Effect of pH on Protein−Ligand Equilibria

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When a protein binds a ligand, hydrogen ions may be liberated or consumed. When this occurs, the apparent equilibrium constant K' for the dissociation of the ligand will be a function of pH. Thus, the study of the dependence of K' on pH can provide information on the acid dissociation constants of acid groups in the binding site of the protein before and after binding a ligand. From a thermodynamic point of view, the acid titration curves of the protein, ligand, and protein−ligand complex are related to the pH dependence of K' through the hydrogen ion binding polynomials P (partition functions) of the three reactants. However, the hydrogen ion binding polynomials of the protein and protein−ligand complex can be factored into a binding polynomial for the site and a binding polynomial for the rest of the protein molecule. When the ligand does not have acid dissociations in the range of pH where the unoccupied site or occupied site does, the pH dependence of K' is given by the ratio of the binding polynomial of the unoccupied site to the binding polynomial of the occupied site. When there are two acid groups in the site, it is shown that all four acid dissociation constants can be calculated from the dependence of K' on pH, even if there is positive cooperativity in the binding site. The pH dependencies of the standard transformed Gibbs energy of formation of the catalytic site in fumarase and for the site occupied by succinate and the three isomers of tartrate are calculated.

Introduction

The binding of a ligand by a protein may be accompanied by the production or consumption of hydrogen ions so that the apparent dissociation constant K' for the biochemical reaction

$$PL_{tot} = P_{tot} + L_{tot} \qquad K' = [P_{tot}][L_{tot}]/[PL_{tot}] \qquad (1)$$

is a function of pH. The abbreviations represent sums of species at a specified pH, and the equilibrium expression is written in terms of concentrations because K' is taken to be a function of ionic strength as well as pH. If the ligand does not have pKs in the pH range studied, hydrogen ions are produced or consumed when there are acid groups in the binding site that have pKs in the pH range studied and the pKs of these groups are changed by the binding of the ligand.

When the pH affects the apparent equilibrium constant of a reaction like reaction 1, the Gibbs energy G does not provide the criterion for spontaneous reaction and equilibrium, and so it is necessary to use a Legendre transform to define a transformed Gibbs energy G' by subtracting the product of the amount $n_c(H)$ of the hydrogen component in the system and the chemical potential $\mu(H^+)$ of hydrogen ions[1].

$$G' = G - n_c(H)\mu(H^+) \qquad (2)$$

The transformed Gibbs energy provides the equilibrium criterion $(dG')_{T,P,pH} \leq 0$. The change in the standard transfomed Gibbs energy in a reaction like reaction 1 at a specified pH is given by

$$\Delta_r G'^o = \sum \nu_i' \Delta_f G_i'^o = -RT \ln K' \qquad (3)$$

where ν_i' is the apparent stoichiometric number of reactant i and $\Delta_f G_i'^o$ is the standard Gibbs energy of formation of reactant i at the specified pH.

This article is concerned with the relationship between the pH dependence of the apparent equilibrium constant K' and the acid titration curves for the protein, ligand, and protein−ligand complex. For simplification, it is assumed that a single molecule of ligand is bound by a protein molecule or that the concentration of the ligand is sufficiently low so that only the binding of the first ligand molecule has to be considered. The binding of hydrogen ions by the three reactants is represented by

$$\begin{array}{ccc} PL = & P + & L \\ || & || & || \\ HPL & HP & HL \\ || & || & || \\ H_2PL & H_2P & H_2L \end{array} \qquad (4)$$

More acid groups may be involved, but this is enough for a discussion of the effect of pH on K'. Charges are not shown, but of course they have to balance in these reactions between species, although they do not have to balance in reaction 1. Through discussion of the effect of changing the hydrogen ion concentration, it is convenient to use the concept[2−7] of the binding polynomials (partition functions) P for P_{tot}, PL_{tot}, and L_{tot}:

$$P = 1 + [H^+]/K_1 + [H^+]^2/K_1K_2 + ... \qquad (5)$$

The K_i values are acid dissociation constants written in terms of concentrations of species; for example, $K_{L1} = [H^+][L]/[HL]$, $K_{L2} = [H^+][HL]/[H_2L]$, ... The dissociation constants are taken to be functions of the ionic strength by incorporating activity coefficients in K_i.

