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Properties and Kinetics of the Reconstitution
Reaction of Apo-Tyrosinase

by

Douglas Max Goltz

A report submitted to the department of
chemistry in partial fulfillment of
the requirements for the degree of
Master of Science.

Carleton University
Department of Chemistry
Ottawa, Ontario
February, 1989

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Thesis Supervisor

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February, 1989

Abstract

The experiments reported in this thesis deal with the properties and kinetics of the reconstitution reaction of apo-tyrosinase with copper (II) ions. The kinetics of reconstitution was pseudo first order with respect to apo-tyrosinase. The kinetics with respect to copper was more difficult to determine because, even at high copper concentrations, the rate of reconstitution depended on the copper concentration. At pH=7.0 and 273 Kelvin, the rate of reconstitution was $8.55 \times 10^{-3} \text{ sec}^{-1}$.

The effects of pH and temperature were also studied. The pH experiments demonstrated that the rate of reconstitution of copper to the active site of tyrosinase is very sensitive to changes in pH, especially in the range of pH 6 to 7.5. The temperature dependence experiments led to the calculation of various activation parameters for the reconstitution of apo-tyrosinase with copper (II) ions. The enthalpy of activation for the reconstitution reaction was calculated to be 84.64 KJ/mole, the Gibbs free energy of activation was calculated to be 81.71 KJ/mole and the entropy of activation was calculated to be 0 KJ/mole/K. A number of possible explanations are discussed for the variation of the rate of reconstitution with varying pH conditions.

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INTRODUCTION

The Role of Metals in Nutrition

Most of the naturally occurring elements have been detected in humans. Exposure may be intentional if the element of interest is essential, such as iron in our diet. On the other hand, exposure to an element may be unintentional if the element is not essential, such as mercury in environmental pollution. The route of exposure of the elements found in the human body is largely oral and, in some cases, by inhalation or through the skin. The elements found in humans may be classified as essential or non-essential. The essential elements can be further subdivided into: bulk and trace elements (3,4,5).

The bulk or constituent elements are given their classification because they occur in substantial quantities in humans. Approximately 99% of human mass is composed of the structural elements: hydrogen, oxygen, carbon, nitrogen, calcium and phosphorus. Included in the bulk elements are the macrominerals which make up roughly 0.7 percent of the mass of living beings. They include: sodium, magnesium, potassium, sulphur and chlorine.

The trace and ultratrace elements are given their classification because they occur only at significantly lower levels in humans. These elements include: iron,

iodine, copper, zinc, manganese, cobalt, chromium, nickel, vanadium, selenium, arsenic, fluorine, silicon and lithium. Although the bulk elements are important structurally as the building blocks of many living things, the trace and ultratrace elements are also essential, acting as catalysts in the active site of many enzymes.

Elements not considered essential presently, but are possible candidates may include: tin, lead, cadmium, and bromine (3). The difficulty in determining whether these possible candidates are essential is due largely to their toxicity, even at trace levels. Also, the ability to create an environment of these possibly essential elements that is low enough to show a deficiency in experimental lab animals, is extremely difficult.

The Role of Copper in Nutrition

Copper was first detected in biological systems over a century and a half ago by Bucholtz (10) and later by Boutigy (9). Both researchers found copper in numerous plant and animal tissues. Fifteen years later, Harless demonstrated that copper was present in the blood proteins of snails (11). Fredricq discovered in 1878 the copper-containing pigment known as hemocyanin (12). In 1925, McHague suggested evidence of the dietary importance of copper in rats (13). Three years later, Hart et al. noted that copper as well as iron was necessary for the prevention of anemia (14).

Defects in the process of pigmentation, keratinization of wool, bone formation, reproduction, mylenation of the spinal cord, cardiac function, and connective tissue formation was all discovered to be related to copper. Beck, in 1941, demonstrated that both copper and iron were required to relieve anemia in pigs (15). It was discovered later that copper plays a major role in iron metabolism including:

- 1) absorption of iron from the Intestine;
- 2) mobilization of iron to transferrin; and,
- 3) synthesis of heme from iron and protoporphryn.

Cohen and Elvehjen established the role of copper as a component of cytochrome oxidase in 1934 (16). Since this catalytic role of copper was discovered, numerous important copper-containing enzymes have been discovered. Examples of these copper-containing enzymes found in humans include: tyrosinase, superoxide dismutase, uricase, hemocyanin, and dopamine B-hydroxylase.

Levels of Copper in Humans

The normal adult human body contains approximately 80 milligrams of copper. Underwood reported in 1977 the highest concentration of copper in the liver which was found to be 14.7 ppm. The next highest concentrations are in the brain, 5.6 ppm; lung, 2.2 ppm; kidney, 2.1 ppm; ovary, 1.2 ppm; and testes, 0.8 ppm. High levels of copper can also

be found in the pigmented part of the eye which has a concentration of roughly 105 ppm (17).

The liver plays a central role in copper metabolism. The liver sequesters and stores copper, converts it into a bile component and produces some of the copper proteins. Liver produces ceruloplasmin, which is the major copper-containing protein in the plasma and, as a result, the presence of copper in the liver is not surprising.

Metabolism of Copper

The most important route for the introduction of copper into the human body, under normal circumstances, is the oral route. Virtually all of the required copper is supplied in our diet. Most mammals absorb copper in the stomach and the small intestine. Although the mechanism for copper is not fully understood, it has been suggested that copper uptake may traverse the intestinal mucosa in the forms of both organic complexes and as the ionic form (18). The amount of copper absorbed depends largely on diet and intestinal pH.

Once copper is absorbed, it is bound by serum albumin, ceruloplasmin or amino acid complexes for transport in the bloodstream to the various organs, such as the liver. Copper in the liver is either stored for future use or is used for incorporation into the various copper-containing enzymes. The most important route for the excretion of ingested copper is in the feces. More than half of the ingested

copper is not absorbed and is passed through the intestine. Absorbed copper is largely excreted in the bile and is thus removed from the body in the feces. Most of the copper circulating in the bloodstream is firmly bound to ceruloplasmin and, as a result, only a small fraction is excreted by the kidney in the urine. Small amounts of copper can be excreted in the sweat of an individual. Women also excrete copper during menstruation and lactation, but these losses are quite small (6).

Copper Deficiency in Humans

Numerous symptoms may appear due to copper deficiency in mammals. These symptoms include:

- 1) Skeletal Defects: spontaneous fractures due to defects in the organic matrix of the bone;
- 2) Cardiac Defects: cardiac lesions which can lead to heart failure;
- 3) Vascular Defects: massive haemorrhage in the walls of the intact aorta due to defective collagen and elastin formation;
- 4) Central Nervous Disorders: symptoms include: uncoordinated movement, nervous disorders, and lack of myelination in the brain stem and spinal cord;
- 5) Steely Wool Hair: "Menke's disease", progressive cerebral degeneration and the presence of twisted hair caused by the failure to absorb copper; and,

6) Iron Metabolism: symptoms include: failure to absorb iron, failure to incorporate iron into transferrin and impaired synthesis of heme.

Copper Enzymes: The Copper Monooxygenases

Presently, there are many well known copper-containing enzymes that take part in redox-type reactions. Most of these types of reactions use molecular oxygen which acts as an electron acceptor.

Monooxygenases incorporate only one atom of molecular oxygen into the substrate while the other oxygen atom is reduced to water. The overall reaction can be described as:

An example of this type of enzyme, as well as being the enzyme of interest in this report, is tyrosinase (19,20).

Tyrosinase catalyzes two reactions:

1) Ortho-hydrogenation of Monophenols:

This is often referred to as MONOPHENOLASE or CRESOLASE activity of tyrosinase. In this reaction, one oxygen atom of

molecular oxygen is incorporated into one monophenol and the other oxygen atom is reduced to water.

2) Oxidation of Ortho-diphenols:

This is often referred to as the DIPHENOLASE or CATECHOLASE activity of tyrosinase. In this reaction, two moles of diphenol are dehydrogenated with molecular oxygen being reduced to water. It should be noted that both the monophenolase and diphenolase activities are closely related in the overall reaction mechanism of tyrosinase.

Tyrosinase has been isolated from many different biological sources including:

- 1) Plants: e.g. potato (21), spinach beet (25).
- 2) Microbial: e.g. neurospora crassa (24,26),
agaricus bispora (mushroom) (23,27)
- 3) Insect: e.g. grasshopper egg (22)
- 4) Mammalian: e.g. all melanin-producing mammals
including humans.

The most frequently studied source of tyrosinase has been the common mushroom, agaricus bispora.

Protein Structure

Early chromatographic studies, using sedimentation techniques, resolved mushroom tyrosinase into four subunits: Alpha, Beta, Gamma and Delta. Their molecular weights vary from 26,000 to 32,000 g/mole and 120,000 to 128,000 g/mole for each isozyme. The assumption was made that each of the subunits were identical in structure and contained one copper atom. Since the molecular weight of the enzyme was determined to be 128,000 g/mole it was thought that the approximate weight of each subunit was probably 30,000 g/mole (28).

Recent studies, however, have shown that the molecular weights of the four subunits are not identical. Using SDS Polyacrylamide Gel Electrophoresis it was found that tyrosinase consisted of two smaller subunits with an approximate molecular weight of 13,400 g/mole and two larger subunits with a molecular weight of 43,000 g/mole (28).

In the past ten years, a substantial amount of research has been performed on tyrosinase isolated from *neurospora crassa*. Recently performed research has led to a much greater understanding of the active site as well as the reaction mechanism of tyrosinase. Tyrosinase isolated from *neurospora crassa* differs structurally from tyrosinase of *agaricus bispora* (common mushroom). For example, the molecular weight is less than half, or roughly 46,000 g/mole. *Neurospora crassa* tyrosinase is a single

polypeptide chain of 407 amino acids and although the active site of both types are thought to be identical, *neurospora crassa* tyrosinase contains only one binuclear coupled pair and tyrosinase isolated from *agaricus bispora* contains two binuclear coupled pairs (29).

Active Site Structure

As stated earlier, the active site of tyrosinase contains a strongly coupled binuclear copper pair. The active site of tyrosinase is able to catalyze both the ortho-hydroxylation of monophenol and the oxidation of ortho-diphenols to ortho-quinones. The binuclear copper pair interacts both with oxygen and substrate. The structure of the active site depends largely on the state of copper and as a result a number of derivatives can be obtained. Studies of these structures have led to a better understanding of the geometric and electronic structure of the binuclear coppers in the active site. The structures which are thought to exist during catalysis are:

- 1) Mettyrosinase,
- 2) Oxytyrosinase,
- 3) Deoxytyrosinase.

Mettyrosinase consists of two tetragonal cupric ions bridged by an endogenous protein ligand R (28,29). Mettyrosinase can be defined as the structure without oxygen bound to the cupric atoms in the active site. Mettyrosinase

has been referred to as "resting tyrosinase" in some papers (26).

The proposed structure for the active site of mettyrosinase is:

where N refers to a nitrogen in histidine, L refers to an exogenous ligand and R refers to an endogenous protein bridge. To date, the author has not found any references as to what exogenous ligand may be. The endogenous ligand is thought to be phenolate, hydroxide or alkoxide (26).

An X-ray absorption fine structure study (EXAFS) by G.L. Woolery et al. estimated the Cu(II)-Cu(II) distance in mettyrosinase as 3.39 angstroms (30). The electron configuration of cupric ions is d^9 and is, therefore, considered to be paramagnetic. As a result, when doing electron paramagnetic resonance spectroscopy, it is expected that a signal for the copper will be seen. However, this is not observed because the protein bridge provides an effective pathway for superexchange between the two cupric ions, which results in an overall diamagnetic state of the copper (28,31).

The resting form of tyrosinase is actually a mixture of roughly 85% mettyrosinase and 15% oxytyrosinase (32). When

peroxide or other reducing agents such as: ascorbic acid, hydroxylamine, or dithionite in the presence of molecular oxygen are added to mettyrosinase, oxytyrosinase is formed (31).

Oxytyrosinase is structurally similar to mettyrosinase in that the binuclear copper site can also be described as two cupric ions bridged by an endogenous protein ligand and the exogenous ligand bridge is a peroxide in a μ -1,2 geometry (33). The proposed active site structure of oxytyrosinase is:

In this structure, N refers to a nitrogen in histidine and R is the endogenous protein ligand. As with mettyrosinase the copper pair are antiferromagnetically coupled and, as a result, the copper is epr silent. Using EXAFS, the distance between the two cupric ions is 3.63 angstroms, which is slightly greater than in mettyrosinase (30).

The lack of epr signal and spectral features in deoxytyrosinase has resulted in the lack of information on the electronic or geometric structure of this form of the active site in tyrosinase. In a separate EXAFS study by T.G. Spiro et al. the copper pair was found to exist in the

cuprous state (28). As a result, the active site structure for deoxytyrosinase has been assigned as:

As with Met and Oxytyrosinase, N refers to a nitrogen of histidine. Unlike Met and Oxytyrosinase, the distance between the cuprous ions has not been determined.

In summary, the active site of tyrosinase is thought to exist as three derivatives in its natural environment:

Derivatives of Tyrosinase

It is possible to synthesize other non-biologically important derivatives of tyrosinase in the presence of molecular species containing only one oxygen atom, instead of two, as with molecular oxygen. These derivatives are known as Dimer and Half-Met tyrosinase (28,29,31). The structure of the binuclear active site was conclusively established using these epr detectable forms.

