We feel that the set of orbital selection rules for radiationless transitions which appear to operate in this complex are only a rough guideline to help indicate which radiationless processes are more and less favorable. For any two given states, vibrational factors as well as factors based upon the orbital nature of the levels will be important in determining the rate of energy transfer between them. It may very well be that in this particular complex, both the orbital and vibrational factors are unfavorable for fast radiationless transitions. In other complexes, it may happen that the vibrational factors are large enough to cause rapid radiationless transitions even though the orbital parentage of the levels may be unfavorable for these processes. Since the vibrational factors which govern radiationless processes are dependent upon the energy gaps between the levels, it may be that the energy gaps between levels of different orbital parentage are particularly unfavorable in $[IrCl_2(phen)(5,6\text{-mephen})]Cl$. We will explore the implications of our proposed selection rules for radiationless transitions in a later publication. At that time we will also report experimental studies of energy transfer in other hetereobischelated complexes of Ir(III).[13]

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Kinetics of the Reduction of *Rhus vernicifera* Laccase by Ferrocyanide Ion

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Abstract: Kinetic studies of the reduction of *Rhus vernicifera* laccase by ferrocyanide ion are reported, and the results are compared with previous findings for hydroquinone as reductant. Observed rate constants for reduction of the laccase "blue" and ESR nondetectable copper sites are identical to within experimental error, and vary with $[Fe(CN)_6^{4-}]$ in a complicated fashion. Relaxation rate data are reported for runs in which laccase was mixed with solutions containing comparable concentrations of ferro- and ferricyanide ions. These results establish that a reversible one-electron transfer from $Fe(CN)_6^{4-}$ to the "blue" copper atom occurs: $Fe(CN)_6^{4-}$ + type 1 Cu(II) $\rightleftharpoons$ $Fe(CN)_6^{3-}$ + type 1 Cu(I) ($k_1 = 24.9\ M^{-1}\ sec^{-1}$, $k_{-1} = 26.8\ M^{-1}\ sec^{-1}$; pH 6.9, $\mu = 0.2$, 25.1°). Comparisons between aerobic ferrocyanide turnover rates and laccase reduction rate constants reveal that four electrons are released to oxygen at a rate proportional to the velocity of the initial laccase reduction step. Fluoride ion fails to inhibit the reaction between ferrocyanide ion and laccase at pH 7.0. The pH dependence results show that a 23-fold laccase reduction rate decrease accompanies ionization of one or more amino acid residues with $pK = 6.41$. It is suggested that the involved residue is histidine, and that ion-pair formation between the highly charged reductant and imidazolium cations may be an integral part of the "blue" copper reduction mechanism. Ionic strength dependence results are in accord with the latter hypothesis.

Laccases are versatile copper-containing enzymes that catalyze the oxidation of a wide variety of phenolic substrates by oxygen, producing quinones, phenol coupling products, and water as the sole oxygen reduction product.[1] Spectroscopic studies of fungal and lacquer tree laccases have revealed that both enzymes possess four tightly bound copper atoms incorporated into "blue" (type 1), ESR detectable, optically nondetectable (type 2), and ESR nondetectable (type 3, 2 Cu/mol) sites.[2] We have undertaken kinetic studies of the reduction and oxidation of *Rhus vernicifera* laccase as part of a program aimed at elucidating biochemical mechanisms for the fixation and reduction of molecular oxygen. Our previously reported stopped flow study of the anaerobic reduction of laccase by hydroquinone[3] suggested that inner-sphere complexes between the phenolate monoanion (HQ^-) and type 2 Cu(II) are intermediates in closely related electron transfer pathways to the type 1 and type 3 copper sites. It was further 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. For comparison with the hydroquinone reduction results, we report here our kinetic observations for the reaction between laccase and a one-electron inorganic reductant, ferrocyanide ion, which typically participates in outer-sphere electron transfer reactions.

Experimental Section

Materials. Reagent grade chemicals were used without further purification, and triply distilled water was used in preparing solutions for kinetic measurements. Nitrogen gas was passed through two chromous scrubbing towers to remove oxidizing impurities. Alfa Inorganics crude $K_3Cr(CN)_6$ was suspended in water, filtered to remove a large amount of green insoluble material, recrystallized from water-ethanol, washed with ethanol, and dried under vacuum. For the recrystallized material: % Cr (calcd) = 15.98; % Cr (found) = 15.34. Sigma Grade III α-D(+)-glucose and Type II glucose oxidase (from *Aspergillus Niger*) were used as supplied. Laccase was extracted from lacquer acetone powder (Saito and Co., Ltd., Tokyo) by the method of Reinhammar.[4] Protein samples with A_{280}/A_{614} ratios of 15.2 to 15.6 were considered acceptably pure for use in kinetics experiments.

