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EPR study of the dinuclear active copper site of tyrosinase from *Streptomyces antibioticus*

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Abstract The [Cu(I)–Cu(II)] half-met form of the dinuclear copper site of tyrosinase has been probed by continuous wave electron paramagnetic resonance (EPR) and hyperfine sublevel correlation (HYSCORE) spectroscopy in the presence and absence of inhibitors. In all cases the EPR spectrum is indicative of a $d_{x^2-y^2}$ ground state for the unpaired electron. From the cross-peaks observed in the HYSCORE spectra, proton hyperfine coupling constants were obtained that are compatible with a hydroxide ion in an equatorial coordination position of the paramagnetic copper. After changing the water solvent to D_2O or after addition of the inhibitors *p*-nitrophenol or L-mimosine, the proton signals disappear. The relevance of these findings for understanding the catalytic cycle is discussed.

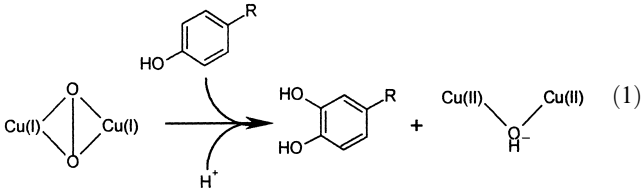
Key words: Tyrosinase; Half-met; Electron paramagnetic resonance; Hyperfine sublevel correlation spectroscopy; Type 3 copper protein

1. Introduction

The enzymes and oxygen transporters that contain a dinuclear copper site as active centre are distinguished in haemocyanins (Hcs), catechol oxidases (COs) and tyrosinases (Tys) according to their activity: Hcs are the oxygen transporting proteins of molluscs and arthropods, COs catalyse the conversion of *ortho*-diphenols to *ortho*-quinones (catecholase activity) and Tys exhibit the same activity as COs but have a broader substrate range in the sense that they can convert not only *ortho*-diphenols but also monophenols into *ortho*-quinones, presumably through an *ortho*-diphenol intermediate.

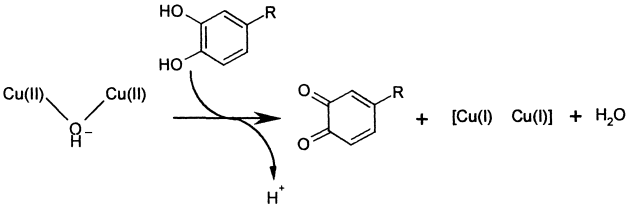
Structural evidence in the case of Hc and CO [1–4], and spectroscopic evidence in the case of Ty [5], shows that the structures of the Cu_2 centres are highly similar and that each Cu is coordinated by at least the Nε atoms of three histidines. Moreover, it has been established by XRD investigations that oxygen binds to the reduced Cu_2 centre in Hc in a μ-η^2:η^2 mode [6]. In CO and Ty oxygen most likely binds in the same fashion. The difference in activity between the three classes of enzymes/proteins is not ascribed to structural differences of the Cu_2 sites, but to differences in the shape and accessibility of the substrate binding cavities. This conjecture was recently placed on a strong footing when the experimentally determined structures of CO and various Hcs could be compared [4].

The present challenge is to understand the catalytic mechanism of the COs and Tys. Mechanistic investigations so far have established that the Ty-catalysed conversion of *monophenols* starts with the binding of the substrate to the oxy-form [Cu(I)–O_2–Cu(I)] of the enzyme and follows reaction 1 [7]:

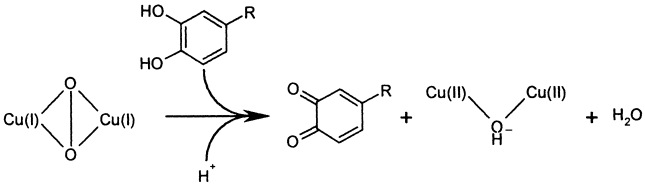


(1)

The reduced form, [Cu(I)–Cu(I)], and the oxidised or met-form, [Cu(II)–OH^-–Cu(II)], do not react with monophenols. The conversion of a *diphenol* to the corresponding quinone can be effected by the met-form as well as by the oxy-form of CO and Ty, according to reactions 2 and 3 [7]:



(2)



(3)

The Ty-catalysed conversion of a monophenol to a quinone can be considered the combined result of reactions 1 and 2. We note that the uptake of a proton in reaction 1 is compensated by the release of a proton in reaction 2. It is conceivable that this proton is never liberated during the reaction cycle. In a similar way the diphenolase cycle of CO and Ty consists of a combination of reactions 2 and 3.

