Fluorescence properties of *Neurospora* tyrosinase

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Some structural properties of *Neurospora* tyrosinase have been studied by fluorescence spectroscopy. The emission spectra observed for oxy-, deoxy-, met- and apo-tyrosinase and the Co^{2+} -substituted form are indicative of a protein containing buried tryptophan residues. By using acrylamide and iodide, part of the emission is quenched, indicating heterogeneity in the tryptophan environment. Upon binding of Cu^{2+} or Co^{2+} to apo-tyrosinase, a marked decrease of the tryptophan quantum yield is observed. A further decrease in emission intensity results from the binding of molecular O_2 to the deoxy form. The fluorescent probe 8-anilidonaphthalene-1-sulphonate binds to tyrosinase only when the metal ions are removed. Reconstitution of apo-tyrosinase with Cu^{2+} completely displaces the probe, suggesting that 8-anilidonaphthalene-1-sulphonate binds to apo-tyrosinase at the active site. The fluorescence properties of *Neurospora* tyrosinase are compared with those of haemocyanin.

Tyrosinase (monophenol mono-oxygenase, EC 1.14.18.1) is a copper-containing enzyme that catalyses both the *o*-hydroxylation of monophenols and the oxidation of *o*-diphenols by dioxygen. The enzyme is widely distributed in micro-organisms, animals and plants, where it is involved in the formation of melanins and other polyphenolic compounds (Mason, 1965; Vanneste & Zuberbühler, 1974; Lerch, 1981). *Neurospora* tyrosinase is a monomeric protein that contains a pair of copper ions in a polypeptide chain of M_r 46 000 (Lerch, 1976, 1978). The metal, which is essential for enzymic activity (Kubowitz, 1938), was found to be e.p.r.-undetectable (Deinum *et al.*, 1976), suggesting antiferromagnetic coupling between the metal ions (Himmelwright *et al.*, 1980), as was previously demonstrated for *Agaricus bisporus* (cultivated mushroom) tyrosinase (Schoot Uiterkamp & Mason, 1973). The 'type 3' copper unit (Fee, 1975) is a feature shared by several metalloproteins (haemocyanin, tyrosinase, laccase, caeruloplasmin and ascorbate oxidase) engaged in different biological functions. In particular, strong similarities exist between tyrosinase and haemocyanin.

Both proteins contain only 'type 3' copper units and both bind oxygen reversibly. Oxy-tyrosinase and oxyhaemocyanin display virtually the same absorption features: an intense band around 340 nm

($\epsilon \sim 20\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$) and a second peak at 570 nm ($\epsilon \sim 1000\text{ M}^{-1}\cdot\text{cm}^{-1}$) (Himmelwright *et al.*, 1980). As demonstrated by Raman resonance studies, the oxygen in oxy-tyrosinase and oxyhaemocyanin is bound as peroxide (Eickman *et al.*, 1978). Moreover, from chemical and spectroscopic studies, the electronic and geometric properties of the *Neurospora* tyrosinase active site were shown to be very similar to those described for haemocyanin (Eickman *et al.*, 1979; Himmelwright *et al.*, 1979, 1980). Additional information on the structure of the active site and the metal ligands in *Neurospora* tyrosinase is available from spectroscopic studies on the Co^{2+} -substituted enzyme (Rüegg & Lerch, 1981) and from photo-oxidation studies (Pfiffner & Lerch, 1981). However, little is known about the overall structure of the protein in relation to the binding of metal and oxygen. Since *Neurospora* tyrosinase contains 12 tryptophan residues (Lerch, 1978), some insight into the structure of the protein might be gained from the study of its fluorescence. In the present work we have studied the emissive properties of different derivatives of *Neurospora* tyrosinase in the presence and absence of fluorescence perturbants. From comparison of the results obtained with oxy($\text{Cu}_2^{2+}\text{O}_2^{2-}$), deoxy(Cu_2^{1+}), met(Cu_2^{2+}), apo- and Co^{2+} -substituted (Co_2^{2+}) *Neurospora* tyrosinase, valuable information was obtained on the effect of metal and oxygen binding on protein structure. The similarity with data obtained on haemocyanins further emphasizes the close structural relationship at the active-site level between the two proteins.