The binding polynomial for hydrogen ions is useful in several ways. The first is that the derivative of ln P for a reactant with respect to ln[H$^+$] is equal to the average number $\bar{N}_H$ of hydrogen ions bound by a molecule of P_{tot}, L_{tot}, and PL_{tot} because[4]

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$$\bar{N}_H = \frac{d \ln P}{d \ln[H^+]} = -\frac{1}{\ln(10)} \frac{d \ln P}{d\ pH} = [H^+] \frac{d \ln P}{d[H^+]} \quad (6)$$

where pH = $-\log[H^+]$. Note that pH is expressed in terms of the concentration of hydrogen ions because the acid dissociation constants incorporate activity coefficients. According to the extended Debye−Hückel theory[8] at 25 °C and $B = 1.6$ $L^{1/2}$ $mol^{-1/2}$, 0, 0.08, 0.11, 0.12, 0.13, and 0.14 need to be subtracted from measured pH values at ionic strengths of 0, 0.05, 0.10, 0.15, 0.20, and 0.25 M to obtain pH = $-\log[H^+]$. The plot of $\bar{N}_H$ versus pH for a reactant is referred to as its titration curve. Substituting eq 5 in eq 6 yields

$$\bar{N}_H = \frac{[H^+]/K_1 + 2[H^+]^2/K_1K_2 + ...}{1 + [H^+]/K_1 + [H^+]^2/K_1K_2 + ...} \quad (7)$$

Use of Binding Polynomials in Protein−Ligand Equilibria

Binding polynomials are also useful because they can be used in expressing the pH dependence of the apparent equilibrium constant K' for the dissociation of a protein−ligand complex. The pH dependence of K' for reaction 1 is given by

$$K' = \frac{K_{ref} P(P_{tot}) P(L_{tot})}{P(PL_{tot})} \quad (8)$$

where $K_{ref} = [P][L]/[PL]$ is written in terms of species and

$$P(P_{tot}) = 1 + 10^{-pH+pK_{P1}} + 10^{-2pH+pK_{P1}+pK_{P2}} + ... \quad (9)$$

$$P(L_{tot}) = 1 + 10^{-pH+pK_{L1}} + 10^{-2pH+pK_{L1}+pK_{L2}} + ... \quad (10)$$

$$P(PL_{tot}) = 1 + 10^{-pH+pK_{PL1}} + 10^{-2pH+pK_{PL1}+pK_{PL2}} + ... \quad (11)$$

where $pK_{L1} = -\log K_{L1}$, etc. The change in binding of hydrogen ions $\Delta_r N_H$ in reaction 1 is obtained by taking the derivative of the logarithm of the apparent equilibrium constant as indicated by

$$\Delta_r N_H = \frac{d \ln K'}{d \ln[H^+]} = -\frac{1}{\ln(10)} \frac{d \ln K'}{d\ pH} = [H^+] \frac{d \ln K'}{d[H^+]} = \bar{N}_H(P_{tot}) + \bar{N}_H(L_{tot}) - \bar{N}_H(PL_{tot}) \quad (12)$$

These relations involve the assumption that no hydrogen ions are produced or consumed in the reference reaction PL = P + L.

The binding polynomial for a reactant can be calculated[9] from its acid titration curve by integrating eq 6:

$$-\ln(10) \int \bar{N}_H \, d\ pH = \ln P + \text{const} \quad (13)$$

$$\int \frac{\bar{N}_H}{[H^+]} d[H^+] = \ln P + \text{const} \quad (14)$$

Equations 13 and 14 indicate that the acid titration curves for P_{tot}, L_{tot}, and PL_{tot} are connected with the pH dependence of K' by eq 8. For example, if the acid titration curves are determined for P_{tot} and L_{tot}, and the pH dependence of K' is determined, the titration curve for PL_{tot} can be calculated.

The problem with this analysis of the effect of pH on K' is that, although it is correct in principle, the pKs of the acid groups in the binding site are buried in the binding polynomials for P_{tot} and PL_{tot}. Therefore, it is of interest to explore the extent to which the binding polynomials of P_{tot} and PL_{tot} can be factored so that there is a separate multiplicative term for the site in the binding polynomials for P_{tot} and PL_{tot}. The factorability of binding polynomials is discussed extensively by Wyman and Gill.[9] A binding polynomial can always be factored into terms for the individual acid groups if the groups are independent. If the acid groups in the protein are independent and some of the acid groups have the same pK, the binding polynomial for the protein can be written as

$$P(P_{tot}) = (1 + 10^{-pH+pK_{P1}})^{n_1}(1 + 10^{-pH+pK_{P2}})^{n_2}... \quad (15)$$

where there are n_1 groups with pK_{P1}, n_2 groups with pK_{P2}, ... Application of eq 6 shows that the average binding of hydrogen ions by the protein when the acid groups are independent is given by the sum of the titration curves of the individual groups.