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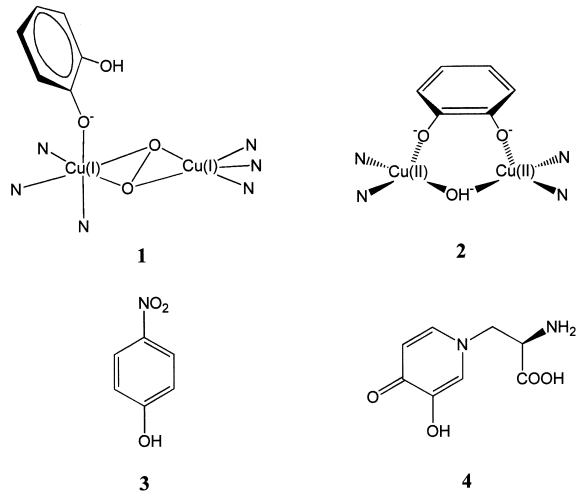
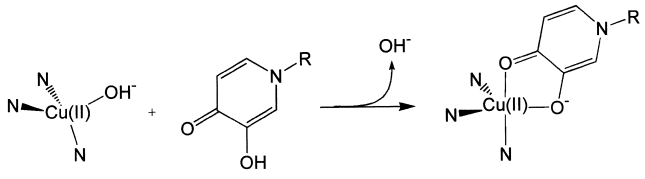


Scheme 1.

On the basis of a recent structure determination of CO, Krebs and co-workers have proposed a molecular mechanism for the *diphenolase* cycle [4]. They suggest that in one of the intermediate steps (reaction 3) the diphenol binds in a monodentate fashion to one of the coppers of the [Cu(I)–O_2–Cu(I)] oxy-form (see Scheme 1, **1**; the His ligands are symbolised by N). For the *monophenol oxygenase* activity of Ty Solomon and co-workers, on the basis of extensive electron paramagnetic resonance (EPR) spectroscopy of inhibitor-bound half-met [Cu(II)–Cu(I)] Ty, have proposed a mechanism whereby the hydroxy group of the incoming phenol binds at an axial coordination position of one copper in the [Cu(I)–O_2–Cu(I)] oxy-Ty and subsequently undergoes a Berry pseudorotation to an equatorial position [4]. The concomitant oxygenation at the *ortho* position is accompanied by stabilisation of the intermediate through a bidentate binding of the diphenol to the two coppers of the active site (Scheme 1, **2**), although in later work binding to a single Cu has not been excluded [7].

Here we report on EPR and hyperfine sublevel correlation spectroscopy (HYSCORE) experiments on the inhibitor-bound half-met tyrosinase of *Streptomyces antibioticus*. Small changes in the continuous wave (cw)-EPR spectrum are observed when the inhibitor is added to the protein solution. In the HYSCORE spectrum of half-met Ty, cross-peaks are observed that derive from a strongly coupled proton. Based on the size of the hyperfine interaction, these cross-peaks are attributed to the proton of an OH^- that is coordinated to Cu(II) in an equatorial position. When the protein is exchanged in D_2O or when an inhibitor is added to the solution, the cross-peaks disappear. These results shed new light on the binding mode of monophenols to the active site and on the conversion of monophenols to diphenols and quinones.



Scheme 2.

