Inhibitor Binding to the Binuclear Active Site of Tyrosinase: Temperature, pH, and Solvent Deuterium Isotope Effects†

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Received November 8, 1993; Revised Manuscript Received February 16, 1994®

ABSTRACT: Competitive inhibition of the monophenolase reaction of tyrosinase by a variety of compounds was investigated with respect to temperature and pH. Derivatives of benzoic acid as well as *p*-nitrophenol were the best inhibitors. Toluene and several N-heterocycles were all weak inhibitors. Thermodynamic parameters of toluene inhibition were qualitatively different from those of aromatic acids and were consistent with a hydrophobic binding site for toluene. Inhibition by both aromatic acids and *p*-nitrophenol was strongly pH-dependent over the range 5.1–8.0, with inhibitor binding favored at lower pH. In contrast, toluene binding and indazole binding were pH-independent while benzimidazole binding was favored at higher pH. For both carboxylic acids and *p*-nitrophenol, inhibitor binding was quantitatively accounted for by assuming the protonated, electrically neutral form of the inhibitor is the reactive species. A solvent deuterium isotope effect on the binding of benzoic acid was observed. A proton inventory study indicated that a single proton transferred from benzoic acid to a site with a fractionation factor of 0.64 ± 0.02 accounts for the isotope effect. The identity of the proton acceptor and possible mechanistic implications are discussed.

Tyrosinase (EC 1.14.18.1; monophenol, dihydroxyphenylalanine:oxygen oxidoreductase) catalyzes the ortho hydroxylation of monophenols (monophenolase activity) and the oxidation of catechols to *o*-quinones (diphenolase activity) (Mason, 1965). The active site contains a pair of antiferromagnetically coupled copper ions (Schoot Uiterkamp & Mason, 1973; Makino et al., 1974) that can exist in either the bicupric (met) or the bicuprous (deoxy) states. Oxytyrosinase is formed by the reaction of the deoxy form with dioxygen or the met form with hydrogen peroxide (Jolley et al., 1972, 1974). Oxytyrosinase is in reversible equilibrium with the deoxy form of the protein and free oxygen and appears to be an intermediate in the catalytic cycle (Makino & Mason, 1973). Oxytyrosinase has electronic and vibrational spectral features which are very similar to those of oxyhemocyanin, the oxygen transport protein found in some molluscs and arthropods (Solomon, 1981). The structure of oxyhemocyanin from the arthropod *Limulus polyphemus* has recently been determined by X-ray diffraction (Magnus & Ton-That, 1992). The oxygen is coordinated to the binuclear site in a side-on (μ-η^2:η^2) peroxide geometry, as first reported for a model compound (Kitajima et al., 1989). The relationship of this structure to the spectroscopic characteristics of oxytyrosinase and the reactivity of the coordinated peroxide have been discussed (Ross & Solomon, 1991; Solomon, 1992; Baldwin et al., 1992).

A mechanism for the ortho hydroxylation of phenols by tyrosinase has been proposed (Figure 1) (Ross & Solomon, 1991; Solomon & Lowery, 1993). In this mechanism, the phenolate oxygen of the substrate coordinates to one copper of the oxytyrosinase binuclear site. This causes a distortion in oxygen binding, resulting in electrophilic attack at the ortho position of the ring by one atom of the coordinated oxygen molecule. The resulting catechol is then oxidized to the *o*-quinone, and the deoxy active site combines with another oxygen molecule, forming oxytyrosinase and completing the catalytic cycle. This mechanism, in which one copper of the binuclear site coordinates with the phenolic substrate, assumes differential reactivity of the two copper ions. Since this proposed model of oxytyrosinase has identical first coordination spheres for the two copper ions, this difference presumably results from the tertiary structure of the protein. Evidence favoring a differential reactivity includes formation of a mononuclear (metapo) derivative, as well as mixed-metal derivatives (Yong et al., 1990) of the binuclear site.

Aromatic carboxylic acids, such as benzoic acid, are potent inhibitors of tyrosinase. These compounds are competitive inhibitors with respect to the phenol substrate and are thought to combine with the oxy form of the enzyme (Menon et al., 1990). Direct interaction with oxytyrosinase has been observed spectroscopically in the absence of the phenolic substrate, and coordination of the carboxylate group to one copper of the binuclear site was suggested (Figure 1) (Wilcox et al., 1985). This would mimic the coordination of the phenolate oxygen of the substrate.

