Stopped-flow Fluorescence Studies of Inhibitor Binding to Tyrosinase from Streptomyces antibioticus*

Received for publication, August 25, 2003, and in revised form, December 19, 2003
Published, JBC Papers in Press, December 29, 2003, DOI 10.1074/jbc.M309367200

Armand W. J. W. Tepper‡, Luigi Bubacco§, and Gerard W. Canters¶

From the ‡Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, Einsteinweg 55, 2333 CC, Leiden, The Netherlands and the §Department of Biology, University of Padua, Via Trieste 75, 30121 Padua, Italy

Tyrosinase (Ty) is a type 3 copper protein involved in the rate-limiting step of melanin synthesis. It is shown that the endogenous Trp fluorescence of tyrosinase from Streptomyces antibioticus is remarkably sensitive to the redox state. The fluorescence emission intensity of the [(Cu(I) Cu(I))] reduced species is more than twice that of the oxygen-bound [(Cu(II)-O2- -Cu(II))] form. The emission intensity of the oxidized [(Cu(II)-OH- -Cu(II))] protein (TYmet) appears to be dependent on an acid-base equilibrium with a pK_a value of 4.5 ± 0.1. The binding of fluoride was studied under pseudo first-order conditions using stopped-flow fluorescence spectroscopy. The kinetic parameters k_on, k_off, and the fraction of fluorescence emission quenched upon fluoride binding show a similar pH dependence as above with an average pK_a value of 4.62 ± 0.05. Both observations are related to the dissociation of Cu_O-bridging hydroxide at low pH. It is further shown that Ty is rapidly inactivated at low pH and that halide protects the enzyme from this inactivation. All results support the hypothesis that halide displaces hydroxide as the Cu_O-bridging ligand in TYmet.

The relevance of the experimental findings for the catalytic cycle is discussed. The data are consistent with the data obtained from other techniques, validating the use of fluorescence quenching as a sensitive and effective tool in studying ligand binding and substrate conversion.

The details of the interaction of inhibitors with the dinuclear copper enzyme tyrosinase (Ty); EC 1.14.18.1) are still far from being completely understood. They are the subject of this report. Tyrosinases are involved in the rate-limiting step in the synthesis of melanin pigments, which fulfill various roles in living organisms. In mammals, melanins are responsible for skin, eye, inner ear, and hair pigmentation, and defects in Ty are related to a range of medical conditions like type I human oculocutaneous albinism (1, 2). In fruits and mushrooms, melanins are related to a range of medical conditions like type I human skin, eye, inner ear, and hair pigmentation, and defects in Ty type 3 site also occurs in the hemocyanins (Hcs), which function as oxygen carriers and storage proteins in arthropods and molluscs, and in the plant catechol oxidases (COs), which oxidize diphenols to the corresponding quinones but lack the Ty hydroxylation activity. Although the Ty type 3 proteins fulfill different functions, the dinuclear active site seems to be highly conserved as witnessed by its characteristic spectroscopic signatures and the available crystal structures for several Hcs (9–11) and a catechol oxidase (12). The differences in functionality are therefore thought to derive from variations in the accessibility of substrates to the active site or the ability of substrates to appropriately dock into the active center (7, 13, 14).

The Ty type 3 site can exist in various forms as follows: (a) the [(Cu(I) Cu(I))]-reduced TYox species; and (b) the [(Cu(II)-O2- -Cu(II))] TYox species, in which molecular oxygen is bound as peroxide in a μ-η^2:η^2 side bridging mode to the Cu_2 center. The TYox form, which results from the binding of molecular oxygen to TYox, is competent to react with both monophenols and diphenols and is characterized by a strong ligand to metal charge transfer (LMCT) band centered around 345 nm and a low O-O stretching vibration frequency of ~750 cm⁻¹ (4). (c) The [(Cu(II)-OH -Cu(II)]-oxidized met form (TYmet) is competent to react with diphenols only. The TYmet form results when TYox reacts with a diphenol to produce the corresponding quinone.

Detailed knowledge of the interaction of Ty with its inhibitors is of paramount importance for understanding the Ty enzyme mechanism, as well as for the development of novel Ty inhibitors relevant to skin treatment and the economically important prevention of the browning of fruits, vegetables, and mushrooms, for example. Consequently, steady-state kinetic studies of Ty inhibition are abundant, and many inhibitors have been identified (e.g. Refs. 15–24). Yet direct spectroscopic investigations of inhibitor binding to tyrosinases have been few, partly because of the scarcity of suitable spectroscopic probes. For example, TYmet shows no strong UV-visible absorption bands and is EPR-silent due to the antiferromagnetic coupling between the two unpaired spins on the copper ions (4). Spectroscopic studies mainly pertained to the half-oxidized...

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Stopped-flow Studies of Inhibitor Binding to Tyrosinase

species, in which one of the copper ions in the active site occurs in the Cu(II) and the other in the Cu(I) oxidation state (Tyhalf-met), rendering the site suitable for study by EPR techniques. Although EPR studies on Tyhalf-met have yielded much information regarding the coordination geometry of the oxidized copper and the changes that occur when ligands bind to the active site, the half-met form does not occur naturally and the technique allows for the investigation of only one of the copper ions in the type 3 site.

It was a step forward recently when the oxidized Tyhalf-met derivative appeared amenable to paramagnetic NMR (8, 25, 26); the S = 1 spin state of Tyhalf-met was found to be thermochemically accessible at room temperature, providing sufficient paramagnetism for the signals of the coordinating histidine protons to shift outside the diamagnetic envelope. The NMR studies demonstrated that also Ty contains a classical type 3 site in which, like in Hcs and CoS, the two copper ions are coordinated by six histidine residues through their NH atoms (8). Furthermore, large changes in the Tymet paramagnetic 1H NMR spectrum are observed when inhibitors bind to the active site (25, 26), allowing us to follow ligand binding and to obtain structural information on the complexes. For instance, it was shown that halides interact with both the oxidized and reduced Ty, where the affinity for halide ion and the mechanism of inhibition depend both on the nature of the halide ion and the oxidation state of the type 3 site (26) \((K_{d}^{red} < F^{<} < Cl^{-} < Br^{-} < Br^{<} < F^{<} ; K_{d}^{met} F^{<} < Cl^{-} < Br^{-} < Br^{<} < F^{<} )\). The binding of halide to Tyhalf-met is strongly pH-dependent, i.e. halide only binds to the acidic form of the enzyme, resulting in stronger inhibition with decreasing pH. As a consequence of the instability of the enzyme at low pH and of the relatively long experimental times required, in the past we were able to estimate only an upper limit of 5.5 for the \(pK_{a}\) of the process modulating the halide binding (26). In the same study it was also proposed that halides bridge the two copper ions in the active site.