The purity of commercial $K_4Fe(CN)_6 \cdot 3H_2O$ was verified by ceric titration using ferroin as the indicator. Laccase concentrations were determined from spectrophotometric analyses for total copper by the method of Broman et al.,[5] assuming 4 Cu/mol. The chromium content of recrystallized $K_3Cr(CN)_6$ was evaluated by oxidizing samples to CrO_4^{2-} with H_2O_2 in basic solution and reading the absorbance at 373 nm ($\epsilon_{373} = 4815\ M^{-1}\ cm^{-1}$).[6]

Measurements. The preparation and handling of anaerobic reducing agent and laccase solutions for kinetics measurements have already been described.[3] Rate measurements were performed using a modified[3] Durrum D-110 stopped flow spectrophotometer, and absorbance-time curves were recorded using a Hewlett-Packard Model 7004 B X-Y recorder or a Tektronix 564 B oscilloscope. Pseudo-first-order conditions for the metalloprotein were used in all kinetic experiments, 100- to 1500-fold excesses of reducing agent typically being present. Laccase concentrations were ca. $1 \times 10^{-5}\ M$, yielding about a 0.1 absorbance change at 614 nm in the 2 cm stopped flow observation chamber.

Kinetics runs were performed at 614, 330, and 420 nm, where reduction of the type 1 and type 3 copper sites and production of $Fe(CN)_6^{3-}$, respectively, may be observed. The difference spectrum (oxidized minus reduced) for the ferri-ferrocyanide system indicates that ($\epsilon_{111} - \epsilon_{11}$) is essentially zero at 614 nm but rises to approximately 500 $M^{-1}\ cm^{-1}$ at 330 nm.[7] Thus kinetic observations at 614 nm reflect changes in the oxidation state of the "blue" site alone; the overall 330 nm absorbance change, however, includes a substantial increase originating from ferricyanide produced in the reduction of type 1 copper in addition to the decrease associated with reduction of the laccase type 3 site ($\Delta\epsilon_{333} = 2800\ M^{-1}\ cm^{-1}$).[8] The 330 nm kinetic results have been interpreted with this complication in mind.

An initial steady state period is observed when traces of oxygen persist in solutions of laccase mixed with ferrocyanide ion. First-order analytical plots of $\log|A_t - A_\infty|$ vs. time based on post steady-state decay curves at 614, 330, and 420 nm were found to be linear for approximately the last 50% of the total absorbance change at all three wavelengths. Observed first-order rate constants (k_{obsd}) were obtained routinely by performing least-squares analyses on the linear regions of $\log|A_t - A_\infty|$ vs. time plots. With hydroquinone as reductant, several control experiments demonstrated that observed rate constants obtained from post steady-state decay curves are equivalent to those that would be obtained under strictly anaerobic conditions.[3] This conclusion was confirmed for ferrocyanide as reductant in an experiment similar to that reported previously[3] employing the glucose, glucose oxidase, oxygen scavenging system. Room temperature k_{obsd} (330, 614) values for $[Fe(CN)_6^{4-}] = 2.5 \times 10^{-3}\ M$, pH 7.0, $\mu = 0.1$, were evaluated from absorbance-time traces in which the steady-state period was quenched. These were found to be indistinguishable (to within ±5%) from observed rate constants we report (vide infra) based on post steady-state decay curves.

Visible and ultraviolet spectra were recorded on Cary 14 and Cary 17 spectrophotometers. Corning Model 12 and Brinkman pH 101 meters were used to make pH measurements.

Results

I. Stoichiometry. The stoichiometry of the reaction between ferrocyanide ion and laccase was investigated under anaerobic conditions at pH 7.0 by means of the 420 nm $Fe(CN)_6^{3-}$ product absorption. Ferrocyanide ($5.0 \times 10^{-3}\ M$) and laccase ($1.69 \times 10^{-5}\ M$) solutions in 0.0463 M phosphate buffer were made rigorously anaerobic following normal deoxygenation procedures by the addition of traces of glucose and glucose oxidase. They were then mixed in the stopped flow apparatus, and absorbance changes at 420 and 614 nm were recorded. The concentration of reduced type 1 copper was obtained from $\Delta A_{614} = 4.5 \times 10^3\ M^{-1}\ cm^{-1}$, $\epsilon_{614}(ox) = 4.7 \times 10^3\ M^{-1}\ cm^{-1}$ (pH 7.0, 0.0463 M phosphate buffer). Ferricyanide production was evaluated using the value $1.01 \times 10^3\ M^{-1}\ cm^{-1}$ as $\Delta\epsilon_{420}$ for the ferri-ferrocyanide system. Using the $\Delta\epsilon_{420}$ value for fully oxidized minus fully reduced laccase,[9] a value of 3.5 ± 0.2 is obtained for the ratio of moles of ferricyanide produced per mole of "blue" copper reduced. Considering the not insignificant difference between our ϵ_{614} value and those reported by other workers,[8,9] this ratio may in fact be as high as 4.3. On the basis of absorbance changes alone, however, it is not possible to rule out a three-electron reduction, as the $\Delta\epsilon_{420}$ for three-electron reduced laccase could be quite different from the number reported for the fully reduced enzyme. To emphasize this point, if we neglect protein contributions to $\Delta\epsilon_{420}$, a value of 2.9 may be obtained as a lower limit for the ratio of moles of $Fe(CN)_6^{3-}$ generated per mole of reduced type 1 copper. The stoichiometric question is perhaps best settled by our spectrophotometric measurements, which indicate that complete reduction of the type 1 and type 3 sites takes place, coupled with Reinhammer's observation that the type 2 Cu(II) ESR signal disappears when *Rhus* laccase is mixed anaerobically with a tenfold excess of $Fe(CN)_6^{4-}$.[10] Considering all the available evidence, then, it would appear that $Fe(CN)_6^{4-}$ reduces the enzyme by four electrons under anaerobic conditions.

