1

OF/DE

1

MicroD

National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

**ACTIVATION AND RECONSTITUTION STUDIES
OF THE ENZYME L-PHENYLALANINE HYDROXYLASE**

SUSAN A. EATOCK

A Thesis submitted to the Faculty of Graduate Studies
In partial fulfilment of the requirements
for the degree of Masters of Science

Department of Chemistry
Carleton University
Ottawa, Ontario
May, 1989.

National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-54424-4

The undersigned hereby recommends to the Faculty of Graduate Studies the acceptance of this thesis submitted by Susan A. Eatock, Hon. B.Sc., in partial fulfilment of the requirements for the degree of Masters of Science.

Dr. D.C. Wigfield
Supervisor

Chairman of the Chemistry
Department

INTRODUCTION

Metals are an essential dietary requirement for proper health and nutrition. Humans require a blood plasma concentration of most metals at ppm levels. It has been recognized for some time, that iron, copper, cobalt and zinc are biologically active. In later years it became apparent that vanadium, chromium, manganese, nickel, molybdenum and tungsten are also necessary elements for some biological systems. Mercury, cadmium and lead have also been found present, but these metals seem to play a toxic role and have been introduced to biological systems by our present day environment. All metals have optimum concentrations and become toxic above approximately 200 ppm.(1) Several metals are antagonistic and interfere with each other's functions. Metals are absorbed through the mucous membrane in the duodenum and are then bound to carrier proteins such as transferrin, ceruloplasmin and the serum globulins.

Metal Activators and Metalloenzymes

Metals play a role as either protein activators, or in metalloenzymes. As protein activators the metal is loosely coordinated and is not an integral part of the enzyme, as is the case with metalloenzymes. The metals in metalloenzymes are tightly coordinated as part of the tertiary protein structure. In both cases the metal is required for protein activity.

The metal in metalloenzymes, may be structural or catalytically active. Structurally they may stabilize the tertiary structure of the protein, or they may hold the subunits of a protein together to form an active complex. The metals most

ACKNOWLEDGEMENTS

I would like to thank Dr. D.C. Wigfield for all his guidance and encouragement during the last two and half years.

Thank you to Dr. C.D. Chakabarti and Mr. Glen Gilchrist for the A.A. Analysis of some of the metal standards.

I would like to especially thank Mr. Doug Goltz for his company and assistance during the lengthy literature search that we performed at the beginning of our masters projects. I would also like to thank Doug for his special skill of keeping everything in its proper perspective and for his sense of humour that made the worst of days enjoyable.

I would like to thank Dr. Crutchley's group for keeping school fun and for the use of their glove box.

Thank you to Mr. Lee Collier for his help in making me a little more computer literate.

And finally I would like to thank my family and my good friend Jamie, who were a constant source of strength and support when things got tough.

TABLE OF CONTENTS

TITLE	PAGE
ABSTRACT	i
ACKNOWLEDGEMENTS	ii
TABLE OF CONTENTS	iii
LIST OF FIGURES	v
LIST OF TABLES	vi
INTRODUCTION	
Metal Activators and Metalloenzymes	1
L-Phenylalanine Metabolism	3
L-Phenylalanine Hydroxylase Structure	8
Enzyme Activation	9
L-Phenylalanine Hydroxylase Reduction	11
The Iron in L-Phenylalanine Hydroxylase	13
EXPERIMENTAL DESIGN	
Enzyme Assay	15
Choice of Assay	18
Fixed Assay	21
Enzyme Purification	25
Controls and Special Considerations	27
MATERIALS AND METHODS	
Buffers and Solutions	28

TABLE OF CONTENTS CONTINUED

TITLE	PAGE
L-Phenylalanine Hydroxylase	29
Enzyme Activation	30
Enzyme Assays	31
Enzyme Purification	34
Metal Stock Solutions	38
Metal Activation Studies	39
Apo-Enzyme Reconstitution Studies	41
Reconstitution by other Metals	42
Anaerobic Reconstitution Studies	42
Atomic Absorption Analysis of Metal Stock Solutions	42
 RESULTS AND DISCUSSION	
Metal Activation Studies	43
Iron Reconstitution	62
Reconstitution by Other Metals	67
Anaerobic Reconstitution	76
In Conclusion	83
 REFERENCES	 81

myelination of the nerves and hyperactive reflexes.(7) This condition has been named phenylketonuria (PKU). It is a recessive autosomal inherited condition, which occurs with a frequency of approximately 1 in 20,000 births.(7) Presently, the only treatment for children suffering from PKU is a low phenylalanine diet.

The high frequency of impaired phenylalanine metabolism and its severe consequences has brought interest and research to the characterization of the enzyme phenylalanine hydroxylase.

L-PHENYLALANINE HYDROXYLASE STRUCTURE

Phenylalanine Hydroxylase is comprised of four equal subunits of molecular weight 50,000. It contains 417 amino acids with a mainly alpha helix structure.(8) There is one mole of a non-heme iron per subunit. The active site is as yet not fully characterized. It has been proposed that it contains three histidine groups, to which the iron is tightly coordinated through the lone pairs of electrons on the nitrogens. PAH contains four sulfhydryl groups. Three are completely buried and one is partially exposed.(9) Iodoacetate does not inactivate the hydroxylase, so it is thought that a sulfhydryl group is not at the active center.(10) It has been found that the exposure of this sulfhydryl group is increased by partial proteolysis, phosphorylation and interaction with some phospholipids such as lysolecithin. (11) Increased hydrophobicity of the enzyme surface occurs concurrently with increased exposure of this sulfhydryl group and increased enzyme activity is observed. An "internal inhibitor model" has been proposed, where the enzyme contains a polypeptide region on each subunit which acts as an inhibitor. Displacement of this segment by charge repulsion results in activation. (9)

LIST OF TABLES

TABLE	TITLE	PAGE
1	Metal Stock Solution Preparation and Analysis	76
2	Summary of Metal Activation Studies	45
3	Properties of Metals Used in Studies	47
	Metal Activation Study using 100 fold Excess of Metals	
4	Trial 1	52
5	Trial 2	53
6	Trial 3	54
7	Trial 4	55
8	Trial 5	56
	Metal Activation Studies Using 10 Fold Excess of Metals	
9	Trial 1	59*
10	Trial 2	60
11	Trial 3	61
12	Summary of Apo-Reconstitution Studies	70
	Apo-Reconstitution By other Metals	
13	Trial 1	72
14	Trial 2	73
15	Trial 3	75
16	Summary of Anaerobic Apo-Enzyme Reconstitution Studies	77
	Anaerobic Apo-Reconstitution Studies	
17	Trial 1	80
18	Trial 2	81

INTRODUCTION

Metals are an essential dietary requirement for proper health and nutrition. Humans require a blood plasma concentration of most metals at ppm levels. It has been recognized for some time, that iron, copper, cobalt and zinc are biologically active. In later years it became apparent that vanadium, chromium, manganese, nickel, molybdenum and tungsten are also necessary elements for some biological systems. Mercury, cadmium and lead have also been found present, but these metals seem to play a toxic role and have been introduced to biological systems by our present day environment. All metals have optimum concentrations and become toxic above approximately 200 ppm.(1) Several metals are antagonistic and interfere with each other's functions. Metals are absorbed through the mucous membrane in the duodenum and are then bound to carrier proteins such as transferrin, ceruloplasmin and the serum globulins.

Metal Activators and Metalloenzymes

Metals play a role as either protein activators, or in metalloenzymes. As protein activators the metal is loosely coordinated and is not an integral part of the enzyme, as is the case with metalloenzymes. The metals in metalloenzymes are tightly coordinated as part of the tertiary protein structure. In both cases the metal is required for protein activity.

The metal in metalloenzymes, may be structural or catalytically active. Structurally they may stabilize the tertiary structure of the protein, or they may hold the subunits of a protein together to form an active complex. The metals most

often involved in these processes are Ca^{2+} , Zn^{2+} , Fe^{3+} and Mn^{2+} .(2) Many other metals are chemically capable but evolution appears to have picked those that were most abundant in nature.

Metals that are involved in the catalytic processes are usually found complexed at or near the enzyme active site. The function of these metals at the active site usually falls into the following categories:

1. Oxidation/Reduction Reactions
2. Acid-Base Reactions
3. Oxygen Activation Reactions

Oxidation-reduction reactions are usually those where the metal acts as an electron carrier or an electron mediator. Those commonly involved are Mn, Mo, Fe, Cu. There have been newly reported cases where Ni and Se have shown to be complexed and active in some enzymes.(3)

In acid-base reactions the metals complex the substrate(s) and bring the enzyme and substrate into close proximity of each other to allow interaction. They often induce the right rotation of the substrate and cause proper alignment. They may polarize the center of attack (i.e. carbonyl group) to make it more susceptible and the reaction more favorable. The enzymes which fit into this category are the transferases, hydrolases, ligases, isomerases and lyases. In all cases there are bonds broken or attacked in a specific manner. Zinc is the most common and important metal in this group. It is readily available in the earth, it may take the strained bipyrimidal form necessary to form the complex and it is strongly polarizing.(2)

The final set of metal enzyme reactions are those where oxygen must be activated in order to be incorporated into a molecule. These include:

1. Oxygen carrying and storing
2. Superoxide dismutase
3. Dioxygenases
4. Monooxygenases
5. Dihydroxylating

The metals that most often take part in these reactions are iron and copper. In most of these cases a cofactor, NADH, FADH or a pterin are required as a reducing agent.(3)

The metalloenzyme investigated in this project falls into this final category of oxygen activators. L-Phenylalanine Hydroxylase (PAH) is the monooxygenase found in the hepatic system, which is responsible for the conversion of excess phenylalanine to tyrosine. PAH requires the pterin cofactor tetrahydrobiopterin. The cofactor is oxidized and regenerated by the enzyme dehydropteridine reductase. The reaction pathway is shown in figure 1.

L-PHENYLALANINE METABOLISM

Phenylalanine Hydroxylase plays a critical role in amino acid metabolism by lowering excess phenylalanine concentrations. Phenylalanine concentrations in the blood above 3.0 mM L^{-1} causes several metabolic pathways to dysfunction. A high phenylalanine concentration will interfere with glycolysis by inhibiting brain hexokinase and brain pyruvate kinase by competitive inhibition.(4) This causes a decreased rate in the production of ATP, GTP and UTP which are necessary for lipid, protein, DNA and RNA synthesis in the brain. This inhibition may be observed

FIGURE 1: REACTION PATHWAY OF L-PHENYLALANINE METABOLISM

by increased concentrations of phosphoenol pyruvate, dihydroxyacetone phosphate and fructose 1,6-diphosphate.(4)

Phenylalanine is metabolized as shown in figure 2. L-phenylalanine may be acetylated to N-acetylphenylalanine, decarboxylated to phenylethylamine or transaminated to phenylpyruvic acid. Phenylpyruvate inhibits gluconeogenesis. The exact place in the pathway where the inhibition is applied has not yet been determined. Phenylacetate effects the decarboxylases and alters lipid metabolism by inhibiting the desaturation of stearyl CoA. This causes a reduction in the amounts of monounsaturated fatty acids in the brain.(5) Phenylacetic acid conjugates with glutamine. This lowers the glutamine levels available in the brain capable of detoxifying ammonia.

Lowered PAH activity dramatically affects metabolism by lowering the concentration of tyrosine available for protein synthesis and other synthetic pathways. The production of the catecholamine hormones adrenalin, noradrenaline and dopamine is dramatically reduced. Again without sufficient levels of these hormones glucose metabolism will be unstable. These hormones are also important neurotransmitters. Amino acids must be actively transported across membranes. High concentrations of phenylalanine competitively inhibits the transportation of tryptophan and tyrosine.(6)

The enzyme tyrosinase which is responsible for the formation of the pigment melanin, found in hair and skin, also requires tyrosine as its substrate. People suffering from low PAH activity suffer the identical symptoms of those with low tyrosinase activity. They are very pale and fair skinned.

These alterations in the metabolic pathways, due to low phenylalanine hydroxylase activity, manifest themselves in severe mental retardation, defective

FIGURE 2: ABNORMAL METABOLITES OF L-PHENYLALANINE

FIGURE 3: INHIBITION OF OF METABOLIC PATHWAYS BY EXCESS L-PHENYLALANINE

myelination of the nerves and hyperactive reflexes.(7) This condition has been named phenylketonuria (PKU). It is a recessive autosomal inherited condition, which occurs with a frequency of approximately 1 in 20,000 births.(7) Presently, the only treatment for children suffering from PKU is a low phenylalanine diet.

The high frequency of impaired phenylalanine metabolism and its severe consequences has brought interest and research to the characterization of the enzyme phenylalanine hydroxylase.