Abbreviations used: GdmCl, guanidinium chloride; ANS, 8-anilidonaphthalene-1-sulphonate; Ac-Trp-CONH₂, *N*-acetyl-L-tryptophanamide.

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Experimental

Neurospora crassa was grown in liquid cultures as described by Horowitz *et al.* (1970). Tyrosinase was prepared from cycloheximide-de-repressed cultures as described by Lerch (1976). Enzymic activity was measured as described by Fling *et al.* (1963). Protein concentration was measured spectrophotometrically at 280 nm by using $A_{280}^{1\%,1\text{cm}} = 22.0$ (Lerch, 1976). All spectroscopic measurements were performed in 50 mM-potassium phosphate, pH 7.5, at $10 \pm 0.2^\circ\text{C}$. Buffer solutions were rendered metal-free by passage through Chelex-100 resin. Chemicals were of the best grade commercially available and used without further purification.

Preparation of tyrosinase derivatives

Since the freshly isolated protein is a mixture of the oxy and met forms, homogeneous preparations of each derivative were made by chemical treatment of the native enzyme. Oxy-tyrosinase was prepared by the method of Lerch (1976) and showed an A_{345}/A_{280} ratio of 0.17. Deoxygenated (reduced) tyrosinase was prepared by a modification of the procedure of Shaklai & Daniel (1970), in which the protein solution is equilibrated with argon. The absorption spectrum of deoxy-tyrosinase showed the loss of the 345 nm band. Re-equilibration with oxygen restored oxy-tyrosinase in 90–95% yield. Met-tyrosinase was obtained by overnight incubation of freshly isolated enzyme in presence of N_3^- (10 mM), which is known to displace the peroxide (Himmelwright *et al.*, 1980). Excess ligand was removed by passage through Sephadex G-25.

Apo- and Co^{2+} -substituted tyrosinase were prepared as described by Rüegg & Lerch (1981). The metal stoichiometry and the spectral properties of Co^{2+} -tyrosinase were in agreement with those reported in the literature (Rüegg & Lerch, 1981). The specific activity measured in solution of oxy-, deoxy-, met- and reoxygenated tyrosinase was 1200 ± 200 units ($\mu\text{mol}/\text{min}$)/mg. The residual specific activity of apo- and Co^{2+} tyrosinase was 10–12 units/mg.

Spectroscopic measurements

Absorption spectra were recorded in a Hitachi/Perkin–Elmer model 340 recording spectrophotometer. Metal analyses were performed on a IL 157 atomic-absorption spectrophotometer (Instrumentation Laboratory, Wilmington, MA, U.S.A.), acid-washed glassware being used. Fluorescence measurements were made with a Perkin–Elmer MPF 2A spectrofluorimeter operating in the ratio mode. If not indicated otherwise, excitation at 294 nm was used. In measurements of intrinsic fluorescence, the protein solutions were diluted to yield an absorbance of 0.04–0.05 at the excitation wavelength in order to

minimize inner-filter effects. The fluorescence quantum yield, Φ , was measured by using the formula (Parker & Rees, 1960):

$$\Phi_x = \Phi_{st.} \cdot \frac{F_x}{A_x} \cdot \frac{A_{st.}}{F_{st.}} \quad (1)$$

where F is the integrated emission intensity, A is the absorbance at the excitation wavelength. The subscripts $_x$ and $_{st.}$ refer, respectively, to solutions of enzyme and Ac-Trp-CONH₂ used as standard.

$\Phi_{st.}$ was recalculated from the results of Lehrer (1971). Assuming $\Phi = 0.13$ at 21°C , the value at 10°C was calculated by comparing the emission intensity observed for the same Ac-Trp-CONH₂ solution at the two different temperatures.