Fluorescence studies on proteins, in which use is made of the emission of endogenous Tyr and Trp residues, have been widely performed to study protein structure and dynamics. Several Hcs (27–39) and one Ty (40) have been the subject of such investigations. Yet, Hcs or Ty protein fluorescence has never been used to study inhibitor binding. This report focuses on the fluorescence properties of the Ty from Streptomyces antibioticus as a function of pH and inhibitor binding. Fluoride was chosen (26) as a model compound to study the transient-state kinetics of its binding to the Tyhalf-met enzyme, as well as its pH dependence. Stopped-flow fluorescence spectroscopy was used to obtain insight into the interaction of fluoride with Ty, as well as to validate the use of fluorescence quenching in studying ligand binding kinetics. The inactivation of Ty was studied at various pH values and under different conditions. To our knowledge, this is the first report on the kinetics of inhibitor binding to oxidized tyrosinase.

MATERIALS AND METHODS

Protein Isolation and Purification—Tyrosinase was obtained from liquid cultures of S. antibioticus carrying the Ty plf703 overexpression plasmid (25). The enzyme was purified from the growth medium according to published procedures (25). The pure enzyme was obtained as a mixture of Tyhalf-met, Tyred, and Tyoxy, and the purity exceeded 95% in all cases as estimated by SDS-PAGE. Protein concentrations were routinely determined optically using a value of 82 m\(^{-1}\) cm\(^{-1}\) for the extinction coefficient at 280 nm (41). The protein was stored at \(-80^\circ\)C at a concentration of 1 mg/ml in 100 mM Pi buffer at pH 6.8 containing 20% glycerol as a cryoprotectant. Prior to further experiments, glycerol was removed from the storage buffer by dialysis at 4 °C against 100 mM Pi, at pH 6.80. The reduced enzyme was prepared by reduction with hydroxylamine as described previously (26) and used immediately.

Optical Spectroscopy—Optical measurements were performed on a PerkinElmer Life Sciences Lambda 800 spectrometer. Steady-state fluorescence measurements were performed on a PerkinElmer Life Sciences 50B fluorometer. For recording the emission spectra, the freshly prepared enzyme was diluted in the measurement buffer immediately prior to the measurement. Anaerobic, air-saturated, or oxygen-saturated buffers were prepared by extensive bubbling with pure argon, air, or oxygen, respectively. The concentration of oxygen was assumed to be 0.27 mM for the air-saturated solutions at 20 °C and 1.32 mM for the oxygen-saturated solutions (42). For the oxygen titration experiments, small aliquots of oxygen-saturated buffer were introduced into the cuvette through an air-tight septum using a 10-μl Hamilton syringe.

Kinetics—Stopped-flow experiments were performed using a computer-controlled Applied-Photophysics SX18MV stopped-flow system equipped with a PBP 05-109 Spectrakinetic monochromator. The system was temperature controlled at 21 °C using a Neslab RTE-111 circulation cryostat. Ty fluorescence was detected using a photomultiplier tube equipped with an optical cut-off filter of 320 nm supplied with the stopped-flow system, thus resulting in the detection of all emitted light above 320 nm. The enzyme was introduced into the reaction chamber from a stock solution kept at 4 °C. Fluoride binding traces were fitted to a single exponential decay function of the form \(F_t = F_0 + A \exp(-k_{a,p}t)\), where \(F_0\) denotes the fluorescence at time \(t\); \(F_0\) is the fluorescence level at completion of the binding reaction; \(A\) is the signal amplitude, and \(k_{a,p}\) is the observed rate constant. The fitting was performed using the least squares fitting algorithm implemented in the stopped-flow software. The reported kinetic parameters \(k_{a,p}\) and \(A\) represent the average of at least four measurements. The standard errors were less than 10% in all cases. Because fluoride ion protonates at low pH-forming HF, effective free fluoride concentrations were calculated using a value of 3.14 for the \(pK_a\) for the HF + H+ equilibrium (42).

RESULTS

pH-dependent Inactivation of Ty—From previous studies (26), it is known that the Ty from S. antibioticus is unstable at low pH. The closely related Ty from Streptomyces glaucescens (93% sequence identity with S. antibioticus Ty) is more stable in Tris-HCl buffer than in phosphate buffer, especially at the lower pH values (43). To investigate the pH stability of S. antibioticus Ty and its dependence on the presence of halide ion, we performed experiments at 21 °C where the protein in 5 mM Pi at pH 7.20 was pH-jumped by rapidly mixing with 200 mM phosphate buffer at the pH of interest in 1:1 volume ratio (end buffer concentration 102.5 mM Pi) using stopped-flow. The reported pH values were measured on the final mixed solutions. The mixture was allowed to incubate for a fixed amount of time after which the solution was rapidly mixed with a 5 mM solution of t-butylcatechol in 5 mM Pi, at the pH of the experiment. After reaching the steady-state *(t < −50 ms)*, linear product formation was observed. The steady-state velocity of formation of the stable reaction product t-butylnquinone was optically measured at 410 nm for 1 s. Fig. 1 shows the normal-
Fluorescence Emission of Ty in Its Three Different Oxidation States—Fluorescence emission spectra of Tyred under anaerobic (Fig. 2A) and air-saturated (Fig. 2D) conditions at pH 6.80 and 21 °C are presented in Fig. 2. Whereas under anaerobic conditions only Tyred is present, under air-saturated conditions the protein exists as a mixture of 94% Tyoxy and 6% Tyred as calculated from $K_{O_2}$ and $[O_2]$ (see below and Fig. 3). Spectra of samples containing the resting form of the enzyme (predominantly Tymet) were recorded under anaerobic (Fig. 2B) and aerobic (Fig. 2C) conditions. All species display a similar line shape for the emission band with a maximum centered at 343 ± 2 nm and a half-width of 60 ± 2 nm. Little fine structure can be distinguished, and the absence of a shoulder at 300–310 nm with all species indicates that the emission is dominated by Trp fluorescence. The partial quenching of the fluorescence by oxygen (see Fig. 2) does not exhibit Stern-Volmer characteristics (in line with what may be expected on the basis of literature data (66)) but is the result of oxygen binding (see below).

When bound oxygen is displaced from Tyoxy by incubation with bromide (26), an increase in fluorescence is observed in which the magnitude parallels the decrease in the optical absorption at 345 nm typical of Tyoxy (data not shown). Therefore, it appears that the Ty fluorescence intensities depend on the oxidation state of the protein, the emission intensities of Tyred and Tyoxy differing by a factor of more than 2. The optical absorption spectra in the 250–300 nm region were identical for all three preparations (resting Ty, Tyred + Tyoxy, and Tyred; not shown).

