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Solvent and Solvent Proton Dependent Steps in the Galactose Oxidase Reaction†

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ABSTRACT: Solvent and solvent proton dependent steps involved in the mechanism of the enzyme galactose oxidase have been examined. The deuterium kinetic solvent isotope effect (KSIE) on the velocity of the galactose oxidase catalyzed oxidation of methyl β-galactopyranoside by O_2 was measured. Examination of the thermodynamic activation parameters for the reaction indicated that the isotope effect was attributable to a slightly less favorable $\Delta H^{\ddagger}$ value, consistent with a KSIE on proton transfer. A detailed kinetic analysis was performed, examining the effect of D_2O on the rate of reaction over the pH range 4.8–8.0. Both pL–rate profiles exhibited bell-shaped curves. Substitution of D_2O as solvent shifted the pK_{es} values for the enzymic central complex: pK_{es1} from 6.30 to 6.80 and pK_{es2} from 7.16 to 7.35. Analysis of the observed shifts in dissociation constants was performed with regard to potential hydrogenic sites. pK_{es1} can be attributed to a histidine imidazole, while pK_{es2} is tentatively assigned to a Cu^{2+}-bound water molecule. A proton inventory was performed (KSIE = +1.55); the plot of k_{cat} vs. mole fraction D_2O was linear, indicating the existence of a single solvent-derived proton involved in a galactose oxidase rate-determining step (or steps). The pH dependence of CN^- inhibition was also examined. The K_i–pH profile indicated that a group ionization, with pK_a = 7.17, modulated CN^- inhibition; K_i was at a minimum when this group was in the protonated state. The inhibition profile followed the alkaline limit of the pH–rate profile for the enzymic reaction, suggesting that the group displaced by CN^- was also deprotonating above pH 7. Consistent with this suggestion was the D_2O-dependent shift in pK_a (+0.17) of the group modulating CN^- inhibition which was similar to the shift observed in pK_{es2}. Nuclear and electron magnetic resonance studies have shown previously that CN^- coordinates equatorially to the enzymic Cu(II), apparently displacing a water molecule [Marwedel, B. J., Kosman, D.J., Bereman, R. D., & Kurland, R. J. (1981) *J. Am. Chem. Soc. 103*, 2842–2847]. The data indicate that this Cu(II)-bound H_2O is required in the protonated aquo state for catalysis and is responsible for the KSIE observed in the pL–rate profile. A mechanism that couples electron transfer to O_2 with the proton transfer step(s) probed by these experiments is discussed.

Galactose oxidase (EC 1.1.3.9) is a copper-containing enzyme that catalyzes the conversion of a primary alcohol and molecular oxygen to an aldehyde and hydrogen peroxide (Kosman et al., 1974; Ettinger & Kosman, 1981). GO[1] is the only known mononuclear Cu(II) protein that catalyzes a two-electron redox reaction involving molecular oxygen without the involvement of another metal ion or organic cofactor. Because of this unique characteristic and because it is the only known type 2 (nonblue) Cu(II) protein possessing a single such prosthetic group, GO has been of considerable interest both spectrally and mechanistically. These features make GO an experimentally attractive choice for the study of the structure and function of Cu(II)-active sites involved in substrate-level oxidation.

One of the least well-characterized aspects of metal-dependent oxidation is the coupling of the requisite H^+- and electron-transfer steps. GO exhibits a bell-shaped pH dependence for V_{max}. The ascending limb of the pH–rate profile has been assigned to a histidine imidazole (pK_a = 6.3) which may provide base catalysis of abstraction of a substrate carbinol proton (Kwiatkowski et al., 1977). The origin of the descending, alkaline limb has not been elucidated. However, a variety of kinetic and spectral observations indicate that this group ionization could be associated with an equatorially coordinated water molecule. Specifically, ^{17}O ESR measurements show H_2O to coordinate equatorially to the Cu(II) (Melnyk, 1979). This H_2O is displaced by CN^-, known also

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[1] Abbreviations: GO, galactose oxidase; ESR, electron spin resonance; DMF, dimethylformamide.

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to coordinate equatorially (Giordano et al., 1974; Bereman & Kosman, 1977; Marwedel et al., 1981), again via direct ESR observation. In addition, CN^-, kinetically, is a noncompetitive inhibitor with respect to alcohol substrate, suggesting a role for this coordination site in turnover (Winkler et al., 1981; Ettinger & Kosman, 1981). The alcohol substrate does not bind at this site (Marwedel & Kurland, 1981). Thus, this H_2O site appears to be of some importance in the reaction mechanism, and its role requires elucidation if the enzyme mechanism, itself, is to be fully understood.

The possible involvement of metal-coordinated H_2O in the reactions catalyzed by a number of metalloenzymes and model complexes has been evaluated. For example, Zn^{2+}–H_2O species in carboxypeptidase may provide general-acid catalysis via proton transfer to the leaving group (Spratt et al., 1983). A similar mechanism may obtain for Cu^{2+}- and Zn^{2+}-catalyzed amide hydrolysis by a model complex (Groves & Chambers, 1984). Also relevant is the pH dependence and kinetic solvent isotope effect observed for laccase turnover (Koudelka & Ettinger, 1985). As noted for GO, the binding of a water molecule to the type 2 (nonblue) Cu(II) in laccase has been detected by using ^{17}O ESR (Deinum & Vänngård, 1975). The reductant independent pH–rate profile (descending) for laccase exhibits (in H_2O) a pK_{a_2} = 5.91, a pK_{a_2} in D_2O of 6.17, and a KSIE of 2.12 (Koudelka & Ettinger, 1985). These values could be attributed to the Cu^{2+}–H_2O species in laccase acting as a proton donor during turnover. In addition, general-acid catalysis of acetol (monohydroxyacetone) oxidation by O_2 catalyzed by Cu(II)–tetrakis(pyridine) has been suggested, on the basis of the pH dependence of the reaction and measured KSIE (Driscoll & Kosman, 1987). Thus, there is accumulating evidence that transition metal ion bound water can act as an effective Brønstëd acid and that general-acid catalysis of O_2 reduction does occur. The question whether or not these are characteristics of the H_2O bound at the active site Cu(II) in GO and of the enzymic reaction mechanism is the focus of the experiments discussed herein.

