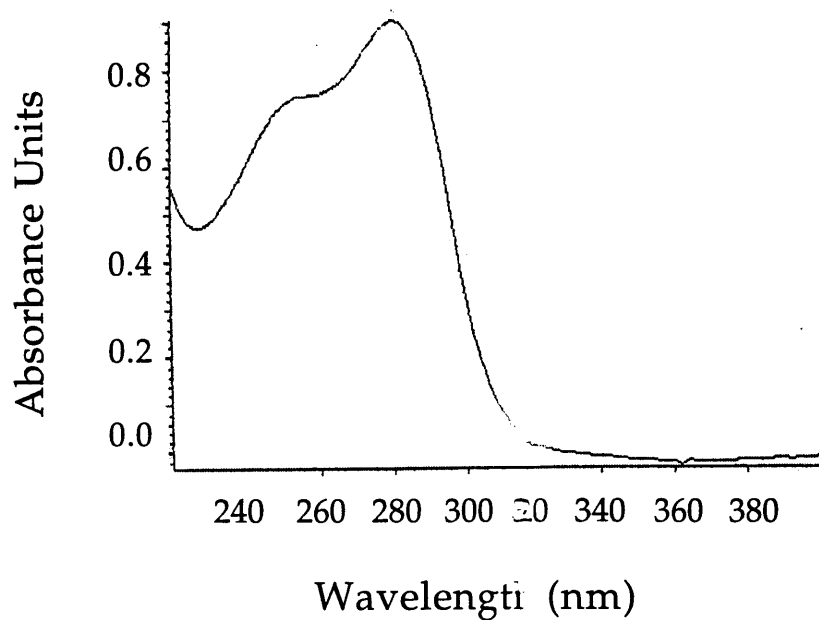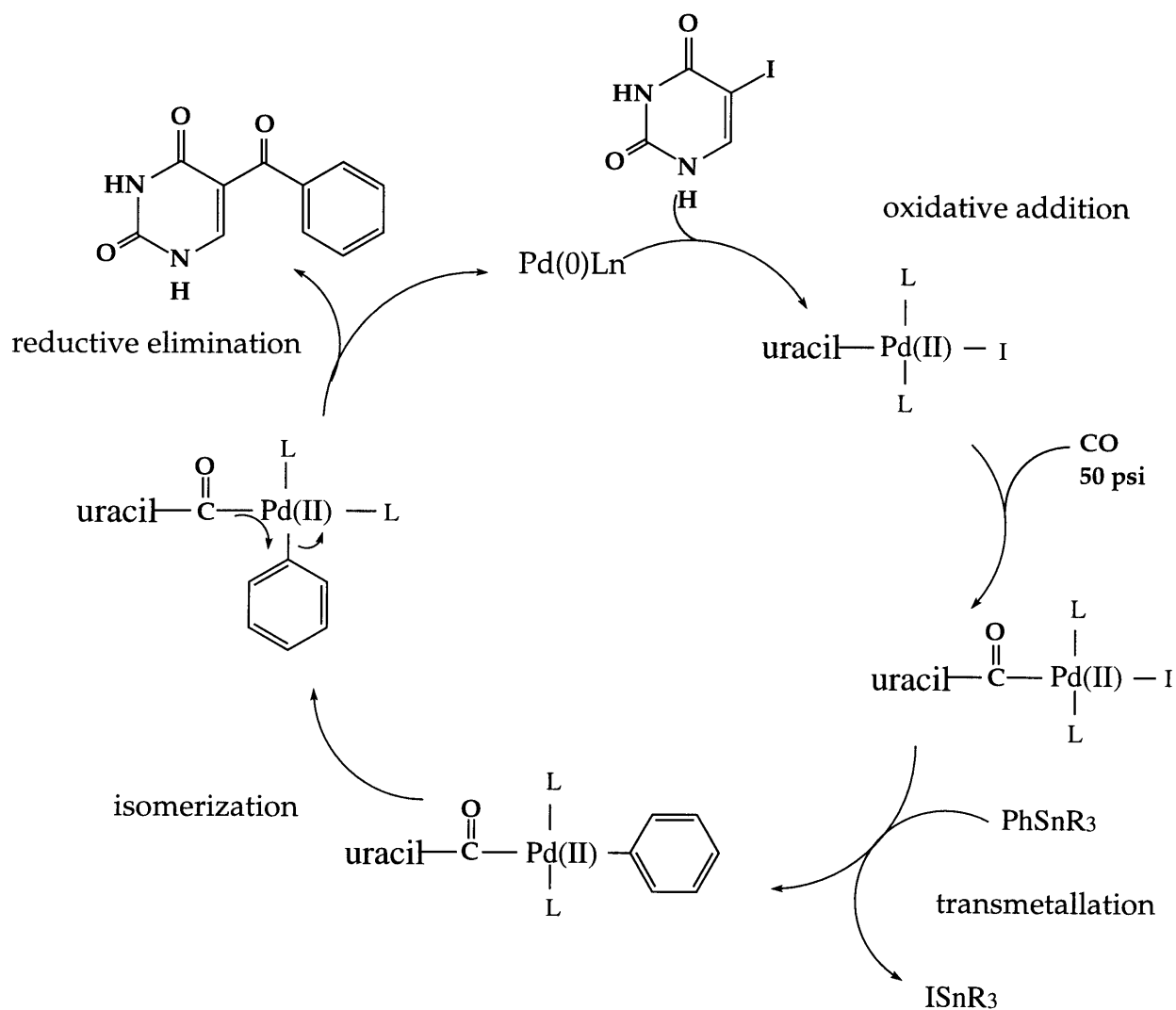
126

