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Electrostatic Control of Enzyme Reactions: Effect of Ionic Strength on the pK_a of an Essential Acidic Group on Glucose Oxidase†

Judith G. Voet,* James Coe,‡ Jeffrey Epstein,§ Viken Matossian, and Thomas Shipley

ABSTRACT: The dissociation constant of an essential acidic group on the reduced form of glucose oxidase from *Aspergillus niger* (K_4) has been found to be extremely sensitive to ionic strength. Increasing the ionic strength from 0.025 to 0.225 causes a decrease in p$K_{4,obsd}$ of 0.9 pH unit, from 8.2 to 7.3. Analysis of the ionic strength dependence of p$K_{4,obsd}$, making the assumption that the enzyme is a homogeneously charged impenetrable sphere [Edsall, J. T., & Wyman, J. (1958) *Biophysical Chemistry*, Vol. 1, pp 282–289, 512–514, Academic Press, New York], predicts that the intrinsic pK_a of the acidic group is 6.7 and that the charge on the protein is −78. The enzyme was titrated from its isoelectric point (pH 4.05) to pH 7.7, the pH at which the ionic strength dependence was determined. It was found to have an actual charge at that pH of −77, in remarkable agreement with the theoretical prediction. Thus, glucose oxidase exerts electrostatic control on p$K_{4,obsd}$ as though it were a uniformly charged sphere. The group responsible for p$K_{4,obsd}$ has not been identified. However, its measured $\Delta H°_{obsd}$ of 8.0 kcal mol^{-1} and $\Delta S°_{obsd}$ of −6.1 cal mol^{-1} K^{-1}, together with its pK_a of 6.7, are consistent with the group being a histidine residue.

Electrostatic potential is an important contributor to the control of the activity of many sorts of enzymes: membrane-bound enzymes such as cytochrome oxidase (Maurel et al., 1978), enzymes such as ribonuclease and lysozyme which have polyelectrolytes as substrates (Irie, 1965; Maurel & Douzou, 1976), and acidic or basic enzymes which as a result of high surface charge create their own electrostatic potential (Valenzuela & Bender, 1971). Douzou & Maurel (1977) have reviewed the general mechanisms by which ionic control may be exerted on enzymatic reactions.

Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) is an acidic enzyme. Its reaction involves the apparent participation of a group whose dissociation occurs in a pH range in which the enzyme is polyanionic. Since fluctuations in ionic strength change the electrostatic potential exerted by the negative charge on the enzyme, they should alter the dissociation constant of this group. These fluctuations thus provide a mechanism for controlling the enzyme's activity.

The kinetics of the glucose oxidase reaction are easily analyzed in terms of individual rate constants according to pH-

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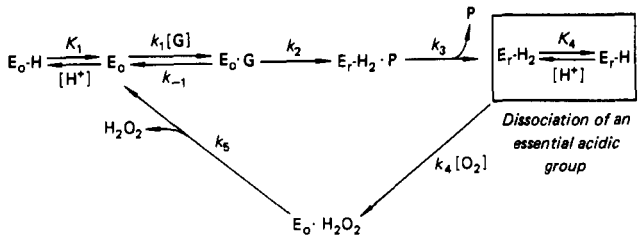
Scheme I



dependent kinetic Scheme I as modified[1] from Bright & Appleby (1969).[2]

The rate equation derived from this scheme is shown below:

$$\frac{E_T}{v} = \frac{(k_{-1} + k_2)(1 + [H^+]/K_1)}{k_1 k_2 [G]} + \frac{1}{k_2} + \frac{1}{k_3} + \frac{1}{k_5} + \frac{1 + K_4/[H^+]}{k_4[O_2]} \quad (1)$$

Stankovich et al. (1978) have reported that the oxidative half-reaction of glucose oxidase is sensitive to ionic strength at pH 9.3 and 10.0. They provided no interpretation for this effect, however. We report here that K_4, the dissociation constant of an as yet unidentified essential acidic group on the reduced enzyme, is extremely sensitive to the ionic strength of the medium. This effect is analyzed in terms of simple electrostatic theory.