2. Materials and methods

The purification of *S. antibioticus* met-tyrosinase, the resting form of the enzyme, has been described elsewhere [8]. Selective reduction of one copper ion in the binuclear site was achieved according to the method given by Wilcox et al. [9] and resulted in the half-met derivative. The procedure entails the incubation of the tyrosinase with sodium nitrite under reducing conditions. The half-met form consists of a reduced Cu(I) and an oxidised Cu(II) ion (see below). The final concentration of the solution of the protein was 0.7 mM in 0.1 M phosphate buffer and the pH was 6.8. *para*-Nitrophenol (Scheme 1, **3**) and L-mimosine (Scheme 1, **4**) were purchased from Sigma. These substrate analogues were dissolved in the same buffer and added to the protein solution to a final concentration of 2 mM.

The microwave frequency for the cw-EPR experiments was 9.40 GHz for half-met tyrosinase and half-met tyrosinase with L-mimosine and 8.95 GHz for half-met tyrosinase with *p*-nitrophenol. The modulation field was 0.5 mT and the modulation frequency 100 kHz. The temperature was 77 K.

The HYSCORE experiments were performed at 6 K on a Bruker FT-EPR spectrometer using a $\pi/2$-τ-$\pi/2$-T_2-π-T_1-$\pi/2$ microwave pulse sequence with pulses of 16 and 24 ns. The magnetic field setting for half-met tyrosinase in H_2O and in D_2O was 335 mT, for half-met tyrosinase in H_2O with L-mimosine 337.5 mT and for half-met tyrosinase in H_2O with *p*-nitrophenol 332.5 mT. These settings correspond to the fields of maximum intensity in the ESE-detected EPR spectra. The microwave frequency was 9.75 GHz for the HYSCORE experiments on half-met tyrosinase in H_2O, in D_2O and in H_2O with L-mimosine and 9.73 GHz for the HYSCORE experiment on half-met tyrosinase with *p*-nitrophenol. The time τ between the first and second microwave pulses was fixed at 192 ns. In total, 512×256 data points were acquired in the T_1 and T_2 directions, respectively. Starting times of T_1 and T_2 were 168 ns and 136 ns and the time increments were 16 ns for both times. The background of the resulting two-dimensional modulation pattern was subtracted by a quadratic fit, first to the slices with fixed T_2 and then to the slices with fixed T_1. Apodisation was performed with a Hamming function. Finally, the data were zero-filled to 1024×512 points and transformed into the frequency domain by a two-dimensional Fourier transformation to obtain magnitude contour plots.

3. Results

3.1. Spectroscopy

The EPR spectra of frozen solutions of half-met tyrosinase and half-met tyrosinase incubated with *p*-nitrophenol or L-mimosine are shown in Fig. 1. Half-met tyrosinase (Fig. 1a) is characterised by an axial EPR spectrum ($g_{\parallel}=2.296$) and four copper hyperfine bands ($A_{\parallel}=13.76$ mT), indicative of a Cu(II) site with a $d_{x^2-y^2}$ ground state. No differences in line width were observed between the EPR spectra of half-met tyrosinase in D_2O and half-met tyrosinase in H_2O. When *p*-nitrophenol is added to the solution, the EPR spectrum (Fig. 1b) resembles that of half-met tyrosinase, with almost identical values for $g_{\parallel}$ (2.300) and $A_{\parallel}$ (14.07 mT). When L-mimosine is added to the solution of the protein, the EPR spectrum (Fig. 1c) shows slightly larger values for $g_{\parallel}$ (2.305) and for the hyperfine interaction at copper (15.32 mT), but the overall appearance of the spectrum remains the same. A nitrogen superhyperfine splitting is visible in the $g_{\perp}$ region of the spectrum and on the copper hyperfine band at low magnetic field.

The proton region in the HYSCORE spectrum of half-met tyrosinase in H_2O measured at the field of maximum intensity of the ESE-detected EPR spectrum is shown in Fig. 2a. Two strong cross-peaks are observed centred at about (12.4, 17.2) MHz and (17.2, 12.4) MHz. These peaks are shifted upward in frequency with respect to the antidiagonal that passes through the proton Larmor frequency of 14.26 MHz, which

reveals the presence of a sizeable anisotropic hyperfine interaction [10]. No intensity is observed at the corresponding diagonal positions of (12.4, 12.4) MHz and (17.2, 17.2) MHz. At slightly lower frequencies two weaker cross-peaks are observed at (11.8, 16.8) MHz and (16.8, 11.8) MHz. The diagonal peak at 15.625 MHz is a spectrometer artefact.