The dimer form of tyrosinase can be synthesized by the addition of nitric oxide. An epr study of this derivative conclusively showed that copper exists in the active site as a binuclear pair. The results from the epr spectra suggest

that the cuprous ions are isolated. The structure of the active site of Dimer tyrosinase is thought to be:

A computer-simulated study has shown the distance between the copper ions to be roughly 5.9 angstroms, which is in agreement with the studies performed on arthropod and molluskan hemocyanin (24). It should be noted that the active site structure of hemocyanin and tyrosinase are quite similar.

Another series of epr detectable forms of the active site were prepared by treating mettyrosinase with an excess of sodium nitrite, a reducing agent such as ascorbic acid and a subsequent ligand substitution reaction of the half Met-NO₂ derivative with different anions (28). Examples of the anions may be cyanate or azide. Depending on the type of exogenous ligand, the endogenous ligand either remains intact or is ruptured. For example, azide will rupture the endogenous bridge. This form of tyrosinase is known as half-met tyrosinase and the structure of this active site is thought to be:

where the dotted line indicates either bonding or no bond at all, depending on the anion.

Reaction Mechanism

Numerous mechanisms for the action of tyrosinase have been proposed in the past (35,36). Many of the mechanisms proposed were largely hypothetical and based on the assumption of only one copper atom in the active site (35). As a result of these assumptions, the mechanism of action of tyrosinase was a controversial one.

More recently, a mechanism has been proposed by Lerch which was based on a binuclear active site for tyrosinase (28,31). The proposed mechanism is shown in figure A.

The diphenolase or catecholase activity is the oxidation of two ortho-diphenols to two ortho-quinones followed by a reduction of molecular oxygen to water. The first step is the binding of ortho-diphenol to the active site of mettyrosinase and the loss of two H^+ . The diphenol is then oxidized to ortho-quinone with the reduction of copper to form deoxy-tyrosinase. Addition of molecular oxygen to the deoxy-tyrosinase oxidizes the binuclear copper in the active site to form oxy-tyrosinase. The second ortho-diphenol binds to the oxy-tyrosinase and is followed by oxidation of the ortho-diphenol and reduction of the peroxide to water. The cycle is thus complete with the formation of mettyrosinase.

Figure A: Mechanistic Scheme of tyrosinase

The monophenolase or cresolase activity is the ortho-hydroxylation of monophenol to a diphenol followed by an oxidation of the ortho-diphenol to ortho-quinone. In the first step, the monophenol binds to the oxy-tyrosinase. This is followed by a rearrangement of the geometry of the copper bound to the monophenol, from tetragonal to trigonal bipyramidal. This results in a stabilization of the monophenolic-copper complex which is more suitable for ortho-hydroxylation. The ortho-diphenol is then oxidized to ortho-quinone with the subsequent reduction of the binuclear copper to form the deoxy-tyrosinase. The cycle is completed with the addition of molecular oxygen to oxidize the copper and form oxy-tyrosinase.

Research on Copper in Tyrosinase

Following Kubowitz's experiments with tyrosinase (21), similar studies were conducted by other groups. Allen and Bodine tested the effects of the removal and later addition of copper to tyrosinase extracted from grasshopper eggs (22). The procedure was similar to Kubowitz's for removing the copper from the active site of the enzyme. An excess of cyanide was added to bind irreversibly to the copper in the active site. The copper and excess cyanide were then removed by dialysis using a cellophane tube.

Allen and Bodine were able to remove most of the copper from the native tyrosinase. Roughly one fifth of the copper

remained in the enzyme preparation. It was also noted that roughly twice as much copper was required for complete restoration of activity. In agreement with Kubowitz, salts of iron, cobalt, nickel, manganese and zinc were unable to replace copper at the active site. This suggests that tyrosinase is dependent, specifically, on copper.

A later study by Lerner et al. was initiated to provide further evidence that mammalian tyrosinase requires copper for activity (37). Tyrosinase prepared from Harding-Passey mouse melanoma was used for their experiments. Using a method similar to Allen and Bodine, Lerner et al. found that the copper content of tyrosinase was 0.19% before and 0.09% after treatment with cyanide. This corresponded to an 85% loss of enzyme activity. The enzyme could be restored to approximately 90% of the original activity of the native enzyme.

The addition of salts of iron, zinc, cobalt, magnesium and manganese failed to restore any activity. Nickelous ions were found to restore some activity but significantly less than cupric ions. In addition to studying the properties of the apoenzyme, a study of tyrosinase inhibitors was also completed. Various inhibitors such as 2,3-dithiopropanol or thiourea exert their action by combining with the copper at the active site. This inhibition can be reversed by adding cupric ions, however, other metals, such as iron, zinc, cobalt, magnesium and manganese, had no effect.

The effect of various anions and cations on the activity of tyrosinase was also studied by Lerner. Various univalent cations, such as lithium, sodium, potassium and ammonium, had no effect on tyrosinase. The anions, fluoride, chloride, acetate and oxalate, inhibited both the tyrosine-tyrosinase reaction and the dopa-tyrosinase reaction. Iodate, persulphate and nitrate also inhibited the activity of tyrosinase, but only in high concentrations.

A second type of experiment was carried out whereby apoenzyme was prepared and small quantities of metal salts were added to the copper free enzyme, along with enough copper to restore 80-100% of the original activity. Of the 24 metal ions tested, only mercury, silver and gold produced an inactive enzyme preparation. It is believed that these ions compete with copper ions for active centers on apo-tyrosinase.

Exchange experiments with radioactive copper and resting tyrosinase was studied by Dressler and Dawson (38). In these experiments, tyrosinase was exposed to radioactive cupric ions for 40-50 minutes and it was found that small amounts of radioactive copper were incorporated in the active site. The amount of incorporated radioactive copper was less than 15% and decreased with increasing purification of the enzyme. This low amount of copper incorporated indicates that the original copper is firmly bound to the protein in the active site. A possible explanation for the incorporation of the small amount of copper may be the

existence of two types of copper-bonding sites: active and inactive. It is thus thought that the inactive sites are the only ones exchanging with radioactive copper ions.

Further research was completed to determine the exchange of radioactive copper ions in functioning tyrosinase (39). In general, it was found that the functioning enzyme incorporated higher amounts of radioactive copper than resting tyrosinase. The amount of copper incorporated in the functioning tyrosinase also depended greatly on the substrate. For example, it was found that enzyme preparations with high catecholase activity incorporated more radioactive copper during catalysis of *O*-dihydricphenols than monophenols. Enzyme preparations containing high cresolase activity retained little radioactivity. Again the possibility of two different exchange sites in tyrosinase are suggested. The copper at the cresolase activity site is non-exchangeable or more tightly bound, and the copper at the catecholase activity site is more exchangeable and, therefore, less tightly bound.

The kinetics of reconstitution of polyphenoloxidase from apo-tyrosinase and copper was studied by D. Kertesz et al., using electron paramagnetic resonance (epr). The copper in tyrosinase is not detectable by epr spectroscopy and, for this reason, it was thought to exist in the cuprous state. The kinetics of reconstitution with copper were studied by rapidly freezing equimolar amounts of cupric ions and apo-tyrosinase. Measurements were made at various stages of the

reconstitution for the disappearance of epr signal and for the recovery of enzymatic activity. Following the addition of cupric ions, the epr signal disappears steadily until the signal intensity of native tyrosinase is approached. A minimum estimate of the second order velocity constant was calculated as " k_{on} " = $5 \times 10^6 \text{ min}^{-1} \text{ sec}^{-1}$. There is some variability in the rate constant between one preparation and another as well as between fresh and aged apoenzyme.

The explanation put forward by Kertesz et al., for the disappearance of the epr signal and the non-homogeneous kinetics of reconstitution, is the existence of two independent sites for the copper atoms in the active site. The mechanism of the reconstitution reaction is thought to occur in two stages. The first stage is the binding of copper to the active site in the enzyme and the second stage is the reduction of the cupric ions.

Kidani et al. studied the binding constant between apo-tyrosinase and copper ions using equilibrium dialysis with various complexing agents (41). The various complexing agents which removed copper from the enzyme were: sodium cyanide, sodium thiosulfate, 2,2' bipyridyl, 8-hydroxy-quinoline-5-sulfonic acid, 4,7-diphenyl-1,10-phenanthroline disulfonic acid disodium salt. Other complexing agents such as EDTA were found to be ineffective in the removal of copper.

The apparent binding constant between copper and apo-tyrosinase using reducing complexing agents such as sodium cyanide is 10^{-15} M. The apparent binding constant between copper and apo-tyrosinase using non-reducing complexing agents such as 2,2-bipyridyl is 10^{-13} M. Because it was observed that non-reducing complexing agents had a binding constant similar to the stability constant calculated for cupric ions and those complexing agents, it is thought that the copper in tyrosinase is in the cupric state. The cupric ion was reduced by the reducing complexing agents to the cuprous state. The apparent binding constant between cuprous ions and apo-tyrosinase was larger than cupric ions and apo-tyrosinase.

The successful replacement of copper in tyrosinase with cobalt was observed by Ruegg and Lerch (19). The cobalt(II) substitution was used to study the geometry and interaction of ligands with the metal site. The apo-tyrosinase was prepared by incubation of the enzyme in cyanide followed by gel filtration to remove the excess cyanide and copper ions.

In agreement with past studies, it was found that tyrosinase was resistant to direct substitution of copper with cobalt. Apo-tyrosinase binds the cobalt much more readily than the holoenzyme and once bound the cobalt is also very resistant to direct substitution with other metals, including copper. Cobalt tyrosinase displays very weak enzymatic activity and it is thought that this activity is due to trace amounts of copper left in the preparation.

There are numerous differences between the properties of cobalt tyrosinase and native tyrosinase. The absorption spectra of cobalt tyrosinase contain four peaks in the visible region of intermediate intensity, indicating a tetrahedral coordination around the cobalt center. There are no peaks in the visible region for native tyrosinase, except when oxygenated, and only one peak at 280nm with a shoulder at 290nm. The geometry of the copper ion at the active site in native tyrosinase is thought to be distorted tetragonal. The addition of competitive inhibitors to the cobalt tyrosinase changes the absorption spectra according to the inhibitor. The addition of inhibitors does not change the absorption spectra of native tyrosinase. Cobalt substituted tyrosinase does not appear capable of binding molecular oxygen, unlike native tyrosinase. One of the cobalt ions can be removed preferentially with cyanide at high pH. This suggests that the two cobalt ions found in the substituted tyrosinase are not in an identical environment.

Beltramini and Lerch studied the reconstitution reaction of neurospora apo-tyrosinase using both cupric and cuprous ions (24). In this manner, they were able to study the influence of metal valency on the reconstitution of apo-tyrosinase. Copper sulphate was used as a source of cupric ions, whereas the complex $\text{Cu}(\text{CH}_3\text{CN})_4\text{ClO}_4$ or copper sulphate in excess ascorbate, was used as a source of cuprous ions. The kinetics for both metals was first order, however, the rate constant depended on the valency of the metal. Using

cupric ions, $K_{app}=0.123 \times 10^{-3} \text{ sec}^{-1}$. For cuprous ions, $K_{app}=25 \times 10^{-3} \text{ sec}^{-1}$. Using $\text{Cu}(\text{CH}_3\text{CN})_4\text{ClO}_4$ or using copper sulfate in excess ascorbate, $K_{app}=80 \times 10^{-3}$.

The reconstitution with cuprous ions was linear with respect to enzymatic activity and copper content. The reconstitution seems to follow an "all or none" mechanism such that the partially reconstituted enzyme preparation contains both fully active and apo-tyrosinase. In the case of cupric ions, the reconstitution was non-linear between enzymatic activity and copper content. The reconstitution of apo-tyrosinase with cupric ions was slower and less complete than with cuprous ions. The non-linearity with cupric ions may indicate that the two copper ions are bound at two different rates and half apo-enzyme species may be present as intermediates during reconstitution. This study proved the reduction mechanism proposed by Kertesz (40) is not essential for the reconstitution of apo-tyrosinase.

The effect of cupric ions on the dopa-oxidase activity of different isoenzymatic forms of tyrosinase isolated from Harding-Passey mouse melanoma was studied by Martinez et al. (42). The initial activity of the various isozymes was taken as 100%. The addition of cupric ions resulted in the activation of both the soluble and the microsomal isozymes, but had no effect on the melanosomal isozymes. The isozymes could be activated from 120 to 180% depending on the concentration of the cupric ions added. This suggests that

tyrosinase occurs naturally as apo and holoenzyme in mammalian melanocytes.

Salts of zinc, cobalt, nickel and manganese were also incubated with the isozymes and it was found that these metals had no effect, either inhibitory or by activation, on the isozymes. The same salts were added in addition to cupric ions and again there was no effect. This suggests that these metals were not able to compete with copper for the active site in the enzyme. The kinetics of reconstitution was also studied and it was found that the rate constant, K_{app} , for the reconstitution of apo-tyrosinase with cupric ions was 0.105×10^{-3} which was slightly lower than reported previously by Beltramini and Lerch. It was concluded that isozymes, such as the soluble and microsomal tyrosinase, may be both apo and holoenzyme, and the melanosomal tyrosinase exists entirely as holoenzyme.

Tyrosinase in Living Systems

As mentioned previously, tyrosinase occurs widely in both the plant and animal kingdom. Probably the single most important function of tyrosinase is the catalytic oxidation of tyrosine to melanin. Tyrosinase is the single most important enzyme involved in the process of melanin production in living systems. The ortho-hydroxylation of L-tyrosine to L-Dopa and the subsequent oxidation of L-Dopa to

Dopachrome are the first two steps in melanin biosynthesis.