L-PHENYLALANINE HYDROXYLASE STRUCTURE

Phenylalanine Hydroxylase is comprised of four equal subunits of molecular weight 50,000. It contains 417 amino acids with a mainly alpha helix structure.(8) There is one mole of a non-heme iron per subunit. The active site is as yet not fully characterized. It has been proposed that it contains three histidine groups, to which the iron is tightly coordinated through the lone pairs of electrons on the nitrogens. PAH contains four sulfhydryl groups. Three are completely buried and one is partially exposed.(9) Iodoacetate does not inactivate the hydroxylase, so it is thought that a sulfhydryl group is not at the active center.(10) It has been found that the exposure of this sulfhydryl group is increased by partial proteolysis, phosphorylation and interaction with some phospholipids such as lysolecithin. (11) Increased hydrophobicity of the enzyme surface occurs concurrently with increased exposure of this sulfhydryl group and increased enzyme activity is observed. An "internal inhibitor model" has been proposed, where the enzyme contains a polypeptide region on each subunit which acts as an inhibitor. Displacement of this segment by charge repulsion results in activation. (9)

The enzyme is capable of utilizing tryptophan as a substrate as well as phenylalanine, but not as efficiently.(12) As well as the natural cofactor the enzyme is capable of utilizing two synthetic analogues of the natural cofactor, 6-methyl-tetrahydropterin and 6,7-methyl-tetrahydropterin.(13) The *pseudomonas* phenylalanine hydroxylase enzyme is activated by mercury, cadmium and copper cations.(14) Mammalian PAH has been found to be inhibited by the copper(II) cation and by the common buffer Tris.(13) The enzyme is not substrate or product inhibited. Some of the catechols will inhibit the enzyme by competing with the cofactor.(13)

This is not a rugged enzyme. The pH optimum of the enzyme differs greatly depending upon the source. Rat liver enzyme has a pH optimum of 6.8-8.0.(13) The enzyme is stable frozen in solution overnight, with a loss of only 10% of its activity. It is stable indefinitely as a lyophilized powder. At 50°C the rat liver enzyme loses 50% of its activity in four minutes.(13) The apo-enzyme however, has a stability of only four minutes at 4°C.(15) L-Phenylalanine, dithiothreitol and agents that increase the hydrophobicity of the enzyme surface increase the stability of the enzyme.

ENZYME ACTIVATION

It has been observed that the enzyme exists in an equilibrium mixture of active and inactive conformations.(17) The enzyme requires several different activation steps before it is fully competent to hydroxylate phenylalanine. This allows careful regulation of phenylalanine hydroxylase activity so it will not completely deplete phenylalanine stores necessary for synthesis. Tourian found that PAH is activated by its substrate L-phenylalanine.(16) Through binding

studies it was found that the enzyme binds one phenylalanine molecule per subunit. L-tryptophan which may act as a substrate does not however, activate the enzyme.(17) It was concluded, that the activation sites are therefore not identical to the active site. Phenylalanine dependent activation changes the conformation of the enzyme forming an activated tetramer with an increased external hydrophobic surface as previously observed by other synthetic means of activation.(17)

The fully active enzyme has a linear rate for the first three to four minutes, at which time it drops to a slower rate as it decays to an inactive form of the enzyme.(16) The rate of activation is directly dependent upon L-phenylalanine concentration such that:

where E_i is the inactive enzyme, E_a is the activated enzyme, E_x is the deactivated enzyme and k_1 is the first order rate constant for the activation process.

This activation by phenylalanine has an Arrhenius activation energy of 33.5 kcal/mol and a temperature dependence which increases 7.2 fold per 10 degree celsius. In comparison the PAH hydroxylation reaction has an activation energy of 12.0 kcal/mol and a temperature increase of only 2.6 fold per 10 degrees celsius.(8) Shiman found that the four enzyme activation sites were non-independent.(8) He postulated a sequential activation of the four subunits, where the first L-phenylalanine bound is the least favorable. PAH is 50% activated by a 0.06 mM phenylalanine concentration at physiological pH of 7.4.(8) This is the phenylalanine level found in normal rat plasma. L-phenylalanine concentrations

provide a direct metabolic control on the enzyme.

L-PHENYLALANINE HYDROXYLASE REDUCTION

The enzyme is still not catalytically competent after complete activation with phenylalanine. It has been found by Wallick et al. and by further studies by Marota et al. that the enzyme requires a stoichiometric prereluction step by the cofactor separate from the hydroxylation.(18,19) Once reduced the activated reduced enzyme (E_4^*) requires an additional cofactor to complete the hydroxylation. The initial electron transferred cannot be used for the hydroxylation. Wallick et al. believes from EPR data that it is the Fe^{3+} being reduced to Fe^{2+} that is being observed. Marota et al. has shown reasons why it may not be that simple. First, during the hydroxylation reaction the cofactor is oxidized to a 4a-OH-6-MPH₄ intermediate that spontaneously dehydrates to the quinoid. During the enzyme reduction step the 4a-OH-6-MPH₄ intermediate observed during the catalytic hydroxylation is not observed. Secondly, the reduction step requires O_2 , but does not release any reduced oxygen species. It would appear that some form of oxygen becomes bound or activated and could be useable during the catalytic hydroxylation. A $Fe^{2+}-O$ complex has been suggested.(19) The reaction scheme for the enzyme reduction and activation process is shown in figure 4.

It has been observed that activated PAH is more rapidly reduced than unactivated PAH. It seems that activation affects the reduction site as well as the active site, or the reduction site and the active site are one and the same. Once reduced the enzyme remains catalytically competent for many cycles.

FIGURE 4: REDUCTION AND ACTIVATION OF PHENYLALANINE HYDROXYLASE

ENZYME + L-PHENYLALANINE → ENZYME ACTIVATED

Note: Enzyme* is the reduced enzyme, MPH₄ is methyl-tetrahydropterin
MPH₂ is the oxidized H₂quinoid

THE IRON IN L-PHENYLALANINE HYDROXYLASE

Iron might also play a role in the physiological control of PAH. In well fed adult rats upto 45% of the PAH is not fully activated. Shiman et al. found that when these same rats were perfused with iron saturated transferrin the PAH activity increased to a maximal level.(20) A significant portion of the in vivo PAH is non-free when the enzyme is in a resting state.

85% of the enzyme activity may be lost by removal of iron from the active site using a metal chelator.(21) Compounds which do coordinate Fe^{3+} like tiron are not capable of removing iron from the active site of the enzyme. Ferene S which is a strong Fe^{2+} chelator did not sequester the metal even with a reducing agent. 1,10 α -phenanthroline did remove the iron but only when used in the presence of the reducing agent dithiothreitol.(21) Compounds that chelate through charged groups may interfere with hydroxylation but are incapable of removal of the iron.

1,10 α -phenanthroline binds via aromatic nitrogens and for this reason is the only chelator capable of stripping the iron from the active site producing an apo-enzyme. A Sephadex G-25 column isolates and separates the apo-enzyme from the α -phenanthroline iron complex and the reducing agent. The apo-enzyme can be reconstituted to 95% of its original activity. A double reciprocal plot of $1/\text{Activity}$ vs $1/\text{Fe}^{2+}$ gives a K_m for Fe^{2+} of 5.6 μM .(21)

Reconstitution with Fe^{3+} resulted in only a 10% increase in enzyme activity. Anaerobically Fe^{3+} was found to reactivate the enzyme to 95% of the original activity.(21) It has been proposed that there is a reversible oxidation of one or more of the apo-protein amino acid residues. Reactivation by Fe^{2+} provides

reducing power and subsequently Fe^{3+} is incorporated. Aerobically Fisher tried to restore activity with mercurous, nickelous, cobalt, manganese, cupric, chromium, cadmium and zinc with no success.(22) Fisher did not attempt to reconstitute the apo-enzyme anaerobically with these metals. The oxidation of the amino acid side group could play a role in these metals inability to reconstitute the apo-enzyme.

OBJECTIVES:

This project used rat liver enzyme as its source of PAH. This source will most closely mimic the toxicological responses of human liver PAH. The sensitivity of the PAH enzyme to common metals in our environment was further studied by activation/inhibition experiments and by reconstitution of apo-L-phenylalanine hydroxylase by other metals.

Activation and inhibition of the native rat liver PAH with metals other than Fe^{2+} has been performed in the past but requires further study and clarification. Reconstitution of the PAH enzyme has been performed aerobically with many of the transition metals, but with no metals outside that period. Reconstitution with other metals has been performed aerobically but there is some evidence that an amino acid residue is being oxidized during native iron removal and this could possibly hinder reconstitution. Therefore, reconstitution was performed anaerobically as well as aerobically to determine if this residue plays any steric role in the substitution process.

EXPERIMENTAL DESIGN

MEASUREMENT OF L-PHENYLALANINE HYDROXYLASE ACTIVITY

The first order of business was to find the appropriate assay for measuring the activity of L-Phenylalanine Hydroxylase. The complications in making this decision will be discussed first.

The standard assay mixture contains L-phenylalanine, the enzyme, the cofactor 6-methyl-tetrahydropterin (6-MPH₄), and a reducing agent to regenerate the cofactor. The enzyme dihydropterin reductase can be used to regenerate the cofactor from the quinoid back to the 6-methyl-tetrahydropterin. The reductase requires the natural reducing agent NADH.(23) Sulfhydryl compounds such as dithiothreitol (DTT), are also reducing agents capable of regenerating the cofactor.(24) DTT is a much simpler system and removes the necessity for NADH and complications arising from a second sensitive enzyme system. DTT inhibits the enzyme in concentrations higher than 12 mM.(25)

There are several complications that arise that must be understood when choosing the appropriate continuous assay system. First, the cofactor spontaneously oxidizes in the presence of oxygen. The cofactor has a half life of two to twenty minutes depending on the buffer being used and the temperature.(27) The oxidation is a free-radical chain process where the H₄pterin forms a radical at the 4a position of the pterin to initiate the reaction. The reaction is propagated by the uptake of oxygen to form the 4a-hydroperoxide radical. In the final step hydrogen peroxide is released.(26) The enzyme is inactivated by this latter product. The reaction scheme is shown in figure 5.

FIGURE 5: AUTOOXIDATION OF THE H₄ TETRAHYDROPTERIN

Catalase, in the amount of 2500 units/mL must be added to every assay mixture to protect the enzyme from this by-product.(25)

The two most commonly employed buffers for enzyme systems, phosphate and Tris-HCl also affect the assay system. Tris competitively inhibits the 6-MPH₄ in both the reduction and catalytic step.(19) The phosphate buffer catalyzes the non-enzymatic oxidation of the cofactor, 6-MPH₄ forming the H₂quinoid and the subsequent rearrangement to the 7,8-H₂pterin occurs.(28) This oxidation can be minimized and measured. Therefore, a 0.10 M phosphate solution is most commonly employed.

The enzyme is active at a pH from 6.8-8.0 and increases in activity two fold as the pH approaches 8.0.(8) However, at the high pH and at low ionic strength the pterin hydroxylation intermediate, 4a-hydroxypterin has a relatively long half-life and is visible at 242 nm. If the intermediate does not dehydrate the cofactor cannot be regenerated and it can become limiting.(29) The advantage of increased activity at higher pH's must be forfeited and care must be taken to pick assay conditions where the intermediate spontaneously dehydrates.

The order that the reagents are added also greatly affects the initial velocity. The cofactor inhibits the activation of the enzyme by L-phenylalanine. The unactivated enzyme with tetrahydrobiopterin (BH₄) bound has a compact or globular conformation making the binding of phenylalanine unfavorable. In contrast the phenylalanine activated enzyme exhibits an unfolded or "open" conformation displacing an inhibitory polypeptide segment.(30) This is consistent with the "internal inhibitor model" being proposed by Parniak et al.(9) Therefore, for conformational reasons it is important to add the L-phenylalanine alone and allow the enzyme and L-phenylalanine to incubate together for about five minutes at 25 °C before the addition of the cofactor.(25)

Under all assay conditions there is a continuous loss of activity of PAH during the hydroxylation reaction due to enzyme inactivation.(8) The inactivation is assumed to be due to a functionally irreversible inactivation of the enzyme due to the accumulation of a reversible inhibitor or the depletion of one of the substrates.(8) This problem is more apparent with purified enzyme than with unpurified due to its higher instability. When the fixed-assay systems are used careful time courses must be investigated to find the true initial rate.

Choice of Assay

All of the compounds in the standard assay mixture absorb light in either the ultra-violet or visible range. The appearance or disappearance of assay components can be monitored spectrophotometrically. This provides quite a choice of continuous assays.