Materials and Methods

Materials

Glass-distilled water was used throughout the experiments. Galactose oxidase was isolated and purified from cultures of the fungus *Dactylium dendroides* according to previously described procedures (Tressel & Kosman, 1980). Methyl β-galactopyranoside and deuterium oxide were purchased from Sigma and used as received. All other materials used were reagent grade products.

Methods

Kinetic Measurements. Initial velocity measurements were obtained under air (0.24 mM oxygen). A biological oxygen monitor (YSI 53), equipped with a Clark-type electrode, was used to record the amount of oxygen uptake upon enzyme addition. The reaction temperature was maintained at 25 ± 0.1 °C unless otherwise noted. Appropriate corrections were made for the solubility of oxygen at temperatures other than 25 °C. Indicated experimental pD values of D_2O-containing buffers were calculated by adding 0.40 to the pH meter reading (Glasoe & Long, 1960).

Titration of Model Complexes. Cu(II)–diethylenetriamine was prepared by mixing 20 mM copper perchlorate (from Alfa) with a stoichiometric amount of diethylenetriamine (obtained from Eastman) (Allison & Angelici, 1971; Morpurgo et al., 1973). Cu(II)–pyr_3 was prepared by titrating copper perchlorate (20 mM) with 60 mM pyridine (obtained from Aldrich). The formation of the complexes was followed by their visible absorbance spectra with λ_{max} values of 620 nm for Cu(II)–diethylenetriamine (Morpurgo et al., 1973) and 670 nm for Cu(II)–pyr_3 (DaCosta, 1982). All model complex solutions were prepared in a mixed solvent containing 20% DMF and either 80% H_2O or 80% D_2O. Potentiometric titrations were performed and titration curves were analyzed as described by Allison and Angelici (1971). The fitting program PENNZYME (Kohn et al., 1979) was used to fit the data to the appropriate pH function.

Data Handling. Initial velocity data were handled by computer fitting to the Michaelis–Menten equation using the computer program MM-FIT (provided by Dr. Eric Dahmer, Department of Biochemistry, State University of New York at Buffalo). Arrhenius plot, proton inventory, and slope and intercept data were analyzed by using the linear regression fitting program POLYFIT (provided by Dr. James Spain, Department of Biology, Clemson University). The pL–rate data were computer fit to individual rate laws by using the PENNZYME program, which optimizes kinetic parameters through a two-stage nonlinear weighted least-squares minimization (Kohn et al., 1979).

Results

Kinetic Solvent Isotope Effect (KSIE): Temperature and pH Dependence. The solvent isotope study is a nonperturbing probe useful in studying enzyme mechanism and transition-state characterization (Schowen & Schowen, 1982). The substitution of deuterium for protium in the hydrogenic sites of water leads to exchange into some positions of the enzyme and its substrates which may affect the kinetic rate constants of the catalyzed reaction. However, one must first establish that this exchange does not alter enzyme conformation or stability or cause a change in the catalytic mechanism. Therefore, the kinetic parameters V_{max}, K_m, and V_{max}/K_m in a solvent containing D_2O were examined as was the effect of D_2O on the thermodynamic activation parameters for the enzymic reaction.

Figure 1 displays linear transformations of kinetic data fit initially to the Michaelis–Menten equation which were obtained for the GO-catalyzed oxidation of methyl β-galactopyranoside. Substitution of D_2O for H_2O altered V_{max} and V_{max}/K_m while K_m for the alcohol substrate remained constant within experimental error. Figure 1 (inset) shows that the ratio of the initial velocities in the two solvent mixtures was constant at all substrate concentrations examined, consistent with the insensitivity of K_m (alcohol) to isotopic substitution.

The kinetic constants for this reaction were examined over the temperature range 10–38 °C with H_2O or D_2O as solvent. From the Arrhenius plots (Figure 2), the thermodynamic activation parameters were determined; these parameters are summarized in Table I. The results show that the free energy of activation $\Delta G^{\ddagger}$ and the activation enthalpy $\Delta H^{\ddagger}$ values were slightly more favorable in H_2O, while the activation entropy values $\Delta S^{\ddagger}$ were equivalent. Furthermore, the inset of Figure 2 shows that the ratio of the maximal rates of catalysis remained constant as a function of temperature. Together, these results indicate that D_2O does not significantly alter enzyme conformation nor does it cause a change in the mechanism (or rate-limiting step).

Figure 3 displays pL–rate profiles for the GO-catalyzed oxidation by O_2 of methyl β-galactopyranoside. Both curves were bell shaped with the data points fitting well to eq 1. The

$$k_{cat,obsd} = k_{cat,max}/\{1 + ([H^+]/K_{es1}) + (K_{es2}/[H^+])\} \quad (1)$$

term $k_{cat,max}$ is the pH-independent value for the turnover rate of the enzyme, while K_{es1} and K_{es2} represent the dissociation