Melanin can be described as a brown to black-colored polymeric pigment. The term "melanin" is a general one since there are numerous types of melanin. Melanins constitute a class of pigments which can be further classified as (43):

- 1) Eumelanins: black in colour, found in the skin, hair and tumors of most animals;
- 2) Phaeomelanins: brown to yellow in colour, found in hair, and feathers of many animals; and,
- 3) Allomelanins: black in colour; found in the darkened skin of many plants such as the potato.

The Biology of Melanin

Numerous factors, such as blood content, can contribute to skin colour, however, the single most important difference between skin colors of different races is the melanin content. In humans, eumelanin is found in cells called melanocytes. The actual synthesis of melanin occurs in special units in the melanocyte called organelles. In the organelle called premelanosome, inactivated tyrosinase is arranged in an ordered pattern. The non-active tyrosinase is then activated, probably by a conformation change, and becomes tyrosinase. At this stage, melanin biosynthesis begins and the organelle at this stage is called melanosome. As melanin is accumulated, the melanosome is transformed

into a dense structureless particle called the melanin granule.

A recent study by Tomita et al. demonstrated the presence of tyrosinase isozymes outside the melanosome in microsomal and soluble fractions (44). More recently, Solano et al. found that the activity of these microsomal and soluble fractions could be increased by incubation with cuprous ions (45). It was then postulated that the extramelanosomal tyrosinase isozymes could be in a partially inactive apoenzyme form and the melanosomal enzyme is in the active or mature form. It was suggested that the inactive form outside the melanosome was such because 1) there was a lower copper concentration than in the melanosome; 2) tyrosinase isozymes have higher affinity for copper outside the melanosome; or, 3) both 1 and 2.

The Role of Melanin in Biological Systems

In mammals, the dark polymeric pigment, melanin, provides protection for skin exposed to the ultraviolet rays of sunlight. One theory for this protection is that melanin can act as an electron exchange polymer capable of trapping free radicals which may be produced by such radiation. (47,48). In marine animals, such as the cuttlefish or squid, melanin is used for defence purposes. The melanin in the ink sac is excreted by the startled squid into the surrounding water which provides a black cloud to hide its retreat. Fish

and some insects use melanin as a camouflage for defence against predators. Melanin found in the external shells of some seeds is thought to act as an absorber of thermal energy from the surroundings. In plants such as potatoes or mushrooms, melanin is found in the blackened skin. The role of tyrosinase in plants is open to speculation at the present time. Melanin may also play a role in neurological disorders. For example, in schizophrenic patients, large deposits of melanin occur in the brain. One possibility for the presence of melanin is that it may act as an inhibitor of nervous depolarization (48).

The Biosynthesis of Melanin

The pathway of melanin biosynthesis was initially worked out by Raper (52) in 1928 and Mason (53) in 1948. In 1951, Lerner and Fitzpatrick proposed a slightly modified version which is widely accepted. Although deviations in their proposal for melanin synthesis do occur, the scheme proposed by Raper and Mason represents the majority of the products formed. A recent study by Garcia-Canovas et al. in 1982 confirms that the Raper-Mason-Lerner pathway is the main one (65). The pathway proposed by Raper-Mason is shown in figure 8.

Figure B: Biosynthesis of Melanin

Objectives of the Study

Originally, the goal of this study was to investigate metal substitution in the copper-containing metalloenzyme, tyrosinase. It was decided, however, that metal substitution was already partially investigated by a number of research groups in the past and that trying different metals would not contribute significantly to the understanding of this enzyme. Therefore, it was decided that the reconstitution reaction itself would be investigated. The objective of this study was to investigate some of the properties and kinetics of the reconstitution reaction of apo-tyrosinase with copper (II) ions.

Some of the properties examined were time, specificity of the active site for copper (II) ions, pH and temperature. The effects of time were thought to give information on the stability of both the native and apo-tyrosinase preparations over a period of time. These same stability experiments provided insight into the reproducibility of the reconstitution reaction over a period of time. The specificity of tyrosinase for copper (II) ions was demonstrated using metal substitution experiments. In these experiments, metals ions other than copper (II) were used in an attempt to reconstitute apo-tyrosinase. The third property investigated was the effect of pH on the reconstitution reaction. Since it is

known that varying the pH affects the action of enzymes, it was thought that varying the pH would affect the rate of reconstitution. The final property investigated was the effect of temperature on the reconstitution reaction. The temperature dependence of the reconstitution reaction led to the calculation of the activation parameters, $\Delta G^\ddagger$, $\Delta H^\ddagger$ and $\Delta S^\ddagger$, for this reaction.

The kinetics for the reconstitution reaction was also investigated for a number of reasons:

- 1) To measure the rate of copper insertion into the active site by determining the rate in which enzyme activity is recovered after the addition of copper to apo-tyrosinase; and
- 2) To determine the order of the reconstitution reaction overall with respect to copper and with respect to apo-tyrosinase. These experiments were completed by varying the concentration of copper and determining the rate of reconstitution.

Once the properties and kinetics were fully examined and documented, the mechanistic implications for the reconstitution reaction were discussed.

RESULTS AND DISCUSSION

Reconstitution Experiments

The objective of the reconstitution experiments was to investigate the effect of the removal of copper from the active site of tyrosinase as well as the effect of the subsequent addition of copper to the enzyme. The reconstitution experiments were similar to many reported in the literature (22,37,40).

Several sulfur-containing organic compounds can act as an inactivator of tyrosinase, presumably by binding to the copper in the active site (37,41,57). These compounds include: thiourea, thiouracil, or 2,3-dithiopropanol (BAL). There are also numerous complexing agents which are capable of removing the copper from the active site of tyrosinase (41). The non-reducing complexing agents include potassium cyanide or sodium thiosulfate. The reducing complexing agents include 8-hydroxyquinoline -5-sulfonic acid or picolinic acid.

It has been demonstrated in numerous past studies that cyanide can remove copper from the active site of tyrosinase and this resulted in a decrease in tyrosinase activity. Cyanide is probably the most common agent used for the removal of copper in tyrosinase (22,37,40). For these experiments, cyanide was chosen for the removal of

copper from the active site of tyrosinase because it has been used considerably and with great success in the literature.

The choice of cyanide as a complexing agent for copper is a logical one when applying the "hard and soft acids and bases" (HSAB) concept. Hard acids and bases tend to be small in size and only slightly polarizable, whereas the soft ligands tend to be larger in size and more polarizable. Theoretically, hard acids prefer hard bases and soft acids prefer soft bases. Copper (I) ions can be classified as soft acids and copper (II) ions, though harder, may also be considered as borderline soft acids (58). Cyanide is also a soft base and, therefore, it readily forms a complex with the copper ions in tyrosinase.

Two properties of cyanide for copper removal from tyrosinase are: 1) The copper ion is removed more linearly from tyrosinase over time than reducing complexing agents such as 2,2 bipyridyl; and 2) The copper removal from tyrosinase is essentially complete with cyanide, whereas with complexing agents, such as picolinic acid, the removal of copper is much less complete (41).

Activity of Tyrosinase and Copper Concentration

The apo-tyrosinase solution was prepared using the procedure described in the experimental section. The

amount of tyrosinase used was 3.6 mg in 0.6 mLs to give a final apo-tyrosinase concentration of 1.25×10^{-5} M in a 2.0 mL volume. The 2.0 mL solution was then separated into 0.1 mL volumes and placed in 0.4 mL centrifuge tubes. Using a 100 uL syringe, 10 uLs of copper sulfate were added to the apo-enzyme preparation and agitated with a vortex apparatus. The apo-tyrosinase - copper solution was allowed to incubate for at least 12 hours. The activity recovered was then determined using the dopachrome formation assay described in the experimental section.

Sample Calculation for Percent Activity Recovered:

Absorbance of Enzyme Preparations = $A(\text{observed}) - A(\text{Blank})$

The absorbance measurements were taken at 280 nm.

$$\frac{\text{Absorbance of Control Tyrosinase}}{\text{Absorbance of Apo-Tyrosinase}} = \text{Correction Factor for Concentration}$$

$$\frac{\text{A/min. of Reconstituted Enzyme}}{\text{A/min. of Control Tyrosinase}} = \text{Ratio of Activity Recovered with Respect to Control}$$

$$\% \text{ Activity Recovered} = \frac{\text{Ratio of Activity}}{\text{Activity}} \times \frac{\text{Correction Factor}}{\text{Factor}} \times 100\%$$

Example:

$$\text{Abs. of Control Tyrosinase} = 0.3540 - 0.0374 = 0.3166$$

$$\text{Abs. of Apo-tyrosinase} = 0.3430 - 0.0386 = 0.3044$$

$$\text{Correction Factor for Concentration} = \frac{0.3166}{0.3044} = 1.0401$$

$$\text{Ratio of activity Recovered} = \frac{0.2882}{0.3044} = 0.8150$$

$$\% \text{ Activity Recovered} = 0.8150 \times 1.0401 \times 100\% = 84.77\%$$

Results for the Reconstitution of Apo-tyrosinase with Copper

Table 1

Concentration of Copper (M)	% Activity Recovered	Standard Deviation
1.0 x 10 ⁻⁶	7.643	1.526
2.5 x 10 ⁻⁶	19.563	3.498
5.0 x 10 ⁻⁶	40.070	5.054
7.5 x 10 ⁻⁶	65.782	6.652
1.0 x 10 ⁻⁵	68.277	6.128
2.5 x 10 ⁻⁵	94.807	8.945
5.0 x 10 ⁻⁵	99.553	6.659
7.5 x 10 ⁻⁵	96.250	3.213
1.0 x 10 ⁻⁴	93.765	5.567
2.5 x 10 ⁻⁴	98.167	9.868
5.0 x 10 ⁻⁴	95.310	6.224
1.0 x 10 ⁻³	97.632	6.706
2.5 x 10 ⁻³	84.761	9.797
5.0 x 10 ⁻³	56.305	9.721
7.5 x 10 ⁻³	39.705	6.659
1.0 x 10 ⁻²	11.838	2.930

The data recorded in table 1 are average values from at least six trials. The results are plotted as percent activity recovered against copper concentration in figure 1.

Figure 1: Percent Activity Recovered vs.
Concentration of Copper in Tyrosinase

The percent activity recovered appears to increase linearly with increasing copper concentration when starting from low concentrations of copper. The percent activity reaches a maximum when approximately 5.0×10^{-5} M copper sulfate is added. This concentration of copper is not equal to the concentration of tyrosinase, which suggests that the copper required for complete recovery of activity must be in a 1:4 ratio of enzyme to copper. When 5.0×10^{-5} M copper sulfate is added, the maximum recovery of activity was reached or approximately 95-100% of the activity was recovered relative to the control tyrosinase. The maximum recovery of activity begins to fall at approximately 1.0×10^{-3} M copper sulfate. This suggests that tyrosinase is able to withstand a considerable excess of copper, or roughly 20-fold excess, without any significant loss of activity. If more than 40-fold excess was added to the apo-tyrosinase preparation, the percent activity recovered began to decrease significantly. At very high concentrations of copper sulfate, the copper precipitated.

A plot of percent activity vs percent copper required is presented in figure 2. This graph demonstrates a near linear relationship between the percent activity of tyrosinase and percent copper added to the apo-tyrosinase. A perfectly linear relationship would indicate that the copper ions follow an "All or None" type of binding mechanism. That is, both copper ions are incorporated in

Figure 2: Percent Activity Recovered
vs. Percent Copper Added

exist for any length of time except possibly as an intermediate. Therefore the population of partially reconstituted enzyme is made up of both apo and fully active tyrosinase (24,40).

The curve obtained in figure 2 is not perfectly linear and is slightly concave, which may indicate that the copper ions do not bind pair-wise in the active site. For example, each copper ion may bind at a different rate in the active site. This type of mechanism has been suggested previously by two research groups, Kertesz et al. (24) and Lerch et al. (40).

Although the mechanism for the reconstitution cannot be concluded from these results, the relationship between copper and tyrosinase activity appears to be close to the experimental results obtained by Kertesz (24). The results from the reconstitution experiments reinforce the essential requirement of copper in the active site for tyrosinase activity.

Enzyme Stability Experiments

The objective of the enzyme stability experiments was to investigate the effect of time on the reproducibility of enzyme activity experiments. During the reconstitution experiments, the enzyme preparations were often left to incubate for at least 12 hours. As well, the reconstitution experiments themselves took between six and 12 hours to complete. In some experimental trials, tyrosinase did not reconstitute completely and, as a result, only 85-90% of the activity was recovered from the apoenzyme. It was then questioned whether time had any effect on the activity of native tyrosinase and whether time had any effect on the reproducibility of the recovery of enzyme activity from apo-tyrosinase. It is thought that the loss of enzyme activity over time is due to denaturation of the protein and not due to the oxidation or loss of metal in the active site of tyrosinase (27).

Three different experiments were used to study the effects of time on the reconstitution of apo-tyrosinase. The first experiment was performed with the native tyrosinase preparation and the enzyme solution was prepared as described in the experimental section. The concentration of tyrosinase was 5.1 mg in 10 mLs of 0.1 M phosphate buffer at pH = 7. The enzyme preparation was separated into two 5.0 mL volumes. One volume was refrigerated, at 273-277 K and the second volume was

unrefrigerated at 293-297 K. Enzyme activity was measured for both enzyme preparations after predetermined lengths of time. The results for the first stability experiment on native tyrosinase can be found in table 2. These results were plotted as percent activity against time in figure 3 with the initial activity being assigned the value 100%.