$$\bar{N}_H(P_{tot}) = \frac{n_1 10^{-pH+pK_{P1}}}{1 + 10^{-pH+pK_{P1}}} + \frac{n_2 10^{-pH+pK_{P2}}}{1 + 10^{-pH+pK_{P2}}} + ... \quad (16)$$

However, it is not necessary to go this far in making assumptions. If the acid groups in the binding site are independent of the acid groups in the rest of the protein molecule, the binding polynomial for the protein is given by

$$P(P_{tot}) = P(P_{nonsite}) P(P_{site}) \quad (17)$$

where $P(P_{nonsite})$ is the binding polynomial for the acid groups outside of the binding site and $P(P_{site})$ is the binding polynomial for the acid groups in the unoccupied binding site. In writing this equation, the acid groups in the binding site are defined as the acid groups that undergo a shift in pK when the ligand is bound. This major step in the treatment of the effect of the binding of a ligand by a protein is possible if the binding of the ligand changes the pKs of only some of the acid dissociations of the protein.

The corresponding binding polynomial for PL_{tot} is given by

$$P(PL_{tot}) = P(P_{nonsite}) P(PL_{site}) \quad (18)$$

where $P(P_{nonsite})$ is the same function that is in eq 16 and $P(PL_{site})$ has the same number of terms as $P(P_{site})$. The nonsite groups can interact with each other, and the site groups in the protein and in the protein−ligand complex can interact with each other.

Substituting eqs 17 and 18 in eq 8 when the ligand does not have pKs in the pH range considered yields a simpler equation for the dependence of K' on pH:

$$K' = K_{ref} P(P_{site})/P(PL_{site}) \quad (19)$$

This equation is important because it shows that the pH dependence of K' is determined entirely by the pKs of the acid groups in the unoccupied ligand-binding site and in the binding site when it is occupied by ligand. If the number of acid groups in the site is small, their pKs in the unoccupied and occupied site can be calculated from the dependence of K' on pH. Thus the determination of the pH dependence of K' can yield important information about the acid groups in the binding site. If the effect of temperature on K' is studied, the standard transformed enthalpies and entropies of the site can also be determined.

Positive Cooperativity (Accentuated Equilibrium Constants)

Before calculations are made on an actual system, it is important to discuss the possibility that positive cooperativity

may be involved in the binding of hydrogen ions in the unoccupied site and in the site occupied by ligand. The term positive cooperativity is somewhat unsatisfactory because it raises the question of negative cooperativity; the designation accentuated equilibrium constants is better. In a general way, positive cooperativity means that after a hydrogen ion is bound, the binding of the next hydrogen ion is accentuated, but this can be made more specific, as we will see below. Positive cooperativity is not observed in the binding of hydrogen ions by small molecules, but it has been observed in the binding of hydrogen ions by the catalytic site of fumarase when it is occupied by the competitive inhibitor L-tartrate.[10] Positive cooperativity has also been observed in the binding of ligands other than hydrogen ions by proteins in several systems described by Klotz.[11] Cooperativity in ligand binding by proteins occurs when the binding of a molecule of ligand causes a structural change so that binding of the next molecule of ligand is accentuated.

When eq 15 is used for a reaction system with positive cooperative effects, Klotz[11] has shown that some of the equilibrium constants may be complex; that is, they may involve the square root of −1. Klotz provides a detailed discussion of these "ghost site" equilibrium constants, as he refers to them. Since he discusses the association of ligands with proteins, he uses association constants, but here the emphasis is on the effect of pH, and so acid dissociation constants and their p*K*s are used. Klotz discusses ghost-site equilibrium constants for diprotic, triprotic, and tetraprotic acids, but only a diprotic acid is discussed here to provide the background for the next section.

