Standard KH_2PO_4–Na_2HPO_4 and borax buffers for the mixed methanol–water titrations were made according to Bates, Paabo, and Robinson,[11] and the electrode was checked for stability and slope in the 16.3, 33.3 and 52.1 wt % methanol buffers. The titration samples were made up by weight with appropriate amounts of degassed, distilled methanol and water and titrated with KOH (J.T. Baker Dilute-It) diluted to the appropriate solvent composition.

^{13}C NMR/pH Studies. For the model compounds the hydrochlorides were dissolved in 50% D_2O–50% H_2O and 2.0 M KOH to provide spectra of the protonated and deprotonated forms. Spectra at intermediate pH values were run for *n*-butylguanidine (**9**) and 2-amino-4-methyl-2-imidazoline (**10**) which showed chemical shift values between the fully protonated and deprotonated values as expected. The 2 M KOH spectra may not reflect the maximum shift possible for these model compounds due to the high p*K* values of normal guanidines. The pH-dependent spectra of **1**, **4** and **5** were run on samples of 126.8, 56.3, and 44.0 mg, respectively, dissolved in 3 mL of 50% D_2O–50% H_2O, and the pH was adjusted with 2.0 M KOH. Reacidification with 2.0 M HCl in each case gave spectra indentical with the original.

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Chemical and Spectroscopic Studies of the Binuclear Copper Active Site of *Neurospora* Tyrosinase: Comparision to Hemocyanins

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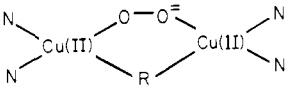
Abstract: This paper reports the results of a series of chemical and spectroscopic studies on derivatives of the binuclear copper active site of *Neurospora crassa* tyrosinase. Parallel results are also reported for *Limulus polyphemus*, the hemocyanin which exhibits spectral properties most similar to those found for tyrosinase. The chemistry and spectroscopy of half met [Cu(II)Cu(I)], 2-mercaptoethanol, dimer (EPR-detectable met) [Cu(II)Cu(II)], met (EPR-nondetectable met) [Cu(II)···Cu(II)], and oxy I derivatives of tyrosinase are presented and shown to be quite similar to the results for hemocyanin in terms of geometric and electronic structure, but with higher accessibility to exogenous ligands. The involvement of the effective structure of the oxy "type 3" copper active site (*Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 2094–2098) in substrate reactions is then discussed.

Introduction

Tyrosinase is one of a series of metalloproteins and enzymes (hemocyanin, tyrosinase, laccase, ceruloplasmin, and ascorbic acid oxidase) which contain a strongly coupled binuclear copper active site[1a] but perform different biological functions. This "type 3" copper unit[1b] is characterized by its lack of any EPR signal for formally cupric ions and as the site of interaction with dioxygen. Particularly strong parallels exist between hemocyanin and tyrosinase in that both contain only one binuclear copper unit[2] (laccase, ceruloplasmin, and ascorbate oxidase contain additional copper sites) and both reversibly bind oxygen to produce oxyhemocyanin and oxytyrosinase.[3] These exhibit very similar absorption features which are unique when compared to simple copper complexes: an intense band at ~350 nm ($\epsilon \approx 20000$ M^{-1} cm^{-1}) and a reasonably intense absorption at ~570 nm ($\epsilon \approx 1000$ M^{-1} cm^{-1}). However, while hemocyanin functions only as an oxygen carrier, tyrosinase is an enzyme which functions both as a monooxygenase (monophenol + O_2 → *o*-diphenol + H_2O) and as a 2-electron oxidase (2 *o*-diphenol + O_2 → 2 *o*-quinone + $2H_2O$). Resonance Raman studies using UV laser excitation into the 350-nm absorption of oxytyrosinase produced a 755-cm^{-1} vibration which shifted upon oxygen isotopic substitution.[4] This peroxide type stretching frequency, as well as the M–L vibrational spectrum was extremely similar to resonance Raman vibrational features observed earlier for oxyhemocyanins.[5] Thus the enzymatic activity of tyrosinase, in contrast to hemocyanin, cannot simply be related to differences in oxygen activation but must be associated with detailed differences in the binuclear copper active site.

Recently, a series of hemocyanin derivatives has been prepared which allows the active site to be systematically varied and subjected to detailed spectroscopic study.[6] These derivatives (met apo [Cu(II)–], half met [Cu(II)Cu(I)], met (EPR-nondetectable met) [Cu(II)···Cu(II)], dimer (EPR-detectable met) [Cu(II)-Cu(II)] and oxy I) allowed an effective structural model of the



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binuclear copper active site to be developed[1a,6a] which also explains

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the unique ground state and optical spectral features of oxyhemocyanin. The oxy site contains two tetragonal copper(II)'s (with nitrogen and oxygen ligation) bridged by an endogenous ligand (either phenolate or oxo) which provides a superexchange pathway for antiferromagnetic coupling and thus the lack of a detectable EPR signal. The exogenous peroxide also bridges the two coppers in a μ-dioxo linkage, producing the unique optical features of oxy (peroxide to metal charge-transfer transitions to two weakly coupled copper(II)'s). Extension of these chemical and spectroscopic studies to hemocyanin derivatives of five molluscs and five arthropods further demonstrated[7] that the "type 3" copper active sites are qualitatively similar in all hemocyanins; however, quantitative differences were observed. A structural distortion was indicated for all arthropods which affects exogenous ligand bridging to the met form and which seems to correlate with the low catalase activity of this phylum (met + $H_2O_2 \rightarrow$ oxy only for the molluscs).[8] Also large differences in exogenous ligand access to the oxy site were indicated by the three order of magnitude range in rates of peroxide displacement by azide (arthropods > molluscs $\gg$ *Limulus*). Finally, an active site instability was observed for the arthropod (but not *Limulus*) hemocyanins with group 2 ligands (those which break the endogenous bridge by binding the coppers with a >5-Å M–M distance).