A)

B)

Figure 4-16. Comparison of the UV Difference Spectrum of the Minor Modified
Peptide with the UV Spectrum of 5-Benzoyluracil

Figure 4-17. Stille Carbonylative Coupling

The second synthetic method employed in the model compound synthesis was based on the procedure Lora Thornburg used to synthesize the related 5-acetyluracil.[6] The commercially available ethyl benzoylacetic acid (in place of the diketene) was condensed with urethane to yield the intermediate N-acetobenzoylurethane compound. The N-acetobenzoylurethane compound could then be reacted with triethylorthoformate in acetic anhydride to form the linear ethoxyacrylamide compound. The ethoxyacrylamide warmed in the presence of 3N ammonia, cooled and then acidified would yield the desired compound (Figure 4-
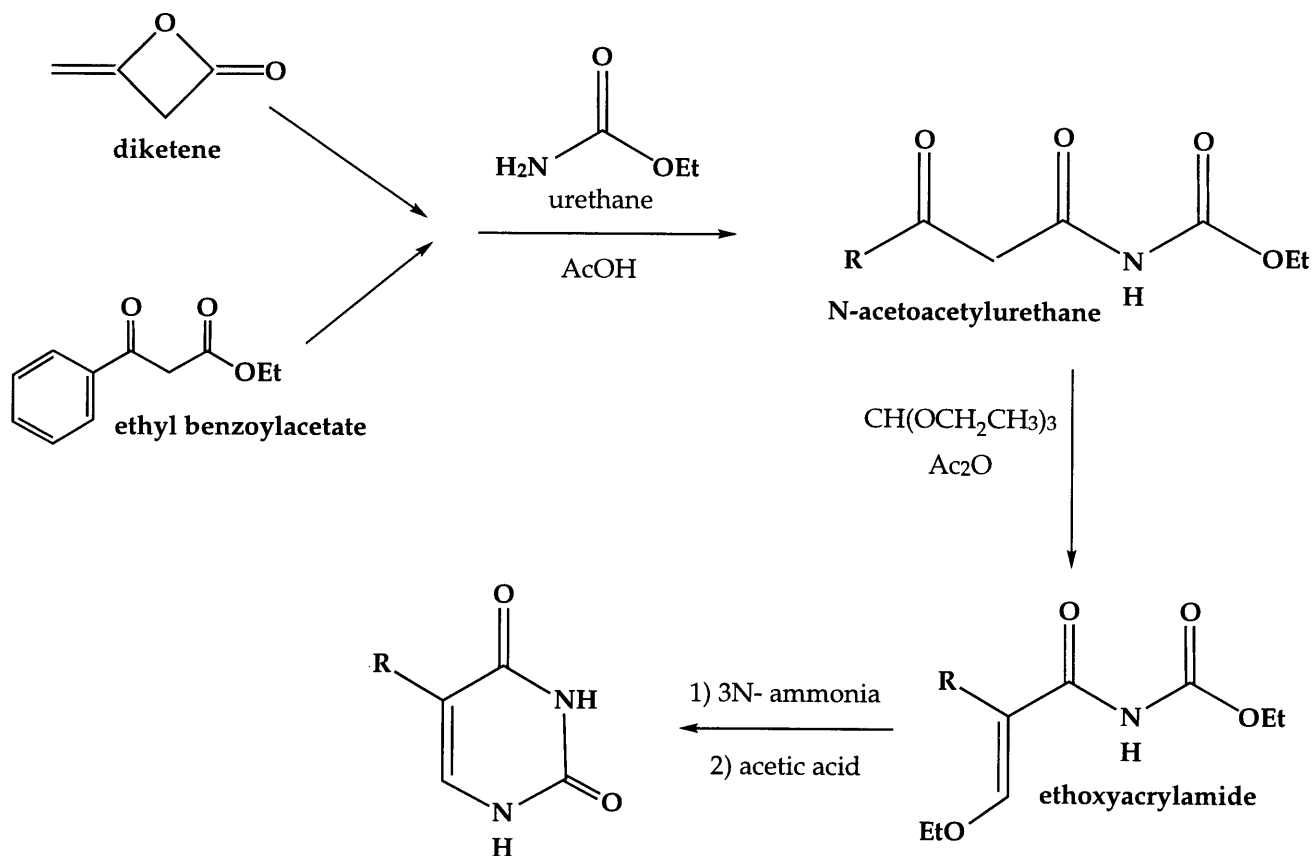
Figure 4-18. Synthesis of 5-Benzoyluracil Based on Synthesis of 5-Acetyluracil