The dissociations for a diprotic acid are represented by

$$HA^- = H^+ + A^{2-} \tag{20}$$

$$H_2A = H^+ + HA^- \tag{21}$$

The binding polynomial for diprotic acid H_2A is given by

$$P(A) = 1 + [H^+]/K_1 + [H^+]^2/K_1K_2 \tag{22}$$

If it is assumed that the two acid groups are independent, the binding polynomial can be written

$$P(A) = (1 + [H^+]/K_\alpha)(1 + [H^+]/K_\beta) = 1 + [H^+](1/K_\alpha + 1/K_\beta) + [H^+]^2/K_\alpha K_\beta \tag{23}$$

where Greek letters are used to designate the equilibrium constants that are complex when positive cooperativity is involved. When the two acid groups are independent, K_α and K_β are the real site acid dissociation constants (sometimes referred to as microscopic constants to distinguish them from the macroscopic constants K_1 and K_2). One way to see what eq 23 involves is to note that it leads to the following relation for the titration curve for diprotic acid H_2A with independent acid groups:

$$\bar{N}_H = [H^+]\frac{d \ln P(A)}{d[H^+]} = \frac{[H^+]/K_\alpha}{1 + [H^+]/K_\alpha} + \frac{[H^+]/K_\beta}{1 + [H^+]/K_\beta} \tag{24}$$

This shows that when the acid groups are independent, the number of hydrogen ions bound by A is simply the sum of the bindings at the two independent acid groups. Comparison of eqs 22 and 23 shows that

$$K_1 = \frac{1}{1/K_\alpha + 1/K_\beta} \tag{25}$$

$$K_1K_2 = K_\alpha K_\beta \tag{26}$$

Solving these two equations for K_α and K_β yields

$$K_\alpha = \frac{K_2 + \sqrt{K_2^{\,2} - 4K_1K_2}}{2} \tag{27}$$

$$K_\beta = \frac{K_2 + \sqrt{K_2^{\,2} - 4K_1K_2}}{2} \tag{28}$$

When $K_2 = 4K_1$, $K_\alpha = K_2/2$, and $K_\beta = K_2/2$, $K_\alpha = K_\beta = K$ so that $K_1 = K/2$ and $K_2 = 2K$, where K is the microscopic constant when the two acid groups are identical and independent. This case provides a boundary between positive cooperativity (with the possibility of ghost constants) and noncooperativity (with no ghost equilibrium constants):

$K_1 > K_2/4$	cooperative	$pK_1 < pK_2 + 0.60$
$K_1 = K_2/4$	identical and independent	$pK_1 = pK_2 + 0.60$
$K_1 < K_2/4$	noncooperative	$pK_1 > pK_2 + 0.60$

When the binding of the two hydrogen ions is positively cooperative ($K_1 > K_2/4$), K_α and K_β will be complex. When K_α and K_β are complex, they cannot be interpreted as site dissociation constants. It should be noted that if eqs 27 and 28 are substituted into eq 24 and it is cleared of fractions, the imaginary terms disappear, and so the correct real average number of hydrogen ions bound by the diprotic acid is obtained, even when the acid groups are positively cooperative and K_α and K_β are used.

The terminology that hydrogen ion binding for a diprotic acid is cooperative if $K_2 < 4K_1$ differs from the terminology that the binding is cooperative when the second dissociation constant is less than the first dissociation constant ($K_2 < K_1$), which is incorrect even though it is given in most books. The boundary between cooperative and noncooperative interaction is the case where the groups are identical and independent. This case is neither cooperative nor noncooperative. The boundary between cooperative and noncooperative is given by the relation between the successive dissociation constants of an *N*-protic acid with identical but independent acid groups.

$$K_i = \frac{iK}{N - i + 1} \tag{29}$$

For a triprotic acid, a cooperative effect is indicated by $K_2 < 3K_1$ or $K_3 < K_2$. For a tetraprotic acid, a cooperative effect is indicated by $K_2 < (^8/_3)K_1$, $K_3 < (^9/_4)K_2$, or $K_4 < (^8/_3)K_3$. These inequalities are derived using eq 29. Complex equilibrium constants always occur in pairs, so for a triprotic acid cooperativity leads to two complex constants and one real constant.[11]

Calculations on Hydrogen Ion Binding at a Catalytic Site

The equilibrium constants for the binding of competitive inhibitors at the catalytic site of an enzyme can be determined by studies of enzyme kinetics. In the case of fumarase,[12,13] the plot of the maximum initial velocity V versus pH is a symmetrical bell-shaped curve that is represented by