This paper reports the results of a series of chemical and spectroscopic studies on derivatitves of *Neurospora crassa* tyrosinase. Parallel results[7] on *Limulus polyphemus* will also be presented as this hemocyanin exhibits spectral properties most similar to those found for tyrosinase. Prior to this work, Mason and co-workers had demonstrated that resting mushroom tyrosinase can be equated with the met[9] derivative (with ~15% intrinsic oxy[3b]) and that a dimer EPR spectrum is obtained from deoxy mushroom tyrosinase by reaction with nitric oxide.[10] However, *Neurospora* tyrosinase was reported[11a] not to react. A new tyrosinase derivative, with unusual EPR spectral properties, obtained from the reaction of 2-mercaptoethanol with the resting enzyme was also reported.[11] The chemistry and spectroscopy of half met, 2-mercaptoethanol, dimer, met and oxy derivatives of tyrosinase are presented and shown to be quite similar to the results for hemocyanin in terms of geometric and electronic structure but with higher accessibility to exogenous ligands. The involvement of the oxy "type 3" copper active[1a] site in substrate reactions is then discussed.

Experimental Section

Neurospora crassa tyrosinase was isolated and purified as previously described[4] and stored either as microcrystals or as an $(NH_4)_2SO_4$ precipitate. The crystals or precipitate were centrifuged for 20 min at 15000 rpm, and the tyrosinase pellets were then dissolved in 0.1 M sodium phosphate pH 6.3. Tyrosinase solutions were stored for periods of longer than 1 week in 10 mM sodium phosphate pH 6.8 containing 0.5 M NaCl. All investigations of tyrosinase were carried out at 4 °C or in an ice bath since even short exposures at room temperature cause some denaturation and browning due to autoxidation of tyrosine residues.[12] Tyrosinase solutions were concentrated from <0.2 mM to ~0.5 mM without significant browning by using a collodion bag apparatus obtained from Schleicher and Schuell, Keene, NH. L-Mimosine was obtained from Sigma, St. Louis, MI.

Except where noted in the Results, the chemistry and spectroscopy were performed as described in detail for the hemocyanins in previous publications.[6] The enzymatic activity was measured according to the method of Fling et al.[12] and determined to be: native enzyme, 1200 units/mg; oxy, 1200 units/mg; half met-NO_2^-, 1140 units/mg.

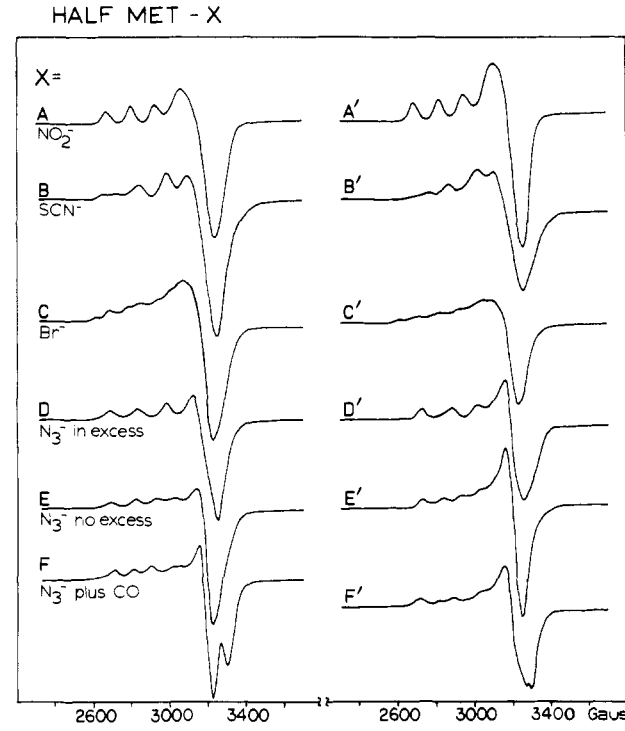

Figure 1. EPR spectra (77 K, pH 6.3, phosphate buffer) of (A) half met-NO_2^-, (B) half met-SCN^- in excess thiocyanate, (C) half met-Br in excess bromide, (D) half met-N_3^- in excess azide, (E) half met-N_3^- after removal of excess azide, and (F) half met-N_3^- after reaction with carbon monoxide of *Neurospora crassa* tyrosinase. Parallel spectra of *Limulus polyphemus* hemocyanin are given in A'–F'.

