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Magnetic Dipole–Dipole Coupled Cu(II) Pairs in Nitric Oxide-Treated Tyrosinase: A Structural Relationship Between the Active Sites of Tyrosinase and Hemocyanin

(O_2 binding/Cu proteins/electron paramagnetic resonance)

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ABSTRACT **The T_r and T''' states of tyrosinase were treated with NO. EPR spectra of the products observed at 14°K and at 113°K showed mixtures of two signals. One had components in the region of g = 2, about 1200 G wide, and in the region of g = 4, showing hyperfine splitting. The other signal was similar to that arising from isolated Cu(II) ions in an axially symmetric environment. The first signal was indicative of $\Delta m = 1$ and $\Delta m = 2$ transitions arising from magnetic dipole–dipole coupled Cu(II) ion pairs. It closely resembled previously reported EPR spectra obtained from NO-treated hemocyanin, which were confirmed in this study. The normal Curie behavior of the signals between 230°K and 14°K ruled out significant exchange coupling between the ion pairs. The $\Delta m = 2$ signals were not saturable up to 350 mW at 14°K. The broad $\Delta m = 1$ signals could be separated from accompanying signals by the saturation characteristics of the latter at about 10 mW at 14°K. The results establish the presence of a pair of copper ions at the active site of tyrosinase, and a close structural relationship between this active site and that of hemocyanin.**

Tyrosinase, an oxygen- and 4-electron transferring oxidase found throughout the plant and animal kingdoms, catalyzes formation of *o*-quinones from monophenols and from catechols (1, 2):

$$\text{Monophenol} + O_2 = o\text{-Quinone} + H_2O$$

$$2\ o\text{-Diphenol} + O_2 = o\text{-Quinone} + 2\ H_2O$$

The enzyme contains copper (4 atoms per 120,000 daltons in potato and mushroom) (3, 4). It may be reconstituted from apo-protein and Cu(II) (3–5), but has no intrinsic EPR absorption in its resting state, T_r; the enzyme binds CO in the presence of substrate (3, 5, 6). While neither the oxidation state of the copper in the resting enzyme nor the number of copper atoms at each active site has been established, the enzyme, T_r, reacts with molar equivalents of H_2O_2 to form a blue compound, T'', from which O_2 can be removed reversibly (7). The O_2-binding nonblue state, T''', resembles the respiratory copper protein, hemocyanin; the absorption spectrum of its oxygenated form, T'', closely resembles that of O_2-hemocyanin (7). Schoot Uiterkamp has reported that NO-treated hemocyanin (*Helix pomatia*) displays an EPR signal characteristic of magnetic dipole–dipole coupled Cu(II) pairs (8). If tyrosinase and hemocyanin are structurally related copper proteins, T''' should then contain contiguous pairs of Cu(I) atoms at its active site, and T_r should contain contiguous pairs of Cu(II) atoms, its catalytic mechanism being similar to one already proposed (2). In the present study, Schoot Uiterkamp's observation with *Helix pomatia* hemocyanin has been repeated, and confirmed with *Cancer magister* hemocyanin. The technique has been extended to an examination of copper atoms in NO-treated resting tyrosinase, T_r, and deoxygenated, H_2O_2-treated tyrosinase, T''', at 14°K.

Abbreviation: EPR, electron paramagnetic resonance.

MATERIALS AND METHODS

Preparation of Tyrosinase and Hemocyanin. Alpha-tyrosinase was prepared from mushrooms, *Agaricus bispora*, by a modification of the method of Nelson and Mason (9). It was a representative high-cresolase enzyme: total Cu, 0.192%; EPR-detectable Cu, less than 1% of total Cu; catecholase activity: 1011 chronometric (10) units/mg of protein; *t*-butylcatecholase, 215 units/mg; *p*-cresolase, 307 units/mg; 10% of the enzyme Cu was oxygenatable in the resting state. Hemocyanin (*Cancer magister*) was prepared according to Thomson *et al.* (11). After clotting, the product was sterilized by ultrafiltration; $A_{350}/A_{280} = 0.175$ at pH 8.5 in the presence of 0.01 M Mg(II); total Cu, 0.175%; EPR-detectable Cu, less than 1%. Alpha-hemocyanin (*Helix pomatia*) was prepared according to Konings *et al.* (14). It contained total Cu 0.240%; EPR-detectable Cu, less than 1% of total Cu; ΔA_{346}/mg = 0.320 at pH 6.8.