The oxidation of the cofactor 6-MPH₄ to the H₂quinoid can be followed by the decrease in absorbance at 330-340 nm with a molar extinction coefficient of 4000. (28) The initial rate may be easily underestimated using this method. The 4a-hydroxypterin intermediate absorbs at about the same wavelength as MPH₄.(29) It's presence would hold the absorbance artificially high, when the intermediate has a significant life time due to the dehydration to the quinoid being slow. The blank rate is also extremely high due to non-enzymatic oxidation of the cofactor. The H₄pterin is not regenerated during this assay and becomes limiting.

The oxidation of the cofactor can be measured indirectly by following the oxidation of NADH at 340 nm.(23) In this assay system the H₄pterin is regenerated and maintained at a constant concentration by dihydropterin reductase. The dihydropterin reductase does not recognize the 4a-hydroxypterin so again

conditions where dehydration is spontaneous are required.(29) The blank is lower in this assay procedure.

Both of these previous assay systems assume that the hydroxylation is stoichiometrically coupled to the cofactor oxidation. This assumption is not always valid, and care must be taken in choosing enzyme conditions. These two assays were not appropriate for this project where the enzyme system could become uncoupled.

The formation of tyrosine is the simplest continuous assay in the literature to date. It was therefore, the first assay procedure explored in this project. Tyrosine production can be directly monitored by an increase in absorbance at 275 nm. (8) All the assay components absorb to some extent at this wavelength and the time zero absorbance observed was found to be high, approximately 0.600 absorbance units. The sulfhydryl compound, dithiothreitol is used to regenerate the cofactor. The oxidized dithiothreitol also absorbs at 275 nm. (8) Therefore, as the assay proceeds there is a constantly increasing blank of about 0.001 absorbance units per minute. Difficulties arise with this assay procedure when a PAH sample of low specific activity is being used, where the change in absorbance being observed at 275 nm was 0.002 absorbance units per minute. In this case the blank is one third of the absorbance being observed.

Further interference of the blank caused abnormalities in the shape of the assay curve making interpretation of the enzyme reaction rate extremely difficult. A sample time drive is shown in figure 6.

FIGURE 6: CONTINUOUS ASSAY MEASURING L-TYROSINE AT 275 NM

The reaction time drive showed a sharp peak in the first twenty seconds of the assay and a broad peaks giving rise to plateaus. This is thought to be due to fluctuation in the concentration of the quinoid. The quinoid absorbs in this region and fluctuations in its concentration will give rise to peaks. The sharp peak is due to the reduction step, and the broader peaks are due to the catalytic step. The cofactor is in 600 fold excess, so at no time is it limiting. If one was using an enzyme preparation with a higher specific activity these spectral interferences by the blank would be insignificant and go unobserved. However, the enzyme sample used in this project was of low specific activity, making these interferences significant. For this reason this continuous assay method was abandoned and other assay methods were explored and employed.

FIXED-ASSAY

A fixed assay was developed by Udenfriend and Cooper which exploits the fact that L-tyrosine forms a yellow complex with 1-nitroso-2-naphthol in the presence of nitric acid.(30) The same standard assay mixture as the continuous assay is used in a 0.500 mL volume. The reaction is stopped by precipitating the enzyme with 30% (w/v) cold Trichloroacetic acid (TCA). 0.01% (w/v) sodium nitrite is added to the nitric acid reagent to prevent the TCA from interfering with the complexation of the L-tyrosine. Excess nitroso-2-naphthol is extracted into an organic phase of dichloroethane and the absorbance of the complex in the aqueous phase is measured at 450 nm.

The blank for this method was found to be low giving an absorbance of approximately 0.040 absorbance units. None of the assay components absorb in this region and therefore do not interfere. The average change in absorbance observed

was 0.1600 absorbance units for a five minute assay as compared to 0.030 for the continuous. A set of L-tyrosine standards provided a linear calibration plot as shown in figure 7, with a correlation coefficient of 0.998. A lower limit of one nanomole provided the sensitivity necessary for an enzyme preparation with a low specific activity.

FIGURE 7: L-TYROSINE NITROSO-2-NAPTHOL STANDARDS

The limitation of using a fixed assay is that it provides limited information and the true rate can be miscalculated. This is especially true in the case of PAH, where the rate slowly decreases due to enzyme inactivation. To avoid the trap of underestimating the activity of the enzyme the fixed assay was run at more than one time interval. Figure 8 shows an example of the enzyme plots obtained using this fixed time assay procedure.

It was found for an enzyme preparation of low specific activity this fixed assay procedure was sufficiently sensitive and provided information that was readily interpretable with few spectral interferences.

FIGURE 8: FIXED ASSAY USING 20 μ g PAH

ENZYME PURIFICATION

It was desirable to try and increase the specific activity of the purchased rat liver enzyme. It was obtained as a lyophilized powder. The powder was quoted as 70% protein with a specific activity of 0.10 units/mg protein. A unit is equal to the production of one micromole of L-tyrosine. Pure fully active PAH has a specific activity of 13 units/mg of protein with an absorbance of 1.0 unit for 1 mg/ml of protein at 280 nm.(34)

Sigma purifies the enzyme by one of the more classical procedures, an ethanol fractionation and an ammonium sulphate fractionation followed by a sephadex G-25 isolation step. This leaves an enzyme preparation of lower specific activity. On average this method of purification provides an enzyme preparation with 50% of the activity obtained by other methods.(32) A purification procedure has been developed based on the observation that the enzyme undergoes a conformation change upon activation with phenylalanine and its external surface becomes hydrophobic. The enzyme will in the presence of phenylalanine therefore absorb to a phenyl-sepharose affinity column. The enzyme may be reversibly eluted by removing drastically lowering the phenylalanine concentration. The enzyme may be concentrated using the ion-exchanger DEAE-cellulose with no loss of activity.(34)

In adult rats 20-50% of the PAH is iron-free when in a resting state. The crude homogenate must be iron-treated with Fe^{2+} prior to further purification. Shiman observed that if this step is not performed before purification of the crude homogenate an enzyme preparation with only 50% of the maximal specific activity will be obtained.(20) This activation step is not performed in the Sigma preparation

of the enzyme. Therefore, it is not possible to obtain fully active pure enzyme.

Attempts were then made at maximizing the activity as much as possible by incubating the enzyme with Fe^{2+} and DTT. It was possible to increase the activity of the enzyme from 0.100 units/min to 0.130 units/min by filling some of the "vacant" iron sites on the enzyme subunits. The enzyme specific activity was further increased to 1.800 units/min by the affinity column purification. This however yielded a highly unstable enzyme that possibly might not withstand the harsh conditions to which it would be exposed, thus making it difficult to know if the loss of activity was due solely to the metals. Since the impurities would not interfere with the reconstitution experiments and an assay for measuring the lower activity enzyme preparation had been found successfully, this time consuming purification step therefore had no experimental advantages and was dropped from the bulk of the work. Fe^{2+} incubation was continued.

CONTROLS AND SPECIAL CONSIDERATIONS

As a source of control the native enzyme was run concurrently with all sets of samples and was used as a standard reference for 100% activity. Experiments were run in triplicate and repeated on several different occasions to determine reproducibility.

Metal solutions were added in 1 μ L volumes so that the enzyme sample was not diluted. Enzyme samples were made 0.10 M using the fact that 70% of the powder was PAH enzyme and that the enzyme absorbs at 278nm with an extinction coefficient of 6.80×10^4 . The enzyme was kept at 0.10 M to ensure that loss of activity observed was due to the metal and not due to dilution of the enzyme. When preparing the apo-enzyme, a sufficient concentration of enzyme was initially prepared such that dilutions of the enzyme during procedure did not provide an apo-enzyme of less than 0.10 M.

Iron contamination was a parameter that had to be controlled or determined analytically so that the results could be properly interpreted. All glassware and plastic vessels were a possible source of metal contamination. They were therefore carefully cleaned with 20% nitric acid and rinsed thoroughly with Millipore distilled water. All buffers and solutions were treated with Chelex 100 to removed any trace metal contaminants. Metal stock solutions were prepared from the highest grade salts available and were furthered analysed for trace iron contamination by Graphite Furnace Atomic Absorption.

The sephadex column used to prepare the apoenzyme was treated with EDTA at 90°C to remove any contaminating metal before every experiment

MATERIALS AND METHODS

ULTRA-VIOLET VISIBLE SPECTRA

All UV-VIS spectra and absorbance measurements were performed on a Beckman DU-7 spectrophotometer. A Nelma 1 milliliter quartz cuvette with a 1 cm path length was used to hold all samples.

DISTILLED WATER

All distilled water used in this project was passed through a Millipore water treatment system (Millipore, Bedford, Mass), consisting of one carbon cartridge followed by two ion-exchange cartridges.

GLASSWARE

All glassware, 0.300 mL polypropylene micro-test tubes (Brinkmann Instruments, Westbury, N.J.) and Eppendorf tips (Fisher Scientific Co, Ottawa) were soaked for 24 hours in 30% nitric acid, to remove any trace metals. The glassware was then rinsed and soaked in distilled water.

BUFFERS

All buffers were made metal free by being passed through a 24 cm x 1.5 cm column of analytical grade 50-100 mesh sodium form, Chelex-100 chelating resin (Bio-Rad Laboratories, Mississauga). The buffers were used at 4°C. Glycerin (Sigma Chemical Co, St Louis, MO.) used in buffers as a stabilizer, was first made

25% (v/v) and then treated with activated charcoal and filtered. All glycerin buffers were made fresh.

A Tris (AnalaR, BDH Chemicals, Toronto) stock solution was prepared by dissolving 24.20 g in 1.00 L of distilled water. HCl (AnalaR, BDH Chemical Co, Toronto) was used to make Tris-HCl buffers.

Sodium Phosphate, monobasic monohydrate (Baker Analysed, J.T. Baker Chemical Co, Phillipsburg, N.J.) stock solution was prepared by dissolving 27.80 g in 1.00 L of distilled water.

Di-Sodium Hydrogen Orthophosphate, Dibasic phosphate decahydrate (AnalaR, BDH Chemical Co, Toronto) was prepared by dissolving 53.65 g in 1.00 distilled water. Phosphate buffers of various pH were prepared by varying the ratios of volumes of the two solutions.

pH MEASUREMENTS

All solutions were pH adjusted using a Corning 135 pH meter (Fisher Scientific Chemical Co, Ottawa), consisting of a glass membrane electrode and a standard calomel reference electrode. The pH meter was calibrated at pH 6.00 and 10.00 with Standard phosphate buffers (Fisher Scientific Co, Ottawa).

L-PHENYLALANINE HYDROXYLASE

The enzyme was obtained (Sigma Chemical Co, St Louis, Mo) as a rat liver lyophilized powder containing approximately 70% protein. The balance was primarily buffer salts such as Tris and dithiotreitol. The enzyme was dissolved in a stabilizing buffer at a concentration of 5 mg/ml. The enzyme was frozen in 0.500 mL aliquots.

Stabilizing buffer: 25 mM Tris-HCl, pH 7.0, containing 1 mM dithiothreitol, 10 mM L-phenylalanine and 25% glycerol.

The stabilizing buffer was prepared by combining 12.50 mL of 0.20 M Tris metal-free stock solution, 15.42 mg of dithiothreitol (Sigma Chemical Co., St Louis MO) 0.1652 g of L-phenylalanine (Sigma Chemical Co, St Louis, MO) and 20.00 mL of 25% glycerol (Fisher Scientific, Ottawa). The pH was adjusted with 0.20 M HCl and the volume brought to 100.00 mL

ENZYME ACTIVATION

A stored enzyme aliquot was thawed and made 0.01 mM in Fe^{2+} and 5 mM in dithiothreitol by adding 10 μL of a 0.25 M dithiothreitol Sigma Chemical Co, St Louis) solution and 10 μL of 0.52 mM Ferrous Ammonium Sulphate (Fisher Scientific Co, Ottawa) solution. 0.25 M dithiothreitol was prepared by dissolving 0.3850 g in 10.00 mL of distilled water. A 0.52 mM solution of ferrous ammonium sulphate was prepared by dissolving 20.39 mg of ferrous ammonium sulphate in distilled water and the solution was brought to 100.00 mL.

The enzyme solution was warmed to 25°C for twenty minutes and then cooled to 0°C for five minutes. The enzyme would then be fully activated and ready for assaying or for further experimentation.

