acting on *nucleus* A and the second term may be said to represent the force acting on the *electrons* μ of atom A. Thus, the force operator in the R coordinate may be considered to represent the force acting on *atom* A.[10b] A remarkable merit is that

$$-(\partial\mathcal{H}^0/\partial\mathbf{R}_A)_R = 0 \qquad \text{(B2)}$$

which means that we can save the required accuracy of the wave function by one order.[10,30] Equation B2 means also that the concept of the AD force disappears in the R coordinate. The operator of the AD force, $-(\partial\mathcal{H}^0/\partial\mathbf{R}_A)_L$, which is an *internal* force in the R-coordinate representation, is exactly canceled by the force the electrons μ receive, $-\Sigma_\mu(\partial\mathcal{H}^0/\partial\mathbf{r}_\mu)_L$. Instead, we have a new operator, $-\Sigma_\mu(\partial\mathcal{H}^1/\partial\mathbf{r}_\mu)_L$, which satisfies

$$\langle\Psi| - \sum_\mu\left(\frac{\partial\mathcal{H}^1}{\partial\mathbf{r}_\mu}\right)_L|\Psi\rangle = \langle\Psi| - \left(\frac{\partial\mathcal{H}^0}{\partial\mathbf{R}_A}\right)_L|\Psi\rangle \qquad \text{(B3)}$$

for the exact wave function Ψ. Namely, the AD force is substituted in the R-coordinate representation by the force the electrons μ receive through the long-range interaction with atom B. This correspondence is possible only for the atom–atom long-range interactions. The concept of the long-range forces in the relative coordinate system will be discussed more fully elsewhere.

(30) Yaris (ref 10a) has discussed further the conditions under which the wave function correct to nth order gives the Hellmann–Feynman force to order $2n + 1$.

Mechanistic Studies of the Reduction of *Rhus vernicifera* Laccase by Hydroquinone

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Abstract: Anaerobic stopped flow kinetic studies of the reaction of hydroquinone with *Rhus vernicifera* laccase have been performed with the objective of elucidating the mechanisms by which the three enzymatic copper sites are reduced. The "blue" and esr nondetectable copper sites are reduced in parallel at comparable rates over a wide range of conditions. Second-order rate constants characterizing the hydroquinone reduction of the laccase type 1 and type 3 sites at 25.6°, pH 7.0, and μ 0.1 (phosphate) are 3.25×10^2 and 4.57×10^2 M^{-1} sec^{-1}, respectively. Terms proportional to $[H^+]^{-1}$ are dominant in the rate laws for reduction of both optically observable copper sites, suggesting participation of the phenolate anion HQ^- in the slow steps governing electron transfer. Fluoride and azide inhibition results at pH 6 strongly suggest that inner-sphere complexes between HQ^- and type 2 Cu(II) are intermediates in the closely related electron-transfer pathways to the type 1 and type 3 copper sites in the native enzyme. It is proposed that the disposition of these intermediates involves (1) reduction of the type 2 copper atom followed by intramolecular electron transfer to the type 3 site and (2) initiation of a protein conformational change permitting conduction of an electron from the coordinated substrate to the type 1 copper atom. Displacement of anion from type 2 coordination in the slow step is proposed to account for saturation in observed 614- and 330-nm rate constants for reduction of the type 2 Cu(II)-F^- laccase complex to the respective values 1.13×10^{-1} and 9.8×10^{-2} sec^{-1} (25.1°, pH 6.0, μ 0.2). Displacement of azide ion from type 2 coordination is not a prerequisite for reduction of the "blue" copper site, however, as decay in the 405-nm absorption peak of the azide derivative (N_3^- to type 2 Cu^{2+} charge transfer) is much slower than loss of blue color. A type 1 Cu(I) to type 2 Cu(II) electron-transfer step catalyzed by a bridging azide ion is postulated to account for the first-order $[N_3^-]$ dependence of observed rate constants for type 2 copper reduction in the laccase–azide complex at pH 6.0 (k_{obsd}-(405) = $0.106[N_3^-]$ sec^{-1}; 25.1°, μ 0.2).

Laccases are copper-containing enzymes which catalyze the oxidation of ortho- and para-aryl diamines and diphenols by oxygen, producing the corresponding quinones and water.[1] Two laccases, those from the fungus *Polyporus versicolor* and the lacquer tree *Rhus vernicifera*, have been the subject of much recent study. Both proteins possess four tightly bound copper atoms distributed in three distinct sites.[2–6] Laccase rapidly reduces oxygen to water only when all of its electron-accepting sites are reduced (four electron equivalents total),[7] and partial removal of enzymatic copper causes destruction of polyphenol oxidase activity.[8]

Rhus laccase is a glycoprotein containing only about 55% protein by weight and having a molecular weight of 110,000.[9] Amino acid analysis of the enzyme shows an excess of 38 basic amino acid residues over acidic residues (arginine + histidine + lysine = 56; glutamic acid + aspartic acid − NH_3 = 18), explaining its high isoelectric point of 8.55.[9] The type 1 (or "blue") copper atom is responsible for the intense blue color of

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the enzyme (ϵ_{614} 5700 M^{-1} cm^{-1})[2] and is characterized by unusually narrow hyperfine splitting in the esr spectrum. Progress toward the elucidation of the ligand environment has been made very recently for several proteins containing a single "blue" copper. An endor study of stellacyanin indicated nitrogen coordination to copper at a solvent-inaccessible site.[10] Titration studies employing *p*-mercuribenzoate have suggested the presence of cysteine in stellacyanin,[11] azurin,[12] and spinach plastocyanin[13] coordination environments. In addition, cysteine has been established as a ligand in a recent investigation of the cobalt(II) derivative of stellacyanin.[14]

A second form of copper found in laccase, type 2, has not been associated with any visible–ultraviolet absorption bands; its presence has been inferred from the appearance of two overlapping components in the esr spectrum, indicating that two paramagnetic forms of copper are present in equal amounts.[2] The esr spectrum of type 2 Cu(II) resembles that of a typical axial copper(II) complex. Type 2 copper is known to function as an anion binding site; fluoride and azide ions cause changes in the *Rhus* laccase type 2 esr spectrum but not in that of the type 1 component.[2]

Type 3 copper is esr nondetectable and is associated with a near-ultraviolet absorption band near 330 nm (ϵ_{333} 4500 M^{-1} cm^{-1} for *Rhus* laccase).[2] Laccase contains 50% of its total copper, or 2 Cu/mol, in an esr nondetectable form, and anaerobic redox titrations have shown that the *Rhus* type 3 site functions as a two-electron acceptor.[6] When laccase is fully reduced, the 614- and 330-nm absorption bands disappear and the esr spectrum vanishes. Some of the structural alternatives for the fungal laccase type 3 site have been discussed.[4] A formulation of two cuprous atoms in conjuction with a reducible disulfide bond was ruled out because the type 3 site appears to be too strong an oxidant to be associated with the disulfide–sulfide couple. The structure of the esr nondetectable unit most likely involves two antiferromagnetically coupled cupric atoms; this site may be involved in oxygen fixation.[15]

A Cu(II)–Cu(I) "valance shuttle" has been suggested many times as the key to laccase polyphenoloxidase activity, but the mechanistic details of enzymatic reduction and reoxidation steps remain obscure. It is well known, for example, that type 2 binding anions cause dramatic inhibition in *Rhus* laccase activity,[16] but the basis for this inhibition has not been explored in detail. Only a few kinetic studies dealing with the reduction of laccase copper sites are available;[5,7,17,18] specific reduction rate constants, when reported, are given for only a limited variety of conditions and thus are of little use in discussing mechanisms.

The objective of studies reported in this paper is to provide an experimental background extensive enough to permit meaningful discussion of the mechanism by which hydroquinone (H_2Q) reduces *Rhus* laccase. A preliminary communication which describes our early kinetic experiments on the reduction of laccase by chromous ion has been published.[19] Rate parameters are reported here for the hydroquinone reduction of both native and anion-inhibited laccase samples. In the absence of structural information pertaining to copper coordination environments, distances between metal atoms, and protein conformation, experiments have been designed and results have been interpreted with emphasis on functional relationships among laccase copper sites. The nomenclature used is as follows; the symbol (2,2,Ox) refers to fully oxidized laccase. The first and second entries give the oxidation states of the type 1 and type 2 copper atoms, respectively. The final notation, either "Ox" or "Red," indicates whether or not the two-electron acceptor is oxidized or reduced.

Experimental Section

Materials. Reagent grade chemicals were used without further purification, as were Sephadex ion exchange and gel filtration resins. Triply distilled water was used in preparing solutions for kinetic measurements. Nitrogen gas was passed through two chromous scrubbing towers to remove oxidizing impurities.

Practical *p*-benzoquinone was recrystallized from absolute ethanol, thoroughly dried under suction on a sintered glass filter, and doubly sublimed. High yields of bright yellow needles with the correct melting point were obtained.

Chelex 100 resin, obtained from Bio-Rad, was purified by successive washings with concentrated NH_4OH, HCl, and NaOH and was rinsed thoroughly with water. Before use it was equilibrated with pH 7 phosphate buffer.

Union Carbide dialysis membrane was boiled extensively in several changes of distilled water to remove sulfurous impurities and was stored immersed in cold distilled water.

Rubber serum caps were soaked in concentrated base before use, removing material which might be reactive with proteins or reducing agents.

Matheson Coleman and Bell "fine chemical" grade NaN_3 was used without further purification after its azide content was found to be better than 99% of the theoretical value. Aldrich 1,4-cyclohexanediol (mixture of cis and trans) and Matheson Coleman and Bell resorcinol were used as received. Sigma Grade III α-D(+)-glucose and type II glucose oxidase (from *Aspergillus Niger*) were also used as supplied.

Analyses. The purity of commercial hydroquinone was verified by ceric titration[20] using ferroin as indicator. Arnold's ceric procedure[21] was used for the analysis of sodium azide.

Laccase concentrations were evaluated from total copper analyses of protein solutions by the spectrophotometric biquinoline method,[22] assuming 4Cu/mol. Varian Techtron 1000 ppm aqueous $CuSO_4$ was used in the preparation of standard solutions and was diluted with dithizone-extracted pH 6 0.01 *M* phosphate buffer. Traces of nonenzymatic Cu were removed from protein solutions by stirring them in the presence of Chelex 100 resin for approximately 30 min.

Protein Purification. Laccase was extracted and purified essentially by the method of Reinhammar.[9] Lacquer acetone powder was obtained from Saito and Co., Ltd., Tokyo. Much of the laccase used in this study was subjected to gel filtration (Sephadex G-

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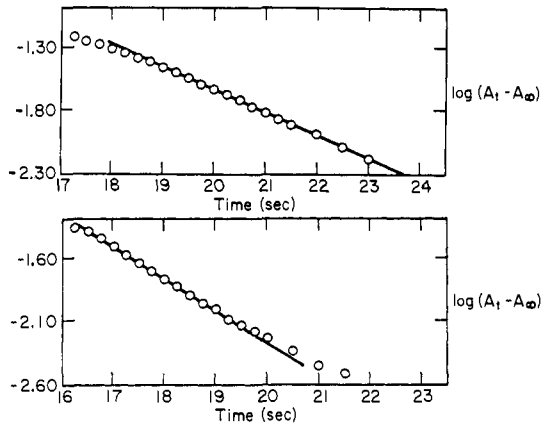

Figure 1. Typical first-order plots for the reaction of hydroquinone with laccase, 25.6°, pH 7.0, μ 0.1, $[H_2Q] = 1.25 \times 10^{-3}$ *M*: top, 614 nm; bottom, 330 nm.

150 equilibrated with 0.01 *M* pH 6 potassium phosphate buffer) as a test of its chromatographic purity and as a final purification step. Chromatographically pure laccase samples typically had A_{280}/A_{614} ratios of 15.2 to 15.6, in good agreement with Reinhammar's result (15.2).[9] Following the final purification step, laccase was shell frozen in polyethylene vials and stored in liquid nitrogen.