Table 2 : The Percent Activity of Native Tyrosinase vs. the Time Stored at Two Temperatures.

Percent Activity		
Time (hours)	Refrigerated (273-277 K)	Unrefrigerated (293-297 K)

0	100.00	100.00
4	91.21	88.94
24	39.56	81.22
72	87.50	76.15
120	74.28	63.32
144	72.44	67.76

As expected, the enzyme preparation stored at room temperature lost activity at a somewhat faster rate than the refrigerated tyrosinase preparation. The unrefrigerated and refrigerated enzyme preparations had remarkably similar activities after 12 hours and it was only after 24 hours that a noticeable difference existed between the two enzyme preparations. Both tyrosinase preparations were still active after one week, the refrigerated tyrosinase having only four to five percent

Figure 3: Stability of Native Tyrosinase
vs. Time at Two Temperatures

more activity. In conclusion, it appears that the loss of activity of native tyrosinase was significant after 12 hours at room temperature or under refrigeration.

The second enzyme stability experiment was designed to determine if passing the native tyrosinase preparation through a sephadex column would have a different effect on the reproducibility of tyrosinase activity over time. The native tyrosinase solution was prepared as described in the experimental section with a concentration of 5.0 mg in 1.0 mL of 0.1 M phosphate buffer at pH = 7. The enzyme preparation was passed through a sephadex G 25-150 column equilibrated with the same phosphate buffer. The native enzyme solution was collected and diluted to give a final volume of 10 mLs. The enzyme preparation was separated into two 5.0 mL groups and, like the first stability experiment, one enzyme preparation was refrigerated at 273-277 K and the second enzyme preparation was unrefrigerated at 293-297 K. Activity was again measured after predetermined lengths of time and the results for this experiment can be found in table 3. These results were plotted as percent activity against time in figure 4 with the initial activity being assigned the value 100%.

Table 3 The Percent Activity of Native Tyrosinase After
Passing Through a Sephadex Column vs.
the Time Stored at Two Different Temperatures

Time (hours)	Percent Activity	
	Refrigerated (273-277 K)	Unrefrigerated (293-297 K)
0	100.00	100.00
6	98.00	81.32
12	95.10	63.12
24	91.90	36.20
48	85.18	32.30
72	93.08	16.51
96	89.28	12.50
168	67.27	1.00

As with the first stability experiment, the enzyme preparation stored at room temperature lost activity at a faster rate than the refrigerated tyrosinase preparation. After passing the tyrosinase through sephadex, however, the enzyme activity was lost at a faster rate than the preparatic not passed through sephadex, especially the unrefrigerated sample. There was no significant loss in enzyme activity after 12 hours for the refrigerated preparation, but the unrefrigerated tyrosinase preparation lost close to 40 percent of its original activity. After one week, the refrigerated enzyme preparation had more than 50 percent of its original activity, whereas the unrefrigerated preparation was essentially inactive.

In conclusion, the rate at which tyrosinase activity is lost is increased after passing the enzyme preparation

Figure 4:
Stability of Tyrosinase vs Time
After Passing through a Sephadex Column

through a sephadex column. The loss of activity is especially great for the unrefrigerated enzyme preparation. It seems likely that passing the tyrosinase solution through the sephadex results in a purification of the protein by the removal of any protein stabilizers. This may result in a subsequent greater loss of enzyme activity over time.

The objective of the final enzyme stability experiment was to determine the effect of time on the reproducibility of the reconstitution of apo-tyrosinase with copper. The apo-tyrosinase solution was prepared as described in the experimental section. The concentration of tyrosinase was 5.0 mg in 1.0 mL of 0.1 M phosphate buffer at pH = 7. The enzyme solution was incubated in 0.1 M KCN for 12 hours to remove the copper from the active site of tyrosinase. The enzyme-KCN preparation was then passed through a sephadex column to remove the copper-cyanide and any excess cyanide. The apo-tyrosinase preparation was collected and diluted to a final volume of 10 mLs with 0.1 M phosphate buffer at pH = 7. The enzyme preparation was then separated into 0.1 mL volumes with 0.4 mL polypropylene centrifuge tubes. As with the previous stability experiments, the apo-tyrosinase solution was separated into a refrigerated group and an unrefrigerated group. After a predetermined length of time, an excess of copper sulfate was added and the reconstituted enzyme-copper mixture was incubated for five

minutes. Activity was again measured for each group with the initial activity recovered being assigned the value of 100 percent. The results for this enzyme stability experiment can be found in table 4 and the percent activity recovered against time plot can be found in figure 5.

Table 4 : The Recovery of Tyrosinase Activity from Apo-Tyrosinase with Copper vs. Time for Two Different Temperatures.

Time (hours)	Percent Activity	
	Refrigerated (273-277 K)	Unrefrigerated (293-297 K)
0	100.00	100.00
1	100.21	93.23
2	101.65	89.85
4	91.99	61.90
6	85.89	55.02
9	85.87	40.79
10	82.63	31.30
12	72.24	31.15
24	65.76	12.72
48	47.43	2.55

The recovery of tyrosinase activity was affected by time. The longer the apo-tyrosinase preparation was left unreconstituted, the lower the percent recovery of activity. Therefore, the ideal situation would be to reconstitute the apo-tyrosinase immediately after its preparation. For the refrigerated enzyme preparation, this effect was significant because approximately ten percent was unrecovered after four hours. As with the previous two

Figure 5: Recoverable Activity of Tyrosinase
vs. Time at Two Different Temperatures

experiments, the loss of recoverable tyrosinase activity was increased greatly with time and, after only four hours, there was a significant decrease in recovered activity. The loss of recoverable tyrosinase activity would be particularly high if the enzyme preparation were unrefrigerated for more than one hour.

In summary, it appears that native enzyme activity was not significantly altered after 12 hours if the enzyme preparation was refrigerated. Secondly, the loss in recoverable enzyme activity from apo-tyrosinase over an eight-hour period was less than five percent if the preparation was refrigerated. It is concluded that the enzyme solution should be kept cold as much as possible for any reconstitution experiments. In future experiments, such as the activation studies, where the apo-tyrosinase is reconstituted above room temperature, the enzyme preparation will be refrigerated until it is required for the experiment.

Metal Substitution Experiments

The main objectives of metal substitution in metalloenzymes are :

- 1) To establish a measure of native metal specificity to the enzyme; and
- 2) To probe into the active site structure of the metalloenzyme.

The specificity of the native metal to the metalloenzyme can be measured by the ability to incorporate different metal ions into the active site. If the native metal is highly specific, then metal substitution may be extremely difficult, if not impossible. If the metal is not highly specific, then metal substitution may be possible with one or more different metals. An example of a metalloenzyme with a metal ion which is not highly specific is carboxypeptidase A, which has a zinc (II) ion in the active site. Cobalt has been substituted, *in vitro*, for the native metal. The cobalt substitution does not deactivate or change significantly the conformation of the active site of the native enzyme.

The role of the metal ion in metalloenzymes can be structural and/or catalytic. A metal ion can be successfully substituted for the native metal, where the metal can occupy the active site and satisfy the structural requirements of the enzyme. If the role of the metal is catalytic, however, the successful replacement of

the native metal with another metal can be much more difficult depending on the active site structure and the type of enzyme.

Successful metal substitution in the active sites of metalloenzymes can then be used as a probe into the active site structure. Various spectroscopic techniques can be used to study the properties of the native and metal-substituted active site. These techniques include: Nuclear Magnetic Resonance, Electron Paramagnetic Resonance and Ultraviolet - Visible spectroscopy.

In the case of tyrosinase, Ruegg and Lerch were successful in replacing copper in the active site with cobalt using $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$. The metal substitution inactivated the tyrosinase completely, which indicates that the copper ions are highly specific for tyrosinase. The successful replacement of copper with cobalt in tyrosinase provided useful information on the active site structure. It supported the idea that the copper binding sites in tyrosinase are slightly different because the cobalt ions in the metal-substituted tyrosinase could be removed differentially at high pH. Experimentally, this finding was unique, since half-apo derivatives had not been isolated in the copper-containing native tyrosinase (19).

The metal substitution experiments performed in the present study were different from past experiments because:

1) A wider range of metals were tried. In past experiments, only Zn, Co, Ni, Fe, Mn and Mg were tried.

2) A method similar to the one employed by Ruegg and Lerch for the successful replacement of copper with cobalt was used.

Past experiments used dialysis with KCN for 12-24 hours to remove the copper from the active site. The present study used a sephadex column which greatly shortened the length of time of the experiment. The metal salts used for the metal substitution experiments were:

1) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	10) $\text{Mn}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$
2) $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	11) $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$
3) $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	12) $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$
4) $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$	13) HgCl_2
5) $\text{Pb}(\text{NO}_3)_2$	14) TlCl
6) $\text{Al}(\text{NO}_3)_3$	15) CsCl
7) $\text{HAuCl}_4 \cdot \text{H}_2\text{O}$	16) TiCl_3
8) $\text{PdSO}_4 \cdot 2\text{H}_2\text{O}$	17) CuSO_4
9) AgNO_3	

In view of the findings by Lerner, the choice of metal salts for the metal substitution experiments was based on the properties of these salts. All of the metal salts were soluble in water. If possible, the metal salt chosen had an anion in solution which was either a sulfate

or a nitrate because Lerner demonstrated that these anions were the least inhibiting towards the native tyrosinase (52).

Three different experiments were used to study the effects of various metals on the native as well as the apo-enzyme for the purpose of metal substitution. The experiments were:

- 1) Direct metal substitution: addition of various metal ions to the native enzyme preparation;
- 2) Addition of copper complexing agent (KCN) and the subsequent addition of various metal ions; and,
- 3) Removal of copper from the active site using KCN and gel filtration followed by a subsequent addition of various metal ions.

In the experiment involving direct metal substitution, the native enzyme solution was prepared according to the method described in the experimental section. The enzyme solution was then separated into 0.1 ml. volumes in 0.4 ml. polyethylene centrifuge tubes. To the enzyme preparation, 10 μ L of the metal salt was added to give a ten-fold excess of metal salt to protein. The activity of the enzyme solution was then assayed using the dopachrome formation procedure. The results of this experiment are summarized in table 5.

From the results in table 5, it appears that a ten-fold excess of all of the metal salts tried have essentially no effect on the activity of the native

tyrosinase, either inhibitory or activating. Therefore, it seems highly unlikely that these metals are capable of directly substituting with copper in the active site. This is not a surprising result since the copper ions in the active site of tyrosinase are strongly bound to the protein. The next logical step, therefore, is the removal of the copper ions with a non-competitive inhibitor such as KCN, followed by the addition of the metal salts of interest.

Table 5 : The Activity of Tyrosinase in the Presence of Different Metal Ions for the Purpose of Direct Metal Substitution in Tyrosinase.

Metal Salt	Abs./min.	% Activity
ZnSO ₄ ·7H ₂ O	.8271 (.0180)	97.40 (2.12)
Pb(NO ₃) ₂	.7577 (.0276)	89.23 (3.25)
HAuCl ₄	.6356 (.0363)	74.85 (4.27)
HgCl ₂	.8778 (.0290)	103.37 (3.41)
CsCl	.8710 (.0245)	102.57 (2.89)
TiCl ₃	.8727 (.0299)	102.77 (3.52)
TlCl	.7983 (.0388)	94.01 (4.57)
Cd(NO ₃) ₂ ·4H ₂ O	.8090 (.0356)	95.27 (4.19)
Al(NO ₃) ₃	.8182 (.0272)	96.35 (3.20)
PdSO ₄ ·2H ₂ O	.8437 (.0226)	99.35 (2.66)
Mn(NO ₃) ₂ ·H ₂ O	.8586 (.0304)	101.11 (3.58)
AgNO ₃	.8149 (.0336)	95.96 (3.96)
Cr(NO ₃) ₃ ·9H ₂ O	.8360 (.0282)	98.45 (3.32)
Fe(NO ₃) ₃ ·9H ₂ O	.8593 (.0257)	101.19 (3.03)
Ni(NO ₃) ₂ ·6H ₂ O	.8320 (.0269)	97.97 (3.17)
Co(NO ₃) ₂ ·6H ₂ O	.8337 (.0516)	98.17 (6.08)
CuSO ₄	.8084 (.0217)	95.20 (2.56)
Native Tyrosinase	.8492 (.0284)	100.00 (3.34)

- Note: The bracketed numbers are standard deviations of the measured values.

In the second experiment, the effects of various metals on tyrosinase were investigated after the native

enzyme was incubated with KCN. It was postulated that the native copper ions could be substituted for other metals without the removal of the non-competitive inhibitor, KCN. This experiment would also supply information on which metals could reverse the inhibitory effects of cyanide. The native enzyme preparation was treated in the same manner as the native enzyme in the first experiment, except that cyanide was also added to the preparation as a 10 μ L aliquot to give a final concentration of 0.1 M KCN. The enzyme-KCN preparation was incubated for six hours at which time a ten-fold excess of one of the metal salts was added. The activity was then determined using the dopachrome assay procedure after a second six-hour incubation in the metal salts. The results for this experiment are summarized in table 6.

All of the metals investigated, except copper, were incapable of reversing the inhibition by KCN. Ultra-violet and visible scans performed on the various enzyme preparations indicated no change in the protein or active site structure as there was no change in spectrum after the various metal salts were added. Therefore, it seems likely that metal substitution with the metals investigated cannot occur without first removing the copper and excess cyanide and secondly, adding the various metal ions.