CONTINUOUS ENZYME ASSAY

Phosphate buffer: A 0.12 M phosphate solution was prepared by combining 30.00 mL of monobasic phosphate stock solution and 90.00 mL of dibasic phosphate stock solution. 0.200 mL of catalase, liver bovine aqueous suspension (Sigma Chemical Co, St Louis, MO) was dissolved in 0.200 mL of 1.0 M Tris (AnalaR, BDH Chemical Co,) and added to the 0.12 M phosphate solution. The solution was diluted to 200.00 mL and the pH confirmed to be 6.80

Dithiothreitol: A 6 mM solution of dithiothreitol was prepared by dissolving 95.52 mg of dithiothreitol (Sigma Chemical Co, St Louis, MO) in 10.00 mL of distilled water. The solution was frozen in 0.500 mL aliquots and thawed just prior to use.

6-Methyl-Tetrahydropterin: A 120 μ M solution was prepared by dissolving 10.16 mg of 6-methyl-tetrahydropterin (Sigma Chemical Co, St Louis) in 0.250 mL of 0.20 M HCl and the solution was diluted to 10.00 mL with distilled water. The solution was frozen in 0.500 mL aliquots and thawed just prior to use. The solution was kept on ice once it was thawed.

L-Phenylalanine: A Solution of L-phenylalanine was prepared by dissolving 0.3304 g of L-phenylalanine (Sigma Chemical Co, St Louis) in 10.00 mL of distilled water.

Assay procedure: 0.700 mL of 0.12 M phosphate buffer pH 6.90, 0.100 mL of 2 mM L-phenylalanine and 0.100 mg of enzyme were added to 1.0 mL quartz cuvette (Nelma, Germany). The total volume was adjusted to 0.885 mL by adding 0.12 M phosphate

buffer and the cuvette was incubated for 10 minutes at 25°C to activate the enzyme. The reaction was initiated by adding 0.100 mL of dithiothreitol and 15 µL of 6-methyl-tetrahydropterin, respectively. The reaction was monitored by measuring the absorbance at 275 nm. A blank solution was run with all components except L-phenylalanine.

FIXED ASSAY

Phosphate Buffer: Prepared the same as for the continuous assay.

6-Methyl-tetrahydropterin: Prepared and treated the same as for the continuous assay.

L-Phenylalanine: Prepared the same as for the continuous assay.

30% (w/v) Trichloroacetic Acid: The solution was prepared by dissolving 30.00 g of trichloroacetic acid (TCA) (Fisher Scientific Co, Toronto) and diluted to 100.00 mL with distilled water.

0.1% 1-Nitroso-2-naphthol in 100% (v/v) Ethanol: The solution was prepared by dissolving 0.250 g of 1-nitroso-2-naphthol (Sigma Chemical Co, St Louis MO) in 250.00 mL of 100% ethanol (Consolidated Alcohol Ltd, Toronto).

Nitric Acid Reagent: 1:5 Nitric acid solution containing 0.5 mg/mL of sodium nitrite. The solution was prepared by dissolving 125 mg of sodium nitrite

(Certified A.C.S., Fisher Scientific Co, Ottawa) and 45.0 mL of nitric acid (AnalaR, BDH Chemical Co, Toronto) in distilled water, and diluting the solution to 250.00 mL. This solution must be made fresh just before use.

L-Tyrosine Standards: Standards were prepared by dissolving 8.812 mg of L-tyrosine (Sigma Chemical Co, St Louis, MO) in 2.00 mL of distilled water to form a 10 μ M stock solution. A series of standards from 5 nmoles to 100 nmoles were prepared from this stock solution. These were treated concurrently with all sample sets.

Assay Procedure: 0.050 mL of L-Phenylalanine solution, enzyme solution 10 - 50 μ g containing 20 μ g of enzyme and enough phosphate buffer to bring the volume to 0.485 mL were incubated together in a 3 mL test-tube at 25°C for 10 minutes. The reaction was initiated by adding 15 μ L of 6-methyl-tetrahydropterin. The reaction was stopped at the desired times by the addition of 0.100 mL of 30% (v/v) cold TCA solution. Each sample was run individually for 1, 3 and 5 minutes in order to determine the initial reaction rate.

After the reaction had been stopped, the test tubes were centrifuged to precipitate the protein. A 0.100 mL of nitric acid reagent, followed by 0.100 mL of 1-nitroso-2-naphthol solution was added to each test tube. The test tubes were then covered with parafilm and inverted. The test tubes were incubated for 30 minutes in a 55°C water bath. After heating 1.00 mL of 1,2-dichloroethane (Spectra grade, Caledon Laboratories Ltd, Georgetown, Ont) was added to each tube and the tubes were vortexed to extract any excess reagent. The test tubes were then

centrifuged to separate the layers. The aqueous layer was then transferred to a 1 mL cuvette and the absorbance was read at 450 nm. The L-tyrosine standard solutions were treated with the complexing agent concurrently.

A calibration line was determined with a computer linear regression program and the concentrations of the L-tyrosine in the sample test tubes interpolated. The reaction rates for the points for 1, 3 and 5 minutes were determined by using the same linear regression program.

ENZYME PURIFICATION

Buffer 1: 0.03 M Tris-HCl pH 7.25, containing 0.20 M KCl, and 0.02 M phenylalanine.

Buffer 1 was prepared by combining 15.00 mL of 0.20 M metal free Tris stock solution with 1.491 g of potassium chloride (AnalaR, BDH Chemical Co, Toronto) and 9.330 g of L-phenylalanine (Sigma Chemical Co, ST Louis, MO). The pH was adjusted to 7.25 with 0.20 HCl and the solution was diluted to 100.00 mL.

Buffer 1 containing 0.1% (v/v) Tween 80: 0.100 mL of Tween 80 (Sigma Chemical Co, St Louis, MO) was added to Buffer 1 before adjusting the pH. The pH was adjusted using 0.20 M HCl and the solution was diluted to 100.00 mL.

Buffer 2: Buffer 1, containing 4.8% (v/v) N,N-dimethylformamide.

4.8 mL of N,N-dimethylformamide (Fisher Scientific Co, Ottawa) was added to Buffer 1 and the pH was adjusted with 0.20 M HCl to a pH of 7.25. The solution was diluted to 100.00 mL.

Buffer 3: 0.03 M Tris-HCl pH 7.25, containing 15% (v/v) glycerol, 10 mM phenylalanine and 50 μ M EDTA.

60.0 mL of 25% (v/v) glycerol solution, 0.1652 g L-phenylalanine, and 1.86 mg of disodium EDTA (Baker Analysed, J.T. Baker, Phillipsburg NJ) was dissolved in 15.00 mL of 0.20 M Tris metal-free stock solution. The pH was adjusted to 7.25 with 0.20 M HCl. The volume was adjusted to 100.00 mL.

Purification Procedure:

Purification was performed with a phenyl-sepharose CL-4B affinity column (Sigma Chemical Co, St Louis MO). Average bed volume used was 1 mL for every 2 mg of protein sample to be applied. The average column was 0.5 cm x 3.0 cm.

Prior to use the column was equilibrated with Buffer 1, containing 0.1% (v/v) Tween 80. The enzyme was activated as described and made 0.1% (v/v) in Tween 80. The enzyme was then applied to the column. The column was washed with one bed volume of Buffer 1, followed by ten bed volumes of Buffer 2, and finally with 10 bed volumes of Buffer 3. The enzyme was eluted from the column with the Elution Buffer. Enzyme appears immediately after the L-phenylalanine concentration falls. The enzyme usually eluted after a 1.50 mL void volume, in an elution volume of 4.0 mL.

The phenyl-sepharose column was regenerated after every usage by rinsing the column with the following solutions: 5% (v/v) sodium dodecyl sulphate (Sigma Chemical Co.), distilled water, ethanol, distilled water, 1-butanol (Fisher

Scientific, Ottawa) distilled water, ethanol and finally distilled water.

The phenyl-sepharose was then made 10 mM with EDTA and heated to 90°C for 20 minutes to remove and trace amounts of metals. The phenyl-sepharose was then poured into the column and rinsed with distilled water. When not in use the column was stored in 2 mM EDTA at 4°C.

ENZYME SOLUTION CONCENTRATION

The enzyme solution was concentrated using a DEAE-cellulose microgranular anion exchanger (Sigma Chemical Co, St Louis, MO). For every milliliter of enzyme solution, one milliliter bed volume was required. Average column size was 0.5 cm x 2.0 cm.

Equilibration Buffer: 0.03 Tris-HCl pH 7.25, containing 15% (v/v) glycerol, 0.1% (v/v) Tween 80 and 50 μ M EDTA.

15.00 mL of 0.20 M metal-free Tris stock solution was combined with 60.0 mL of 25% (v/v) glycerol stock solution, 0.100 mL Tween 80 and 1.86 mg of EDTA. The pH was adjusted with 0.20 M HCl to a pH of 7.25. The solution was diluted to 100.00 mL.

Elution Buffer: 0.03 Tris-HCl pH 7.25, containing 15% (v/v) glycerol, 0.1% (v/v) Tween 80, 50 μ M EDTA and 0.4 M Potassium Chloride.

15.00 mL of 0.20 M metal-free Tris stock solution was combined with 60.0 mL of 25% (v/v) glycerol stock solution, 0.100 mL Tween 80, 1.86 mg of EDTA and 4.17

g of potassium chloride (AnalaR, BDH Chemical Co, Toronto). The pH was adjusted with 0.20 M HCl to 7.25. The volume was adjusted to 100.00 mL.

Procedure: The column was equilibrated with 10 bed volumes of equilibration buffer. The purified enzyme solution was then applied to the column and the column was washed with 5 bed volumes of equilibration buffer. The enzyme was eluted with the elution buffer. The enzyme eluted immediately after the void volume in a volume of about 0.500 mL.

METAL STOCK SOLUTIONS

Stock solutions of 0.100 M of metals of interest were prepared by dissolving the mass listed into 100.00 mL of distilled water. Purity of each metal and the supplier is also listed in table 1.

Table 1: Specifics of Metals Used for Reconstitution and Activation Studies

METAL SALT	% PURITY	MASS DISSOLVED (g)	CONTAMINATING IRON(ppm)
Metals obtained from AESAR (Johnson Matthey Ltd, Toronto):			
Mn(NO ₃) ₂ H ₂ O	99.99	1.96940	1.7
Fe(NO ₃) ₂ 9H ₂ O	99.50	2.79840	
Cd(NO ₃) ₂ H ₂ O	98	1.92400	2.0
Al(NO ₃) ₃ H ₂ O	99.999	2.30980	5.0
Pb(NO ₃) ₂	99.999	2.69200	
Co(NO ₃) ₂ 6H ₂ O	99.999	2.90930	2.0
Ni(NO ₃) ₂ 6H ₂ O	99.7	1.20700	1.5
TiNO ₃	99.5	2.66300	0.75
RbCl	99.0	1.20920	3.0
Metals obtained from Aldrich Chemical Co Inc, Milwaukee, Wi:			
ZnSO ₄ 7H ₂ O	99.0	2.87380	1.75
Cr(NO ₃) ₃ H ₂ O	99.0	2.55990	120
HgCl ₂	Reagent	2.71400	
CuSO ₄	98.5	1.59540	
Fe(NH ₄) ₂ SO ₄	99.0	1.87840	

METAL ACTIVATION STUDIES

The native enzyme was activated with L-phenylalanine as previously described and divided into 60 μ g aliquots which was contained in 40 μ L. The enzyme was transferred to 0.300 mL polypropylene micro-test tubes. 1 μ L of each of the metal stock solutions was added to each of the enzyme aliquots. The enzyme metal solutions were incubated at 4°C for twenty minutes. Each 60 μ g sample was split into three equal portions and was added to each of three test tubes containing the standard assay mixture. The test tubes were assayed for 1, 3 and 5 minutes respectively.

This activation study was repeated at a metal concentration 10 fold smaller. The stock solutions were diluted 1:10 prior to their addition. Such that Enzyme:metal was 1mmol enzyme : 10 mmoles metal.

APO-ENZYME RECONSTITUTION STUDIES

PREPARATION OF APO-ENZYME:

Apo Preparation Buffer: 0.10 M Tris-HCl pH 6.8, containing 10 mM o-phenathroline, 10 mM dithiotheitol, 10 mM L-phenylalanine, 0.1 M potassium chloride and 15% (v/v) glycerol.

12.50 mL of 0.20 M Tris metal-free stock solution was combined with 99.1 mg of o-phenathroline (Sigma Chemical Co, St Louis,MO), 77.1 mg of dithiothreitol, 82.6 mg of L-phenylalanine, 1.118 g of potassium chloride and 7.5 mL of glycerol. The pH of the solution was adjusted with 0.20 M HCl. The solution was diluted to 25.00 mL. This solution was made fresh before each apo-preparation.

Sephadex Equilibration Buffer: 0.03 M Tris-HCl pH 7.25, containing 15% (v/v) glycerol, 50 μ M EDTA, 0.10 M potassium chloride, 2% (v/v) N,N-dimethylformamide and 10 mM L-phenylalanine.

