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SUBSTITUENT EFFECTS ON THE ELECTRON TRANSFER REACTIVITY OF HYDROQUINONES WITH LACCASE BLUE COPPER *

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Summary

Stopped-flow kinetic studies of the anaerobic reduction of *Rhus vernicifera* laccase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) type 1 copper by 25 mono- and disubstituted hydroquinones-(H_2Q-X) have been performed at 25°C and pH 7.0 in 0.5 M phosphate. All of the data are compatible with a mechanism involving rapid enzyme-substrate complex formation followed by rate-limiting intra-complex electron transfer. ES complex formation constants (Q_p) for many substrates are strikingly insensitive to the electronic characteristics of the substituent X, falling within the range 5—50 M^{-1}. It is shown that this result may be accounted for if only the singly ionized forms of the substituted hydroquinones are bound by the enzyme. All of the substrates exhibiting exceptionally high Q_p values (>50 M^{-1}) have X groups capable of functioning as ligands; substituents with lone pairs of electrons may facilitate enzyme-substrate complex formation by enabling hydroquinone to function as a bidentate bridging ligand between the type 2 and type 3 copper sites. Intra-complex electron transfer rate constants for most substrates are remarkably insensitive to the thermodynamic driving force for the oxidation of H_2Q-X to the corresponding semiquinone, the average value for ten substrates being 30 ± 10 s^{-1}. The electron transfer reactivity of polyphenols with laccase blue copper therefore appears to be controlled largely by protein-dependent activation requirements rather than by the oxidizability of the substrate.

* Supplementary data to this article are deposited with, and can be obtained from Elsevier Scientific Publishing Company, BBA Data Deposition, P.O. Box 1345, Amsterdam, The Netherlands. Reference should be made to BBA/DD/100/68742/568(1979)307. The data contain the rate constants for the reduction of laccase blue copper by substituted hydroquinones (Table I).

Introduction

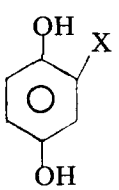
The metalloprotein laccase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) catalyzes the four-electron reduction of molecular oxygen by polyphenols. The activity of this enzyme is linked to the presence of four tightly bound copper atoms, distributed in type 1 (blue, ESR detectable), type 2 (non-blue, ESR detectable), and type 3 (two Cu/molecule, ESR non-detectable) sites [1]. While the mechanism of electron transfer from reduced laccase to O_2 has received considerable attention recently [2–5], comparatively little is known about the factors which facilitate enzyme-substrate complex formation between polyphenols and laccase, and which promote electron transfer within this complex, yielding reduced enzymatic copper sites and a semiquinone.

Several recently published papers have demonstrated that rate constants for the one-electron oxidation of substituted hydroquinones by transition metal ion oxidants vary systematically with the standard free energy change for the redox step. Marcus theory for outer-sphere electron transfer predicts that the excess free energy of activation for a cross-reaction (ΔG^*) should be related to the standard free energy change for the redox step (ΔG°) by the equation [6]:

$$\Delta G^* = w_r + \lambda \left(1 + \frac{\Delta G^\circ}{\lambda}\right)^2 /4 \qquad (1)$$

where λ is a function of the activation free energies for the self-exchange electron transfer reactions of the reactants. The w_r term accounts for the work required to bring the reactants together into the activated complex. This equation successfully correlates ΔG^* and ΔG° values for the reactions of hydroquinone and its derivatives with iron(III) phenanthroline complexes [7], $IrCl_6^{2-}$ [8], Np(VI) [9], Fe(III) [9], $Mo(CN)_8^{3-}$ [10], and the 12-tungstocobaltate(III) ion [11].

Preliminary kinetic results for the reduction of the blue copper site in *Rhus vernicifera* laccase by monosubstituted hydroquinones of the form:



$= H_2Q\text{-}X$

have suggested that factors other than the thermodynamic driving force strongly influence the value of the electron transfer rate constant [12]. Thus, wide variations in enzyme-substrate complex formation constants were reported for seven hydroquinones (X = -H, $-CH_3$, $-C(CH_3)_3$, -OH, -Cl, $-COO^-$, and $-SO_3^-$), and it was noted that the highest binding constants are found for substrates with substituents possessing at least one lone pair of electrons [12]. To better understand the substrate-dependent factors which affect the rate of reduction of laccase blue copper by polyphenols and influence binding of the substrate prior to its oxidation, we have now extended our kinetic studies to

include a family of 25 mono- and disubstituted hydroquinone derivatives. These studies have allowed us to systematically correlate binding constants with pK_a values for the various substrates, and also to determine whether the dependence of intra-complex electron transfer rate constants on thermodynamic driving force is in accord with the predictions of Marcus theory.

Materials and Methods

Bromohydroquinone and 2,5-dichlorohydroquinone were used as supplied by Eastman. Methoxyhydroquinone (Aldrich) was also used without further purification. Trimethylhydroquinone (Aldrich), 2,5-dihydroxybenzaldehyde (Aldrich) and 2,5-dihydroxyphenyl acetic acid (Eastman) were purified by vacuum sublimation.