The proton region in the HYSCORE spectrum of half-met tyrosinase in D_2O is shown in Fig. 2b. The intensity in this region stems from the non-exchangeable protons as the contribution of the deuteriums is expected around the deuterium Larmor frequency of 2.2 MHz. Notably, the strong cross-peaks observed for half-met tyrosinase in H_2O are absent. Furthermore, the total intensity in the area around 14 MHz is considerably less than the corresponding intensity in the spectrum for half-met tyrosinase in H_2O (cf. Fig. 2a), which shows that a significant number of protons have been exchanged.

The proton regions in the HYSCORE spectra of half-met tyrosinase in H_2O with *p*-nitrophenol (Fig. 2c) or L-mimosine (Fig. 2d) also lack the strong cross-peaks. In the spectrum of half-met tyrosinase with L-mimosine, the weak correlation features that were observed for half-met tyrosinase in H_2O are still present, albeit distributed slightly asymmetrically around the Larmor frequency because of interference with the spectrometer artefact. In both cases, the signal to noise ratio in the area around 14 MHz is comparable to that of the HYSCORE spectrum for half-met tyrosinase in H_2O (Fig. 2a).

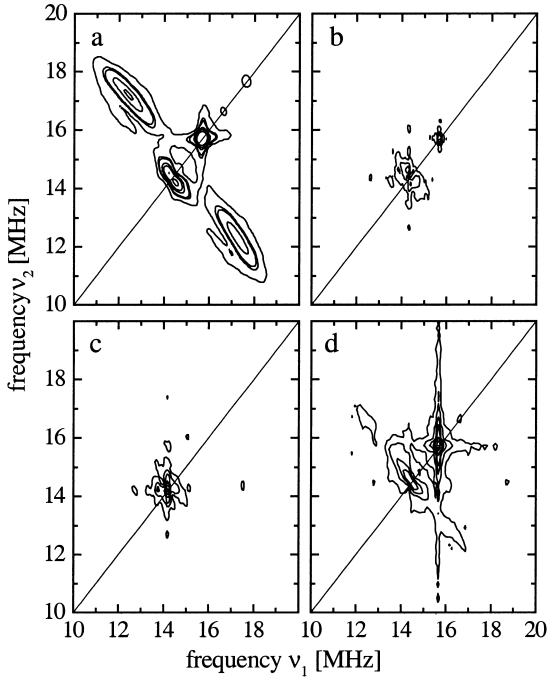


Fig. 2. Proton regions of HYSCORE spectra ($T = 6$ K) for frozen solutions of (a) half-met tyrosinase, (b) half-met tyrosinase in D_2O, (c) half-met tyrosinase with *p*-nitrophenol and (d) half-met tyrosinase with L-mimosine. The levels of the contours in the different plots are not comparable.

3.2. Analysis

The changes in the EPR spectrum of half-met tyrosinase that are observed when either *p*-nitrophenol or L-mimosine is added to the solution (cf. Fig. 1) are indicative of the binding of the inhibitors to the copper site, in line with earlier reports on the binding of inhibitors to *Neurospora crassa* Ty, although slight differences in the EPR traces are observed [11]. Under the assumption that the anisotropic part of the hyperfine interaction between the unpaired electron ($S = 1/2$) and a nucleus with $I = 1/2$ may be described by the point dipole–dipole approximation, the anisotropic hyperfine tensor is axial with eigenvalues (−T, −T, 2T) and the shape of the cross-peaks is given by Eq. 4 [12]

$$\nu_1^2 = Q\nu_2^2 + G \tag{4}$$

where Q and G are constants given by

$$Q = (T + 2a \mp 4\nu_1)/(T + 2a \pm 4\nu_1) \tag{5}$$

and

$$G = \pm 2\nu_1(4\nu_1^2 - a^2 + 2T^2 - aT)/(T + 2a \pm 4\nu_1). \tag{6}$$

Here a represents the isotropic coupling constant, T the dipolar coupling constant and ν_I the nuclear Larmor frequency [12].