Measurements. Kinetics measurements were performed on a Durrum Model D-110 stopped flow spectrophotometer. Solutions to be mixed were contained in glass drive syringes submerged in water circulated from a Forma Scientific temperature control unit (temperature control ±0.2°). Viton O rings fitted to the plunger heads provided a reasonably satisfactory seal against oxygen leakage into the drive syringes. Output voltages obtained from the stopped flow apparatus were displayed *vs.* time on a Tektronix 564B oscilloscope, or, for slower reactions, on a Hewlett-Packard Model 7004B *x-y* recorder.

The reactant inlet ports on the Durrum instrument were modified to accept gas-tight fittings for a Teflon needle, through which anaerobic solutions were drawn directly into the drive syringes. Solutions resting in the drive syringes were allowed at least 20 min to come to temperature equilibrium with the bath water before a kinetic determination was made.

Artifacts may be observed in stopped flow kinetics if mixed solutions resting in the observation chamber diffuse back through the flow system to interact with unmixed reactants on the drive syringe side of the mixing jet. As a precaution against this occurrence, valves permitting flow between the drive syringes and the mixing jet were closed immediately following mixing. As a test of the reliability of the stopped flow apparatus for runs requiring 5 to 10 min to be complete, several determinations were made using the slow redox reaction between chromous ion and the glycinatopentaamminecobalt(III) ion as a standard. The room temperature rate constant for μ 1.0 was found to be in excellent agreement with the literature value [$k(25°) = 0.064\ M^{-1}\ sec^{-1}$][23] measured on a Cary 14 spectrophotometer.

Visible and ultraviolet spectra were recorded on a Cary 17 spectrophotometer. Routine measurements on protein solutions and for analyses were performed on a Cary 14 instrument. Corning Model 12 and Brinkman pH 101 meters were used to make pH measurements.

Preparation of Solutions. After removal from liquid nitrogen storage, copper protein solutions were dialyzed against two changes of distilled water, the last being triply distilled. Phosphate buffer and other solid components were then added, and a final minor adjustment of pH, if necessary, was made with sodium hydroxide or phosphoric acid. Buffered laccase solutions are stable to decomposition for at least several weeks at 5°, but most measurements were performed on freshly prepared solutions.

Protein solutions were deoxygenated by evacuation through a needle inserted into a serum-capped bottle. Freeze-pump and N_2 bubbling techniques were avoided; repeated freezing and thawing causes denaturation, as does the foaming induced by bubbling a gas through the solution. Evacuated protein samples were packed in an ice bath and purged with a stream of nitrogen flowing over the top of the liquid.

Hydroquinone solutions were made up in serum bottles either by weight or by diluting fresh stock solutions using Hamilton gas-tight syringes and stainless steel needles. Before introduction of the reducing agents, buffer solutions were purged with nitrogen for at least 30 min. Reducing agent solutions were always used on the same day as their preparation.

Results

An initial steady state period is observed when laccase and hydroquinone are mixed aerobically.[24] For this reason analysis of our absorbance–time curves is complicated by the presence of small amounts of oxygen in solutions mixed in the stopped flow apparatus. As reoxidation of the metalloenzyme by O_2 is much faster than reduction,[25] the steady state absorbance levels at 614 and 330 nm are close to those expected for fully oxidized enzyme. After oxygen is completely consumed, plots of log $(A_t - A_\infty)$ *vs.* time based on 330 and 614 nm absorbance–time data are invariably linear (A_t and A_∞ represent the absorbance at time t and after completion of the reaction, respectively). Observed first-order rate constants (k_{obsd}) were obtained routinely by performing least-squares analyses on the linear regions of log $(A_t - A_\infty)$ plots. At least two duplicate runs were performed and analyzed for each pair of solutions considered.

An important point to consider in analyzing the kinetic data is whether or not the 614- and 330-nm decay curves are truly independent. Redox titrations have demonstrated that the *Rhus* laccase 614- and 330-nm absorption peaks correspond to distinct copper sites,[6] but absorbance changes due to the conversion of hydroquinone to benzoquinone may indirectly couple rate processes observed at the two wavelengths. It is easily seen that benzoquinone-induced coupling between 614- and 330-nm kinetic results may be neglected; the benzoquinone minus hydroquinone extinction coefficient is nearly zero at 614 nm and also is very small at 330 nm ($\Delta\epsilon < 100\ M^{-1}\ cm^{-1}$)[26] compared with the protein absorbance change at this wavelength.

Typical analytical plots for the reduction of laccase by hydroquinone at pH 7.0, μ 0.1, are given in Figure 1. Logarithmic plots were usually found to be linear for at least the last 50% of the total absorbance change at both wavelengths. In no case was evidence detected for apparent autocatalysis due to the reaction of semiquinone with the protein.

The relationship between the 614- and 330-nm observed rate constants and the hydroquinone concentration was evaluated at several temperatures using phosphate buffer (0.0463 *M*, pH 7.0) alone to maintain the ionic strength at 0.1. The rate data are collected in Tables I and II, and the room temperature results are pictured in Figure 2. The variation of $k_{obsd}(614)$ and $k_{obsd}(330)$ with $[H_2Q]$ is first order within the interval $5 \times 10^{-4} \leq [H_2Q] \leq 1 \times 10^{-2}\ M$, and the rate law for laccase reduction may be written

$$\frac{-d[Cu(330 \text{ or } 614)]_{tot}}{dt} = k_1[H_2Q][Cu(330 \text{ or } 614)]_{tot}$$

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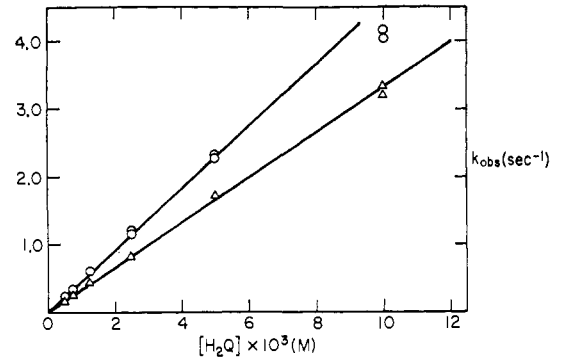

Figure 2. Plot of k_{obsd} *vs.* $[H_2Q]$ for the reduction of laccase by hydroquinone, 25.6°, pH 7.0, μ 0.1: (○) 330 nm; (△) 614 nm.

Table I. Observed Rate Constants for the Reaction of Hydroquinone with Laccase (614 nm, pH 7.0, μ 0.1)

$[H_2Q] \times 10^3$, M	k_{obsd}, sec^{-1} 11.7°	17.1°	25.6°	35.7°
0.50			0.167	
			0.167	
0.75			0.247	
			0.246	
1.25	0.072	0.156	0.418	0.951
	0.074	0.156	0.430	0.962
2.50	0.142		0.818	1.70
	0.145		0.806	1.75
5.00	0.292	0.614	1.692	3.53
	0.295	0.637		
7.50	0.450			5.15
	0.423			
10.00	0.576	1.24	3.19	
	0.577	1.18	3.32	

Table II. Observed Rate Constants for the Reaction of Hydroquinone with Laccase (330 nm, pH 7.0, μ 0.1)

$[H_2Q] \times 10^3$, M	k_{obsd}, sec^{-1} 11.7°	17.1°	25.6°
0.50			0.223
			0.225
0.75			0.343
			0.334
1.25	0.125		0.582
	0.125		0.599
2.50	0.233	0.476	1.14
	0.232	0.471	1.19
5.00	0.464		2.26
	0.457		2.30
7.50	0.690	1.38	
	0.700	1.38	
10.00	0.923		4.02
	0.919		4.14

The quantities [Cu(330)] and [Cu(614)] refer, respectively, to total concentrations of oxidized protein species absorbing at 330 and 614 nm. The slopes of the linear plots in Figure 2 yield the following values of k_1 (25.6°).

$$k_1(614) = 3.25 \pm 0.16 \times 10^2\ M^{-1}\ sec^{-1}$$

$$k_1(330) = 4.57 \pm 0.23 \times 10^2\ M^{-1}\ sec^{-1}$$

Unfortunately, the plots in Figure 2 could not be quantitatively extended to higher reducing agent concentrations. The extent of the post steady state absorbance change decreases markedly as $[H_2Q]$ increases, severely limiting the absorbance interval usable in extracting rate parameters.

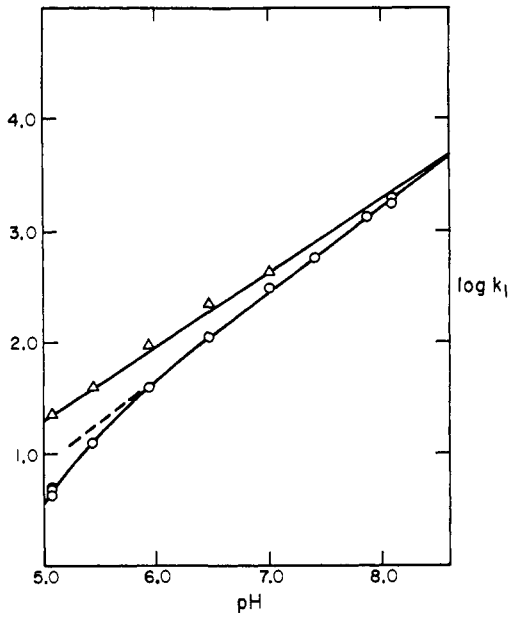

Figure 3. Plot of log k_1 *vs.* pH for the reaction of hydroquinone with laccase, 25.1°, μ 0.1: (○) 614 nm; (△) 330 nm.

Several experiments were performed to confirm that the observed rate constants obtained from post steady state decay curves are equivalent to those that would be obtained under strictly anaerobic conditions. The steady state period is quenched through the addition of trace amounts of glucose and glucose oxidase to oxidant and reductant solutions, and observed rate constants at 330 and 614 nm (pH 7.0, μ 0.1, $[H_2Q] = 1.25$, 2.50×10^{-3} M) were found to be in agreement (to within ±10%) with those calculated from runs where no oxygen scavenger other than laccase itself was present. In another experiment, anaerobic conditions were achieved by adding laccase to reducing agent solutions (1.6×10^{-6} M) and titrating the oxidase solution with H_2Q to consume residual O_2. Laccase reduction rate constants calculated from runs where the steady state period was quenched are indistinguishable from those evaluated by the usual method (average values of runs with $[H_2Q] = 1.25$, 5.00×10^{-3} M, pH 7.0, μ 0.1, 25.1°: $k_1(614) = 3.1 \pm 0.2 \times 10^2\ M^{-1}\ sec^{-1}$, $k_1(330) = 4.4 \pm 0.2 \times 10^2\ M^{-1}\ sec^{-1}$; extrapolated for 25.1° from an Eyring plot of data based on post steady state decay curves: $k_1(614) = 3.1 \times 10^2\ M^{-1}\ sec^{-1}$, $k_1(330) = 4.3 \times 10^2\ M^{-1}\ sec^{-1}$).

pH Dependence. A series of ionic strength 0.1 phosphate buffers in the range pH 5–8 was employed in determining the hydrogen ion dependence of the rate of laccase reduction by hydroquinone. Rate constants were measured at room temperature for two different hydroquinone concentrations at each pH. Separate protein solutions were made up in the appropriate buffer for each pH considered to make certain that hydrogen ion equilibration would not compete with the redox steps of interest. Results are illustrated in Figure 3 as a plot of log k_1 *vs.* pH.[27] The two k_1 values for each pH (calculated as $k_{obsd}/[H_2Q]$ for each hydroquinone concentration) are in good agreement, indi-

(27) See paragraph at end of paper regarding supplementary material.