TABLE 1: p*K*s of Acid Groups in the Catalytic Site in Fumarase at 25 °C and an Ionic Strength of 0.01 M

ligand	pK_1	pK_2	K_{ref}
unoccupied	6.9	6.3	
succinate	7.5	6.5	1.2×10^{-3}
D-tartrate	7.8	6.9	2.5×10^{-3}
L-tartrate	7.5	7.4	4.1×10^{-3}
meso-tartrate	7.1	5.7	4.6

$$V = \frac{k[E]_0}{1 + \frac{K_1}{[H^+]} + \frac{[H^+]}{K_2}} = \frac{k[E]_0[H^+]/K_1}{1 + \frac{[H^+]}{K_1} + \frac{[H^+]^2}{K_1K_2}} \quad (30)$$

which shows that there are two acid groups in the catalytic site and that the catalytically active form contains one hydrogen atom. In this equation, k is the first-order rate constant for the production of product from the enzyme−substrate complex and $[E]_0$ is the concentration of enzymatic sites. The p*K*s of the two acid groups when the site is not occupied can be determined from the kinetics of the catalyzed reaction in the absence of a competitive inhibitor. The p*K*s of the unoccupied catalytic site of fumarase and the site occupied by a competitive inhibitor that have been determined[10] from kinetic measurements are given in Table 1. The competitive equilibrium constant K_{ref} in the limit of high pK is also given, where concentrations are expressed on the molar scale.

In all of these cases except one, the p*K*s are increased by the binding of the inhibitor, as would be expected for the binding of a negative ion. However, it is important to keep in mind that Laskowski and Scheraga[14] pointed out that the dissociation constants of acid groups in proteins can be increased or decreased by the presence of hydrogen bonds as well as by electrostatic effects. Similarly, cooperative effects may result from hydrogen bonding as well as partial unfolding of the protein molecule. The objective in this paper is to provide a thermodynamic representation of the experimental data rather than to analyze specific equilibrium constants in terms of the structures of the inhibitors. It was pointed out in 1960 that the p*K*s for the unoccupied catalytic site indicate that the groups can be considered to be identical and independent. It was also pointed out that when L-tartrate is bound, the addition of the first hydrogen ion increases the affinity of the site for the second hydrogen ion. Thus K_α and K_β for the binding site in the fumarase−L-tartrate are complex.

To quantitatively discuss the pH dependence of the apparent equilibrium constant for the binding of L-tartrate by the catalytic site of fumarase, the binding polynomial for the unoccupied site is given by

$$P(P_{site}) = 1 + 10^{-pH+pK_{PS1}} + 10^{-2pH+pK_{PS1}+pK_{PS2}} \quad (31)$$

The binding polynomial for the site when it is occupied by L-tartrate is given by

$$P(PL_{site}) = 1 + 10^{-pH+pK_{PLS1}} + 10^{-2pH+pK_{PLS1}+pK_{PLS2}} \quad (32)$$

Substituting eqs 31 and 32 in eq 19 yields

$$K' = K_{ref}\frac{1 + 10^{-pH+pK_{PS1}} + 10^{-2pH+pK_{PS1}+pK_{PS2}}}{1 + 10^{-pH+pK_{PLS1}} + 10^{-2pH+pK_{PLS1}+pK_{PLS2}}} \quad (33)$$

As indicated in Table 1, the p*K*s for the two acid groups in the unoccupied catalytic site of fumarase are $pK_{PS1} = 6.9$ and $pK_{PS2} = 6.3$. These two acid groups can be considered to be identical and independent. The p*K*s for site-L-tartrate are $pK_{PLS1} = 7.5$ and $pK_{PLS2} = 7.4$, indicating there is a cooperative effect. The acid dissociations of L-tartrate are ignored in these calculations because this paper is primarily concerned with what happens in the neighborhood of pH 7.

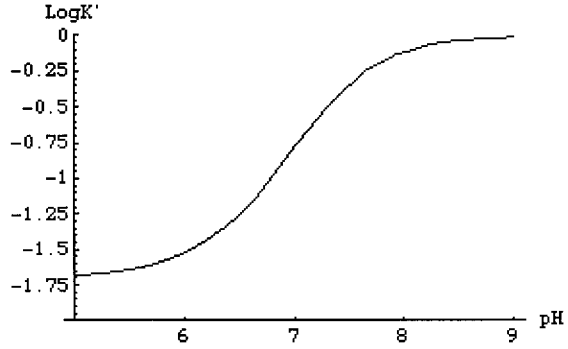

Figure 1. Plot of log K' for the reaction site−L-tartrate (site + L-tartrate over a range of pH at 25 °C and an ionic strength of 0.01 M).

