Agaricus bisporus Metapotyrosinase: Preparation, Characterization, and Conversion to Mixed-Metal Derivatives of the Binuclear Site

Grace Yong, Christine Leone, and Kenneth G. Strothkamp*
Department of Chemistry, Bryn Mawr College, Bryn Mawr, Pennsylvania 19010
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ABSTRACT: The α, β, and γ isozymes of *Agaricus bisporus* tyrosinase undergo inactivation in the presence of oxalate. The inactivation rate law is first order in enzyme and second order in oxalate. On a more rapid time scale than inactivation, oxalate acts as a competitive inhibitor of the catecholase reaction of tyrosinase. After removal of oxalate by dialysis, the inactivated enzyme is found to contain 50% of the original copper, all of which is present as paramagnetic, mononuclear copper sites. The ESR parameters of this copper indicate a tetragonal environment with nitrogen or nitrogen and oxygen ligands. The product of oxalate inactivation has lost one copper from each binuclear site and is thus a metapo derivative. Addition of Cu(II) to metapotyrosinase results in complete recovery of copper and catalytic activity. Prolonged storage of metapotyrosinase, in the absence of any additional Cu(II), results in copper migration, producing a 50% recovery of the original specific activity, expressed on a protein basis. Copper migration converts metapo sites into equal numbers of reconstituted, holo sites and fully apo sites. Both copper migration and copper reconstitution follow apparent first-order kinetics and are pH dependent. The involvement of two ionizable groups accounts for the observed pH dependence of each process. For copper migration pK_a values of 6.0 and 8.8 were found, while for copper reconstitution the pK_a values were 5.4 and 6.9. Addition of either Co(II) or Zn(II) to metapotyrosinase results in the formation of enzymatically inactive, mixed-metal derivatives of the binuclear copper site having one Cu(II) and one Co(II) or Zn(II) ion. These derivatives cannot be made by direct displacement of Cu(II) from native tyrosinase. The relative affinity of metal ions for the vacant metal binding site of metapotyrosinase is Cu(II) > Co(II) > Zn(II).

Tyrosinase (EC 1.14.18.1, monophenol,dihydroxyphenylalanine:oxygen oxidoreductase) is a copper-containing enzyme that catalyzes the ortho-hydroxylation of monophenols (cresolase reaction) and the oxidation of catechols to *o*-quinones (catecholase reaction) (Mason, 1965). The enzyme contains a binuclear copper site and is related to the oxygen transport protein hemocyanin, which also has a binuclear copper site. Most studies of tyrosinase have used enzyme from either the bread mold *Neurospora crassa* or the mushroom *Agaricus bisporus*. *Neurospora* tyrosinase consists of a single polypeptide chain of molecular weight 4.6×10^4 with a single binuclear copper active site (Lerch, 1982; Deinum et al., 1976). The presence of a binuclear copper site in tyrosinase and the formation of oxytyrosinase were first demonstrated with the *Agaricus* enzyme (Makino et al., 1974; Schoot Uiterkamp & Mason, 1973; Jolley et al., 1972, 1974). This protein has a molecular weight of 1.2×10^5 (Bouchilloux et al., 1963), is composed of two H subunits (M_r 4.3×10^4) and two L subunits (M_r 1.3×10^4), and contains two binuclear active sites per molecule (Strothkamp et al., 1976). *Agaricus* tyrosinase can also be fractionated into three isozymic forms, α, β, and γ, which differ in amino acid composition (Bouchilloux et al., 1963) and catalytic properties, including inhibitor binding (Menon et al., 1990).

The binuclear site of both proteins can exist in met ([Cu(II)Cu(II)]), deoxy or reduced ([Cu(I)Cu(I)]), and oxy ([Cu(II)Cu(II)O_2^{2-}]) forms (Solomon, 1981). In both cases, the copper ions are believed to be coordinated to histidine residues and an endogenous bridging ligand, which is responsible for the strong antiferromagnetic coupling seen in their cupric forms. Spectroscopic studies of native oxyhemocyanin and derivatives where the binuclear site has been altered [metapo ([Cu(II)-]), half-met ([Cu(II)Cu(I)]), and dimer (ESR[1]-detectable met) ([Cu(II)Cu(II)]) forms] have resulted in a "spectroscopically effective" model for the binuclear site of that protein (Eickman et al., 1979). The similarity of the spectroscopic features of oxytyrosinase and its derivatives to those of oxyhemocyanin (Eickman et al., 1978) has suggested an extension of the oxyhemocyanin model to oxytyrosinase (Himmelwright et al., 1980) and resulted in proposals for the structures of the phenol and catechol complexes of the binuclear copper site and the catalytic mechanism (Winkler et al., 1981; Wilcox et al., 1985). Monophenols are believed to coordinate to one copper of the binuclear site of oxytyrosinase via the phenolate oxygen while catechols bind to both copper ions of the binuclear site in both the met and oxy forms of tyrosinase. Each oxygen of the catechol is coordinated to one of the copper ions. The spectroscopic properties of the half-met, metapo, and dimer forms of *Neurospora* tyrosinase as well as their interaction with substrate analogues provided essential information in the construction of these models.