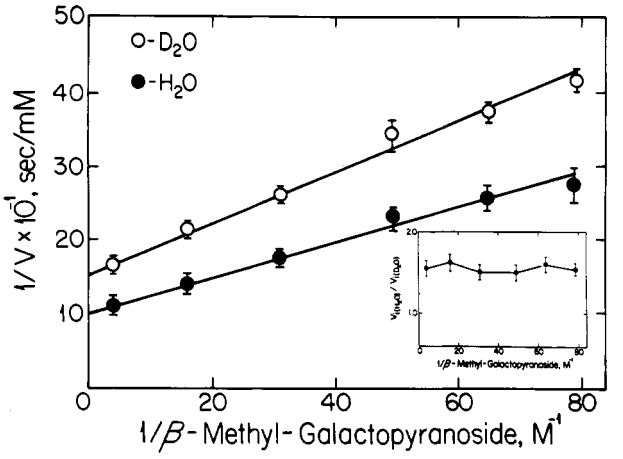


FIGURE 1: Lineweaver–Burk double-reciprocal plot for the GO-catalyzed oxidation of methyl β-galactopyranoside. Assay buffers were used at either pH 6.70 or pD 7.10. Data points are the average of triplicate measurements fit to the Michaelis–Menten equation. The lines are based on these fits. Experimental points were determined under air (0.24 mM oxygen) in solvents containing either H_2O (●) or D_2O (○). The inset displays the ratio of the rates of oxidation of methyl β-galactopyranoside by GO in either solvent plotted vs. reciprocal substrate concentration.

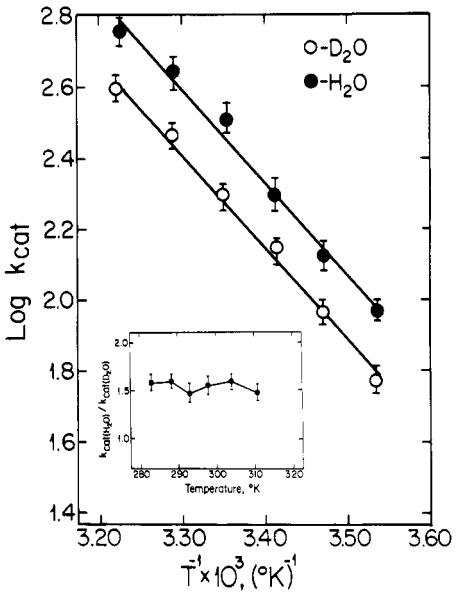


FIGURE 2: Arrhenius plot for the GO-catalyzed oxidation of methyl β-galactopyranoside. Assay buffers were used at either pH 6.70 or pD 7.10. Experimental k_{cat} values were calculated from maximal rate values obtained under air (0.24 mM oxygen) at each indicated temperature in solvents containing either H_2O (●) or D_2O (○). The lines are based on a fit of the data using POLYFIT. The inset displays the ratio of the rates of oxidation of methyl β-galactopyranoside by GO in either solvent plotted vs. temperature.

Table I: Thermodynamic Activation Parameters Determined for GO-Catalyzed Oxidation of *O*-Methyl β-Galactopyranoside[a]

solvent	k_{cat} (s)	$\Delta G^{\ddagger}$ (kcal/mol)	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu)
H_2O	325.0 ± 15.0	13.98 ± 0.10	11.04 ± 0.40	9.84 ± 1.68
D_2O	210.0 ± 18.0	14.24 ± 0.11	11.30 ± 0.40	9.87 ± 1.68

[a] Determined at pH 6.70 and pD 7.10, and under air (0.24 mM oxygen) in a buffer solution containing either H_2O or D_2O as solvent. Temperature ranged from 10 to 38 °C. Parameters were determined through the use of POLYFIT, a linear regression analysis program.

constants for the prototropic groups which modulate the observed k_{cat} values (Kwiatkowski et al., 1977). The pK_{es} values associated with the enzyme central complex (E·Alc·O_2) were pK_{es1} = 6.30 ± 0.05 and pK_{es2} = 7.16 ± 0.05.

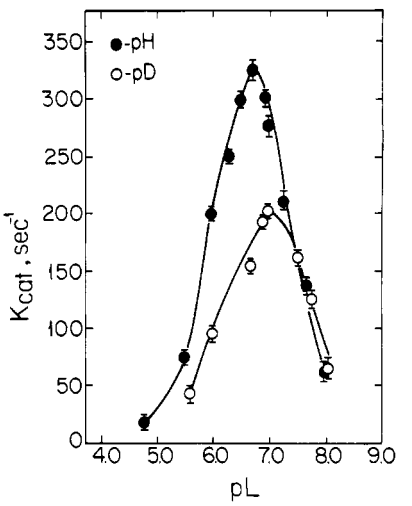


FIGURE 3: pL–k_{cat} profile for the GO-catalyzed oxidation of methyl β-galactopyranoside. Reactions were performed under air (0.24 mM oxygen) at indicated pH (●) or corrected pD (○) values. Bell-shaped curves are based on the fits of the maximal rate values to the pH-dependent rate equation used (see text).

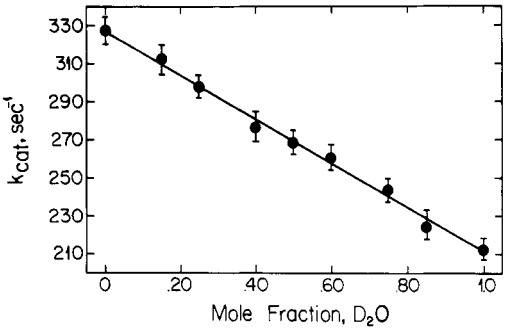


FIGURE 4: Proton inventory data conducted under air (0.24 mM oxygen) for the GO-catalyzed oxidation of methyl β-galactopyranoside performed at equivalent pL (pH 6.70, pD 7.10). The pH meter readings were adjusted appropriately to correct for isotopic dilution. Data points are the average of triplicate measurements obtained at each indicated pH. The simulated fit was determined by using the polynomial regression analysis program POLYFIT. The same curve was generated by simulation (see Discussion).