Results

A. Half Met Tyrosinase. When met (resting) tyrosinase is reacted with a 100-fold excess of $NaNO_2$ in the presence of 20-fold excess ascorbic acid in pH 6.3 phosphate buffer for 15 min, the EPR spectrum shown in Figure 1A is obtained. This spectrum accounts for ~45% of the total copper and arises from mononuclear copper(II) ions. Half met-NO_2^- hemocyanin, also obtained by reaction with excess $NaNO_2$ in ascorbic acid,[6b] has the EPR spectrum shown in Figure 1A' (for all tyrosinase derivatives the parallel form of *Limulus polyphemus* hemocyanin will be included as Figure X' for spectral comparison). The close spectral similarity between these forms (hemocyanin, $g_{\|} = 2.302$, $A_{\|} = 125 \times 10^{-4}$ cm^{-1}, and $g_{\perp} = 2.096$; tyrosinase $g_{\|} = 2.296$, $A_{\|} = 131 \times 10^{-4}$ cm^{-1}, and $g_{\perp} = 2.078$) allows the tyrosinase derivative to be assigned as a half met-NO_2^-.

Upon dialysis of the half met-NO_2^- tyrosinase in pH 6.3 phosphate buffer to remove reagents, the EPR signal was significantly reduced in intensity (to 18%) and browning of the enzyme occurred. For this reason, a different technique was necessary for reagent removal and ligand-substitution chemistry. After preparation of half met-NO_2^-, excess ascorbic acid and nitrite were removed by passing through Sephadex G-25. This procedure prevents any loss of the intensity of the EPR signal and eliminates the browning of the enzyme. Further, as only slight dilution occurred, this method was used exclusively in performing reactions on the half met *Neurospora* tyrosinase.

The half met-NO_2^- derivative undergoes ligand-substitution reactions as shown by changes in the EPR spectrum. The nitrite ligand can be displaced by addition of 300-fold excess SCN^-, Br^-, and N_3^- (Figure 1B–D). Removal of excess N_3^- produces a new EPR signal (Figure 1E) which corresponds to half met-N_3^- as one azide remains tightly bound at the half met site. This is demonstrated by the presence of a low-energy $N_3^- \rightarrow$ Cu(II) charge-transfer transition for this derivative. The 400–600-nm region of the optical spectrum of the half met-N_3^- form is given in Figure 2. In the presence of 300-fold excess of N_3^- a transition at 530 nm ($\epsilon \approx 1300$ M^{-1} cm^{-1}) is observed along with additional transitions to higher energy (Figure 2A). After removal of excess

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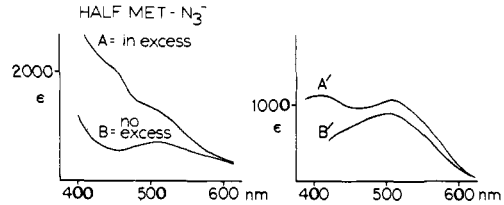

Figure 2. Optical absorption spectra (room-temperature difference spectrum pH 6.3, phosphate buffer) of (A) half met-N_3^- in excess azide and (B) half met-N_3^- after removal of excess azide of *Neurospora* tyrosinase. Parallel spectra of *Limulus* hemocyanin are given in A′ and B′.

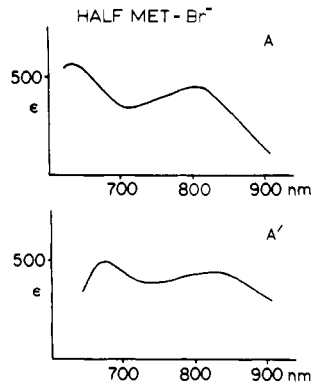

Figure 3. Optical absorption spectrum (~15 K, pH 6.3, phosphate buffer in 1:1 sucrose glass) of (A) half met-Br^- *Neurospora* tyrosinase and (A′) half met-Br^- *Limulus* hemocyanin.

azide, the transition at 530 nm remains (Figure 2B), and the excess N_3^- EPR spectrum (Figure 1D) is replaced by a spectrum exhibiting a low $A_\parallel$ splitting of 112×10^{-4} cm^{-1} (Figure 1E). This behavior completely parallels that observed for half met-N_3^- hemocyanin[6,7] (Figures 1D′, E′ and 2A′, B′), where the $N_3^- \rightarrow$ Cu(II) charge-transfer transition at ~500 nm along with the small $A_\parallel$ splitting are characteristic of a tightly bound, bridging N_3^- ligand.

The half met-N_3^- form of tyrosinase reversibly binds carbon monoxide as shown in Figure 1F. The EPR spectrum of half met-N_3^- is perturbed on coordination of carbon monoxide at the site when treated with 30 psig CO for 30 min. Evacuation and flushing with nitrogen yield recovery of the original half met-N_3^- EPR spectrum.