30.00 mL of 0.20 M Tris metal-free stock solution was combined with 3.72 mg of EDTA, 1.491 g potassium chloride, 4.00 mL of N,N-dimethylformamide, 0.0330 g L-phenylalanine and 120.0 mL of 25% (v/v) glycerol stock solution. The pH of the solution was adjusted to 7.50 with 0.20 M HCl and diluted to 200.00 mL.

Sephadex G-25-100: A 15.0 cm x 0.5 cm column of sephadex was used. The sephadex was dissolved in 5 mM EDTA and heated to 90°C for 8 hours to activate. The sephadex was then degassed on an aspirator and the column was poured. The void volume of the column was measured using blue dextran (Sigma Chemical Co., St Louis, MO)

After each use the Sephadex was washed with 100 mL of 0.20 M NaOH. The column was then washed with distilled water until the column was neutral. The Sephadex was equilibrated with the equilibration buffer and cooled to 4°C. After the column has been used approximately 6 times the column becomes permanently contaminated with Fe²⁺ and fresh Sephadex must be activated and used.

Apo-preparation Procedure:

0.300 mL of the apo-preparation buffer was added to 0.500 mL of purified and concentrated enzyme solution previously harvested. (The purification step may be dropped and activated enzyme preparation used directly) This preparation was incubated at 4°C for one half hour to allow the complex to form. The concentration of the iron- α -phenanthroline complex could be measured if desired at 510 nm with a molar extinction coefficient of 11,000. The apo-preparation was made 2% (v/v)

with N,N-dimethylformamide and applied to the Sephadex column. The enzyme was run with the equilibration buffer and eluted immediately after the void volume of 2.5 mL. A 0.500 mL apo-enzyme fraction was collected on ice. The apo-enzyme has a half life of 2.5 minutes at 21°C.(21) It therefore, was kept cold and used within one hour of harvesting. For expediency the enzyme concentration was calculated by measuring the absorbance of the enzyme at 278 nm using a 0.400 mL quartz cuvette with a path length of 0.10 cm. The extinction coefficient for the enzyme at this wavelength is 6.8×10^4 . The absorbance from the equilibration buffer was subtracted as the blank.

0.300 mL polypropylene sealable micro-test tubes were equilibrated to 4°C. 60 µg of apo-enzyme was transferred to each test-tube.

Fe²⁺ CONCENTRATION RECONSTITUTIONS

Using the ferrous ammonium sulphate stock solution previously prepared a set of seven iron(II) solutions were prepared in 100.00 mL volumetrics for the concentrations of 0.05 M to 5×10^{-7} M. 10 µL of each of these concentrations was added to 60 µg of the apo-enzyme in 40 µL to provide effective concentrations of 10^{-3} M to 10^{-9} M iron. The apo-enzyme and metal solutions were incubated at 4°C for 30 minutes. The 60 µg samples were split into three equal 20 µg aliquots and added to each of three 3 mL test tubes containing the standard assay mixture. The tubes were assayed for 1, 3 and 5 minutes as previously described for the fixed enzyme assay.

RECONSTITUTION BY OTHER METALS

0.100 mL of the metal stock solutions were diluted in 1.0 mL of distilled water. 1 μ L of these solutions was then added to 60 μ g of the apo-enzyme as described for the Fe^{2+} reconstitutions. The enzyme metal solutions were incubated for 20 minutes at 4°C. Each sample was assayed for 1, 3 and 5 minutes.

ANAEROBIC METAL RECONSTITUTION

All solutions, Sephadex, and buffers were degassed on an argon vacuum line. The solutions were purged and evacuated three times for 3 minute cycles. The apo-enzyme was prepared, isolated and reconstituted in a glove box under an argon atmosphere. The micro-test tubes were sealed and then the samples were removed from the glove box. They were incubated for 30 minutes on ice and then assayed as previously described.

ATOMIC ABSORPTION ANALYSIS OF METAL STOCK SOLUTIONS

Iron analysis was performed by Mr. Glen Gilchirist using Perkin-Elmer HGA 76B graphite furnace, a Varian Techtron AA6 0.5 m monochromator, a Westinghouse hollow cathode lamp and a laboratory made detection system with lock-in amplifier modulated at 843 Hz. 1% (w/v) metal solutions were prepared and dissolved in a 1% (v/v) solution of nitric acid. Standard additions of 50-150 picograms of a standard iron solutions were performed and peak areas calculated.

RESULTS AND DISCUSSION

METAL ACTIVATION

Many metals activate enzymes by transiently binding to proteins and altering their conformations to a more active form. For example, Mg^{2+} activates hexokinase by binding with ATP.(34) Metals can also be inhibitory to enzymes by competitively binding to the active site, or oxidizing important residues near the active site. They may also bind elsewhere on the protein altering its conformation to a less active form. Finally, the metals can also uncouple the electron transfer necessary for activity and thus quench the reaction.

Guroff in 1965 isolated and began characterizing PAH from *pseudomonas*. He found that Hg^{2+} , Cu^{2+} and Cd^{2+} activated the enzyme.(35) In fact, *pseudomonas* PAH in its resting state is totally devoid of Fe^{2+} and these metals were not activating in the true sense but were substituting for the iron. Other metals tested were Al^{3+} , Ba^{2+} , Co^{2+} , Ca^{2+} , Fe^{3+} , Mg^{2+} , Sn^{2+} , Sn^{4+} , Zn^{2+} , Pb^{2+} , Mn^{2+} , Ni^{2+} , Cr^{2+} , and Mo^{2+} to see if they would activate the enzyme.(14) They were found to be totally unable to activate. Ca^{2+} , Al^{3+} , Zn^{2+} , Sn^{2+} and Co^{2+} were found to inhibit the *pseudomonas* PAH.(14) The thought has been proposed that this inhibition is by competitively binding at the active site, but not being catalytically competent to perform the hydroxylation.(25)

Bublitz in 1971 incubated mammalian PAH with Na^{+} , K^{+} , Mg^{2+} , Fe^{3+} , Cd^{2+} , Cu^{2+} and Zn^{2+} at a 10^{-4} M concentration and found no effect on the enzyme by these metals.(25) Guroff on the contrary found Cd^{2+} , and Cu^{2+} to inhibit the rat liver enzyme by 25%.(14)

It is surprising to find Hg^{2+} and Cd^{2+} activating the *pseudomonas* PAH enzyme. and also to find Cd^{2+} then activating the rat liver PAH enzyme. Both of these metals are very toxic and Cd^{2+} has only the one valence state available.

Clarification of the results with rat liver enzyme seemed necessary and additional metals also common to our environment needed to be studied. In the present studies the fully activated native enzyme from rat liver was exposed to a 10 fold and 100 fold excess of each metal (1mmole enzyme:10mmole metal, 1mmole enzyme: 100mmole metal). Activation or inhibition was measured as the percentage of activity found with the metal treated enzyme as compared to a fully active native control PAH sample. The native control stated as 100% activity gave a specific activity of 0.170 micromoles of tyrosine/min/mg of PAH.

Metals were picked from the three main groups of the periodic table, the alkali metals, the alkaline and the transition metals. Samples were run in duplicate on several different occasions providing different trials, to ensure reproducibility of the results. The results from these experiments are summarized in table 2.

TABLE 2: SUMMARY OF METAL ACTIVATION STUDIES

METAL	% ACTIVITY RETAINED METAL CONCENTRATION	
	10 FOLD EXCESS	100 FOLD EXCESS
MANGANESE	44.36 $\pm$ 11.38	48.16 $\pm$ 18.61*
NICKEL	69.84 $\pm$ 14.08	100.28 $\pm$ 9.24
ALUMINUM	92.03 $\pm$ 13.52	76.11 $\pm$ 15.71
CHROMIUM	71.01 $\pm$ 9.28	50.23 $\pm$ 14.40
LEAD	65.30 $\pm$ 6.08	107.30 $\pm$ 12.76
CADMIUM	86.85 $\pm$ 15.10	71.92 $\pm$ 8.58
ZINC	93.85 $\pm$ 5.20	39.39 $\pm$ 19.94
THALIUM	100.11 $\pm$ 12.06	151.57 $\pm$ 20.09
RUBIDIUM	120.17 $\pm$ 21.77	136.18 $\pm$ 14.40
COPPER	38.62 $\pm$ 20.19	33.88 $\pm$ 7.84
IRON(II)	171.00 $\pm$ 14.00	149.23 $\pm$ 16.70
IRON(III)	73.14 $\pm$ 10.90	122.11 $\pm$ 40.81
COBALT	62.64 $\pm$ 17.63	88.50 $\pm$ 9.98
MERCURY	11.02 $\pm$ 5.51	17.34 $\pm$ 1.54

*Note: Error calculated from accumulated trials

At a 10 fold excess of metal Al^{3+} , Zn^{2+} and Tl^{1+} showed no significant effect to the enzyme activity. Zinc and aluminum both became inhibitory when their concentrations were increased to a 100 fold excess. The zinc treated enzyme dropped to 39.39% of the activity found in the native enzyme and aluminum treated enzyme dropped to 76% of the activity of the native enzyme.

Iron(II) was the only true enzyme activator. Thallium and Rubidium both showed an increase in enzyme activity above that of the native when they were in a 100 fold excess as compared to the amount of enzyme. Both of these metals were purchased as 99.0% pure and contained some Fe^{2+} which was actually performing the activation. This small amount as will be shown later in the iron-reconstitution experiment is all that is required to raise the activity by 10%. Mn^{2+} , Ni^{2+} , Cr^{2+} , Pb^{2+} , Cd^{2+} , Cu^{2+} , Co^{2+} and Hg^{2+} were all found to be inhibitory to the enzyme. Mn^{2+} , Hg^{2+} , and Cu^{2+} inhibit the enzyme drastically by 66%, 89% and 62% respectively. The inhibition was felt immediately at the 10 fold excess and does not deteriorate any further when any additional metal is added. The other metals mentioned depressed the enzyme activity approximately 20% with a 10 fold excess and a further 10% activity was lost with a 100 fold excess of metal was added. In the case of lead and nickel some recovery of activity was observed with the 100 fold excess over that obtained with the 10 fold excess of metal. This is due to iron contamination of these metal standards. Cu^{2+} is thought to competitively inhibit the enzyme and be antagonistic to iron(II).⁽²⁵⁾ It is roughly the same size as iron(II) having an ionic radius of 0.96 Å as compared to 0.74 Å. Both are intermediate in their hardness and have similar binding properties and will take an octahedral conformation. However, copper has a reduction potential (E°) of only +0.15 where iron has an E° of +0.77

TABLE 3: PROPERTIES OF METALS USED IN STUDIES

METAL		OXIDATION STATE	IONIC RADII Å°	E V
Rb	5s ¹	1+	1.147	N/A
Cr	3d ⁴	2+/3+	0.89	-0.41
Mn	3d ⁵	2+/3+	0.80	1.59
Fe	3d ⁶	2+/3+	0.74	0.77
Co	3d ⁷	2+/3+	0.72	1.84
Ni	3d ⁸	2+	0.69	N/A
Cu	3d ⁹	2+/1+	0.96	0.15
Zn	3d ¹⁰	2+	0.74	N/A
Cd	4d ¹⁰	2+	0.97	N/A
Hg	5d ¹⁰	2+/1+	1.27	
Al	3p ¹	3+	0.51	N/A
Tl	6p ¹	1+	0.95	N/A
Pb	6p ²	2+	1.20	N/A

Copper is possibly not sufficiently oxidizing to perform this hydroxylation.

Hg^{2+} also drastically effects the enzyme. Hg^{2+} has a high affinity for sulfhydryl groups. There is in PAH a partially buried sulfhydryl group, whose oxidation state and movement seems to be critical to the formation of the activated conformation. Hg^{2+} probably interferes with the sulfhydryl group and could hinder the formation of the more open active complex, possibly blocking the active site.

The activation data is graphed several times grouping the metals by their place in the periodic table, increasing ionic radii and by the oxidation state that they hold. This was performed in the hope of observing a pattern or trend to the successful activators and inhibitors. No pattern is observed for size or placement in the periodic table but subtle trends appear when the oxidation states are grouped by trivalent, and divalent, and by the different oxidation couples that the metals may hold.