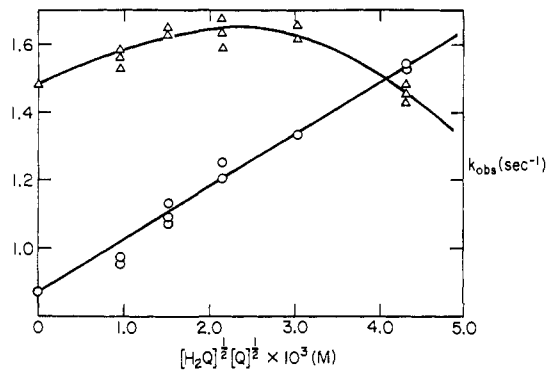

Figure 4. Plot of k_{obsd} *vs.* $[H_2Q]^{1/2}[Q]^{1/2}$ for the reaction of hydroquinone with laccase in the presence of added benzoquinone, 26.1°, pH 7.0, μ 0.1, $[H_2Q] = 2.50 \times 10^{-3}$ *M*: (○) 614 nm; (△) 330 nm.

cating that a first-order $[H_2Q]$ dependence is observed over the entire pH range.

Second-order rate constants for reduction of the type 3 site obey the relationship

$$k_1 = (2.2 \pm 0.2 \times 10 + 7.15 \pm 0.45 \times 10^{-5}[H^+]^{-1})\ M^{-1}\ sec^{-1}$$

over the interval pH 5 to 6.5 (25.1°, μ 0.1). Measurements above pH 7 were complicated by the appearance of slow downward instability in the base line following decay of most of the 330-nm absorbance. To check the possibility that the downward drift might correspond to a slowly reacting high pH form of laccase, a series of runs was performed at pH 7.8. Native laccase was reduced quickly enough to allow observation of most of the absorbance change for the slow component. Linear log $(A_t - A_\infty)$ *vs.* time plots were obtained for the slow process, suggesting it may correspond to reduction of an alternative form of laccase which interconverts slowly with the native enzyme. Results of several runs at varying $[H_2Q]$ (Table III) indicate that the reduction rate of the high pH form of laccase is independent of the reducing agent concentration over the range $2.5 \times 10^{-3} \leq [H_2Q] \leq 1.0 \times 10^{-2}$ *M* ($k_{obsd} = 0.161 \pm 0.008$ sec^{-1}; 25.1°, pH 7.8, μ 0.1). Approximately 30% of the overall 330-nm absorbance change is attributable to the high pH form at pH 7.8. The extent of this component increases with increasing pH, and its reduction rate appears at first inspection to be nearly pH independent.

Table III. Rate Data for the Reaction of Hydroquinone with the High pH Form of Laccase (330 nm, 25.1°, pH 7.8, μ 0.1)

$[H_2Q] \times 10^3$, *M*	k_{obsd}, sec^{-1}	$[H_2Q] \times 10^3$, *M*	k_{obsd}, sec^{-1}
2.50	0.164	7.50	0.167
	0.153		0.162
5.00	0.141	10.00	0.172
	0.173		0.158

Between pH 6.5 and 8.1 the rate law for reduction of laccase "blue" copper is

$$\frac{-d[Cu(614)]_{tot}}{dt} = (1.5 \pm 0.1 \times 10^2 + 1.53 \pm 0.09 \times 10^{-5}[H^+]^{-1})[H_2Q][Cu(614)]_{tot}$$

(25.1°, μ 0.1). Below pH 6.5 hydrogen ion inhibition is even stronger. At high pH, no indication of a slower reacting form of laccase was found in 614-nm measurements.

It is suggested that ionization of hydroquinone is responsible for the appearance of $[H^+]^{-1}$ terms in the rate laws for reduction of the type 1 and type 3 copper sites.

$$H_2Q \rightleftharpoons HQ^- + H^+ \qquad K_{H_2Q}$$

$$HQ^- + Cu(614) \xrightarrow{k_1'(614)} \text{products}$$

$$HQ^- + Cu(330) \xrightarrow{k_1'(330)} \text{products}$$

The value of K_{H_2Q} under conditions similar to those employed here (25°, $\mu \sim 0.04$) is 9.95×10^{-11} *M*.[26] The predicted rate law characterizing pathways involving the phenolate anion HQ^- thus is

$$\frac{-d[Cu(330 \text{ or } 614)]_{tot}}{dt} = \frac{k_1'(330 \text{ or } 614)K_{H_2Q}[H_2Q][Cu(330 \text{ or } 614)]_{tot}}{[H^+]}$$

Estimates of k_1' values may be obtained assuming the coefficients of the $[H^+]^{-1}$ terms to be of the form $k_1' \cdot K_{H_2Q}$

$$k_1'(614) = 1.5 \pm 0.1 \times 10^5\ M^{-1}\ sec^{-1}$$

$$k_1'(330) = 7.2 \pm 0.5 \times 10^5\ M^{-1}\ sec^{-1}$$

(25.1°, μ 0.1).

It is clear that the simple expression $k_1 = k_0 + k_{-H^+}/[H^+]$ will not adequately describe rate data for laccase reduction obtained outside the narrow pH ranges cited above. Consequently, the kinetic significance of the hydrogen ion independent terms in the stated rate laws, obtained by extrapolating k_1 *vs.* $[H^+]^{-1}$ plots to $[H^+]^{-1} = 0$, is open to question. The k_0 terms may include contributions from laccase reduction pathways involving un-ionized hydroquinone, but it is not possible to assign these contributions with certainty on the basis of present evidence. In any case, it seems clear that terms varying with $[H^+]^{-1}$ are dominant in the rate laws for reduction of the type 1 and type 3 sites within the region where laccase activity is optimal.

Effect of Benzoquinone. The effect of benzoquinone on the rate of laccase reduction by hydroquinone was evaluated at pH 7.0, μ 0.1. Reoxidation of reduced copper sites by benzoquinone is not expected on the basis of the comparatively low (*ca.* 0.27 V)[28] reduction potential for the Q/H_2Q couple at pH 7. Benzoquinone may, however, compete for enzymatic binding sites needed by hydroquinone, and the rapid equilibrium between H_2Q and Q producing semiquinone (SQ)[29] must also be accounted for in interpreting kinetic results.

$$H_2Q + Q \rightleftharpoons 2SQ \qquad K_{SQ}$$

Pseudo-first-order conditions for semiquinone as reductant are established by maintaining constant high

(28) W. M. Clark, "Oxidation-Reduction Potentials of Organic Systems," The Williams and Wilkins Co., Baltimore, Md., 1960.

(29) T. Nakamura, "Free Radicals in Biological Systems," M. S. Blois, Jr., H. W. Brown, R. M. Lemmon, R. O. Lindblom, and M. Weissbluth, Ed., Academic Press, New York, N. Y., 1961, p 169.

concentrations of benzoquinone and hydroquinone simultaneously.

Observed rate constants obtained holding $[H_2Q]$ constant at 2.5×10^{-3} M and varying [Q] are given in Figure 4 as a plot of k_{obsd} *vs.* $[H_2Q]^{1/2}[Q]^{1/2}$.[27] Benzoquinone causes modest increases in both 614 and 330 nm rate constants; inhibition at high [Q] was found at 330 nm but not at 614 nm. The "blue" copper reduction results may be interpreted attributing benzoquinone-induced rate increases to the reaction of semiquinone with laccase. The rate law expected for this circumstance is

$$\frac{-d[Cu(614)]_{tot}}{dt} = (k_1[H_2Q] + k_2[SQ])[Cu(614)]_{tot} = (k_1[H_2Q] + k_2K_{SQ}^{1/2}[H_2Q]^{1/2}[Q]^{1/2})[Cu(614)]_{tot}$$

For constant $[H_2Q]$, a plot of k_{obsd} *vs.* $[H_2Q]^{1/2}[Q]^{1/2}$ should be linear with slope $k_2K_{SQ}^{1/2}$ and intercept $k_1[H_2Q]$. A linear plot is indeed obtained with the experimental data and the intercept (0.874 sec^{-1}) agrees very closely with the $k_1[H_2Q]$ value predicted from earlier measurements (0.872 sec^{-1}). From the slope of the line (152 M^{-1} sec^{-1}; 26.1°, pH 7.0, μ 0.1) and the literature value[28] of K_{SQ} (*ca.* $\times 10^{-6}$) at pH 7.1, room temperature, k_2 is estimated to be 1.5×10^5 M^{-1} sec^{-1}.

The 330-nm data may reflect a combination of rate enhancement from semiquinone contributions to laccase reduction and rate inhibition from association of Q with the type 3 site. A rate law may be derived for this situation, but no attempt was made to fit the data to it quantitatively in view of the small magnitude of the benzoquinone effect at 330 nm (10% maximum acceleration). Again, the apparent intercept of the k_{obsd} *vs.* $[H_2Q]^{1/2}[Q]^{1/2}$ plot agrees with the predicted $k_1[H_2Q]$ value.

The benzoquinone experiments show that small concentrations of Q generated in kinetic runs with large excesses of H_2Q have negligible influence on observed rates. This is indicated by the excellent agreement between $k_1[H_2Q]$ values evaluated from runs with no added benzoquinone and from extrapolating the kinetic benzoquinone dependence data to [Q] = 0. Furthermore, it is evident that semiquinone does not contribute significantly to the reduction of laccase "blue" and esr nondetectable copper sites in experiments with no added benzoquinone. This confirms Nakamura's conclusion[29] based on esr studies of SQ formation and decay.

Effect of Potential Competitive Inhibitors and EDTA. Organic molecules structurally similar to hydroquinone potentially may inhibit laccase activity by competing for binding sites involved in substrate oxidation. This possibility was checked in several experiments at pH 7.0, 25°, with added 1,4-cyclohexanediol and 1,3-dihydroxybenzene (resorcinol). However, with $[H_2Q] = 1.25 \times 10^{-3}$ M and concentrations of added organic molecules up to and over 100-fold in excess, no appreciable variations in rate were found at either 614 or 330 nm.

The possibility of trace metal catalysis of protein redox reactions is distinct, especially with a two-electron organic reductant such as hydroquinone. Inhibition with EDTA has pointed to Fe(III), Fe(II) catalysis of ceruloplasmin redox reactions,[30] but no effect was observed in an analogous experiment with *Rhus* laccase.[16] This result was confirmed by finding that 1×10^{-4} M Na_2H_2EDTA has no influence on 330 and 614-nm k_{obsd} values with $[H_2Q] = 1.25 \times 10^{-3}$ M at pH 7.0, 25°. Consequently, no special precautions were taken against trace metal contamination of kinetics solutions.

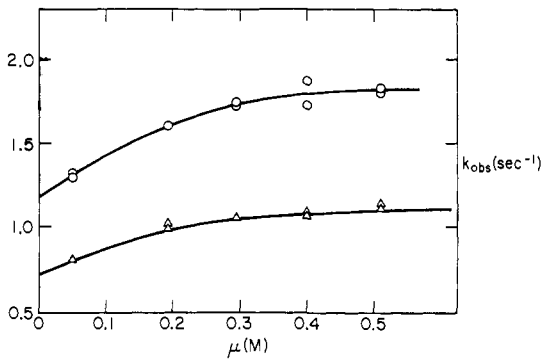

Figure 5. Ionic strength dependence of observed rate constants for the reduction of laccase by hydroquinone, 25.5°, pH 7.0, $[H_2Q] = 2.5 \times 10^{-3}$ M: (○) 330 nm; (△) 614 nm.

Ionic Strength Dependence. A series of pH 7.0 phosphate buffers in the range $0.024 \leq [phosphate]_{tot} \leq 0.211$ M was used in a study of the ionic strength dependence of laccase reduction rate constants. In view of the sensitivity of laccase to anion binding and the fact that phosphate is by no means a noncomplexing anion,[31] these experiments may also be seen as a phosphate concentration dependence study. Substantial stability constants have been reported[31] for the association of mono- and dihydrogen phosphate ions with aqueous copper(II) (log $K_{H_2PO_4^-} = 1.3 \pm 0.1$, log $K_{HPO_4^{2-}} = 3.3 \pm 0.1$; 37°, 0.15 M KNO_3).

Rate data collected over the range $0.05 \leq \mu \leq 0.50$ are given in Figure 5.[27] Laccase in ionic strength 0.05 buffer was mixed with hydroquinone solutions containing enough phosphate to give the final ionic strengths indicated in Figure 5. As the ionic strength increases from 0.05 to 0.50, 330- and 614-nm observed rate constants for $[H_2Q] = 2.5 \times 10^{-3}$ M both are enhanced by approximately 40%.