The low-temperature optical spectrum of half met-Br^- is presented in Figure 3A. In addition to the ligand field transitions observed at ~630 nm, a rather intense transition at lower energy (820 nm) is also observed. This low-energy transition coupled with the complex EPR spectrum of half met-Br^- (Figure 1C) showing more than four hyperfine lines in the $g_\parallel$ region classifies this derivative, in parallel with half met-Br^- hemocyanin[6,7] (Figures 3A′ and 1C′), as a class II mixed-valent form[13] with the 820-nm band assigned as an intervalent transfer (IT) transition. This IT assignment has been previously confirmed for the hemocyanin derivatives by the presence of this band in the half met-X, [Cu(II)Cu(I)], but not met apo-X, [Cu(II)–], forms and by the correlation of its intensity with the electron delocalization capabilities of X over the series X = F^-, Cl^-, Br^-, I^-, and N_3^- (see ref 6).

B. 2-Mercaptoethanol Tyrosinase. It has been previously reported[11] that a biphasic reaction occurred when met (resting) tyrosinase was treated with 2-mercaptoethanol producing the unusual EPR spectrum shown in Figure 4A which accounts for 70% of the total copper. The lack of a resolvable copper hyperfine splitting has raised the possibility that this signal is associated with a sulfur radical, similar to that believed to be responsible for the "EPR-detectable copper" in cytochrome *c* oxidase.[14] The complete optical spectrum of this derivative is presented in Figure 4B. This optical spectrum has strong similarities to a number

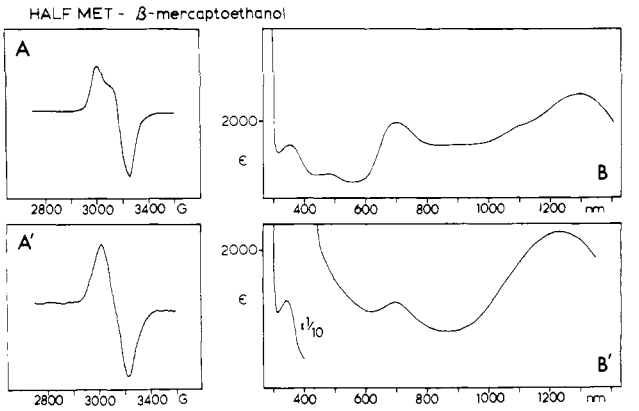

Figure 4. (A) EPR spectra (77 K, pH 6.3, phosphate buffer) and (B) absorption spectra (4 °C in optical, ~15 K in infrared regions, pH 6.3, phosphate buffer) of half met-2-mercaptoethanol *Neurospora* tyrosinase. Parallel spectra for *Busycon* hemocyanin are given in A′ and B′. Spectrum A was recorded at 9.21-GHz and 2-mW microwave power. Spectrum A′ was recorded at 9.188-GHz and 10-mW microwave power.

of previously[6] characterized class II mixed-valent derivatives of hemocyanin. First, d–d transitions of a tetragonal cupric site are present at ~700 nm. Next, charge-transfer transitions (sulfur to copper(II)) are observed at 355 and 460 nm, energies similar to those expected from tetragonal cupric model studies.[15] Finally, an intense transition is observed in the near IR (abs = 1300 nm, ϵ = 3800 M^{-1} cm^{-1}) which in half met hemocyanins and half met-Br^- tyrosinase is assigned as an intervalence transfer transition.

Interpretation of this 2-mercaptoethanol form as a half met-2-mercaptoethanol derivative requires a one-electron reduction of the met (resting) form by the first mercaptan followed by coordination of a second. This mechanism can be tested by direct treatment of a half met derivative with 2-mercaptoethanol. The EPR spectrum obtained by the addition of 50-fold excess 2-mercaptoethanol to half met-acetate *Busycon canaliculatum* hemocyanin is shown in Figure 4A′. The EPR intensity amounts to 70% conversion from the original half met-acetate hemocyanin, with approximately 20% of the remaining protein observed as a gain in the intensity of the 345-nm oxy band in the optical spectrum. Reaction of 50-fold excess 2-mercaptoethanol with the met hemocyanin derivative produces an analogous EPR signal which accounts for 20% of the total copper and a 30% increase in the oxy absorption spectrum. (Data on *Busycon* are presented here as no reaction was observed with half met or met *Limulus* hemocyanin for 2-mercaptoethanol concentrations kept low enough to limit protein denaturation. This is in accord with the much lower ligand substitution reactivity generally exhibited by *Limulus* derivatives and interpreted in terms of limited active site accessibility.[7]) The *g* values for *Neurospora* tyrosinase ($g_\parallel$ = 2.19, $g_\perp$ = 2.05) compare favorably with those obtained for *Busycon* ($g_\parallel$ = 2.17, $g_\perp$ = 2.10) as do the electronic absorption spectral features. The almost quantitative direct conversion of the half met-acetate form to the 2-mercaptoethanol derivative of the met reaction strongly supports its assignment as the half met-2-mercaptoethanol form.

C. Dimer Tyrosinase. The met (resting) form of *Neurospora* tyrosinase (0.5 mM) was treated with a twofold excess of H_2O_2 in pH 6.3 phosphate buffer to obtain oxytyrosinase.[3] Approximately 5 min after addition of peroxide the pale yellow color of the met (resting) enzyme was replaced by a reasonably intense bluish green color, indicating the conversion to oxy. This protein solution was then evacuated and flushed with oxygen-free nitrogen several times over a period of ~15 min during which the bluish green color of oxy was gradually lost by removal of oxygen, yielding the deoxy form. (All manipulations of tyrosinase were carried out in an ice bath or in a 4 °C cold room.) The deoxytyrosinase was equilibrated with 1 psig of nitric oxide for 5 min.