FIGURE 10: METAL ACTIVATION STUDY, ARRANGED BY INCREASING IONIC RADII

FIGURE 9: METAL ACTIVATION STUDY, METALS ARRANGED BY PERIODIC TABLE

FIGURE 11: METAL ACTIVATION ARRANGED BY
OXIDATION STATES

Rubidium which is a monovalent ion with no other stable available oxidation states does not affect the enzyme. This is also the case with the trivalent ion aluminum. Zinc, cadmium and mercury contain full d^{10} shells and only mercury due to its specific affinity shows any significant inhibitory affect. In the block of the transition metals that were studied Mn, Cr and Co are all $3+/2+$ oxidation couples the same as iron. All three were inhibitory to the enzyme. Their ionic radii are 0.80°A , 0.89°A and 0.72°A respectively. All are capable of fitting into the active site and will bind with nitrogen in an octahedral conformation. The E° are 1.59, -0.41 and 1.84 for Mn, Cr, and Co respectively. The Cr^{3+} is not sufficiently oxidizing. The Co^{2+} and Mn^{2+} could be both in the lower oxidation states and would not oxidize any amino acid residues. However, more than 1/3 of the protein is aspartic and glutamic acids which have carboxylate groups that would be deprotonated at a pH of 7.0 and both of these metals could complex with these residues and alter the protein conformation.

Additional studies are required to determine the true nature of the inhibition by these metals. These experiments are out of the time scope of this project.

TABLE 4: METAL ACTIVATION STUDY USING 100 FOLD EXCESS OF METALS,
TRIAL 1

METAL	ACTIVITY MICROMOLES/MIN	SPECIFIC ACTIVITY MICROMOLES/MIN/MG	% RECOVERY
NATIVE	0.0034	0.1707	100.00
IRON(II)	0.0057	0.2787	168.25
IRON(III)	0.0011	0.0520	30.46
MANGANESE	0.0078	0.0383	22.44
COBALT	0.0013	0.0670	39.25
CHROMIUM	0.0011	0.0567	33.22
COPPER	0.0014	0.0707	41.42
RUBIDIUM	0.0026	0.1305	76.45
LEAD	0.0098	0.2589	151.67
ZINC	0.0044	0.9221	12.95
MERCURY	0.0063	0.0315	18.45
	0.0059	0.0298	17.46
CADMIUM	0.0024	0.1193	69.81
	0.0022	0.1093	64.03

TABLE 5: METAL ACTIVATION STUDY USING 100 FOLD EXCESS METAL, TRIAL 2

METAL	ACTIVITY MICROMOLES/MIN	SPECIFIC ACTIVITY MICROMOLES/MIN/MG	% RECOVERY
NATIVE	0.0019	0.0933	
	0.0018	0.0910	
AVERAGE		0.0922	100.00
MANGANESE	0.0012	0.0586	63.56
NICKEL	0.0018	0.0889	96.42
ALUMINUM	0.0013	0.0674	73.10
CHROMINUM	0.0016	0.0812	88.07
ZINC	0.0013	0.0566	61.39
COPPER	0.0018	0.0883	95.77
IRON(II)	0.0025	0.1263	136.98
IRON(III)	0.0017	0.0827	89.70

TABLE 6: METAL ACTIVATION STUDY USING 100 FOLD EXCESS METAL, TRIAL 3

METAL	ACTIVITY MICROMOLES/MIN	SPECIFIC ACTIVITY MICROMOLES/MIN/MG	% RECOVERY
NATIVE	0.0026	0.1305	100.00
RUBIDIUM	0.0031	0.1557	119.31
	0.0040	0.2007	153.79
NICKEL	0.0026	0.1294	99.16
	0.0024	0.1202	92.11
CHROMIUM	0.0011	0.0544	
	0.0013	0.0644	49.35
	0.0013	0.0644	49.35
COBALT	0.0025	0.1363	96.78
	0.0022	0.1090	83.52
	0.0019	0.0968	74.18
ALUMINUM	0.0015	0.0750	57.47
	0.0023	0.1164	89.20
THALIUM	0.0023	0.1166	89.35
	0.0039	0.1961	150.27
	0.0027	0.1339	127.52
MERCURY	0.0003	0.0194	14.87
	0.0005	0.0246	18.80

TABLE 7: METAL ACTIVATION STUDY USING 100 FOLD EXCESS OF METALS, TRIAL. 4

METAL	ACTIVITY MICROMOLES/MIN	SPECIFIC ACTIVITY MICROMOLES/MIN/MG	% RECOVERY
NATIVE	0.0035	0.1734	100.00
IRON(II)	0.0065	0.3247	142.46
IRON(III)	0.0046	0.2290	132.06
	0.0058	0.2919	168.34
	0.0041	0.2052	118.34
MANGANESE	0.0016	0.081	46.71
COBALT	0.0062	0.3119	179.87
	0.0034	0.1707	98.44
	0.0031	0.1553	89.56
THALIUM	0.0061	0.3027	174.57
	0.0050	0.2513	144.93
NICKEL	0.0039	0.1967	113.44
CHROMIUM	0.0024	0.1187	68.45
ALUMINUM	0.0023	0.1142	65.86
	0.0033	0.1646	94.93
COPPER	0.0009	0.0447	25.78
	0.0012	0.0597	33.43
MERCURY	0.0006	0.0298	17.10
RUBIDIUM	0.0048	0.2419	139.50
	0.0046	0.2291	132.12
LEAD	0.0022	0.1091	62.92
CADMIUM	0.0024	0.1209	69.72
	0.0029	0.1459	84.14
ZINC	0.0014	0.0722	41.64
	0.0014	0.0718	41.41

TABLE 8: METAL ACTIVATION STUDY USING 100 FOLD EXCESS OF METALS, TRIAL 5

METAL	ACTIVITY MICROMOLES/MIN	SPECIFIC ACTIVITY MICROMOLES/MIN/MG	% RECOVERY
NATIVE	0.0028	0.1105	100.00
MANGANESE	0.0021	0.0844	76.46
NICKEL	0.0032	0.1262	114.33
ALUMINUM	0.0027	0.1091	98.90
CHROMIUM	0.0022	0.0898	80.82
LEAD	0.0022	0.0096	81.16
COPPER	0.0031	0.0126	11.10
	0.0009	0.0394	35.68
IRON(II)	0.0032	0.1263	114.37
IRON(III)	0.0045	0.1811	164.03

LEAVES 57 AND 58 OMITTED IN PAGE
NUMBERING.

FEUILLETS 57 ET 58 NON INCLUS DANS
LA PAGINATION.

National Library of Canada
Canadian Theses Service.

Bibliothèque nationale du Canada
Service des thèses canadiennes.

TABLE 9: METAL ACTIVATION STUDY USING 10 FOLD EXCESS OF METALS TRIAL 1

METAL	ACTIVITY MICROMOLES/MIN	SPECIFIC ACTIVITY MICROMOLES/MIN/MG	% RECOVERY
NATIVE	0.0033	0.1662	100.00
MANGANESE	0.0015	0.0772	46.46
	0.0012	0.0606	36.46
NICKEL	0.0018	0.0893	53.73
	0.0017	0.0853	51.32
ALUMINUM	0.0034	0.1707	102.71
	0.0028	0.1422	85.56
CHROMIUM	0.0020	0.0996	59.93
	0.0025	0.1239	74.55
LEAD	0.0027	0.1351	81.29
CADMIUM	0.0021	0.1031	62.03
ZINC	0.0030	0.1499	90.17
	0.0032	0.1621	97.59
THALIUM	0.0038	0.1919	115.46
RUDIDIUM	0.0031	0.1557	93.60
	0.0033	0.1647	99.10
COPPER	0.0010	0.0510	30.61
IRON(III)	0.0022	0.1124	67.63
	0.0021	0.1053	63.36
IRON(II)	0.0100	0.5023	302.23
COBALT	0.0033	0.1520	91.46
	0.0021	0.1030	61.97

TABLE 10: METAL ACTIVATION STUDY USING 10 FOLD EXCESS OF METALS, TRIAL 2

METAL	ACTIVITY MICROMOLES/MIN	SPECIFIC ACTIVITY MICROMOLES/MIN/MG	% RECOVERY
NATIVE	0.0040	0.1995	100.00
THALIUM	0.0042	0.2113	105.91
	0.0040	0.2017	101.10
RUBIDIUM	0.0053	0.2634	132.03
	0.0060	0.2997	150.23
ALUMINUM	0.0029	0.1448	72.58
	0.0034	0.1692	84.91
CHROMIUM	0.0027	0.1341	67.22
	0.0033	0.1654	82.91
LEAD	0.0028	0.1412	70.78
	0.0028	0.1404	70.83
MANGANESE	0.0024	0.1221	61.20
	0.0021	0.1068	53.53
NICKEL	0.0031	0.1572	78.79
	0.0029	0.1469	73.63
	0.0034	0.01724	86.42
COBALT	0.0024	0.1213	60.80
	0.0022	0.1098	55.04
MERCURY	0.0003	0.0156	7.84
	0.0003	0.0156	7.84
	0.0007	0.0347	17.39

TABLE 11: METAL ACTIVATION STUDY USING 10 FOLD EXCESS OF METALS, TRIAL 3

METAL	ACTIVITY MICROMOLES/MIN	SPECIFIC ACTIVITY MICROMOLES/MIN/MG	% RECOVERY
NATIVE	0.0035	0.1756	100.00
RUBIDIUM	0.0040	0.2002	114.10
	0.0046	0.2317	131.95
CADMIUM	0.0039	0.1961	111.67
LEAD	0.0024	0.1235	70.33
	0.0030	0.1489	84.79
	0.0028	0.1389	79.10
ALUMINUM	0.0030	0.1523	86.73
	0.0040	0.1984	113.90
THALIUM	0.0029	0.1462	83.26
	0.0033	0.166	94.82
CHROMIUM	0.0021	0.1048	59.68
	0.0025	0.1264	71.98
COBALT	0.0015	0.0771	43.91
	0.0011	0.0556	31.66
MANGANESE	0.0013	0.0639	36.31
	0.0011	0.0565	32.18
NICKEL	0.0022	0.1096	62.41
	0.0029	0.1452	82.61
COPPER	0.0021	0.1972	61.05
	0.0020	0.0981	55.87
IRON(III)	0.0026	0.1286	73.23
	0.0031	0.1551	88.33
IRON(II)	0.034	0.1693	96.41

IRON RECONSTITUTION

All metals have an optimum concentration where they are catalytically most efficient. Above this optimum even the natural metal may be destructive and inhibitory. Gottschall found that the amount of iron present in the enzyme was directly proportional to the catalytic activity observed.(21) He found iron reconstitution was extremely fast. The enzyme regains 50% of it's activity in approximately three minutes.(21) This makes kinetic measurements as to the rate of iron reconstitution almost impossible with conventional equipment. The K_m of the iron was found to be $5.6 \mu\text{M}$.(22) The apo-enzyme was assayed at 5 different iron concentrations (figure 13) and the data was plotted as $1/\text{Activity}$ vs $1/[\text{Fe(II)}]$ was plotted as shown in figure 14. The K_m was found to be in the same order of magnitude at $3.4 \mu\text{M}$. The V_{max} was found to be $5.16 \mu\text{M}/\text{min}$ and the optimum iron concentrations for reconstitution was found to be $100 \mu\text{M}$. All these figures are consistent with previous work by Gottschall and what is expected for a physiological process.

This project was most interested in the efficiency of other metals with the enzyme PAH. The iron was used as a control. For reasons of testing our reconstitution method, iron reconstitution was performed at 8 different iron concentrations. The reproducibility of the experiments was tested from day to day by running samples in duplicate, and triplicate if the amount of apo-enzyme harvested allowed. The samples were run on four different occasions to ensure that there was no bias from one days experiments.

The enzyme was initially fully active and this native was the control 100%

activity with which the reconstituted PAH was compared and it was measured concurrently and is shown in the top of all tables. Only a 5% loss due to the sephadex column was observed. The apo-enzyme was isolated with 20% of its metal still complexed. A percent activity of 20% was the lowest that could be obtained. It is difficult to remove all the iron from the enzyme because of the numerous places that iron may be a contaminant and reconstitute the enzyme. The enzyme was found not to be inhibited by iron concentration as high as 10^{-3} M. An addition of Fe^{2+} as small as 10^{-6} M raised the activity 10%. A maximum recovery of 81% of the original activity with 10^{-4} M was obtained. (Figure 12) This experiment was run on several different occasions with approximately the same results. The method for iron removal and reconstitution was therefore considered efficient.

The apo-enzyme was prepared with o-phenanthroline and the reducing agent dithiothreitol. Only the chelator o-phenanthroline is capable of actually removing the iron. o-Phenanthroline chelates by aromatic nitrogens and is hydrophobic enough to enter the active site. (21) The purpose of the reducing agent has not been elucidated, however it is absolutely necessary for the iron removal. It could play a role in the oxidation state of the sulfhydryl group which moves in the process of forming the active open conformation and it probably ensures the $2+$ state of the iron metal for chelation.