The Reactions of Tyrosinase and Hemocyanin with NO. All experiments were performed in 0.1 M Na phosphate (pH 6.8). EPR spectra were recorded with a Varian V-4500 spectrometer at a modulation amplitude of 9.7 G and modulation frequency of 100 kc. Low temperatures at the sample were produced and controlled by a Varian variable temperature accessory and dewar, using N_2 or He gas cooled by liquid N_2 or liquid He, respectively. Reactions between NO and tyrosinase or hemocyanin were performed (8) for 20 min, except that the anaerobic EPR reaction tubes were kept at 0° in an ice–water bath to prevent frothing of protein and undue evaporation of solution as a result of evacuation; evaporation was kept to less than 5%. After the reaction was complete, samples were frozen rapidly in liquid N_2. EPR intensities were calculated (8), unless otherwise noted, by use of standard Cu(II), 1.00 mM, containing 10.0 mM EDTA.

NO Derivatives of Resting Tyrosinase, and of T'''. Untreated resting tyrosinase at 14°K showed a signal around g = 2 corresponding to less than 1% of the total copper concentra-

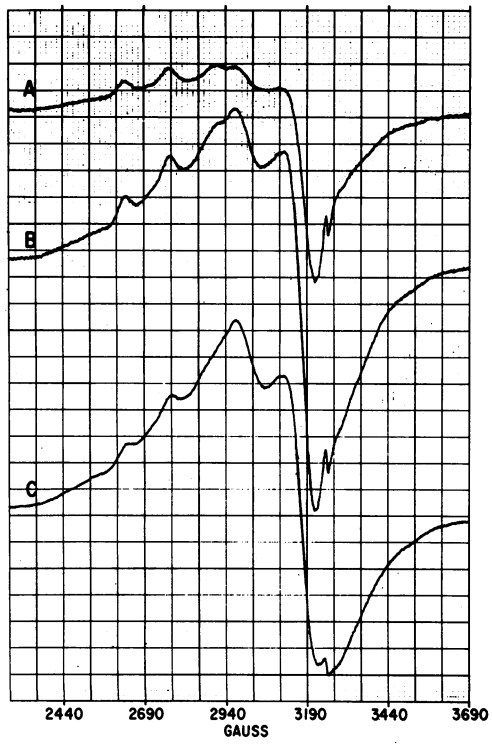

FIG. 1. EPR spectra around g = 2 of the nitric oxide–T'''-tyrosinase reaction product. T = 14°K; power (A) 0.1 mW; (B) 1 mW; (C) 10 mW.

tion; no signal was observed in the g = 4 region. Resting tyrosinase (0.2 ml, total Cu = 1.22 mM; oxygenatable Cu = 0.12 mM), faint green, gave no visible color change upon reaction with NO, and showed qualitatively the same EPR spectra around g = 2 and g = 4 as observed with T''' (described below), although the signal amplitudes corrected for differences in concentration were 30% or less, and probably due to intrinsic T'''.

For the preparation of the NO derivative of T''', 0.2 ml of deoxygenated resting tyrosinase (total Cu, 2.0 mM; oxygenatable Cu, 0.2 mM) was converted into T''' at room temperature by addition of 7.5 μl of 31 mM H_2O_2 under N_2 and a counterflow of N_2. After H_2O_2 addition, the sample was kept deoxygenated for 15 min before being cooled to 0° for the reaction with NO. It was shown in a separate experiment that aerobic reaction conditions, but without NO, led to essentially complete formation of T''. The NO compound had a yellow–green color.

NO Derivatives of Cancer magister and Helix pomatia Hemocyanins. The hemocyanins were treated with NO in the manner described in the previous section, using 0.3 ml, 64 mg/ml (*Cancer magister*) or 0.2 ml, 70 mg/ml (*Helix pomatia*). The products had a yellow–green color as reported (8).

RESULTS

The reaction products of NO with the T''' state of tyrosinase, and with the hemocyanins, consistently showed EPR spectra similar to those already described for *Helix pomatia* hemocyanin–NO (8): broad signals 1200 or more gauss wide in the g = 2 region, and characteristic signals with much smaller amplitude (about 1.5%) around g = 4. Figs. 1*A* and 2*A* depict the signals around g = 2 from the T''' state of tyrosinase (1*A*) and from *Helix pomatia* hemocyanin (2*A*) taken at 14°K and 0.1 mM. Both show mixtures of two types of copper signals, one of which, about 1400 G wide, we ascribe to magnetic dipole–dipole coupled pairs of Cu(II) ions and the other, with characteristic hyperfine splitting and about 750 G wide, to single, i.e., isolated, Cu(II) ion sites. The absorption due to isolated Cu(II) ion sites was estimated to arise from 20 ± 5% of the copper in the protein, that due to coupled ion pairs has not been quantitated. In addition to the copper signals, Figs. 1 and 2 show the presence of an unknown radical at g = 2, in an amount much less than 1% of the copper in the protein. The g = 2 signal from *Cancer magister* hemocyanin-NO at 113°K and 10-mW incident power is shown in Fig. 3. It comprises Δm = 1 signal from a predominant coupled $Cu(II)_2$ site, admixed with a small amount of signal from isolated Cu(II) ion. Incubation of *Cancer magister* hemocyanin with NO for less than 20 min produced products showing higher ratios of isolated Cu(II) to coupled $Cu(II)_2$.