Quenching experiments

Acrylamide and potassium iodide were used as quenchers of tryptophan fluorescence. In a typical quenching experiment, portions of a stock solution of acrylamide (5 M) or KI (3 M) were added to the protein solution and the emission spectrum was recorded. After each addition of iodide, the sample volume and the salt concentration were kept constant by adding portions of buffer solution and 3 M-KCl. In order to overcome the formation of I_3^- , a small amount ($\sim 10^{-4}$ M) of $\text{S}_2\text{O}_3^{2-}$ was added to the iodide solution (Lehrer, 1971). As acrylamide has a molar absorption coefficient (ϵ) of $0.28 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 294 nm (recalculated from results published by Eftink & Ghiron, 1976a), the emission intensity was corrected for the inner-filter effect by using the coefficient (Chignell, 1972):

$$D = \text{antilog} \left(\frac{A_{\text{exc.}} + A_{\text{emiss.}}}{2} \right) \quad (2)$$

where $A_{\text{exc.}}$ and $A_{\text{emiss.}}$ indicate the absorbances of the solution at the wavelengths of excitation and emission respectively. The quenching constants obtained for apo-tyrosinase in 6 M-GdmCl were corrected for the viscosity effect. The correction factor was calculated as the ratio between the quenching constant calculated for Ac-Trp-CONH₂ in buffer and in the presence of 6 M-GdmCl.

ANS binding

The fluorescence spectra of the magnesium salt of ANS (64 μM) in the presence and in the absence of tyrosinase (27 μM) were obtained by excitation at 390 nm and were corrected for the protein scattering and inner-filter effect. ANS concentrations were calculated by using $\epsilon = 4.95 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 350 nm in 0.1 M-potassium phosphate, pH 6.8 (Azzi, 1974).

Results

Tyrosinase intrinsic fluorescence

Upon excitation at 294 nm, where 94% of light is absorbed by tryptophan residues, tyrosinase exhibits fluorescence with maximum intensity near 330 nm. In Fig. 1 the emission spectra for apo-, met-, and oxy-tyrosinase normalized to the same absorbance are shown. In addition, the spectra obtained for deoxy- and Co^{2+} -tyrosinase (not shown) overlap, within the error of measurement, with that of

Fig. 1. Fluorescence emission spectra obtained for different tyrosinase derivatives

The spectra refer to apo- (a), met- (b) and oxy- (c) tyrosinase in 50 mM-potassium phosphate buffer, pH 7.5, at $10 \pm 0.2^\circ\text{C}$. The spectra obtained for deoxy- and Co^{2+} -tyrosinase overlap, within the error, that of met-tyrosinase. All the spectra are recorded under excitation at 294 nm and are normalized for the same protein concentration. Insert: emission spectrum obtained for apo-tyrosinase in 6 M-GdmCl at 294 nm (—) and 275 nm (----) excitation. The fluorescence intensity is in arbitrary units.

met-tyrosinase. The position of the maxima and the shape of the spectra do not change as a function of the wavelength of excitation. Moreover, from the difference between the spectra obtained under excitation at 275 and 294 nm (Longworth, 1971), the contribution of tyrosine residues was found to be extremely low in apo-, met- and oxy-tyrosinase. For instance, in oxy-tyrosinase, where the tryptophan fluorescence is strongly quenched, the contribution of the tyrosine residues to the total emission is less than 10%. A distinct shoulder attributable to the tyrosine emission is, however, observed upon exciting at 275 nm a solution of apo-tyrosinase in buffer containing 6 M-GdmCl (Fig. 1, insert). The emissive properties of different tyrosinase derivatives are reported in Table 1. Note that the values for deoxy- and Co^{2+} -tyrosinase are very similar to that of the met form.