18). Unfortunately, the ethyl benzoylacetate did not react with the urethane to give the desired product. This is most likely due to the relative reactivity of ethyl benzoylacetate compared to the diketene used in the 5-acetyluracil synthesis.

The procedure used to successfully synthesize BU was based on reports in the literature of the condensation of alkoxymethylene esters with ureas to yield acyclic pyrimidine intermediates.[6-10] The acyclic pyrimidines are then cyclized to yield a variety of substituted pyrimidines. The first step in the synthesis of BU was the reaction of ethyl benzoylacetate with triethyl orthoformate by a Knoevenagel condensation-like mechanism to yield alkylidene **1** (Figure 4-3).[Dewar, 1961 #68] Urea was then added to **1** via a Michael-type addition to afford both isomers of the acyclic uracil, urea derivative **2**. The crude uracil derivative was obtained after

heating in base and then adding 1 equivalent acid.[Buerger, 1934 #67] The synthetic procedure for preparing BU is straightforward and reproducible through the synthesis of urea derivative **2**. The cyclization of **2** to yield the desired product proved to be troublesome; however, a protocol was finally developed which is reproducible.

Initially, cyclization was accomplished by the literature procedure for the synthesis of 1-methyl-5-acetyluracil. Compound **2** was dissolved in the 5% aqueous KOH at 75°C before acidifying the reaction mixture with glacial acetic acid (final pH 5-6 [NOTE: pH value not mentioned in published procedure]).[9] ^{1}H-NMR analysis of the reaction mixture revealed the presence of the desired product plus potassium acetate. Removal of the potassium acetate by acidifying the sample with HCl and then lyophilizing the sample to remove volatile acetate did not work as well as hoped so an alternate procedure for cyclization was developed. Instead of acidifying with acetic acid, a single equivalent of HCl was added to the cyclization reaction. This procedure worked well and is reproducible. One other observation regarding the cyclization is the importance of heat to the reaction. The acid should be added to the aqueous base while the reaction is heating and should be allowed to stir several minutes on the heat before cooling the flask to room temperature.