Formation of Azide and Fluoride Complexes of Laccase. Addition of a large excess of NaN_3 to laccase causes a fast color change from blue to green, but no significant change in the protein visible–ultraviolet spectrum results when NaF is added (pH ≥6, phosphate buffer). Difference spectra between azide-complexed and native laccase (Figure 6) reveal an intense new band with a maximum at 405 nm. Little absorbance change in the vicinity of 330 nm occurs, but the "blue" absorption becomes appreciably more intense in the presence of azide ion. Formation of the laccase–azide complex is reversible, as the new absorption peak is abolished by dialysis against a large volume of distilled water.

Preliminary kinetic studies of the reaction of laccase with azide at pH 7.0 showed very complicated behavior. For $0.01 \leq [N_3^-] \leq 0.05$ M and [laccase] $\cong 1 \times 10^{-5}$ M, very small absorbance changes occur at

(30) J. A. McDermott, T. C. Hunter, S. O. Saki, and E. Frieden, *Biochim. Biophys. Acta*, **151**, 541 (1968).
(31) C. W. Childs, *Inorg. Chem.*, **9**, 2465 (1970).

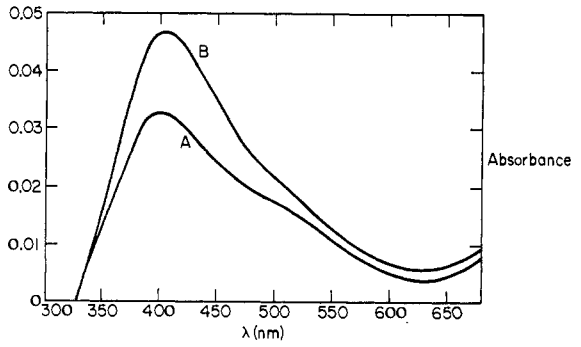

Figure 6. Azide complex minus native laccase difference spectra, pH 6.1 (3.87 × 10^{-2} *M* phosphate), 25°, $[laccase]_{tot}$ = 1.83 × 10^{-5} *M*, 1-cm path length: (A) $[N_3^-]$ = 0.02 *M*; (B) $[N_3^-]$ = 0.1 *M*.

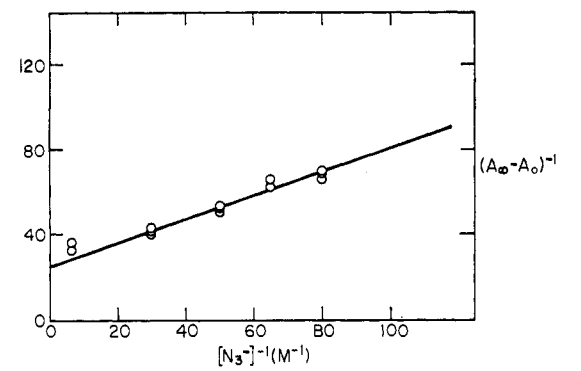

Figure 7. Plot of $(A_\infty - A_0)^{-1}$ *vs.* $[N_3^-]^{-1}$ for the anation of laccase by azide ion, 405 nm, 25.1°, pH 6.1, μ 0.2, $[laccase]_{tot}$ = 1.3 × 10^{-5} *M*.

405 nm at an azide-independent rate of 0.26 ± 0.01 sec^{-1}. Both fast and slow rate processes are found at 405 nm for higher azide concentrations and the total absorbance changes at this wavelength are not consistent with formation of a 1:1 laccase–azide complex.

The kinetics and stoichiometry of azide binding to laccase are much more straightforward at pH 6.1. A single first-order relaxation process is observed at 405 nm for all azide concentrations up to at least 0.15 *M*. Observed rate constants were obtained from the least-squares slopes of log $(A_e - A_t)$ *vs.* time plots, where A_e and A_t represent absorbances at equilibrium and at time *t*, respectively. Total absorbance changes, $A_e - A_0$ (*A* at time 0), were evaluated from the intercepts of log $(A_e - A_t)$ *vs.* time plots. All solutions were made up in 0.0387 *M* phosphate buffer, pH 6.1, and the ionic strength was maintained at 0.2 using sodium nitrate. Azide ion contributes over 96% of the total azide concentration at pH 6.1 (pK_a = 4.75 for HN_3).[32]

It may be easily shown that a plot of $(A_e - A_0)^{-1}$ *vs.* $[N_3^-]^{-1}$ is expected to be linear only if laccase and azide form a 1:1 complex exclusively over the entire range of azide concentrations employed. Figure 7 indicates that the pH 6.1 data are consistent with 1:1 stoichiometry over the concentration range of $0.0125 \leq [N_3^-] \leq 0.15$ *M* used in the kinetic measurements. The formation constant K_f of the complex (K_f = 45 ± 2 M^{-1}; 25.1°, pH 6.1, μ 0.2) is given by the ratio of intercept to slope in the $(A_e - A_0)^{-1}$ *vs.* $[N_3^-]^{-1}$ plot. It was possible

(32) D. Yost and H. Russell, Jr., Systematic Inorganic Chemistry," Prentice-Hall, New York, N. Y., 1946.

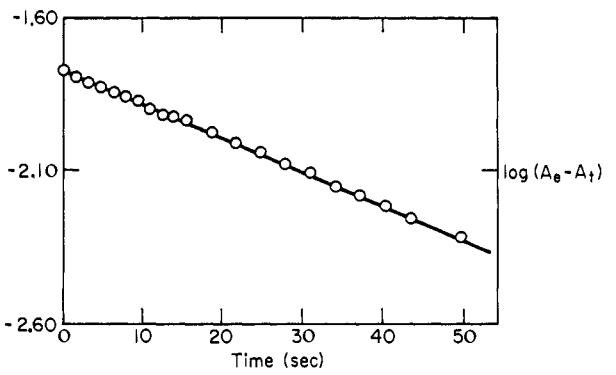

Figure 8. Typical first-order plot for the anation of laccase by azide ion, 405 nm, 25.1°, pH 6.1, μ 0.2, $[N_3^-]$ 0.02 *M*.

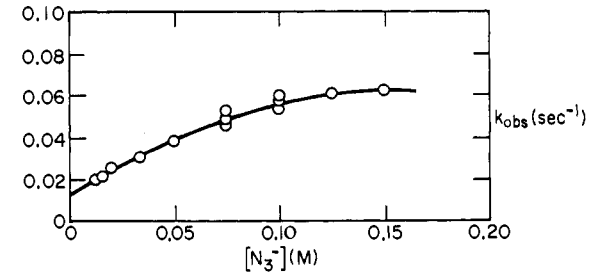

Figure 9. Plot of k_{obsd} *vs.* $[N_3^-]$ for the anation of laccase by azide ion, 405 nm, 25.1°, pH 6.1, μ 0.2.

to estimate $\Delta\epsilon_{405}$, the azide complex minus native protein molar extinction coefficient at 405 nm ($\Delta\epsilon_{405}$ = 1.6 ± 0.1 × 10^3 M^{-1} cm^{-1}), using the relationship: slope = $K_f l \Delta\epsilon_{405}[laccase]_{tot}$ where *l* is the spectrophotometric path length.

A typical log $(A_e - A_t)$ *vs.* time plot for the azide anation of laccase is shown in Figure 8. Observed rate constants are displayed as a function of azide concentration in Figure 9.[27] For an anation mechanism of the form

$$M^{n+} + L^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ML^{(n-1)+}$$

the expression characterizing the observed rate constants is[33]

$$k_{obsd} = k_1[L^-] + k_{-1}$$

Figure 9 shows that the anation of laccase by azide ion is not susceptible to this simple analysis. As $[N_3^-]$ increases, k_{obsd} values tend toward a saturation limit. Sutin and Yandell[34] have pointed out two mechanistic alternatives compatible with this behavior in discussing anation reactions of cytochrome *c*. The first possibility requires a protein activation step, most likely dissociation of a ligand, followed by attack of the incoming anion

$$\text{laccase} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{laccase*}$$

$$\text{laccase*} + N_3^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{laccase-}N_3^-$$

Laccase* represents the activated intermediate and laccase-N_3^- the species absorbing at 405 nm. The ob-

(33) A. A. Frost and R. G. Pearson, "Kinetics and Mechanisms," 2nd ed, Wiley, New York, N. Y., 1961, p 186.
(34) N. Sutin and Y. Yandell, *J. Biol. Chem.*, 247, 6932 (1972).

served rate constant expected for a reaction following this scheme is

$$k_{obsd} = \frac{k_1k_2[N_3^-] + k_{-1}k_{-2}}{k_1 + k_{-1} + k_2[N_3^-] + k_{-2}}$$

Making the steady state approximation for laccase* yields the analytically useful expression

$$\frac{1}{\alpha k_{obsd}} = \frac{1}{k_1} + \frac{1}{k_{-2}K_f[N_3^-]}$$

where $\alpha = K_f[N_3^-]/(1 + K_f[N_3^-])$. Plotting (αk_{obsd}) *vs.* $[N_3^-]^{-1}$ then should give a line with slope $(k_{-2}K_f)^{-1}$ and intercept k_1^{-1}.

The other mechanism consistent with saturation in k_{obsd} requires fast preequilibrium formation of an outer-sphere complex (laccase·N_3^-) followed by rate-determining dissociative interchange of azide with a native laccase ligand to give the inner-sphere complex laccase-N_3^-.

$$\text{laccase} + N_3^- \rightleftharpoons \text{laccase}\cdot N_3^- \qquad K_{os}$$

$$\text{laccase}\cdot N_3^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{laccase-}N_3^-$$

The apparent first-order rate constant implied by this mechanism is easily shown to be

$$k_{obsd} = k_{-2} + \frac{k_2K_{os}[N_3^-]}{1 + K_{os}[N_3^-]}$$

if a large excess of azide over laccase is present. This expression may be rearranged to yield the same relationship for $(\alpha k_{obsd})^{-1}$ as was derived for the alternative anation mechanism.

Figure 10 illustrates the $(\alpha k_{obsd})^{-1}$ *vs.* $[N_3^-]^{-1}$ plot for the laccase-azide complex. The excellent straight line obtained indicates that the data are consistent with either one of the proposed mechanisms. Least-squares analysis of the linear plot allows estimation of the dissociation rate of the azide complex ($k_{-2} = 1.36 \pm 0.08 \times 10^{-2}$ sec^{-1}) and k_1 (or k_2 for the outer-sphere complex interpretation) ($1.94 \pm 0.10 \times 10^{-1}$ sec^{-1}).

Reduction of the Laccase-Azide Complex by Hydroquinone, pH 6.1. The reaction of the laccase-azide complex with hydroquinone at pH 6.1 was followed at 330, 405, and 614 nm using the same conditions as were employed in the anation study. Laccase was preequilibrated with NaN_3 or NaF in all experiments described in this and subsequent sections. In view of the limited stability of laccase-azide solutions against copper reduction, these were used within several hours after their preparation.

Hydroquinone solutions in the range 2.5×10^{-3} to 1.0×10^{-1} *M* were used in making kinetics measurements. Native laccase is reduced rapidly using reducing agent concentrations in this range, leaving a slower reacting component at 614 nm presumably attributable to reduction of the azide-complexed protein. Observed rate constants for "blue" copper reduction are presented in Table IV. The rate is essentially independent of the reducing agent concentration and the azide concentration ($0.02 \leq [N_3^-] \leq 0.15$ *M*). Actually, k_{obsd} reaches a shallow maximum at *ca.* $[H_2Q] = 2.0 \times 10^{-2}$ *M*, but deviations from the average value of the observed rate constants over the 20-fold concentration range (0.13 ± 0.02 sec^{-1}) are not large enough to be considered outside the limits of experimental error.

