Anation of Laccase Type 2 Copper by Azide and Thiocyanate Ions†

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ABSTRACT: A mechanistic study of the anation of type 2 Cu(II) in fully oxidized laccase by azide and thiocyanate ions (X^-) is reported. The rate data support a mechanism involving rapid formation of an outer-sphere complex (laccase·X^-) followed by rate-limiting dissociative interchange to give the inner-sphere complex (laccase–X^-) product:

$$\text{laccase} + X^- \overset{K_{os}}{\rightleftharpoons} \text{laccase}\cdot X^- \overset{k'_2}{\underset{k'_{-2}}{\rightleftharpoons}} \text{laccase}-X^-$$

Rate parameters for the laccase–azide reaction are $k'_2 = 1.25 \times 10^{-1}$ s^{-1} and $k'_{-2} = 1.50 \times 10^{-2}$ s^{-1}; those for the laccase–thiocyanate reaction are $k'_2 = 2.0 \times 10^{-2}$ s^{-1} and $k'_{-2} = 1.1 \times 10^{-2}$ s^{-1} (25° C, pH 6.1 phosphate buffer, $I = 0.5$ M). Although the rate law for anation of type 2 Cu(II) by N_3^- in 2-(*N*-morpholino)ethanesulfonic acid (Mes) or acetate medium ($k_{obsd} = k_3 + k_4[N_3^-]$) differs from that observed in phosphate buffer, the data may still be accounted for in terms of the above mechanism, with $K_{os}[N_3^-] \ll 1$ [in Mes, $k_3 = 2.2 \times 10^{-2}$ s^{-1} and $k_4 = 3.3 \times 10^{-1}$ M^{-1} s^{-1}; in acetate, $k_3 = 2.1 \times 10^{-2}$ s^{-1} and $k_4 = 3.4 \times 10^{-1}$ M^{-1} s^{-1} (25° C, pH 6.0, $I = 0.25$ M)]. The equilibrium and spectroscopic characteristics of the laccase type 2 Cu(II)–N_3^- complex are compared with those of low molecular weight copper(II)–azide species, and the factors responsible for the very low substitutional reactivity of the type 2 cupric ion are discussed.

The type 2 copper atom of *Rhus vernicifera* laccase is thought to be important as a binding site for polyphenolic substrates (Holwerda & Gray, 1974; Clemmer et al., 1979) or as an electron acceptor from reduced type 1 copper (Andréasson & Reinhammar, 1976). This site has also been implicated in the rapid conversion of O_2 to H_2O by fully reduced laccase (Bränden & Deinum, 1977). As type 2 Cu(II) is not optically observable, conclusions regarding the role of this site have been based largely on inhibition experiments with fluoride, cyanide, azide, and thiocyanate ions, which are known, from electron spin resonance (ESR) studies, to interact strongly with the "nonblue" copper atom (Malmström et al., 1970; Morpurgo et al., 1974; Desideri et al., 1979). An intense ligand to metal charge-transfer transition near 400 nm permits the optical detection of complexation between laccase type 2 Cu(II) and N_3^- or NCS^- ions (X^-) (Holwerda & Gray, 1974; Morpurgo et al., 1974) and has been utilized to follow the reduction of this copper atom in azide-inhibited laccase by hydroquinone (Holwerda & Gray, 1974).

Our previous work (Holwerda & Gray, 1974) demonstrated that the anation of *Rhus vernicifera* laccase type 2 Cu(II) by azide ion is atypically slow for substitution at Cu(II) but did not clearly define the mechanism of this reaction. Thus, the rate data could be accounted for in terms of "activated intermediate" (eq 1) or "outer-sphere complex" (eq 2) mechanisms where laccase*, laccase·N_3^-, and laccase–N_3^-

$$\text{laccase} \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} \text{laccase}^* \qquad (1a)$$

$$\text{laccase}^* + N_3^- \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} \text{laccase}-N_3^- \qquad (1b)$$

$$\text{laccase} + N_3^- \overset{K_{os}}{\rightleftharpoons} \text{laccase}\cdot N_3^- \qquad (2a)$$

$$\text{laccase}\cdot N_3^- \overset{k'_2}{\underset{k'_{-2}}{\rightleftharpoons}} \text{laccase}-N_3^- \qquad (2b)$$