Photoinactivation of *Agaricus* tyrosinase in the presence of citrate resulted in the irreversible formation of the metapo form of this enzyme (Fry & Strothkamp, 1983). These experiments also demonstrated that destruction of histidine residues on the heavy subunit occurred with inactivation. This suggested that the heavy subunit contained the binuclear copper site and that the destroyed histidines may serve as ligands to the copper ion that is labilized during photoinactivation. A convenient re-

* To whom correspondence should be addressed at the Department of Chemistry, Drew University, Madison, NJ 07940.

[1] Abbreviations: ESR, electron spin resonance; r_f, formal mole ratio of metal ion added to protein.

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versible method for preparing metapo *Agaricus* tyrosinase would open up new avenues for investigation of the binuclear site and possibly the preparation of new derivatives. Such investigations are not possible with citrate photoinactivation due to the irreversible damage to the protein. This paper describes the reversible formation, characterization, and reconstitution of *Agaricus* metapotyrosinase as well as its use in the preparation of two mixed-metal derivatives of the binuclear site.

Materials and Methods

The α, β, and γ isozymes of *Agaricus bisporus* tyrosinase were isolated as previously described (Nelson & Mason, 1970; Jolley et al., 1974). The α and β isozymes were homogeneous on nondenaturing polyacrylamide gel electrophoresis (Davis, 1969), while some γ preparations showed a trace (<5%) of a second component lacking tyrosinase activity. Gels were stained for protein with Coomassie blue and for tyrosinase activity (Jolley & Mason, 1965). Protein concentrations were determined by the Lowry procedure (Lowry et al., 1951), with crystalline bovine serum albumin as the standard. Metal ion analyses were done by graphite furnace atomic absorption spectroscopy. Enzymatic activity was measured with a Gilson Oxygraph and Clark oxygen electrode at 25.0 ± 0.2 °C by use of 4-*tert*-butylcatechol (catecholase activity) or *p*-cresol (cresolase activity) as substrates (Nelson & Mason, 1970).

p-Cresol and 4-*tert*-butylcatechol were obtained from Aldrich and were purified by sublimation. Chelex-100 was from Bio-Rad Laboratories. Bovine erythrocyte Cu,Zn-superoxide dismutase was from Sigma Chemical Co. Distilled, deionized water was used in the preparation of all aqueous solutions. All pH measurements were at 25 °C. Reactions of metapotyrosinase employed polyethylene or polypropylene labware, and all buffers were treated with Chelex-100 before use to prevent contamination by adventitious metal ions. ESR spectra were obtained with a Varian E-line X-band spectrometer interfaced with an IBM System 9000 computer. Quartz ESR tubes were acid washed and exhaustively rinsed with distilled, deionized water. Spectra were recorded with 100-kHz field modulation and a modulation amplitude of 10 G. The power was 20 mW, and the temperature was 77 K. The magnetic field was calibrated with the signal of Mn(II) naturally present as an impurity in strontium oxide (Bolton et al., 1972). ESR parameters were calculated as previously described (Malmström & Vänngård, 1960; Peisach & Blumberg, 1974). Quantitation of the metapo ESR signal was done by double integration with Cu,Zn-superoxide dismutase as a standard.

Reaction of Tyrosinase with Oxalate. Tyrosinase was incubated in 100 mM Na_2HPO_4 containing varying concentrations of oxalic acid (0–50 mM) at the desired pH. Samples were kept at 5 °C in the dark, and aliquots were removed periodically and assayed for catecholase activity. These experiments investigated the effect of oxalate concentration and pH on the rate of inactivation of each isozyme. The inactivated enzyme was dialyzed against 5 × 1 L of 5.0 mM Na_2HPO_4, pH 7.00, at 5 °C over 24 h and then assayed for protein, copper, and enzymatic activity.

Inhibition of the catecholase reaction by oxalate was investigated by standard Michaelis–Menton kinetic analysis. The initial rate of catechol oxidation was measured at different concentrations of the organic substrate at a fixed oxygen concentration (air-saturated solution) in the presence of different concentrations of oxalate. Reactions were carried out at 25.0 ± 0.2 °C and pH 5.60. Data were analyzed by double-reciprocal plots of initial velocity vs initial substrate concentration. Initial rates were obtained within 30 s of the start of the reaction. Over this brief time period, the previously described inactivation did not result in a detectable loss of enzymatic activity.

Standard Procedure for Metapotyrosinase Preparation. On the basis of the characteristics of oxalate inactivation, the following standard procedure for metapo preparation was employed in subsequent work. Enzyme was incubated in Chelex-100-treated 56 mM oxalic acid–100 mM Na_2HPO_4, pH 5.60, at 5 °C in the dark for 5 h. The sample was then dialyzed against 5 × 1 L of 5.0 mM Na_2HPO_4, pH 7.00, at 5 °C over 24 h. The metapo sample was characterized and used immediately for experiments.

Reconstitution of Metapotyrosinase with Cu(II). Aliquots of metapoenzyme were incubated with varying concentrations of copper sulfate, up to a 12-fold molar excess of Cu(II), in 5.0 mM Na_2HPO_4, pH 7.00, at 5 °C for 24 h. This was followed by an additional 24-h incubation at 5 °C in the presence of Chelex-100 to remove Cu(II) not tightly bound to the protein. Controls consisted of metapotyrosinase and native tyrosinase incubated under identical conditions in the absence of Cu(II). Following the Chelex-100 treatment, all samples were analyzed for copper, protein, and catecholase activity.