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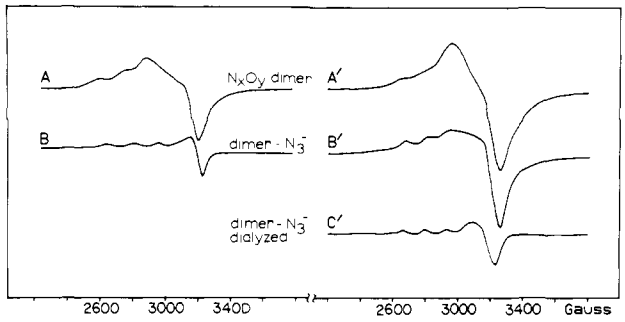

Figure 5. EPR spectra (77 K, pH 6.3, phosphate buffer) of (A) dimer tyrosinase and (B) dimer tyrosinase after addition of excess azide. Parallel spectra for *Limulus* hemocyanin are given in A′ and B′, and the spectrum for *Limulus* dimer-N_3^- after dialysis is given in C′.

This was followed by admitting 100 μL of air with a syringe to the 10-mL vessel containing 1-mL of protein solution. After 60 s of vigorous stirring the pale yellow color became bright gold and all traces of nitric oxide and nitrogen dioxide were removed by evacuation (taking care to prevent foaming of the protein) and flushing with nitrogen.

The EPR spectra of the *Neurospora* tyrosinase after reaction with NO is shown in Figure 5A. This intense broad spectrum, which corresponds to 45% of the sites, is characteristic of dipolar coupled copper(II) ions as found in the dimer forms of hemocyanin[6,7,10,16] and mushroom tyrosinase.[10] The EPR spectrum of the dimer form of *Limulus polyphemus* hemocyanin is presented in Figure 5A′. The dimer EPR spectrum of *Neurospora* tyrosinase confirms other results[2] classifying this protein as containing a binuclear copper active site. The inability of earlier studies[11a] to prepare dimer tyrosinase from *Neurospora* may have been a result of not providing trace amounts of oxygen which have been shown to be necessary in preparing the dimer form of hemocyanin.[6,7,16c]

Dimer tyrosinase rapidly reacts with a 50-fold excess of NaN_3 as shown in Figure 5B. The broad intense dimer EPR signal is replaced by a weak copper(II) EPR spectrum corresponding to 10% of the sites. This signal is too low in intensity to determine if it is a dimer-N_3^- form or if complete conversion to met has occurred (with a small residual half met signal). For hemocyanin, addition of N_3^- to the dimer form produces[6,7] an intermediate dimer-N_3^- derivative with a ~30 % decrease in intensity (Figure 5B′). This derivative further converts to the EPR-nondetectable met with a small mononuclear cupric EPR signal remaining, associated with half met, which is also produced in the NO reaction (Figure 5C′).

D. Oxy and Met Tyrosinase. The room- and low-temperature absorption and circular dichroism spectra of oxy and met (resting) *Neurospora* tyrosinase in the 300–800-nm region are presented in Figure 6. As low-temperature spectra must be obtained by dialysis against sucrose (followed by addition of 2–5-fold excess H_2O_2 to generate oxy) for an optical quality glass some enzyme browning was unavoidable. This produces an absorption background increasing into the UV; however, reasonable low-temperature spectra could still be obtained in the 425-nm region. Parallel oxy and met spectra for *Limulus* hemocyanin are presented in Figure 6A′–D′.

Comparision between the met (resting) and oxytyrosinase show that both exhibit ligand field contributions to the absorption and CD spectra at 680 nm. No other features can be distinguished in the met spectra to higher energy. Therefore, the dominant oxy spectral features can be assigned as $O_2^{2-} \rightarrow Cu(II)$ charge-transfer transitions. These include the intense peak in absorption and CD at 345 nm, the 590-nm band in absorption which appears as a minimum at this energy in the positive CD spectrum, and the CD peak at 520 nm which contributes to the absorption overlapping the high-energy side of the 590-nm band. All these features are

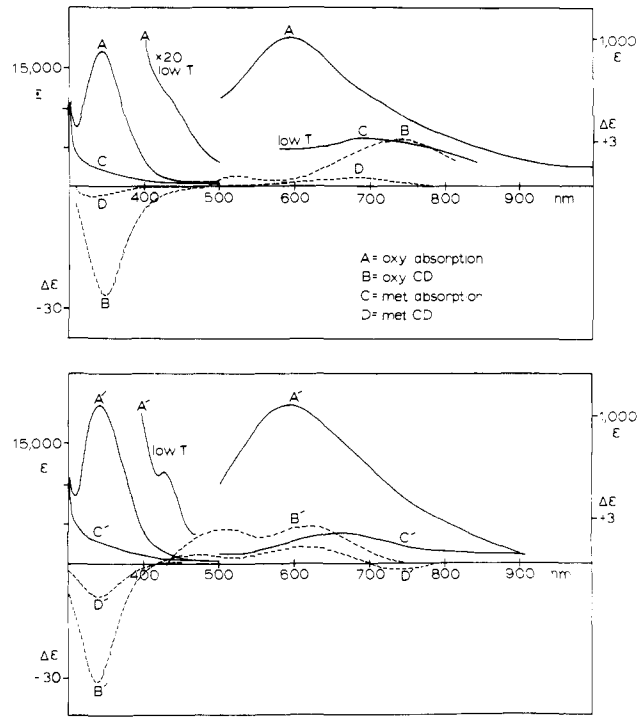

Figure 6. Optical absorption (—, room temperature and ~15 K in the 425-nm region) and circular dichroism (---, room temperature) spectra of (A, B) oxytyrosinase and (C, D) met tyrosinase. Parallel spectra of *Limulus* hemocyanin are given in A′–D′.