correspond, respectively, to an activated (steady-state intermediate) form of laccase, an outer-sphere, intermediate laccase–azide complex, and the ultimate inner-sphere laccase–azide product responsible for the strong absorption at 405 nm. In order to provide a definitive basis for choosing between these two mechanisms, new kinetic results on the anation of laccase type 2 copper by azide and thiocyanate ions have been obtained. This distinction is important, considering the probable similarity between mechanisms for binding of pseudohalide ions and the active phenolate anion forms of laccase substrates (Holwerda & Gray, 1974) to the type 2 copper atom. The equilibrium and spectroscopic characteristics of the laccase type 2 Cu(II)–N_3^- complex are compared with those of low molecular weight copper(II)–azide species, and the factors responsible for the dramatically low substitutional reactivity of the type 2 cupric ion are discussed.

Experimental Procedures

Reagents. Reagent grade chemicals were used throughout, and solutions for kinetic measurements were prepared with triply distilled water. Eastman sodium azide was used without further purification, as ceric titrations (Arnold, 1945) showed that the salt was better than 99% pure. As sodium thiocyanate is hygroscopic, a stock solution of this reagent was standardized by the Volhard method (Skoog & West, 1963). Laccase was extracted from *Rhus vernicifera* lacquer acetone powder and purified as described by Reinhammar (1970). Cu(dien)$(NO_3)_2$[1] (Bew et al., 1972), [Cu(terpy)(H_2O)]$(ClO_4)_2$ (Harris & Lockyear, 1970), and Cu(Me_5dien)$(ClO_4)_2 \cdot 2CH_3CN$ (Kolks et al., 1981) were prepared by literature methods.

Solution Preparation. Laccase was prepared in pH 6 sodium acetate, phosphate, or Mes buffers following dialysis against triply distilled water and was stored at 5 °C until use. The metalloprotein concentration was determined from the 614-nm absorbance ($\epsilon_{614} = 5700$ M^{-1} cm^{-1}) (Malmström et al., 1970). Sodium azide or thiocyanate solutions were pre-

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[1] Abbreviations: dien, diethylenetriamine; terpy, 2,2′,2″-terpyridine; Me_5dien, 1,1,4,7,7-pentamethyldiethylenetriamine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; tren, 2,2′,2″-triaminotriethylamine; Me_6tren, 2,2′,2″-tris(*N*,*N*-dimethylamino)triethylamine; LMCT, ligand to metal charge transfer.

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pared in the same buffer as the metalloprotein (or copper complex) and contained sufficient sodium nitrate to maintain constant ionic strength within a series of runs. In kinetic studies, the laccase concentration was maintained at ca. 10 μM, 1000 times smaller than the minimum pseudohalide concentration.

Kinetic and Equilibrium Measurements. Kinetic measurements were carried out at 25.0 ± 0.2 °C on a Durrum D-110 stopped-flow spectrophotometer, using Teflon needles for the transfer of solutions into the drive syringes. As the reactions studied required more than 5 min to be complete, precautions were taken (Holwerda & Gray, 1974) against back-diffusion of mixed solutions in the stopped-flow apparatus, and absorbance–time traces were displayed on a Hewlett-Packard 7004-B X-Y recorder. Formation of azide and thiocyanate complexes of laccase type 2 copper was followed at 405 and 400 nm, respectively. Equilibrium measurements on the formation of $[Cu(dien)N_3]^+$ and $[Cu(terpy)N_3^+]$ were carried out at 346 and 370 nm, respectively, by using a Spectronic 100 spectrophotometer with thermostated cell block. Observed pseudo-first-order relaxation rate constants (k_{obsd}) were derived from the least-squares slopes of ln $(A_e - A_t)$ vs. time plots, where A_e and A_t represent the absorbances at equilibrium and time t, respectively. Reported values are the average of two or three determinations. Reliable A_e values could not be obtained in kinetic studies of the laccase–N_3^- reaction in acetate media, possibly owing to the presence of denatured protein. The Guggenheim (1926) method was employed to derive k_{obsd} in these runs.