The results reported in Table 1 show that the position of emission maximum and the half-band width are characteristics shared by all derivatives of tyrosinase examined. However, the decrease in the quantum yield on going from apo- to met- (or deoxy-) tyrosinase discloses a large effect of copper binding on fluorescence intensity. The same reduction in fluorescence intensity is observed in Co^{2+} -tyrosinase. Oxygenation brings about a further decrease in emission intensity. This reversible effect directly correlates with the presence of the characteristic absorption band at 345 nm generated on oxygen binding.

Denaturation of apo-tyrosinase by addition of 6 M-GdmCl produces a red shift of 18 nm of the fluorescence emission wavelength with a concomitant increase of half-band-width value (Fig. 1, insert; Table 1).

Tyrosine-tryptophan energy transfer

An evaluation of tyrosine→tryptophan energy-transfer efficiency is made from the wavelength-

Table 1. Emissive properties of different derivatives of *Neurospora* tyrosinase

The protein concentration was about 0.1 mg/ml, in 50 mM-potassium phosphate, pH 7.5, the temperature was $10 \pm 0.2^\circ\text{C}$, and the excitation wavelength was 294 nm. λ_{em} is the emission wavelength.

Derivative	λ_{em} * (nm)	Half-band width $\Delta\lambda$ (nm)	Quantum yield† (Φ)	Quenching‡ (%)
Oxy-	331	50	0.026	80
Met-	332	51	0.051	60
Deoxy-	332	53	0.046	64
(Co^{2+}) ₂ -	332	50	0.050	60
Apo-	330	50	0.129	0
Apo-(6 M-GdmCl)	348	60	0.131	—

* Values relative to the maximum of emission.

† Values calculated assuming 0.17 as quantum yield for Ac-Trp-CONH₂ at 10°C .

‡ Values calculated with respect to the quantum yield of apo-tyrosinase.

dependency of the relative quantum yield of tyrosinase. For this purpose, the protein quantum yield, $\Phi(\lambda)$, is measured as a function of wavelength of excitation and is compared with the value obtained at $\lambda_{\text{exc.}} 294 \text{ nm}$ (Φ_{Trp}) where tryptophan absorbs almost exclusively. The experimental data are fitted as described by Eisinger (1969) using the equation:

$$\Phi(\lambda) = \Phi_{\text{Trp}} [f_{\text{Trp}}(\lambda) + e f_{\text{Tyr}}(\lambda)] \quad (3)$$

where $f_{\text{Trp}}(\lambda)$ and $f_{\text{Tyr}}(\lambda)$ are the fractional ab-

Fig. 2. Tyrosine-tryptophan energy transfer. The wavelength-dependencies of the relative quantum yield (Φ/Φ_{Trp}) observed for apo-(O), oxy-(●) and met-(▲) tyrosinase are shown. The continuous lines refer to the theoretical points calculated from the Eisinger's (1969) equation assuming values of $e = 0$, $e = 0.2$ and $e = 0.3$. All the theoretical curves and the experimental points are normalized at $\lambda = 294 \text{ nm}$. Conditions were as in Fig. 1.

sorptions of tryptophan and tyrosine respectively at the excitation wavelength λ , calculated from their molar absorption coefficients and molar ratio in the protein, and e is the transfer efficiency. Fig. 2 shows plots of the measured relative quantum yields [$\Phi(\lambda)/\Phi_{\text{Trp}}$] of apo-, met- and oxy-tyrosinase versus the wavelength of excitation. The data are normalized to 1 at $\lambda = 294 \text{ nm}$. The best fitting to the Eisinger's (1969) equation is obtained by assuming $e = 0$ for apo-tyrosinase and $e = 0.2$ – 0.3 for met- and oxy-tyrosinase. These results indicate that, in met- and oxy-tyrosinase, 20–30% of light absorbed by tyrosine is transferred to tryptophan, whereas in apo-tyrosinase this process is ineffective.