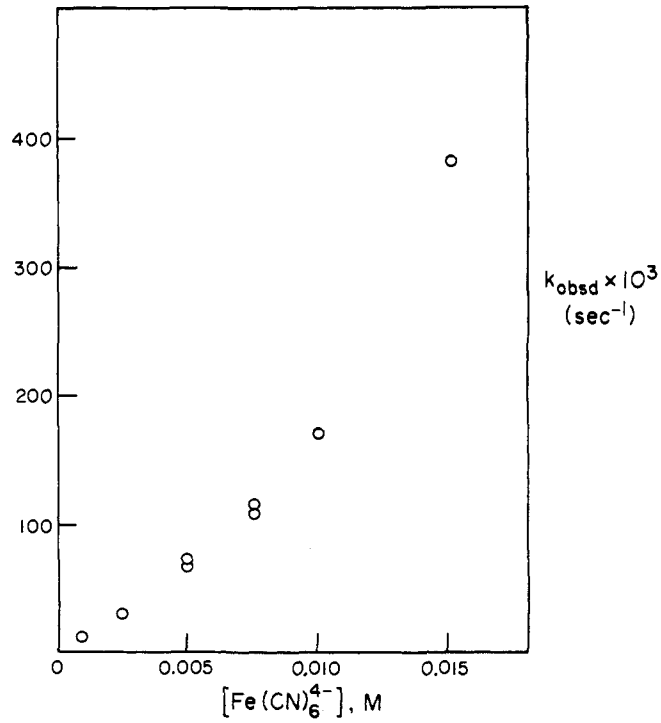

Figure 1. Plot of k_{obsd} vs. $[Fe(CN)_6^{4-}]$ for the reaction of ferrocyanide with laccase: 614 nm, 24.9°, pH 6.9, $\mu = 0.5$.

II. Ferrocyanide Concentration Dependence. Observed rate constants for reduction of laccase "blue" copper were obtained at 24.9°, $\mu = 0.5$, for six ferrocyanide concentrations in the range $1.0 \times 10^{-3} \leq [Fe(CN)_6^{4-}] \leq 1.5 \times 10^{-2}\ M$. All solutions were prepared with 0.133 M sodium phosphate buffer, pH 6.9, and sodium nitrate was used to maintain the ionic strength. The kinetic results are illustrated in Figure 1.[11] The variation of $k_{obsd}(614)$ with $[Fe(CN)_6^{4-}]$

Table I. Rate Data for the Reduction of Laccase by Ferrocyanide Ion (pH 7.0, μ = 0.1)

	k_{obsd}, sec^{-1} (330 nm)		
$[Fe(CN)_6^{4-}] \times 10^3$, M	12.8°	25.3°	36.0°
1.5	0.010	0.039	
	0.010	0.042	
2.0		0.058	0.222
		0.063	0.194
			0.216
2.5	0.018	0.081	
	0.019	0.081	
3.0		0.103	0.332
			0.332
4.0	0.037	0.147	0.463
	0.037	0.147	0.483
	k_{obsd}, sec^{-1} (614 nm)		
	12.8°	25.7°	35.3°
1.5		0.048	
		0.047	
2.0	0.016	0.067	0.188
	0.015	0.070	0.180
2.5		0.086	0.258
		0.085	0.262
3.0	0.027	0.109	0.298
			0.310
4.0	0.037	0.155	
	0.036		
	k_{obsd}, sec^{-1} (420 nm)		
		25.7°	
1.5		0.048	
		0.048	
2.0		0.081	
		0.082	
		0.080	
2.5		0.107	
		0.103	
3.0		0.132	
		0.130	
		0.130	
4.0		0.169	
		0.170	

clearly is complex. An attempt was made to fit the data to a rate law containing terms both first and second order in the reducing agent concentration, but this was not successful.