Formation constants (K_f) of N_3^- and NCS^- complexes were calculated as the ratio of least-squares intercept to slope in plots of $(A_e - A_0)^{-1}$ vs. $[X^-]^{-1}$, based on the relationship (Holwerda & Gray, 1974)

$$(A_e - A_0)^{-1} = (lC_0\Delta\epsilon)^{-1} + (lC_0\Delta\epsilon K_f)^{-1}[X^-]^{-1} \quad (3)$$

where A_0, l, C_0, and $\Delta\epsilon$ correspond, respectively, to the initial absorbance, the spectrophotometric path length, the total copper concentration, and the differential extinction coefficient associated with formation of the anion complex. The linearity of these plots was found to be excellent, indicating that only 1:1 Cu(II)–anion complexes are formed under our conditions. $A_e - A_0$ values for laccase–anion complexes were obtained from the intercepts of ln $(A_e - A_t)$ vs. time plots.

UV-visible spectra were acquired on a Cary 17 spectrophotometer, and pH measurements were obtained with Ionalyzer 801 or Brinkmann pH-104 meters.

Results

Anation of Laccase by N_3^- and NCS^- in Phosphate Buffer. The reactions of laccase with N_3^- and SCN^- were studied at 25 °C; pH 6.1, I = 0.5 M (0.2 M contributed by the sodium phosphate buffer). The concentration ranges covered were 0.020–0.200 M (N_3^-) and 0.033–0.250 M (SCN^-). Formation constants for the azide (20 ± 2 M^{-1}) and thiocyanate (18 ± 2 M^{-1}) complexes of type 2 Cu(II) are nearly identical under these conditions (Figure 1), but the reaction of N_3^- with laccase is substantially faster than that of SCN^-. $\Delta\epsilon_{405}(N_3^-)$ and $\Delta\epsilon_{400}(SCN^-)$ values are (1.8 ± 0.2) × 10^3 and (6.4 ± 0.5) × 10^2 M^{-1} cm^{-1}, respectively. Nonlinear dependences of k_{obsd} on anion concentration were observed for both reactions, with k_{obsd} approaching a saturation limit at high concentrations. This behavior, found also in our previous studies of the laccase–N_3^- reaction (25 °C, pH 6.1 phosphate buffer, I = 0.2 M) (Holwerda & Gray, 1974), is consistent with both the

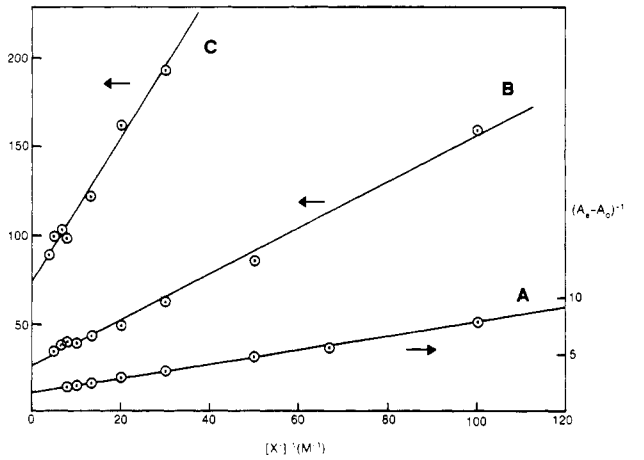

FIGURE 1: Plots providing a basis for the calculation of K_f; 25 °C, pH 6.1 phosphate buffer, l = 1 cm. (A) $[Cu(dien)N_3]^+$, C_0 = 0.25 mM, I = 0.2 M. (B) Laccase–N_3^-, C_0 = 10.8 μM, I = 0.5 M. (C) Laccase–NCS^-, C_0 = 10.8 μM, I = 0.5 M.

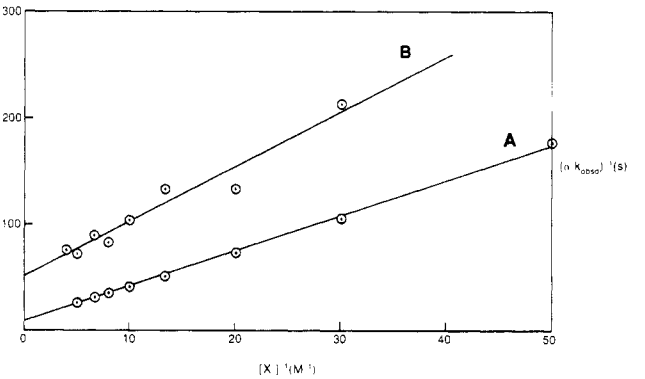

FIGURE 2: Plots of $(\alpha k_{obsd})^{-1}$ vs. $[X^-]^{-1}$ for the anation of laccase type 2 Cu(II) by N_3^- (A) and NCS^- (B) ions in phosphate buffer; 25 °C, pH 6.1, I = 0.5 M.