Table 6 : The Activity Recovered After the Addition of Various Metal Ions to the Apoenzyme-Cyanide Mixture.

Metal Salt	Abs./min	% Activity
ZnSO ₄ ·7H ₂ O	.0022 (.0020)	1.42 (1.29)
Pb(NO ₃) ₂	.0012 (.0011)	0.77 (0.71)
HAuCl ₄	.0016 (.0020)	1.03 (1.29)
HgCl ₂	.0012 (.0021)	0.71 (1.35)
CsCl	.0015 (.0010)	0.97 (0.64)
TiCl ₃	.0062 (.0009)	3.99 (0.58)
TlCl	.0022 (.0020)	1.42 (1.29)
Cd(NO ₃) ₂ ·4H ₂ O	.0007 (.0008)	0.45 (0.51)
Al(NO ₃) ₃	.0081 (.0105)	5.21 (6.76)
PdSO ₄ ·2H ₂ O	.0004 (.0003)	0.26 (0.19)
Mn(NO ₃) ₂ ·H ₂ O	.0004 (.0005)	0.26 (0.32)
AgNO ₃	.0008 (.0011)	0.51 (0.71)
Cr(NO ₃) ₃ ·9H ₂ O	.0189 (.0073)	12.16 (4.70)
Fe(NO ₃) ₃ ·9H ₂ O	.0116 (.0047)	7.46 (3.02)
Ni(NO ₃) ₂ ·6H ₂ O	.0152 (.0017)	9.78 (1.09)
Co(NO ₃) ₂ ·6H ₂ O	.0197 (.0116)	12.68 (7.46)
CuSO ₄	.1096 (.0137)	70.55 (8.82)
Native Tyrosinase	.1554 (.0057)	100.00 (3.67)

Note: The numbers in brackets are the standard deviations of the measured values.

In the third experiment, the apo-enzyme was prepared with KCN and then gel filtration was used to remove the copper-KCN and any excess cyanide. The native and apo-enzyme solutions were prepared in a manner similar to that used in the second experiment. After a 12-hour incubation in cyanide, the 0.5 mL. tyrosinase-KCN solution was loaded onto a sephadex column. Approximately 1.0 mL was collected and then diluted to 2.0 mL. with cold buffer. As with the previous experiments, the enzyme preparation was separated into 0.1 mL volumes and a ten-fold excess of the metal salts was added. The enzyme-metal preparation was then incubated for 12 hours and the activity of the enzyme

preparation assayed using the dopachrome formation procedure. The results for this experiment are summarized in table 7.

Table 7 : The Activity of Tyrosinase After the Removal of Copper Ions Followed by a Subsequent Addition of Various Metal Ions.

Metal Salt	Abs./min.	% Activity
ZnSO ₄ ·7H ₂ O	.0085 (.0009)	1.53 (0.16)
Pb(NO ₃) ₂	.0062 (.0014)	1.12 (0.25)
HAuCl ₄	.0044 (.0009)	0.79 (0.16)
HgCl ₂	.0166 (.0013)	2.99 (0.25)
CsCl	.0148 (.0045)	2.67 (0.81)
TiCl ₃	.0094 (.0047)	1.69 (0.85)
TlCl	.0110 (.0012)	1.98 (0.22)
Cd(NO ₃) ₂ ·4H ₂ O	.0038 (.0014)	0.69 (0.25)
Al(NO ₃) ₃	.0063 (.0035)	1.14 (0.63)
PdSO ₄ ·2H ₂ O	.0104 (.0019)	1.88 (0.34)
Mn(NO ₃) ₂ ·2H ₂ O	.0058 (.0007)	1.05 (0.13)
AgNO ₃	.0013 (.0010)	0.32 (0.18)
Cr(NO ₃) ₃ ·9H ₂ O	.0072 (.0004)	1.30 (0.07)
Fe(NO ₃) ₃ ·9H ₂ O	.0108 (.0037)	1.95 (0.67)
Ni(NO ₃) ₂ ·6H ₂ O	.0632 (.0039)	11.40 (0.70)
Co(NO ₃) ₂ ·6H ₂ O	.0521 (.0032)	9.39 (0.58)
CuSO ₄	.5123 (.0109)	92.37 (1.97)
Native tyrosinase	.5546 (.0127)	100.00 (2.29)
Apo-tyrosinase	.0291 (.0048)	5.24 (0.87)

Note: The numbers in brackets are standard deviations of the measured values.

The removal of copper ions and excess cyanide prior to the addition of the various metal ions did not result in the successful reconstitution of apo-tyrosinase with metals other than copper. All of the metals investigated were incapable of functionally replacing the copper ions in the active site as there was no recovery of enzyme activity. The ultra-violet and visible scans performed on each of the enzyme preparations also indicated no change

in the protein or active site structure since there were no changes in the spectra obtained after incubation of the apo-tyrosinase in the presence of the various metal ions.

To ensure that the loss of enzyme activity in the metal-substituted apo-tyrosinase preparation was not due to the presence of the various metal ions, copper was added to the metal-apoenzyme mixture. These results are summarized in table 8.

Table 8 : The Recovery of Activity of Tyrosinase After the Addition of Copper Ions Following the Addition of Various Metal Ions to the Apo-Tyrosinase.

Metal Salt	Abs./min.	% Activity
ZnSO ₄ ·7H ₂ O	.4037 (.0099)	72.79 (1.79)
Pb(NO ₃) ₂	.4076 (.0099)	73.49 (1.79)
HAuCl ₄	.0170 (.0041)	3.07 (0.74)
HgCl ₂	.0128 (.0042)	2.31 (0.76)
CsCl	.3845 (.0325)	69.33 (5.86)
TiCl ₃	.3305 (.0106)	59.59 (1.91)
TiCl	.4017 (.0240)	72.43 (4.33)
Cd(NO ₃) ₂ ·4H ₂ O	.1610 (.0053)	29.03 (0.96)
Al(NO ₃) ₃	.4217 (.0156)	76.04 (2.81)
PdSO ₄ ·2H ₂ O	.3568 (.0245)	64.33 (4.42)
Mn(NO ₃) ₂ ·2H ₂ O	.4887 (.0194)	88.12 (3.50)
AgNO ₃	.4862 (.0199)	87.67 (3.59)
Cr(NO ₃) ₃ ·9H ₂ O	.3739 (.0168)	67.42 (3.03)
Fe(NO ₃) ₃ ·9H ₂ O	.3523 (.0186)	63.52 (3.35)
Ni(NO ₃) ₂ ·6H ₂ O	.3038 (.0077)	54.78 (1.39)
Co(NO ₃) ₂ ·6H ₂ O	.3912 (.0081)	70.54 (1.46)
Native tyrosinase	.5546 (.0127)	100.00 (2.29)
Apo-tyrosinase	.0754 (.0081)	13.59 (1.07)

Note : Numbers in brackets are standard deviations of measured values.

Significant enzyme activity was recovered for all of the enzyme preparations except for mercury, gold, cadmium and palladium. Mercury and gold severely affected the reconstitution of apo-tyrosinase with copper ions. Cadmium

and palladium had a much smaller effect. This experimental result is consistent with previous experiments reported by Lerner (52).

In summary, a wide range of metals was used to investigate metal substitution in tyrosinase. One method used was similar to the method used for the successful replacement of copper with cobalt in tyrosinase by Lerch et al.(19). These observations provide further evidence of the high specificity of the active site in tyrosinase for copper ions.

Some insight as to why the active site in tyrosinase is so specific to copper ions can be gained by looking at the physical properties of the metals investigated for substitution. As stated earlier, the metal salts chosen for the present study were soluble in water and had an anion in solution which was either a sulfate or a nitrate since these were the least inhibitory to tyrosinase.

One of the most, if not the most, important property of metal ions for metal substitution in tyrosinase is the size or the ionic radius. The ionic radius of the cuprous ion is 0.96 angstroms whereas the ionic radius of the cupric ion is 0.72 angstroms. Some metal ions are clearly unsuitable for replacing copper in the active site due to their large size. For example, rubidium (+1) is probably too large to fit in the active site of tyrosinase because it has an ionic radius of 1.47 angstroms. Metal ions such as aluminum (+3) would probably be too small since the

ionic radius is only 0.51 angstroms. Many metals, like copper, can have more than one oxidation state. In some metals, such as thallium, one of the ionic species is close in size to copper, Tl (+1), and one is not, Tl (+3). Most metals have at least one ionic species similar in size to one of the copper ions, for example, Iron (+2), cobalt (+2), nickel (+2), cadmium (+2), and gold (+3). Only a small number of metals have all ionic species similar to the copper ions. They are: tin (+2,+4), zinc (+1,+2) and titanium (+1,+2,+3).

Another important property of metal ions for successful metal substitution is the existence of more than one oxidation state. The copper ions in the active site exist in both the cuprous and cupric states depending on which reaction tyrosinase is catalyzing. Metals such as silver which have ions of only one oxidation state are clearly not a suitable replacement for copper in tyrosinase. It may be possible for another metal to occupy the site of the native metal, however, with enzymes containing a metal ion for oxidation and/or reduction, it can be extremely difficult to recover enzyme activity. Since metal substitution is being attempted in an enzyme which is a monooxygenase, the reduction and oxidation of the copper is accompanied by the loss or gain of one electron from each of the two copper ions. Many metals have more than one ionic species, however, for many of the metals, oxidation and reduction is accompanied by the gain

of two electrons. This property makes metal such as tin unsuitable for metal substitution because two electrons are required to oxidize stannous ion to stannic ion.

Another property to consider, since tyrosinase is an oxidoreductase enzyme is the reduction potential. This property rules out almost all of the metals as suitable substitutes for copper in the active site. None of the metals investigated had a reduction potential similar to that of copper ions except tin. However, the reduction of stannic to stannous ions involves the transfer of two electrons rather than one which is the case for the cupric to cuprous ion.

The last property considered in this discussion is the geometry of the metal. The geometry of the copper ions in the active site of tyrosinase has been described as distorted tetragonal. For a metal to simply occupy the site of the native metal, geometry may not be very important. For example, Lerch et al. successfully substituted the copper in tyrosinase with cobalt. It was determined that the cobalt had a tetrahedral geometry, which is unlike the copper ions. The cobalt-substituted tyrosinase was also totally inactive, unlike the native copper-containing tyrosinase.

The Kinetics of Reconstitution

The procedure for determining the kinetics of the reconstitution of apo-tyrosinase with copper (II) ions begins with the tyrosinase activity assay. The assay used for all of the experiments was the dopachrome formation assay described in the experimental section. In this assay procedure, the substrate is L-Dihydroxyphenylalanine (L-Dopa) and the activity was measured by the increase in absorbance due to the formation of dopachrome at 475 nm. The rate-determining step of this assay is the oxidation of L-Dopa to Dopachromone and dopachrome. Therefore, the increase in absorbance at 475 nm corresponds to the rate of oxidation of L-Dopa and, since a large enough excess of substrate was used, the enzyme was working at a maximum rate or V_{max} . The rate can then be described as:

$$\text{Rate} = k_e [\text{enzyme}]$$

Consistent with this equation, the increase in absorbance at 475 nm was found to be proportional to time and the slope of this line is, therefore, proportional to k_e for a given enzyme concentration.

For the reconstitution experiments, the apo-enzyme is left in contact with copper for various lengths of time. The same assay was conducted after various lengths of time to determine the amount of tyrosinase activity recovered.

Experimentally, this was valid because the rate of reconstitution is slow with respect to the rate of L-Dopa oxidation. Also, when the 0.1 mL enzyme-copper preparation was placed in the 3 mL of substrate and buffer, further reconstitution was not significant because of the 30-fold dilution. The rate of recovery of the enzyme activity was measured relative to the native enzyme. That is, the rate of reconstitution is normalized to 100% and the native enzyme used to prepare the apo-tyrosinase was used as a control in each experiment.

At the beginning of the kinetic experiments, it was not obvious which type of kinetics would be observed. For example, the binding of the copper to the active site might be enzymatically catalyzed and, therefore, might follow enzyme kinetics. This was not likely, however, since the reconstitution reaction would appear to be a chemical reaction and, indeed, the kinetics actually observed turned out to be chemical kinetics.

The kinetic order with respect to apo-tyrosinase was considered most likely to be first order. This was tested by using a very large excess of copper to apo-tyrosinase ratio such that the rate of activity recovered or the reconstitution would not be affected by changing copper concentration during the course of the reconstitution reaction.

The kinetic order with respect to copper was much less obvious since there were a number of possibilities.

For example, if the rate of recovery of activity were dependent only on a conformation change prior to the insertion of copper into the active site, the order of the kinetics would be zero with respect to copper. The second possibility was a kinetic order which was first order with respect to copper and enzyme which would result in second order kinetics overall for the reconstitution reaction. This was expected since a second order rate constant for the reconstitution of apo-tyrosinase was reported by Kertesz et al. (40). The third possibility was a kinetic order which was second order with respect to copper and first order with respect to enzyme. This would result in an overall kinetic order of three for the reconstitution reaction. Third order kinetics might be expected since there are two copper atoms per active site in the enzyme.

The order of the reconstitution reaction with respect to copper was checked by varying the concentration of copper added to the apo-tyrosinase. The results of these kinetic experiments are summarized in table 8.1

At low copper concentrations, third order kinetics was observed since changes in the copper concentration resulted in marked changes in the recovery of activity in tyrosinase. At higher concentrations of copper, the order with respect to copper was less than two since the reaction was beginning to approach first order with respect to enzyme and pseudo first order kinetics overall.