Fluorohydroquinone and trifluoromethylhydroquinone were prepared by the method of Feiring and Sheppard [13]. The reaction of Cl_2 with hydroquinone in glacial acetic acid was used to prepare 2,3-dichlorohydroquinone [14]. 2,6-Dichloro-*p*-benzoquinone and 2,5-dimethyl-*p*-benzoquinone (Eastman) were reduced with $SnCl_2$ [15] to give the corresponding hydroquinones. The reaction of tri-*n*-butylborane with *p*-benzoquinone in diethyl ether was used to prepare *n*-butylhydroquinone [16]. The methyl ester of 2,5-dihydroxybenzoic acid was obtained by refluxing the parent acid (Eastman) in CH_3OH saturated with HBr [17]. α-2,5-Trihydroxytoluene was prepared through the reduction of 2,5-dihydroxybenzaldehyde with sodium amalgam [18]. Nitrohydroquinone was obtained through the nitration of hydroquinone monobenzoate, followed by saponification of the ester in basic solution [19]. The method of Wallenfels et al. [20] was followed to prepare cyanohydroquinone. Potassium *S*-2,5-dihydroxyphenyl thiosulfate was obtained through the condensation of thiosulfate ion with *p*-benzoquinone in glacial acetic acid [21]. Finally, Kvalnes' procedure [22] was followed for the most part in synthesizing iodohydroquinone. The iodobenzoquinone intermediate was purified through vacuum sublimation, and reduced to the corresponding hydroquinone with $SnCl_2$. Iodohydroquinone was obtained by recrystallization from $CHCl_3$.

Vacuum sublimation was the final purification step for all compounds synthesized except *n*-butylhydroquinone, 2,3-dichlorohydroquinone, α-2,5-trihydroxytoluene, iodohydroquinone and potassium *S*-2,5-dihydroxyphenyl thiosulfate. The purity of all compounds except the thiosulfate derivative was confirmed through comparisons of melting points with the literature values. Excellent agreement in infrared spectra was found with those measured for hydroquinones used in our kinetic studies and other published work, and proton NMR spectra of methyl-2,5-dihydroxyphenyl benzoate and *n*-butylhydroquinone confirmed the presence of $-COOCH_3$ and $-C_4H_9$ substituents, respectively.

R. vernicifera laccase was isolated from lacquer acetone powder and purified by the method of Reinhammar [23]. Other materials and methods used in preparing anaerobic solutions of laccase and reducing agents for use in kinetic studies were identical with those described previously [24]. Sodium phosphate buffers containing 100 μM Na_2H_2EDTA were used throughout to maintain the

pH at 7.0 and the ionic strength at 0.5 M. Kinetics measurements were performed at 25.0 ± 0.1°C using a Durrum D-110 stopped-flow spectrophotometer. Reduction of laccase type 1 copper was followed at 614 nm, employing metalloprotein concentrations of approx. 10 μM and 40—400-fold excesses of the organic reductants. Observed first-order rate constants (k_{obsd}) were obtained as before [24] by performing least-squares analyses on the linear regions of $\ln(A_t - A_\infty)$ vs. time plots based on post-steady state, 614-nm absorbance changes. Reported rate constants are, in most cases, the average of at least three determinations.

Infrared spectra (KBr pellet) were recorded using a Perkin-Elmer 457 spectrophotometer, and NMR spectra (acetone d_6 solvent) were obtained using a Varian A-60 spectrometer.

Results

Observed first-order rate constants for the reduction of laccase type 1 copper by the various hydroquinone derivatives are given in Table I (see footnote p. 307). At least a 10-fold range in reducing agent concentration was covered for all substrates except H_2Q-2, 5-Cl_2, H_2Q-*n*-C_4H_9, H_2Q-$(CH_3)_3$, H_2Q-S_2O_3K, and H_2Q-CHO. The limited solubility of the former three substrates precluded our attaining concentrations higher than 4 mM. Instability in the final absorbance value was observed for concentrations of H_2Q-CHO higher than 1 mM, and the slowness of the reaction of H_2Q-$S_2O_3^-$ with laccase blue copper limited the concentration range which could be covered with the stopped-flow apparatus. Upward instability in the 614 nm A_∞ value was observed for the reactions of H_2Q-NO_2 and H_2Q-CH_2COOH with laccase, presumably because of secondary reactions of the benzoquinone products. The upward drift was slow on the time scale of the electron transfer processes, however, and did not interfere with the evaluation of A_∞ values.

Plots of K_{obsd} vs. concentration for the reduction of laccase blue copper [L-Cu(II)] by H_2Q-$COOCH_3$, H_2Q-CH_2OH, H_2Q-NO_2, H_2Q-CF_3, H_2Q-2, 5-$(CH_3)_2$, H_2Q-$(CH_3)_3$, and H_2Q-*n*-C_4H_9 are linear with intercepts within experimental error of zero, consistent with the rate law:

$$\frac{-d[\text{L-Cu(II)}]_{tot}}{dt} = k[H_2Q\text{-X}][\text{L-Cu(II)}]_{tot} \qquad (2)$$

Second-order rate constants derived from the slopes of these plots are given in Table II along with parameters obtained previously for other hydroquinones whose reactions with laccase blue copper are cleanly first order in H_2Q-X. Observed rate constants for all of the other substrates considered approach a limiting value with increasing hydroquinone concentration. We have previously [12] attributed this behavior to a mechanism (intermediate complex mechanism) involving rapid complexation between the enzyme and substrate followed by a rate-limiting electron transfer step:

$$\text{L-Cu(II)} + H_2Q\text{-X} \overset{Q_p}{\rightleftharpoons} \text{laccase-hydroquinone complex} \overset{k_2}{\rightarrow} \text{L-Cu(I)} + \text{SQ-X} \qquad (3)$$

The semiquinone one-electron oxidation product of H_2Q-X is represented by

TABLE II

RATE PARAMETERS FOR THE REDUCTION OF LACCASE BLUE COPPER BY SUBSTITUTED HYDROQUINONES

25°C; I, 0.5 M phosphate, pH 7.0. Standard deviations are given in parentheses.