The relative fluorescence intensity of the resting form of the enzyme (Fig. 2, B and C) depends on the presence of oxygen in the sample. This can be explained by considering that the resting form of the enzyme contains a percentage of Tyred and Tyoxy in addition to a predominant amount of Tymet (4, 5). Under anaerobic conditions only Tymet and Tyred are present, and the total fluorescence is given by Equation 1,

$$F_1 = xF_{met} + (1 - x)F_{red}$$

where $F_1$ is the observed emission intensity; $x$ is the fraction Tymet in the sample; $F_{met}$ is the normalized fluorescence intensity of Tymet; and $F_{red}$ is the normalized fluorescence intensity of Tyred. When oxygen is present, an equilibrium between Tyred and Tyoxy is established, the ratio of the two species depending on the oxygen dissociation constant $K_{O_2}$ and $[O_2]$. The total emission is then given by Equation 2,

$$F_2 = xF_{met} + (1 - x) \left(1 - \frac{K_{O_2}}{[O_2] + K_{O_2}}\right)F_{oxy} + (1 - x) \left(1 - \frac{K_{O_2}}{[O_2] + K_{O_2}}\right)F_{oxy}$$

From the measured emission intensities at $\lambda_{max}$ and Equations 1 and 2, the fraction of Tyoxy as well as the relative Tyoxy emission intensity can be calculated according to the following Equations 3 and 4,

$$x = \frac{(F_1 - F_2)K_{O_2} + (F_1 - F_2 - F_{oxy} + F_{oxy}[O_2])}{(F_{oxy} - F_{oxy}[O_2])}$$

and

$$F_{oxy} = \frac{(F_{oxy}F_2 - F_{oxy}F_1)K_{O_2} + (F_{oxy}F_1 - F_{oxy}F_2)[O_2]}{(F_2 - F_2K_{O_2} + (F_2F_1 - F_2F_1 + F_{oxy}F_2)[O_2])}$$

By using 0.27 mM for $[O_2]$, 16.5 μM for $K_{O_2}$ (see below and Fig. 3), 1 for $F_{oxy}$, 0.41 for $F_{oxy}$ (see below), and the measured intensities $F_1$ and $F_2$, amounting to 0.76 and 0.60, respectively, a value of 0.71 is obtained for the fraction Tyoxy in the sample ($x$) and a value of 0.66 for the fractional emission intensity $F_{oxy}$ relative to that of Tyred. In a resting Ty sample in air-saturated buffer at pH 6.80 and 21 °C, it is calculated that Tyoxy and Tyred contribute a fraction of 0.21 to the total fluorescence, whereas 0.79 of the emission originates from the Tymet species. We found the fraction of Tymet in our fresh preparations to be constant under the used experimental conditions.

Oxygen Binding—We have studied the steady-state binding of molecular oxygen to Tyred, rendering Tyoxy, by following the
changes occurring in the Ty Trp fluorescence as described above ($\lambda_{\text{ex}}$ 290 nm and $\lambda_{\text{em}}$ 345 nm). For this, 3.0 ml of a freshly prepared anaerobic solution of 0.1 $\mu$m Tyred in 100 ml $P_0$ at pH 6.80 was introduced in a sealed 3.5-ml quartz cuvette, after which this solution was titrated with microliter amounts of a 100 ml $P_0$ buffer saturated with oxygen. The fluorescence emission intensity at 345 nm at given $[O_2]$ was measured using a 5-s instrument integration time. The resulting measured intensities, expressed as $F/F_0$ versus $[O_2]$, are represented in Fig. 3. The data could be accurately fitted assuming the binding of a single oxygen using Equation 5,

$$F/F_0 = 1 - f_q [L]/[L] + K_d$$  \hspace{1cm} (Eq. 5)

where $F$ denotes the observed fluorescence intensity; $F_0$ denotes the fluorescence intensity in the absence of ligand; $[L]$ denotes the ligand concentration; $K_d$ denotes the dissociation constant for ligand $L$; and $f_q$ denotes the fractional decrease in fluorescence upon complete saturation with the ligand, i.e. at $[L] \gg K_{dp}^{\text{eq}}$. Values of 16.5 ± 1.2 $\mu$m for the oxygen dissociation constant and of 0.59 for $f_q$ were obtained. The latter value means that the fluorescence intensity of the Tyoxy species amounts to 0.41 of the Tyred fluorescence intensity.

The pH Dependence of Ty Fluorescence—Because we found that Ty is unstable at low pH (see Fig. 1), we measured the pH dependence of Ty fluorescence by pH jump stopped-flow at a time scale where the protein has not decayed to any measurable extent. For this, we rapidly mixed an $\approx 2 \mu$m Ty solution in 1 mm $P_0$ at pH 7.20 with a 200 mm air-saturated $P_0$ buffer at the pH of interest in a 1:1 volume ratio ($[Tyr] \approx 1 \mu$m; 3.8 $\leq$ pH $<$ 9.0). The fluorescence level was then measured for 0.25 s. The measurements were performed with three Ty preparations: resting Ty (71% Tyred, 27% Tyoxy, and 2% Tyred), reduced Ty (94% Tyoxy and 6% Tyred), and resting Ty in the presence of 100 mm fluoride. In the time period of the measurement, the fluorescence was stable. The actual pH values were measured on the mixed solutions. The obtained emission intensity data, expressed as the fractions of emission intensity relative to that of Tyred, are presented in Fig. 4. For the reduced sample containing Tyoxy and Tyred, there is no detectable pH dependence of the emission intensity. For the resting Ty sample, the emission intensity decreases at low pH. Because the emission of the reduced sample was not sensitive to the pH, the observed variation in emission must be due to changes in the contribution of Tymet. Accordingly, the experimental data were corrected for the contribution of Tyoxy and Tyred to the emission (0.21 of $F_0$ at pH 6.80) and normalized using the calculated value of $F_{\text{red}}$ of 0.66 at pH 6.80. The corrected data are plotted in Fig. 4. The Tyoxy emission data can be interpreted by assuming that the fluorescence level is under control of a single acid/base equilibrium according to Equation 6,

$$F_{\text{obs}} = F_A[H^+] + F_K[H^+] + F_B[H^+]$$  \hspace{1cm} (Eq. 6)

where $F_{\text{obs}}$ denotes the observed fluorescence level at given pH; $F_A$ denotes the fluorescence level of the acidic form of the protein, and $F_B$ the fluorescence level of the basic form of the protein. Fitting the data to Equation 6 yields a $K_{\text{eq}}$ value of 4.5 ± 0.1 and a value of 0.42 for $F_A$, i.e. the relative fluorescence level of the acidic form of the enzyme relative to the fluorescence level of Tyred. Remarkably, the fluorescence level of resting Ty + fluoride is independent of the pH between pH 3.75 and pH 6.25. Here a $[F^-]$ of 100 mm was present in both the enzyme and the buffer solution. At this $[F^-]$, the Tymet enzyme can be considered to be fully saturated with $F^-$ at all pH values used in the experiment, whereas fluoride does not interact with Tyred and Tyoxy (see Ref. 26 and see below). Depending on the pH, the relative fluorescence intensities of Tymet and Tyoxy differ significantly, showing that the protein fluorescence can be exploited as a probe to study the fluoride binding.