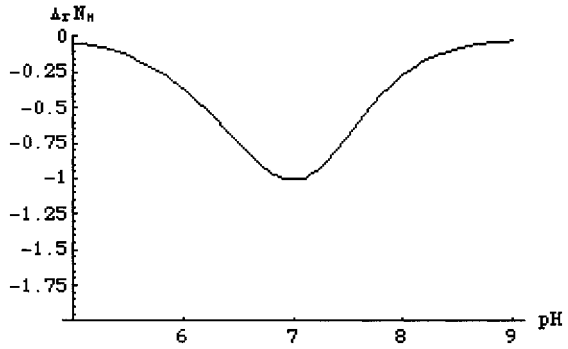

Figure 2. Plot of Δ_rN_H for the reaction site−L-tartrate (site + L-tartrate over a range of pH at 25 °C and an ionic strength of 0.01 M).

With the indicated values of the p*K*s, the pH dependence of the apparent equilibrium constant for the dissociation of L-tartrate from the complex is given by

$$K' = K_{ref}\frac{1 + 10^{-pH+6.9} + 10^{-2pH+13.2}}{1 + 10^{-pH+7.5} + 10^{-2pH+14.9}} \quad (34)$$

The base 10 logarithm of K' is given as a function of pH in Figure 1. The constant $K_{ref} = 4.1 \times 10^{-3}$ has been omitted in making Figure 1 because it is not involved in the pH dependence. The change in binding of hydrogen ions in the dissociation of the site−L-tartrate complex can be calculated by taking the derivative of log K' with respect to pH (eq 12). The pH dependence of Δ_rN_H is shown in Figure 2. Since the products (unoccupied site plus L-tartrate) bind hydrogen ions less strongly than the complex, Δ_rN_H is negative at all pH values. Another way to express this is that hydrogen ions are produced in the dissociation, except in the limit of very high and very low pH values where $\Delta_rN_H = 0$.

To show how the p*K*s of acid groups in the binding site of fumarase might be determined without using enzyme kinetics, values of K' were calculated using eq 33 at intervals of 0.5 pH, and these values were fit using NonlinearFit in *Mathematica*[15] to the function in eq 33. This calculation yielded the expected result, that is, eq 34. A calculation more like the interpretation of experimental data was made by introducing random errors between 0 and 0.05 into the pH values used to calculate K' at a series of pH values. When this was done, the values of the four p*K*s (pK_{PS1}, pK_{PS2}, pK_{PLS1}, and pK_{PLS2}) obtained using NonlinearFit were as follows: first trial, 6.37, 6.67, 7.39, and

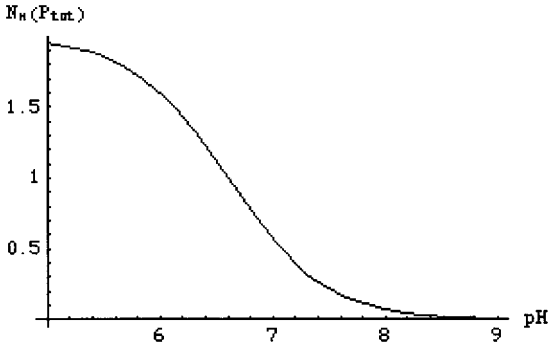

Figure 3. Plot of the average number of hydrogen ions bound $\bar{N}_H$ by the unoccupied catalytic site of fumarase at 25 °C and an ionic strength of 0.01 M.

7.28; second trial, 6.91, 6.24, 7.51, and 7.36; third trial, 6.28, 6.55, 7.48, and 7.01; in comparison with the experimental values 6.9, 6.3, 7.5, and 7.4. A more realistic calculation would use random errors that follow a Gaussian distribution. These computer experiments indicate that all four p*K*s can be calculated from the dependence of K' on pH.

Binding Capacities of the Catalytic Sites

This section is concerned with the average numbers of hydrogen ions bound by the catalytic site of fumarase (unoccupied and occupied by L-tartrate) and with a related quantity, the binding capacity named by Di Cera, Gill, and Wyman.[16] The binding capacity for hydrogen ions is defined by

$$\frac{d\bar{N}_H}{dpH} = -\frac{1}{\ln(10)}\frac{d^2 \ln P}{d\,pH^2} \qquad (35)$$

This name for the second derivative of ln *P* with respect to pH was adopted because this quantity is analogous to the heat capacity, which is given by the second derivative of the Gibbs energy *G* with respect to temperature. They pointed out that the binding capacity is a measure of cooperativity.