The effect of pH on the rate of reconstitution was investigated by incubating metapotyrosinase in 5.0 mM Na_2HPO_4 containing a 12-fold molar excess of Cu(II) at various pH values. Incubations were at 5 °C, and aliquots were removed at various times, treated with Chelex-100, and assayed for copper, protein, and enzyme activity. Controls again consisted of native tyrosinase and metapotyrosinase incubated under identical conditions in the absence of Cu(II). Apparent first-order rate constants for copper binding and activity recovery were determined at each pH value.

Copper Migration in Metapotyrosinase. Freshly prepared metapotyrosinase has no catalytic activity. However, on prolonged storage at 5 °C, a slow recovery of activity is observed. To investigate the pH and time dependence of this phenomenon, metapoenzyme was incubated at 5 °C in Chelex-100-treated 200 mM Na_2HPO_4 at pH values from 5 to 10. Aliquots of each sample were removed periodically and assayed for catecholase activity. The initial and final aliquots were also assayed for copper content. Controls consisted of native tyrosinase incubated under identical conditions. An apparent first-order rate constant for activity recovery was determined at each pH value.

Preparation of Mixed-Metal Derivatives. Freshly prepared metapotyrosinase in Chelex-100-treated 5.0 mM Na_2HPO_4, pH 7.00, was treated with varying amounts of $CoCl_2$ (up to a 250-fold molar excess) or $ZnSO_4$ (up to a 350-fold molar excess) at 5 °C for 24 h. This was followed by treatment with Chelex-100 for an additional 24 h at 5 °C and then metal ion analyses by atomic absorption spectroscopy and measurement of catecholase activity.

Direct displacement of metal ions from native, [Cu(II)-Co(II)], and [Cu(II)Zn(II)] tyrosinase was also investigated. Each form of the protein was incubated with a 500-fold molar excess of either Cu(II), Co(II), or Zn(II) in 5.0 mM Na_2HPO_4, pH 7.00, at 5 °C for 5 h. Samples were then incubated with Chelex-100 as described above and analyzed for metal ion content and catalytic activity.

Results

Characterization of Oxalate Inactivation. Incubation of tyrosinase in an oxalate–phosphate buffer of pH 5.60 at 5 °C in the dark results in a slow loss of both catecholase and

Table I: Metal Ion Content of *Agaricus* Tyrosinase Derivatives[a]

sample	mol of metal/mol of protein		
	Cu	Co	Zn
native enzyme	4.3 ± 0.3		
metapo	2.2 ± 0.3		
[Cu(II)Co(II)]	1.7 ± 0.2	1.8 ± 0.3	
[Cu(II)Zn(II)]	1.9 ± 0.1		2.0 ± 0.1

[a] Values shown were determined after exhaustive treatment with Chelex-100 to remove adventitious metal ions. Each value is the mean ± 1 standard deviation of four separate analyses.

cresolase activity. No loss of catalytic activity is observed in the absence of oxalate. A plot of log (percent activity remaining) vs time is linear, indicating an apparent first-order process. A 10-fold increase in the enzyme concentration, at the same concentration of oxalate, produces no change in k_{obs}, the apparent first-order rate constant. The linearity of the first-order kinetic plots holds over the entire duration of an experiment: up to five half-lives in some cases. All three isozymes of tyrosinase show the same behavior, but the rate of inactivation varies between isozymes in the order $\alpha > \beta > \gamma$.

The rate of inactivation is actually pseudo first order since it is a function of the concentration of oxalate. The apparent first-order kinetics results from the high molar concentration of oxalate relative to enzyme. To determine the order of the reaction with respect to oxalate, measurements of k_{obs} as a function of oxalate concentration were made at pH 5.00, 5.60, and 6.00. Plots of log k_{obs} vs log [oxalate] at each pH were linear with slopes of 2.0 ± 0.2, 2.2 ± 0.2, and 2.6 ± 0.7, respectively. The average slope was 2.3 ± 0.3. The reaction is thus second order in oxalate at each pH, yielding the rate equation

$$\text{inactivation rate} = k[\text{enzyme}][\text{oxalate}]^2 \quad (1)$$

The inactivation rate, at a constant total concentration of oxalate in all its ionic forms, is strongly pH dependent with the rate increasing as pH decreases over the range 4.0–6.0. At pH 8.00, no inactivation occurs, thus suggesting that the dianion of oxalate, the only form present at pH 8.00, is not responsible for the observed inactivation (pK_a values of oxalic acid: 1.2 and 4.2). Attempts to account for the pH dependence of k_{obs} in terms of a requirement for either the monoanionic or fully protonated form of oxalic acid in the process were unsuccessful. The data could also not be satisfactorially fit by assuming both the monoanion and neutral species of oxalate cause inactivation but at different rates. Thus, the pH dependence of k_{obs} is complex and may reflect the effect of pH not only on the relative amounts of the various forms of oxalic acid present in solution but also on the overall charge on the protein or ionization state of one or more specific amino acid side chains.

Oxalate functions as a weak simple competitive inhibitor of the catecholase reaction of γ-tyrosinase at pH 5.60, with a K_i value of 0.30 M. This finding is based on initial rate measurements obtained within 30 s of addition of enzyme to the reaction mixture. Inhibition thus results from an interaction between enzyme and oxalate occurring on a much faster time scale than that of inactivation. A replot of the slopes of the Lineweaver–Burke plots vs the oxalate concentration was linear, consistent with a 1:1 stoichiometry between active site and inhibitor.