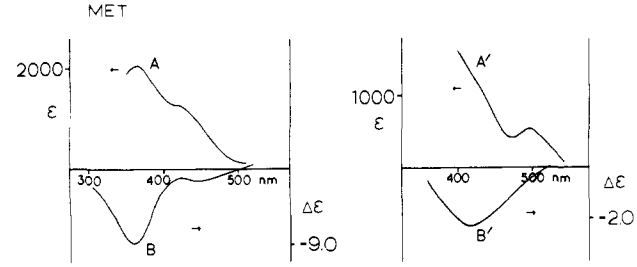

Figure 7. (A) Optical absorption and (B) circular dichroism (room-temperature difference spectrum, pH 6.3, phosphate buffer) of the charge-transfer region of met-N_3^- *Neurospora* tyrosinase. Parallel spectra of *Limulus* hemocyanin are given in A′ and B′.

also contained in the hemocyanin spectrum.[6] Some spectral variation is observed in the visible CD region; however, this is simply associated with the ligand field transitions being to higher energy and the 520- and 590-nm bands being more split in energy and less mixed in absorption and CD intensity in the hemocyanins. Finally, the hemocyanins[6a] exhibit a shoulder at 425 nm assignable as phenolate (or oxo) to Cu(II) charge transfer from an endogenous ligand. This band can also be resolved in the oxy tyrosinase absorption spectrum at low temperature.

Addition of azide to met (resting) tyrosinase (Figure 7) generates the met-N_3^- derivative. Small spectral shifts in the ligand field region are observed (abs = 690 nm) and an $N_3^- \rightarrow Cu(II)$ charge-transfer transition develops in the near UV. This transition has peaks in the absorption and CD spectra at 360 nm (ϵ = 2100 M^{-1} cm^{-1}, $\Delta\epsilon$ = −8.7 M^{-1} cm^{-1}) and at 420 nm (ϵ = 1400 M^{-1} cm^{-1}, $\Delta\epsilon$ = −2.1 M cm^{-1}) and can be used to determine the azide-met tyrosinase binding constant (k_{tyros} = 3000 M^{-1}). Analogous met-N_3^- absorption and CD spectra for *Limulus* hemocyanin[7] are presented in Figure 7A′,B′ (k_{Lim} = 10 M^{-1}).

Finally, it was observed that addition of azide to the met (resting) enzyme initially eliminates absorption at 345 nm. This is associated with the displacement of peroxide from the 20% intrinsic oxytryosinase present in the met (resting) enzyme. For a quantitative comparison to earlier results on oxyhemocyanins,[6,7] the pseudo-first-order rate constants for azide (×100-fold) displacement of peroxide from oxytyrosinase at 4 °C was measured

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as $k_{N_3^-\text{-tyros}} = 0.95\ h^{-1}$. Ligand displacement of peroxide (not oxygen, as determined by an oxygen electrode) from oxytyrosinase was also observed with the organic substrate inhibitor *l*-mimosine with a pseudo-first-order rate constant $k_{\text{mimosine-tyros}} = 162\ h^{-1}$. Representative values for pseudo-first-order rates for peroxide displacement from oxyhemocyanins are as follows:[7] azide, $k_{\text{Lim}} \ll 10^{-4}\ h^{-1}$, $k_{\text{Busycon}} = 0.002\ h^{-1}$, $k_{\text{Cancer}} = 0.04\ h^{-1}$; mimosine, $k_{\text{all hemocyanins}} \ll 10^{-4}\ h^{-1}$.