The preceding discussion has been concerned with the apparent dissociation constant of a protein−ligand complex and the change in binding of H^+ in the dissociation, but it is also of interest to consider the hydrogen ion binding of the unoccupied site of fumarase and especially the site occupied by L-tartrate. The average number of hydrogen ions bound by the catalytic site is given by $\bar{N}_H = -(1/\ln 10)\mathrm{d} \ln P(P_{site})/\mathrm{d}\ pH$. This quantity for the unoccupied site is plotted in Figure 3. This plot has the same shape as the titration curve of a single site because $K_2 = 4K_1$ but with the ordinate multiplied by 2. The slope of this plot (see eq 35) is given in Figure 4. This case corresponds with zero cooperativity as defined here.

The number of hydrogen ions bound by the catalytic site in the fumarase−L-tartrate complex is plotted in Figure 5. This plot is steeper than the plot in Figure 3 because of positive cooperativity, that is, $K_1 > K_2/4$. This is shown more clearly by the corresponding plot for the binding capacity, which is shown in Figure 6. Comparison of this plot with Figure 4 confirms that the binding capacity is greater when there is positive cooperativity.

Calculation of Standard Transformed Gibbs Energies of Formation of the Catalytic Site of Fumarase

Equation 3 indicates that the apparent equilibrium constant for a biochemical reaction at a specified pH can be calculated

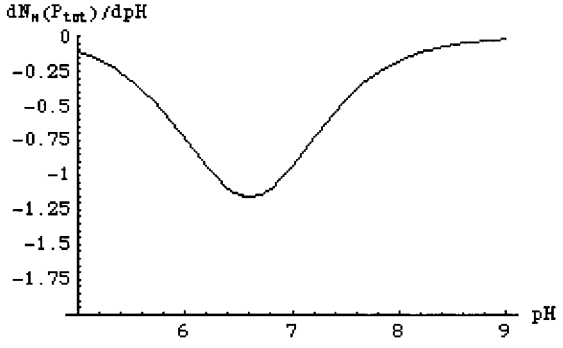

Figure 4. Plot of the binding capacity (rate of change of the average number of hydrogen ions bound with respect to the pH) for an unoccupied catalytic site of fumarase at 25 °C and an ionic strength of 0.01 M.

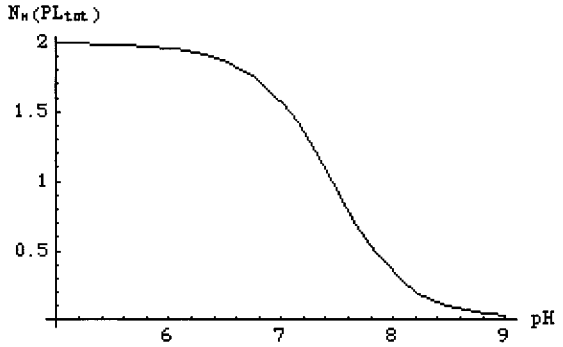

Figure 5. Plot of the average number of hydrogen ions bound $\bar{N}_H$ by the catalytic site of fumarase occupied by L-tartrate at 25 °C and at an ionic strength of 0.01 M.

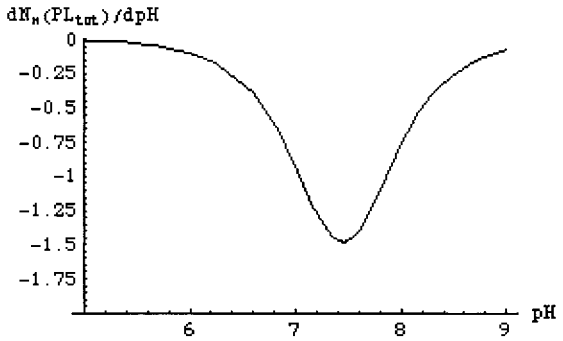

Figure 6. Plot of the binding capacity (rate of change of the average number of hydrogen ions bound with respect to the pH) for the catalytic site of fumarase occupied by L-tartrate at 25 °C and an ionic strength of 0.01 M. The slope of the binding curve is steeper than in Figure 4.

from the standard transformed Gibbs energies of formation of the reactants. A recent article[17] has shown that $\Delta_f G_i'^{o}$ for a biochemical reactant is given by

$$\Delta_f G_i'^{o} = \Delta_f G_1'^{o} - RT \ln P \qquad (36)$$

where $\Delta_f G_1'^{o}$ is the standard transformed Gibbs energy of formation for the species with the fewest hydrogen atoms and *P* is the binding polynomial for the reactant. This equation can be applied to the enzymatic site for fumarase and to the complexes formed with competitive inhibitors.