Stopped-flow Studies of Fluoride Binding—We studied the binding of fluoride to Tymet and its pH dependence between pH 4.0 and pH 7.9 by using stopped-flow fluorescence spectroscopy under pseudo first-order conditions ($[F^-] \gg [Tyr]$). Representative fluoride binding traces obtained by mixing $-2.5 \mu$m resting Ty with various $[F^-]$ (0–120 mm) at pH 6.80 in 1:1 volume ratio and 21°C are represented in Fig. 5A, where a decrease in fluorescence is observed upon binding of $F^-$. At all pH values and at all $[F^-]$, traces could be fitted with good accuracy to a mono-exponential decay function, yielding values for the apparent binding rate ($k_{\text{obs}}$) and signal amplitude (A) at each $[F^-]$. At pH 6.80, no change in protein fluorescence was observed when 50 mm fluoride was mixed with Tyoxy and Tyred, showing that the observed kinetics were solely due to the
The plots of $k_{\text{obs}}$ versus $[F^-]$ at given pH were linear in all cases, in agreement with a two-state binding scheme where a single fluoride ion binds to the enzyme. Representative data obtained at pH 6.03 are shown in Fig. 5B. The apparent first-order fluoride binding rate ($k_{\text{app}}$) and the $K_{\text{app}}$ fluoride dissociation rate constant ($k_{\text{off}}$) can be determined from the slope and intercept of $k_{\text{obs}}$ versus $[F^-]$, according to Equation 7 (44),

$$k_{\text{obs}} = k_{\text{app}} [F^-] + k_{\text{off}} \quad \text{(Eq. 7)}$$

At pH 6.03 (Fig. 5B), values of 11.7 $\text{mm}^{-1} \text{s}^{-1}$ and 13.9 $\text{s}^{-1}$ for the $k_{\text{app}}$ and $k_{\text{off}}$ were obtained, respectively. The signal amplitudes, expressed as $F_0/F_{\text{eq}}$, are also represented in Fig. 5B. As with the $k_{\text{obs}}$ values, the data are in agreement with the binding of a single fluoride ion and were fitted to Equation 5 with $L = F^-$. For the data at pH 6.03 in Fig. 5B, values of 0.90 mm and 0.10 were obtained for $K_{\text{app}}$ and $F_0$, respectively.

Because the protein appeared unstable at the lower pH values (see Fig. 1), the fluoride binding at the two lowest pH values was studied using pH jump sequential stopped-flow where an ~5 $\mu$M protein solution in 1 mM P$_i$ at pH 7.2 was mixed with 200 mM P$_i$ buffer at the pH of interest, after which the solution was allowed to equilibrate for 0.5 s. The resulting solution was then mixed with a fluoride solution in 100 mM P$_i$ at the pH of the experiment after which the fluorescence trace was recorded. The reported pH values were measured in the final mixed solutions. The sequential flow method gave identical results to those obtained through single mixing experiments at pH 6.0 as used for the higher pH values. At pH 4.04, an increase in fluorescence was observed, in agreement with the pH dependence of native Ty$_{\text{met}}$ and Ty$_{\text{met}}F$ fluorescence as depicted in Fig. 4 and indirectly illustrating the reversibility of the pH dependence of the native Ty$_{\text{met}}$ emission. We did not measure the fluoride binding kinetics in the pH range of 4.2–5.0, because in this region the fluorescence levels of the native and the fluoride-bound Ty$_{\text{met}}$ do not differ enough (see Fig. 4). The log values of the obtained $k_{\text{app}}$ values are represented in Fig. 6. It appears that the log($k_{\text{app}}$) is directly proportional to the pH in the range 6–8, whereas it levels off at pH < 5.5. This can be explained by Scheme 1, where fluoride ion only binds to the acidic form of the enzyme, as was proposed earlier on the basis of paramagnetic NMR and fluoride inhibition studies (26).
Fig. 6. The pH dependence of the kinetic parameters of fluoride binding to Ty met showing the apparent association rates $k_{app}^p$ (■) and the apparent dissociation constants $K_{a,app}$ determined from amplitude data (○) and from $k_{off}/k_{on}$ (□) versus the pH. For $k_{app}^p$, the solid line represents a fit to Equation 8 with $pK_a = 4.57$ and $k_{on} = 3.3 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$. For $K_{a,app}$ and $k_{off}/k_{on}$, the solid line was obtained from fitting the $K_{app}^p$ data with a $pK_a$ value of 4.68 and $K_d = 48 \mu\text{M}$. The inset shows the pH dependence of the fractional change in Ty met fluorescence $f_{max}$ upon complete saturation with fluoride as obtained from stopped-flow $F/F_0$ versus $[F^-]$ data. The solid line represents a fit of the data with a $pK_a$ value of 4.57. In all measurements, the conditions were 100 mM Pi, 21°C, $\chi_{max}$ 285 nm and $\chi_{em}$ > 320 nm.

chloride binding to Ty met shows analogous behavior to that of fluoride binding. It is therefore unlikely that the observed pH dependence of the apparent $k_{on}$ for fluoride originates from the exclusive binding of HF (pK a 3.14), because HCl (pK a -7) occurs fully dissociated in the pH range of the experiment.

The finding that the fluoride binding traces follow monomolecular behavior, as well as linear dependence of $k_{off}$ on [F -] at all pH values, shows that the pH-dependent step is fast in comparison with the fluoride binding step. In that case, the measured $k_{app}^p$ values are proportional to the fraction of Ty met that is in the low pH form (44) according to Equation 8,

$$k_{app}^p = \frac{[H^+]}{[H^-] + K_a}$$  \hspace{1cm} (Eq. 8)

Fitting the data in Fig. 6 to this function yielded a $pK_a$ of 4.6 ± 0.1, which is within error of the $pK_a$ found for the pH dependence of native Ty met fluorescence as depicted in Fig. 4. The fit also yielded an estimate for the $k_{on}$ of fluoride binding to the low pH form of the enzyme, amounting to $(3.3 \pm 0.7) \times 10^7 \text{ M}^{-1} \text{s}^{-1}$. The determined values for $k_{off}$ are much less dependent on the pH, in agreement with the mechanism in Scheme 1 (44), and varied between 8 s$^{-1}$ and 16 s$^{-1}$ without showing a clear correlation with the pH (not shown). From the $k_{on}$ and the value of $k_{off}$ determined at the lowest pH used (pH 4.04; 12.3 ± 0.8 s$^{-1}$), the $K_d$ for fluoride binding to the low pH enzyme can be estimated from the relation $K_d = k_{off}/k_{on}$, amounting to 37 ± 8 μM.