Substrate	k ($M^{-1} \cdot s^{-1}$)
H_2Q-CH_2OH	$1.55\ (0.02) \cdot 10^3$
H_2Q-CHO	$7.65\ (0.20) \cdot 10^2$ *
H_2Q-NO_2	$7.35\ (0.15) \cdot 10^2$
H_2Q-$(CH_3)_3$	$6.68\ (0.15) \cdot 10^2$
H_2Q	$4.5\ (0.1) \cdot 10^2$ **
H_2Q-CH_3	$4.49\ (0.02) \cdot 10^2$ ***
H_2Q-2,5-$(CH_3)_2$	$4.33\ (0.02) \cdot 10^2$
H_2Q-CF_3	$3.05\ (0.01) \cdot 10^2$
H_2Q-n-C_4H_9	$2.41\ (0.04) \cdot 10^2$
H_2Q-$C(CH_3)_3$	$1.54\ (0.07) \cdot 10^2$
H_2Q-$COOCH_3$	$9.33\ (0.12) \cdot 10^1$
H_2Q-SO_3^-	$4.02\ (0.01) \cdot 10^1$ ***
H_2Q-$S_2O_3^-$	$2\ (1) \cdot 10^0$ *

* Calculated as the average value of $k_{obsd}/[H_2Q\text{-}X]$.
** Ref. 24.
*** Ref. 12.

SQ-X. Under conditions where $[H_2Q\text{-}X]$ is much larger than $[L\text{-}Cu(II)]_{tot}$, the total concentration of all species absorbing at 614 nm, the rate law implied by this mechanism is:

$$\frac{-d[L\text{-}Cu(II)]_{tot}}{dt} = \frac{k_2 Q_p [H_2Q\text{-}X][L\text{-}Cu(II)]_{tot}}{1 + Q_p[H_2Q\text{-}X]} \quad (4)$$

Enzyme-substrate complex formation constants (Q_p) and first-order electron transfer rate constants (k_2), derived as before [12] from the least-squares slopes ($(k_2Q_p)^{-1}$) and intercepts (k_2^{-1}) of linear k_{obsd}^{-1} vs. $[H_2Q\text{-}X]^{-1}$ plots, are listed in

TABLE III

RATE PARAMETERS BASED ON THE INTERMEDIATE COMPLEX MECHANISM

25°C, I, 0.5 M phosphate, pH 7.0. Standard deviations are given in parentheses. k is calculated from $k = k_2Q_p$.

Substrate	k_2 (s^{-1})	Q_p (M^{-1})	k ($M^{-1} \cdot s^{-1}$)
H_2Q-F	65 (17)	$3.4\ (0.9) \cdot 10^1$	$2.2 \cdot 10^3$
H_2Q-Cl	32 (3) *	$1.37\ (0.16) \cdot 10^2$ *	$4.3 \cdot 10^3$
H_2Q-Br	30 (4)	$1.93\ (0.30) \cdot 10^2$	$5.9 \cdot 10^3$
H_2Q-I	12 (2)	$2.14\ (0.39) \cdot 10^2$	$2.6 \cdot 10^3$
H_2Q-2,3-Cl_2	17 (1)	$1.40\ (0.16) \cdot 10^3$	$2.4 \cdot 10^4$
H_2Q-2,5-Cl_2	20 (2)	$1.92\ (0.39) \cdot 10^3$	$3.9 \cdot 10^4$
H_2Q-2,6-Cl_2	27 (3)	$1.65\ (0.18) \cdot 10^2$	$4.6 \cdot 10^3$
H_2Q-OH	27 (1) *	$1.00\ (0.09) \cdot 10^3$ *	$2.7 \cdot 10^4$
H_2Q-OCH_3	43 (9)	$1.21\ (0.30) \cdot 10^2$	$5.3 \cdot 10^3$
H_2Q-COO^-	0.25 (0.04) *	$2.84\ (0.44) \cdot 10^1$ *	$7.2 \cdot 10^0$
H_2Q-CH_2COO^-	0.89 (0.14)	$2.04\ (0.39) \cdot 10^2$	$1.8 \cdot 10^2$
H_2Q-CN	29 (11)	$8.0\ (3.3) \cdot 10^1$	$2.3 \cdot 10^3$

* Ref. 12.

Table III, together with parameters calculated previously for H_2Q-Cl, H_2Q-COO^-, and H_2Q-OH.