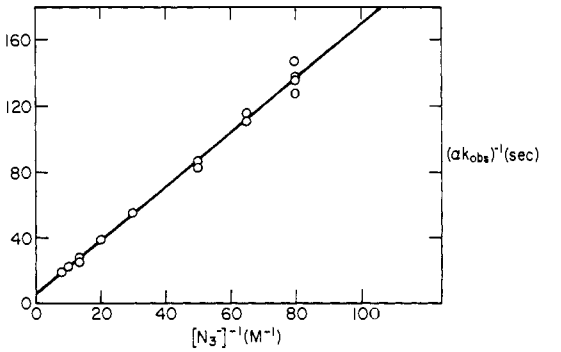

Figure 10. Plot of $(\alpha k_{obsd})^{-1}$ *vs.* $[N_3^-]^{-1}$ for the reaction of azide ion with laccase, 405 nm, 25.1°, pH 6.1, μ 0.2.

Table IV. Observed Rate Constants for the Reduction of the Laccase-Azide Complex (614 nm, 25.1°, pH 6.1, μ 0.2)

$[H_2Q] \times 10^2$, *M*	$[N_3^-] \times 10^2$, *M*	k_{obsd}, sec^{-1}
0.25	5.0	0.098
		0.095
0.50	5.0	0.122
		0.123
1.0	5.0	0.157
2.5	5.0	0.161
		0.155
5.0	5.0	0.132
		0.140
1.0	2.0	0.160
		0.163
1.0	7.5	0.168
		0.175
1.0	10.0	0.175
		0.165
1.0	15.0	0.153
		0.162

Following a fast small decrease, most of the 405-nm absorbance decays slowly by a first-order process when hydroquinone is mixed with the laccase-azide complex. Rate data are accumulated in Table V, and a plot of k_{obsd} as a function of $[N_3^-]$ is given in Figure 11 for $[H_2Q] = 1.0 \times 10^{-1}$ *M*. The 405-nm rate is independent of reducing agent concentration over the interval $2.5 \times 10^{-3} \leq [H_2Q] \leq 1.0 \times 10^{-1}$ *M* ($k_{obsd} = 5.6 \pm 0.4 \times 10^{-3}$ sec^{-1}; 25.1°, pH 6.1, μ 0.2, $[N_3^-] = 0.05$ *M*) but first order in the azide concentration. The rate law for bleaching of the 405-nm absorbance may be written

$$\frac{-d[Cu(405)]_{tot}}{dt} = k_2[N_3^-][Cu(405)]_{tot}$$

where $k_2 = 1.06 \pm 0.06 \times 10^{-1}$ M^{-1} sec^{-1} (25.1°, pH 6.1, μ 0.2).

The small initial absorbance decrease at 405 nm takes place on the same time scale as 614-nm absorbance decay. It probably appears because the absorbance changes at the two wavelengths are not totally independent of each other. A small decrease in A_{405} does accompany reduction of native laccase. Absorbance changes at 614 nm were complete before the first data points for the considerably slower 405-nm reaction were taken, so these do not pose a potential interference in the evaluation of 405-nm results.

The 330-nm absorbance actually begins to rise after a small initial decrease when hydroquinone reacts with the laccase-azide complex. Absorbance changes at

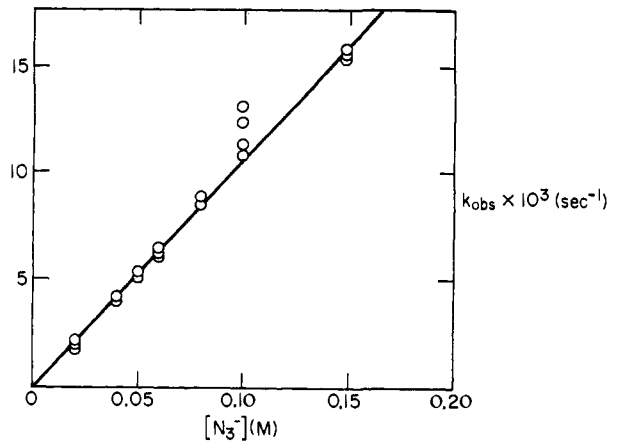

Figure 11. Plot of k_{obsd} *vs.* [N_3^-] for the reduction of the laccase–azide complex by hydroquinone, 405 nm, 25.1°, pH 6.1, μ 0.2, [H_2Q] = 0.1 *M*.

Table V. Observed Rate Constants for the Reduction of the Laccase–Azide Complex (405 nm, 25.1°, pH 6.1, μ 0.2)

[H_2Q] × 10^2, *M*	[N_3^-] × 10^2, *M*	k_{obsd} × 10^3, sec^{-1}
0.25	5.0	5.0
		5.1
0.50	5.0	5.4
		5.3
1.0	5.0	6.1
		6.3
2.5	5.0	5.6
		5.9
5.0	5.0	6.0
		6.1
7.5	5.0	5.3
		5.2
10.0	5.0	5.3
		5.0
10.0	2.0	2.2
		1.9
		1.8
10.0	4.0	4.0
		4.1
10.0	6.0	6.0
		6.2
		6.4
10.0	8.0	8.8
		8.9
		8.5
		8.8
10.0	10.0	13.0
		10.7
		11.3
		12.3
10.0	15.0	15.5
		15.7
		15.4

this wavelength appear to follow those at 405 nm very closely, but slow upward absorbance drifts continue long after the decay in 405-nm absorbance is complete. No attempt was made to quantitatively derive rate parameters from 330-nm absorbance–time curves. An increase in 330-nm absorbance also occurs if NaN_3 is added to laccase already fully reduced by hydroquinone.

Reduction of the Laccase–Fluoride Complex by Hydroquinone, pH 6.0. Reduction of the laccase–fluoride complex by hydroquinone was followed at 614 and 330 nm, again using the same conditions as were employed in the pH 6 anation experiments. Absorption at both wavelengths decreases normally upon the addition of hydroquinone; Table VI contains observed rate constants derived from the absorbance–time data. The absence of any fast reduction component in the absorbance–time profiles suggests that laccase is present only as the fluoride complex for all concentrations of NaF employed.

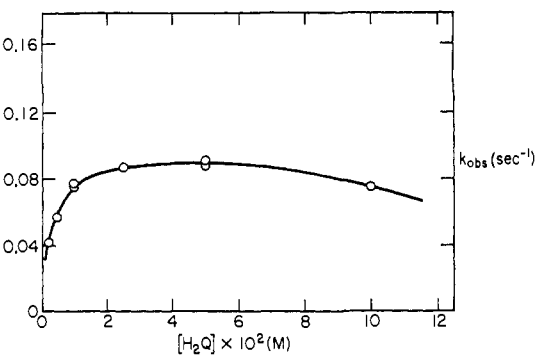

Figure 12. Plot of k_{obsd} *vs.* [H_2Q] for the reduction of the laccase–fluoride complex by hydroquinone, 330 nm, 25.1°, pH 6.0, μ 0.2, [F^-] = 0.05 *M*.

Table VI. Rate Data for the Reaction of Hydroquinone with the Laccase–Fluoride Complex (25.1°, pH 6.0, μ 0.2)

[H_2Q] × 10^2, *M*	[F^-] × 10^2, *M*	—k_{obsd} × 10^2, sec^{-1}— 614 nm	330 nm
0.25	5.0	3.0	4.1
		2.8	4.2
0.50	5.0	5.1	5.7
		4.8	5.7
1.0	5.0	7.2	7.7
		7.5	7.6
2.5	5.0	8.7	8.7
		8.8	8.7
5.0	5.0	10.5	9.0
		9.2	8.8
		9.5	
		8.0	
10.0	5.0		7.4
			7.4
10.0	2.0		7.2
			7.5
10.0	7.5		7.1
			7.2
10.0	10.0		7.1
			7.3
10.0	15.0		5.2
			5.2
1.0	2.0	7.7	
		7.6	
1.0	10.0	7.4	
		7.0	
1.0	15.0	7.6	
		7.6	

Figure 12 illustrates the dependence of k_{obsd}(330) on [H_2Q] for [F^-] = 0.05 *M*. Rate saturation with increasing hydroquinone concentration occurs, and slight apparent substrate inhibition is evident as [H_2Q] is raised above the minimum level required to saturate the rate. Observed rate constants at 614 nm approach a a similar saturation rate, but k_{obsd}(614) values at lower reducing agent concentrations invariably are somewhat smaller than corresponding 330-nm results. Reduction rates at both wavelengths are essentially inde-

pendent of the fluoride ion concentration over the interval $0.02 \leq [F^-] \leq 0.15\ M$.

Rate saturation with increasing substrate concentration in enzyme systems has been treated in many instances using the Michaelis–Menten formalism.[35] A mechanism based on the Michaelis–Menten approach is proposed here to quantitatively account for the laccase–fluoride complex reduction results.

$$(2,2F^-,Ox) + HQ \rightleftharpoons [(2,2F^-,Ox)\cdot HQ]_1 \qquad K_s(1)$$

$$(1,2F^-,Ox) + HQ \rightleftharpoons [(1,2F^-,Ox)\cdot HQ]_1$$

$$(2,2F^-,Ox) + HQ \rightleftharpoons [(2,2F^-,Ox)\cdot HQ]_2 \qquad K_s(2)$$

$$(2,2F^-,Red) + HQ \rightleftharpoons [(2,2F^-,Red)\cdot HQ]_2$$

$$[(2,2F^-,Ox)\cdot HQ]_1 \xrightarrow{k_1} (2,2HQ^-,Ox) + F^-$$

$$HQ + F^- + (2,2HQ^-,Ox) \xrightarrow{\text{fast}} (2,2F^-,Red) + 2SQ$$

$$[(1,2F^-,Ox)\cdot HQ]_1 \xrightarrow{k_1} (1,2HQ^-,Ox) + F^-$$

$$HQ + F^- + (1,2HQ^-,Ox) \xrightarrow{\text{fast}} (1,2F^-,Red) + 2SQ$$

$$[(2,2F^-,Ox)\cdot HQ]_2 \xrightarrow{k_2} (2,2HQ^-,Ox) + F^-$$

$$F^- + (2,2HQ^-,Ox) \xrightarrow{\text{fast}} (1,2F^-,Ox) + SQ$$

$$[(2,2F^-,Red)\cdot HQ] \xrightarrow{k_2} (2,2HQ^-,Red) + F^-$$

$$F^- + (2,2HQ^-,Red) \xrightarrow{\text{fast}} (1,2F^-,Red) + SQ$$

The species $(2,2F^-,Ox)$ refers to the oxidized laccase type 2 fluoride complex, and the binding constants $K_s(1)$ and $K_s(2)$ refer respectively to facile enzyme–substrate complex preequilibria leading to reduction of the type 3 and type 1 copper sites. The symbol HQ is used to indicate either H_2Q or HQ^-, as it is not possible to state which form of the reductant is participating on the basis of experiments at constant pH.

The rate laws derived from the above mechanism are

$$\frac{-d[Cu(330)]_{tot}}{dt} = \frac{k_1K_s(1)[HQ][Cu(330)]_{tot}}{1 + K_s(1)[HQ]} = k_{obsd}(330)[Cu(330)]_{tot}$$

$$\frac{-d[Cu(614)]_{tot}}{dt} = \frac{k_2K_s(2)[HQ][Cu(614)]_{tot}}{1 + K_s(2)[HQ]} = k_{obsd}(614)[Cu(614)]_{tot}$$

Rearranging these equations yields the analytically useful expressions

$$\frac{1}{k_{obsd}(330)} = \frac{1}{k_1K_s(1)[HQ]} + \frac{1}{k_1}$$

$$\frac{1}{k_{obsd}(614)} = \frac{1}{k_2K_s(2)[HQ]} + \frac{1}{k_2}$$

Linear plots of $k_{obsd}(330)^{-1}$ and $k_{obsd}(614)^{-1}$ *vs.* $[H_2Q]^{-1}$ are obtained with the experimental data (Figure 13), as expected from the proposed mechanism. The kinetic parameters derived from the least-squares slopes and intercepts of these plots are: $k_1 = 9.8 \pm 0.2 \times 10^{-2}$ sec^{-1}, $K_s(1) = 298\ M^{-1}$; $k_2 = 1.13 \pm 0.05 \times 10^{-1}$ sec^{-1}, $K_s(2) = 142\ M^{-1}$ (25.1 ± 0.1°, pH 6.0, μ 0.2).