The pH optimum was 6.70. When D_2O was substituted for H_2O as solvent, a shift in these pK_{es} values as well as a decrease in the overall rate of reaction was observed. The k_{cat} values determined in D_2O as solvent also fit well to eq 1. This fit yielded the following dissociation constants: pK_{es1} = 6.80 ± 0.05 and pK_{es2} = 7.35 ± 0.06. The pD optimum was 7.10. Thus, pK_{es1} exhibited a shift of +0.50 pH unit, which is "standard" for weak, heteroatom general acids, i.e., imidazolium (Schowen & Schowen, 1982). This indicated also that isotopic substitution in the solvent had minor effects on protein conformation. On the other hand, the value for ΔpK_{es2}, +0.19, is atypical of oxygen or nitrogenous weak acids (e.g., carboxyl or imidazole). It does fall in the range characteristic of sulfhydryl acids or a di- or trivalent metal-bound water molecule (Schowen & Schowen, 1982).

KSIE in Mixed Isotopic Waters: Proton Inventory. A proton inventory was conducted in a series of mixed isotopic waters to characterize the protic events responsible for the observed KSIE. When comparing kinetic constants in isotopic waters, one must use comparable positions on the pH–rate profile. Thus, pH 6.70 and pD 7.10 represent equivalent pL, that is, the pL at which equal fractions of catalytically active species are present in the two solvents. The magnitude of the KSIE was 1.55 ± 0.02, and as Figure 4 shows, the rate data were best fit to a linear equation for which n = 1. The

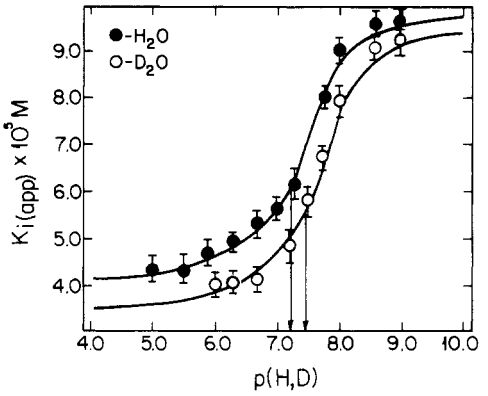


FIGURE 5: Apparent K_i values for the inhibition of the GO reaction by CN^- plotted as a function of pH. Reactions were performed under air (0.24 mM oxygen) at a constant temperature of 25 °C in a solvent containing either H_2O (●) or D_2O (○). The pK_{a_i} values determined in either solvent by fitting to eq 2 are indicated by the arrows. The theoretical curves were generated by the fit to this equation.

(non)linear regression fitting program POLYFIT was used to analyze these data; the coefficient for an x^2 term was 10^{-5}. The linear shape of the k_{cat} vs. mole fraction of deuterium plot indicated the existence of a single solvent-derived proton in a rate-determining step (or steps) of the reaction. That is, the isotope effect seen is generated by a single such hydrogenic site associated with the transition state of the reaction mechanism (Schowen, 1972).

Inhibition by CN^-: pH Dependence and Solvent Isotope Effect. The dissociation constants determined from the pL–rate profile data, and the shifts in these dissociation constants, are consistent with the observation that the groups responsible for the observed pL dependence of the GO reaction are a histidine imidazole (Kwiatkowski et al., 1977) and another group with a $pK_a = 7.16$, which could be a Cu-bound H_2O molecule. To probe further the identity of the latter group, the pH dependence of CN^- inhibition was examined to determine whether the H_2O molecule bound at the Cu(II) (Melnyk, 1979) and displaced by CN^- (Melnyk, 1979; Marwedel et al., 1981) is deprotonating in the neutral pH region during catalytic turnover.

The pH vs. K_i profile shown in Figure 5 illustrates the effect of pH and solvent composition on CN^- inhibition of the GO reaction. Apparent K_i values were determined over the pH range 5.0–8.9[2] and then fit to eq 2. Equation 2 describes the

$$K_{i,app} = K_i'(1 + K_{a_i}/[H^+])/(1 + \beta K_{a_i}/[H^+]) \quad (2)$$

pH-dependent behavior of the apparent CN^- inhibition constant ($K_{i,app}$). The term K_i' represents the pH-independent value for this inhibition constant, while K_{a_i} represents the group dissociation constant that modulates the pH-dependent behavior of $K_{i,app}$. The symbol β (fitted value, 0.12) is a solvent-independent factor that describes the limiting value for $K_{i,app}$ as $[H^+] \rightarrow 0$. This limit was demonstrably not due to a term representing the ionization of HCN. Although the pK_a of HCN is 9.3, it is assumed that CN^-, not HCN, is bound to the metal center. Although this would indicate that K_i should increase with decreasing pH, that it does not is most reasonably due to enzymic buffering. Possibly, CN^- binding results in endogenous ligand displacement and an associated ligand protonation.

The experimentally determined $K_{i,app}$ values vary with pH in a manner consistent with titration of a group having a pK_{a_i} = 7.17 ± 0.05 (Figure 5). This indicates that CN^- more readily displaced the protonated form of the group at this coordination site than it did the deprotonated form. Figure 5 also shows the results of experiments in which the effect of D_2O on the inhibition by CN^- was determined. These data also were fit to eq 2. With D_2O as solvent the pK_a value of the observed group ionization was 7.34 ± 0.06, indicating a shift in pK_{a_i} of 0.17 pH unit. Note that the pK_{a_i} value and the shift in pK_{a_i} seen in the CN^- inhibition study were extremely similar to the values determined for the descending limb of the pL–rate profile.