A comparison of 614, 330, and 420 nm rate parameters for the reduction of laccase by ferrocyanide ion was made under the same conditions (pH 7.0, ionic strength 0.1) as in our earlier kinetic study.[3] All solutions for these runs were prepared with 0.016 M phosphate buffer, and sodium nitrate again was used to maintain the ionic strength. The pH 7.0 and ionic strength 0.1 kinetic results are set out in Table I. Observed rate constants based on 614 and 330 nm absorbance-time curves do not follow a simple first-order ferrocyanide concentration dependence over the interval $1.6 \times 10^{-3} \leq [Fe(CN)_6^{4-}] \leq 4.0 \times 10^{-3}$ M, as least squares lines through the data points have smaller than zero intercepts. From Table I it is evident that 330 and 614 nm rate parameters at a given temperature are the same within experimental error over the range 13 to 36°. These parameters vary strongly with temperature. At 25°, reduction of the laccase type 1 and type 3 sites by $Fe(CN)_6^{4-}$ is not particularly fast ($k_{obsd}(330, 614) = 0.08 \pm 0.01$ sec^{-1} for $[Fe(CN)_6^{4-}] = 2.5 \times 10^{-3}$ M). That linear first-order analytical plots are obtained from 330 nm absorbance-time traces is itself an indication that the reduction rates of the type 1 and type 3 sites are identical. Recalling the several contributions to 330 nm absorbance changes, it is clear that nonlinear analytical plots would have been found were the reduction rates

Table II. Turnover Rates for the Laccase-Catalyzed Oxidation of $Fe(CN)_6^{4-}$ by O_2 (420 nm, 24.9°, pH 6.9, μ = 0.5)[a]

$[Fe(CN)_6^{4-}] \times 10^3$, M	Turnover rate $\times 10^6$, M sec^{-1}	$k_{obsd}(420, O_2)$ sec^{-1}	$k_{obsd}(420, O_2)/k_{obsd}(614)$
2.5	1.15	0.138	4.6
	1.17	0.140	4.7
5.0	2.44	0.292	4.1
	2.45	0.293	4.1
7.5	3.90	0.466	4.1
	3.96	0.474	4.2
10.0	5.85	0.700	4.1
	5.66	0.677	4.0
15.0	11.81	1.413	3.6
	12.36	1.480	3.8
	11.64	1.393	3.7

[a] $[laccase]_{tot} = 8.36 \times 10^{-6}$ M.

of the two optically observable copper sites very different in value.

If the same slow step governs electron transfer to all the enzymatic copper sites reduced by $Fe(CN)_6^{4-}$, then it is expected that observed rate constants for $Fe(CN)_6^{3-}$ production should agree with those for reduction of the type 1 and type 3 copper sites. Inspection of Table I shows reasonable agreement among k_{obsd}(420, 614, 330) values evaluated under nearly identical conditions.

III. Ferrocyanide Turnover Rates in the Presence of Oxygen (pH 6.9, μ = 0.5, 24.9°). Solutions of ferrocyanide and laccase left open to the air rapidly turn bright yellow, and spectrophotometric observations at 420 nm show continuing increases in absorbance. The solutions prepared for the ionic strength 0.5 concentration dependence study were also used for measurements of aerobic ferrocyanide turnover rates at 420 nm. Oxygen was not excluded in these runs, and turnover rates were evaluated from the slopes of A_{420} vs. time traces shortly after mixing. These traces were linear initially before oxygen consumption was significant, indicating a constant rate of ferricyanide generation.

Table II lists turnover rates, values of the observed turnover constant $k_{obsd}(420, O_2)$ = (turnover rate)/$[laccase]_{tot}$, and compares $k_{obsd}(420, O_2)$ with average $k_{obsd}(614)$ values found under the same conditions. For each of the five ferrocyanide concentrations considered, the ratio $k_{obsd}(420, O_2)/k_{obsd}(614)$ is very nearly equal to four (average value 4.1 ± 0.2). The value of this ratio does, however, follow a slight increasing trend as $[Fe(CN)_6^{4-}]$ decreases from 1.5×10^{-2} to 2.5×10^{-3} M.

IV. Ionic Strength Dependence. A series of pH 7.0 sodium phosphate buffers was used in a study of the ionic strength dependence of 330 and 614 nm observed rate constants for the reaction of laccase with ferrocyanide ion. Phosphate buffer and potassium ferrocyanide alone contributed to the ionic strength, and $[Fe(CN)_6^{4-}]$ was 2.5×10^{-3} M throughout. Before mixing, laccase was maintained in ionic strength 0.05 buffer.