Discussion

Several mechanistic models have been proposed for the reduction of the laccase type 1 copper site by hydroquinone and related electron donors. Holwerda and Gray [24] suggested that a protein conformational change permits electron transfer to occur from a type 2 Cu(II)-bound hydroquinone monoanion to the less exposed type 1 copper site. These authors claim that the type 1 and type 3 sites are reduced in parallel, and that the reduction rate of the blue copper atom is not dependent on the oxidation state of the type 3 site. Andreasson and Reinhammar [25] proposed that reduction of the laccase type 3 copper site involves an electron shuttle of the blue copper atom; in this case, final reduction of type 1 Cu(II) would occur only after full reduction of the type 3 site.

We were not able to obtain reliable rate data for the reduction of the laccase type 3 site by substituted hydroquinones, as large 330-nm absorbance increases associated with oxidation of many of these substrates effectively couple rate processes for all of the enzymatic copper sites at this wavelength. Semi-quantitative observations suggest, however, that substituted hydroquinones typically reduce the type 3 site substantially faster than the blue copper atom, as is the case for hydroquinone itself [24]. Rate parameters based on post-steady-state 614-nm absorbance changes, therefore, most reasonably correspond to the anaerobic reduction of blue copper sites in laccase molecules possessing reduced type 3 copper sites. Such an occurrence would be consistent with either one of the mechanistic models outlined above. It should be noted that these rate parameters pertain only under special conditions (absence of O_2, type 3 site reduced) and are not intended to reflect the reactivity of the blue copper site under physiological conditions (O_2 present, oxidation state of types 3 site variable). Indeed, at pH 7.4 hydroquinone reduces type 1 Cu(II) approx. 3 times faster in the presence of O_2 than it does under anaerobic conditions [25].

The most striking feature of the data presented in Table III is the insensitivity of intra-complex electron transfer rate constants to the nature and number of substituents linked to the aromatic ring system of 1,4-dihydroxybenzene. Thus, the average value of k_2 for ten of the twelve substrates listed is $30 \pm 10\ s^{-1}$, excluding only the hydroquinones with carboxylate-containing substituents. By contrast, room temperature rate constants for the reduction of $Cu(dmp)_2^{2+}$ (dmp = 2,9-dimethyl-1,10-phenanthroline) by the singly ionized forms of these same ten substrates [$(HQ\text{-}X)^-$] span a 500-fold range (Clemmer, J.D. et al. unpublished data). The carboxylate anions H_2Q-COO^- and H_2Q-CH_2COO^- follow a markedly different reactivity pattern, yielding k_2 values lower than the mean value calculated for the other substrates by factors of 120 and 35, respectively.

The intermediate complex mechanism accounts well for the kinetic results for the reduction of laccase blue copper by substituted hydroquinones, but it should be noted that the data are also consistent with a mechanism requiring

rate-limiting formation of an activated form of laccase (L-Cu(II)*) followed by rapid reduction of type 1 Cu(II) within this intermediate by the substrate:

$$\text{L-Cu(II)} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{L-Cu(II)}^*$$

$$\text{L-Cu(II)}^* + \text{H}_2\text{Q-X} \xrightarrow{k_2'} \text{SQ-X} + \text{L-Cu(I)} \qquad (5)$$

A linear plot of k_{obsd}^{-1} vs. $[\text{H}_2\text{Q-X}]^{-1}$ is expected for both the activated intermediate and intermediate complex mechanisms, assuming that L-Cu(II)* may be trated as a steady-state intermediate; the parameters obtained from (intercept)$^{-1}$ and intercept/slope for the activated intermediate mechanism are k_1 and k_2'/k_{-1}, respectively, while the corresponding parameters for the intermediate complex alternative are k_2 and Q_p. The activated intermediate mechanism implies that (intercept)$^{-1}$ = k_1 values should be independent of the substituent X, in fair agreement with results for the ten substrates exhibiting values in the range 12—65 s^{-1}. The markedly different (intercept)$^{-1}$ values obtained for the hydroquinones with carboxylate-containing substituents, however, argue strongly against this mechanism. Further support for the intermediate complex mechanism comes from the linear free energy relationship observed between pK_a values for the various substrates and binding constants based on the relationship: intercept/slope = Q_p (vide infra). Such a correlation is not readily understood in terms of the activated intermediate mechanism, for which intercept/slope is not an enzyme-substrate binding constant, but rather is a ratio of rate constants which would not be expected to follow the observed dependence on the acidity of hydroquinone substrates.

Recently completed calculations for the $(\text{HQ-X})^-$-Cu(dmp)_2^{2+} system (unpublished data) indicate that ΔG^* values for 15 of the monosubstituted hydroquinones employed in the present study exhibit the dependence on ΔG°

TABLE IV

COMPARISON OF FREE ENERGY CHANGES FOR INTRA-COMPLEX ELECTRON TRANSFER

Free energies expressed in kcal/mol. ΔG_2° is calculated from $\Delta G_2^\circ = -RT \ln K_2$; the calculation of K_2 is described in the text. ΔG_2^* is calculated from $k_2 = k_B T/h \exp(-\Delta G_2^*/RT)$ [34]. ΔG_2^* (calc.) is calculated from Eqn. 1 using λ = 60 kcal/mol.