Reduction of the Laccase–Fluoride Complex by Hydroquinone, pH 7.0. The reactivity of laccase–anion complexes with hydroquinone changes dramatically as the pH is altered only one unit from 6.0 to 7.0. A series of runs at $[F^-] = 0.02\ M$, μ 0.1, pH 7.0 is discussed in this section; phosphate buffer and NaF alone maintained the ionic strength.

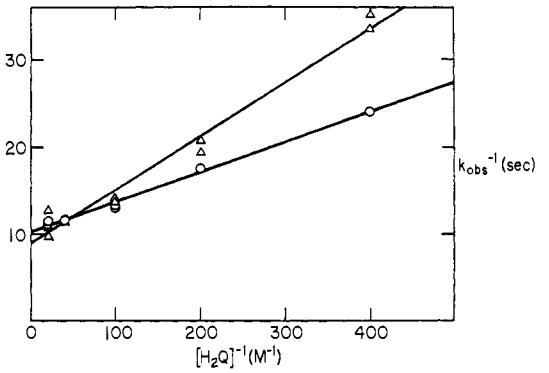

Figure 13. Plot of k_{obsd}^{-1} *vs.* $[H_2Q]^{-1}$ for the reduction of the laccase–fluoride complex by hydroquinone, 25.1°, pH 6.0, μ 0.2, $[F^-] = 0.05\ M$: (○) 330 nm; (△) 614 nm.

A biphasic absorbance change is observed at 614 nm when hydroquinone reacts with laccase in the presence of NaF at pH 7.0. The absorbance decays rapidly to a first base line and then, following a short period, again begins to drop, ultimately reaching the final base line. We suggest that the initial absorbance drop corresponds to reduction of "blue" copper in a laccase–fluoride complex which does not dissociate rapidly to give the native enzyme on the time scale of reduction; the second component is assigned to reduction of native laccase. This interpretation implies that the reduced laccase–fluoride complex is not rapidly reoxidized by oxygen, permitting reduction of the oxidized laccase–fluoride derivative to be observed while the native enzyme is participating in steady state oxygen consumption.

Laccase absorbance typically drops to a steady state level in the presence of oxygen and a reducing agent and then falls to the value characteristic of fully reduced protein when oxygen is fully consumed. Absorbance decay toward the steady state level is exponential, so it is conceivable that this phenomenon might be mistaken for reduction of a nonoxidizable laccase species. It is easily shown that the steady state 614-nm absorbance value will decrease as the hydroquinone concentration increases if the initial oxygen concentration is held constant from one experiment to the next. No variation in the absorbance level of the initial base line was observed as the hydroquinone concentration was varied over a tenfold range in making stopped flow kinetic measurements of the reduction of laccase in the presence of added fluoride at pH 7.0. The initial absorbance decrease always accounts for 73 ± 3% of the total 614-nm absorbance change at room temperature, ruling out the possibility that relaxation to the steady state condition accounts for the observed two-step reduction pattern.

Accurate rate parameters for reduction of the slower reacting laccase component could not be obtained in view of the small extent of the second A_{614} decrease. Approximate observed rate constants are in reasonable agreement with values expected for native laccase at the same temperature, ionic strength, and pH.

(35) H. R. Mahler and E. H. Cordes, "Biological Chemistry," Harper and Row, New York, N. Y., 1966, Chapter 6.

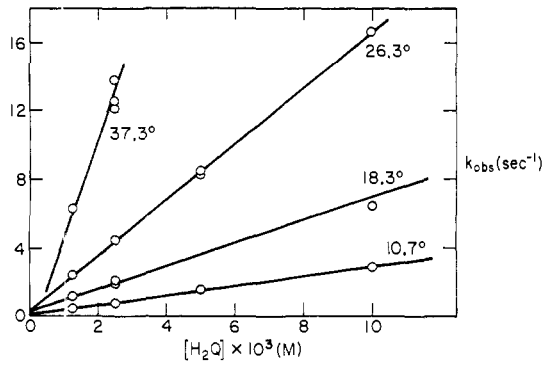

Figure 14. Plot of k_{obsd} *vs.* [H_2Q] for the reaction of hydroquinone with the laccase–fluoride complex, fast phase, 614 nm, pH 7.0, μ 0.1, [F^-] = 0.02 *M*.

Analytical plots based on the fast absorbance change yield the observed rate constants plotted as a function of hydroquinone concentration in Figure 14.[27] The linear plots of $k_{obsd}(614)$ *vs.* [H_2Q] for data taken at several temperatures show small positive intercepts. These are large enough to be considered outside the limits of experimental error from zero but too small to justify firmly assigning them to a parallel reduction pathway zeroth order in [H_2Q]. The room temperature coefficient of the hydroquinone-dependent term is approximately five times larger than that found for the native protein under the same conditions: $k_{obsd}(614) = 0.394 + 1.62 \times 10^3[H_2Q]$ sec^{-1} (26.3°, pH 7.0, μ 0.1).

The reactivity of laccase "blue" copper is almost unchanged in the presence of fluoride ion at pH 7.0. In contrast, reduction of the type 3 site is severely inhibited and the rate law for reduction is considerably altered. Following the usual steady state period, a fast absorbance decrease accounting for *ca.* 25% of the total 330-nm absorbance change occurs. Considering observations at 614 nm, this fast change most logically corresponds to reduction of native laccase. The remaining 330-nm absorbance change occurs by a very slow first-order process, attributed to reduction of the esr nondetectable copper site in fluoride-inhibited laccase. The kinetic results based on the slow process show an essentially zeroth order variation of k_{obsd}(330) with [H_2Q] at several temperatures.[27] Observed rate constants actually fall off significantly with increasing hydroquinone concentration. The reduction rate at 25.5° is 1.0 ± 0.2 × 10^{-2} sec^{-1} for 1.25 × $10^{-3} \leq$ [H_2Q] $\leq$ 7.5 × 10^{-3} *M*.

Reduction of the Laccase–Azide Complex by Hydroquinone, pH 7.0. In view of the apparent complexity of laccase–azide equilibria at pH 7.0, only a few hydroquinone reduction experiments were attempted with azide-inhibited protein at this pH value. One set of kinetic runs with [N_3^-] = 0.02, μ 0.1, was performed for comparison with the fluoride results at the same anion concentration. Reduction of 614-nm absorbance again takes place in two steps, but the base line for the initial fast process is not well defined. Observed rate constants were estimated by the Guggenheim method,[36] and an order of magnitude value for the room-temperature second-order rate constant was obtained ($k_1 \cong 1.0 \times 10^3\ M^{-1}$ sec^{-1}).

The similarity between results of kinetic experiments with added NaF and NaN_3 at pH 7 extends to observations at 330 nm. Although an absorbance increase develops after hydroquinone is mixed with dilute laccase containing 0.02 *M* NaN_3 at pH 6.1, normal reduction of 330-nm absorbance occurs at pH 7.0 using the same azide concentration. The rate of absorbance decay at 330 nm is independent of [H_2Q] over the interval of 1.25 × $10^{-3} \leq$ [H_2Q] $\leq$ 1.0 × 10^{-2} *M* with k_{obsd} = 9.1 ± 0.2 × 10^{-2} sec^{-1} (25.1°).[27]

Finally, a few points were taken at 25°, μ 0.2, pH 7.0 for azide concentrations higher than 0.02 *M*. Under conditions where the concentration of the species absorbing at 405 nm is appreciable, pH 7.0 results for the reaction of hydroquinone with azide-inhibited laccase strongly resemble the pH 6.1 observations presented earlier. Increases in 330-nm absorbance are again noted, and 405-nm absorbance decay takes place even slower than at pH 6.1. For example, with [H_2Q] = [N_3^-] = 1.0 × 10^{-1} *M*, $k_{obsd}(405)$ is 3.1 × 10^{-3} sec^{-1} at pH 7.0 compared with 1.2 × 10^{-2} sec^{-1} at pH 6.1. Observed 405-nm rate constants increase with increasing azide concentration, but the dependence on [N_3^-] is greater than first order.

Discussion

All of the experimental evidence clearly points to closely similar reduction mechanisms for the type 1 and type 3 sites in native laccase. Trends in observed rate constants for reduction of the protein found at one wavelength invariably are reflected in results at the other, and the absolute values of 614- and 330-nm rate constants are always comparable.[37] It is particularly significant, however, that the reduction rates of the "blue" and esr nondetectable copper sites are *not* identical. This allows us to conclude that the pathways leading to reduction of the type 1 and type 3 copper sites must involve at least slightly different activated complexes.

The hydrogen ion dependences of 614- and 330-nm rate constants agree with Omura's observation[38] that *Rhus* laccase activity rises with increasing pH up to pH 7.5. Variations in the oxidizing strengths of laccase copper sites are not a likely contributor to the kinetic pH dependence since the standard reduction potential of "blue" copper actually decreases as solutions become more basic.[39] Furthermore, the reduction rate laws are not of the form expected if ionization of laccase amino acid side chains was responsible for the rate increase with increasing pH. Inverse first-order kinetic hydrogen-ion dependences have been reported for the hydroquinone reduction of ferric ion[26] and cytochrome *c*,[40] and in both cases the result was interpreted in terms of participation of the hydroquinone monoanion in the rate-determining step for electron transfer. This assignment seems most reasonable for the $[H^+]^{-1}$-dependent pathways in the reduction of the laccase type 1 and type 3 sites, but a kinetically indistinguishable mechanistic alternative, hydrogen atom

(36) Reference 33, p 49.

(37) It is evident from the data in Tables I and II that the temperature coefficients of 330- and 614-nm rate constants also are comparable in value. The temperature variation data given in this paper, however, are insufficient to separately evaluate activation parameters for the k_0 and k_{-H^+} terms in the rate laws for reduction of the type 1 and type 3 sites. These activation parameters will be evaluated and discussed in a subsequent paper.

(38) T. Omura, *J. Biochem.* (*Tokyo*), **50**, 264 (1961).

(39) T. Nakamura, *Biochim. Biophys. Acta*, **30**, 44 (1958).

(40) G. R. Williams, *Can. J. Biochem. Physiol.*, **41**, 231 (1963).

transfer within an activated complex of the form CuL-$(-H^+)\cdots H_2Q$, cannot be completely ruled out. Support for the hypothesis that rate increases with increasing pH are linked to ionization of the substrate is found in the observation that $k_{obsd}(614)$ varies only slightly with pH in the region $5 \leq pH \leq 8$ for the $Fe(EDTA)^{2-}$ reduction of laccase "blue" copper.[41]

Several other experimental observations support the assignment of HQ^- as the reductant interacting with laccase in the $[H^+]^{-1}$-dependent pathways for electron transfer to the type 1 and type 3 copper sites. It is expected that the phenolate anion HQ^- and the free radical SQ ought to have similar reactivities with laccase. The "blue" copper reduction rate constant calculated assuming HQ^- to be the electron donor contributing to the $[H^+]^{-1}$-dependent pathway is indeed in close agreement with that found for semiquinone as reductant in experiments with added benzoquinone. The ionic strength dependence of $k_{obsd}(330, 614)$ is additionally consistent with a mechanism requiring HQ^- to be the dominant and perhaps only active reductant involved in the slow step for electron transfer to the type 1 and type 3 sites. Thus the observed rate changes may be interpreted in a straightforward manner considering variations in the hydroquinone acid ionization constant induced *via* the secondary salt effect. Baxendale and Hardy[42] have reported $K_{H_2Q} = 1.41 \times 10^{-10}$ M at 25°, μ 0.65. Comparing this value with the one given above for μ 0.04 reveals a 42% increase over the interval $0.04 \leq \mu \leq 0.65$. This increase is in excellent accord with the 40% enhancement in $k_{obsd}(614, 330)$ values found over approximately the same range in ionic strength.