ΔpK_a Values for Equatorial H_2O: Cu(II)–Tris(pyridine) and –Diethylenetriamine. Since little experimental evidence exists regarding the magnitude of the shift in pK_a for hydrated metal ion sites, the potentiometric titration profiles of two simple copper complexes, Cu(II)–diethylenetriamine and Cu(II)–pyr_3, were studied to examine the effect of D_2O on the ionization of Cu-bound H_2O molecules. These complexes have an exchangeable equatorial coordination site to which water is bound in the absence of added ligand (Morpurgo et al., 1973; DaCosta, 1982). Initial absorbance experiments indicated that the complexes were stable over the pH range of the titration. In the alkaline pH range, Cu(II)–diethylenetriamine exhibited a pK_a of 8.42 and a pK_a shift of +0.38 in D_2O, while Cu(II)–pyr_3 displayed a pK_a of 7.11 and a pK_a shift of +0.34. The pK_a value for the former complex was in good agreement with that determined by Allison and Angelici (1971), 8.62, whose titration procedure was employed herein. The increase in the acidity of the equatorial H_2O in the pyridine complex was consistent with the expected effects of triamine and pyridine on the binding of and transfer of positive charge to the water molecule (Allison & Angelici, 1971; Cotton & Wilkinson, 1980). Although these pK_a values are similar to the galactose oxidase kinetic pK_a, pK_{es2}, the shifts were not. The latter were, however, less than those observed for common, weak organic acids.

DISCUSSION

The effect of hydrogen ion concentration on an enzymic reaction may yield information regarding the identity of active site groups involved in catalysis through the characterization of specific ionization constants (Tipton & Dixon, 1983). The pH–k_{cat} profile (Figure 3) and other data (Kwiatkowski et al., 1977) indicate that catalysis by GO requires an unprotonated histidine imidazole. In addition, the pH dependence shows that another protic group having a pK_a greater than 7.0 is required in the protonated state for a catalytically competent enzyme. Thus, the pH-dependent ionization of these two protic groups controls the interconversion of the GO central complex.

Since the dissociation constants associated with an enzymic reaction are dependent upon the isotopic composition of the solvent, the pL–rate profile obtained for such a reaction should change with substitution of deuterium for protium (Schowen, 1972). The observed shift in pK_{es1} of +0.50 is consistent with the shift expected for a histidine imidazole group (Schowen & Schowen, 1982), an assignment in accord with other evidence for such a group at the enzymic active site (Kwiatkowski et al., 1977; Weiner et al., 1977). The descending portion of the same curve was dependent on the ionization of a hydrogenic site, $pK_{es2} = 7.16$. The assignment of this to a Cu-bound water molecule is based on four facts. (1) An H_2O molecule is coordinated to the single, solvent-accessible, equatorial Cu(II) site in galactose oxidase (Melnyk, 1979). (2) The value for pK_{es2} is similar to pK_a values for other hydrated metal ion complexes. (3) The displacement of this H_2O molecule by CN^- has been directly observed by spectral measurement

[2] Enzyme instability precluded kinetic measurement above this pH value (Kwiatkowski et al., 1977).

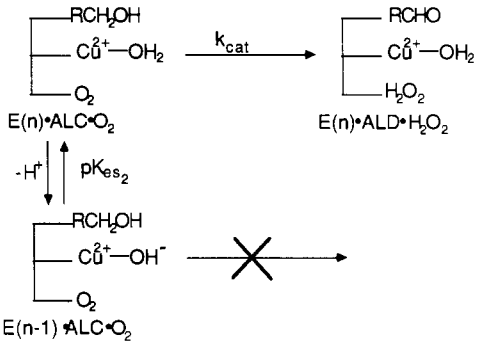
(Bereman & Kosman, 1977; Melnyk, 1979; Kosman et al., 1980; Marwedel et al., 1981); CN^- inhibition kinetically exhibits a pH dependence (pK_{a_i} = 7.17) mimicking the descending limb of the pH–rate profile. (4) The shift of these two pK_a values in deuterium oxide is identical, +0.19 for pK_{es2} and +0.17 for pK_{a_i}.

The proton ionization constants for hydrated metal ions vary considerably and depend markedly on the overall coordination and metal oxidation state (Schowen & Schowen, 1982). The two Cu^{2+} complexes choosen as models exhibited rather typical pK_a values for a single water molecule equatorially coordinated to Cu^{2+} or Zn^{2+}, values that are nearly always lower than those expected or observed for the corresponding hexaaqua complexes (Sillen & Martel, 1964). For example, Groves and Chambers (1984) have reported a similar pK_a value (7.2) for the ionization of an H_2O molecule bound at the Cu^{2+} center of a tridentate azlactam which catalyzes amide hydrolysis. These investigators suggest that such a Cu^{2+} atom can facilitate deprotonation of the coordinated H_2O molecule while also properly positioning the resulting metal–hydroxide for nucleophilic attack. Similarly, the proposed Zn^{2+}–H_2O species involved in the carbonic anhydrase reaction, as well as in the carboxypeptidase A reaction, displays a pK_a of 7–8 (Coleman, 1984; Makinen et al., 1979). A Zn^{2+}-containing complex, biomimetic of the catalytic action of carbonic anhydrase, displays a pK_a of 7.71 (Brown et al., 1982). Allison and Angelici (1971), in addition to Cu(II)–diethylenetriamine, examined several other similar complexes, all of which exhibited pK_a values for the equatorial water of 7.6–9.0. In all of these examples, the metal ion was otherwise three-coordinate, with primarily nitrogenous ligation in a ligand field that was pseudo square planar (and rhombically distorted, in some cases). This is true of the Cu(II) site in galactose oxidase (Giordano et al., 1974; Bereman & Kosman, 1977; Kosman et al., 1980), as apparently it is of all type 2 Cu(II) sites (Peisach & Blumberg, 1974; Boas, 1984). Thus, the assignment suggested for pK_{es2} is completely consistent with the ionization behavior expected of the water molecule bound at the Cu(II) in this enzyme.