Experimental Procedures

Chemicals. Glucose oxidase from *Aspergillus niger* was either purchased from Sigma Chemical Corp. or purified from Dee-O concentrate (a generous gift of Miles Laboratories) by the procedure of Swoboda & Massey (1965). The enzyme was stored frozen in distilled water as described by Swoboda & Massey (1965).

The following buffers were used: 0.01 M potassium citrate for the pH range 4–6; 0.01 M potassium phosphate for the pH range 6–8; 0.01 M sodium pyrophosphate for the pH range 8–10. Ionic strength was adjusted with either potassium chloride or potassium sulfate. All enzyme kinetic reactions contained 0.1 M glucose. All chemicals used were reagent grade and obtained from standard chemical suppliers.

Enzyme Concentration. Glucose oxidase is a dimer containing one molecule of FAD per subunit. Enzyme concentrations are expressed in kinetic analyses as molarity of bound FAD and determined spectrophotometrically by using a molar absorptivity of 1.41×10^4 M^{-1} cm^{-1} for the oxidized enzyme subunit at 450 nm (Swoboda & Massey, 1965). For the determination of the charge on the enzyme as a function of pH, enzyme concentration is expressed as molarity of native dimer with a molar absorptivity of 2.82×10^4 M^{-1} cm^{-1} at 450 nm. When necessary, the concentration of enzyme in milligrams per milliliter was determined spectrophotometrically at 280 nm where $\epsilon^{1\%}_{280nm} = 1.67$ (Swoboda & Massey (1965).

Glucose Oxidase Activity. The enzyme was assayed for activity by the measurement of oxygen uptake with a Yellow Springs Instrument Co. Model 53 biological oxygen monitor attached to a Linear strip-chart recorder. The 3-mL reaction mixture contained 0.1 M glucose in air-saturated 0.13 M phosphate buffer, pH 5.6, at 25 °C. Under the conditions of the assay, purified glucose oxidase had a specific activity of 155 μmol min^{-1} mg^{-1}. The purified preparation was found to have approximately 10 "Sigma units" per mg of catalase.[3] Rates of oxygen uptake were independent of added cyanide ion, however, indicating that these levels of catalase did not interfere with glucose oxidase kinetic measurements.

Determination of pK_4. The pH was determined at 25.0 °C by using a Fisher Accumet Model 620 pH meter calibrated with Fisher Scientific Co. certified buffer solutions. The kinetic data obtained on the oxygen monitor thermostated at 25.0 °C were amplified 50-fold and digitized by using a Vector Graphics precision analog to digital converter interfaced to a Commodore PET 2001 computer. Reactions were conducted in 3 mL of reaction mixture of appropriate pH and ionic strength which was 0.1 M in glucose and $(1–3) \times 10^{-8}$ M in glucose oxidase. Oxygen concentration in an air-saturated solution was taken to be 2.58×10^{-4} M under these conditions (Cooper, 1977). Kinetic constants were obtained by using a nonlinear least-squares regression program to fit the data to the integrated form of rate eq 1:

$$-E_T(t_2 - t_1) = A([O_2]_2 - [O_2]_1) + B \ln \frac{[O_2]_2}{[O_2]_1} \quad (2a)$$

where

$$A = \frac{(k_{-1} + k_2)(1 + [H^+]/K_1)}{k_1 k_2 [G]} + \frac{1}{k_2} + \frac{1}{k_3} + \frac{1}{k_5} \quad (2b)$$

and

$$B = \frac{1 + K_{4,obsd}/[H^+]}{k_4} = \frac{1}{k_{4,app}} \quad (2c)$$

$K_{4,obsd}$ and k_4 were determined by measuring $k_{4,app}$ as a function of pH. Once k_4 had been determined and found to be independent of ionic strength, $k_{4,app}$ was determined at several ionic strengths, at a fixed pH of 7.7, and $pK_{4,obsd}$ calculated by use of eq 3:

$$pK_{4,obsd} = pH - \log\left(\frac{k_4}{k_{4,app}} - 1\right) \quad (3)$$

Titration of Glucose Oxidase. Type VII glucose oxidase from Sigma Chemical Co., 40 mg, was dissolved in 1 mL of deionized, distilled water and dialyzed overnight against deionized, distilled water. The concentration of the enzyme was determined spectrophotometrically to be 2.95×10^{-5} M dimer. Enzyme (1.00 mL) was adjusted to pH 3.9 with HCl and then titrated with 0.0954 M NaOH. The isoelectric point of glucose oxidase was taken to be at pH 4.05.[4]

Results and Discussion

The pH dependence of $k_{4,app}$ in eq (2c) is shown in Figure 1 for ionic strengths of 0.025 and 0.225. The pH at the midpoint of the curves corresponds to $pK_{4,obsd}$, the pK_a of an essential acidic group on the enzyme. It may be seen that an increase in ionic strength causes a sharp decrease in $pK_{4,obsd}$. This decrease has a dramatic effect. At a constant pH of 7.8, increasing the ionic strength from 0.025 to 0.225 causes a 3-fold decrease in $k_{4,app}$, from 1.2×10^6 to 4×10^5 M^{-1} s^{-1}.

This behavior is a generalized salt effect rather than a specific ion effect since the same behavior is obtained whether potassium chloride or potassium sulfate is used to increase the ionic strength. Using potassium sulfate, $pK_{4,obsd}$ was deter-

[1] The complete pH-dependent scheme includes additional ionizations which have no effect on the kinetic constants measured in this paper.

[2] Abbreviations used: G, D-glucose; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

[3] Sigma Chemical Co., Feb 1981 Catalog, p 170.

[4] Bio-Rad Catalog G, 1981, p 95.

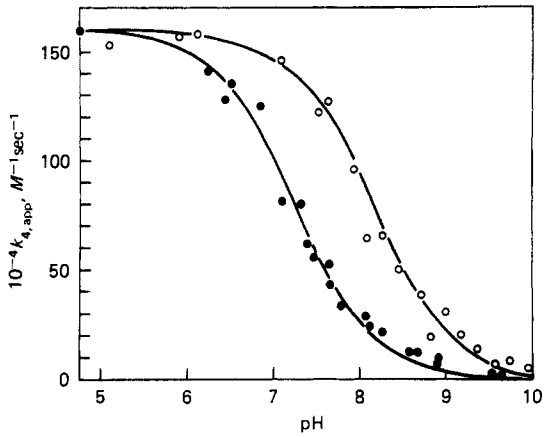


FIGURE 1: pH dependence of $k_{4,app}$ at ionic strengths of 0.025 (○) and 0.225 (●). Data were collected and analyzed as described under Experimental Procedures. The solid lines correspond to theoretical curves calculated from eq 2c for $k_4 = 1.6 \times 10^6$ M^{-1} s^{-1} and $pK_{4,obsd}$ = 8.2 (○) and 7.3 (●).

mined for several different ionic strengths as described under Experimental Procedures.

The model used to analyze the data is summarized by Edsall & Wyman (1958): The pK_a of an acid, HA, is represented as

$$pK_{obsd} = pK_{int} + \log \gamma_{A^-} - \log \gamma_{HA}$$

where pK_{obsd} is the observed pK_a of the acid, pK_{int} is the intrinsic (thermodynamic) pK_a of the acid, and γ_{HA} and γ_{A^-} are the activity coefficients of the acid and its conjugate base. If it is assumed that the only difference in the activity coefficient between the acid and its conjugate base is due to the difference in charge and that the acid is a uniformly charged, impenetrable sphere of radius b, then the environmental dependence of the activity coefficient of each ion may be expressed in terms of the Debye–Hückel theory as

$$\log \gamma = \frac{Z^2\epsilon^2}{4.6DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) \quad (4a)$$

where

$$\kappa = \left(\frac{8\pi N\epsilon^2}{1000kDT}\right)^{1/2} I^{1/2} \quad (4b)$$