The inactivated enzyme, after dialysis to remove oxalate, was found to have no catalytic activity and a 50 ± 5% reduction in copper content (Table I). This copper-depleted enzyme derivative was paramagnetic with an ESR spectrum characteristic of a mononuclear copper complex, in contrast to native tyrosinase which does not show an ESR spectrum. The signal accounted for 92% of the copper present in the enzyme as determined by atomic absorption analysis and was undiminished by repeated treatment of the sample with Chelex-100. Oxalate inactivation thus removes 50% of the active site copper, leaving the remainder as magnetically isolated Cu(II) ions tightly bound to the protein. This is accompanied by a total loss of catalytic activity. Thus, each binuclear active site must lose one copper ion, resulting in the formation of a metapo ([Cu(II)–]) derivative of the enzyme. The ESR parameters of the copper in the metapo derivative are given in Table II along with those of other tyrosinase and hemocyanin derivatives for comparison.

Reconstitution of Metapotyrosinase with Cu(II). Titration of metapotyrosinase with cupric sulfate in 5.0 mM Na_2HPO_4, pH 7.00, resulted in the parallel recovery of specific activity and copper content to the values characteristic of the native enzyme. Thus, the oxalate method of preparing metapotyrosinase is completely reversible. Addition of copper to native tyrosinase under identical conditions had no effect on the specific activity of copper content of the enzyme. In these and all subsequent metal-addition experiments, the enzyme was treated exhaustively with Chelex-100 before metal ion and activity determinations in order to remove metal ions not tightly bound to the protein. Control experiments verified that the conditions of Chelex-100 treatment would selectively remove all copper from buffer without removing copper from the active sites of native or metapotyrosinase.

The rate of copper reconstitution of metapotyrosinase incubated with a 12-fold molar excess of Cu(II) was found to be pH dependent. Reconstitution was monitored by removing aliquots of the reaction mixture at various times and assaying for both catecholase activity and copper content of the protein. At each pH value studied, reconstitution followed apparent first-order kinetics and an apparent first-order rate constant, k_{obs}, was determined. A plot of k_{obs}, based on measurements of activity recovery, vs pH is shown in Figure 1. The k_{obs} values based on measurements of the rate of copper binding to the protein were virtually identical and yielded a plot showing the same pH dependence. Attempts to fit the data

Table II: ESR Parameters of Metapotyrosinase and Related Proteins

protein	buffer (pH)	$g_\perp$ (or g_m)	$g_\parallel$	$A_\parallel$ (cm^{-1})
Agaricus metapotyrosinase by the oxalate method	phosphate (7.0)	2.06	2.30	0.0158
Agaricus metapotyrosinase by the citrate method[a]	phosphate–citrate (5.6)	2.06	2.25	0.0142
Streptomyces metapotyrosinase by site-directed mutagenesis[b]				
His-62		2.05	2.23	0.0202
His-189		2.04	2.23	0.0202
Busycon metapohemocyanin[c]	acetate (5.7)	2.08	2.31	0.0131
Neurospora half-mettyrosinase[d]	phosphate (6.3)	2.08	2.32	0.0131
Busycon half-methemocyanin[c]	acetate (5.7)	2.08	2.32	0.0141

[a] Fry and Strothkamp (1983). [b] Huber and Lerch (1988). Two metapo derivatives were prepared, one lacking His-62 and the other lacking His-189. [c] Himmelwright et al. (1978). [d] Himmelwright et al. (1980).

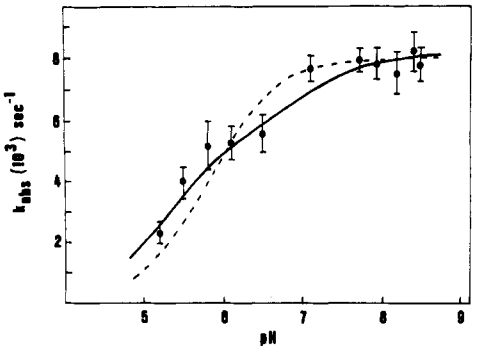

FIGURE 1: pH dependence of the apparent first-order rate constant, k_{obs}, for reconstitution of activity to metapotyrosinase in the presence of a 12-fold molar excess of Cu(II) in 100 mM Na_2HPO_4 at 5 °C. Error bars show ±1 standard deviation. The dashed line is the best fit of the data to a model where ionization of a single group accounts for the pH dependence. The solid line, which is a better fit to the data, is based on a model where two ionizations effect k_{obs} (eq 3; see text for details). The best-fit values to this model are $k_1 = 5.4 \times 10^{-3}$ s^{-1}, $k_2 = 8.0 \times 10^{-3}$ s^{-1}, $pK_{a1} = 5.3$, and $pK_{a2} = 6.9$.