Quenching experiments

Addition of acrylamide or iodide to tyrosinase brings about a decrease of fluorescence emission intensity. Oxy- and met-tyrosinase are found to be fully active in the presence of both quenchers up to a concentration of 0.6 M and at substrate concentrations below K_M (Eftink & Ghiron, 1981). In oxy-tyrosinase, the exposure to iodide and acrylamide leads to a decrease of the 345 nm band, with a consequent increase of fluorescence emission intensity. Addition of a small excess of hydroxylamine stabilizes the 345 nm band up to an iodide concentration of about 0.2 M , but is completely inefficient in the case of acrylamide. The experiments with oxy-tyrosinase could therefore be performed only at low iodide concentrations ($< 0.2 \text{ M}$). The quenching data for different tyrosinase derivatives were analysed in terms of the Stern-Volmer relationship (Lehrer & Leavis, 1978):

$$F_0/F = 1 + K_Q[Q] \quad (4)$$

Fig. 3. Fluorescence quenching on tyrosinase

Stern-Volmer plots obtained with acrylamide (a) and iodide (b) as fluorescence quenchers. The symbols are relative to the data for apo-tyrosinase in buffer (O), apo-tyrosinase in 6 M-GdmCl (▲), met-(●) and oxy-tyrosinase (□). The values for apo-tyrosinase in 6 M-GdmCl are not corrected for the viscosity effect. Conditions were as in Fig. 1.

where F_0 and F are the fluorescence intensities at the emission λ_{max} in the absence and presence of quencher, $[Q]$ is the quencher concentration, and K_Q is the quenching constant. The plots of F_0/F against $[Q]$ obtained for apo- and met-tyrosinase with acrylamide are presented in Fig. 3(a). The plots obtained with the native proteins are curve-downwards, but a linear relationship is observed with apo-tyrosinase dissolved in buffer containing 6M-GdmCl. The results obtained with iodide are presented in Fig. 3(b). In this case, too, downward-curved plots are observed with apo- and met-tyrosinase, but deviation from linearity is also observed under conditions of denaturation. The downward curvature indicates that the quenching process cannot be described by eqn. (4). Since in a multi-tryptophan protein with a heterogeneous distribution of fluorescing groups each fluorophor may have different quenching constants, the measured decrease in fluorescence emission can be a non-linear function of the concentration of the quenching agent. The quenching effect is in this case better described

by a modified Stern–Volmer equation (Lehrer, 1971), which, at low quencher concentrations, has the form:

$$\frac{F_0}{F_0 - F} = \frac{1}{[Q]} \cdot \frac{1}{\sum f_i K_{Q_i}} + \frac{\sum K_{Q_i}}{\sum f_i K_{Q_i}} \tag{5}$$

were F_0 , F and $[Q]$ are as defined above, f_i and K_{Q_i} represent respectively the fractional fluorescence and the quenching constant relative to the i th tryptophan residue. Plotting $F_0/(F_0 - F)$ against $1/[Q]$, the ratio intercept/slope is $\sum K_{Q_i} = (K_Q)_{\text{eff}}$, the effective quenching constant, and $1/\text{intercept}$ gives $\sum f_i K_{Q_i} / \sum K_{Q_i} = (f_a)_{\text{eff}}$, the effective fraction of fluorescence accessible to the quencher (Lehrer, 1971).

The modified Stern–Volmer plots obtained with acrylamide and iodide are shown in Figs. 4(a) and 4(b) respectively, where the straight lines were obtained by the least-squares method. The data calculated for different tyrosinase derivatives with acrylamide and iodide are presented in Table 2. The $(f_a)_{\text{eff}}$ values obtained for apo- and met-tyrosinase

Fig. 4. Fluorescence quenching on tyrosinase

Modified Stern–Volmer plots obtained for fluorescence quenching by acrylamide (a) and iodide (b). The symbols used are relative to the data for apo-tyrosinase in buffer (○), apo-tyrosinase in 6M-GdmCl (▲) and met-tyrosinase (●). The values for apo-tyrosinase in 6M-GdmCl are corrected for the viscosity effect. Conditions were as in Fig. 1. The straight lines were obtained by the least-squares method.