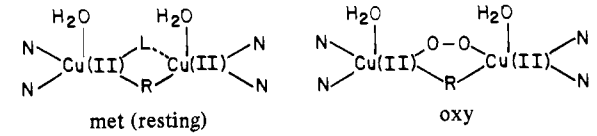
Discussion

The chemistry and spectroscopy of the tyrosinase derivatives demonstrate that the geometric and electronic structure of the active site of this enzyme is extremely similar to that of oxyhemocyanin, the quantitative differences being no more significant than those found among five mollusc and five arthropod hemocyanins previously studied.[7] Exogenous ligand bridging at the binuclear copper active site of tyrosinase is demonstrated through the half met studies. First, ligands are tightly bound by the half met site as illustrated by half met-NO_2^- and N_3^- after removal of excess ligand. Further, coordination of CO to the copper(I) directly perturbs the copper(II) EPR spectrum of half met-N_3^- tyrosinase. Finally, a low-energy $N_3^- \rightarrow$ Cu(II) charge-transfer transition is present in half met-N_3^- and class II mixed-valent properties dominate the spectrum of half met-Br^- tyrosinase (delocalized copper hyperfine in the EPR spectrum and an intervalence transfer transition at ~800 nm). Extensive chemical and spectroscopic studies on the half met series of hemocyanins have demonstrated[6b] that these properties require the exogenous ligand to bridge the two coppers. The unusual EPR signal of the 2-mercaptoethanol derivative of resting tyrosinase[11] and of met hemocyanin can now be interpreted. Its chemistry and spectroscopy define this form as a member of the half met series (the half met-2-mercaptoethanol derivative) with very delocalized mixed-valent properties. It is a class II form as copper(II) ligand field and sulfur to copper(II) charge-transfer transitions are still present; however, a very low-energy intense intervalence transfer (IT) transition is also observed. The half met-N_3^- derivative of mollusc hemocyanins also exhibits very delocalized class II mixed-valent optical properties and has a similar unresolvable hyperfine splitting in its EPR spectrum.[6b]

On the basis of detailed studies on hemocyanin derivatives, the results for tyrosinase also require the presence of an endogenous bridge between the coppers. First, for the half met derivatives, a group 1–group 2 ligand behavior is indicated by half met-NO_2^- and N_3^-. The half met-NO_2^- EPR spectrum does not change upon addition of 100-fold excess NO_2^-, while for half met-N_3^- addition of excess N_3^- results in EPR spectral changes associated with coordination of a second N_3^- to the Cu(II) site. From detailed hemocyanin studies,[6] NO_2^- is a group 1 ligand with a <3.5-Å Cu(II)–Cu(I) bridging distance, while azide is a group 2 ligand which bridges end-to-end keeping the coppers >5 Å apart. The >5-Å Cu–Cu distance ruptures an endogenous bridge and provides a second coordination position on the copper(II). An analogous effect associated with variation in the endogenous bridge is found for the binuclear cupric derivatives in the conversion of dimer to met (resting) tyrosinase. The dipolar nature (essentially no exchange coupling) of the dimer tyrosinase EPR signal requires a >5-Å Cu(II)–Cu(II) distance and the rupture of the endogenous bridge by an N_xO_y coupling product of the NO + O_2 reaction (a group 2 ligand effect). In analogy to reactions observed for dimer hemocyanin, azide displaces the N_xO_y ligand, allowing the coppers to eventually reform the endogenous bridge. This provides an effective pathway for superexchange between the copper(II)'s producing the EPR-nondetectable met (resting) derivative.

It is interesting to note that for *Neurospora crassa* met (resting) and half met tyrosinase, excess group 2 ligand (N_3^-) does not lead to irreversible disruption of the binuclear copper unit. This disruption was observed[6g,7,17] for the arthropod (but not *Limulus*) hemocyanins and was associated with the protein ligand either on a tertiary or quaternary level. The fact that *Neurospora crassa* tyrosinase, in contrast to the highly aggregated hemocyanins, is a monomeric protein may be significant with respect to this active site stability.

Chart I



Studies of azide binding to met hemocyanins were particularly informative[7] in understanding the large differences in catalase activity between the arthropods and molluscs. This ability of the hemocyanins to dismutate peroxide is associated with the peroxide binding to the met[7,18] to regenerate oxy, which can be considered a met-peroxide form. Arthropod and mollusc met-N_3^- derivatives exhibited very different charge-transfer and ligand field spectra with the arthropod also having a low azide binding constant, all consistent with a structural distortion of the arthropod site. This directly correlates with its low affinity for peroxide as the arthropods have very low catalase activity.[8] The met-N_3^- charge-transfer spectrum of tyrosinase is very different from that of *Limulus* (Figure 7) and the equilibrium constant for azide binding is the highest of the mets ($K_{\text{Lim}} = 10\ M^{-1}$, K_{Busycon}[7] $= 500\ M^{-1}$, $K_{\text{tyros}} = 3000\ M^{-1}$). This should relate to the ability of met (resting) tyrosinase to bind peroxide and, in fact, oxytyrosinase is strongly favored in the reaction of met with peroxide.[3] There are also smaller charge-transfer spectral differences between met-N_3^- tyrosinase and mollusc hemocyanin. For tyrosinase, the absorption and CD spectra show only two bands in the charge-transfer region and thus do not require the azide to bridge. (Bridging would generate a total of four transitions.) The details of these structural differences are presently being probed by resonance Raman studies into these $N_3^- \rightarrow$ Cu(II) charge-transfer transitions.[19] Spectral differences are also observed for oxytyrosinase as compared to the oxyhemocyanins, particularly in the visible CD spectral region. These differences are, however, straightforwardly interpreted in terms of the transition dipole vector coupling model applied to the hemocyanins.[6a] Two $\pi_v^* \rightarrow$ Cu(II) charge-transfer transitions are still present (CD peak at 520 nm and absorption peak at 590 nm), demonstrating a bridging mode for the peroxide, and the $\pi_\sigma^* \rightarrow$ Cu(II) charge-transfer transition at 345 nm is comparable to that of oxyhemocyanin, indicating a μ-dioxo linkage. The spectral differences in the visible region suggest limited mixing of the π_v^* components. In parallel to the met-N_3^- charge-transfer spectrum of tyrosinase, this spectral perturbation in oxy is consistent with a small distortion of the site relative to the hemocyanins, but not significantly larger than variations observed among the hemocyanins from different phyla.