Analysis of the strong cross-peaks for half-met tyrosinase in H_2O in Fig. 2a by means of Eqs. 4–6 reveals that these signals are mainly anisotropic with $a = 0.7 \pm 0.2$ MHz and $T = 5.0 \pm 0.2$ MHz. The size of the hyperfine coupling constants is close to that found by electron nuclear double resonance spectroscopy for the equatorial waters of the $Cu(H_2O)_6^{2+}$ complex ($T \approx 5.2$ MHz for equatorial water, $T \approx 3.7$ MHz for axial water) [13]. The strong cross-peaks disappear upon incubation of the protein in D_2O, which

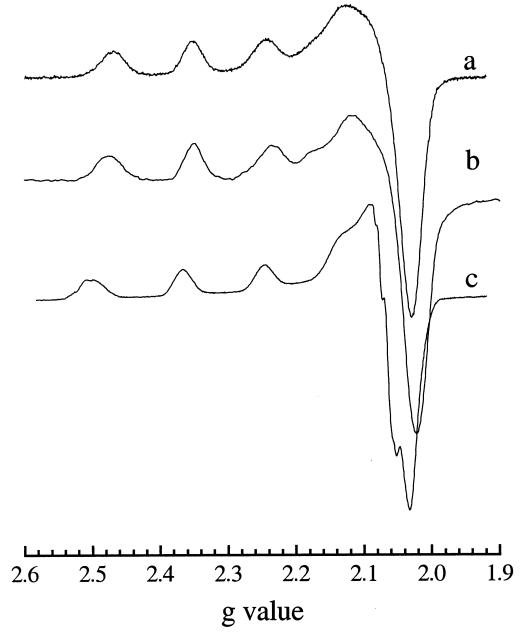
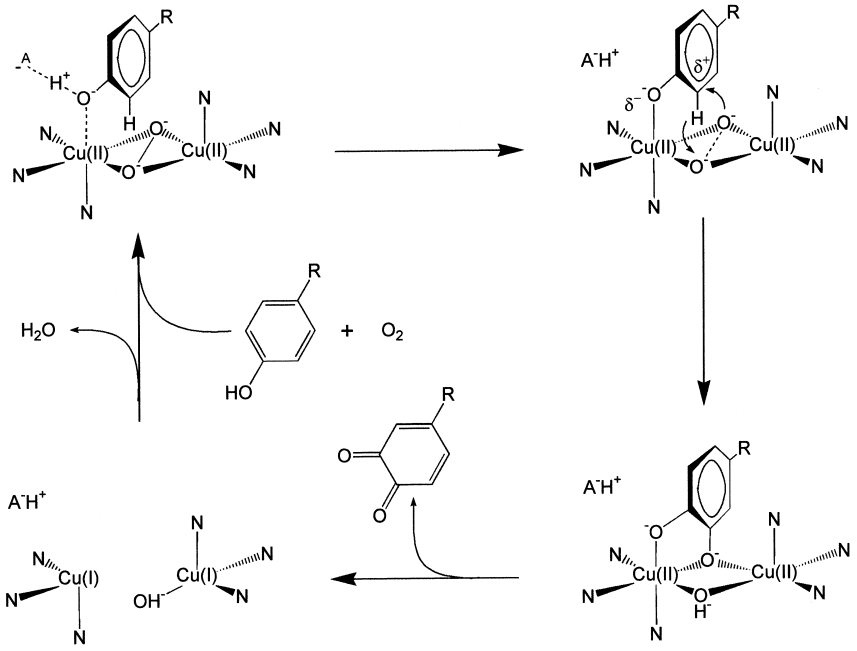


Fig. 1. EPR spectra ($T = 77$ K) for frozen solutions for (a) half-met tyrosinase, (b) half-met tyrosinase with *p*-nitrophenol and (c) half-met tyrosinase with L-mimosine. The copper hyperfine splitting is 13.76 mT for a, 14.07 mT for b and 15.32 mT for c. The $g_{\parallel}$ values are 2.296, 2.300 and 2.305 for a, b and c, respectively. In c, a superhyperfine splitting of 1.3 mT is present on the copper hyperfine line at low magnetic field.