We report a systematic study of the inhibition of tyrosinase by several types of compounds. Inhibitor binding was studied as a function of temperature and pH. In addition, a solvent deuterium isotope effect on inhibitor binding was observed. These results indicate that inhibitor binding involves proton transfer and suggest a role for such a transfer in the catalytic mechanism.

MATERIALS AND METHODS

Tyrosinase was isolated from mushrooms as previously described (Nelson & Mason, 1970; Jolley et al., 1974). Some experiments employed commercially available enzyme (Sigma Chemical Co.). Inhibitor binding constants obtained using the commercial enzyme and enzyme prepared in this laboratory were the same within experimental error. L-Tyrosine and deuterium oxide (99.9% D) were obtained from Sigma. All inhibitors were from Aldrich. Buffers were prepared using reagent-grade citric acid and dibasic sodium phosphate in deionized water.

Unless stated otherwise, all experiments utilized 0.200 M dibasic sodium phosphate/0.100 M citric acid, pH 5.60, and

† Supported by NIH Grant DK 41550.
® Abstract published in *Advance ACS Abstracts,* April 15, 1994.

0006-2960/94/0433-5739$04.50/0

were performed at 25.0 ± 0.2 °C. The buffer pH was measured with an Orion Model 801A pH meter before and after preparation of substrate and inhibitor solutions and readjusted if necessary. In experiments at temperatures other than 25.0 °C, the buffer pH was adjusted at the temperature of the experiment. Since the heat of ionization of benzoic and *o*-toluic acids is almost zero (Fasman, 1976), the ratio of acid/base forms of the inhibitor was constant in the temperature variation experiments. In experiments where the pH was other than 5.60, the total buffer concentration was adjusted as necessary to keep the ionic strength of the buffer constant at 0.771, the value at pH 5.60. Thus, the effect of pH on inhibitor binding was measured at constant ionic strength. In D_2O solutions, pD was calculated as the pH meter reading + 0.4 (Schowen & Schowen, 1982).

Enzyme inhibition was measured as a function of the tyrosine concentration in air-saturated solutions either by measuring the rate of oxygen consumption using a Gilson K-IC Oxygraph and Clark oxygen electrode (Menon et al., 1990) or by measuring the formation of product spectrophotometrically at 280 nm using a Hewlett Packard 8452A spectrophotometer (Duckworth & Coleman, 1970). For given inhibitor and solution conditions, both methods gave identical inhibition constants (K_i).[1] Double-reciprocal plots of initial velocity vs initial tyrosine concentration were used, along with appropriate replots, to determine the inhibition constants (Segel, 1975). Best-fit lines were determined by the method of least squares.

The pK_a of *p*-nitrophenol in the buffer employed for the kinetics experiments was determined spectrophotometrically at a wavelength of 465 nm. At this wavelength, only the basic form absorbs. A linear Beer's Law plot was obtained by adjusting the pH of a 1.00 mM solution of the compound in buffer to pH 11 and making a series of dilutions with pH 11 buffer. The same extinction coefficient was obtained in buffer and in pure water adjusted to pH 11 with NaOH. This extinction coefficient was used to calculate the concentration of the basic form of the compound at each of the different buffer pHs used for the kinetics experiments. Knowing the total concentration of compound, the concentration of the acidic form was determined by difference. Using these concentrations and the known exact pH of each solution, the data were plotted according to the Henderson–Hasselbach equation:

$$\mathrm{pH} = \mathrm{p}K_a + \log([\text{basic form}]/[\text{acidic form}])$$

and the pK_a was determined.

The solvent deuterium isotope effect on the K_i for benzoic acid was determined as a function of the atom fraction of deuterium in the solution. Stock solutions of buffer, tyrosine, and benzoic acid in H_2O and D_2O were prepared and combined in various proportions to give different atom fractions (n) of deuterium. The atom fraction of deuterium in the stock D_2O solutions was determined to be 0.985 by NMR (JEOL JNM-PMX60 spectrophotometer) using acetonitrile as the internal reference (Schowen & Schowen, 1982). D_2O buffers were prepared by lyophilizing the corresponding H_2O buffer (pH 5.60) and dissolving the solid in D_2O. The pD of the resulting buffer was 6.09. The difference, pD − pH, of 0.49 exactly matches the change in pK_a of benzoic acid in going from H_2O to D_2O (Lowe & Smith, 1975). Thus, the ratio of acid to base forms of benzoic acid was the same in the H_2O and D_2O solutions. Enzyme samples were prepared to have the same percent D_2O as the reaction mixture by combining stock solutions of enzyme in H_2O and D_2O in the appropriate proportions. Use of enzyme in H_2O did not change the value of the deuterium isotope effect on K_i. Thus, equilibration of enzyme in advance with the percent D_2O being used in a given experiment was not actually necessary.