Figure 2 gives a plot of $k_{obsd}(330, 614)$ against ionic strength. The rate data for the two wavelengths are indistinguishable to within experimental error for five different μ values, and the laccase reduction rate drops by 56% as the ionic strength increases from 0.075 to 0.536.

V. pH Dependence. The effect of $[H^+]$ on the rate of laccase reduction by ferrocyanide ion was evaluated at 614 nm using ionic strength 0.3 sodium phosphate buffers in the range pH 5–8. A constant ferrocyanide ion concentration of 0.02 M brought the total ionic strength up to 0.5 in each case. Separate protein solutions were made up in the appropriate buffer for each pH considered.

A plot of $k_{obsd}(614)$ vs. pH (Figure 3) strongly resembles a titration curve, suggesting that ionization of a particular

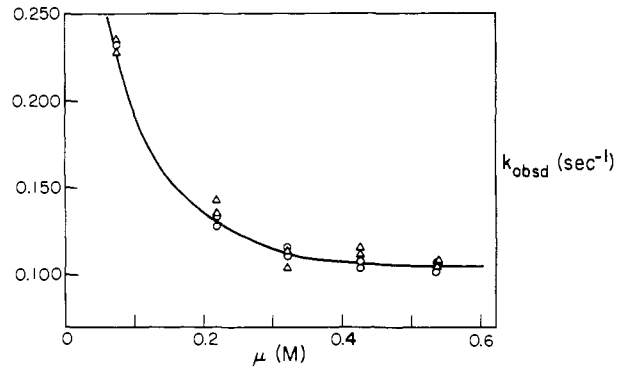

Figure 2. Ionic strength dependence of observed rate constants for the reduction of laccase by ferrocyanide; 25.5°, pH 7.0, $[Fe(CN)_6^{4-}] = 2.5 \times 10^{-3}$ *M*; O, 614 nm; Δ, 330 nm.

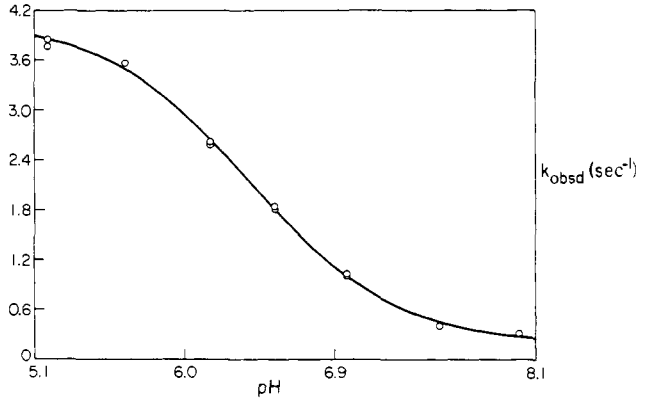

Figure 3. Plot of k_{obsd} vs. pH for the reaction of ferrocyanide with laccase: 614 nm, 24.9°, $\mu = 0.5$, $[Fe(CN)_6^{4-}] = 0.02$ *M*.

amino acid side chain is responsible for the observed decreasing trend in laccase reduction rate with increasing pH. Assuming this to be the case, the following scheme was used to treat the data quantitatively.

$$Fe(CN)_6^{4-} + \text{laccase} \xrightarrow{k_u} \text{products}$$

$$Fe(CN)_6^{4-} + \text{laccase}\cdot H^+ \xrightarrow{k_p} \text{products}$$

$$\text{laccase}\cdot H^+ \overset{K_H}{\rightleftharpoons} \text{laccase} + H^+$$

The species "laccase" and "laccase·H^+" represent unprotonated and protonated forms of the metalloprotein, respectively, with k_u and k_p the associated reduction rate constants. The equilibrium between unprotonated and protonated forms, governed by the acid ionization constant K_H, is assumed to be rapid.

The rate law derived for decolorization of laccase "blue" copper is:

$$\frac{-d[Cu(614)]_{tot}}{dt} =$$

$$[Fe(CN)_6^{4-}](k_u[\text{laccase}] + k_p[\text{laccase}\cdot H^+]) =$$

$$\left(\frac{k_p[H^+] + k_uK_H}{[H^+] + K_H}\right)[Fe(CN)_6^{4-}][Cu(614)]_{tot} =$$

$$k_{obsd}(614)[Cu(614)]_{tot}$$

The experimental data were fit, in a least squares sense, to the equation:

$$k_{obsd}(614) = \left(\frac{k_p'[H^+] + k_u'K_H}{[H^+] + K_H}\right)$$

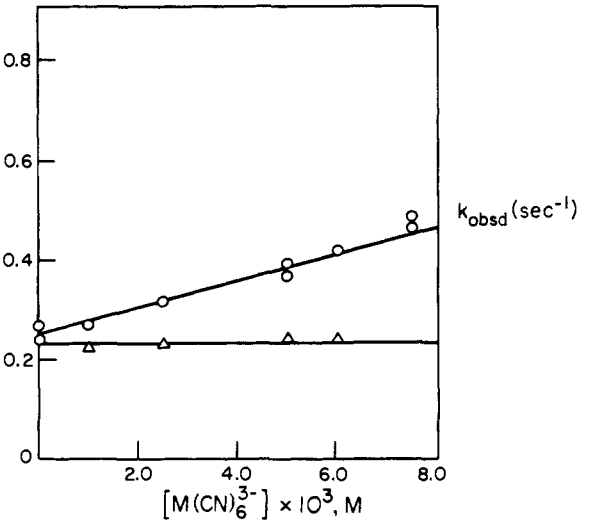

Figure 4. Effect of hexacyanometalate ions on k_{obsd} for the reaction of ferrocyanide with laccase: 614 nm, 25.3°, pH 7.0, $\mu = 0.2$, $[Fe(CN)_6^{4-}] = 0.01$ *M*; O, $Fe(CN)_6^{3-}$; Δ, $Cr(CN)_6^{3-}$.

where k_p' and k_u' are $[Fe(CN)_6^{4-}]$-dependent rate constants associated, respectively, with reduction of the protonated and unprotonated forms of the metalloenzyme.[12]

The parameters of the best fit and their standard errors are:

$$k_u' = 0.175 \pm 0.032 \text{ sec}^{-1}$$

$$k_p' = 4.09 \pm 0.04 \text{ sec}^{-1}$$

$$pK_H = 6.41 \pm 0.01$$

VI. Effect of Ferricyanide. The standard reduction potentials of laccase copper sites[10] are so close to that of the ferri-ferrocyanide couple[13] that it ought to be possible to reoxidize reduced laccase with $Fe(CN)_6^{3-}$ under appropriate concentration conditions. Solutions containing comparable concentrations of ferro- and ferricyanide were mixed with oxidized laccase in experiments described in this section, and relaxation to redox equilibrium was followed at 614 nm. All solutions were prepared with 0.0185 *M* phosphate buffer, pH 6.9, and the total ionic strength was adjusted to 0.2 with $NaNO_3$.

Holding $[Fe(CN)_6^{4-}]$ constant at 0.01 *M*, the extent of 614 nm absorbance changes was found to decrease markedly with increasing $[Fe(CN)_6^{3-}]$, consistent with the reaction between $Fe(CN)_6^{4-}$ and laccase coming to equilibrium short of full reduction of available "blue" copper sites. Decay of 614 nm absorbance to its equilibrium value (A_{eq}) is first order, as plots of log ($A_t - A_{eq}$) vs. time were found to be linear. Relaxation rate constants based on these plots are plotted against ferricyanide concentration in Figure 4.[11] As a check that any rate effects induced by $Fe(CN)_6^{3-}$ are related to redox processes, some control runs were performed keeping the conditions constant but substituting the redox-inert chromicyanide ion for ferricyanide. The results of these runs are also presented in Figure 4.

For $[Fe(CN)_6^{4-}] = 0.01$ *M*, observed relaxation rate constants increase linearly with $[Fe(CN)_6^{3-}]$ over the interval $1.0 \times 10^{-3} \leq [Fe(CN)_6^{3-}] \leq 7.5 \times 10^{-3}$ *M*. Full reduction of laccase 614 nm absorbance is still achieved in the presence of added $K_3Cr(CN)_6$, and no variation in k_{obsd} with $[Cr(CN)_6^{3-}]$ was found over the same concentration range used in the ferricyanide experiments. It seems safe to conclude, then, that increases in k_{obsd} with added $Fe(CN)_6^{3-}$ are redox related and not attributable to some

other interaction between laccase and hexacyanometalate(III) ions.

The data may be understood in terms of a one-step mechanism for the approach of $Fe(CN)_6^{4-}$ and type 1 Cu(II) to redox equilibrium.

$$Fe(CN)_6^{4-} + \text{type 1 Cu(II)} \rightleftharpoons Fe(CN)_6^{3-} + \text{type 1 Cu(I)}$$

$$\frac{-d[Cu(614)]}{dt} = (k_1[Fe(CN)_6^{4-}] + k_{-1}[Fe(CN)_6^{3-}])([Cu(614)]_t - [Cu(614]_{eq}) = k_{obsd}(614)([Cu(614)]_t - [Cu(614)]_{eq})$$