to a model based on the ionization of a single group (Fersht, 1985) resulted in a best fit curve shown by the dashed line in Figure 1. Alternatively, assuming that k_{obs} is influenced by the ionization of two acidic groups (solid curve in Figure 1) results in a significantly better fit to the data. In this model, EH_2, EH, and E represent metapoenzyme in three different states of protonation (eq 2). The data were best accounted

$$EH_2 \underset{pK_{a1}}{\rightleftharpoons} EH \underset{pK_{a2}}{\rightleftharpoons} E \qquad (2)$$

for by assuming that EH_2 would not bind added copper while EH and E would undergo reconstitution with rate constants k_1 and k_2, respectively ($k_1 < k_2$). This yields an expression for the pH dependence of k_{obs} given by eq 3 (Fersht, 1985).

$$k_{obs} = (k_1K_{a1}[H^+] + k_2K_{a1}K_{a2})/([H^+]^2 + K_{a1}[H^+] + K_{a1}K_{a2}) \qquad (3)$$

K_{a1} and K_{a2} are the acid dissociation constants of EH_2 and EH, respectively. The best fit of this equation to the experimental data resulted in pK_a values of 5.3 and 6.9 from activity recovery measurements and 5.4 and 6.9 from copper binding measurements.

Copper Migration in Metapotyrosinase. When initially prepared, metapotyrosinase has no catalytic activity. On prolonged storage at 5 °C in 5.0 mM Na_2HPO_4, pH 7.00, however, a slow recovery of catalytic activity, up to a specific activity of 50% of the native enzyme value on a protein basis, is observed. This recovery occurs even when enzyme is kept in plastic containers in buffer previously treated with Chelex-100 to remove traces of Cu(II). Furthermore, there is no change in the total copper to protein mole ratio during the recovery process. This indicates a migration of copper ions from metapo sites to form fully reconstituted, catalytically active binuclear sites and fully apo sites (eq 4). This accounts

$$\underset{\text{metapo sites}}{2[Cu(II)-]} \rightarrow \underset{\text{holo site}}{[Cu(II)Cu(II)]} + \underset{\text{apo site}}{[--]} \qquad (4)$$

for the maximum recovery of 50% of the specific activity since half of all active sites are reconstituted while the other half are fully devoid of copper and catalytic activity.

The rate of copper migration in metapotyrosinase, like that of copper reconstitution, is pH dependent and follows apparent first-order kinetics. At each pH value, a plot of log (percent inactive enzyme) vs time is linear with an apparent first-order rate constant, k_{obs}. The rate constant, k_{obs}, is a complicated function of pH (Figure 2), suggesting the involvement of two

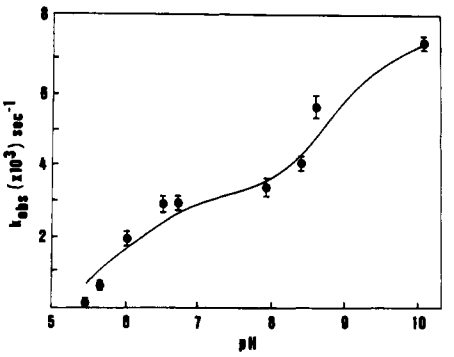

FIGURE 2: pH dependence of the apparent first-order rate constant, k_{obs}, for copper migration in metapotyrosinase in 200 mM Na_2HPO_4 at 5 °C. The error bars are ±1 standard deviation. The solid line represents the best fit to the data of a model assuming that two ionizations account for the pH dependence (eq 3; see text for details). The best-fit values to this model are $k_1 = 3.1 \times 10^{-3}$ s^{-1}, $k_2 = 7.5 \times 10^{-3}$ s^{-1}, $pK_{a1} = 6.0$, and $pK_{a2} = 8.8$.

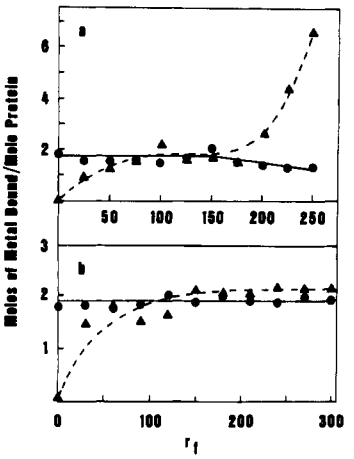

FIGURE 3: Moles of copper (●) and cobalt (▲) bound per mole of protein vs formal mole ratio (r_f) of Co(II) added per mole of metapotyrosinase (a). Samples were incubated for 5 h in 5.0 mM Na_2HPO_4, pH 7.00, at 5 °C, treated with Chelex-100, and analyzed for copper and cobalt by atomic absorption spectroscopy. Moles of copper (●) and zinc (▲) bound per mole of protein vs formal mole ratio (r_f) of Zn(II) added per mole of metapotyrosinase (b). Samples were incubated for 5 h in 5.0 mM Na_2HPO_4, pH 7.00, at 5 °C, treated with Chelex-100, and analyzed for copper and zinc by atomic absorption spectroscopy.

ionizable groups in the migration mechanism. The data are best fit by assuming two ionizations (eq 2) and also that the protonated form of the protein, EH_2, does not undergo the migration process. Assuming EH and E undergo migration with rate constants k_1 and k_2, respectively, results in an expression for k_{obs} identical with eq 3. When the data are fit to eq 3, the pK_a values are found to be 6.0 and 8.8.