Thus, the met and oxy hemocyanin "type 3" copper effective structural models[6a] can now be applied to met (resting) and oxy tyrosinase (Chart I). The question, then, which must now be addressed is what makes tyrosinase an enzyme. The rates for ligand displacement of peroxide from the oxy site by N_3^- and *l*-mimosine (organic substrate inhibitor) seem to directly relate to this question. Tetragonal copper(II) ligand displacement is known[20] to proceed through associative axial substitution. Although the hemocyanins and tyrosinase have very similar sites, large decreases in the rates of peroxide displacement are observed for the hemocyanins.

This would seem to be a kinetic effect and relate to access to the axial position of the "type 3" site.[7] This then suggests a picture for tyrosinase as a hemocyanin "type 3" copper site with high accessibility for organic substrates. Observations in the literature[11,21] of the relative reactivity of tyrosinase as compared to

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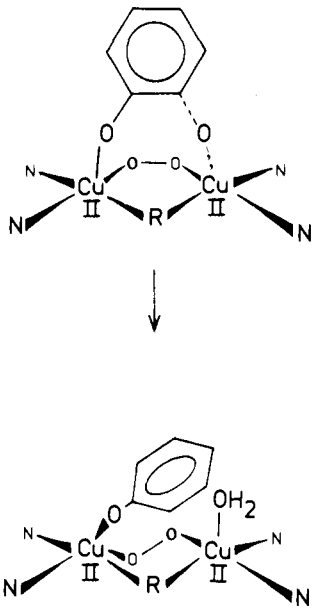

Figure 8. Proposed interaction of the oxy site effective structure with monophenolic (—) and diphenolic (---) substrates.

hemocyanins are consistent with this picture and certain hemocyanins exhibit low phenolase activity.[22] Finally, ligand displacement of peroxide by a substrate inhibitor supports the idea that a ternary complex is formed between the oxy "type 3" copper site and the substrate. Figure 8 demonstrates how the effective structure of the oxy "type 3" site is well-suited for reactions[23] with *o*-diphenol and monophenol under normal enzymatic turnover. In the case of catecholase activity, the "type 3" site is geometrically correct for coordination of both phenolic oxygens in ortho positions axially to both coppers with a M-M distance of ~3.6 Å. Oxidase activity is not observed with *m*- and *p*-phenols.[24,25] For cresolase activity, coordination of the monophenol to one copper would be followed by a rearrangement to a trigonal-bipyramidal intermediate as generally associated with square-planar and tetragonal associative substitution chemistry. This has the effect of labilizing the peroxide from one copper leaving a geometrically correct ortho-substrate-position-activated peroxide complex. Orientation of the monophenolic substrate by amino acid side chains constituting the active site is certainly another important factor for the observed specificity in hydroxylation. This deprotonated peroxide coordinated to only one copper should be extremely reactive and either directly hydroxylate the substrate or provide an oxene intermediate through the heterolytic polarization of the peroxide by coordination to one copper(II).

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Stereospecificity of Enzymatic Dehydrogenation during Tiglate Biosynthesis

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Abstract: (2*RS*,3*S*,4*S*)-[2-^{14}C,4-$^{3}H_1$]Isoleucine has been prepared by stereospecific synthesis, using LiAl[$^{3}H_4$] reduction of optically pure 2-butene oxide to introduce tritium stereospecifically at the prochiral methylene group of 2-butanol. The tritiated alcohol was carried forward in a malonic ester synthesis with ^{14}C-labeled dimethyl malonate, followed by Schmidt reaction of the *sec*-butyl malonic acid, to afford doubly labeled isoleucine. Feeding this substrate to *Datura innoxia* plants led to isolation of 3α,6β-ditigloyloxytropane, from which tiglic acid was obtained by hydrolysis and degraded to locate the labels. More than 95% of the tritium was retained in the tiglic acid, allowing the conclusion that the enzymatic dehydrogenation involves antiperiplanar elimination of the hydrogen at C-2 and the *pro-R* hydrogen at C-3 of (2*S*)-2-methylbutanoic acid.

Metabolism of isoleucine,[2] like that of other branched-chain amino acids, begins with transamination to the α-ketoacid followed by oxidative decarboxylation, yielding 2-methylbutanoyl coenzyme A (scheme I). The next metabolic step is enzymatic dehydrogenation to tigloyl coenzyme A. This dehydrogenation occurs in plants and microorganisms as well as in animals. It has been shown in the laboratories of Leete[3] and Wooley[4] that the tiglic acid component of the tropane ester alkaloids is derived specifically from isoleucine and 2-methylbutanoic acid in *Datura meteloides*, and Crout has shown[5] that isoleucine is the precursor of the geometric isomer angelic acid in *Cynoglossum officinale*. Neither the 2-hydroxy nor the 3-hydroxy derivatives of 2-methylbutanoic acid serve as precursors to tiglate,[4a,6] and angelate is not converted

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