The purification of BU by HPLC was developed and the compound was isolated for 1D ^{1}H- and ^{13}C-NMR spectroscopies. BU is not very soluble in H_2O and so the ^{13}C-NMR spectrum was taken of a sample in DMSO. The ^{1}H-NMR sample was prepared in D_2O for comparison to the minor peptide adduct. Comparison of the spectroscopic data of the model compound to that of the modified peptide is consistent with the original assignment of the minor adduct structure (Table 4-1). The spectrum of BU also shows a downfield shift for the C-6 proton in D_2O at 8.28 ppm referenced to TSP (Figure 4-12). This is almost identical to the resonance at 8.24

ppm seen in the minor adduct spectrum which has been assigned as the uracil C-6 proton.

The aromatic resonances for BU and the minor adduct are, not suprisingly, different since BU is a monosubstituted system (1 electron withdrawing-substituent) and the peptide is a disubstituted system (1 electron-withdrawing and 1 electron-donating substituent). In addition, the resonances for the minor adduct between 7.7 and 7.1 ppm contain the unmodified phenylalanine resonances overlapping the modified phenylalanine resonances which complicate assignment of the peaks. The upfield shift of the minor adduct resonances with respect to BU can in part be explained by the electron donating nature of the peptide chain which is absent in the model compound. A simple example of an upfield shift caused by an electron-donating substituent is illustrated by the comparison of the chemical shifts of the aromatic protons of benzene (7.15 ppm) and toluene (7.09, 7.0 and 6.98 ppm).

The ^{13}C NMR spectrum was obtained for BU in DMSO because of the compound's limited solubility in H_2O (Figure 4-14). As referenced to the methyl carbon of TSP, the bridging carbonyl carbon resonance appeared at 191.6 ppm. The UV absorbance of the model compound appears at 280 nm, close to the absorbance at 284 nm seen in the UV difference spectrum between the minor modified and unmodified peptides (Figure 4-14).

The focus of the project was directed next toward obtaining evidence for the mechanism of the minor adduct's formation. Precedence for this type of partitioning between oxidation and cleavage reactions exists in heme-iron dependent systems (Figure 4-19). Akhtar and co-workers have studied several P-450 enzymes including aromatase, 14α-demethylase and 17α-hydroxylase-17,20-lyase which catalyze not only hydroxylations but also the oxidation of an alcohol to a carbonyl compound and an acyl-carbon bond cleavage reaction.[11] Shown below is

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the series of reactions catalyzed by sterol 14α-demethylase (Figure 4-20). Based on

Table 4-1. Comparison of the Spectroscopic Data for the Minor Adduct and BU

| Spectroscopic Technique	Minor Enzyme Adduct	Model Compound (BU)
^{13}C-NMR Bridging Carbonyl	196.8 ppm	191.6 ppm
^{1}H-NMR uracil C-6 H	8.24 ppm	8.28 ppm
Uv-vis Spectroscopy	$\lambda_{max} = 284$	$\lambda_{max} = 280$