[1] Abbreviations: K_i, apparent dissociation constant of the enzyme–inhibitor complex; K_i^*, pH-independent dissociation constant of the enzyme–inhibitor complex; K_f (=$1/K_i$), apparent formation constant of the enzyme–inhibitor complex; K_f^* (=$1/K_i^*$), pH-independent formation constant of the enzyme–inhibitor complex.

Table 1: Competitive Inhibitors of Tyrosinase[a]

inhibitor	K_i[b]
HA-type acids	
benzoic acid	18 (±1) μM
o-toluic acid	6.8 (±0.3) μM
m-toluic acid	9.0 (±0.7) μM
p-toluic acid	80 (±20) μM
2,6-dimethylbenzoic acid	noninhibitory
4-methylthiobenzoic acid	8.3 (±0.8) μM
1-naphthoic acid	140 (±5) μM
2-naphthoic acid	13 (±2) μM
4-hydroxybenzaldehyde	37 (±10) μM
4-nitrophenol	62 (±6) μM
BH^+-type acids	
benzimidazole	12 (±1) mM
indazole	0.23 (±0.09) mM
isoquinoline	1.5 (±0.1) mM
nonacidic	
toluene	23 (±9) mM

[a] With respect to tyrosine. All data at pH 5.60 and 25 °C. [b] Value in parentheses is the standard deviation.

RESULTS AND DISCUSSION

Classes of Inhibitors. All of the compounds used in this study are simple linear competitive inhibitors with respect to tyrosine, as indicated by double-reciprocal plots at different inhibitor concentrations that intersect on the $1/v$ axis and linear replots of the slopes of the double-reciprocal plots vs the inhibitor concentration. The measured inhibition constant, K_i, is thus the dissociation constant of the enzyme–inhibitor complex:

$$EI \rightleftharpoons E + I$$

Table 1 gives the K_i values for a variety of inhibitors. Aromatic carboxylic acids are the best inhibitors. Small changes in structure (*o*-toluic vs benzoic acid, 1- vs 2-naphthoic acid, the isomeric toluic acids) produce significant changes in K_i. Although *o*-toluic acid is a better inhibitor than benzoic acid, 2,6-dimethylbenzoic acid is noninhibitory. In the previously proposed model for inhibitor binding (Figure 1), the inhibitor, like the substrate, is coordinated to one copper and is positioned over the binuclear site. Substitution of both ortho positions of benzoic acid with a methyl group may sterically prevent such binding. Thus, these data support the proposed model of inhibitor binding. The large difference in K_i for the two isomeric naphthoic acids also suggests that the position of the aromatic ring affects the stability of the complex. Inhibitor binding may involve interactions of the aromatic ring with hydrophobic groups in the enzyme active site, and similar interactions may also stabilize substrate binding.

Competitive inhibition of tyrosinase by toluene is consistent with a hydrophobic binding pocket in the active site that normally accommodates the aromatic ring of the substrate and may serve to orient it properly for hydroxylation. Thermodynamic parameters for benzoic acid, *o*-toluic acid, and toluene were determined from the temperature dependence of K_i. Table 2 gives K_f (=$1/K_i$), the formation constant of the enzyme–inhibitor complex, and $\Delta G°$, $\Delta H°$, and $\Delta S°$ for