Table 2. Quenching parameters calculated for tyrosinase

Conditions: protein concentration ~0.2 mg/ml in 50 mM-potassium phosphate, pH 7.5; temperature = 10 ± 0.2 °C; excitation wavelength 294 nm. For further details, see the Experimental section.

Tyrosinase derivative	Quencher	Parameter	Acrylamide		Iodide	
			$(f_a)_{\text{eff}}$	$(K_Q)_{\text{eff.}} \text{ (M}^{-1}\text{)}$	$(f_a)_{\text{eff.}}$	$(K_Q)_{\text{eff.}} \text{ (M}^{-1}\text{)}$
Met-			0.32	13.2	0.39	1.6
Apo-			0.40	18.0	0.42	1.5
Apo- (6 M-GdmCl)			1	18.9*	0.65	10.3*
Ac-Trp-CONH ₂			1	19.7	1	11.4

* Value corrected for the viscosity.

at low quencher concentrations are ~ 0.4 in the presence of either acrylamide or iodide. With apo-tyrosinase in 6 M-GdmCl, acrylamide and iodide give $(f_a)_{\text{eff}}$ values of 1 and 0.65 respectively.

The $(K_Q)_{\text{eff}}$ values for the acrylamide quenching of met- and apo-tyrosinase in buffer are 13.2 and 18.0 M^{-1} respectively. The latter value is very similar to that obtained for apo-tyrosinase in 6 M-GdmCl and Ac-Trp-CONH₂ in buffer (18.9 and 19.7 M^{-1} respectively). With iodide, however, the data calculated for met- and apo-tyrosinase in buffer (1.6 and 1.5 M^{-1} respectively) are very low in comparison with those calculated for apo-tyrosinase in 6 M-GdmCl (10.3 M^{-1}) and Ac-Trp-CONH₂ in buffer (11.4 M^{-1}). For oxy-tyrosinase, no decrease of protein fluorescence was observed.

ANS binding

Excitation of a solution of ANS in buffer at 390 nm yields a broad emission band with a maximum at 515 nm. The same fluorescence spectrum is also observed when native or Co^{2+} -substituted tyrosinase is added to the ANS solution. However, addition of apo-tyrosinase to the ANS solution leads to a drastically changed fluorescence spectrum characterized by a higher intensity of emission and blue shift of the emission maximum to

Fig. 5. Interaction between tyrosinase and ANS. Fluorescence emission spectra of ANS ($64 \mu\text{M}$) in 50 mM-potassium phosphate, pH 7.5 (temperature $10 \pm 0.2^\circ\text{C}$) (----) and in the presence of $27 \mu\text{M}$ -apo-tyrosinase in the same buffer (—) are shown. The broken line also describes the spectra of ANS in the presence of met-, Co^{2+} , oxy-tyrosinase and apo-tyrosinase after addition of CuSO_4 ($95 \mu\text{M}$). Insert: emission spectrum of ANS in presence of apo-tyrosinase corrected for the contribution of ANS in buffer. Spectra were recorded under an excitation wavelength of 390 nm and corrected for the protein scattering and inner-filter effect.

490 nm (Fig. 5). The difference between the fluorescence spectra of ANS recorded in the presence and in the absence of apoprotein shows the net shift is even larger ($\lambda_{\text{max.}} = 475 \text{ nm}$) (Fig. 5, insert). The effect of apo-tyrosinase on ANS fluorescence is completely reversed by the addition of CuSO_4 (~ 4 -fold excess with respect to the protein concentration).