extensive studies of sterol 14α-demethylase and related systems they have proposed that these enzymes use the $Fe^{III}OOH$ species, which is normally generated as a precursor to the oxo derivative postulated to be reponsible for hydrogen-atom abstraction, to carry out nucleophilic addition to a carbonyl carbon (Figure 4-21).
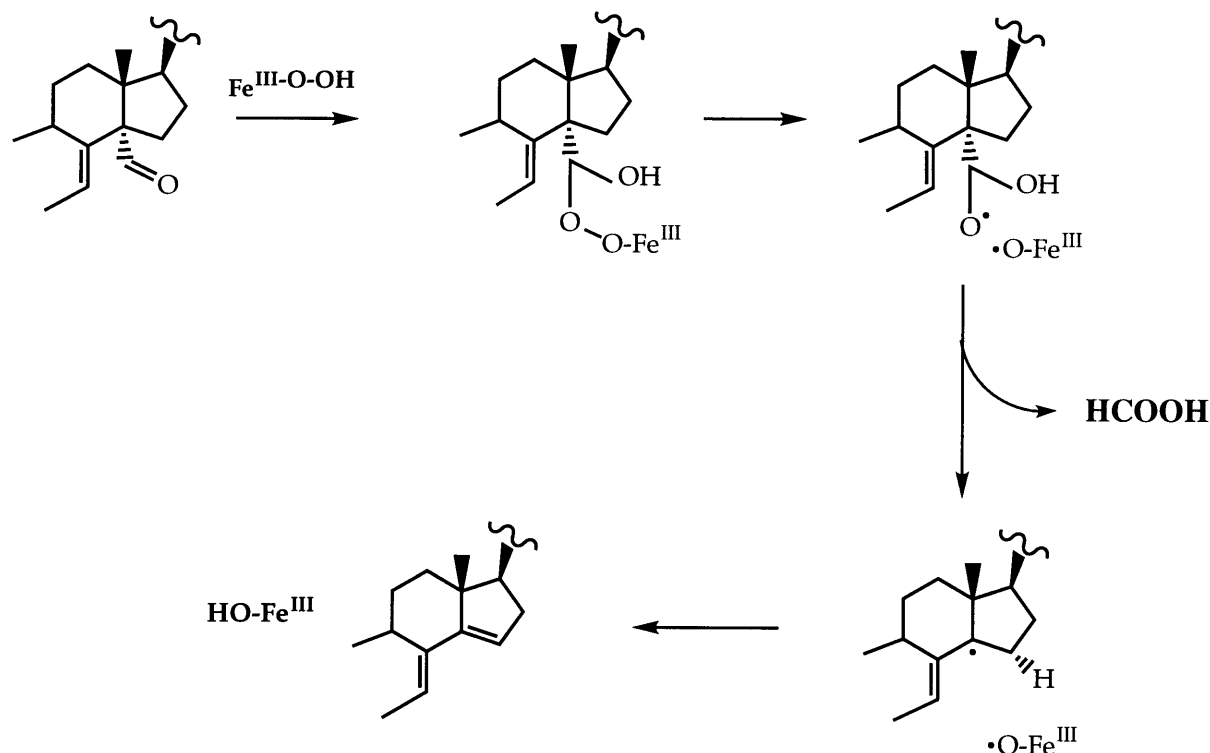
FRAGMENTATION
PATH
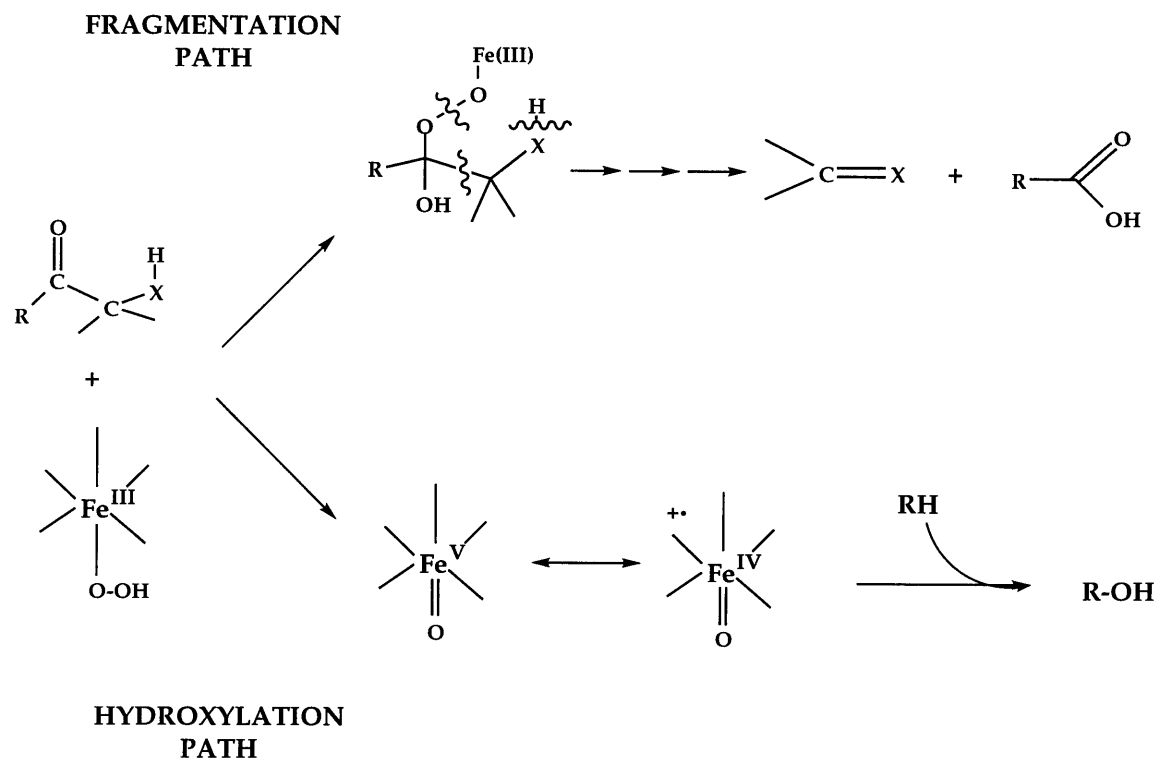
Figure 4-19. Hydroxylation and Fragmentation Reactions Catalyzed by P-450
Enzymes
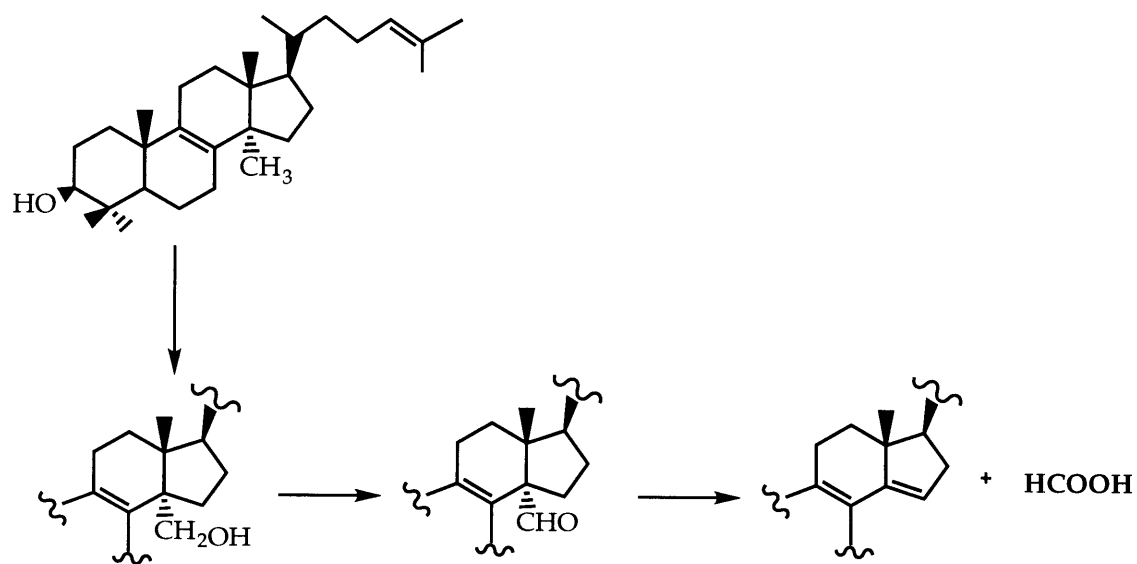
Figure 4-20. Sequence of Reactions Catalyzed by Sterol 14α-demethylase