activated intermediate (eq 4) and outer-sphere complex (eq 5) mechanisms (Sutin & Yandell, 1972).

$$k_{obsd} = \frac{k_1k_2[X^-] + k_{-1}k_{-2}}{k_1 + k_{-1} + k_2[X^-] + k_{-2}} \quad (4)$$

$$k_{obsd} = k'_{-2} + \frac{k'_2K_{os}[X^-]}{1 + K_{os}[X^-]} \quad (5)$$

Rate parameters associated with these kinetically indistinguishable mechanisms were derived, as before (Holwerda & Gray, 1974), from the least-squares slopes and intercepts of linear $(\alpha k_{obsd})^{-1}$ vs. $[X^-]^{-1}$ plots (Figure 2), where $\alpha = K_f[X^-]/(1 + K_f[X^-])$. Results for the laccase–azide reaction are k_1 (or k'_2) = (1.25 ± 0.19) × 10^{-1} s^{-1} and k_{-2} (or k'_{-2}) = (1.50 ± 0.16) × 10^{-2} s^{-1}. For the laccase–thiocyanate reaction, k_1 (or k'_2) = (2.0 ± 0.3) × 10^{-2} s^{-1} and k_{-2} (or k'_{-2}) = (1.1 ± 0.2) × 10^{-2} s^{-1}. The linearity of the $(\alpha k_{obsd})^{-1}$ vs. $[X^-]^{-1}$ plot for NCS^- clearly is not as satisfactory as that for N_3^-, as the comparative smallness of ΔA_{400} resulted in larger uncertainties in $k_{obsd}(SCN^-)$.

Anation of Laccase by N_3^- in Mes and Acetate Buffers. To determine whether buffer anions have any effect on the anation mechanism, we studied the reaction of N_3^- with laccase at 25 °C, pH 6.0, in I = 0.1 M Mes and acetate buffers containing sufficient $NaNO_3$ to maintain the total ionic strength at 0.25 M. As shown in Figure 3, plots of k_{obsd} vs. $[N_3^-]$ are linear, consistent with the expression

$$k_{obsd} = k_3 + k_4[N_3^-] \quad (6)$$

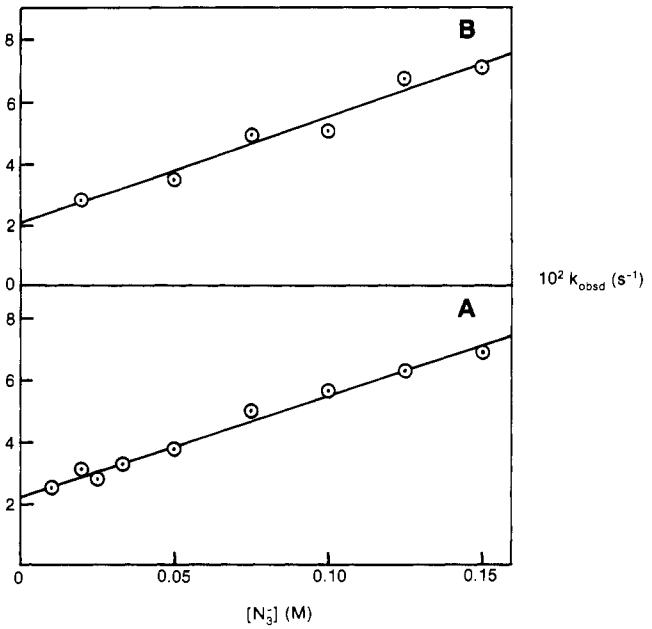

FIGURE 3: Plots supporting the relationship $k_{obsd} = k_3 + k_4[N_3^-]$ for the anation of laccase type 2 Cu(II) by N_3^- in Mes (A) and acetate (B) buffers; 25 °C, pH 6.0, I = 0.25 M.

K_f and $\Delta\epsilon_{405}$ values determined for the reaction in Mes buffer are 31 ± 2 M^{-1} and (1.5 ± 0.1) × 10^3 M^{-1} cm^{-1}, respectively. The least-squares parameters derived from the data in Figure 3 are the following: in Mes, k_3 = (2.2 ± 0.1) × 10^{-2} s^{-1} and k_4 = (3.3 ± 0.2) × 10^{-1} M^{-1} s^{-1}; in acetate, k_3 = (2.1 ± 0.3) × 10^{-2} s^{-1} and k_4 = (3.4 ± 0.2) × 10^{-1} M^{-1} s^{-1}.