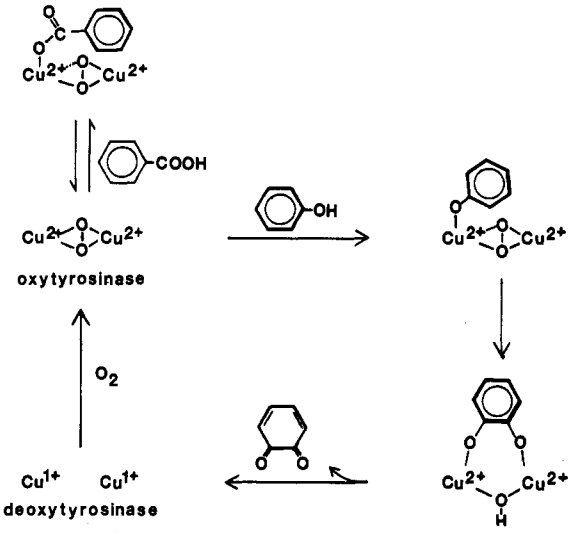

FIGURE 1: Monophenolase reaction of tyrosinase and benzoic acid inhibition. The two copper ions of the binuclear site are shown, but protein ligands are omitted for clarity. In oxytyrosinase, the oxygen is proposed to bind in a side-on μ-η^2:η^2 peroxide fashon. Phenols coordinate to one copper of oxytyrosinase and are hydroxylated to catechols (Ross & Solomon, 1991; Solomon & Lowery, 1993). Electron transfer then produces the quinone and deoxytyrosinase. Binding of dioxygen regenerates oxytyrosinase. Benzoic acid binds to oxytyrosinase and inhibits the reaction competitively with respect to the phenolic substrate.

Table 2: Thermodynamic Data for Inhibitor Binding to Tyrosinase[a]

inhibitor	K_f	$\Delta G°$ (kcal)	$\Delta H°$ (kcal)	$\Delta S°$ (cal/K)
benzoic acid	5.6×10^4	−6.5	−32	−85
o-toluic acid	1.5×10^5	−7.1	−25	−60
toluene	1.4×10^2	−2.9	+13	+54

[a] K_f ($=1/K_i$) is the formation constant of the enzyme–inhibitor complex at 25 °C and pH 5.60. $\Delta G°$ is calculated from K_f, $\Delta H°$ is calculated from the slope of the linear van't Hoff plot, and $\Delta S°$ is calculated from the relationship $\Delta S° = (\Delta H° - \Delta G°)/T$.

complex formation. $\Delta G°$ was calculated from the K_f value at 25.0 °C, $\Delta H°$ from linear van't Hoff plots of log K_f vs $1/T$, and $\Delta S°$ from $\Delta S° = (\Delta H° - \Delta G°)/T$. Both *o*-toluic acid binding and benzoic acid binding are driven by the large negative $\Delta H°$, which overcomes the negative $\Delta S°$ of binding. Interestingly, the stronger binding of *o*-toluic acid results from a less negative $\Delta S°$ of binding, which more than compensates for the less favorable $\Delta H°$ for this compound. Numerous factors may contribute to the observed entropy of binding. One important factor is the positive contribution to the entropy of binding from transfer of the hydrophobic portion of the inhibitor from the aqueous environment to a hydrophobic pocket in the active site (Tanford, 1980). The larger hydrophobic surface of *o*-toluic acid may make this contribution more positive than for benzoic acid, thereby making the overall $\Delta S°$ less negative. The standard free energy of transfer of toluene from water to pure toluene is 0.8 kcal more negative than the free energy of transfer of benzene from water to pure benzene. This difference, due to the toluene methyl group, is similar to the 0.6 kcal difference seen in $\Delta G°$ between *o*-toluic and benzoic acids.

The thermodynamic parameters for toluene binding are qualitatively different than for the two carboxylic acids. The $\Delta H°$ for toluene binding is positive, but this is more than compensated for by the very positive $\Delta S°$ of binding. The positive $\Delta S°$ of binding is consistent with removal of toluene from contact with solvent on binding and supports the proposed hydrophobic pocket in the active site. The negative $\Delta H°$ of binding for the carboxylic acids presumably reflects, in part, the coordination of the carboxylate group to copper.

Table 3: pH Dependence of the Inhibition of Tyrosinase[a]

inhibitor	pK_a	pH	K_i	K_i* (μM)
HA-type acids				
2-naphthoic acid	4.2	5.60	8.2 (0.1) μM	0.28
		6.30	26 (1) μM	0.18
		7.00	130 (30) μM	0.19
4-methylthiobenzoic acid	5.7	5.10	6.3 (0.6) μM	5.0
		5.60	8.4 (0.8) μM	4.7
		7.00	72 (10) μM	3.4
BH^+-type acids				
benzimidazole	5.4	5.60	12 (1) mM	
		6.30	4.4 (0.6) mM	
		7.00	3.6 (0.2) mM	
indazole	1.2	5.60	0.23 (0.09) mM	
		6.30	0.29 (0.04) mM	
		7.00	0.20 (0.01) mM	
nonacidic				
toluene		5.60	13 (3) mM	
		6.30	8 (2) mM	
		7.00	13 (1) mM	

[a] All data are at 25.0 °C and constant ionic strength. The K_i* values are the pH-independent constants calculated assuming the acid form is the true inhibitor (see text). pK_a values are for pure water (Fasman, 1976). Value in parentheses is the standard deviation.