The values for the apparent dissociation constants $K_{a,app}^p$ as determined from signal amplitudes and Equation 5 follow similar behavior as the $k_{off}$ values, as expected from the relative insensitivity of $k_{off}$ to the pH, as shown in Fig. 6. Here the data could be fitted to Equation 8 (with $k_{on}$ substituted for by $K_a$) with a $pK_a$ value of 4.7 ± 0.2, whereas the dissociation constant for the Ty met F complex was estimated at 54 ± 22 μM by extrapolation to low pH. As depicted in Fig. 6, there is good agreement between the $K_{a,app}^p$ values determined from amplitude data and those calculated from $k_{max}^p = k_{off}/k_{on}$. From the latter data, values of 4.7 ± 0.2 for the $pK_a$ and 48 ± 10 μM for the $K_d$ for the Ty met F complex were obtained.

The pH dependence of the determined $f_o$ values, i.e. the fractional changes in fluorescence upon complete saturation of Ty met with fluoride, is shown in Fig. 6. The obtained values are in good agreement with the data obtained for the pH dependence of the Ty met and Ty met F fluorescence intensities as described above (Fig. 4). The data could be fit assuming that $f_o$ is dependent on a single acid/base equilibrium, yielding an estimate for the $pK_a$ value of 4.6 ± 0.1.

**DISCUSSION**

Ty Fluorescence Properties and Emission Quenching—Ty from *S. antibioticus* contains 12 tryptophans and 6 tyrosines on a total of 272 amino acids. Consistent with the Trp/Tyr ratio and the excitation wavelength employed, all Ty species (Ty met, Ty oxy, and Ty red) display emission spectra dominated by Trp fluorescence. The positions of the emission maxima (343 ± 2 nm for all species) are the highest reported for a type 3 copper protein and indicate that a significant fraction of the Trp residues is exposed to the solvent. As was found earlier for several hemocyanins (27–39) and *Neurospora crassa* tyrosinase (40), it appears that the Ty fluorescence emission intensities are markedly sensitive to the oxidation state of the dinuclear copper center; the emission quantum yields of Ty red and Ty oxy differ by more than a factor of 2.

The mechanism of quenching has not been unambiguously established for any He or Ty. For Ty oxy, the fluorescence quenching possibly occurs through Förster energy transfer because the Ty emission spectrum ($\lambda_{max} = 343$ nm) overlaps with the strong LMCT transition absorption band characteristic of Ty oxy ($\epsilon_{max} = 18.5 \text{ M}^{-1} \text{cm}^{-1}$). The energy transfer mechanism may also contribute to the fluorescence quenching in the Ty met species considering the weak LMCT between the Cu(II) and the ligand nitrogens in the emission region ($\epsilon_{max} = 2 \text{ M}^{-1} \text{cm}^{-1}$). The relatively high quantum yield of the Ty oxy species may be due to the absence of Förster quenching consistent with the absence of absorption bands in the emission region. The fluorescence changes that occur upon fluoride binding to Ty met might be associated with electronic changes at the active site. To elucidate the mechanism of quenching, further study is needed. For the purpose of the present investigations it is sufficient to note that the large differences between the emission intensities of the various Ty species can be used as a sensitive probe of ligand binding and of interconversion between the Ty redox states.

The Composition of Resting Ty and Oxygen Binding—The resting form of the enzyme in our preparations is a mixture of oxidized (Ty met) and reduced (Ty red + Ty oxy) enzyme. From the fluorescence data in Fig. 2, it was possible to calculate that 71% of the enzyme occurs in the oxidized form, whereas the remainder is a mixture of Ty oxy and Ty red, the ratio of the latter two species depending on the concentration of oxygen in the sample. In the literature, it is usually reported that more than 85% of the enzyme occurs in the oxidized form in resting Ty (5). The ratio of oxidized and reduced Ty in resting preparations determines in part the length of the lag phase observed in the conversion of monophenolic compounds (5). The stable percentage of Ty met in resting preparations may reflect a natural
equilibrium between the three Ty species present in solution. Establishment of this equilibrium may be connected with the dissociation of peroxide from Ty\textsubscript{oxy} resulting in the formation of Ty\textsubscript{met}, as observed for \textit{N. crassa} Ty (45). This would be consistent with the observed slow decay of Ty\textsubscript{oxy} to Ty\textsubscript{met} in fresh preparations of \textit{S. antibioticus} Ty\textsubscript{met}.

The large difference between the Ty\textsubscript{red} and Ty\textsubscript{oxy} quantum yields has been used to study the binding of molecular oxygen to the reduced enzyme (Fig. 3). The obtained dissociation constant for the oxygenated Ty complex of 16.5 \(\mu\text{M}\) is lower than that reported for mushroom Ty (47 \(\mu\text{M}\) (46)) and \textit{Octopus vulgaris} hemocyanin (90 \(\mu\text{M}\) (47)). This higher affinity of Ty\textsubscript{red} for oxygen may reflect adaptation to an oxygen-poor environment, as experienced by soil bacteria like \textit{Streptomyces} in their natural environment.

The Kinetics of Fluoride Binding—Recently a structural and mechanistic study of the interaction of halide ions with \textit{S. antibioticus} Ty was reported (26). The interaction of fluoride with Ty was the most extensively studied. In contrast to the other halides, fluoride solely interacts with the Ty\textsubscript{met} form of the protein and acts as a simple competitive inhibitor in the conversion of l-DOPA, whereas the other halides interact with both Ty\textsubscript{met} and Ty\textsubscript{red}.

It appears that the Ty\textsubscript{met} and the Ty\textsubscript{met}F fluorescence emission levels differ significantly and that this difference is dependent on the pH as depicted in Figs. 4 and 6 (inset). This feature has been exploited in studying the transient state kinetics of fluoride binding to Ty\textsubscript{met} by stopped-flow fluorescence spectroscopy. All data are in agreement with the binding of a single fluoride ion and can be explained by adopting a simple binding mechanism. Likewise, titrations of Ty\textsubscript{met} with fluoride followed by \(^{1}\text{H}\) paramagnetic NMR (26) could also be explained by assuming a binding mechanism where a single fluoride ion binds to Ty\textsubscript{met}. From that work, it was proposed that a single halide ion binds to Ty\textsubscript{met} at the Cu\textsubscript{2} bridging position. The findings presented here are consistent with this hypothesis. The presence of a Cu\textsubscript{2} bridging ligand in Ty\textsubscript{met} is also corroborated by extended x-ray absorption fine structure studies on \textit{S. antibioticus} Ty\textsuperscript{3} and oxidized catechol oxidase (48), indicating 3N/1O coordination for each copper ion, as well as with the crystal structure of oxidized sweet potato catechol oxidase, where a single Cu\textsubscript{2} bridging ligand (possibly chloride) was identified in the electron density map upon refinement (12, 49).