Preparation of Mixed-Metal Derivatives. When incubated in the presence of varying amounts of Co(II) for 24 h at 5 °C in 5.0 mM Na_2HPO_4, pH 7.00, metapotyrosinase binds Co(II) (Figure 3a, Table I). Exhaustive treatment with Chelex-100 does not remove the bound Co(II), indicating strong binding. A plateau occurs in Co(II) binding in the range of 75–175 mol of Co(II) added per mole of protein under these experimental conditions. At this plateau, two Co(II) ions are bound per protein molecule, with no loss of the original copper. The one to one stoichiometry of tightly bound Cu(II) and Co(II) suggests the formation of a binuclear site, [Cu(II)Co(II)], in which the Co(II) occupies the vacant metal binding site in metapotyrosinase. At higher mole ratios of added Co(II), additional Co(II) is bound, and a slight decrease in copper is seen. The titration curve shown in Figure 3a thus indicates

the presence of two high-affinity Co(II) binding sites per molecule in metapotyrosinase and a larger number of weaker binding sites. In contrast, native *Agaricus* tyrosinase binds Co(II) with no loss of copper from the binuclear active sites, indicating the presence of the weaker but not the high-affinity binding sites.

Similarly, addition of Zn(II) to metapotyrosinase results in binding of up to 2 mol of Zn(II)/mol of protein with no loss of copper (Figure 3b, Table I). This indicates the formation of a mixed-metal binuclear site [Cu(II)Zn(II)]. Unlike Co(II), higher ratios of added Zn(II) do not result in additional Zn(11) binding. Both the [Cu(II)Co(II)] and [Cu(II)Zn(II)] derivatives of tyrosinase were catalytically inactive. This is consistent with a mixed-metal active site since catalytic activity requires the presence of a binuclear copper site.

Direct displacement of copper from native or reconstituted tyrosinase did not occur even when the enzyme was incubated with a 500-fold molar excess of Co(II) or Zn(II). Thus, formation of mixed-metal binuclear sites requires the use of the metapo derivative. Addition of Co(II) or Zn(II) to either native or reconstituted tyrosinase had no effect on catalytic activity. The relative stability of [Cu(II)Co(II)] and [Cu(11)Zn(11)] sites was investigated by incubating each derivative with a 500-fold molar excess of Cu(II), Co(II), or Zn(II) followed by exhaustive Chelex-100 treatment. A 40% loss of Co(II) from [Cu(II)Co(II)] tyrosinase occurred in the presence of copper. This replacement of Co(II) with Cu(II) resulted in a 48% recovery of catalytic activity. The recovery of activity in parallel with displacement of Co(II) by Cu(II) is further evidence that the Co(II) was bound in the binuclear site. There was, however, no loss of Co(II) when [Cu(II)-Co(II)] tyrosinase was incubated in the presence of excess Zn(II). In contrast, Zn(II) was displaced from [Cu(II)Zn(II)] tyrosinase by both Cu(II) and Co(II). Displacement of Zn(II) by Cu(II) produced a parallel recovery of catalytic activity. Thus, the relative affinity of metal ions for the vacant binding site in metapotyrosinase is Cu(II) > Co(II) > Zn(II).

Discussion

Reaction of Tyrosinase with Oxalate. The rate law for oxalate inactivation suggests a two-step mechanism for the reaction between enzyme active site (E) and oxalate (OX) leading to inactivation (eq 5). In the rapid, reversible first

$$\begin{aligned} &\text{fast:} && E + OX \rightleftharpoons E\text{–}OX \\ &\text{slow:} && E\text{–}OX + OX \rightarrow E_{inact} \end{aligned} \quad (5)$$

step, one oxalate molecule combines with a catalytically active binuclear site, producing a catalytically inactive complex (E–OX), which accounts for the competitive inhibition observed with oxalate. In a subsequent slow step, E–OX reacts with a second oxalate molecule, resulting in the loss of one Cu(II) from the binuclear site, possibly as the bis-oxalate complex, and forming a metapo site with no catalytic activity (E_{inact}). This second step is irreversible to the extent that activity can be restored only by removal of the oxalate and reintroduction of Cu(II) into the metapo site. The demonstration of both a 50% loss of copper content and complete loss of catalytic activity on inactivation, along with the finding that all of the remaining copper is present in an ESR-detectable form, indicates formation of metapotyrosinase ([Cu(II)–]). The alternative possibility, that both copper ions of half of the binuclear sites are lost, is incompatable with the data. The demonstration of full recovery of catalytic activity on reintroduction of Cu(II) indicates that treatment with oxalate does not irreversibly alter the protein structure. This is in contrast to the irreversible formation of metapotyrosinase by citrate photoinactivation (Fry & Strothkamp, 1983).