Substrate	E° (Q-X) (V)	ΔG_2°	ΔG_2^*	ΔG_2^* (calc.)
$\text{H}_2\text{Q-F}$	0.693 [a]	+1.6	15.0	15.8
$\text{H}_2\text{Q-Cl}$	0.712 [b]	+1.6	15.4	15.8
$\text{H}_2\text{Q-Br}$	0.712 [b]	+1.9	15.5	16.0
$\text{H}_2\text{Q-I}$	0.706 [c]	+1.7	16.0	15.9
$\text{H}_2\text{Q-OH}$	0.601 [d]	—0.6	15.5	14.7
$\text{H}_2\text{Q-OCH}_3$	0.594 [e]	—1.3	15.2	14.4
$\text{H}_2\text{Q-CN}$	0.747 [f]	+3.1	15.5	16.6

[a] Estimated from half-wave potential [13].
[b] Ref. [35].
[c] Ref. [22].
[d] Ref. [36].
[e] Ref. [37].
[f] Estimated from plot of E° vs. Hammett *para* substituent constant for monosubstituted hydroquinones.

predicted by the equation for Marcus theory Eqn. 1. It is of interest, therefore, to determine whether the insensitivity of k_2 values for the reduction of laccase type 1 copper to the nature of hydroquinone substituents may be understood within the context of Marcus theory. The overall electron transfer reactivity of substituted hydroquinones with laccase type 1 copper clearly is not a simple function of the thermodynamic driving force for oxidation of the substrate, as wide variations in the value of the binding constant Q_p among the various hydroquinones are reflected in the second-order rate constant k [12].

Table IV presents the results of an attempt to use Eqn. 1 in correlating activation free energies based on k_2 (ΔG_2^*) with standard free energy changes for the redox step (ΔG_2°), presumably transfer of an electron from type 2 Cu(II)-bound monoionized hydroquinones (vide infra) to the blue copper atom. The work term w_r has been neglected, and it is assumed that ΔG_2° essentially is the free energy change for the reaction:

$$(\text{HQ-X})^- + \text{L-Cu(II)} \overset{K_2}{\rightleftharpoons} \text{SQ-X} + \text{L-Cu(I)} \tag{6}$$

For the purpose of estimating ΔG_2°, therefore, perturbations in the thermodynamic oxidizability of $(\text{HQ-X})^-$ linked to ES complex formation are assumed to be negligible. It is easily seen that the equilibrium constant K_2 may be calculated from the equation:

$$K_2 = \frac{\sqrt{K_0 K_{SQ}}}{K_1} \tag{7}$$

where K_0 and K_{SQ}, respectively, are equilibrium constants for the reactions:

$$\text{H}_2\text{Q-X} + 2\ \text{L-Cu(II)} \rightleftharpoons 2\ \text{H}^+ + \text{Q-X} + 2\ \text{L-Cu(I)} \tag{8}$$

(Q-X = 2-X-1,4-benzoquinone)

and

$$\text{H}_2\text{Q-X} + \text{Q-X} \rightleftharpoons 2\ \text{SQ-X} \tag{9}$$

K_1 is the first ionization constant of $\text{H}_2\text{Q-X}$ (-OH group ionized); a tabulation of pK_1 values is given in Table V. K_0 is readily calculated knowing standard reduction potentials for Q-X (Table IV) and for laccase type 1 Cu(II) (+432 mv) [26]. The method of Pelizzetti et al. [8] was followed to obtain semiquinone formation constants (K_{SQ}). Activation free energies calculated on the basis of Eqn. 1 [ΔG_2^* (calc.)] are compared with experimental values in Table IV. The former were obtained using $\lambda = 60$ kcal/mol, the average value obtained upon substituting ΔG_2°, ΔG_2^* pairs into Eqn. 1.

Although our ΔG_2° values are at best only approximations, comparisons of trends within the free energy changes (Table IV) allow us to conclude that Marcus theory does not provide a useful framework for understanding the electron transfer reactivity of substituted hydroquinones with laccase type 1 Cu(II). ΔG_2^* and ΔG_2^*(calc) values span ranges of 1.0 and 2.2 kcal/mol, respectively. Stated in another way, k_2 values for the substrates shown in Table IV span only a 5-fold range, whereas Eqn. 1 predicts a 40-fold reactivity range. Marcus theory clearly cannot account for the extremely narrow interval spanned by intra-complex electron transfer rate constants. More importantly,

TABLE V

COMPARISON OF BINDING CONSTANTS FOR THE ASSOCIATION OF SUBSTITUTED HYDROQUINONES WITH LACCASE

25°C, I, 0.5 M phosphate, pH 7.0. pK_a is based on first ionization constant for phenolic OH group.