Discussion

Tyrosinase contains 12 tryptophan and 20 tyrosine residues per molecule (Lerch, 1978). A detailed analysis of the distribution of the fluorescing groups is therefore quite complicated. Nonetheless, the present study shows that fluorimetric methods can give valuable information about their environment. The emission properties of tyrosinase, such as the position of the emission maximum and the half-band width of the spectra, are typical for a protein containing tryptophan residues buried within the protein molecule (Burstein *et al.*, 1973; Longworth, 1971). This conclusion is also supported by the red shift and the increase in half-band width occurring upon exposure of apo-tyrosinase to denaturing conditions. However, the quenching studies with acrylamide and iodide reveal substantial environmental heterogeneity. The downward curvatures of the Stern-Volmer plots indicate different accessibility of the quenchers to the tryptophan residues (Lehrer, 1971; Eftink & Ghiron, 1976b). The modified Stern-Volmer plots show that, in apo- and met-tyrosinase, about 40% of the total fluorescence is quenched at low acrylamide concentrations. This fluorescence fraction can be associated with a class of solvent-exposed tryptophan residues, as suggested by the similarity of the corresponding $(K_Q)_{\text{eff}}$ values with those calculated for Ac-Trp-CONH₂ and apo-tyrosinase in 6 M-GdmCl, where all the tryptophan residues are exposed [$(f_a)_{\text{eff.}} = 1$]. The same conclusion is supported by the similarity of the $(f_a)_{\text{eff.}}$ values obtained with acrylamide to those measured with iodide, an agent known to quench preferentially the external tryptophan residues (Lehrer, 1971) and by the small blue shift ($\sim 2 \text{ nm}$) of the residual tyrosinase emission maximum measured upon addition of the quenchers. Since iodide is an anion and acrylamide an uncharged molecule, the large differences of $(K_Q)_{\text{eff.}}$ values observed for the same tyrosinase derivative with two different quenchers may reflect electrostatic hindrance from negatively charged residues located near the tryptophan residues or a situation in which the tryptophan residues are partially shielded in the protein structure. This effect could explain the fact that only 65% of the fluorescence can be quenched by iodide under denaturing conditions. At this point it is noteworthy that, according to the sequence of the protein

(Lerch, 1978), one-third of the tryptophan residues are juxtaposed to an aspartic acid residue. Hence the theoretical fraction of the tryptophan residues expected to be quenched by iodide is 67%, a number in good agreement with the experimental result. The marked decrease in quantum yield observed between apo-, met- and oxy-tyrosinase, an effect reported previously for several haemocyanins (Shaklai & Daniel, 1970; Bannister & Wood, 1971; Ma *et al.*, 1977; Shaklai *et al.*, 1978; Ricchelli *et al.*, 1980), strongly indicates the presence of tryptophan residue(s) in the proximity of the active site. However, the constancy of spectral shape and position of maximum do not allow further consideration of the class of the tryptophan residue(s) specifically affected by the quenching.

For the metal-containing derivatives (met-, deoxy- and Co^{2+} -tyrosinase), the metal bound could quench the emission intensity of the neighbouring fluorophores, either by acting as a paramagnetic centre or via a heavy-atom effect (Hercules, 1966). However, since in met-tyrosinase the two bivalent ions bound at the active site are strongly spin-coupled, resulting in a completely diamagnetic state (Makino *et al.*, 1974; Himmelwright *et al.*, 1980; Rügge & Lerch, 1981), a paramagnetic effect is unlikely. This conclusion is also supported by the fact that, upon reduction of the copper ions (met $\rightarrow$ deoxy), the fluorescence yield does not change. As shown for azurin (Finazzi-Agrò *et al.*, 1970) and in haemocyanin (Klarman *et al.*, 1977; Ma *et al.*, 1977), a heavy-metal effect provides a more probable explanation. It can also account for the fluorescence quenching observed upon cobalt binding to apo-tyrosinase.