Preferential binding of azide and fluoride to *Rhus* laccase type 2 copper has been suggested on the basis of perturbations in the esr spectrum of this component induced by the anions.[2] That type 2 coordination occurs with the addition of fluoride seems clear from experiments in which ^{19}F superhyperfine splitting was detected in the fungal laccase type 2 esr signal.[43] This assignment has also been made for ceruloplasmin.[44] Esr spectra of tree laccase in the presence of 1 equiv of NaF indicate that more than 60% of the fluoride is bound to type 2 Cu(II) at pH 5.5 (0.05 M acetate buffer).[43b] Alterations in the ceruloplasmin type 2 spectrum induced by azide, however, have been attributed to anion binding at a type 3 site closely linked to a type 2 copper atom.[45] There seems to be disagreement on whether or not fluoride and azide compete for the same coordination position in ceruloplasmin. Byers, *et al.*,[45] claim they do not and suggest that the new visible spectral features accompanying azide coordination have their origin in drastic structural perturbations in the type 3 site. Andreasson and Vänngård,[44] on the other hand, state that the addition of large excesses of fluoride to the ceruloplasmin–azide complex yields the esr spectrum characteristic of the fluoride derivative. A correlation was found between the appearance of the new absorption band around 390 nm and changes in the type 2 Cu(II) esr spectrum, and this was taken as strong evidence for type 2 Cu(II)–azide complex formation. Recent experiments by Morpurgo, *et al.*,[46] have demonstrated that a similar correlation exists for *Rhus* laccase at relatively high azide concentrations; type 2 Cu(II) again is thought to be involved in the appearance of the new band. Correct assignment of the 405-nm absorption peak of the laccase–azide complex is essential to the interpretation of kinetic results reported here; the available evidence definitely favors the type 2 Cu(II)–N_3^- formulation.

The pH 6.1 study of laccase anation by azide shows that absorbance changes at 405 nm are associated with formation of a 1:1 complex over the entire range of azide concentrations employed. This finding, coupled with the observation[47] that fluoride and azide compete for the same binding site, indicates that the pH 6 results for hydroquinone reduction of anion-inhibited laccase may be safely interpreted in terms of coordination at the type 2 site. As will be seen later, this is apparently no longer true when the pH is changed to 7.0 and low concentrations of anions are used.

Our finding of a new absorption band when NaN_3 is added to laccase is in good agreement with other recent results for *Rhus* laccase,[46] and similar absorptions have been reported for fungal laccase[3] and ceruloplasmin.[44,45,48] Analogous absorptions in low molecular weight pseudohalide complexes have been assigned as ligand-to-metal charge transfer,[49] and this assignment seems most reasonable for the laccase type 2 Cu(II)–N_3^- complex as well, considering the high band intensity and requirement for cupric copper. Our value for the formation constant of the type 2 Cu(II)–N_3^- complex at pH 6.1, μ 0.2, is considerably smaller than that obtained by Morpurgo, *et al.* (4×10^2 M^{-1}, pH 5.5 (acetate)),[46] but the apparent discrepancy probably may be satisfactorily accounted for considering the pH and medium sensitivity of K_f documented by the Italian workers.[46] The K_f value reported here also is about an order of magnitude smaller than stability constants for simple azido–copper(II) complexes.[50]

The fluoride inhibition study at pH 6.0 is fully consistent with the proposal that the reduction mechanisms of the type 1 and type 3 sites are closely related and provides an important clue to the structural nature of the activated complexes for electron transfer. Reduction rates of the type 1 and type 3 sites are still similar when hydroquinone attacks the type 2 Cu(II)–F^- laccase complex, but saturation in observed rate constants at 330 and 614 nm indicates that new protein-dependent slow steps are operating. In view of the apparent preference of laccase for HQ^-, we propose that fluoride ion inhibits laccase under these circumstances by occupying an inner-sphere coordination position of the type 2 copper which is readily accessible

(41) R. A. Holwerda, S. Wherland, and H. B. Gray, unpublished results.

(42) J. Baxendale and H. R. Hardy, *Trans. Faraday Soc.*, **49**, 1140 (1953).

(43) (a) R. Malkin, B. G. Malmström, and T. Vänngård, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, 1, 50 (1968); (b) R. Bränden, B. G. Malmström, and T. Vänngård, *Eur. J. Biochem.*, **36**, 135 (1973).

(44) L.-E. Andreasson and T. Vänngård, *Biochim. Biophys. Acta*, 200, 247 (1970).

(45) W. Byers, G. Curzon, K. Garbett, B. E. Speyer, S. N. Young, and R. J. P. Williams, *Biochim. Biophys. Acta*, 310, 38 (1973).

(46) L. Morpurgo, G. Rotilio, A. Finazzi-Agro, and B. Mondovi, *Biochim. Biophys. Acta*, **336**, 324 (1974).

(47) Preliminary kinetic measurements at pH 6.0, 25°, have shown that the addition of fluoride ion to the laccase–azide complex causes a marked decrease in the 405-nm absorbance, indicating that F^- and N_3^- do compete for a common binding site.

(48) C. B. Kasper, *J. Biol. Chem.*, **243**, 3218 (1968).

(49) P. Day, *Inorg. Chem.*, **5**, 1619 (1966).

(50) L. G. Sillen and A. E. Martell, *Chem. Soc., Spec. Publ.*, No. 17 (1964).

to the phenolate anion in the native enzyme. The slow steps in the presence of fluoride then probably involve intramolecular dissociative interchange, releasing fluoride back into the solvent as HQ^- takes its place in the first coordination sphere of type 2 Cu(II). It has already been shown that the experimental results are consistent with rate laws calculated on the basis of a mechanism requiring slow breakdown of two distinct enzyme–substrate complexes between the type 2 Cu(II)–F^- derivative and hydroquinone.

The results for reduction of the type 2 laccase–azide complex at pH 6.1 offer a marked contrast with findings for the fluoride derivative under virtually the same conditions. Displacement of anion from type 2 coordination reasonably could be a prerequisite to reduction of the other two copper sites in fluoride-inhibited laccase, but this clearly is not the case in the azide derivative. Type 1 copper in the type 2 Cu(II)–N_3^- derivative is fully reduced before significant loss of 405-nm absorbance occurs, and its reduction rate is nearly an order of magnitude higher than the dissociation rate of azide from type 2 copper in the fully oxidized enzyme. Thus it is clear that an intramolecular type 2 Cu(I) to type 1 Cu(II) intramolecular electron-transfer step is not required to reduce the "blue" copper.

The stoichiometry of the reaction of hydroquinone with the laccase–azide complex at pH 6.1 is still uncertain. The hydroquinone-induced absorbance rise at 330 nm casts some doubt on the reduction of the type 3 site, and the origin of this effect is not readily understood. Reduction of the type 3 site in the pH 6.1 laccase–azide complex, if indeed this occurs, is certainly no faster than electron transfer to the anion-binding site. The best explanation of the 330-nm results, assuming that reduction of type 3 copper does occur, is that reduction of the esr nondetectable site *is* dependent on formation of type 2 Cu(I).

The kinetic results for the reaction of hydroquinone with azide-inhibited laccase at pH 6.1 may be interpreted through the following mechanism.

$$\mathrm{HQ} + (2,2N_3^-,\mathrm{Ox}) \rightleftharpoons (2,2N_3^-,\mathrm{Ox})\cdot\mathrm{HQ} \qquad K_s$$

$$(2,2N_3^-,\mathrm{Ox})\cdot\mathrm{HQ} \xrightarrow{k_1} (1,2N_3^-,\mathrm{Ox}) + \mathrm{SQ}$$

$$(1,2N_3^-,\mathrm{Ox}) + N_3^- \xrightarrow{k_2} (1\text{-}N_3^-\text{-}2,\mathrm{Ox})\,(N_3^-)$$

$$(1\text{-}N_3^-\text{-}2,\mathrm{Ox}) \overset{\text{fast}}{\rightleftharpoons} (2\text{-}N_3^-\text{-}1,\mathrm{Ox})\quad (N_3^-)\quad (N_3^-)$$

$$\mathrm{HQ} + (2\text{-}N_3^-\text{-}1,\mathrm{Ox}) \overset{\text{fast}}{\longrightarrow} (1\text{-}N_3^-\text{-}1,\mathrm{Ox}) + \mathrm{SQ}\quad (N_3^-)\quad (N_3^-)$$

The species $(2,2N_3^-,\mathrm{Ox})$, $(2,2N_3^-,\mathrm{Ox})\cdot\mathrm{HQ}$, and $(1\text{-}N_3^-\text{-}2\text{-}N_3^-,\mathrm{Ox})$ refer respectively to the fully oxidized type 2 azide complex, an enzyme–substrate complex, and a type 1 to type 2 azide-bridged intermediate. The zeroth order $[H_2Q]$ and $[N_3^-]$ dependences of observed rate constants for type 1 reduction suggest rate-limiting electron transfer within an enzyme–substrate complex resulting in direct reduction of the "blue" copper site.

The first-order azide dependence and zeroth-order hydroquinone dependence of k_{obsd} values for bleaching of 405-nm absorbance strongly suggest that an intramolecular electron-transfer reaction is taking place. The azide dependence rules out a mechanism requiring dissociation of N_3^- from type 2 coordination and is best accounted for considering azide to be a bridging group establishing rapid redox equilibrium between type 1 Cu(I) and type 2 Cu(II). Introduction of an azide ion linking type 1 Cu(I) and type 2 Cu(II) may explain the observation[46] that the affinity of the type 2 cupric atom for N_3^- appears to increase as the "blue" copper is reduced. The substitutional lability of laccase copper in the cuprous form is indicated by its fast exchange with radioactive Cu(I) in the medium.[51] Since one azide ion is already bound to laccase before addition of the reducing agent, the data indicate that two azides are present in the activated complex for the electron transfer step which results in disappearance of 405-nm absorbance. The data do not demand that both azides be involved in bridging roles, but this is possible. A number of studies[52–55] of simple inorganic systems have demonstrated that the μ-diazido bridged configuration is particularly effective in promoting electron transfer between metal centers. It is particularly interesting that an azide bridge apparently suffices to establish redox communication between the type 1 and type 2 copper sites. This is an excellent indication that these two functional units are close enough together in laccase for a hydroquinone molecule coordinated at type 2 Cu(II) to be positioned for electron transfer to the "blue" copper atom.