The slope of the ferricyanide dependence plot is thus assigned to k_{-1} and the intercept to $k_1[Fe(CN)_6^{4-}]$ (k_1 = 24.9 M^{-1} sec^{-1}, k_{-1} = 26.8 M^{-1} sec^{-1}, pH 6.9, μ = 0.2, 25.1°). Using the relationship $K_{eq} = k_1/k_{-1}$ the equilibrium constant for the reaction between $Fe(CN)_6^{4-}$ and type 1 Cu(II) is calculated to be 0.93. Knowing K_{eq} and the standard reduction potential for the ferri-ferrocyanide couple under the present experimental conditions (E^0 = 0.42 V, 25°, μ = 0.2, sodium phosphate media),[13] the E^0 value for laccase type 1 copper may be estimated at 0.42 ± 0.02 V. This value is in reasonable agreement with that obtained via potentiometric titration under conditions similar to ours (E^0 = 0.432 V, pH 6.8, μ = 0.35, [hexacyanoferrate]/[laccase] = 100).[14]

Considering the results given in section II, the intercept of the k_{obsd} vs. $[Fe(CN)_6^{3-}]$ plot cannot be unambiguously equated with $k_1[Fe(CN)_6^{4-}]$, as this assumes a simple first-order reducing agent dependence. Nevertheless, the agreement between the K_{eq} value predicted from well-established reduction potentials and that obtained from our analysis of the kinetic results strongly suggests that the given k_1 value is in fact a reasonable estimate of a second-order rate constant for the $Fe(CN)_6^{4-}$ reduction of type 1 Cu(II). In view of the complicated variation of k_{obsd} values with $[Fe(CN)_6^{4-}]$, our k_1 value is considered to be of quantitative value only insofar as it establishes a reference point for reactivity comparisons.

VII. Effect of Fluoride Ion. The effect of 0.02 M NaF on the rate of reaction between $Fe(CN)_6^{4-}$ and laccase was investigated under the conditions described in section II. No significant perturbation in 330 and 614 nm observed rate constants was found for several values of $[Fe(CN)_6^{4-}]$ at pH 7.0, μ = 0.1.

Discussion

The kinetic results for the reaction of *Rhus* laccase with ferrocyanide ion are complicated in a number of ways. In contrast to findings for hydroquinone as reductant,[3] the variation of k_{obsd}(330, 614) values with the ferrocyanide concentration deviates from first-order behavior at both low and high concentrations. Indeed, the data do not justify the firm assignment of any part of the k_{obsd} vs. $[Fe(CN)_6^{4-}]$ profile as corresponding to a first-order $[Fe(CN)_6^{4-}]$ dependence. The kinetic results for high reducing agent concentrations do not support the formulation of authentic terms in the rate law higher than first order in the hexacyanoferrate(II) concentration; re-expressing the data given in Figure 1 as a plot of $k_{obsd}(614)/[Fe(CN)_6^{4-}]$ vs. $[Fe(CN)_6^{4-}]$ still gives a nonlinear graph. Rather, it is suspected that the anomalous, high-concentration rates reflect, in part, effective decreases in the ionic strength accompanying substitution of a 1:4 electrolyte ($K_4Fe(CN)_6$) for a 1:1 electrolyte ($NaNO_3$) at nominally constant ionic strength.

Hydroquinone reduces the laccase "blue" and ESR nondetectable copper sites in parallel at comparable rates over a wide range of conditions, but anion inhibition experiments clearly indicate that the pathways to reduction of the two sites must involve at least slightly different activated complexes.[3] In contrast, comparison of 330 and 614 nm observed rate constants for the reaction of laccase with ferrocyanide ion reveals that the electron transfer pathways to the type 1 and type 3 sites now share a common rate-determining step. Pecht found a single chemical relaxation at the fungal laccase 610 nm chromophore in temperature jump studies of the equilibria between the enzyme and the redox couples $Mo(CN)_8^{4-/3-}$ and $Ru(CN)_6^{4-/3-}$.[15] Similarly, we find that the approach to equilibrium between *Rhus* laccase type 1 copper and the $Fe(CN)_6^{4-/3-}$ couple may be described in terms of a single electron transfer pathway. As Pecht has pointed out,[15] these observations allow the important conclusion that sequential electron transfer from external reductant to type 1 copper to type 3 copper does not take place.

The role of type 2 Cu(II) in the reduction of laccase by $Fe(CN)_6^{4-}$ is not easily defined. As the optically nondetectable copper atom is known to bind anions,[2] it seems distinctly possible that outer-sphere complex formation between enzymatic Cu(II) and the highly charged reducing agent may occur. Interaction between $Fe(CN)_6^{4-}$ and type 2 Cu(II) is evident not only from changes in the copper(II) ESR signal induced by the reducing agent, but also from marked variations in the E^0 values of the type 1 and type 3 sites with the concentration of hexacyanoferrate added as an electron mediator.[10] Complex formation between laccase and $Fe(CN)_6^{4-}$ may in fact be partially responsible for the dramatic upswing in k_{obsd} values at high ferrocyanide concentrations, but there is as yet no additional experimental evidence in support of this hypothesis.