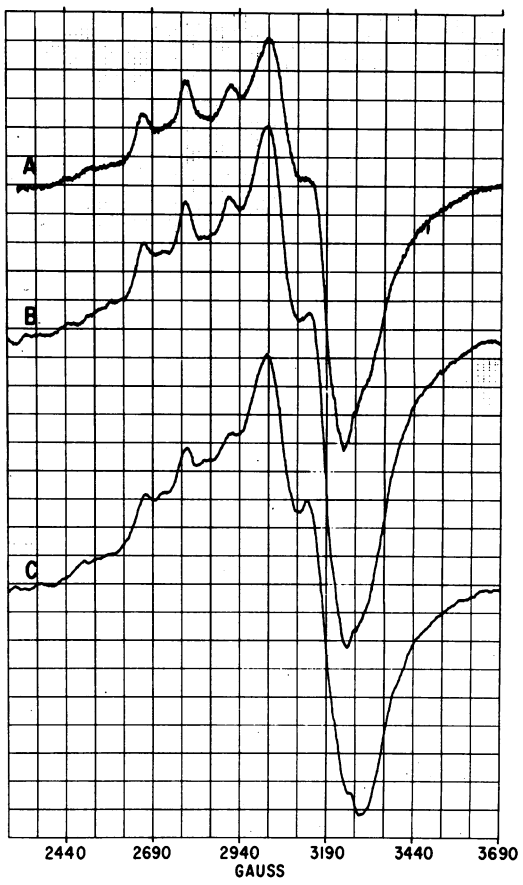

FIG. 2. EPR spectra in the g = 2 region of the *Helix pomatia*–NO reaction product. T = 14°K; power (A) 0.1 mW; (B) 1 mW; (C) 5 mW.

The g = 2 signals arising from the NO derivatives of T''' and the hemocyanins from *Cancer magister* and *Helix pomatia* measured at 230°K and 10-mW incident power, 113°K and 10-mW incident power, and at 14°K and 0.1-mW power, showed normal Curie behavior of the signal intensities. However, at 14°K the Δm = 1 signals from the coupled $Cu(II)_2$ site, and from the lone Cu(II) site could be readily distinguished by saturating the latter. In addition, the Δm = 2 signals were much better resolved at 14°K than at 113°K. The Δm = 2 signals arising from the NO products of T''' and the hemo-

cyanins, at 14°K and 200-mW power, are depicted in Fig. 4. These spectra contain more- or less-well-resolved 7-line hyperfine structure, differences in resolution being due to differences in line widths. A power dependence study of the $\Delta m = 2$ signal of the NO derivative of *Cancer magister* hemocyanin at 14°K showed no saturation between 10 and 355 mW. Comparison with the same $\Delta m = 2$ signal at 113°K and 200 mW showed normal Curie behavior of the signal intensities.

The $\Delta m = 1$ signal arising from the NO product of T''' at 14°K and 1-mW or 0.1-mW power and from *Helix pomatia* hemocyanin at 14°K, showed no saturation between 10 and 355 mW. Comparison with the same $\Delta m = 2$ signal at 113°K and 200 mW showed normal Curie behavior of the signal intensities.

The $\Delta m = 1$ signal arising from the NO product of T''' at 14°K and 1-mW or 10-mW power and from *Helix pomatia* hemocyanin at 14°K and 1-mW or 5-mW power, are depicted in Fig. 1, *B* and *C* and Fig. 2, *B* and *C*. Comparison of these spectra with the corresponding spectra observed at 14°K and 0.1 mW (Fig. 1*A* and Fig. 2*A*) shows a considerable decrease in intensity of the narrow signal attributed to isolated Cu(II) with increase of incident power; the predominant $\Delta m = 1$ signal from the NO product of *Helix pomatia* hemocyanin resembles that obtained by manual subtraction of the isolated Cu(II) ion signal from the spectrum measured at 96°K and 10-mW incident power (ref. 8, Fig. 3).