The following mechanism is one of several closely related possibilities for reduction of the laccase type 1 and type 3 sites by hydroquinone.

$$\mathrm{H_2Q} \rightleftharpoons \mathrm{HQ^-} + \mathrm{H^+} \qquad K_{\mathrm{H_2Q}}$$

$$\mathrm{HQ^-} + (2,2,\mathrm{Ox}) \rightleftharpoons (2,2,\mathrm{Ox})\cdot\mathrm{HQ^-} \qquad K_s$$

$$(2,2,\mathrm{Ox})\cdot\mathrm{HQ^-} \xrightarrow{k_1} (2,1,\mathrm{Ox}) + \mathrm{SQ}$$

$$\mathrm{HQ} + (2,1,\mathrm{Ox}) \xrightarrow{\text{fast}} (2,2,\mathrm{Red}) + \mathrm{SQ}$$

$$(2,2,\mathrm{Ox})\cdot\mathrm{HQ^-} \xrightarrow{k_2} (1,2,\mathrm{Ox}) + \mathrm{SQ}$$

$$\mathrm{HQ^-} + (1,2,\mathrm{Ox}) \rightleftharpoons (1,2,\mathrm{Ox})\cdot\mathrm{HQ^-} \qquad K_s$$

$$(1,2,\mathrm{Ox})\cdot\mathrm{HQ^-} \xrightarrow{k_1} (1,1,\mathrm{Ox}) + \mathrm{SQ}$$

$$\mathrm{HQ} + (1,1,\mathrm{Ox}) \xrightarrow{\text{fast}} (1,2,\mathrm{Red}) + \mathrm{SQ}$$

$$\mathrm{HQ^-} + (2,2,\mathrm{Red}) \rightleftharpoons (2,2,\mathrm{Red})\cdot\mathrm{HQ^-} \qquad K_s$$

$$(2,2,\mathrm{Red})\cdot\mathrm{HQ^-} \xrightarrow{k_2} (1,2,\mathrm{Red}) + \mathrm{SQ}$$

The species $(2,2,\mathrm{Ox})\cdot\mathrm{HQ^-}$, $(1,2,\mathrm{Ox})\cdot\mathrm{HQ^-}$, and $(2,2,\mathrm{Red})\cdot\mathrm{HQ^-}$ all refer to enzyme–substrate complexes with HQ^- presumably bound somewhere in the vicinity of the oxidized type 2 copper atom. This mechanism predicts the rate laws

$$\frac{-\mathrm{d[Cu(330)]_{tot}}}{dt} = \frac{k_1 K_{\mathrm{H_2Q}} K_s [\mathrm{H_2Q}][\mathrm{Cu(330)}]_{\mathrm{tot}}}{([\mathrm{H^+}] + K_s K_{\mathrm{H_2Q}}[\mathrm{H_2Q}])}$$

$$\frac{-\mathrm{d[Cu(614)]_{tot}}}{dt} = \frac{k_2 K_{\mathrm{H_2Q}} K_s [\mathrm{H_2Q}][\mathrm{Cu(614)}]_{\mathrm{tot}}}{([\mathrm{H^+}] + K_s K_{\mathrm{H_2Q}}[\mathrm{H_2Q}])}$$

where $[Cu(330)]_{tot}$ and $[Cu(614)]_{tot}$ now include all pro-

(51) T. Omura, *J. Biochem. (Tokyo)*, **50**, 305 (1961).
(52) R. Snellgrove and E. L. King, *J. Amer. Chem. Soc.*, **84**, 4609 (1962).
(53) R. Snellgrove and E. L. King, *Inorg. Chem.*, **3**, 288 (1964).
(54) A. Haim, *J. Amer. Chem. Soc.*, **88**, 2324 (1966).
(55) W. Beck and K. Schorpp, *Angew. Chem., Int. Ed. Engl.*, **9**, 735 (1970).

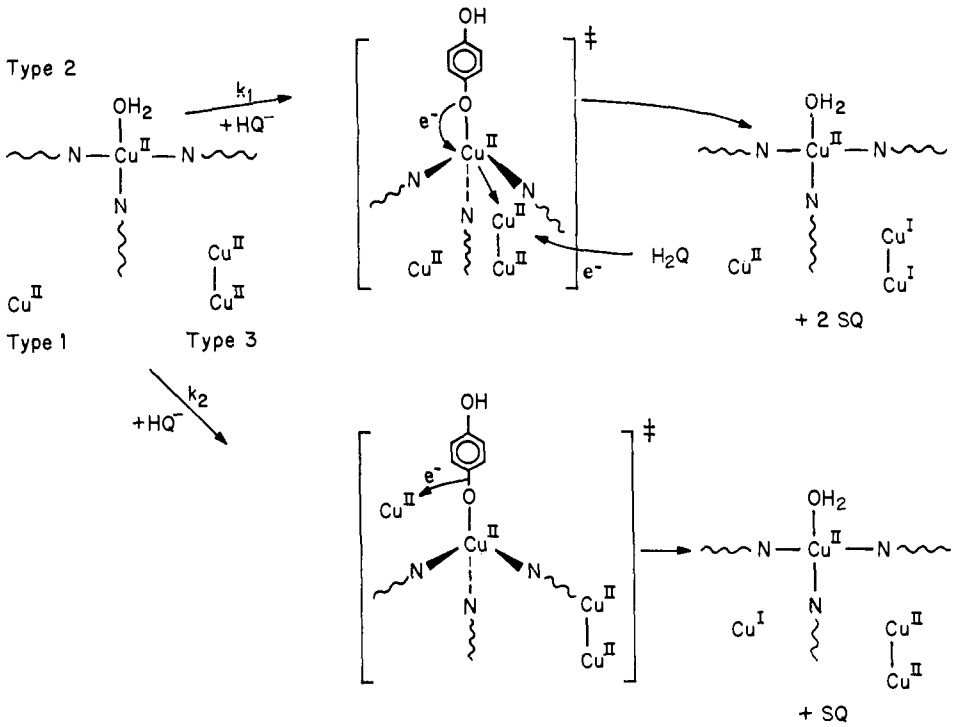

Figure 15. Proposed electron-transfer pathways from hydroquinone to the laccase type 1 and type 3 copper sites.

tein species containing oxidized type 3 and type 1 copper sites, respectively, complexed by substrate or not. It is essential to note that the suggested mechanism implies that the oxidation state of the type 3 copper site has no influence on the reduction rate of the type 1 copper atom and *vice versa*. This remarkable conclusion is demanded by the observation that the reduction rates of the type 1 and type 3 copper sites are different yet first order in $[Cu(614)]_{tot}$ and $[Cu(330)]_{tot}$, respectively. That the electron transfer pathways to the two sites are independent is additionally clear from the observation that inhibitor-induced changes in 330-nm rates at pH 7.0 are not always reflected in 614-nm results.

Although saturation in observed rate constants with increasing hydroquinone concentration is predicted from the theoretical rate laws, it is easily seen that this will not be observed if $K_s K_{H_2Q}[H_2Q] \ll [H^+]$. For $[H^+] = 1 \times 10^{-7}$ *M*, a typical hydroquinone concentration (1×10^{-3} *M*), and using $K_{H_2Q} \cong 1 \times 10^{-10}$ *M*, K_s would have to be *ca.* 1×10^6 M^{-1} for the terms $[H^+]$ and $K_s K_{H_2Q}[H_2Q]$ to be comparable in value. For a K_s value even as large as 10^3–10^4 *M*, the $[H_2Q]$ term in the denominator of the rate expressions may be neglected compared with $[H^+]$, and second-order kinetics will be found. Thus it is entirely possible that rate saturation in the hydroquinone–laccase system is not observed up to $[H_2Q] = 0.01$ *M* simply because the concentration of HQ^- in solution is much smaller than the total reducing agent concentration.

There is no information available on the ligand environment of the laccase type 2 copper atom, but Rotilio and coworkers[56] have pointed out the possible structural and functional correspondence between this site and the copper atoms in superoxide dismutase. The evidence suggests the presence of three to four nitrogen donor ligands as well as a water molecule in the first coordination sphere of bovine superoxide dismutase.[57] Analogous to our proposal for laccase, the fluoride and azide binding position in superoxide dismutase is suspected to be the site of attachment of the substrate.[56]

Figure 15 gives our proposed laccase reduction mechanism in schematic form, emphasizing characteristics that may distinguish the activated complexes for electron transfer to the type 1 and type 3 sites. Considering the pH 6.1 azide inhibition results, we have described the type 1 copper reduction pathway for the native enzyme in terms of a substrate-induced conformational transition which is assisted by coordination of HQ^- to type 2 Cu(II) but does not depend on the reduction of this unit. A protein conformational change coupled to movement of the type 2 copper coordination geometry away from axial could position the coordinated reducing agent in a spatial configuration allowing direct attack at the "blue" copper atom. Substrate-induced protein structural changes are an integral part of the induced fit theory of enzyme action initially proposed by Koshland,[58] and crystallographic, chemical, and kinetic evidence has been found for these transitions in a number of instances.[59,60] Our results for the anation of laccase type 2 copper by azide ion are not definitive for the mechanism of anion binding but do indicate that this process is considerably slower and more complicated than would be expected on the basis of the well-known high substitutional lability of simple Cu(II) complexes.

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Reduction of *Rhus* laccase by hydroquinone evidently takes place by a mechanism somewhat different from that preferred by the fungal enzyme. Type 1 Cu(II) in fungal laccase A oxidizes H_2Q much more rapidly than does its *Rhus* counterpart ($k = 7.7 \times 10^6\ M^{-1}$ sec^{-1}, 25°, pH 5.4 (acetate buffer)),[7] and the reduction rate of the fungal laccase B 330-nm chromophore is independent of the substrate concentration at high concentrations ($k(330) = 1.0\ sec^{-1}$ at 25°, pH 5.5).[17] At least one of the electrons necessary for reduction of the type 3 site is thought to be transferred intramolecularly from type 1 Cu(I).[17] Formation of the type 2 Cu(II)–F^- complex does not affect the "blue" copper reduction rate in either chromatographic form of fungal laccase,[7,17] but the rate of reduction of the 330-nm chromophore is slowed by about two orders of magnitude.[17] Fungal laccase "blue" copper apparently is susceptible to direct attack by hydroquinone; conversely, we have argued that the reactivity of the tree laccase type 1 copper atom is controlled largely by an interaction involving type 2 Cu(II). That the fungal "blue" copper atom is well suited to be the initial electron acceptor is indicated by its extraordinarily high reduction potential (ϵ_0 +785 mV, pH 5.5, μ 0.2).[61]

The mechanism suggested to account for cupric and cupric poly-L-histidine complex catalysis of the reduction of O_2 to H_2O_2 by hydroquinone[62] is similar to that proposed here for H_2Q reduction of the laccase type 3 copper site in several respects. For cupric ion the proposed mechanism is

$$Cu^{2+} + H_2Q \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Cu(HQ)^+ + H^+$$

$$Cu(HQ)^+ \xrightarrow{k_2} Cu^+ + SQ$$

$$Cu^+ + O_2 \xrightarrow{\text{fast}} Cu^{2+} + O_2^-$$

and an analogous scheme has been outlined for the polyhistidine complex.[62] The existence of a catalyst–substrate complex is clearly indicated in the case of polyhistidine–Cu^{2+} catalysis, as Michaelis–Menten type substrate saturation was observed.

Azide binding at the type 3 copper site has been suggested to account for spectroscopic observations on laccase solutions containing low concentrations of azide.[46] Our pH 7.0 fluoride and azide inhibition results are compatible with this hypothesis. Azide ion dramatically affects the reactivity of the type 3 site under conditions where the extent of formation of the species absorbing at 405 nm is minimal and activation requirements for reduction of the type 1 site are essentially unchanged from those for the native enzyme. Considering our proposed mechanism for reduction of native laccase, the latter observation suggests that type 2 Cu(II)-anion binding does not take place for low anion concentrations at pH 7.0. Further studies of laccase anation reactions clearly are required to clarify the vastly different anion inhibition patterns observed at pH 6 and 7.

Inhibition of electron transfer to the type 3 site at high pH follows the same pattern as rate effects induced by low concentrations of F^- and N_3^- at pH 7. A laccase species reacting by a hydroquinone-independent type 3 reduction pathway is present at pH 7.8, but observations at 614 nm indicate that its "blue" copper reduction rate must not be very different from that of the native enzyme. The high pH form of laccase may be the hydroxide analog of the fluoride and azide complexes formed at pH 7.0 when the concentrations of the inhibitory anions are low. It is also possible, however, that hydroxide-induced changes in ligands to laccase copper atoms are responsible for the inhibition of type 3 reduction at high pH. The presence of this species in appreciable concentrations above pH 7.5 perhaps explains why *Rhus* laccase activity reaches a maximum at this pH value and declines appreciably in more basic solutions.[38]

At least two mechanistic possibilities may be considered for reduction of the type 3 azide, fluoride, and high pH complexes by hydroquinone. The zeroth-order reducing agent dependencies observed at 330 nm are compatible with a rate-determining intramolecular electron-transfer step, perhaps type 2 Cu(I) to type 3 Cu(II)–Cu(II). Anion binding at the type 3 site might perturb the protein structure enough to considerably change the characteristics of an intramolecular electron transfer step in laccase which ordinarily is fast. Another possibility is that the zeroth-order dependencies on $[H_2Q]$ reflect rate-determining displacement of the anions from type 3 coordination, permitting direct attack at the 330-nm chromophore by hydroquinone.

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Supplementary Material Available. Complete tabulation of k_{obsd} values will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number JACS-74-6008.

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