This is followed by homolytic cleavage of the Fe-O bond, fragmentation resulting in the production of formate and finally, disproportionation resulting in the production of an olefin.

Figure 4-21. Postulated mechanism for the acyl-carbon bond cleavage reaction by sterol 14α-demethylase

A key prediction of the proposed mechanism for the formation of the minor adduct during the inactivation of TH by EU is that formate is produced. Work was begun to determine if formate is released during the reaction. Although the reaction and work-up procedures are straightforward, the small quantities of formate expected from the inactivation make formate detection and reaction analysis difficult. Akthar and co-workers have used a procedure in their studies developed to detect small quantities of formate by derivatizing the formate produced biosynthetically with PDM.[3] The resulting benzyl formate is then analyzed by GC-MS.

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Preliminary studies were undertaken to examine the detection of small quantities of formate. Two different approaches were studied: the chemical derivatization of formate for GC-MS analysis and the enzymatic detection of formate by formate dehydrogenase (FDH).

Investigations into the sensitivity of GC analysis were undertaken with authentic samples of benzyl formate. Measurements made showed that the response of the detector was linear for samples with concentrations ranging from 0.002- 2 nmol/µL. This suggested the potential to measure quantities of biosynthetically formed formate as low as fractions of a ng per µL. Samples of formate derivatized with PDM as described in the experimental section were also analyzed by GC-MS. Benzyl formate was detected in these samples, however, the GC-MS analysis revealed the presence of numerous additional species. At this point the condition for the purification of PDM by distillation were worked out to try to clean up the derivatization reaction. Derivitization reactions were re-done with purified PDM. These analyses were much cleaner. The other products seen in the trace (benzyl methyl ether, benzyl chloride and benzyl alcohol) were well-separated from the benzyl formate.

The FDH end-point assay was tested with both authentic samples of sodium formate and formate samples collected in mock reaction distillations. Results from assays done with authentic samples showed the expected absorbance changes in concentrations ranging from 0.006-0.044 mM. Some issues that were not addressed in these experiments is whether under reaction conditions the conversion of formate to CO_2 is is reversible and if the reaction is at equilibrium. If use of this assay is to be pursued in the future, this information must be determined.

Although the standards appeared to work well, unfortunately, quantitation of samples collected from mock distillations was not straightforward and the experimental values did not correlate well with the amount of formate expected.

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One possibility is that the treatment of the bulb-to-bulb distilled sample is affecting the assay reaction mixture. In early FDH assays with the mock distilled formate, excess base was used to basify the collection flask contents prior to lyophilization. The excess base caused the reduction of NAD^+ in the assay mixtures and a falsely elevated change in absorbance. This problem appeared to be corrected by adjusting the distillate pH with the FDH buffer (minimal amount to achieve required pH of 6-7) instead of with 0.5 M NaOH. Another potential problem is that the recovery from the distillate flask was not consistant, either because of poor recoveries from the collection flask walls or from loss of formate during lyophilization. With the small amounts of formate being analyzed, this technique appeared only sufficient to determine whether formate was present, and was not useful for quantitative purposes.