Aromatic N-heterocycles were expected to be strong inhibitors of tyrosinase since they possess both an aromatic ring and a nitrogen capable of coordinating to copper. Stability constants for coppper–N-heterocycle complexes are generally larger than for copper–carboxylic acid complexes.[2] However, the compounds studied, although they do inhibit tyrosinase, have considerably larger K_i values than the carboxylic acids (Table 1) and are closer to the K_i of toluene. The pH dependence of inhibition by these compounds also differs from that of carboxylic acids (see below).

Two phenols with strongly electron-withdrawing groups in the para position were also found to be competitive inhibitors of tyrosinase (Table 1). Neither of these compounds served as a substrate, even when incubated with large amounts of enzyme for extended periods of time. The aromatic ring is apparently sufficiently deactivated to prevent electrophilic attack by oxygen. However, active-site binding does occur as evidenced by K_i values in the general range found for aromatic carboxylic acid inhibitors.

pH Dependence of Inhibition. The best inhibitors of tyrosinase from Table 1, aromatic carboxylic acids and phenols, are both weak acids in which the acidic form is electrically neutral (HA-type acids). In contrast, the aromatic N-heterocycles are cationic in their acidic form (BH^+-type acids). Benzoic acid was found to be a stronger inhibitor at pH 5.60 than at pH 7.00 (Menon, 1989). We investigated the pH dependence of inhibition of tyrosinase by a number of compounds over the pH range of 5.1–8.0, a range over which the enzyme is stable. With one exception, all of the carboxylic acids that inhibit tyrosinase have pK_a values below 5.0, and thus can be studied only at pH values where they exist predominantly in their basic form. As shown in Table 3, 2-naphthoic acid becomes a better inhibitor as the pH is lowered from 7.00 to 5.60, a behavior characteristic of all HA-type acid inhibitors. The data were analyzed by assuming that only the acid form of the compound could bind to the

[2] For example: for the reaction $Cu^{2+} + L \rightleftharpoons CuL$, $K = [CuL]/[Cu^{2+}][L]$; for L = acetic acid, $K = 240$ M^{-1}; for L = benzoic acid, $K = 32$ M^{-1}; for L = benzimidazole, $K = 1800$ M^{-1}; for L = imidazole, $K = 16\,000$ M^{-1} (Perrin, 1979).

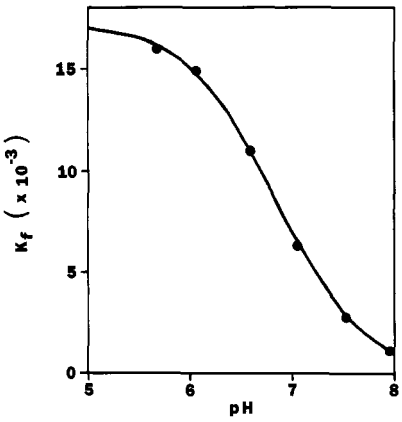

FIGURE 2: Inhibition of tyrosinase by *p*-nitrophenol as a function of pH. The apparent formation constant of the enzyme–*p*-nitrophenol complex, K_f, is plotted against pH. The curve is the best fit to the data according to eq 2 (see text), with $K_f^* = 1.73 \times 10^4$ M^{-1} and $pK_a = 6.82$.

enzyme. Thus, the reaction for complex formation is

$$E + HA \rightleftharpoons E(HA)$$

and the apparent K_i is pH-dependent because it is calculated using the *total* inhibitor concentration in the reaction solution and not the concentration of the HA form. It can be shown that the apparent K_i is related to K_i^*, the inhibitor dissociation constant calculated using the concentration of the acidic form of the inhibitor, the $[H^+]$ of the solution, and the acid dissociation constant, K_a, of the inhibitor, by the equation:

$$K_i = K_i^*[([H^+] + K_a)/[H^+]] \quad (1)$$

The K_i^* values for 2-naphthoic acid, shown in Table 3, were calculated with eq 1, using the pK_a value given. The K_i^* values at the three pH values are in fairly good agreement. If the model is correct, the K_i^* values should be pH-independent. The data for 2-naphthoic acid have two limitations: K_i can be measured only over a small part of the inhibitor titration curve, and the literature K_a used in the calculation is measured in pure water and would be slightly different in our buffer because of the effect of ionic strength on K_a. The data for 4-methylthiobenzoic acid can be analyzed more satisfactorily because of the higher pK_a of this compound. Equation 2 is derived from eq 1 using the relationship $K_f = 1/K_i$. A plot of K_f vs $K_f/[H^+]$ is a straight line from which K_f^* is 1.80 (±0.04) × 10^5 M^{-1} and the pK_a for 4-methylthiobenzoic acid is 5.9. This value is in fairly good agreement with the literature value of 5.74 (Fasman, 1976).