Substrate	Q_p (M^{-1})	pK_a (H_2Q-X)
1. H_2Q-2,5-Cl_2	1920	7.90 [b]
2. H_2Q-2,3-Cl_2	1400	
3. H_2Q-OH	1000	9.08 [c]
4. H_2Q-I	214	8.81 [e]
5. H_2Q-CH_2COO^-	204	
6. H_2Q-Br	193	8.53 [e]
7. H_2Q-2,6-Cl_2	165	7.30 [d]
8. H_2Q-Cl	137	8.90 [b]
9. H_2Q-OCH_3	121	9.91 [b]
10. H_2Q-CN	80	7.50 [e]
11. H_2Q-CH_2OH	52 [a]	
12. H_2Q-F	34	8.66 [e]
13. H_2Q-COO^-	28	10.50 [f]
14. H_2Q-CHO	26 [a]	
15. H_2Q-NO_2	25 [a]	7.47 [c]
16. H_2Q-$(CH_3)_3$	22 [a]	10.8 [b]
17. H_2Q	15 [a]	9.85 [d]
18. H_2Q-CH_3	15 [a]	10.05 [d]
19. H_2Q-2,5-$(CH_3)_2$	14 [a]	10.38 [b]
20. H_2Q-CF_3	10 [a]	8.66 [e]
21. H_2Q-n-C_4H_9	8 [a]	10.25 [e]
22. H_2Q-$C(CH_3)_3$	5 [a]	10.22 [e]
23. H_2Q-$COOCH_3$	3 [a]	9.63 [c]
24. H_2Q-SO_3^-	1 [a]	9.57 [b]
25. H_2Q-$S_2O_3^-$	<0.1 [a]	

[a] Calculated assuming $Q_p \cong k/30$ M^{-1}.
[b] 25°C, I, 0.375 M; Ref. 38.
[c] 20°C, I, 0.1 M; Ref. 36.
[d] 25.0°C (H_2Q), 25.1°C (H_2Q-CH_3), 26.1°C (H_2Q-2,6-Cl_2), I, 0.65 M; Ref. 39.
[e] 25°C, I, 0.2 M.
[f] Ref. 29.

ΔG_2^* values do not vary in any consistent way with ΔG_2°, while Eqn. 1 predicts that activation free energies for substrates yielding favorable standard free energy changes (H_2Q-OH, H_2Q-OCH_3) shoiuld be substantially smaller than those for less oxidizable substrates. In fact, ΔG_2^* values for H_2Q-OCH_3 and H_2Q-CN are virtually identical in spite of a 4.4 kcal/mol difference in ΔG_2°. Admittedly, the range in ΔG_2° covered by the substrates in Table IV is not large and the effect of ES complex formation on this parameter is unknown. Nevertheless, it seems clear that there is no basis for concluding that the electron transfer reactivity of hydroquinones with laccase blue copper is controlled to any significant extent by the oxidizability of the substrate. Quite to the contrary, the insensitivity of ΔG_2^* to variations in ΔG_2° strongly supports our previous suggestion [12,24,27] that protein-dependent activation requirements are dominant in the rate-limiting step for electron transfer to type 1 Cu(II). Protein conformational movement in the k_2 step reasonably could be a prerequisite for hydroquinone bound on the surface of the enzyme to penetrate towards the solvent-inaccessible blue copper atom.

While intra-complex electron transfer rate constants are essentially identical

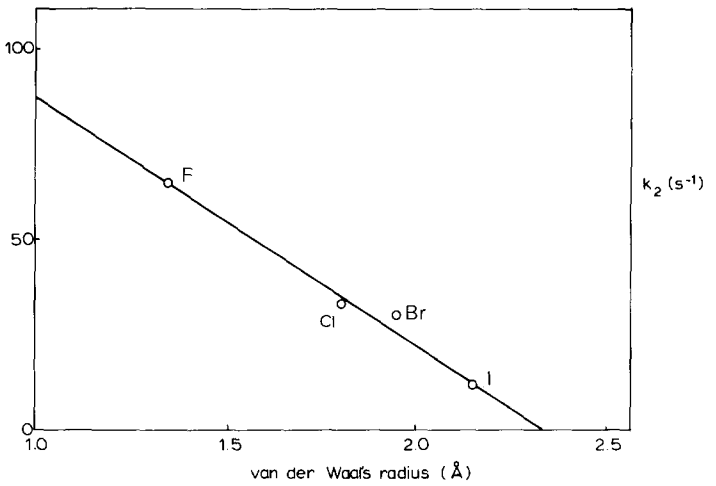


Fig. 1. Effect of substituent size on the intra-complex electron transfer rate constant for monohalohydroquinone substrates.

for most of the substrates examined, it may be noted that k_2 values for the monohalohydroquinones follow a slight decreasing trend, with $k_2(H_2Q\text{-}F) > k_2(H_2Q\text{-}Cl) \approx k_2(H_2Q\text{-}Br) > k_2(H_2Q\text{-}I)$. Fig. 1 shows that k_2 values for this series of hydroquinones vary linearly with the van der Waals radius of the halogen atom, suggesting that the rate of the electron transfer step is somewhat sensitive to the size of the ring substituent. This would not be surprising if the enzyme-bound substrate is, in fact, required to penetrate towards the buried type 1 copper site in the rate-limiting k_2 step.