At pH 6.80, the \(K_{qF}^{pp}\) value as determined from amplitude data is 6.0 \(\text{mM}\), whereas the \(K_{qF}^{pp}\) amounts to 7.4 \(\text{mM}\) by using the relation \(k_{qF}^{pp} = K_{qF}^{pp}\). These values, taking into account the notable pH sensitivity of the fluoride binding, compare reasonably well with the competitive inhibition constant for fluoride of 11.8 \(\text{mM}\) obtained at the same pH and temperature and the dissociation constant of 5.1 \(\text{mM}\) for the fluoride Ty\textsubscript{met} complex determined at pH 7.06 and 4 °C by using paramagnetic NMR (26). The relatively low value of the fluoride dissociation rate, \(k_{qF}^{pp} (13.7 \pm 0.5 \text{ s}^{-1}\) at pH 6.80), is in agreement with the previous finding that fluoride is in slow exchange on the (paramagnetic) NMR shift time scale (26). The observation that no changes in fluorescence are observed when Ty\textsubscript{red} or Ty\textsubscript{oxy} are mixed with 50 \(\text{mM}\) fluoride at pH 6.80 is in agreement with the previous finding that fluoride solely interacts with the Ty\textsubscript{met} protein.

The pH Dependence of the Kinetic Parameters of Fluoride Binding to Ty\textsubscript{met}—It appears that the fluoride binding to Ty\textsubscript{met} is strongly dependent on the pH, as was observed earlier by paramagnetic NMR of Ty\textsubscript{met} and its halide-bound species and by kinetic studies of the inhibition of the conversion of l-DOPA by halide ion (26). The latter data could be explained by assuming that the halide binding to Ty\textsubscript{met} is under control of a single acid/base equilibrium where the ion only binds to the acidic form of Ty\textsubscript{met}. Due to the experimental limitations imposed by the instability of Ty\textsubscript{met} at low pH and the relatively long experimental times required, it was only possible to determine an upper limit for the \(pK_{a}\) of this acid/base equilibrium (\(pK_{a} < 5.5\)). By using the faster method of stopped-flow fluorimetry, the problem of protein instability has been circumvented in the present study.

The kinetic parameters of fluoride binding corroborate the earlier finding that fluoride ion only binds to the acidic form of the enzyme as witnessed by the pH dependence of \(k_{qF}^{pp}\) and \(K_{qF}^{pp}\) (Fig. 6) and the relative insensitivity of \(k_{qF}^{pp}\) to the pH. The data obtained for the pH dependence of \(K_{qF}^{pp}\) could be fitted using a \(pK_{a}\) value of 4.6 \(\pm 0.1\), which is equal, within the experimental error, to the \(pK_{a}\) values obtained from the pH dependence of \(K_{qF}^{pp}\) and \(k_{qF}^{pp}\) (4.7 \(\pm 0.2\)). Similar \(pK_{a}\) values are obtained from the pH dependence of the Ty\textsubscript{met} fluorescence (Fig. 4; \(pK_{a} = 4.5 \pm 0.1\)) and of \(f_{q}\), i.e., the fractional changes in Ty\textsubscript{met} emission upon complete saturation with fluoride (Fig. 6; \(pK_{a} = 4.6 \pm 0.1\)). This provides a total of 5 \(pK_{a}\) values, the average amounting to 4.59 \(\pm 0.05\).

Fluoride Replaces Hydroxide as the Bridging Ligand in Ty\textsubscript{met}—Fluoride is known to inhibit many metalloenzymes (e.g., superoxide dismutase (50), urease (51), laccase (52), peroxidase (53), and enolase (54)), often with apparent inhibition constants in the micromolar to millimolar range. In most cases, the fluoride binding is pH-dependent, and it is thought that fluoride replaces a metal-bound hydroxide ion or water ligand. The results presented here suggest that the same occurs with Ty. We propose that the pH dependence of fluoride binding derives from the pH-dependent dissociation of Cu\textsubscript{2} bridging hydroxide according to the mechanism outlined in Scheme 2. Direct dissociation of OH\textsuperscript{−} or dissociation of water after protonation of the bound hydroxide are indistinguishable at present. As established earlier on the basis of paramagnetic NMR of Ty\textsubscript{met} (26), it can be excluded that the pH dependence of fluoride binding results from a protonation and subsequent dissociation of a coordinating histidine residue at low pH. Such a mechanism was proposed earlier to explain the pH dependence of halide inhibition (22, 55, 56). A pH-dependent dissociation of Cu\textsubscript{2} bridging hydroxide has been shown to occur for several small dinuclear copper model compounds (57–59). Therefore,
pK_a values between 4.0 and 5.5 have been obtained for the dissociation process, comparable with the average pK_a of 4.59 ± 0.05 found in the present case. This low pK_a value can be ascribed to the charge of the two copper ions and the relatively low basicity of the coordinating histidine ligands. The linear dependence of k_{obs} on [F^-] at all pH values implies that both the hydroxide dissociation and association processes are much faster than the corresponding steps for fluoride.

The pH Dependence of the Tymet Fluorescence Emission.—The pH dependence of the fluorescence emission intensity of Ty was studied under various conditions by pH jump stopped-flow fluorimetry (Fig. 4). It appeared that the emission level of the Tymet enzyme is dependent on an acid/base equilibrium with an apparent pK_a value of 4.5 ± 0.1, which is identical to the experimental pK_a values found for fluoride binding. This strongly suggests that the pH dependence of Tymet emission is associated with pH-dependent changes occurring directly at the oxidized dinuclear copper center. If it would reflect, for example, a protonation of a carboxylic acid residue in close vicinity to a fluorescent Trp or pH-dependent changes in protein structure, a pH dependence...
of the emission would also be expected for the other species, contrary to what is observed. Thus, in view of the current proposal that bridging hydroxide dissociates at low pH, it is likely that this process is responsible for the decrease in quantum yield. We note that this would lead to loss of the antiferromagnetic coupling between both copper ions, as has been shown to occur for Cu2 model compounds. Therefore, a marked change in paramagnetism is expected upon loss of the bridging ligand, which could be related to the lower quantum yield of the enzyme at low pH.