Characterization of Metapotyrosinase. Both citrate photoinactivation and oxalate inactivation of *Agaricus* tyrosinase produce a metapo derivative. Failure of either method to remove *both* Cu(II) ions from the binuclear site suggests two possible explanations. Either (1) the coordination environment or accessibility of the two copper ions is different such that either reagent can attack only one copper of the binuclear site, or (2) the two copper ions are initially identical in every respect, but removal of one causes some change in the protein that renders the remaining copper inaccessible. The former proposal is consistent with the expected lack of perfect 2-fold symmetry in protein tertiary structure and the observed lack of symmetry in other metalloproteins having binuclear metal sites (Richardson et al., 1975; Stenkamp et al., 1981). The X-ray crystal structure of *Panularis interruptus* deoxyhemocyanin indicates nonequivalence of the two copper ions (Gaykema et al., 1984). Comparison of amino acid sequences of *Neurospora crassa* (Lerch, 1978) and *Streptomyces glaucesens* (Huber, 1975) tyrosinases with that of *P. interruptus* hemocyanin shows sequence homology in regions containing the ligands of the Cu_B site of *Panularis* hemocyanin. Both *Neurospora* and *Streptomyces* tyrosinase share another homologous region containing two conserved histidines. Site-directed mutagenesis on the *Streptomyces* enzyme implicated this region in the binding of Cu_A (Huber & Lerch, 1988). Despite the general similarity of tyrosinase and arthropod hemocyanin, the Cu_A site appears to be different in the two proteins, which may account for their vastly different functions. These comparisons suggest an inherent structural and perhaps functional distinction between the two copper ions of the binuclear site of tyrosinases. Such a distinction is also a feature of the model of monophenol binding to the binuclear site of tyrosinase, which proposes coordination of the monophenol to one, presumably specific, copper ion (Wilcox et al., 1985). Formation of metapotyrosinase most likely results from selective rather than random removal of one of the two copper ions of the binuclear site.

A number of paramagnetic derivatives of hemocyanin and tyrosinase have been prepared (Table II). All have ESR spectra suggestive of distorted tetragonal coordination involving nitrogen or nitrogen and oxygen ligands (Peisach & Blumberg, 1974), consistent with other spectroscopic data on tyrosinase and hemocyanin and the known structure of *Panularis* deoxyhemocyanin, in which the copper ions are bound to histidine ligands. The observed differences in ESR parameters among the derivatives may reflect either real differences in copper coordination or the effects of their method of preparation.

Addition of Cu(II) to metapotyrosinase results in complete recovery of both specific activity and copper content. Storage of metapotyrosinase, in the absence of any source of Cu(II), leads to slow recovery of catalytic activity. This results from migration of Cu(II) from metapo sites to form fully reconstituted catalytically active holo sites and fully apo sites. An analogous phenomenon occurs during the storage of the zinc-free form of Cu,Zn-superoxide dismutase, where Cu(II) migrates to form [Cu(II)Cu(II)] binuclear sites and fully apo sites (Valentine et al., 1979). The spontaneous migration of Cu(II) in metapotyrosinase suggests that the fully occupied binuclear site is more stable than the metapo site.

Both the rate of copper reconstitution of metapotyrosinase and the rate of copper migration are pH dependent. Both of the observed apparent first-order rate constants show a pH dependence implicating two ionizable groups in these processes. In both cases, the model resulting in the best fit to the data

assumes that the enzyme form in which both groups are protonated, EH_2, does not undergo reconstitution or copper migration. Deprotonation of the more acidic of the two groups, forming EH, allows both processes to occur while deprotonation of the second group enhances the rate of each process. The best-fit pK_a values obtained for the two processes are different, especially in the case of the higher pK_a value. This may reflect actual differences in the groups involved in the rate-determining steps of the two processes. Both pK_{a1} values are consistent with a histidine residue and may reflect the deprotonation of a histidine ligand in the vacant metal binding site essential for the binding by either added copper or copper migration. The pK_{a2} value of 6.9 of the copper reconstitution reaction may reflect deprotonation of a second histidine ligand in the vacant site, which enhances the rate of copper binding. The pK_{a2} of 8.8, observed in the copper migration studies, may represent another residue besides histidine that is involved in this latter process. Further work will be required to clarify the identity of these groups. Ionization of Cu(II) aquo or phosphate complexes could also contribute to the observed pH dependence. However, since k_{obs} *increases* with pH whereas ionization of coordinated water or phosphate should produce more inert Cu(II) complexes, this explanation seems less likely.

Preparation of Mixed-Metal Derivatives. Replacement of the naturally occurring metal ion by another element has been widely employed in the study of metalloproteins. Classic examples include the substitution of Co(II) for Zn(II) in many zinc enzymes and the many metal-substitution studies on Cu,Zn-superoxide dismutase. Bis-Co(II) derivatives of both hemocyanin (Suzuki & Nakahara, 1982; Lorosch & Haase, 1986) and tyrosinase (Ruegg & Lerch, 1981) have been prepared in which both copper ions of the binuclear site are replaced by Co(II). The reversible formation of metapotyrosinase by our method suggests that mixed-metal derivatives of the binuclear site, in which one copper ion is replaced by a different element, can be prepared. As shown in Figure 3 and Table I, metapotyrosinase will bind one Co(II) or Zn(II) ion per binuclear site with no loss of Cu(II). Cu(II) will slowly replace the nonnative metal ion from either [Cu(II)Co(II)] or [Cu(II)Zn(II)] tyrosinase, resulting in recovery of catalytic activity. These results indicate that the Co(II) or Zn(II) occupies the vacant metal binding site in metapotyrosinase, forming mixed-metal derivatives of the binuclear site, both of which are catalytically inactive. The observed order of affinity of metal ions for the apo site may reflect, in part, the preferred coordination geometry of the metal ions. In native tyrosinase, both Cu(II) are believed to be in distorted tetragonal environments (Solomon, 1981). Co(II) and Zn(II) tend to form tetrahedral complexes, as does four-coordinate Cu(I). Under physiological conditions, copper cycles between the 2+ and 1+ oxidation states, and some rearrangement of ligand number and/or geometry may accompany these changes. However, *both* copper ions of the binuclear site are always in the same oxidation state. The mixed-metal derivatives differ in that one metal ion prefers tetrahedral geometry while the other tetragonal geometry. This unnatural situation may make the Co(II) or Zn(II) ion more labile than the native Cu(II), facilitating their displacement by copper. The previously reported bis-cobalt derivative of tyrosinase, in which both metal ions are expected to prefer tetrahedral geometry, may be an analogue of the reduced [Cu(I)Cu(I)] form of tyrosinase, analogous to the suggestion for the bis-Co(II) derivative of hemocyanin (Lorosch & Haase, 1986). *Neurospora* [Co(II)Co(II)] tyrosinase was resistant to displacement of Co(II) by Cu(II) (Ruegg & Lerch, 1981), suggesting that the [Cu(II)Co(II)] site may be strained by the different preferential geometries of the two metal ions, facilitating Co(II) replacement. These mixed-metal derivatives may resemble the nonphysiological half-met, [Cu(II)Cu(I)], tyrosinase. Binding of exogenous ligands (substrates and substrate analogues) can also perturb metal coordination, and such perturbations are proposed to be important in the catalytic mechanism of tyrosinase (Wilcox et al., 1985). Thus, further studies of these mixed-metal derivatives and their interaction with various ligands will contribute to our understanding of the structure and mechanism of tyrosinase.