Table 8.1: Summary of Kinetic Experiments with Various Copper Concentrations.

Excess Copper	Theoretical Rate Increase			Observed Rate Increase
	1st Order (x=0)	2nd Order (x=1)	3rd Order (x=2)	
2-fold	1.0	1.0	1.00	1.00
5-fold	1.0	2.5	6.25	5.36
10-fold	1.0	5.0	25.00	8.93
30-fold	1.0	15.0	225.00	12.57
100-fold	1.0	50.0	2500.00	21.37

note: Rate = $k [\text{apo}]^1 [\text{Cu(II)}]^x$

At very high concentrations of copper, the kinetic order with respect to copper was less than one and appeared to approach zero. However, establishing the order with respect to copper was difficult since the order was never exactly zero at very high copper concentrations. This was evident because, even at very high concentrations of copper, the rate of reconstitution was still somewhat dependent on the copper concentration.

Experimentally, it was possible to check pseudo first order kinetics by adding a large excess of copper ions to the apo-tyrosinase. When a large enough excess of copper (II) ions was used, the concentration of copper during the reconstitution reaction should not change significantly and, therefore, the rate of reconstitution would simply depend on the rate in which apo-tyrosinase was converted to reconstituted tyrosinase. For example:

Therefore, at time zero, the concentration of apo-tyrosinase is a_0 and the concentration of reconstituted tyrosinase would be zero. After the apo-tyrosinase has incubated in excess copper (II) ions for a predetermined length of time, the concentration of reconstituted tyrosinase is x and the concentration of apo-tyrosinase then becomes $a_0 - x$. For a first order reaction, the rate of reconstitution is:

$$\frac{dx}{dt} = k(a_0 - x) \quad \text{or} \quad \frac{dx}{(a_0 - x)} = k dt \quad (1)$$

Integration of (1) gives:

$$-\ln(a_0 - x) = kt + I$$

where I is a constant of integration. When A_0 is zero, time is zero and, therefore, $-\ln a_0 = I$, and

$$-\ln(a_0 - x) = kt + a_0$$

or

$$\ln \frac{a_0}{(a_0 - x)} = kt$$

Therefore, the first order equation can be evaluated and the first order rate constant k can be determined by plotting the relationship:

$$\ln \frac{a_0}{(a_0 - x)} \quad \text{vs.} \quad \text{time}$$

where the slope of this line is k , the first order rate constant.

An example of the pseudo first order calculation for the reconstitution of apo-tyrosinase with copper (II) ions can be found in table 9. The fraction of apo-tyrosinase at time zero is 1.0 and the fraction of reconstituted tyrosinase in the same reaction would, therefore, be zero. The temperature at which this data was recorded was 273 kelvin and the pH was 7.0.

Table 9: Summary of Calculations for the Reconstitution of Apo-Tyrosinase with Copper (II) Ions.

Time (sec)	Total Tyrosinase(A_0)	Reconstituted Tyrosinase (x)	$\ln \frac{a_0}{(a_0 - x)}$
0	1.0	0	0
20	1.0	0.3217	0.3882
30	1.0	0.3906	0.4953
40	1.0	0.5020	0.6972
50	1.0	0.6020	0.9213
60	1.0	0.6294	0.9926
75	1.0	0.6746	1.1227
90	1.0	0.7197	1.2719
120	1.0	0.7446	1.6160
300	1.0	0.8013	1.6160

The percent activity recovered $[(x) \times 100\%]$ vs. time was plotted in figure 6. The example shown in this figure was apo-tyrosinase at pH=7.0 and 273 kelvin. A ten-fold excess of copper to protein was used in this example.

Figure 6: Activity Recovered from Apo-Tyrosinase vs. Time

Temperature = 273 Kelvin

Figure 7: First Order Plot of Recovered Activity vs Time

Temperature= 273 Kelvin

The first order rate constant was determined by plotting the $\ln [a_0/(a_0-x)]$ vs. time and this plot can be found in figure 7. The near linearity of this plot in figure 7 indicates that the reaction is pseudo first order with respect to enzyme. Both graphs show that the rate at which copper binds to the active site of tyrosinase is fast. The rate of reconstitution at this pH and temperature was calculated to be $8.55 \times 10^{-3} \text{ sec}^{-1}$. The rate of reconstitution reported by Lerch et al. was $1.23 \times 10^{-4} \text{ sec}^{-1}$ which was much slower. However, it should be noted that the ionic strength and pH conditions in Lerch's experiments were much different than in the present study. Lerch also used a different source of enzyme which was isolated from *neurospora crassa*. The source of tyrosinase in the present study was isolated from *agaricus bispora* or the common mushroom.

The Effects of pH on Apo-Tyrosinase Reconstitution

The objective of the pH experiments was to gain a better understanding of the role of pH on the rate of reconstitution. The effects of pH on the rate of reconstitution was one property which has not been reported in the literature. It was hypothesized that differences in pH may account for the variation in the rate constant of apo-tyrosinase reconstitution with copper (II) ions reported in the literature. For example, Lerch reported a first order rate constant for the reconstitution of apo-tyrosinase with copper (II) ions of $1.23 \times 10^{-4} \text{ sec}^{-1}$ at pH=7.5 and 0.15M potassium phosphate (24). In the same study, Lerch reported a much higher rate of reconstitution using copper (I). The rate reported was $25 \times 10^{-3} \text{ sec}^{-1}$, however, this reconstitution reaction was performed at pH=5.8 using 1.5 M acetonitrile. Since the reconstitution experiments performed with different ions had much different rates of reconstitution, it was questioned whether pH played a role in this difference in the rate of reconstitution.

To study the effects of pH, apo-tyrosinase was prepared according to the procedure described in the experimental section. The amount of tyrosinase used was 3.6 mg. in 0.6 mL. of 50 mM phosphate buffer. The pH of the buffer was adjusted with 0.1 M sodium hydroxide to the desired pH. The native, apo and reconstituted enzyme

solutions were all prepared in the buffer of the desired pH. For these experiments, the pH of the buffer and protein solutions varied from 5.5 to 9.0. It was found that below pH=5.5 the enzyme denatured during the removal of copper from the apo-tyrosinase on the sephadex column. This was evident when the protein separated into two distinct bands, neither of which had any activity. Above pH=9.0, the recovery of tyrosinase activity was extremely slow and incomplete. When the apo-tyrosinase was prepared, it was loaded onto a sephadex column which was also equilibrated at the desired pH. Therefore, during the course of the reconstitution experiment, the pH was kept constant during all stages of the experiment. The apo-enzyme preparation was then collected and diluted to 2.0 mls using the same buffer and separated into 0.1 ml. volumes in polypropylene centrifuge tubes. A ten-fold excess of copper to protein was added to ensure pseudo first order kinetics was followed.

The rate of recovery of enzyme activity for each enzyme solution was determined by assaying the enzyme preparation after a desired length of time at pH=7.0. In this way, the removal and addition of copper to the tyrosinase was performed at the desired pH and the assay used to determine the recovery of enzyme activity was performed at pH=7.0 for each experiment.

Some of the results for the reconstitution of apo-tyrosinase under various pH conditions can be found in

figures 8 to 11. The results of all the reconstitution experiments at various pH conditions are summarized in table 9 and figure 12. The results of these experiments clearly show that pH has a pronounced effect on the rate of reconstitution of apo-tyrosinase with copper ions. The effects of pH on the rate of reconstitution are summarized as follows:

1) Acidic pH (pH=5.5)

The rate of reconstitution is lowered only slightly compared to the fastest recorded rate of reconstitution. It was found that lowering the pH further to 5.0 resulted in denaturation of the apo-enzyme. Therefore, it is thought that the rate is lowered slightly because of some protein denaturation. This is consistent with a study performed by Tripathi et al. which showed one form of tyrosinase at pH=5.2 to be less stable than tyrosinase under more neutral pH conditions (59).

2) Slightly Acidic pH (pH=6.0)

The highest rate of reconstitution was found at this pH. It should be noted that the rate of reconstitution is much higher than the rate found in more alkaline conditions (pH=6.5), but not much higher than the rate found in more acidic conditions (pH=5.5).

3) Neutral pH (pH=7.0)

The rate of reconstitution is much lower than at pH=6.0. The rate of reconstitution has decreased fairly steadily from pH=6.0.

Figure 8a: The Recovery of Tyrosinase Activity After the Addition of Copper vs Time at pH=5.5

Temperature- 273 Kelvin

Figure 8b: First Order Plot of the Recovery of Tyrosinase Activity vs. Time at pH=5.5

Temperature- 273 Kelvin

Figure 9a: The Recovery of Tyrosinase Activity
after the Addition of Copper vs.
Time at pH=6.5

Temperature= 273 Kelvin

Figure 9b: First Order Plot of the Recovery of
Tyrosinase Activity vs. Time at pH=6.5

Temperature= 273 Kelvin

Figure 10a: The Recovery of Tyrosinase Activity
after the Addition of Copper
vs. Time at pH=7.5

Temperature= 273 Kelvin

Figure 10b: First Order Plot of the Recovery of
Tyrosinase Activity vs. Time at
pH=7.5

Temperature= 273 Kelvin

Figure 11a: The Recovery of Tyrosinase Activity
after the Addition of Copper
vs. Time at pH=8.5

Temperature- 273 Kelvin

Figure 11b: First Order Plot of the Recovery of
Tyrosinase Activity vs. Time at
pH=8.5

Temperature- 273 Kelvin

Figure 12: pH vs. the Pseudo First Order
Rate Constant for the Reconstitution
Reaction of Tyrosinase

Temperature= 273 Kelvin

4) Basic pH (pH=7.5-9.0)

The rate of reconstitution decreases more gradually with increasing pH and appears to level off after pH=7.5. Because the rate of reconstitution seemed to level off, it seems reasonable to conclude that no further changes in protein conformation were occurring after pH=7.5.

The Variation of the Rate of Reconstitution and pH

A number of possible explanations were explored for assessing why the rate of reconstitution of apo-tyrosinase with copper (II) ions is affected by changes in pH.

Initially, it was questioned whether or not copper was completely removed from the active site of native tyrosinase with cyanide at all pH conditions. In order to assess this question experimentally, it was determined by graphite furnace atomic absorption that copper is completely removed from the active site of native tyrosinase in all pH conditions. These results are summarized in table 10.

Table 10: Percent Copper in Apo and Native Tyrosinase Preparations in Various pH Conditions.

Percent Copper		
pH	Apo-tyrosinase	Native Tyrosinase

6.0	0.0028	0.0480
6.5	0.0021	0.0521
7.5	0.0030	0.0494
8.5	0.0030	0.0476

The results in table 10 show that pH did not play a role in the removal of copper from the active site with cyanide. This is evident because at least 90% of the copper was removed from the active site of tyrosinase in all pH conditions. Therefore, pH did not play a role in changing the extent of copper removal from the active site with cyanide.

Secondly, it was questioned whether the higher pH conditions had any effect on the availability of the copper ions to the apo-tyrosinase. If this were the case, then possibly there would be less copper available for the apo-tyrosinase at higher pH conditions. For example:

If copper were precipitating in the higher pH conditions, then increasing the concentration of copper at the higher pH would not increase the rate of reconstitution; rather, more copper would precipitate. Experimentally, when a three-fold increase in copper concentration was tried at pH=7.0, an increase in the rate of reconstitution of 1.7 resulted. At pH=9.0 a similar three-fold increase in the copper concentration was added to the apo-tyrosinase and the rate of reconstitution increased by 2.0, an even higher factor. These results suggest that the copper available to tyrosinase is not greatly affected by pH and

the possible precipitation of copper in higher pH conditions is not a major factor.

A third possibility for the differences seen in the rate of reconstitution at different pH conditions is that the amino groups in the enzyme may be binding the copper and also decrease the amount of copper available in higher pH conditions. This might have been expected since, in higher pH conditions, there would be more electro-negatively charged amino groups on the protein. Experimentally, this is unlikely since the reconstitution experiments were all performed in a large excess of copper to protein. Also, adding additional copper to the apo-tyrosinase in different pH conditions resulted in similar changes in the rate of reconstitution.

Fourthly, the fastest rates of reconstitution were found near pH=6.0. There are four histidine groups in each active site which have nitrogens capable of binding the copper ions in the active site. The pKa of histidine is 6.0 and probably a little higher when it is bound to a protein structure.

Experimentally, this may be a coincidence, since it would be expected that, in higher pH conditions, the histidine group would be more negatively charged which could then be expected to encourage the binding of copper (II) ions in the active site. This effect is not seen since, as the protein proceeds to more basic conditions, the rate of reconstitution decreases.

Conformational Changes:

It is possible that the differences in the rate of reconstitution in different pH conditions are related to conformational changes in the protein. If this were the case, then the ideal conformation is at pH=6.0 or lower. In higher pH conditions, the active site and/or the protein structure would be less suitable for insertion of the copper ion into the active site. If conformational changes can account for the differences in the rate of reconstitution under different pH conditions, then this might suggest that the process of reconstitution is not dependent on copper concentration, except at lower copper concentrations when pseudo first order kinetics is not being observed. The process of reconstitution would then greatly depend on protein conformation.

Before any conclusions can be drawn about conformational changes during the reconstitution process, other properties such as the activation parameters will be examined to give evidence either for or against this idea.