The NO derivative of resting tyrosinase, T_r, showed the same qualitative spectra around g = 2 and g = 4 as described for the derivative from the T''' state, although the signal amplitudes corrected for differences in concentration were less than 30% as large. We have observed that resting tyrosinase contains variable amounts of oxygenatable T''' without H_2O_2 treatment (R. L. Jolley, Jr., L. Evans, and H. S. Mason, unpublished observation), and we ascribe the signal obtained from NO-treated resting tyrosinase to this source.

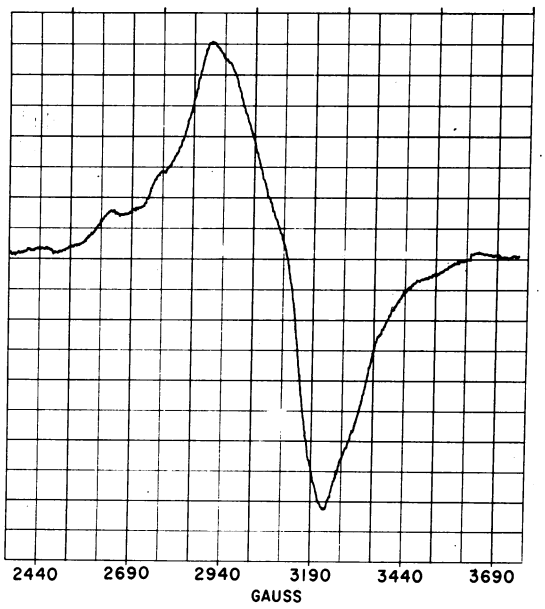

FIG. 3. EPR spectrum in the g = 2 region of the *Cancer magister* hemocyanin–NO reaction product. T = 113°K; power = 10 mW.

DISCUSSION

The present study has confirmed and extended with *Cancer magister* hemocyanin (arthropodal) and with fungal tyrosinase results previously reported with *Helix pomatia* hemocyanin (molluscan), namely that the NO reaction products of the O_2-binding forms show EPR signals with broad absorption in the g = 2 region, and well-resolved signals with seven hyperfine lines in the g = 4 region, interpreted as arising from a pair of magnetic dipole–dipole coupled Cu(II) atoms at the NO-binding site (8, 12, 13) that give rise to $\Delta m = 1$ and $\Delta m = 2$ transitions around g = 2 and g = 4, respectively. The NO is presumably bound at the O_2-binding sites. The binding ratios, Cu/O_2 and Cu/CO for hemocyanin and substrate-reduced tyrosinase, are 2 (3, 15–17), a value consistent with the present observations that two copper atoms are present at the binding site. In addition to the $\Delta m = 1$ and $\Delta m = 2$ signals leading to an inference of contiguity, a signal typical of Cu(II) ions in an axially symmetric environment was observed. The EPR characteristics were estimated as: $g_\perp = 2.08$; $g_{||} = 2.30$; $A_\perp = 5 \times 10^{-4}$ cm^{-1}; $A_{||}$ (tyrosinase) = 145×10^{-4}cm^{-1}; $A_{||}$ (*Cancer magister*) = 140×10^{-4}cm^{-1}; $A_{||}$ (*Helix pomatia*) = 132×10^{-4}cm^{-1}.

The normal Curie behavior of the *Helix pomatia* signals between 230 and 96°K (8) has now been shown to be valid for both NO-treated hemocyanins and NO-treated T''' tyrosinase down to 14°K, putting an upper limit upon the exchange coupling constant J = 10 cm^{-1} (assuming an error of 5% made in comparison of the signal amplitudes). Therefore, exchange coupling contribution can be neglected in computer simulations that give the distance between the coupled Cu(II)