Some of the factors which may influence binding of substituted hydroquinones by laccase prior to their oxidation are: (1) the ionization state of the phenolic -OH groups; (2) the size and electronic characteristics of the substituent; (3) the ability of the X group(s) to function as a ligand, and (4) the hydrogen-bonding capability of the substituent. A term proportional to $[H^+]^{-1}$ dominates the rate law for reduction of laccase blue copper by hydroquinone in the range pH 5—8, indicating that the monoanion form HQ^- is the active species [24]. Fluoride and azide inhibition studies have further suggested that type 2 Cu(II), the solvent-accessible [5] non-blue copper atom, is the binding site preferred by HQ^- [24]. Table V summarizes binding constants and first ionization constants, where available, for the various hydroquinones. Q_p values for the substrates included in Table II have been estimated from $Q_p = k/k_2$, assuming that k_2 remains essentially invariant at 30 s^{-1} throughout the entire series. A better understanding of the factors promoting enzyme-substrate complex formation is achieved by separately considering the properties of three classes of substrates. Thus, we designate weak, intermediate, and strong binding classifications according to $Q_p < 5\ M^{-1}$, $5 < Q_p < 50\ M^{-1}$, and $Q_p < 50\ M^{-1}$, respectively.

Apparent Q_p values for substrates falling within the intermediate binding classification are strikingly insensitive to the electronic characteristics of the

substituent. Thus, the electron-withdrawing or donating capability of the substituent has little effect on binding constants even though inductive effects result in substantial differences in the acidities of the substrates. This result is not surprising if the monoionized forms of the various substrates are the species which bind to the enzyme.

For most of the substrates considered, the concentration of the singly ionized form is very small at pH 7.0. The binding equilibrium, therefore, is best represented as:

$$\text{L-Cu(II)} + \text{H}_2\text{Q-X} \rightleftharpoons \text{L-Cu(II)} - (\text{HQ-X})^- + \text{H}^+ \tag{10}$$

$$K = \frac{[\text{L-Cu(II)} - (\text{HQ-X})^-][\text{H}^+]}{[\text{H}_2\text{Q-X}][\text{L-Cu(II)}]} = Q_p[\text{H}^+] \tag{11}$$

assuming that only the ionized hydroquinone is bound by the enzyme. This equilibrium can be expressed as the sum of two related equilibria:

$$\text{H}_2\text{Q-X} \overset{K_a}{\rightleftharpoons} (\text{HQ-X})^- + \text{H}^+ \tag{12}$$

$$(\text{HQ-X})^- + \text{L-Cu(II)} \overset{K'}{\rightleftharpoons} \text{L-Cu(II)} - (\text{HQ-X})^- \tag{13}$$

such that $K = K_a K'$. Linear free energy relationships of the form:

$$\log K' = a\ \text{p}K_a + b \tag{14}$$

successfully correlate binding constants with acid ionization constants for many equilibria of this type involving the interaction of structurally similar ligands with a common metal center; the implication is that the Lewis basicity of the ligand towards the metal ion is proportional to its basicity towards to proton [28]. The parameters a and b are constants when considering the interaction of a family of structurally similar ligands with a particular metal ion. Assuming that a similar linear free energy relationship applies to the binding of ionized hydroquinones at the laccase type 2 site, it is easily shown that the observed binding constant Q_p is related to K_a through the equation:

$$Q_p = \frac{K}{[\text{H}^+]} = \frac{e^b}{[\text{H}^+]} K_a^{(1-a)} \tag{15}$$

A plot of log K' vs. pK_a for substituted hydroquinones falling within the intermediate binding category is indeed linear (Fig. 2), yielding an 'a' value of 0.93 ± 0.08. Since the 'a' value is close to 1.0 and $[\text{H}^+]$ is held constant in our studies at $1 \cdot 10^{-7}$ M, it is not surprising that Q_p values for substrates falling within the intermediate binding class are essentially independent of K_a and thus the electronic characteristics of the ring substituent. The existence of a linear free energy relationship between log K' and pK_a for ten intermediate binding substrates provides strong support for the hypothesis that enzyme-substrate complex formation involves a coordinative interaction between enzymatic

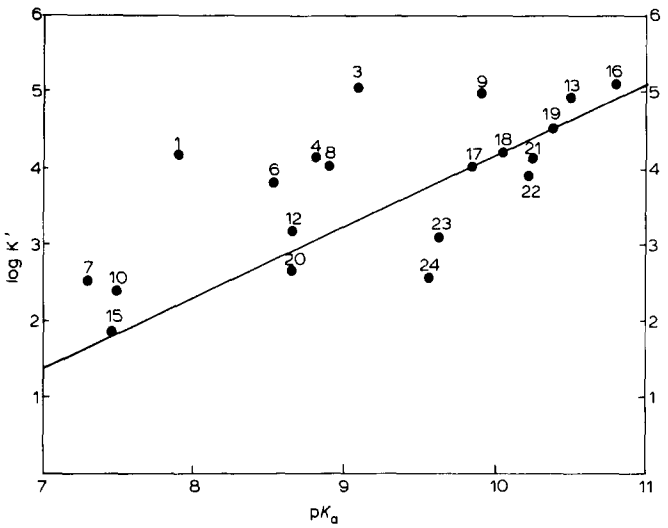


Fig. 2. Correlation of binding constants with the basicity of singly ionized substituted hydroquinones. Substituent key is identical with that in Table V. Least-squares correlation line has been drawn on the basis of substrates 12, 13, 15—22 (Table V).

copper and the deprotonated oxygen atom of monoionized hydroquinones *. Desai and Milburn [30] have demonstrated that an analogous linear free energy relationship exists for the interaction of monosubstituted phenolate anions with Fe^{3+}.