Activation Parameters

The main objective of studying temperature dependence on the rate of reconstitution was to calculate the activation parameters of metal incorporation into the active site. The process of reconstitution in tyrosinase is strongly temperature dependent. This temperature dependence has been demonstrated by two groups in the literature. Both Kertesz et al.(40) and Solano et al.(45) have calculated the activation energy for the reconstitution of apo-tyrosinase. Using Arrhenius plots, the activation energy for the reconstitution of apo-tyrosinase was calculated by both groups to be 20.23 Kcal/mole or 87.19 KJ/mole. Neither group reported any other activation parameters.

To study the effects of temperature, apo-tyrosinase was prepared according to the procedure described in the experimental section. The amount of tyrosinase used was 3.6 mg. in 0.6 ml. of 50 mM phosphate buffer. The pH was 8.2 for all of the temperature experiments because, in lower pH conditions, the rate of reconstitution was extremely fast and, therefore, it was impossible to determine the rate of reconstitution at temperatures higher than 278 kelvin. The temperatures used for the activation experiments ranged from 278 to 303 kelvin. For these reconstitution experiments, the apo-tyrosinase preparation was refrigerated until needed. Before the

reconstitution reaction, the copper sulfate, apoenzyme and syringes were equilibrated to the desired temperature. The buffer and substrate for the assay procedure were all kept at a constant temperature of 293 kelvin. In this way, the reconstitution of apo-tyrosinase was performed at one specific temperature and the activity recovered for each temperature was measured at 273 kelvin. Therefore, any differences in temperature of the different apoenzyme preparations would not affect the measurement of the assay.

As with previous reconstitution experiments, the apo-tyrosinase was loaded onto a sephadex column to separate the metal and excess cyanide from the apo-tyrosinase. Approximately one ml. was collected and diluted to 2.0 mls with the phosphate buffer. The enzyme preparation was then separated into 0.1 ml volumes using polypropylene centrifuge tubes. A ten-fold excess of copper to protein was added such that the rate of reconstitution would give pseudo first order kinetics.

A sample of the results for the reconstitution of apo-tyrosinase in various temperatures can be found in figures 13 to 16. The results of all the reconstitution experiments at various temperatures are summarized in table 11 and figure 17.

Resolution test chart showing patterns of vertical and horizontal lines with numerical values: 1.0, 1.1, 1.25, 1.4, 1.6, 1.8, 2.0, 2.2, 2.5, 2.8, 3.2, 3.6, 4.0, 4.5, 5.0, 5.6, 6.3, 7.1, 8.0, 9.0, 10.0, 11.2, 12.5, 14.0, 16.0, 18.0, 20.0, 22.5, 25.0, 28.0, 32.0, 36.0, 40.0, 45.0, 50.0, 56.0, 63.0, 71.0, 80.0, 90.0, 100.0.

Figure 13a: The Recovery of Tyrosinase Activity
after the Addition of Copper vs.
Time at 278 Kelvin

Figure 13b: First Order Plot of the Recovery of
Tyrosinase Activity vs. Time at
278 Kelvin

Figure 14a: The Recovery of Tyrosinase Activity
after the Addition of Copper vs. Time
at 283 Kelvin

Figure 14b: First Order Plot of the Recovery of
Tyrosinase Activity vs. Time at
283 Kelvin

Figure 15a: The Recovery of Tyrosinase Activity after the Addition of Copper vs. Time at 293 Kelvin

Figure 15b: First Order Plot of the Recovery of Tyrosinase Activity vs. Time at 293 Kelvin

Figure 16a: The Recovery of Tyrosinase Activity after the Addition of Copper vs. Time at 303 Kelvin

Figure 16b: First Order Plot of the Recovery of Tyrosinase Activity vs. Time at 303 Kelvin

Table 11: Summary of the Effects of Different Temperatures on the Rate of Reconstitution of Tyrosinase

Temperature (K)	Rate of Reconstitution(sec ⁻¹)
278	0.001321
283	0.002652
288	0.004958
293	0.011034
298	0.019861
303	0.029317

Figure 17: Rate of Reconstitution of Tyrosinase vs Temperature

Calculation of Activation Parameters

In order to calculate the activation parameters, transition state theory will be used rather than the Arrhenius plots which have been used in the past. For the reaction:

$$\ln k_1 = \ln \frac{kT}{h} - \frac{\Delta G^\ddagger}{RT}$$

where k is the Boltzman constant, $1.381 \times 10^{-23} \text{ J K}^{-1}$, h is Planck's constant, $6.626 \times 10^{-34} \text{ J s}$, R is the gas constant, $8.314 \text{ J K}^{-1} \text{ mol}^{-1} \text{ dm}^3$ and T is the temperature. But,

$$\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$$

therefore,

$$\ln k_1 = \ln \frac{kT}{h} - \frac{\Delta H^\ddagger}{RT} + \frac{\Delta S^\ddagger}{R}$$

and

$$\ln \frac{k_1}{T} = \ln \frac{k}{h} - \frac{\Delta H^\ddagger}{RT} + \frac{\Delta S^\ddagger}{R}$$

A plot of $\ln k_1 / T$ vs. $1/T$ will then have a slope of $-\Delta H^\ddagger / R$. Using this real plot, the enthalpy of activation

can be calculated. The results for which these calculations were derived can be found in table 12 and the plot of $\ln k_1/T$ vs. $1/T$ is found in figure 18. The slope, $-\Delta H^\ddagger/R$, of the curve in figure 18 was calculated to be -10210.2 K^{-1} . Therefore, the enthalpy of activation for the reconstitution of apo-tyrosinase with copper (II) ions is calculated to be 84.64 KJ/mole . The Gibbs free energy of activation can also be calculated in a similar manner using the relationship:

$$\ln \frac{k_1}{T} = \ln \frac{k}{h} - \frac{\Delta H^\ddagger}{RT} + \frac{\Delta S^\ddagger}{R}$$

and since $\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$

$$\ln \frac{k_1}{T} = \ln \frac{k}{h} - \frac{\Delta G^\ddagger}{RT} \quad (10)$$

At any given temperature, the rate of reconstitution is known, therefore, equation (10) can easily be solved to give a value for the Gibbs free energy of activation. The Gibbs free energy of activation for the reconstitution of apo-tyrosinase with copper (II) ions was calculated to be 81.71 KJ/mole . The entropy of activation can then be calculated since both the enthalpy and free energy of activation are known. The entropy of activation for the reconstitution of apo-tyrosinase with copper (II) ions was calculated to be $+0.0047 \text{ KJ mol}^{-1} \text{ K}^{-1}$. The mechanistic

Table 12: Summary of Calculations for the Transition State Plot

Temperature (K)	$1/T$ ($\times 10^3$)	k (sec $^{-1}$)	k/T ($\times 10^5$)	$-\ln k/T$
278	3.595	0.00132	0.475	12.258
283	3.533	0.00265	0.936	11.578
288	3.470	0.00496	1.721	10.970
293	3.411	0.01103	3.764	10.187
298	3.335	0.01986	6.666	9.617
303	3.298	0.02932	9.670	9.244

Figure 18: Plot of $-\ln k/\text{Temperature}$ vs $1/\text{Temperature}$

implications of the pH effects and the activation parameters will be discussed in the following section.

Mechanistic Implications

At the outset of this work, the mechanism for the reconstitution reaction of apo-tyrosinase with copper was not a major consideration. It was only after the pH experiments were performed that the mechanism was questioned. The pH experiments demonstrated that the rate of reconstitution of tyrosinase was greatly affected by pH. Several explanations were considered for the dependence of the rate of reconstitution on pH. Copper analysis revealed that pH did not change the extent of copper removal from the active site using cyanide. Various pH conditions did not affect the availability of copper to the active site since varying the copper concentration in different pH conditions gave similar changes in the rate of reconstitution.

The most likely explanation for the pH dependence of the reconstitution reaction may be conformation changes in the protein, in the active site, or in both. It was only at this stage that the mechanism for the reconstitution reaction was in question because, if conformation changes can account for the differences in the rate of reconstitution, then it was postulated that the rate of reconstitution is dependent on the rate of change of enzyme conformation. Therefore, the rate of conformation change may be the rate-determining step in the reconstitution of apo-tyrosinase with copper, as opposed

to simple chemical kinetics where the rate is dependent only on the rate of copper binding to the active site.

Conformation can be defined as "the three dimensional arrangement of atoms in a molecule" (62). The three dimensional arrangement is maintained by a number of interactions which include:

- 1) hydrogen bonding,
- 2) electrostatic forces, and
- 3) van der Waals forces.

Conformation changes can be described as any change in molecular geometry which does not involve the breaking and remaking of primary bonds (63). Subtle conformation changes are termed as such because their detection and physical measurement is difficult. An example of a subtle conformation change is the swelling of a protein in solution. Conformation change is applied to a process in which one or more observable physical properties is so large it suggests that the alteration in the protein involves more than a few amino acid residues in an isolated area (64). Entropy is as convenient as any physical property and is an attractive measure of conformation change because folding and unfolding processes are accompanied by large changes in entropy. Conformation changes in proteins can arise from variations in ionic strength, pressure, temperature and pH. Even subtle conformation changes are accompanied by changes in entropy.

Increases in entropy are due to increased motion in the protein such as unfolding of the protein and decreases in entropy are a result of folding to a more efficient, compact, or dense form of the protein. In general, the better folded a protein, the lower the entropy of that protein in solution. These properties make entropy a reasonably attractive parameter as an index of the integrity of the folding.

The activation parameters for the reconstitution of apo-tyrosinase with copper (II) ions were calculated to be:

Enthalpy of Activation = 84.64 KJ/mole

Gibbs Free Energy of Activation = 81.71 KJ/mole

Entropy of Activation = 0 KJ/mole

Because of the lack of entropy change during the reconstitution reaction, it does not appear that a significant conformation change is occurring in tyrosinase. Although there was no entropy change detected during the reconstitution reaction, this does not rule out the possibility of the enzyme unfolding (gain of entropy) and then refolding (loss of entropy) during the incorporation of the copper in the active site. These processes are extremely difficult to detect because the overall changes may be extremely small. The net result in the case of the reconstitution reaction may ultimately be zero entropy. Other possibilities can include any number

of combinations from the following list:

Positive Entropy	Negative Entropy
- Folding.	- Unfolding.
- Loss of water surrounding the cupric ion in an aqueous buffer.	- Incorporation of the cupric ion in the active site.

There is no doubt that a conformation change is occurring in tyrosinase over the pH range in which the reconstitution experiments were performed. In a recent paper by Tripathi et al., two forms of tyrosinase were found in human skin. One form (or conformation) existed at pH=5.2 and the other conformation existed at pH=6.8 (59). These two forms are shown to be interconvertable by appropriate gradual change in pH by dialysis. Rapid changes in pH from 6.8 to 5.2 resulted in an irreversibly modified form of tyrosinase, whereas a gradual decrease in pH resulted in a completely reversible form. The less acidic form appeared to be a more stable form. It was concluded that the two forms of tyrosinase differ in conformation and it is this conformation change that is responsible for the increased stability at pH=6.8. The results from this paper are in agreement with the results obtained from the pH experiments. For example, if we look at the graph of K vs pH (figure 12), this type of curve may suggest that there are two possible conformations of tyrosinase and that the conformation which exists at or

below pH=6.0 is better suited for incorporating copper(II) ions in the active site than the conformation which exists above pH=6.0. As with Tripathi's results, it was found that at pH conditions of 5.0 the enzyme solution was very unstable and, as a result, the reconstitution of tyrosinase with copper(II) ions was impossible. For example, when the apo-tyrosinase preparation was loaded onto the Sephadex column, a number of bands of protein were eluted and the apo-tyrosinase collected from the column failed to reconstitute with copper. Therefore, it was decided that removal of the metal under these experimental conditions resulted in denaturation of the protein.

In conclusion, it appears that there is indeed a conformation change occurring when tyrosinase is present in different pH conditions. It was questioned whether the rate of reconstitution was actually a measure of the rate of conformation change. This does not appear likely because, if this were the case, then the greatest conformation change would be expected to occur under pH conditions where the rate of reconstitution was the slowest. Under these pH conditions, the entropy of activation would also be quite large, indicating a conformation change. This was not observed, however, and it appears that any change in conformation arises from variations in pH and not incorporation of copper in the active site.

It is, therefore, concluded that the rate of reconstitution is a chemical process dependent only on the rate in which copper binds to the active site. Further proof for this hypothesis is found in the experiments where the copper concentration was varied because it was observed that the rate of reconstitution always depended to some degree on the concentration of copper and not the enzyme concentration alone. Kinetic experiments, which were performed to establish the order of the reconstitution reaction with respect to copper, revealed the dependence of the rate of reconstitution on the copper even at high concentrations. At low copper concentrations, the order appeared to be third order overall and second order with respect to copper. At higher copper concentrations, the kinetics observed for the reconstitution reaction was pseudo first order. The order with respect to enzyme was between first order and an order of less than two with respect to copper. Even at very high concentrations of copper, however, the rate of reconstitution depended to some degree on the concentration of copper because the order with respect to copper did not reach zero. Establishing the order with respect to copper is clearly difficult. A conclusive order with respect to copper was never obtained from the kinetic experiments, however, the reaction appears to be approaching zero order with respect to copper at high copper concentrations. This rate dependence on copper

that the rate of reconstitution is dependent on copper rather than on conformation changes.