Spectroscopic and Equilibrium Studies of the Anation of $[Cu(dien)(H_2O)]^{2+}$, $[Cu(terpy)(H_2O)]^{2+}$, *and* $[Cu(Me_5dien)(H_2O)]^{2+}$ *by* N_3^-. Spectroscopic and equilibrium properties were determined for three azide complexes formed through the displacement of H_2O from an equatorial position on Cu(II) to provide a basis for comparison with the laccase type 2 Cu(II)–N_3^- complex. In each case, the other three equatorial positions were occupied by strongly bound nitrogen atoms of a tridentate ligand, diethylenetriamine, 2,2′,2″-terpyridine, or 1,1,4,7,7-pentamethyldiethylenetriamine. Difference spectra (Cu(II)–N_3^- complex – (Cu(II)–H_2O complex) showed that λ_{max} values for the azide to copper(II) charge-transfer transitions of $[Cu(dien)N_3]^+$, $[Cu(terpy)N_3]^+$, and $[Cu(Me_5dien)N_3]^+$ are 346, 348, and 375 nm, respectively.

The formation constant of the laccase type 2 Cu(II)–N_3^- complex at 25 °C, pH 6.1 ± 0.1, I = 0.2 M (0.05 M from sodium phosphate buffer), is 45 M^{-1} (Holwerda & Gray, 1974). Under these conditions, K_f for $[Cu(dien)N_3]^+$ (based on the data in Figure 1) was found to be 27 ± 1 M^{-1}; $\Delta\epsilon_{346}$ = (2.4 ± 0.1) × 10^3 M^{-1} cm^{-1}. Increasing the ionic strength to 0.5 M (0.10 M from phosphate buffer) has very little effect on K_f (25 ± 3 M^{-1}).

The temperature dependence of K_f for $[Cu(dien)N_3]^+$ and $[Cu(terpy)N_3]^+$ was examined in the ranges 18.6–60.1 °C (six temperatures) and 17.5–64.2 °C (five temperatures), respectively. The azide concentration was varied from 0.03 to 0.15 M in I = 0.10 M acetate buffer, pH 6.0, holding the total ionic strength constant at 0.25 M; C_0 values for the dien and terpy complexes were 0.4 and 0.08 mM, respectively. Linear van't Hoff plots of log K_f vs. $1/T$ (data not shown) yielded least-squares thermodynamic parameters associated with the formation of $[Cu(dien)N_3]^+$ [K_f(25 °C) = 36 ± 1 M^{-1}, $\Delta H°$ = −2.2 ± 0.3 kcal/mol, $\Delta S°$ = 0 ± 1 eu] and $[Cu(terpy)N_3]^+$ [K_f(25 °C) = 68 ± 3 M^{-1}, $\Delta H°$ = −2.9 ± 0.4 kcal/mol, $\Delta S°$ = −1 ± 1 eu]; $\Delta\epsilon_{346}$(dien) = (3.1 ± 0.2) × 10^3 M^{-1} cm^{-1}, $\Delta\epsilon_{348}$(terpy) = (3.1 ± 0.2) × 10^3 M^{-1} cm^{-1}.

Unfortunately, complex kinetic behavior was observed for the laccase type 2 Cu(II)–N_3^- reaction above 30 °C and below 15 °C, and $(A_e - A_0)^{-1}$ vs. $[N_3^-]^{-1}$ plots no longer were linear, preventing the measurement of standard and activation enthalpy or entropy changes. The reaction of N_3^- with $[Cu(dien)(H_2O)]^{2+}$ was found to be complete within the mixing time (3 ms) of the stopped-flow apparatus, even at low azide concentrations.

Discussion

Proton and oxygen-17 magnetic resonance relaxation studies of fully oxidized laccase have shown that the type 2 copper atom is bound to a single, slowly exchanging water molecule (pK = 6.2) at an equatorial coordination position (Goldberg et al., 1980). In oxidized laccase, the type 2 and type 3 copper atoms evidently occupy a common cavity (Bränden & Deinum, 1977) connected to the bulk medium through a small opening which permits rapid proton exchange, but blocks the passage of H_2O or anions in the medium (Goldberg et al., 1980). A slow, crevice-opening conformational change with an activation energy of 12–15 kcal/mol was proposed to account for the slow association and dissociation of type 2 Cu(II) ligands (Goldberg et al., 1980). Our kinetic data are fully consistent with this model of the type 2 Cu(II) site but indicate that the requirement for a conformational change is not the only factor governing the substitutional reactivity of this site.