Oxy-tyrosinase displays typical absorption features at 345 and 600 nm (Jolley *et al.*, 1972; Lerch, 1976). As the near-u.v. band largely overlaps with the emission band centred at 330 nm, the quenching effect of oxygen bound can be related to a non-radiative energy-transfer process. However, since in oxy-tyrosinase it is not possible to relate the quenching of intrinsic fluorescence to a single tryptophan residue or to exclude the possibility of tryptophan $\rightarrow$ tryptophan energy transfer, a calculation of the interchromophore distance as previously performed for the mollusc *Levantina* (Shaklai & Daniel, 1970) and for *Limulus* (horseshoe crab) (Ma *et al.*, 1977) oxyhaemocyanin is excluded.

The low tyrosine/tryptophan ratio in tyrosinase makes this protein also not a good candidate for the resolution of tyrosine fluorescence. However, in *Carcinus* (crab) oxyhaemocyanin, characterized by a ratio similar to that of tyrosinase but with a higher tyrosine $\rightarrow$ tryptophan energy-transfer efficiency (Ricchelli *et al.*, 1980) the tyrosine emission could be resolved. Therefore the very low tyrosine

emission in oxy-tyrosinase, where the tryptophan quantum yield is comparable with that of *Carcinus* oxyhaemocyanin, would suggest that, in oxy-tyrosinase, the tyrosine residues are strongly quenched (Brand & Witholt, 1967). Moreover, the low tyrosine $\rightarrow$ tryptophan energy-transfer efficiency indicates that such a mechanism of tyrosine quenching is not very important in the case of tyrosinase. A comparison of e values calculated for apo-, met- and oxy-tyrosinase indicates a probable conformational change occurring upon metal binding, as reflected also from the difference in $(K_Q)_{\text{eff}}$ obtained for the apo and met derivatives in the acrylamide quenching studies.

Iodide is not an efficient quencher for tyrosinase and hence the poor discrimination between apo- and met-tyrosinase may be related to the low quenching constant. In agreement with the similar e values observed for met- and oxy-tyrosinase, a minor conformational change is expected upon oxygen binding. Therefore the lack of iodide quenching in oxy-tyrosinase suggests that the fluorophores quenched upon binding of oxygen probably correspond to the fraction that is accessible to iodide and acrylamide in met-tyrosinase (Shaklai *et al.*, 1978).

Several proteins have been found to contain binding sites for the naphthalenesulphonate fluorescence probes (Stryer, 1968; Brandt & Gohlke, 1967). In particular, ANS was reported to be specifically bound at the active site of apohaemoglobin, apomyoglobin (Stryer, 1965) and apo-haemocyanin (Ricchelli & Salvato, 1979). The changes in emissive properties of ANS in the presence of apo-tyrosinase are very similar to those reported previously and indicate that the probe is bound to the protein. Furthermore, the displacement of ANS from the apoprotein by Cu^{2+} ions and the lack of binding to met-, Co^{2+} - and oxy-tyrosinase strongly suggest that the dye is bound at the active site. The enzymic activity is not immediately restored after displacement of ANS by copper; however, the results reported by Kertesz *et al.* (1972) indicate that the regain of enzymic activity is a rather slow process (10–15 h), whereas the Cu^{2+} ions are bound to the apoprotein very quickly ($k_{\text{on}} > 5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$). The observed changes in the emissive properties of ANS upon binding to apo-tyrosinase suggest a hydrophobic character for the binding sites. This conclusion is supported by Raman resonance data on oxy-tyrosinase (Eickman *et al.*, 1978) and oxyhaemocyanin (Loehr *et al.*, 1974), which are indicative of a peroxo complex in a hydrophobic environment. Alternatively the change in the ANS fluorescence properties could be interpreted in terms of a loss of flexibility of the ANS molecule bound in a rigid site (Ainsworth & Flanagan, 1969).

In conclusion, the present data provide further

evidence for the strong structural similarities between the active sites of tyrosinase and haemocyanin. Thus, in addition to the virtually identical copper-binding mode, both proteins contain tryptophan residues in the proximity of the binuclear copper site, whose emissive properties are strongly affected by the presence of metal and dioxygen.

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