Possible Physiological Implications

The rate of reconstitution is very sensitive to small changes in pH, especially when the enzyme is in an environment when the pH is between 6 and 7.5, the latter of which is close to "cellular pH". Since the apo-tyrosinase exists in the melanocyte as well as native or reconstituted tyrosinase, it can be suggested that one possible way in which the melanocyte can produce tyrosinase is through changes in the cellular pH. In this way, a supply of tyrosinase can be utilized when needed simply by a small change in pH which in turn would result in a more favorable conformation for increased production of native tyrosinase from the "dormant" supply of apo-tyrosinase. In this way, the supply of tyrosinase can be switched on and off whenever needed. This does not suggest that changes in melanocyte pH is the only factor responsible for the mobilization of active tyrosinase in the same manner that a light switch is not the only factor responsible for turning on a light bulb.

EXPERIMENTAL

Reagents and Solutions

TYROSINASE

Tyrosinase was obtained from the Sigma Chemical Company as a dry powder. Solutions were freshly prepared by dissolving the desired amount of enzyme in cold buffer.

BUFFER SOLUTIONS

- 1) Tris-HCl Buffer: analytical grade tris-hydroxymethylamine (BDH Chemicals) was dissolved in the desired amount of water. The pH was adjusted using analytical grade hydrochloric acid (0.2M).
- 2) Phosphate Buffer: analytical grade sodium phosphate, monobasic, monohydrate (J.T. Baker Chemical Company), was dissolved in the desired amount of water. The pH of the buffer was adjusted using sodium hydroxide (0.2M). All buffers were passed through a chelex column to remove any trace metals and primarily copper.

WATER

All solutions were prepared using distilled water which was further purified using a millipore ion exchange apparatus to deionize the water. The level of copper in the purified water was undetectable using graphite furnace atomic absorption.

GLASSWARE

All glassware used for solution preparation or storage was cleaned by soaking in 35% nitric acid for at least 24 hours. The glassware was then rinsed thoroughly with distilled deionized water.

METAL SOLUTIONS

All metal solutions were prepared by dissolving the desired amount of the salt in water. Titanium chloride was prepared by weighing the desired amount of salt in a nitrogen-filled atmosphere bag and dissolved in water. The metal salts used were: copper (II) sulfate, titanium (III) chloride, cesium (I) chloride, thallium (I) chloride, mercury (II) chloride, cadmium (II) nitrate, chromium (II) nitrate, manganese (II) nitrate, silver (I) nitrate, palladium (II) sulfate, hexachlorohydroaurate, aluminum (III) nitrate, lead (II) nitrate, iron (III) nitrate, nickel (III) nitrate, cobalt (II) nitrate and zinc (II) nitrate.

CYANIDE SOLUTIONS

Analytical grade potassium cyanide, (Sigma Chemical Company), was weighed into a five mL sterile, polystyrene test tube and dissolved in distilled water to make a 0.1M solution when mixed with the tyrosinase solution. Any unused cyanide was disposed of by adding the cyanide to an alkaline solution of calcium hypochlorite.

An excess of sodium hydroxide and calcium hypochlorite was maintained while the solution was left for 24 hours. The solution could then be flushed down the drain with copious amounts of water.

CHELEX COLUMN

The chelex column was prepared using analytical grade chelex-100 (Bio-Rad Laboratories). The chelex-100 (50-100 mesh, sodium form) was suspended directly in a chosen buffer and the slurry was used to pack the column. The eluant was collected after 250 mLs was passed through the column. Over 20 L of buffer could be purified through the 120 mL (220mm x 25mm) column. The column could easily be regenerated by adding two bed volumes of hydrochloric acid (1N) and then five bed volumes of water followed by five bed volumes of sodium hydroxide (1N) and finally, five bed volumes of water. The regeneration was performed outside the column because the chelex undergoes volume changes when its ionic form is altered.

SEPHADEX COLUMN

The gel filtration column was prepared using Sephadex G 25-150, (Sigma Chemicals). The sephadex column was prepared by suspending the sephadex in distilled water at 90 C for five hours or at room temperature for 72 hours, to allow for swelling of the beads. The slurry was then cooled, if necessary, and poured into a seven mL (180mm x

0.7mm) column. The sephadex could be cleaned with two bed volumes of sodium hydroxide (0.2N) and at least five bed volumes of distilled water. The sephadex was cleaned after each run to remove any proteins and low molecular weight impurities remaining on the column.

Experimental Methods

PREPARATION OF APO-TYROSINASE

The desired amount of tyrosinase was weighed into a five mL, sterile, polystyrene test tube. The native enzyme was then dissolved in 0.6 mL of cold tris-HCl buffer. Once dissolved, 0.1 mL of the native enzyme solution was extracted into another test tube and later diluted with cold buffer to 1.0 mL and then used as a control. The remaining 0.5 mLs of enzyme solution was then incubated in potassium cyanide solution (0.1M) for at least 12 hours.

Following the incubation, the enzyme-KCN solution was loaded onto a sephadex column which has been equilibrated with the appropriate tris-HCl buffer. Passing the enzyme-KCN solution through the column removes both the copper from the native tyrosinase as $\text{Cu}(\text{CN})_2$ and any excess cyanide. Approximately one mL of apo-enzyme solution was collected from the column into a five mL volumetric flask and brought up to volume with the appropriate Tris-HCl buffer. The activity and concentration of the apo-tyrosinase solution can then be compared to the control or

the native tyrosinase solution prepared earlier.

RECONSTITUTION OF APO-TYROSINASE

The five mL solution of apo-tyrosinase was separated into 0.1 mL volumes with a 100 uL syringe. The 0.1 mL solution was placed in 0.4 mL polypropylene centrifuge tubes. A 10 uL volume of cupric sulfate or other metal solution was then added. The centrifuge tube was capped and the solution agitated for five seconds with a vortex mixer. The solution was then allowed to incubate for the desired length of time at the desired temperature.

COPPER ANALYSIS

Two procedures were used for the analysis of copper in the native and apo-tyrosinase solutions. Method I was used for the initial copper analysis following the early apo-tyrosinase reconstitution experiments with varying amounts of copper. Method II was used following the rate of reconstitution vs. pH experiments.

Copper Analysis (Method I)

Native tyrosinase (5.0mg) was weighed into a ten mL volumetric flask and dissolved with tris-HCl buffer (50mM, pH=7.0). After dissolving the enzyme, the solution was brought to volume using the tris-HCl buffer. The appropriate standards were prepared from a stock solution of 1000 ug/ml copper in 0.1% nitric acid. Serial dilutions

from this solution were freshly made for the analysis. The method of standard addition was used for the analysis. The enzyme-copper solutions were prepared with 100 μL of enzyme, 10 μL of copper standard, and diluted with the appropriate amount of water to 1.0 mL. Using an Eppendorff pipette, 10 μL of the copper-enzyme solution was introduced into the graphite furnace for analysis. The stages for the heating of the graphite tube were:

- 1) Drying Stage: 90°C for 90 seconds.
- 2) Charring Stage: 1000°C for 30 seconds.
- 3) Atomization: 2800°C for 5 seconds.

The equipment used for the initial copper analysis was:

- a) Perkin-Elmer HGA 76B programmer furnace.
- b) Locally manufactured detection system.
- c) Modulated lamp supply of 285 Hz, with a deuterium arc background correction.
- d) Varian Techtron AAG monochromator, wavelength = 324.5nm.
- e) Hollow cathode lamp.
- f) Argon purge.
- g) Signals were recorded with a Nicolet 4094 digital storage oscilloscope.

COPPER ANALYSIS (Method II)

The procedure for the second copper analysis was

modified and the equipment was upgraded for a superior analysis of both native and apo-tyrosinase samples. In this procedure, native and apo-tyrosinase solutions were prepared in the procedure outlined previously. The appropriate standards were prepared from a stock solution of 1000 ug/ml copper in 0.1% nitric acid. Serial dilutions from this solution were freshly made for the analysis. The method of standard additions was again used for the analysis, except the enzyme and copper solutions were kept separate for the analysis. Using an Eppendorff pipette, 10 uL of the enzyme solution was placed onto a platform inside the graphite furnace and dried for 45 seconds at 90 C. Once dried, 10 uL of the appropriate copper standard was placed on top of the enzyme inside the furnace. The stages for heating the furnace were:

Stage	Temperature (°C)	Time (s)	Ramp Time (s)
Drying	90	45	10
	120	6	2
Charring	350	20	10
	1200	30	10
Atomization	2800	6	0

The equipment used for the second copper analysis was:

- Perkin-Elmer HGA 500 programmable furnace
- Perkin-Elmer 5000 atomic absorption spectrophotometer with Zeeman effect background correction.
- Hollow cathode lamp (324.5nm).

- d) Data was recorded using a Perkin-Elmer HGA graphics package on the P.E. 7500 professional computer.
- e) New pyrolytically-coated graphite tube with platform.
- f) Nitrogen purge.

SPECTRAL STUDIES

All time drives were performed in a Beckman DU-7 u.v.-visible spectrophotometer. The scans were done with a 3 mL quartz cuvette at a wavelength of 475 nm at a rate of 60 absorbance readings per minute for three minutes. The relative concentration of tyrosinase was determined by measuring the absorbance at a 280 nm.

ASSAY PROCEDURES

A number of assay procedures for tyrosinase have appeared in the literature (53,54,55,56). Because tyrosinase is thought to be the only enzyme involved in the biosynthetic pathway of melanin formation, it directly or indirectly catalyzes the formation of more than one product. As a result, it is not surprising that a number of spectrophotometric assay procedures for tyrosinase have been developed. To date, spectrophotometric procedures have been the most widely used method for determining tyrosinase activity. As well as spectrophotometric methods, radiometric methods have also been developed based on tritiated water liberated from labeled tyrosine

or the tritium released from radioactively labeled 3,4 dihydroxyphenylalanine (L-DOPA) when leucodopachrome is formed (20). An electrometric method has also been developed using a potentiometric set-up to monitor changes in the hydrogen ion concentration liberated from L-Dopa when leucodopachrome is formed (54).

Four spectrophotometric assay procedures were investigated for the purpose of finding a simple reliable method for determining tyrosinase activity. These four assays are:

- 1) Tyrosinase activity,
- 2) Polyphenoloxidase activity,
- 3) Catechol oxidase activity, and
- 4) Dopachrome formation.

The first three assay procedures were provided by the Sigma Chemical Company and the dopachrome formation procedure was found from numerous papers in the literature (24,40,61).

TYROSINASE ACTIVITY

The principle of this assay is:

This assay procedure requires tyrosine dissolved in distilled water as a substrate. The reaction is performed in a three mL quartz cuvette with 1.0 mL of phosphate buffer (50mM, pH=6.5), 0.9 mLs of water, 1.0 mL of

substrate (10mM) and 0.1 mL of enzyme solution (500-1000 units/mL). The enzyme activity is measured by an increase in absorbance at 280 nm with the formation of dopaquinone.

The catalysis of tyrosine to L-Dopa and subsequently to dopaquinone is characterized by a lag period that occurs in the tyrosine-hydroxylating activity of tyrosinase (42). Numerous experimental conditions such as: pH, substrate concentration or enzyme concentration can affect the length of this characteristic lag period (53). Certain chemical agents such as ascorbate can shorten the lag period, but L-Dopa is the most effective in eliminating it. This assay procedure was not used because the characteristic lag was found to be lengthened after the reconstitution of apo-tyrosinase was completed. It was felt that the initial measurements of activity were the most important in the case where apo-tyrosinase was reconstituted with an excess of copper. Therefore, the presence of the characteristic lag was undesirable. The characteristic lag could have been eliminated using a catalytic amount of L-Dopa, but the simpler dopachrome formation assay procedure was chosen instead.

POLYPHENOLOXIDASE ACTIVITY

The principle of this procedure is:

This assay procedure requires L-dopa as a substrate and the buffer is potassium phosphate (50mM, pH=6.5). The procedure also requires 2.8 mLs of buffer containing 2.1 mM ascorbic acid and EDTA (0.065mM), 0.1 mL of L-dopa (10mM) and 0.1 mL of tyrosinase (500-1000 units/mL). The enzyme activity is determined by coupling ascorbic acid oxidation with dopaquinone production. The activity is measured by the decrease in ascorbic acid concentration which had a corresponding decrease in absorbance at 265 nm.

This assay procedure requires EDTA in the procedure, which made it unsuitable for reconstitution experiments with copper or other metals.

CATECHOL OXIDASE ACTIVITY

The principle of this assay is:

This assay requires 0.1 mL of 5.0 mM catechol as the substrate. The buffer used was potassium phosphate (50 mM, pH=6.5). The substrate, ascorbic acid (2.1 mM) and EDTA (0.065mM), are all dissolved in the buffer. As with the polyphenoloxidase assay, the three mL cuvette contained 2.6 mLs of buffer, 0.1 mL ascorbic acid,

0.1 mL of EDTA and 0.1 mL of enzyme solution (500-1000 units/ml). The enzyme activity is again determined by recording the decrease in ascorbic acid concentration which had a corresponding decrease in absorbance at 265nm.

DOPACHROME FORMATION

The principle of this procedure is:

L-3,4 Dihydroxy phenylalanine(L-Dopa) ----> Dopachrome

This assay procedure requires 1.0 mL of 5.0mM L-Dopa as the substrate. The buffer used was potassium phosphate (50mM, pH=7.0). The enzyme concentration was 5.0-6.0 mg/ml of tyrosinase in buffer. The procedure requires 1.0 mLs of buffer, 1.0 mL of L-Dopa, 0.9 mL of water and 0.1 mL of enzyme. The enzyme activity was measured spectrophotometrically by the formation of Dopachrome which had a corresponding increase in absorbance at 475 nm.

This assay procedure offered many attractive features such as simplicity and reproducibility. It also offered advantages over other spectrophotometric procedures such as the absence of a lag period.

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