Considering the arguments presented above, it seems clear that factors other than the basicity of $(HQ\text{-}X)^-$ must be invoked to account for Q_p values substantially larger than 50 M^{-1} or lower than 5 M^{-1}. Fig. 2 shows that log K' values for eight strong-binding hydroquinones are significantly higher than would be expected on the basis of the correlation line. Similarly, log K' values for $H_2Q\text{-}COOCH_3$ and $H_2Q\text{-}SO_3^-$ are substantially smaller than would be expected on the basis of their pK_a values. Steric requirements for the accomodation of substituted hydroquinones at the binding site reasonably could account for the finding of anomalously low K' values for the weak-binding substrates, all of which have bulky substituents. Plots of log K' vs. pK_a have been used in several instances to identify ligands for which groups other than that involved in the K_a equilibrium also coordinate to the metal atom, enhancing the stability of the resulting complex [28]. Examination of the substrates falling within the strong-binding classification confirms our previous suggestion [12] that unusually large Q_p values are found only when the substituent has at least one lone pair of electrons and, thus, presumably is capable of functioning as a ligand. It is now apparent, however, that not all of the substrates which satisfy this criterion exhibit anomalously large binding con-

* Second-order kinetics is observed for the reduction of laccase blue copper by ascorbic acid under the conditions employed in this study (k = 110 ± 2 $M^{-1} \cdot s^{-1}$; 25° C, pH 7.0, I 0.5 M). Thus, strong association between laccase and this substrate is not observed even though ascorbic acid (pK_1 = 4.3) [29] is completely ionized to its monoanion form at pH 7. A small Q_p value for the reaction of ascorbate with laccase seems reasonable considering the comparatively low basicity of this substrate and the observed correlation between log K' and pK_a.

stants (i.e. X = NO_2, $-CF_3$, -F, $-COO^-$, -CHO). Unfortunately, the relative ligating capabilities of hydroquinone substituents have not been documented even for reactions with simple transition metal ion oxidants, making it impossible at this time to account for the comparatively weak binding observed for these substrates. Linck and Taube's report [31] of a binuclear chromium(III)-H_2Q-2,5-$(OH)_2$ complex in which each chromium atom is chelated by two oxygen atoms makes it clear, however, that hydroquinone substituents are in fact capable of functioning as ligands. Fig. 2 shows that log K' values for most of the strong-binding substrates (H_2Q-2,6-Cl_2, H_2Q-Br, H_2Q-I, H_2Q-OCH_3) fall higher than expected from the correlation line by 0.9—1.1 log units (—1.2—1.5 kcal/mol in ΔG°). Substantially larger stabilizations are evident for H_2Q-2,5-Cl_2 and H_2Q-OH (—2.7 and —2.4 kcal/mol, respectively). Considering the large uncertainties inherent in constructing the correlation line and the small apparent enhancement in log K' for H_2Q-CN, it is not clear whether any special stabilization of the ES complex exists for this substrate.

Coordination of hydroquinone substituents at type 1 Cu(II) seems unlikely considering ESR evidence for the absence of complexation between F^- and N_3^- and the blue copper atom, even at anion concentrations as high as 0.1 M [32]. One possibility is that ES complex stability enhancements for strong-binding substrates reflect chelation of the type 2 copper atom by the X group and the *ortho* oxygen atom. Alternatively, X groups with lone pairs might facilitate complex formation by enabling hydroquinone to function as a bidentate bridging ligand between the type 2 and type 3 copper sites [12]. The data unfortunately do not provide a basis of unambiguously choosing between these alternatives, or excluding other possibilities. Nevertheless, the close agreement between binding constant enhancements for H_2Q-Cl and H_2Q-2,6-Cl_2 (log K' — log K' [correlation line] = 0.9) provides some support for the bidentate bridging hypothesis. Chelation of type 2 Cu(II) reasonably could occur with H_2Q-Cl as the substrate, but steric considerations suggest that for 2,6-dichlorohydroquinone the oxygen atom *ortho* to the two chlorine substituents should be blocked from coordinating to this or other enzymatic copper sites. The similarity between Q_p values for H_2Q-Cl and H_2Q-2,6-Cl_2, therefore, cannot be understood in terms of chelation of one copper site by chlorine and the *ortho* oxygen atom. By contrast, this result is expected if for both substrates a chlorine atom *meta* to the type 2 Cu(II)-bound oxygen atom interacts with the type 3 site. The observed sensitivity of Q_p to the X-to-OH distance [12] [$Q_p(H_2Q\text{-}CH_2OH) \ll Q_p(H_2Q\text{-}OH)$] also is consistent with the bidentate bridging hypothesis.

Intra-complex electron transfer rate constants for H_2Q-COO^- and H_2Q-CH_2COO^- fall far below the value of approx. 30 s^{-1} characteristic of the other substrates. Hydroquinones with carboxylate-containing substituents, therefore, may bind to the enzyme differently and utilize an alternate electron transfer pathway. One possibility is that the carboxylate group enhances binding through hydrogen bonding with lysine or tyrosine residues of the protein. Consistent with this hypothesis is the observation that the substrate with the more basic carboxylate function, H_2Q-CH_2COO^- [pK_a(-COOH) = 4.40] [32], binds more strongly to laccase than H_2Q-COO^- [pK_a(-COOH) = 2.97] [29], which is less basic by a factor of 40.

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