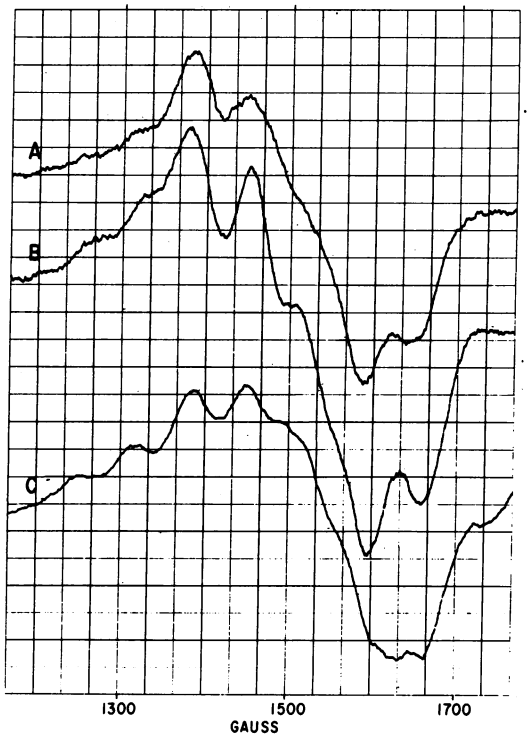

FIG. 4. EPR spectra around g = 4 ($\Delta m = 2$ dimer signals) from nitric oxide derivatives of tyrosinase and hemocyanin. T = 14°K, power, 200 mW. (*A*) Nitric oxide–tyrosinase, T'''; (*B*) nitric oxide–hemocyanin, *Helix pomatia*, (*C*) nitric oxide–hemocyanin, *Cancer magister*.

ions (12, 13). Dr. W. E. Blumberg has kindly pointed out to us that the case of two dipole-coupled spins having isotropic g values and not coupled to nuclear moments is shown to have a splitting, $\Delta H = g\beta/g_e r^3$, where g and g_e are the g values of the paired spins and the free electron, respectively, and r is the separation of the spins. He has calculated from our data for *Cancer magister* hemocyanin–NO (Fig. 3), ignoring the effects of $g_{||}$ and $A_{||}$, that r is approximately 3.9–4.7Å and that the vector joining the two copper atoms lies approximately parallel to $g_{||}$.

The $\Delta m = 2$ signals did not saturate up to 350-mW incident power at 14°K. This is understandable because of the very small transition probability of the $\Delta m = 2$ signals. Lack of saturation of $\Delta m = 2$ transitions makes it possible to detect dipolar coupling in solutions of Cu(II) ions at low concentrations at temperatures around 14°K and at high power. Signals arising from the $\Delta m = 1$ transition at 14°K should saturate at a very low power, making detection difficult. On the other hand, the differences in saturation behavior of the isolated Cu(II) ion signal, and the $\Delta m = 1$ transition of coupled Cu(II) ions, makes it possible to obtain well-resolved signals of the coupled form even in situations in which the signal arises principally from the isolated Cu(II) ions. Comparison of the spectra of the NO product of *T'''* at 14°K and at incident powers of 0.1, 1, and 10 mW (Fig. 1 *A*, *B*, and *C*, respectively) and the NO product of hemocyanin (*Helix pomatia*) at powers of 0.1, 1, and 5 mW (Fig. 2*A*, *B*, and *C*, respectively) shows a considerable decrease of the signal of the isolated Cu(II) ion upon an increase in incident power from 0.1 to 10 mW, or to 5 mW. At 14°K, the spectra arising from the isolated Cu(II) ions were suppressed at 300 mW; signals from coupled Cu(II) ion pairs, $\Delta m = 1$, were increasingly distorted at higher powers for both tyrosinase and *Helix pomatia* hemocyanin. The possibility that signal distortion arose from too-high modulation amplitudes (18) was ruled out by a study of the corresponding signal from *Cancer magister* hemocyanin. Modulation amplitudes between 0.1 and 9.7 G gave the same distortion.

It is not possible to interpret these results in terms of a mechanism of reaction between NO and hemocyanins, or *T'''*-tyrosinase, because a precursor–product relationship has not been demonstrated for the two types of EPR signal we have observed. The kinetics of the reactions giving rise to the two forms is not known, nor the stoichimetries, and spectral or susceptometric studies have not been made on the systems. The presence of other exchange coupled Cu(II) ion pairs cannot be completely excluded, nor is it known with certainty that any product of the NO reactions contains NO or NO^- associated with copper.

However, there is a striking resemblance of the product of the NO reaction with the *T''''*-tyrosinase and the product from two hemocyanins. Therefore, the evidence is strong that in all three proteins there are copper-containing sites that, upon reaction with NO, are converted to sites containing contiguous Cu(II) ions. In the light of the stoichiometry of the reactions between tyrosinase and hemocyanin with CO and O_2, the complete lack of paramagnetism of oxyhemocyanin and of resting tyrosinase at temperatures down to 4.3°K (T. Moss, G. Gould, A. Ehrenberg, J. Loehr, and H. S. Mason, unpublished results), and a lack of an intrinsic EPR signal from hemocyanin, oxyhemocyanin, and resting tyrosinase down to 14°K, as well as a close similarity between the absorption spectra of oxyhemocyanin and oxytyrosinase, *T''*, it appears very likely that the active sites of these copper proteins contain pairs of contiguous copper atoms. The magnetic similarities of tyrosinase and hemocyanins, and their spectral and functional likenesses point to a related evolutionary origin.

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