Spectral studies have shown that CN^- inhibits GO by coordinating to the Cu(II) center located at the active site (Marwedel et al., 1981; Winkler et al., 1981). In binding at the Cu(II) site, CN^- displaced the equatorially bound H_2O molecule (Melnyk, 1979). The inhibition by CN^- also displayed a pH dependence, yielding a pK_{a_i} of 7.17 which was within experimental error of pK_{es2} determined in the pH–V_{max} study. With D_2O as the solvent a shift in the pK_a modulating the CN^- inhibition was observed. The magnitude of this shift was essentially identical with that associated with the kinetic solvent isotope effect. Thus, at the least, the results indicate that the binding of CN^- to the Cu(II) and the distribution of the enzymic central complex between prototropic forms are associated with the ionization of the same group(s). Since the Cu-bound H_2O molecule is known to be displaced by CN^- and reasonably may have a pK_a of 7.16, the assignment of both pK_a values to this species appears justified.

Scheme I depicts the acid group ionization responsible for controlling catalysis by GO. The fit of the pH–rate data to eq 1 indicates that a single central complex [denoted as E-(n)·Alc·O_2] is capable of proceeding to products. Deprotonation of complex E(n)·Alc·O_2 to E(n − 1)·Alc·O_2 leads to a catalytically inactive enzyme form. Although OH^- would bind more tightly to the Cu^{2+} center than H_2O, the inactivation of the enzyme ascribed to this ionization cannot be due to enhanced ligation alone. This view is supported by the observation that the $K_{i,app}$ term describing CN^- inhibition increased by a factor of only 2 over the pH range 5.0 (fully protonated) to 8.9 (fully deprotonated). This small increase in $K_{i,app}$ is consistent with the notion that the dissociation of the HO(H) ligand from the Cu^{2+} is not as crucial to catalysis as is the ability of the Cu^{2+}–H_2O to donate a proton.

Scheme I



The suggestion that CN^- annation results in the displacement or weakening of the bond to an endogenous Cu^{2+} ligand is not without precedent. The metal ion in carbonic anhydrase is coordinated by three histidine imidazoles (Kannan et al., 1975), yet in the CN^- complex of Cu^{2+}–carbonic anhydrase, only two equivalent nitrogen nuclei are coupled to the unpaired spin density (Haffner & Coleman, 1973). Similarly, the rhombic ESR spectrum of Cu,Zn superoxide dismutase becomes axial in the CN^- complex (Rotilio et al., 1972b; Van Camp et al., 1982), as it does in the absence of CN^- at pH values below 4.5 (Rotilio et al., 1972a). A variety of evidence indicates that both treatments result in the virtual displacement of one of the four histidine imidazoles that coordinate the metal ion (Van Camp et al., 1982). Not surprisingly, buffering of the H^+ released upon (H)CN binding was not observed in these two systems since the pK_a of the displaced group, an imidazole, was below the pH of the titration experiment (Coleman, 1967; Fee et al., 1982). If a ligand in GO is displaced by CN^-, it is not an imidazole (Kosman et al., 1980). A tyrosine phenol might be displaced, however. This possibility is consistent with the Cu^{2+}-associated near-UV and visible electronic transitions (Ettinger, 1974) which are characteristic of phenolate–Cu^{2+} charge transfer (Amundson et al., 1977). Such a group could bind the H^+ resulting from the reaction Cu·H_2O + HCN.

The shift of 0.19 unit in pK_{es2} determined from the pL–rate data also can assist in identifying the prototropic group responsible for this ionization. The magnitude of a pK_a shift is related to the fractionation factor values given in eq 3 (Schowen & Schowen, 1982).

$$\Delta pK_a = \log \frac{(\phi BH_y)^y(\phi H_2O)^2}{(\phi H_3O)^3(\phi BH_{y-1})^{y-1}} \quad (3)$$

The value y represents the number of equivalent, dissociable protons (hydrogenic sites) in the conjugate acid of species BH_y; for Cu–OH_2, y = 2. The standard fractionation factor for H_2O is 1.0, and for H_3O^+ it is 0.69. The value ΔpK_{es2} = +0.19 allows calculation of the ratio $(\phi BH_y)^y/(\phi BH_{y-1})^{y-1}$; this ratio is 0.51. If a Cu-bound H_2O is the group responsible for pK_{es2}, then the fractionation factor ϕBH_y for this group would depend upon the amount of positive charge transfer δ from the metal to the coordinated H_2O molecule. An estimate of this fractionation factor can be calculated from eq 4.[3]

$$\phi BH_y = (+0.69)^\delta \quad (4)$$

Should no charge be transferred from the metal ($\delta = 0$), the fractionation factor would be $(0.69)^0 = +1.0$, $(\phi BH_y)^y$ would be +1.0, and $(\phi BH_{y-1})^{y-1}$ would be 2.0. Conversely, upon maximal charge transfer, in this case $\delta = +2$, the fractionation factor value would be $(0.69)^2 = +0.48$, $(\phi BH_y)^y = +0.24$, and $(\phi BH_{y-1})^{y-1} = +0.47$. Should the charge on the water oxygen be +1, $(\phi BH_y) = 0.69$, $(\phi BH_y)^y = 0.48$, and $(\phi BH_{y-1})^{y-1} = 0.94$.

The charge on the galactose oxidase Cu(II) is not known precisely. Two of the ligands are neutral: histidine imidazoles (Bereman & Kosman, 1977; Kosman et al., 1981). The third endogenous equatorial ligand appears ESR silent and thus could be either –O– or –S–, although a weakly coupled nitrogenous ligand cannot be excluded. Tyrosine phenol, as noted above, or methionine thioether coordination is consistent with available optical and magnetic data (Ettinger, 1974; Kosman & Bereman, 1977; Kosman et al., 1980; Amundson et al., 1977). Methionine thioether ligation has been observed in other Cu(II) centers, albeit in those classified as type 1 (Ryden, 1984; Loehr & Loehr, 1984). The spin-Hamiltonian parameters (Bereman & Kosman, 1977; Giordano et al., 1977) and magnitude of the linear electric field effect on the **g** tensors (Kosman et al., 1980) indicate that sulfhydryl coordination is extremely unlikely. Thus, the evidence suggests that there could be either a 1+ or 2+ charge on the metal at the enzyme active site, a suggestion consistent with the enzymic Cu(II) $\mathbf{A}_\parallel$ and $\mathbf{g}_\parallel$ values (Giordano et al., 1974; Peisach & Blumberg, 1974). Transfer of 1+ charge to the water molecule would give it the ionization characteristics of a lyonium ion ($\phi BH = 0.69$). Supportive of this possibility is the value thus attributed to ϕBH_{y-1}, 0.94. The largest fractionation factor for a solvated HO^- is 1.25 while the smallest is 0.70 (Gold & Grist, 1972); the latter value is the one experimentally assigned (Schowen & Schowen, 1982). Coordination to Cu^{2+} could only lead to an increased acidity of HOH, which would have the effect of increasing the value of ϕBH_{y-1} (ϕOH^-). A value near unity, e.g., 0.9, is quite reasonable for the copper hydroxo species.