If the anation of laccase type 2 Cu(II) was controlled entirely by a conformational change, then the activated intermediate mechanism would pertain, with laccase* corresponding to the crevice-opened intermediate. On this basis, the value of k_1 would be independent of the incoming anion. This is clearly not the case for the anation of type 2 Cu(II), as apparent k_1 values for N_3^- and NCS^- differ by a factor of six. Assigning these rate constants to k'_2 in the outer-sphere complex mechanism provides a reasonable basis for understanding the higher reactivity of the more basic anion [pK_a-(HN_3) = 4.75] (Yost & Russel, 1946).[2] Thus, the nucleophilicity of the incoming group will have some effect on the rate of H_2O displacement in a dissociative interchange (I_d) mechanism, and an even larger effect in the case of associative interchange (I_a) (Wilkins, 1974). While the nature of the outer-sphere interaction between laccase and pseudohalide ions is not known, K_{os} values of ca. 2 (N_3^-) and 10 (NCS^-) M^{-1} (I = 0.5 M) may be estimated from the kinetic data.

Further support for mechanism 2 comes from the change in the rate low for the laccase–N_3^- reaction in Mes or acetate buffers compared with phosphate media. For mechanism 1, a rate law consistent with eq 6 will be observed only if $k_2[N_3^-] \ll k_1 + k_{-1} + k_{-2}$. The parameters k_1, k_{-1}, and k_{-2} should be nearly invariant with changes in the medium, being controlled largely by the protein-dependent activation requirements for the generation of laccase*. Since there is no reason to expect k_2 to be smaller in Mes or acetate rather than phosphate buffer, mechanism 1 cannot account for the change in rate law.

Mechanism 2 is consistent with eq 6 under conditions when

[2] While mechanism 2 provides the most straightforward explanation of our kinetic results, it should be noted that a scheme of the form

$$\text{laccase} + N_3^- \overset{\text{fast}}{\rightleftharpoons} \text{laccase}\cdot X^-$$

$$\text{laccase} + N_3^- \rightleftarrows \text{laccase}\text{–}X^-$$

cannot be completely ruled out, where laccase·X^- represents an outer-sphere, dead-end complex.

K_{os} is very small, i.e., $K_{os}[N_3^-] \ll 1$, such that $k_3 = k'_{-2}$ and $k_4 = k'_2K_{os}$. Electrostatic interactions with anions may strongly perturb the surface charge distribution of redox-active metalloenzymes, resulting in large changes in K_{os} for precursor complex formation with redox agents and, ultimately, in the electron transfer rate constant (Taborsky, 1972; Cookson et al., 1980; Yoneda & Holwerda, 1978). Variations in K_{os} with changing medium are expected for the laccase–azide reaction, therefore, when the different affinities of the phosphate, acetate, and Mes buffer anions for electrostatic binding to the protein surface are considered. The similarity between k_3 (Mes and acetate) and k'_{-2} (phosphate) values may also be cited in support of mechanism 2. Only minor changes in rate parameters for anation by azide result from an increase in ionic strength from 0.2 ($k'_2 = 1.94 \times 10^{-1}$ s^{-1}, $k'_{-2} = 1.36 \times 10^{-2}$ s^{-1}, $K_{os} \simeq 3$ M^{-1}; 25.1 °C, pH 6.1) (Holwerda & Gray, 1974) to 0.5 M in phosphate buffer, as k'_2 decreases by 36% while k'_{-2} increases by 10%.