$$K_f = K_f^* - K_a(K_f/[H^+]) \quad (2)$$

p-Nitrophenol, another HA-type inhibitor, is much better suited to a pH study because its pK_a of approximately 7 allows it to be studied over essentially its entire titration curve, and the visible absorbance of the *p*-nitrophenolate ion permits a simple determination of the actual pK_a of the inhibitor under the buffer conditions of the enzyme kinetics experiment. The data for *p*-nitrophenol were analyzed by eq 2. An excellent fit to that equation was obtained, yielding a K_f^* of 1.73 (±0.01) × 10^4 M^{-1} and a pK_a of 6.82 ± 0.03. Figure 2 shows the experimental K_f values plotted vs solution pH. The line is the theoretical curve using the K_f^* and pK_a values given above. The actual experimental pK_a of *p*-nitrophenol under the conditions of the experiment was found to be 6.98 ± 0.04. The excellent agreement between the pK_a values determined from the pH dependence of K_f and those measured directly supports the model according to which the protonated form of HA-type acid inhibitors combines with the enzyme.

The aromatic N-heterocycle inhibitors are BH^+-type acids and show different pH-dependent behavior than the other inhibitors. Indazole, with a pK_a of 1.22 (Fasman, 1976), is entirely in its electrically neutral, basic form throughout the pH range studied, and there is no pH dependence in its K_i value. This is consistent with the lack of any pH effect on toluene binding and supports the conclusion that pH changes, in the range employed, do not alter the enzyme in any way that changes inhibitor binding affinity. Benzimidazole, with a pK_a of 5.4 (Fasman, 1976), shows stronger binding at higher pH, an effect exactly opposite to that observed for the HA-type acids. In this case, it is the basic, electrically neutral form of the compound that binds more tightly to the enzyme. The relatively large K_i values for the N-heterocycles are surprising: an explanation is offered below.

In general, the pH dependence of inhibition could result from changes in the ionization of inhibitor or enzyme, or both. For example, stronger inhibitor binding at acid pH might be explained by stronger binding of the acid form of the inhibitor to the enzyme or stronger binding of the anionic form of the inhibitor to a protonated form of the enzyme. For a single inhibitor, the pH dependence of binding could be equally well explained by either interpretation. We favor the former interpretation for the pH effects we have observed. Three types of pH effects were observed in the present work: (1) stronger binding at acid pH for the HA-type acids; (2) no pH effect on binding for toluene and indazole; and (3) stronger binding at higher pH for benzimidazole. The data for 2-naphthoic acid, 4-methylthiobenzoic acid, and *p*-nitrophenol can be satisfactorily fit by assuming the acid form of the compound is responsible for the inhibition. For *p*-nitrophenol in particular, which could be studied over essentially its entire titration curve, the data agree with the experimentally determined pK_a value. These three compounds have pK_a values from 4.2 to 7.0, and in each case the data are consistent with the pK_a of the inhibitor. If the effect of pH were due to a group on the enzyme, the same pK_a would be expected in each case. The data for toluene and indazole ($pK_a = 1.22$) further support this conclusion since when the inhibitor either is not a weak acid or ionizes well outside the pH range employed, there is no pH effect in binding. Finally, benzimidazole, a BH^+-type acid with a pK_a value in the pH range studied, shows slightly stronger binding at the higher pH values, the reverse of the effect seen with the HA-type acids.

The data indicate that inhibitors of tyrosinase must be electrically neutral to bind to the enzyme. For the strongest inhibitors of tyrosinase, the HA-type acids, this is the protonated form. Since inhibitor binding is believed to involve coordination to copper (Wilcox et al., 1985), and since both carboxylic acids and phenols must lose a proton upon such binding, transfer of a proton from the inhibitor to some other group in the active site likely occurs. One interesting possibility is that a ligand to the copper ion which binds the inhibitor is protonated and displaced from the copper, thus keeping the coordination number of the copper ion the same. The deuterium isotope study described below supports this hypothesis. The inability of the N-heterocycles to displace a copper ligand by proton transfer may account for their weak binding relative to carboxylic acid and phenol inhibitors.