Low concentrations of fluoride ion strongly inhibit reduction of the type 3 site by hydroquinone at pH 7.0,[3] but no inhibition is apparent with ferrocyanide as reductant. Specific interaction of F^- with the 330 nm chromophore has been suggested to account for the hydroquinone result; one possible explanation is that the rate of a type 2 Cu(I) to type 3 Cu(II)-Cu(II) intramolecular electron transfer step is drastically decreased by structural rearrangements accompanying fluoride binding.[3] It is possible, then, that the absence of F^- inhibition reflects nonparticipation of the type 2 copper atom as an electron mediator in the reduction mechanism of the ESR nondetectable site by $Fe(CN)_6^{4-}$. Competitive inhibition of F^- binding by $Fe(CN)_6^{4-}$, however, may equally well account for this result.

The pH dependence results[16] show that a 23-fold "blue" copper reduction rate decrease accompanies ionization of a group (or groups) with pK = 6.41. In contrast, the rates of reduction of the type 1 and type 3 sites by hydroquinone increase sharply with increasing pH, and ionization of the substrate appears to be the dominant influence.[3] Acid-base titrations of a number of proteins have indicated that the histidine imidazole group typically ionizes with $6.4 \leq pK \leq 6.9$, and no other side chain is expected to have a pK value falling between 6 and 7.[17] *Rhus* laccase contains about 17 histidine residues per mole.[4]

The strong influence of the ionization state of histidine on the rate of reaction between laccase and ferrocyanide ion suggests that ion-pair formation between the highly charged reductant and at least one imidazolium cation plays an important part in the "blue" copper reduction mechanism. The observed marked decrease in rate with increasing ionic strength supports this hypothesis. Bennett has distinguished between normal outer-sphere electron transfer and outer-sphere reactions involving pre-equilibrium ion-pair formation in discussing the mechanistic alternatives for reactions between metalloproteins and inorganic

redox agents.[18] Rate-determining electron transfer within an ion-pair intermediate has been documented for the reaction between $Fe(CN)_6^{4-}$ and $Co(NH_3)_5OH_2^{3+}$,[19] and evidence for 1:1 ion-pair formation between ferrocyanide ion and ferricytochrome *c* has been obtained in an elegant NMR study by Stellwagen and Shulman.[20] Rate saturation with increasing reducing agent concentration is expected under circumstances where the ion-pair formation constant is large, but the complicated rate behavior at high $[Fe(CN)_6^{4-}]$ prevents the detection of such an effect in the present case.

Ferrocyanide ion reduces the *Rhus* laccase type 1 and type 3 sites about an order of magnitude more slowly than does hydroquinone [$k_1(330) = 4.57 \times 10^2$, $k_1(614) = 3.25 \times 10^2\ M^{-1}\ sec^{-1}$; 25.6°, pH 7.0, $\mu = 0.1$)][3] in neutral solution. The fungal laccase A and B type 1 copper atoms oxidize $Fe(CN)_6^{4-}$ at specific rates of the order of $10^6\ M^{-1}\ sec^{-1}$,[21,22] and high reduction rates of fungal "blue" copper by $Mo(CN)_8^{4-}$ and $Ru(CN)_6^{4-}$ have also been reported.[15] Thus the present results confirm our previous observation[3] that the reactivity of tree laccase "blue" copper with external reductants is much lower than that of its fungal counterpart. Rate parameters for the reaction of ferrocyanide ion with spinach [$k(20°) = 2.0 \times 10^4\ M^{-1}\ sec^{-1}$, $\Delta H^{\ddagger} = 8.8$ kcal/mol, $\Delta S^{\ddagger} = -9.1$ cal/(mol deg)][23] and bean [$k(25°) = 1.9 \times 10^4\ M^{-1}\ sec^{-1}$, $\Delta H^{\ddagger} = 8.4$ kcal/mol, $\Delta S^{\ddagger} = -10.6$ cal/(mol deg)][23] plastocyanins at $\mu = 0.2$, pH 6.0 (acetate), are much different from those for the $Fe(CN)_6^{4-}$ reduction of *Rhus* laccase, even though the driving forces for reduction of the laccase and plastocyanin type 1 copper atoms are about the same (for spinach plastocyanin: $E^0 = 0.37$ V, $5.4 \leq$ pH ≤ 9.9).[24] Evidently the tree laccase "blue" copper is buried deep within the polypeptide structure, necessitating considerable conformational movement to expose it to attack by external redox agents.

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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 Business Office, Books and Journals Division, American Chemical Society, 1155 16th St., N.W., Washington, D.C. 20036. Remit check or money order for $4.00 for photocopy or $2.50 for microfiche, referring to code number JACS-75-6036.

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