A useful perspective on our results may be gained through a comparison with kinetic studies of the anation of $[Cu(tren)(H_2O)]^{2+}$ and $[Cu(Me_6tren)(H_2O)]^{2+}$ by N_3^- and NCS^- (Coates et al., 1979). A dissociative interchange mechanism was proposed, with dissociation rate constants (k'_{-2}) for $[Cu(Me_6tren)N_3]^+$ (1.98 s^{-1}) and $[Cu(Me_6tren)NCS]^+$ (2.03 s^{-1}) (25 °C, pH 6.80, I = 1.0 M) being nearly identical and relatively slow for substitution at Cu(II), analogous to our type 2 Cu(II)–N_3^- and Cu(II)–NCS^- results. Loss of N_3^- from $[Cu(tren)N_3]^+$ was found to be nearly 5 orders of magnitude faster ($k'_{-2} = 1.477 \times 10^5$ s^{-1}, 25 °C) than that from the Me_6tren analogue, in which the azide ion lies within a considerably more hydrophobic environment (Coates et al., 1979). This remarkable rate difference was attributed, in part, to the inaccessibility of the N_3^- leaving group in $[Cu(Me_6tren)N_3]^+$ to solvation while situated within a sterically constrained, hydrophobic pocket. These results suggest that the hydrophobicity of the cavity containing laccase type 2 Cu(II) contributes significantly to the slowness of azide dissociation, independently of conformational activation requirements associated with crevice opening. As Pecht and co-workers have pointed out (Goldberg et al., 1980), the low pK value of 6.2 associated with H_2O coordinated to type 2 Cu(II) reflects a relatively hydrophobic environment. This point is amplified by comparisons of the type 2 Cu(II)–OH_2 pK value with those for $[Cu(tren)(H_2O)]^{2+}$ (9.37) and $[Cu(Me_6tren)(H_2O)]^{2+}$ (8.52) (Coates et al., 1974).

Formation constants of the type 2 Cu(II)–N_3^- complex are comparable to those measured for $[Cu(dien)N_3]^+$ and $[Cu(terpy)N_3]^+$ under identical or similar conditions. $\Delta G°$ for these anation reactions must also be similar, therefore, in spite of the considerably higher activation free energy required in the formation of the laccase–azide complex. Caution must be used in making these comparisons, however, as both spectroscopic (Morpurgo et al., 1974) and kinetic (Wherland et al., 1975) evidence indicates that azide binds more strongly to an alternate laccase site, probably type 3 copper, than it does to type 2 Cu(II). These additional strongly bound azide ions certainly could influence K_f for type 2 Cu(II)–N_3 and, for that matter, the anation mechanism as well.

ESR studies of partially reduced laccase have shown that the symmetry of the type 2 copper coordination sphere changes from axial (Malmström et al., 1970) to pseudotetrahedral or 5-coordinate upon anation by N_3^- (Desideri et al., 1979). This structural change evidently is transmitted through the polypeptide structure, as a new CD band at 355 nm is observed and the reduction potential of type 1 Cu(II) increases, even though the "blue" cupric atom is not directly ligated by N_3^- (Morpurgo et al., 1974). Comparisons of λ_{max} values for N_3^- (σ) → Cu(II) ($d_{x^2-y^2}$) LMCT transitions reported in this paper provide additional evidence that a major structural change at type 2 Cu(II) accompanies azide binding. Thus, the $d_{x^2-y^2}$ orbital, highest in energy of the 3d orbitals in a square-planar complex, will be stabilized as the square-planar structure is perturbed toward tetrahedral or trigonal bipyramidal (Ballhausen, 1962). Such stabilization will be reflected in a shift to lower energy (and longer λ_{max}) of the azide to copper(II) charge-transfer transition. The LMCT band of the laccase type 2 Cu(II)–N_3^- complex is observed at considerably longer wavelength than those of the square-planar $[Cu(dien)N_3]^+$ (Morpurgo et al., 1973) and $[Cu(terpy)N_3]^+$ ions, while the positions of the bands for $[Cu(Me_5(dien)N_3]^+$ [probably intermediate between square and trigonal pyramidal (Felthouse & Hendrickson, 1978)] and $[Cu(Me_6tren)N_3]^+$ [385 nm (Coates et al., 1979), probably trigonal bipyramidal (Di Vaira & Orioli, 1968)] are substantially closer to that (405 nm) characteristic of the laccase–azide complex.