Scheme 3.

indicates that they derive from one or more protons that exchange with the solvent. Following the conclusions from the X-ray study on met-CO [4], we assign the signal to an OH^- bound equatorially to the paramagnetic copper ion.

4. Discussion

The half-met form of Ty contains a half-reduced centre. From the pattern of hyperfine lines in the $g_{\parallel}$ region of the EPR spectrum it is clear that the unpaired electron interacts with only one Cu nucleus. Apparently, we are dealing with a localised valence state in which the unpaired electron is located on one Cu, while the other Cu is reduced. The EPR features observed for the half-met Ty make it clear, furthermore, that the unpaired electron occupies a $d_{x^2-y^2}$ orbital, in agreement with an earlier study on tyrosinase of *N. crassa* [11]. As we know from the present study that an equatorial OH^- binds to the Cu(II), a structure of the Cu(II) site that is compatible with the present spectroscopic evidence and with the available structural data on Cu_2 centres in Hcs and CO would look as depicted in Scheme 2. Upon binding, nitrophenol or mimosine displaces the equatorial OH^- almost without disturbance of the electronic features. The reaction indicated in Scheme 2 for the case of L-mimosine accounts for these observations.

When we now turn to the monophenolase reaction pathway of Ty, it should be realised that the only form of the active centre that is able to convert a monophenol is the oxy form of the active site, which can be written as Cu(I)–O_2–Cu(I), but which usually and more appropriately is represented by the peroxo-form of the oxidised site, Cu(II)–O_2^{2-}–Cu(II), with the oxygens located in the equatorial plane of the copper atoms [14]. Assuming the phenyl ring of the incoming monophenol is positioned in the substrate cavity in a similar way as the inhibitors in the half-met form, the binding of the incoming substrate would occur as indicated in the upper left corner of Scheme 3. The concomitant uptake of the hydroxyl proton by a negative group in the substrate cavity, as proposed by Krebs and co-workers [4], promotes charge transfer from the phenolate oxygen to the peroxide and leads to a further weakening of the O–O bond, poising one of the oxygens for nucleophilic attack on the aromatic *ortho* position [4] (Scheme 3, upper right corner). The transfer of the *ortho* proton to the other oxygen of the peroxide would assist in the formation of the catechol intermediate (Scheme 3, lower right corner). The driving force for this redox process derives in part from the relatively high redox potential of the peroxo dianion. The reaction is catalysed, in this scheme, by the presence of the negative group (A^-). In the subsequent oxidation step the diphenol is converted to the quinone while the two coppers in the dinuclear site are reduced and one of them becomes three-coordinate in accordance with the XRD structure of reduced CO (Scheme 3, lower left corner). It is known from mechanistic studies on blue copper protein variants that a change from four- or five-coordinate to three-coordinate may raise the redox potential of the Cu site considerably [15]. This contributes to the driving force for the second step in the conversion of monophenols to the corresponding quinones.

In summary, EPR and HYSCORE spectroscopy has allowed the identification of an OH^- equatorially bound to the paramagnetic copper of half-met tyrosinase of *S. antibioticus*. The signals from this species disappear upon incubation of the protein in D_2O or upon inhibitor binding. The mode of binding of inhibitors to *S. antibioticus* Ty seems to differ from *N. crassa* Ty [11]. In our case, the minor changes observed in the EPR spectrum in combination with the loss of the signals from OH^- are consistent with a binding mode by which the hydroxyl oxygen of the inhibitor takes the position of the OH^- and leaves the coordination geometry around the paramagnetic copper relatively unaffected. The suggested reaction mechanism (Scheme 3) emphasises the importance of proton movement during the catalytic cycle. This movement may be

as important for the course of the reaction as the electronic charge redistribution.

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