Solvent Deuterium Isotope Effect on K_i. If binding of HA-type inhibitors results in transfer of a proton from the inhibitor to some other group, a solvent deuterium isotope effect on K_i might be expected (Schowen & Schowen, 1982; Schowen,

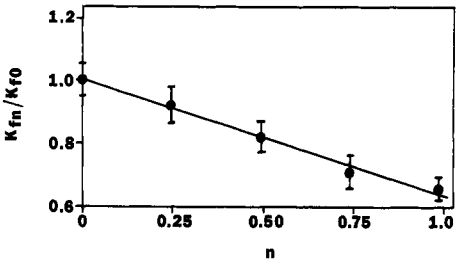

FIGURE 3: Plot of the ratio of formation constants of the enzyme–benzoic acid complex, K_{fn}/K_{f0}, vs the atom fraction of deuterium, n, in the buffer. The data are fit with a straight line of slope –0.36 ± 0.02. Error bars show ±1 standard deviation.

1977; Albery, 1975). Inhibition of tyrosinase by benzoic acid was studied in H_2O/D_2O buffers of different atom fraction deuterium. The ratio of acid to base forms of benzoic acid was the same in all solutions. In each case, simple, linear competitive inhibition was observed and the K_i calculated. The formation constant for the enzyme–inhibitor complex ($1/K_i$) at different values of n, K_{fn}, was determined. The ratio of K_{fn} to the formation constant in H_2O buffer, K_{f0}, is plotted vs n in Figure 3. This "proton inventory" plot indicates that the affinity of enzyme for inhibitor is reduced in D_2O. The best fit to the data is a straight line of slope –0.36 (±0.02). The general dependence of K_{fn}/K_{f0} on n is given by (Schowen & Schowen, 1982)

$$K_{fn}/K_{f0} = \frac{\prod_i^{\nu}(1 - n + n\phi_i^P)}{\prod_j^{\nu}(1 - n + n\phi_j^R)} \quad (3)$$

where ϕ_i^P and ϕ_j^R are the fractionation factors for deuterium in the ith site of the product and the jth site of the reactant, respectively. Since the products are taken over all sites contributing to the solvent isotope effect, the relationship between the ratio of formation constants and n will, in general, be nonlinear. The linear plot obtained in this work indicates that a single proton is responsible for the isotope effect. That proton is assumed to be the carboxyl proton of the benzoic acid, which has a fractionation factor of 1.0 (Lowe & Smith, 1975). Thus, the denominator in eq 3 is 1, and the equation reduces to

$$K_{fn}/K_{f0} = 1 - (1 - \phi^P)n \quad (4)$$

from which the fractionation factor for the proton in the product is found to be $\phi^P = 0.64 \pm 0.02$.

There are many potential proton acceptors in the inhibitor binding reaction. The value of ϕ^P, along with the involvement of only one proton in the isotope effect, eliminates many of these possibilities. Transfer of the proton to a group which already carries one or more exchangeable hydrogens would result in a nonlinear proton inventory plot. This eliminates an active-site water molecule or hydroxide ion, along with a number of protein functional groups (such as amino groups). In addition, the fact that some of these groups have unit fractionation factors eliminates them from consideration. These include protein nitrogen functional groups and carboxyl groups (Schowen & Schowen, 1982). A water molecule coordinated to copper is also ruled out since this would result in a nonlinear dependence on n, and water coordinated to both aquocobalt(II) and cobalt(II) carbonic anhydrase has been shown to have a unit fractionation factor (Silverman, 1981). The fractionation factor for the thiol group of cysteine is 0.40–

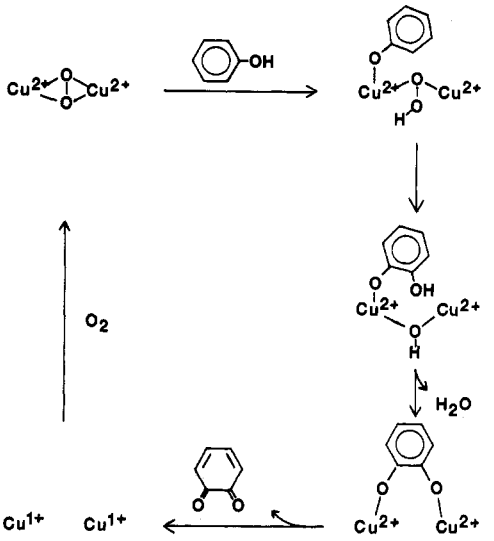

FIGURE 4: Proposed mechanism for proton transfer on substrate binding. The actual substrate is the acid form of the phenol. Coordination of the phenol to one copper of the binuclear site is accompanied by proton transfer to the bound peroxide. Electrophilic attack of the coordinated hydroperoxide on the ring and cleavage of the oxygen–oxygen bond produce the catechol and a bridging hydroxide ion. Bidentate coordination of the catechol is accompanied by a second proton transfer, releasing water. Electron transfer from the catechol gives the quinone and the deoxy binuclear site. Oxidative addition of dioxygen regenerates oxytyrosinase.