Finally, we consider the mechanistic implications of our anation studies for the interaction of phenolate anion laccase substrates with the metalloenzyme. Both aerobic turnover (Andréasson & Reinhammar, 1976) and post-steady-state studies (Clemmer et al., 1979) have demonstrated that reduction of laccase type 1 Cu(II) by hydroquinones is considerably faster than the limiting rate constant ($k'_{-2} + k'_2$) for anation of type 2 Cu(II) in the fully oxidized metalloprotein by NCS^- or N_3^-. Even taking its substantial basicity advantage over N_3^- into account, it appears unlikely that $HOC_6H_4O^-$ anates type 2 Cu(II) in fully oxidized laccase rapidly enough for this to be a prerequisite to the reduction of type 1 Cu(II). It should be noted, however, that the affinity of type 2 Cu(II) for N_3^- increases dramatically upon partial reduction of laccase (Morpurgo et al., 1974), such that water and extrinsic ligand exchange at this site become much more efficient (Goldberg et al., 1980). Rapid preequilibrium binding of hydroquinones at type 2 Cu(II) documented for the post-steady-state reduction of type 1 Cu(II) (Clemmer et al., 1979) thus is not inconsistent with the present kinetic results. Under these conditions, a slow conformational change evidently is not associated with the anation of type 2 Cu(II) but is required to permit access of the coordinated substrate to the blue copper atom (Clemmer et al., 1979).

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Proton Release during the Pre-Steady-State Oxidation of Aldehydes by Aldehyde Dehydrogenase. Evidence for a Rate-Limiting Conformational Change†

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ABSTRACT: A transient release of protons with an amplitude corresponding to one proton per active site has been observed for the oxidation of propionaldehyde, acetaldehyde, and benzaldehyde by sheep liver cytoplasmic aldehyde dehydrogenase at pH 7.6 with phenol red as indicator. At saturating substrate levels, the rate constants for the proton burst are in each case the same, and for acetaldehyde and propionaldehyde show the same dependence on the concentrations of the substrates, as the rate constants for the transient production of NADH reported previously [MacGibbon, A. K. H., Blackwell, L. F., & Buckley, P. D. (1977) *Biochem. J. 167*, 469–477]. Although, with propionaldehyde as a substrate, a full proton burst is also observed at pH 6.0, no proton burst is observed at pH 9.0. For 4-nitrobenzaldehyde, there is no burst in NADH production, but a burst in proton release is observed, showing that proton release precedes hydride transfer. No protons were released during the binding of the substrate analogues acetone and chloral hydrate nor on reaction of the enzyme with the inhibitor tetraethylthiuram disulfide (disulfiram). A model is proposed in which the rate-limiting step in the pre-steady-state phase of the reaction is a conformational change which occurs after the binding of aldehydes to the enzyme. As a result of the conformational change, the environment of a functional group on the enzyme, which initially has a pK_a of about 8.5, is perturbed to give a final pK_a value for the group of less than 5. Computer simulations were used to show that the model accurately reproduces all of the experimental data. The lack of observation of a second transient proton release, as required by the overall stoichiometry, argues that its release occurs in a slow step prior to NADH dissociation.

Extensive transient kinetic studies have been carried out on the oxidation of aldehydes by the cytoplasmic aldehyde dehydrogenase from sheep liver (MacGibbon et al., 1977a,b) and have led to the following kinetic scheme (Scheme I). In Scheme I, release of NADH contributes significantly to the rate-limiting step in the steady state, and the observation of a burst in the production of NADH (in both nucleotide fluorescence and absorbance) is consistent with the first appearance of enzyme intermediates containing NADH occurring before the rate-determining step in the enzyme-catalyzed reaction. It is assumed that the ternary complexes E·NAD$^+$·Ald and E·NADH·acid are rapidly interconverted and that the concentration of E·NADH·acid is low so that no transient results from this intermediate. Computer simulations

Scheme I

$$E \underset{k_{-1}}{\overset{k_1,\ NAD^+}{\rightleftharpoons}} E{\cdot}NAD^+ \underset{k_{-2}}{\overset{k_2,\ Ald}{\rightleftharpoons}} E{\cdot}NAD^+{\cdot}Ald \xrightarrow{k_3} E{\cdot}NADH \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + NADH$$

based on Scheme I, using rate constants determined from pre-steady-state and steady-state studies (MacGibbon et al., 1977a,c), give a good approximation to the experimental data although it has been noted (MacGibbon et al., 1977a) that additional steps are required to explain some of the experimental data.

When saturating concentrations of NAD$^+$ are premixed with enzyme before reaction with propionaldehyde, Scheme I may be treated as a system of two consecutive first-order reactions (MacGibbon et al., 1977a), and an equation relating the observed burst rate constant to the kinetic constants and the concentration of aldehyde has been derived (eq 1). The

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