Reported shifts in dissociation constant for many hydrated metal ions are less than the 0.4–0.6pK_a shift expected for weak organic acids. Unfortunately, while the pK_a values for several hydrated metal ion species are reported in the literature (vide supra), corresponding isotopic shifts have not been measured. Those values measured range from +0.14 (for the hydrolysis of Gd^{3+}; Amaya et al., 1973) to +0.48 [aquapentaamminecobalt(III); Splinter et al., 1968]. In the example cited of a Cu^{2+} chelate due to Groves and Chambers (1984), a shift of 0.5 pH unit was determined. The shifts for the model Cu^{2+} complexes studied herein were intermediate between this latter value and that for pK_{es2}. However, the variability of the "polyproticity" of hydrated metal ions (cf. eq 3, in which y varies with the number of equivalent hydrogenic sites) makes the analysis of shifts in terms of fractionation factors difficult and comparisons of shifts for dissimilar metal ion species potentially meaningless.

The values for pK_{es2} and its isotopic shift fall within the ranges expected for sulfhydryl acids (Schowen & Schowen, 1982). Chemical modification studies on the holo- and apogalactose oxidase, together with the amino acid composition of the protein, indicate the absence of solvent-accessible, free sulfhydryl groups in the native, holo enzyme (Kosman et al., 1974). Even in the apoenzyme, the single free –SH group is titratable only after denaturation of the protein (Kosman et al., 1974). The apparent inaccessability of this group to solvent makes unlikely its contribution to a kinetically detected solvent isotope effect.

[3] Equation 3 follows from the assumption that $M-O^+H_2$ is equivalent to $H-O^+H_2$ for which $\phi BH = 0.69$ (Schowen & Schowen, 1982).

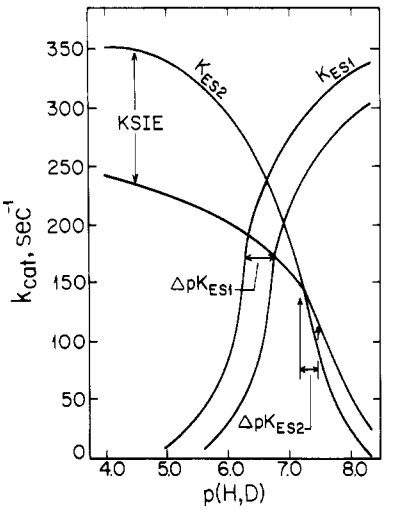


FIGURE 6: Computer simulations obtained from fitting the pH(D) data to the appropriate rate equations by using the following isotope-dependent parameters: $\Delta pK_{es1} = +0.5$ (no KSIE) and $\Delta pK_{es2} = +0.19$, with a KSIE of +1.55 (see Discussion).

Computer simulations of the p(H,D) vs. k_{cat} data were performed in an attempt to determine whether the KSIE could be attributed specifically to an effect on either K_{es1} or K_{es2}. A limiting k_{cat} value was assigned to one or the other limb of the pH–k_{cat} profile; these limiting values were equivalent to the $k_{cat,max}$ value (550 s^{-1}) obtained from the PENNZYME computer analysis. The dissociation constants (K_{es1} and K_{es2}) associated with these limits were those determined experimentally in H_2O. Since at lower pH values the term $K_{es2}/[H^+]$ in eq 1 becomes negligible, the equation becomes

$$k_{cat,obsd} = k_{cat,max}/(1 + [H^+]/K_{es1}) = k_{cat,K_{es1}\,limit}/(1 + [H^+]/K_{es1})$$

Similarly, at high pH values, the $[H^+]/K_{es1}$ term becomes negligible, and the rate equation simplifies to

$$k_{cat,obsd} = k_{cat,max}/(1 + K_{es2}/[H^+]) = k_{cat,K_{es2}\,limit}/(1 + K_{es2}/[H^+])$$

Computer simulations were obtained for three sets of constraints:

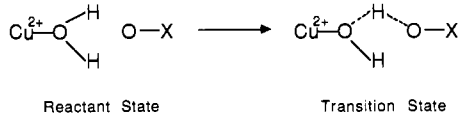
(1) A KSIE of +1.55 was applied to either the K_{es1} limit or the K_{es2} limit, and both K_{es1} and K_{es2} experienced standard pK_a shifts in D_2O of +0.50.

(2) A KSIE of +1.55 was applied to either the K_{es1} limit or the K_{es2} limit, and K_{es1} experienced a standard shift in pK_a of +0.50 and K_{es2} experienced a shift in pK_a of +0.19 in D_2O.

(3) A KSIE was applied to neither limit, and a standard shift was experienced by the pK_a associated with K_{es1} and a nonstandard shift of +0.19 was experienced by the pK_a associated with K_{es2}.