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Substrate Specificities and Mechanism in the Enzymatic Processing of Vitamin A into 11-*cis*-Retinol†

Francisco J. Cañada, Wing C. Law, and Robert R. Rando*
Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115
Toshihiro Yamamoto, Fadila Derguini, and Koji Nakanishi*
Department of Chemistry, Columbia University, New York, New York 10027
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ABSTRACT: The biosynthesis of 11-*cis*-retinol in the retinal pigment epithelium requires two consecutive enzymatic reactions. The first involves the esterification of *all-trans*-retinol by lecithin retinol acyltransferase (LRAT). The second reaction involves the direct conversion of an *all-trans*-retinyl ester into 11-*cis*-retinol by an isomerase-like enzyme. This latter reaction couples the free energy of hydrolysis of an ester to the thermodynamically uphill trans to cis conversion, thus providing the energy to drive the latter process. In this paper both enzymes are studied with respect to their substrate specificities to provide information on mechanism. The isomerase is shown to be highly specific with respect to the ionylidene ring system and substitution at C15, whereas sterically bulkier substituents at C9 and C11 are permitted. C5 and C13 demethyl retinoids are isomerized, removing from consideration isomerization mechanisms involving C-H abstraction at the C5 or C13 methyl groups of the retinoid. On the other hand, C9 demethyl retinoids are not isomerized. A C-H abstraction mechanism is unlikely at the C9 methyl group as well, because no kinetic deuterium isotope effect is found with *all-trans*-19,19,19-trideuterioretinoids and isomerization of unlabeled retinoids occurs without the incorporation of deuterium when the isomerization is performed in D_2O. LRAT proved to be broadly specific for retinols but was relatively inert with other hydrophobic alcohols including cholesterol. The enzyme is also highly specific for phosphatidylcholine analogues versus other potential membranous acyl donors such as phosphatidylethanolamine and phosphatidylserine.

Vision begins with the photoisomerization of the 11-*cis*-retinal Schiff base chromophore of rhodopsin to its *all-trans*-retinal congener (Hubbard & Wald, 1952). *all-trans*-Retinal is reduced in the rod outer segments to *all-trans*-retinol (vitamin A) and carried off to the pigment epithelium where it is esterified and isomerized to produce 11-*cis*-retinol (Deigner et al., 1989). In warm-blooded mammals, at least, the 11-*cis*-retinol is oxidized to 11-*cis*-retinal in the pigment epithelium (Jones et al., 1989) and returned to the rod outer segments, where it recombines with opsin to form rhodopsin.

The retinoid isomerization process is an intriguing one because it involves the input of chemical energy to drive the thermodynamically uphill trans to cis isomerization reaction. 11-Cis retinoids are approximately 4 kcal/mol higher in energy than their all-trans isomers (Rando & Chang, 1983). This problem is solved in the visual system by a mechanism that involves the initial esterification of vitamin A, catalyzed by a lecithin retinol acyltransferase (LRAT)[1] (Barry et al., 1989; Saari & Bredberg, 1989). This enzyme transfers a saturated acyl group from the *sn*-1 position of lecithin, thereby producing a retinyl ester and a 2-acyl lysophospholipid as products (Barry et al., 1989; MacDonald & Ong, 1988; Saari & Bredberg, 1989). In a separate reaction, the retinyl ester is isomerized with hydrolysis to produce 11-*cis*-retinol and a fatty acid (Deigner et al., 1989). In this isomerase- (or more properly isomerohydrolase-) mediated reaction, the required energy is provided by the free energy of hydrolysis of the retinyl ester (Deigner et al., 1989). Thus, retinyl ester synthesis is obligate

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* To whom correspondence should be addressed.

[1] Abbreviations: LRAT, lecithin retinol acyltransferase; RPE, retinal pigment epithelium; MS, mass spectroscopy; BSA, bovine serum albumin; BHT, butylated hydroxytoluene; THF, tetrahydrofuran; PC, phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA, phosphatidic acid.

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