In eq 4a, Z is the charge on the ion, ϵ is the electronic charge, k is the Boltzmann constant, D is the dielectric constant, T is the absolute temperature, a is the sum of the ionic radius of the acid (b) and its counterion, I is the ionic strength, and N is Avogadro's number. Therefore, if HA is presumed to have charge Z and A^- to have charge $Z - 1$, eq 4a becomes

$$pK_{obsd} = pK_{int} - \frac{(2Z - 1)\epsilon^2}{4.6kTD}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) \quad (5)$$

Figure 2 shows the results when $pK_{4,obsd}$ at several ionic strengths is plotted according to eq 5. The radius of the enzyme, b, was calculated from its diffusion coefficient by using the relationship

$$b = \frac{kT}{6\pi\eta D_{20,w}}$$

where k is the Boltzmann constant, T is the absolute temperature, η is the viscosity of water at 20 °C, and $D_{20,w}$ is the diffusion coefficient of the enzyme in water at 20 °C. Since $D_{20,w}$ for glucose oxidase is 4.12×10^{-7} cm^2 s^{-1} (Swoboda & Massey, 1965), b is 5.2×10^{-7} cm. The pK_{int} of the acidic group of glucose oxidase was found to be 6.7 from this experiment and the charge Z on the enzyme −78.

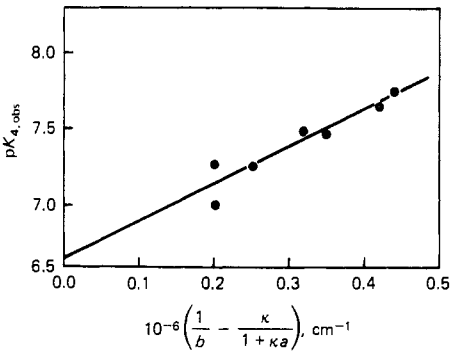


FIGURE 2: Dependence of $pK_{4,obsd}$ on ionic strength according to eq 3. The parameter κ was calculated from eq 2a. a and b were calculated as described in the text to be 5.4×10^{-7} and 5.2×10^{-7} cm. The y intercept corresponds to a pK_{int} of 6.7. Z, the average charge on the protein, was calculated from the slope of the line to be −78.

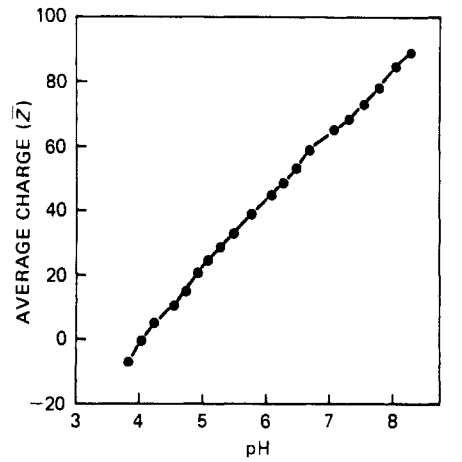


FIGURE 3: Average charge, Z, on glucose oxidase as a function of pH, assuming an isoelectric point of 4.05.[4] The experimental details are described in the text.

A more sophisticated theory has been developed which takes into account nonspherical molecules, nonhomogeneous charge distribution, and the burial of charges (Tanford & Kirkwood, 1957). The interior of the molecule may then be assigned a dielectric constant different from that of water. Lysozyme has been analyzed in this manner (Tanford & Roxby, 1972) as have myoglobin (Shire et al., 1974) and hemoglobin (Matthew et al., 1979). A comparison of the ability of the uniformly charged sphere model and the more complex model to predict the ionic strength dependence of the dissociation constant of the iron-bound water molecule in sperm whale ferrimyoglobin (Shire et al., 1974) shows that the simple theory accounts remarkably well for the data. The ionic strength dependence of the dissociation constant of His-159 on papain-S-SCH_3 has also been analyzed in terms of the simple homogeneously charged sphere model with results which accurately predict the known charge on that enzyme (Johnson et al., 1981). A comparison of the charge predicted by the simple theory with the actual charge on glucose oxidase should therefore show the degree to which this theory can account for the behavior of this enzyme.