Prior to running the formate-release experiment, a reaction and control were run to test the time-scale of the inactivation under the conditions being used for the experiment. Shown in Figure 4-22 is the activity present in the reaction and control vials over 11 minutes. Aliquots were withdrawn from the reaction and control and the activity measured using the $^{14}CO_2$ assay. Assays were run both with thymine and without thymine to determine reaction background.
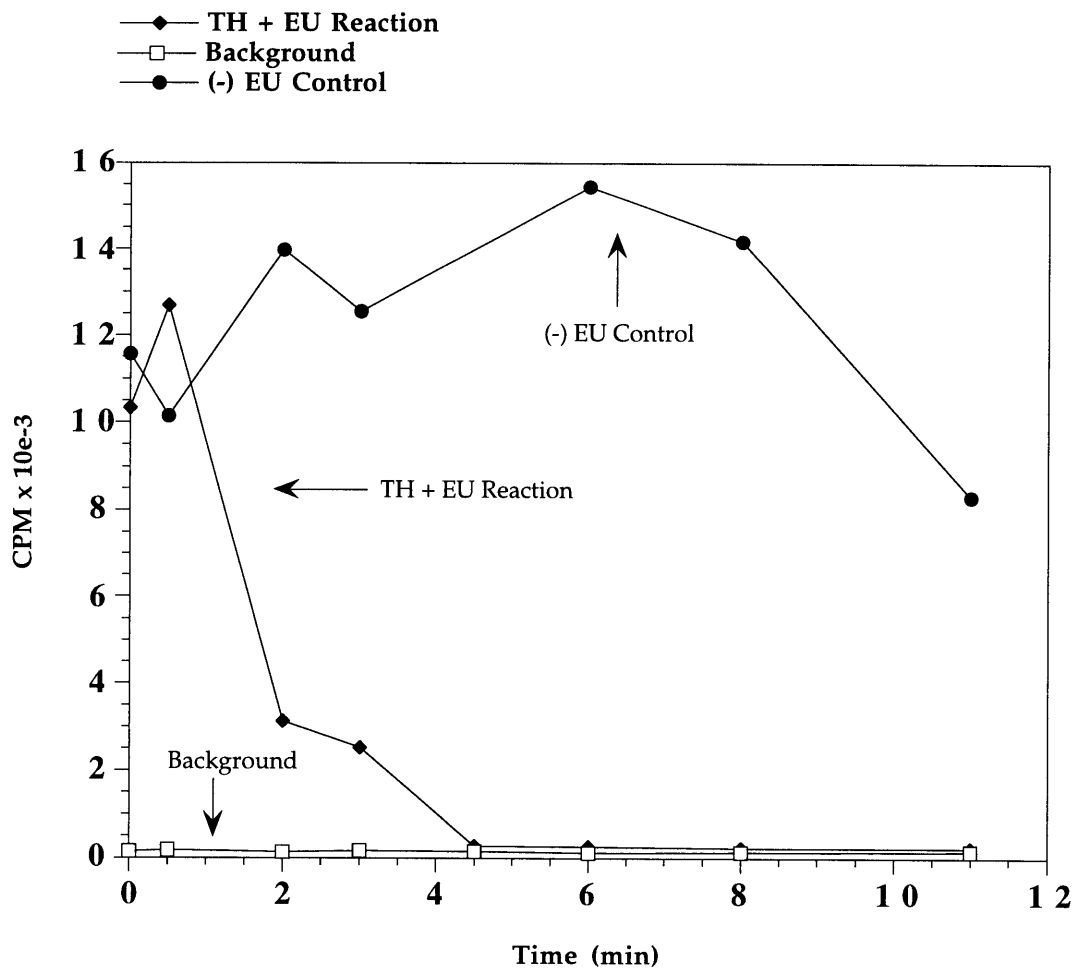
Figure 4-22. Time-Dependence of the Inactivation of TH by EU

In the formate-release experiment, 11 nmol TH was reacted with 49 nmol EU. Based on the reported value of 3:1 ratio of formation of major and minor adduct, 2-4 nmol formate was expected.[12] Taking into account the distillation efficiency, approximately 1.6-3.2 nmol formate could potentially have been isolated from the reaction. The efficiency of the PDM derivatization had not been determined. To account for the possibility of less than 100% derivatization, a known amount of ^{13}C-formate (determined enzymatically by the FDH assay) was added to the reaction mixture as an internal control before the acidification and bulb-to-bulb distillation.

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Quantitation of the ^{13}C-benzyl formate peak would allow for determination of the efficiency of distillation and derivitization. If 1.6 nmol formate was collected, after derivitization, solutions with concentrations of at least 0.016 nmol/μL were expected. Two controls were run in addition to the reaction-- one minus enzyme and one minus inhibitor. Following the distillations, the samples were lyophilized to dryness and then derivatized as described in the experimental section. GC-MS analysis of the reaction and control samples revealed that a side product formed in the derivitization reaction obscured any signal which may have appeared from benzyl formate (either [13C]- or [12C]-formate).

The identity of this product remains to be established and the proper conditions for the PDM derivatization worked out before the experiment can be repeated. Although much less sensitive, the FDH assay may be an alternative to examination of the inactivation. To see an absorbance change of at least 0.1 absorbance units, 4 nmol formate have to be formed. This would mean running the reaction with 26 nmol TH or 1 mg protein. Inactivation reactions run with several mgs TH could produce enough formate to be detected, although most likely not quantitated.

In conclusion, the work accomplished on the structural aspect of the project was successful. Spectroscopic data suggests that 5-benzoyluracil is a good model for the minor adduct. Whether formate is produced during the inactivation remains to be determined. Before this can be examined, however, the lower limits of detection of the formate quantitation assay, either the PDM derivatization or the formate dehydrogenase assay, must be determined accurately and under identical conditions to be used in the final experiment.

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