FIGURE 12: IRON RECONSTITUTION OF THE APO-ENZYME

FIGURE 13: ASSAY OF APO-PAH WITH DIFFERENT [Fe(II)]

FIGURE 14: DETERMINATION OF MICHAELIS MENTEN EQUATION

RECONSTITUTION BY OTHER METALS

In our modern society we subject living organisms and ourselves to a great number of toxic and foreign metals. Metalloenzymes grown on high excesses of foreign metals have been found to adapt and begin to utilize these metals. The enzyme RNA polymerase, a zinc metalloenzyme has been successfully substituted with cobalt by this method.(37)

It is possible to reconstitute many zinc enzymes such as alkaline phosphatase and angiotensin-converting enzyme with Co^{2+} .(38,39) In these cases the metal acts as an acid-base catalyst. In the case of PAH we are looking at the activation and transfer of oxygen. Several oxygen activating enzymes have been successfully reconstituted with Co^{2+} but these enzymes all contained a heme prosthetic group which was capable of being oxidized and reduced thus stabilizing the resonance intermediates.(40) To date no non-heme enzymes have been successfully reconstituted with any metal other than iron.

In this project it was found possible to remove reversibly using a chelator upto 80% of the iron in PAH. Is it then possible to substitute other metals in place of the iron in Phenylalanine Hydroxylase?

It is known that outside of an enzyme iron can hydroxylate aromatic compounds by way of a free radical reaction referred to as Fenton's reaction.(41) Co^{2+} , Cu^{2+} and Ni^{2+} are also capable of performing this reaction.(42) Could these and possibly others be capable of substituting in the PAH enzyme? However when the metal acts as a bridge or an electron transfer intermediary between a reducing agent and the substrate it is known that only Fe^{2+} and Cu^{2+} are competent.(43) Does

the iron form a bridge between the tetrahydropterin and phenylalanine in order for the transfer of an oxygen to occur?

Guroff found that Hg_2^{2+} , Cd^{2+} and Cu^{2+} were capable of restoring activity to the *pseudomonas* PAH.(14) He found the $V_{\max}$ to be 30, 3, 4 μM for Hg_2^{2+} , Cd^{2+} and Cu^{2+} respectively as compared to 50 μM for Fe^{2+} . This prompted studies by Fisher to see if the mammalian PAH would react similarly. Fisher attempted to reconstitute a purified PAH enzyme sample with Hg_2^{2+} , Ni^{2+} , Co^{2+} , Cu^{2+} , Cr^{2+} and Zn^{2+} .(22) He found that none of these metals were capable of substituting for Fe^{2+} . Fisher used a sucrose gradient to purify the PAH enzyme and he did not preincubate the enzyme with phenylalanine before performing the reconstitution. It has been found recently that the enzyme acts as if it has a hinge. When L-phenylalanine binds the enzyme opens on this hinge exposing the active site.(43) It is therefore would seem necessary to activate the enzyme with phenylalanine exposing the active site before performing a reconstitution. In this project the reconstitution was repeated using fully activated enzyme with the metals Fisher attempted plus several outside the transition metal group to see if in fact oxidation-reduction was the role of the metal or if it was closer to a proton transfer almost similar to nonredox enzyme reactions.(44)

Results from reconstitution studies performed on several different days are shown in tables 13-15. A summary of these results are shown in table 12. Pb^{2+} , Ni^{2+} , Cu^{2+} , Co^{2+} , Cr^{3+} and Fe^{3+} showed approximately 8% reconstitution. The metal stock solution were analysed by atomic absorption and found to contain approximately 3 ppm of iron. The results of the analysis are shown in table 1. This would provide an effective Fe^{2+} concentration of 10^{-9}M in the reconstitution experiments. This concentration of iron would provide approximately 5-10% recovery

of activity to the enzyme.

Unlike Guroff's experiments with *pseudomonas* the rat liver PAH is completely inhibited by Hg^{2+} and even the remaining enzyme activity due to iron contamination was destroyed.

Manganese, Chromium and Cobalt did not reconstitute the enzyme. There are several reasons why the cobalt was incapable of reconstituting this enzyme. The rate of interconversion of Co(III)-complex/Co(II)-complex is slow making cobalt not an efficient metal for electron transfer reactions. Also the Co(III) complexes are usually ligand substitution inert making Cobalt unsuitable for the reversible binding of substrates. Chromium(III) is also quite substitution inert. The chromium metal stock solution contained 120 ppm of iron. This would provide an effective concentration of 10^{-7} M iron to the sample which would restore approximately 60% of activity to the enzyme. However, only 5% was observed. Chromium must therefore inhibit the very unstable apo-enzyme.

Cd^{2+} showed 22% recovery which was found to be entirely due to iron contamination of the metal stock solution. Aluminum and thallium, which are both trivalent ions provided 31% and 15% restoration of activity. They too contained 5ppm and 0.75 ppm of iron. The iron contamination of the Al^{3+} would contribute approximately 15% of the activity observed. The iron contamination of the thallium was so low that it would not be observed. Therefore, both of these metals appear to provide approximately 15% restoration of activity.

TABLE 12: SUMMARY OF APO-RECONSTITUTION STUDIES AEROBICALLY AND ANAEROBICALLY USING 10 FOLD EXCESS OF METALS

METALS	% ACTIVITY RECOVERED
ALUMINUM	31.68
LEAD	7.39
CADMIUM	21.99
MERCURY	-3.70
MANGANESE	2.46
NICKEL	6.41
COPPER	8.23
ZINC	2.18
COBALT	7.69
CHROMIUM	1.54
THALIUM	15.02
IRON(III)	8.23
IRON(II)	79.76

Note: 1) Recoveries have been corrected for Apo-enzyme residual activity of 20-24% aerobically

2) Approximately 10% activity can be attributed to ppm iron contamination of metal stock solution as determined by A.A. analysis

FIGURE 15: APO-RECONSTITUTION USING 10 FOLD MOLAR EXCESS OF METALS

METALS

TABLE 13: APO-RECONSTITUTION STUDY USING 10 FOLD
EXCESS OF METALS, TRIAL 1

SAMPLE	ACTIVITY Micromoles/min	SPECIFIC ACTIVITY Micromoles/min/mg	%RECOVERY
NATIVE	0.0028	0.184	100.00
APO	0.000	0.000	0.00
ALUMINUM	0.0011	0.0744	40.43
	0.0006	0.0410	22.28
	0.0006	0.0426	23.15
	0.0009	0.0638	34.67
CHROMIUM	0.0000	0.000	0.00
	0.0002	0.0159	8.64
	0.0000	0.000	0.00
	0.0006	0.0638	22.28
LEAD	0.0005	0.0353	19.18
	0.0000	0.000	0.00
	0.0003	0.0231	12.55
	0.0000	0.000	0.00
ZINC	0.0000	0.000	0.00
	0.0000	0.000	0.00
	0.0000	0.000	0.00
NICKEL	0.0001	0.0085	4.62
	0.0003	0.0173	9.40
	0.0005	0.0357	19.40
	0.0003	0.0222	12.07
MANGANESE	0.0000	0.000	0.00
	0.0004	0.0312	16.96
IRON(III)	0.0005	0.0313	17.01
	0.0008	0.0524	28.48
IRON(II)	0.0010	0.0665	36.13
	0.0012	0.0817	44.40
COPPER(II)	0.0004	0.0291	15.82
	0.0004	0.0245	13.32
CADMIUM	0.0004	0.0237	12.88
	0.0003	0.0170	9.24

TABLE 14: APO-RECONSTITUTION STUDY USING
10 FOLD EXCESS OF METAL, TRIAL 2

SAMPLE	ACTIVITY Micromoles/min	SPECIFIC ACTIVITY Micromoles/min/mg	% RECOVERY
NATIVE	0.0028	0.1840	100.00
APO	0.0009	0.0455	23.24
	0.0008	0.0399	20.38
	0.0007	0.0375	19.15
ALUMINUM	0.0020	0.0959	48.98
	0.0024	0.1184	60.47
	0.0021	0.1030	52.60
LEAD	0.0010	0.0506	25.84
	0.0009	0.0435	22.22
	0.0016	0.0784	40.04
CADMIUM	0.0010	0.0511	26.10
	0.0010	0.0511	26.10
	0.0012	0.0611	31.21
	0.0016	0.0791	40.40
	0.0009	0.0453	23.26
MANGANESE	0.0008	0.0419	23.40
	0.0012	0.0565	23.89
	0.0009	0.0445	22.73
NICKEL	0.0013	0.0666	34.01
	0.0006	0.0323	16.49
	0.0014	0.0679	34.68
COPPER	0.0010	0.0526	26.86
	0.0014	0.0713	36.41
	0.0013	0.0653	33.35
	0.0009	0.0488	24.92
ZINC	0.0008	0.0412	21.04
	0.0012	0.0603	30.79
COBALT	0.0015	0.0761	38.87
	0.0011	0.0554	28.29
	0.0013	0.0666	34.01
	0.0008	0.0423	21.60
IRON(III)	0.0013	0.0631	32.23
	0.0008	0.0405	20.68

TABLE 14: APO-RECONSTITUTION STUDY USING
10 FOLD EXCESS OF METAL, TRIAL 2 CONT'D

SAMPLE	ACTIVITY Micromoles/min	SPECIFIC ACTIVITY Micromoles/min/mg	% RECOVERY
IRON(II)	0.0026	0.1304	66.60
	0.0023	0.1142	58.32
	0.0022	0.1102	56.28
RUBIDIUM	0.0018	0.0915	46.73
	0.0013	0.0657	33.55
	0.0014	0.0709	36.21
CHROMIUM	0.0015	0.0771	39.38
	0.010	0.0512	26.16
	0.0012	0.0595	30.39
	0.0011	0.0555	23.34
	0.0011	0.0555	23.34
THALIUM	0.0020	0.1006	51.38
NATIVE	0.0039	0.1958	100.00

TABLE 15: APO-RECONSTITUTION STUDY USING 10 FOLD EXCESS, TRIAL 3

METAL	ACTIVITY Micromoles/min	SPECIFIC ACTIVITY Micromoles/min/mg	% RECOVERY
NATIVE	0.0063	0.3162	
APO	0.0008	0.0482	18.26
	0.0011	0.0569	21.52
NICKEL	0.0007	0.0388	14.67
	0.0017	0.0849	32.11
COBALT	0.0009	0.0435	16.45
MANGANESE	0.0012	0.0578	21.86
CADMIUM	0.0019	0.0965	36.50
LEAD	0.0014	0.0724	27.38
	0.0019	0.0956	36.16
MERCURY	0.0007	0.0342	12.97
	0.0012	0.0635	24.02
	0.0007	0.0355	13.46
ZINC	0.0011	0.0530	20.05
	0.0010	0.0499	18.87
CHROMIUM	0.0011	0.0562	21.26
ALUMINUM	0.0029	0.1445	56.65
	0.0022	0.1101	41.64
	0.0029	0.1449	54.80
THALIUM	0.0028	0.1421	53.74
	0.0017	0.0823	31.47
RUBIDIUM	0.0022	0.1083	40.96
COPIER	0.0005	0.0500	18.91
	0.0016	0.0845	31.96
IRON(III)	0.0017	0.0874	55.06
	0.0016	0.0769	29.00
IRON(II)	0.0032	0.1579	59.72

ANAEROBIC RECONSTITUTION OF PAH WITH OTHER METALS

In common with the findings of Gottschall it was found that Iron(III) would restore about 10% of the original activity while over 80% could be restored using Fe^{2+} . It was proposed by Gottschall that when removing the iron the sulfhydryl group near the active site is oxidized. Upon incubation of the apo-enzyme with Fe(II) the sulfhydryl group is reduced back by the iron which has the extra electron to lend. The enzyme is then sitting in the resting state Fe(III) , which is pre-reduced by the cofactor exactly as previously observed in many EPR studies.(19)

If this is in fact the case, then it should be possible to remove the metal anaerobically without oxidizing the sulfhydryl group, and it should also be possible to restore activity with Fe^{3+} . This is indeed the case, in both this project and Gottschall's work. 80% of the activity could be restored if the apo-enzyme was prepared and incubated in with Fe^{3+} in an argon atmosphere. In this project, other metals were also tested for their ability to reconstitute the enzyme anaerobically since it is possible that this oxidized sulfhydryl group was hindered their ability to reconstitute the enzyme as well. The results are summarized in table 17.