The Role of the Bridging Ligand in the Diphenolase Mechanism—The role of bridging hydroxide in the oxidation of diphenols has yet to be established. Previously it was shown that halide is displaced from Tynett if inhibitors that mimic the transition state (e.g. mimosin, Kojic acid) are bound to the enzyme. On this basis a model for diphenol coordination was proposed whereby one of the phenolic oxygens bridges the two enzyme. On this basis a model for diphenol coordination was proposed whereby one of the phenolic oxygens bridges the two copper ions by displacing the bridging hydroxide that is present in native Tynett (26). The displacement of an equatorial water ligand by bidentate ligands has also been shown to occur for halide-oxidized Ty (Tynett

Enzyme Inactivation and the Protective Effect of Halide Ion—As depicted in Fig. 1, Ty is rapidly inactivated at low pH where the rate of inactivation is pH-dependent. It is likely that this inactivation originates from the loss of the Cu, bridging hydroxide in Tynett, i.e. one negative charge, at low pH, which illustrates the importance of charge compensation in the stability of protein metal sites. Support for this is provided by the observation that protein inactivation proceeds much slower when fluoride or chloride, which are able to substitute for OH ligand, are present in solution. Furthermore, the stabilizing effect of fluoride and the finding that this ion specifically interacts with Tynett suggest that the inactivation is specifically due to the decay of the Tynett form. Tynett and Tynett do not seem to be susceptible to rapid protein inactivation, providing further support for the loss of bridging OH− as the cause for the inactivation of Tynett.

Physiological Relevance of the Halide Inhibition—It has been proposed that the large variations in the extent of pigmentation in different human skin types originate from variations in the melanosomal pH (63–65). These pH variations would influence the Ty activity, a lower pH value being associated with a lower Ty activity and, thus, with a lower pigment level. For S. antibioticus Ty, we found that the apparent chloride inhibition constant (0.16 mM at pH 6.80) is in the order of physiological chloride concentrations (5–200 mM). Similar values for the apparent chloride inhibition constant, as well as strong pH dependence of halide inhibition, have been found for other Tys (22, 55, 56). Thus, depending on the [Cl−] to KCl ratio, a significant fraction of the enzyme may exist as the inactive chloride-bound form at low pH, which is, however, protected from protein degradation. This might provide an explanation of the sensitivity of Ty activity to melanosomal pH.

Conclusion—In conclusion, the use of Ty fluorescence quenching as a probe in studying the kinetics of fluoride binding to the type 3 copper center yields results that are consistent with previously obtained kinetic and spectroscopic data. The method provides a direct spectroscopic probe of fluoride binding to Tynett. Stopped-flow fluorimetric experiments can be performed with high sensitivity and speed, thereby avoiding the need for large amounts of protein and the experimental difficulties arising from the intrinsic instability of the enzyme, the latter being important especially at the lower pH values. The quenching of the Tynett emission upon the binding of exogenous ligands may not be limited to the Ty from S. antibioticus; a similar behavior for other type 3 copper proteins would allow for a systematic comparison of the ligand binding properties of different tyrosinases, catechol oxidases, and hemocyanins, potentially providing further insight into the mechanisms that underlie the variation in the functionality of these proteins. The notable sensitivity of the Ty emission quantum yield to the state of the type 3 copper center holds promise for further studies. For example, the Ty fluorescence could be used to study the kinetics of the conversion of monophenolic and diphenolic substrates. The thermodynamics and pH dependence of inhibitor binding could be investigated by stopped-flow fluorescence kinetics. A systematic investigation of the binding kinetics of a range of organic inhibitors to Tynett is underway.

REFERENCES

1. Oetting, W. S., and King, R. A. (1983) J. Investig. Dermatol. 183, S131–S136
2. Oetting, W. S. (2000) Pigg. Cell Res. 13, 320–325
3. van Gelder, C. W., Flurkey, W. H., and Wichers, H. J. (1997) Phytochemistry 45, 1299–1323
4. Solomon, R. I., Sundaram, U. M., and Mackinnon, T. E. (1996) Chem. Rev. 96, 2563–2605
5. Sánchez-Ferrer, A., Rodriguez-López, J. N., García-Cánovas, F., and García-Cánovas, F. (1995) Biochim. Biophys. Acta 1247, 1–11
6. S., Sharma, V. K., and Sharma, N. (2003) J. Agric. Food Chem. 51, 2837–2853
7. Decker, H., Dilling, R., and Tuczcz, F. (2000) Angew. Chem. Int. Ed. Engl. 39, 1587–1591
8. Babcock, L., Salgado, J., Tepper, A. W., Vigenboon, E., and Canters, G. W. (1999) FEBS Lett. 422, 215–220
9. Cuff, M. E., Miller, K. I., van Holde, K. E., and Hendrickson, W. A. (1998) J. Mol. Biol. 278, 855–870
10. Hazes, B., Magnus, K. A., Bonavaudant, C., Bonavaudant, J., Dauter, Z., Kalk, A., and Hol, W. J. H. (1993) J. Biol. Chem. 268, 9125–9132
11. Magnus, K. A., Hazes, B., Bonavaudant, C., Bonavaudant, J., and Hol, W. G. (1994) Proteins 19, 302–309
12. Kobunde, T., Ricken, C., Scatichetti, J. C., and Krebs, B. (1998) Nat. Struct. Biol. 5, 1084–1090
13. Decker, H., and Tuczcz, F. (2000) Trends Biochem. Sci. 25, 392–397
14. Cano-Castaño, C., and Krebs, B. (2002) Acc. Chem. Res. 35, 183–191
15. Cabanes, J., Garcia-Carmona, F., Garcia-Canoas, F., Ibora, J. L., and Lozano, J. A. (1984) Biochim. Biophys. Acta 790, 101–107
16. Espin, J. C., and Wichers, H. J. (1999) J. Agric. Food Chem. 47, 2638–2644
17. Hasabu, H., and Takahashi, H. (1977) Mol. Pharmacol. 13, 362–367
18. Healey, D. F., and Strothkamp, K. G. (1981) Arch. Biochem. Biophys. 211, 86–91
19. Jimenez, M., Chazarras, S., Escribano, J., Cabanes, J., and Garcia-Carmona, F. (2001) J. Agric. Food Chem. 49, 4060–4063
20. Kubo, I., and Kinst-Hori, I. (1999) J. Agric. Food Chem. 47, 4121–4125
21. Madsalson, J. F., and Paul, K. P. (1988) Experientia (Basel) 44, 885–887
22. Martínez, J. H., Solano, F., Peñuelas, R., Galindo, J. D., Ibora, J. L., and Lozano, J. A. (1986) Comp. Biochem. Physiol. B Comp. Biochem. Mol. Biol. 84, 633–636
23. Mein, S., Fleck, R. W., Yong, G., and Strothkamp, K. G. (1990) Arch. Biochem. Biophys. 280, 27–32
24. No, J. K., Soung, D. Y., Kim, Y. J., Shin, H. K., Jun, Y. S., Bhee, S. H., Yokosawa, T., and Chang, H. Y. (1999) Life Sci. 65, 1241–1246
25. Babcock, L., Vigenboon, E., Gobin, C., Tepper, A. W. J., Salgado, J., and Canters, G. W. (2000) J. Mol. Catal. B Enzymatic 8, 27–35
26. Tepper, A. W. J., Babcock, L., and Canters, G. W. (2002) J. Biol. Chem. 277, 30436–30444
27. Lippitz, M., Erker, W., Decker, H., van Holde, K. E., and Basche, T. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 2772–2777
28. Shutat, J., Dolashka-Angelova, P., Abrashiev, R., Nicolov, P., and Voelter, W. (2001) Biochim. Biophys. Acta 1546, 325–336
29. Dolashka-Angelova, P., Hristova-Angelova, L., Stoeva, S., Schwarz, H., and Voelter, W. (2000) Spectrochim. Acta A Mol. Spectrosc. 56, 1985–1999
30. Dolashka-Angelova, P., Schick, M., Stoeva, S., and Voelter, W. (2000) Int. J. Biochem. Cell Biol. 32, 529–538
31. Hristova, R., Dolashka-Angelova, P., Giga, M., and Voelter, W. (2000) Oxid. Commun. 23, 145–152
32. Pervanova, K., Ilakieva, K., Stoeva, S., Genov, N., and Voelter, W. (2000)
Stopped-flow Studies of Inhibitor Binding to Tyrosinase