The actual charge on the enzyme as a function of pH was determined by titration as described under Experimental Procedures. The number of equivalents of NaOH necessary to bring the enzyme to a specific pH from its isoelectric point corresponds to the negative charge on the enzyme at that pH. The results are shown in Figure 3. At pH 7.7, the pH at which the ionic strength dependence of $pK_{4,obsd}$ was determined,

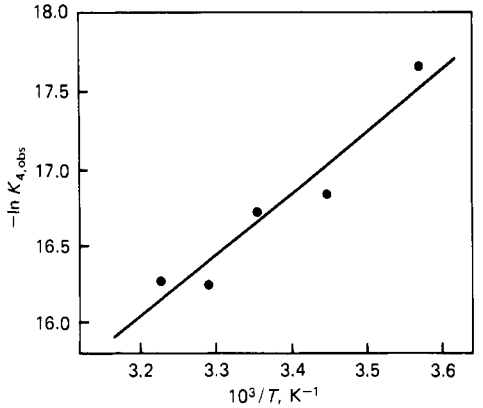


FIGURE 4: van't Hoff plot of the temperature dependence of $K_{4,obsd}$ at $I = 0.225$ according to the equation $-\ln K_{4,obsd} = \Delta H°_{obsd}/(RT) - \Delta S°_{obsd}/R$. Least-squares analysis yields $\Delta H° = 8.0$ kcal mol^{-1} and $\Delta S° = -6.1$ cal mol^{-1} K^{-1}.

the enzyme was found to have a charge of −77. This actual charge agrees remarkably well with the charge of −78 predicted by the simple electrostatic theory. This enzyme, like many others, exerts electrostatic control on $pK_{4,obsd}$ as though it were a uniformly charged sphere.

The essential group on the reduced enzyme responsible for pK_4 has yet to be identified. It has been suggested (Bright & Appleby, 1969) that it may, in fact, be due to the ionization of $FADH_2$. $FMNH_2$ has a pK_a in nonenzymatic systems of 6.7 (Lowe & Clark, 1956). The argument which Bright & Appleby (1969) present against their suggestion is that the anionic species, $FADH^-$, is expected to be more reactive with oxygen than $FADH_2$ whereas the loss of a proton in the enzymatic system results in loss of activity. Another argument against $FADH_2$ ionization causing the observed pH-dependent kinetic behavior is that this ionization involves a spectral change, and no spectral change has been observed for reduced glucose oxidase in the region of pH 6–10 (Ghisla et al., 1974; J. G. Voet and J. Epstein, unpublished results). Spectral arguments are not conclusive, however, since in the case of flavodoxin a pK_a for $FMNH_2$ of 5.8 has been assigned on the basis of redox potential changes where no pH-dependent change in spectral characteristics could be observed (Mayhew et al., 1969).

In an attempt to further characterize the group responsible for $pK_{4,obsd}$, we have studied the effect of temperature on this dissociation constant as shown in Figure 4. The enthalpy of ionization at an ionic strength of 0.225 was found to be +8 kcal mol^{-1}. This enthalpy change is consistent with the groups being a histidine residue on the enzyme (Greenstein & Winitz, 1961). Enthalpies of ionization vary with conditions, however, and therefore cannot provide anything but suggestive evidence. The enthalpy of ionization has not been reported for $FADH_2$ or $FMNH_2$ in either enzymatic or nonenzymatic systems so no comparison may be made with these ionizations at present. Chemical modification and solvent perturbation experiments (Cleland, 1977) and experiments with flavin analogues having different dissociation constants from those of $FADH_2$ are now in progress in an attempt to provide positive identification of the essential acidic group on reduced glucose oxidase.

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