TABLE 16: SUMMARY ANAEROBIC APO-ENZYME RECONSTITUTION STUDIES

METAL	% ACTIVITY RECOVERED
RUBIDIUM	40.51 $\pm$ 15.23
NICKEL	15.69 $\pm$ 10.35
ZINC	13.65 $\pm$ 3.10
CADMIUM	13.05 $\pm$ 2.01
LEAD	11.30 $\pm$ 1.34
THALIUM	29.26 $\pm$ 16.92
ALUMINUM	52.81 $\pm$ 14.72
COPPER	13.03 $\pm$ 9.99
MERCURY	15.00 $\pm$ 10.00
MANGANESE	32.44 $\pm$ 10.86
CHROMIUM	38.94 $\pm$ 8.81
IRON(II)	56.64 $\pm$ 10.54
IRON(III)	86.05 $\pm$ 18.54
COBALT	30.93 $\pm$ 16.47
APO-ENZYME	17.73 $\pm$ 2.24

Note: Approximately 10% of the recovered activity can be attributed to ppm contamination of metal stock solutions as determined by A.A. analysis

FIGURE 16: ANAEROBIC RECONSTITUTION OF APO-PAH

Manganese, Chromium and Cobalt like iron are 3+/2+ couples. These metals did not reconstitute the enzyme aerobically. Anaerobically it was found that Mn^{3+} and Co^{3+} could restore 14.71%, and 13.20% respectively. Only about 5% of this activity is due to iron contamination. These metals are not efficient catalysts but there is some residual capability unlike aerobically where there was no restoration as shown by -4.47% and 0.00% activity for Mn and Co respectively. Manganese of the three metals is the most similar to iron and can replace it in heme-enzymes. However, Mn^{2+} is d^5 and being half-filled does not benefit from ligand stabilization like Fe^{2+} and it will therefore form weaker complexes than Fe^{2+} . Manganese is also not capable of the low-spin configuration and the enzyme seems to change between low spin and high spin. This could make it less efficient and possibly not allow it to reconstitute the enzyme.

It is interesting that anaerobically Cr is not as inhibitory. 21.21% restoration by the contaminating iron is possible as compared to 8% aerobically. Aluminum restored 35.08% of the original activity of the native. Approximately 10% of this activity is due to iron contamination of the metal stock solution. Therefore, 25% of the activity is due to the Al^{3+} , which is about the same as that observed aerobically. It would seem that Aluminum even though it is not a transition metal is capable of replacing the Fe^{2+} to a small extent. The reaction must not be totally "red/oxid" in character.

The capabilities of most of the metals to reconstitute the enzyme increased anaerobically. It would seem that the reduced state of the sulfhydryl group allows these other metals some ability to enter the active site and provide some restoration of activity.

TABLE 17: ANAEROBIC APO-ENZYME RECONSTITUTION
WITH A 10 FOLD EXCESS OF METAL

METAL	ACTIVITY Nanomoles/min	SPECIFIC ACTIVITY Micromoles/min/mg	% RECOVERY
Native	2.600	0.1305	100.00
Apo-enzyme	0.520	0.026	19.84
Mercury	0.0354	0.000	0.00
Cobalt	1.228 0.835	0.061 0.042	46.74 32.18
Copper	0.030	0.002	1.53
Cadmium	0.3027 0.3929	0.015 0.020	11.49 15.32
Zinc	0.384 0.462	0.192 0.023	14.50 17.56
Nickel	0.410 1.924	0.020 0.096	15.38 74.85
Aluminum	1.856 1.984	0.093 0.099	71.54 76.15
Rubidium	1.529 1.151	0.076 0.058	58.46 44.62
Thallium	0.5699 0.975	0.028 0.049	21.54 37.69
Manganese	0.651 0.757	0.033 0.038	25.38 29.23
Chromium	0.994 0.719	0.050 0.036	38.46 27.69
Iron(III)	2.254 2.548	0.113 0.127	86.92 97.69

TABLE 18: ANAEROBIC APO-ENZYME RECONSTITUTION WITH 10 FOLD EXCESS OF METAL, TRIAL 2

METAL	ACTIVITY Nanomoles/min	SPECIFIC ACTIVITY Micromoles/min/mg	%RECOVERY
NATIVE	35.16	0.173	100.00
APO-ENZYME	0.542	0.027	15.61
COBALT	0.489	0.024	13.87
	0.259	0.013	7.51
MANGANESE	0.092	0.046	26.58
	1.687	0.084	48.55
ALUMINUM	0.929	0.046	26.58
	1.270	0.064	36.99
ZINC	0.425	0.021	12.14
	0.362	0.018	10.40
CHROMIUM	1.404	0.070	40.46
	1.707	0.085	49.13
THALIUM	0.333	0.017	9.83
	1.668	0.083	47.98
RUBIDIUM	0.769	0.038	21.96
	1.282	0.064	36.99
MERCURY	0.740	0.037	21.39
COPPER	0.625	0.031	17.92
	0.682	0.034	19.65
LEAD	0.414	0.021	12.34
	0.407	0.020	11.56
CADMIUM	0.426	0.021	12.34

TABLE 18: ANAEROBIC APO-ENZYME RECONSTITUTION
WITH 10 FOLD EXCESS OF METALS, TRIAL 2 CONT'D

METAL	ACTIVITY Nanomoles/min	SPECIFIC ACTIVITY Micromoles/min/mg	PERCENTAGE ACTIVITY
NICKEL	1.039	0.052	30.05
	0.402	0.020	11.56
IRON(II)	2.144	0.107	61.84
	2.319	0.116	63.58
IRON(III)	2.056	0.103	59.53
	3.461	0.173	100.00

IN CONCLUSION:

The non-heme metalloenzyme Phenylalanine Hydroxylase can be activated by only its natural metal iron. The enzyme is inhibited by Mn^{3+} , Cu^{2+} , Cr^{3+} , Zn^{2+} and Hg^{2+} . It was proposed that Zn^{2+} and Cu^{2+} competitively bind to the enzyme but are not capable of performing the "oxidation/reduction". Hg^{2+} also possibly inhibits by binding to the sulfhydryl group near the active site and altering the open active conformation of the enzyme. Mn^{2+} and Cr^{3+} are capable of complexing with the deprotonated carboxylate groups of the aspartic acid and glutamic acid residues of the enzyme, altering the conformation of the enzyme. Further studies would be necessary to determine the exact inhibition that each of these metals is exerting on the enzyme. However, these experiments are outside the time scope of this project.

On removing the iron aerobically from the enzyme it is found that only Fe^{2+} is capable of restoring the majority of the enzyme activity. Aluminum was found to restore 25% of the original activity. It is possible that this enzyme reaction is not totally "oxid/red" in character but is more similar to a proton transfer like non-redox enzymes. Aluminum holding the active players in close proximity of each other.

Anaerobically it was found that Fe^{3+} was capable of restoring activity. It would seem anaerobically the sulfhydryl group near the active site does not become oxidized and the reducing power of Fe^{2+} is no longer essential for restoration of the enzyme activity. Al^{3+} was again found to be capable of

substituting for the iron to the same extent aerobically and anaerobically.

Anaerobically when the sulfhydryl group is not oxidized, the transition metals Co^{2+} and Mn^{2+} were also capable to substitute residually for the natural metal. Manganese is the closest to iron (ionic radius, reduction potential, etc.) however it is not capable of the high spin-low spin transition which seems to possible be a critical component.

It would be interesting to investigate if this partial recovery by Co^{2+} and Mn^{2+} and Al^{3+} could be increased by biosynthesis of the PAH enzyme under enriched conditions of these metals.

Competition studies with equal amount of these other metals and iron would possibly elucidate some interesting liver metal toxicity information. These metals could be competitively replacing the iron in the liver and low PAH activity be a symptom of metal toxicity.

REFERENCES

1. Underwood, E.J., Trace Elements in Human and Animal Nutrition, 2nd edition, Academic Press, New York, (1971).
2. Ochai, Ei-Ichiro, General Principles of Biochemistry of the Elements, Plenum Press, New York, (1987).
3. Frieden, E., Biochemistry of the Essential Ultratrace Elements, Plenum Press, New York, (1984).
4. Webber, G., Advances in Enzyme Regulation, vol. 7, p. 14-39, (1969).
5. Snah, S.N., Johnson, R.C., FEBBS LETTERS, vol. 49, p.404-406, (1975).
6. Agrawal, H.C., Bone, A.H., Davison, A.W., Biochemical Journal, Vol 117, p. 325-331, (1970).
7. Youdim, M., Aromatic Amino Acid Hydroxylases and Mental Disease, Plenum Press, New York, (1982).
8. Shiman, R., Gray, D.W., Journal of Biological Chemistry, vol 255, p. 4793-4880, (1980).
9. Parniak, M.A., Kaufman, S., Journal of Biological Chemistry, vol 256, p. 6879-6882, (1981).
10. Hasagawa, H., Kaufman, S., Journal of Biological Chemistry, Vol 257, p. 3084-3089, (1982).
11. Abita, J-P., Parniak, M., Journal of Biological Chemistry, vol 259, p.14560, (1984).
12. Renson, Journal of Biological Chemistry, vol 237, p.2260-2264, (1962).
13. Blakley, R.L., Benkovic, S.J., editors, Folates and Pterins, Vol 2., John Wiley and Sons, New York, (1985).
14. Guroff, G., Rhoads, C., Journal of Biological Chemistry, vol 242, p. 2641-3645, (1967).
15. Fisher, D.B., Kirkwood, R., Kaufman, S., Journal of Biological Chemistry, vol 247, p. 5161-5167, (1972).
16. Tourian, A., Biochimica Et Biophysica Acta, vol 242, p. 345-354, (1971).
17. Shiman, R., Gray, D.W., Pater, A., Journal of Biological Chemistry, vol 254, p. 11300-11306, (1979).

18. Wallick, D.E., Bloom, L.M., Gaffney, B.J., Benkovic, S.J., *Biochemistry*, Vol 23, p.1295-1302, (1984).
19. Marota, J.J., Shiman, R., *Biochemistry*, vol 23, p. 1303-1311, (1984).
20. Shiman, R., Jefferson, L.S., *Journal of Biological Chemistry*, vol 257, p.839-844, (1982).
21. Gottschall, D.W., Dietrich, R.F., Benkovic, S.J., *Journal of Biological Chemistry*, vol 257, p. 345-349, (1982).
22. Fisher, D.B., Kirkwood, R., Kaufman, S., *Journal of Biological Chemistry*, vol 247, p. 5161-6167, (1972).
23. Nielsen, K.H., Simonsen, V., Lind, K.E., *European Journal of Biochemistry*, vol 9, p. 497-502, (1969).
24. Kaufman, S., *Journal of Biological Chemistry*, vol 234, p. 2677-2682, (1959).
25. Bublitz, C., *Biochimica et Biophysica Acta*, vol 191, p. 249-256, (1969).
27. Blair, J.A., Pearson, A.J., *Tetrahedron Letters*, Nos. 3 , p.203, (1973).
23. Nielsen, K.H., *European Journal of Biochemistry*, vol 7, p. 260-369, (1969).
29. Shiman, R, Kaufman, S., *Journal of Biological Chemistry*, vol 246, p.1330-1334, (1971).
30. Lazarus, R.A., Dietrich, R.F., Wallick, D.E., Benkovic, S.J., *Biochemistry*, vol 20, p. 6834-6841, (1981).
31. Phillips, R.S., Iwaki, M., Kaufman, S., *Biochemical and Biophysical Research Communication*, Vol 110, p. 919-925, (1983).
32. Undenfriend, S., Cooper, J.R., *Journal of Biological Chemistry*, vol 196, 227-233, (1954).
33. Kaufman, S., *Methods in Enzymology*, Vol 142, p. 3-17, (1987).
34. Shiman, R., Gray, D.W., Pater, A., *Journal of Biological Chemistry*, vol 254, p. 11300-11306 (1979).
35. Styer, L., *Biochemistry*, W.H. Freeman and Co, San Francisco, (1981).
36. Guroff, G., Ito, T., *Journal of Biological Chemistry*, vol 240, p. 1175, (1965).
37. Cotton, F.A., Wilkinson, G., *Basic Inorganic Chemistry*, Wiley, New York, (1976).

38. Banci, L., Journal of Inorganic Biochemistry, Vol 30, p. 77-85, (1987).
39. Bunning, P., Riordan, J.F., Journal of Inorganic Biochemistry, Vol 24, p. 183-197, (1985).
40. Wagner, G.C., Gunsalus, I.C., Journal of Biological Chemistry, Vol 256, p.6266-6273, (1981).
41. Walling, Acc. Chem Res., Vol 8, p. 125-131, (1975).
42. Bender, M.L., Bergeron, R.J., Komiyama, M., Bioorganic Chemistry of Enzymatic Catalysis, John Wiley & Sons, New York, (1984).
43. Phillips, R.S., Parniak, M.A., Kaufman, S., Biochemistry, Vol 23, p. 3836-3842, (1984).
44. Hamilton, G., Advances in Enzymology, vol 32, p. 82-88, (1969).

END

1|8|09|90

FIN