The simulations that best described the behavior determined experimentally were those in which pK_{es1} shifted by +0.50 and experienced no KSIE, while pK_{es2} had a small pK_a shift of +0.19 and a solvent isotope effect of +1.55 (Figure 6). The simulations indicated that the KSIE could not be accounted for solely by a nonstandard shift in pK_{es2}, nor could any satisfactory simulation be achieved when the K_{es1}, k_{cat} limit experienced a KSIE of +1.55. Thus, these simulations are consistent with the interpretation that the KSIE is attributable to a protic group required in the undissociated state for catalysis, pK_a = 7.16. The data also indicate that the histidine

Scheme II



imidazole group does not experience a KSIE. This is consistent with the proposal that this group's catalytic function is the abstraction of a proton from the carbinol carbon undergoing oxidation (Kwiatkowski et al., 1977). The interpretation that the Cu-bound water molecule is responsible for the isotope effect, i.e., a single hydrogenic site, is also consistent with the proton inventory results fitting best to a linear equation.

The magnitude of the KSIE is not large (1.55) but is comparable to KSIE values reported for Cu^{2+}-promoted amide hydrolysis (1.6) (Groves & Chambers, 1984), the lipoprotein lipase reaction (1.70) (Quinn, 1985), the hydrolysis of pyrophosphate by inorganic pyrophosphatase (1.9) (Konsowitz & Cooperman, 1976), and the reaction catalyzed by the multicopper oxidase laccase (2.1) (Koudelka & Ettinger, 1985). The KSIE on k_{cat} can be related to the ratio of the fractionation factors for the reactant state [ϕ(RS)] and the transition state [ϕ(TS)] (Kresge, 1977). Although ϕ(TS) could not be measured directly, its value was estimated by knowing that $k_{cat,H_2O}/k_{cat,D_2O}$ was +1.55 and that a reasonable value of ϕ(RS) for the transferred proton on the Cu^{2+}–OH_2 was +0.69. The ϕ(TS) value determined in this manner is +0.46.

It is reasonable to ascribe the proton transferred in a transition state of the GO reaction as a bridge between the oxygen of the Cu-bound water molecule and a recipient oxygen atom as illustrated in Scheme II. Hydrogenic sites like this one at which the transferred proton serves as a bridge between O, N, or S atoms have been implicated in a number of enzymic processes involving acid–base catalysis (Jencks, 1972). These reactions characteristically display a KSIE on k_{cat} no greater than 2–3 (Laughton & Robertson, 1969). Potentially, the low KSIE value seen in the GO reaction may reflect a transition state in which the transferred proton is asymmetrically displaced towards the donor atom rather than toward the acceptor and/or with this proton's "line-of-flight" being bent (Kresge, 1977).

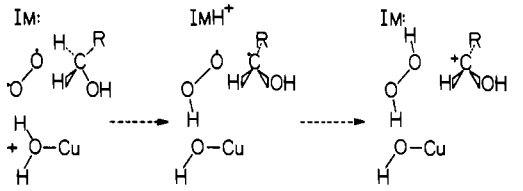
The relative values of ϕ(RS) and ϕ(TS) are consistent with this suggestion and Scheme II above. Specifically, ϕ(RS) > ϕ(TS) indicates that the proton transferred is bound more tightly in the transition state than in the reactant state, i.e., there is an inverse isotope effect (Schowen & Schowen, 1982). The values for ϕ(RS) and ϕ(TS) can be used in eq 5 to simulate the proton inventory data (Figure 4). Although a slight

$$k_n = k_0[1 - n + n\phi(\mathrm{TS})]/[1 - n + n\phi(\mathrm{RS})] \quad (5)$$

bowing upward of the calculated values is discernible, they are within 5% of the best-fit straight line; i.e., this putative inverse isotope effect is small. Nonetheless, the chelation of the bridging proton by the oxygen atoms as illustrated in Scheme II would represent a tighter binding in the transition state. In addition, the geometry depicted is consistent with the relatively small KSIE.

The putative oxygen atom acceptor could be associated with the dioxygen substrate or its one-electron reduction product, O_2^-. An attractive hypothesis based on thermodynamic considerations is that proton transfer (acid catalysis) from the Cu-bound H_2O to O_2 occurs concurrent with or following a one-electron transfer from substrate alcohol. Protonation of O_2 makes O_2 a better oxidant by 6.4 kcal/mol (+280 mV vs. $O_2 + e^- \rightarrow O_2^-$ only) (Fee, 1982). The data are consistent with a reaction pathway involving a transfer of one electron

Scheme III



to O_2 in a transition state which is stabilized by a hydrogen bond from the Cu^{2+}–OH_2, i.e., a rate-determining electron transfer that is catalyzed by partial (asymmetric) proton transfer. The proton transfer is completed in a second transition state which leads formally to $HO_2^{\bullet}$ and an alcohol substrate radical. The second electron transfer from this latter radical would be highly exergonic; the $\Delta G^{\circ\prime}$ for this process can be estimated at −15 kcal/mol. This model is depicted in Scheme III. This scheme is meant only to illustrate the species described and not to specify their identity as transition states or intermediates. The feature emphasized is the kinetic potential afforded by coupling a thermodynamically favorable process, e.g., proton transfer, to an electron-transfer step. To the extent that protonation of O_2^- occurs either during its formation or as a thermodynamic trap subsequent to the first electron transfer, the initial one-electron reduction of dioxygen can be acid catalyzed. Indeed, the pH dependence, KSIE, and sensitivity to added general acids of the reaction between O_2 and monohydroxyacetone (and other α-ketoalcohols) catalyzed by Cu(II)–tetrakis(pyridine) are all consistent with general-acid catalysis of electron transfers to dioxygen from organic, alcohol substrates (Driscoll & Kosman, 1987). Similar behavior has been well documented for the dopamine-β-monooxygenase reaction (Miller & Klinman, 1983, 1985). Support for the hypothesis that general-acid catalysis is a consistent feature of O_2 reduction can come from complementary kinetic evaluation of potential half-reactions rather than the steady-state turnover studied herein.

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