33. Dolashka-Angelova, P., Hristova, R., Stoeva, S., and Voelter, W. (1999) Spectrochim. Acta Part A Mol. Spectrosc. 56, 615–622.

34. Ali, S. A., Stoeva, S., Abbasi, A., Georgieva, D. N., Genov, N., and Voelter, W. (1999) Comp. Biochem. Physiol. A 122, 65–74.

35. Hristova, R., Dolashka, P., Stoeva, S., Voelter, W., Salvato, B., and Genov, N. (1997) Spectrochim. Acta Part A 53, 471–478.

36. Dolashka, P., Genov, N., Parvanova, K., Voelter, W., Geiger, M., and Stoeva, S. (1996) Biochem. J. 315, 139–144.

37. Idakieva, K., Stoeva, S., Voelter, W., and Genov, N. (1995) Comp. Biochem. Physiol. A 108, 877–883.

38. Stoeva, S., Dolashka, P., Bankov, B., Voelter, W., Salvato, B., and Genov, N. (1995) Spectrochim. Acta Part A Mol. Spectrosc. 51, 1965–1974.

39. Shaklai, N., Gufni, A., and Daniel, E. (1978) Biochemistry 17, 4438–4442.

40. Beltramini, M., and Lerch, K. (1982) Biochem. J. 205, 173–180.

41. Jackman, M. P., Hajnal, A., and Lerch, K. (1991) Biochem. J. 274, 707–713.

42. Dean, J. A. (1999) Lange's Handbook of Chemistry, 15th Ed., section 5.6, McGraw-Hill, New York.

43. Lerch, K., and Ettlinger, L. (1972) Eur. J. Biochem. 21, 427–437.

44. Fersht, A. R. (1999) Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding, W. H. Freeman & Co., New York.

45. Wilcox, D. E., Porras, A. G., Hwang, Y. T., Lerch, K., Winkler, M. E., and Miller, M. A. (1998) Inorg. Chem. 37, 677–683.

46. Eicken, C., Zippel, F., Buldt-Karentzopoulos, K., and Krebs, B. (1998) FEBS Lett. 436, 293–299.

47. Salvato, B., Santamaria, M., Beltramini, M., Alzuet, G., and Casella, L. (1998) Biochemistry 37, 14065–14077.

48. Eicken, C., Zippel, F., Buldt-Karentzopoulos, K., and Krebs, B. (1998) FEBS Lett. 436, 293–299.

49. Eicken, C., Krebs, B., and Sacchettini, J. C. (1999) Curr. Opin. Struct. Biol. 9, 677–683.

50. Meier, B., Scherk, C., Schmidt, M., and Parak, F. (1998) Biochem. J. 331, 403–407.

51. Todd, M. J., and Hausinger, R. P. (2000) Biochemistry 39, 5389–5396.

52. Xu, F. (1997) J. Biol. Chem. 272, 924–928.

53. Neri, F., Kok, D., Miller, M. A., and Smulevich, G. (1997) Biochemistry 36, 8947–8953.

54. Lebdia, L., Zhang, E., Lewinski, K., and Brewer, J. M. (1993) Proteins Struct. Funct. Genet. 16, 219–225.

55. Martinez, J. H., Solano, F., Garcia-Borrón, J. C., Iborra, J. L., and Lozano, J. A. (1985) Biochem. Int. 11, 729–738.

56. Peñafiel, R., Galindo, J. D., Solano, F., Pedreno, E., Iborra, J. L., and Lozano, J. A. (1984) Biochem. Biophys. Acts 889, 327–332.

57. Bell, C., Beguin, C., Gautier-Luneau, I., Hamman, S., Philouze, C., Pierre, J. L., Thomas, J., and Torelli, S. (2002) Inorg. Chem. 41, 479–491.

58. Monzani, E., Quinti, L., Perotti, A., Casella, L., Gullotti, M., Randaccio, L., Geremia, S., Nardini, G., Faleschini, F., and Tabbi, G. (1998) Inorg. Chem. 37, 553–562.

59. Torelli, S., Belle, C., Gautier-Luneau, I., Pierre, J. L., Saint-Aman, E., Latour, J. M., Le Pape, L., and Luneau, D. (2000) Inorg. Chem. 39, 3526–3536.

60. Babacco, L., van Gastel, M., Groenen, E. J., Vigreux, E., and Canters, G. W. (2003) J. Biol. Chem. 278, 7381–7389.

61. van Gastel, M., Babacco, L., Groenen, E. J., Vigenboum, E., and Canters, G. W. (2000) FEBS Lett. 474, 228–232.

62. Amudha, P., Akilan, P., and Randaswamy, M. (1999) Polyhedron 18, 1355–1362.

63. Naeyaert, J. M., Eller, M., Gordon, P. R., Park, H. Y., and Gleichrest, B. A. (1991) Br. J. Dermatol. 125, 297–303.

64. Iwata, M., Corn, T., Iwata, S., Everett, M. A., and Fuller, B. B. (1990) J. Investig. Dermatol. 95, 9–15.

65. Mizukami, K., Hoganson, G. E., Pennella, R., Everett, M. A., and Fuller, B. B. (1993) J. Investig. Dermatol. 100, 806–811.

66. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy, 2nd Ed., pp. 242, 460, and 537, Kluwer/Plenum, Dordrecht, The Netherlands.
Stopped-flow Fluorescence Studies of Inhibitor Binding to Tyrosinase from
Streptomyces antibioticus
Armand W. J. W. Tepper, Luigi Bubacco and Gerard W. Canters

J. Biol. Chem. 2004, 279:13425-13434.
doi: 10.1074/jbc.M309367200 originally published online December 29, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M309367200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 59 references, 6 of which can be accessed free at
http://www.jbc.org/content/279/14/13425.full.html#ref-list-1