0.46 (Schowen & Schowen, 1982), which is outside the uncertainty for ϕ^P in the present work. Earlier work assumed the binuclear site in oxytyrosinase and oxyhemocyanin contained a bridging group in addition to the oxygen, and a hydroxide ion was considered a likely candidate (Solomon, 1981). The fractionation factor for such a group is not known. However, there is no evidence of such a bridging hydroxide ion in the crystal structure of oxyhemocyanin (Magnus & Ton-That, 1992), and protonation of such a group would be expected to result in a nonlinear proton inventory plot.

The proton acceptor involved in inhibitor binding must meet the dual requirements of a fractionation factor of 0.64 and have no additional exchangeable hydrogens. As detailed above, none of the groups likely to be in the active site meet these requirements. This led us to consider the possibility that the bound oxygen is the proton acceptor. Coordination of benzoic acid to one copper of the binuclear site may be accompanied by concerted proton transfer to one end of the coordinated dioxygen, resulting in rearrangement of the copper coordination sphere. In this model, HA-type inhibitors, whether carboxylic acids or phenols, would react in this way. With benzimidazole, and other BH^+-type acid inhibitors, the basic, electrically neutral form of the inhibitor is the reactive species. Since these inhibitors cannot protonate the bound oxygen, they may either not bind to copper at all or only coordinate weakly to an axial position. Thus, the BH^+-type inhibitors have K_i values similar to toluene.

Like the HA-type inhibitors, the phenolic hydroxyl of the substrate tyrosine is a weak HA-type acid. The pK_a of the tyrosine hydroxyl is 10.0 (Fasman, 1976), and thus under both physiological conditions and the pH of the kinetic studies in this work, the substrate hydroxyl exists in its acid form. If, as proposed (Solomon, 1993), substrate binding to the active site involves coordination to one copper as a phenolate ion, transfer of the hydroxyl proton to some other group must occur. Thus, like the HA-type inhibitors, the substrate may protonate the bound oxygen, which may activate the oxygen

toward electrophilic attack at the ortho position of the ring. Karlin has suggested a role for protonation of bound oxygen to generate a reactive hydroperoxide species, based upon his study of model compounds (Tyeklar & Karlin, 1989).

We propose that binding of the organic substrate results in proton transfer to bound oxygen and that this is a necessary prerequisite to hydroxylation of the aromatic ring. Figure 4 illustrates the proposed scheme. Phenol coordination to the proposed side-on bridged peroxide structure of oxytyrosinase is accompanied by proton transfer, generating an electrophilic hydroperoxide species. Insertion of oxygen into the ortho carbon–hydrogen bond of the phenol is accompanied by heterolytic cleavage of the oxygen–oxygen bond, leaving a bound hydroxide ion, perhaps bridging the two coppers (Solomon, 1981). Coordination of the second hydroxyl group of the catechol in a bridging fashion is accompanied by both proton and electron transfer, producing the quinone, water, and the deoxy binuclear site. Oxidative addition of dioxygen completes the cycle. The two proton-transfer steps result in release of both products, quinone and water, as neutral molecules. The formation of neutral species is consistent with the proposed hydrophobic nature of the active site. The monodentate catechol indicated in Figure 4 may not exist as an actual intermediate. This structure is drawn to emphasize the point that the phenolic substrate must lose *two* protons in forming the quinone: the hydroxyl proton and the ring proton at the site of hydroxylation. Transfer of both protons to oxygen avoids the production of a hydroxide ion. This mechanism links together proton transfer, hydroxylation of the aromatic ring, and electron transfer in the tyrosinase reaction.

ACKNOWLEDGMENT

We thank Professor Robert Fenstermacher for assistance in data analysis, Jason Karnes for obtaining the data on 4-methylthiobenzoic acid, and Jody L. Kujovich for advice in the preparation of the manuscript.

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