The apparent dissociation constant of the fumarase site−succinate complex to yield unoccupied site and succinate is represented by the following function of pH:

$$K' = 1.2 \times 10^{-3}\frac{1 + 10^{6.9-pH} + 10^{13.2-2pH}}{1 + 10^{7.5-pH} + 10^{14.0-2pH}} \qquad (37)$$

TABLE 2: Standard Transformed Gibbs Energies of Formation in kJ mol^{-1} at 25 °C and Ionic Strength 0.01 M[a]

	pH 5	pH 6	pH 7	pH 8	pH 9
site	−18.39	−7.96	−1.66	−0.19	−0.02
succinate	−576.28	−553.45	−530.62	−507.78	−484.95
site−succinate	−615.86	−582.23	−551.35	−525.15	−501.70
D-tartrate	114.16	136.99	159.83	182.66	205.49
site−D-tartrate	72.45	106.43	138.75	166.52	190.48
L-tartrate	114.16	136.99	159.83	182.66	205.49
site−L-tartrate	72.56	106.71	140.02	168.21	191.78
meso-tartrate	114.16	136.99	159.83	182.66	205.49
site−*meso*-tartrate	101.51	133.36	161.52	186.15	209.24

[a] This table is based on the convention that $\Delta_f G^o = 0$ at 25 °C and zero ionic strength for the doubly charged ions of D-tartrate, L-tartarate, and *meso*-tartrate. In addition, the convention is that $\Delta_f G'^o = 0$ for the binding site at high pH.

According to eq 3, this K' is given by

$$-RT \ln K' = \Delta_f G'^o(\text{site}) + \Delta_f G'^o(\text{succ}) - \Delta_f G'^o(\text{site}-\text{succ}) \quad (38)$$

The value of $\Delta_f G^o(\text{succ}^{2-})$ at 25 °C and zero ionic strength is −690.44 kJ mol^{-1}, and the pH dependence of $\Delta_f G'^o$(succ) is given by $-690.44 - 4RT \ln 10^{-\text{pH}}$, neglecting the effect of the binding hydrogen ions at lower pH values.[18] This value is independent of the ionic strength because $z_i^2 = N_H$. $\Delta_f G'^o$(site) is taken as zero in the limit of high pH by convention so that

$$\Delta_f G'^o(\text{site}) = -RT \ln(1 + 10^{6.9-\text{pH}} + 10^{13.3-2\text{pH}}) \quad (39)$$

Equation 3 can be written as

$$-RT \ln K' = -RT \ln(1 + 10^{6.9-\text{pH}} + 10^{13.3-2\text{pH}}) - 960.44 - 4RT \ln 10^{-\text{pH}} - \Delta_f G'^o(\text{site}-\text{succ}) \quad (40)$$

Substituting eq 37 yields

$$\Delta_f G'^o(\text{site}-\text{succ}) = -707.11 - 4RT \ln 10^{-\text{pH}} - \text{RT} \ln(1 + 10^{7.5-\text{pH}} + 10^{14.0-2\text{pH}}) \quad (41)$$

The values of $\Delta_f G'^o$ for the catalytic site, succinate, and site−succinate complex calculated in this way are shown in Table 2. Similar calculations have been made for D-tartrate, L-tartrate, and *meso*-tartrate using data from Table 1. Since the $\Delta_f G^o$values for these three reactants are not known, the convention has been adopted that they are equal to zero.

This table is important because it shows that standard transformed Gibbs energies of formation at specified pH values can be calculated for an unoccupied binding site or a binding site occupied by a ligand.

Discussion

This article shows that when the dependence of the apparent equilibrium constant for the dissociation of a site−L-tartrate complex is determined as a function of pH, the p*K*s for the acid groups in the unoccupied site and the occupied site can be determined. The binding site includes all of the acid groups that undergo a change in p*K* when the ligand is bound. The calculations with the competitive inhibition constants of four inhibitors of fumarase show that this can be done even when there is positive cooperativity between the acid groups in the binding site. These studies yield important information about the chemical nature of the binding site. Knowledge of the p*K*s of the acid groups in the binding site and the site occupied by various ligands can be used to calculate standard transformed Gibbs energies of formation, as shown in Table 2.

The importance of Table 2 is that this approach can be used to include proteins as well as reactants with low molecular masses in tables of standard transformed thermodynamic properties at various pH values. If the pH dependence of the apparent equilibrium constant for an enzyme-catalyzed reaction is studied over a range of pH values and the thermodynamic properties of the other reactants are known, the standard Gibbs energies of the site in the protein that is involved in the enzyme-catalyzed reaction can be calculated, put in a table, and used for calculating the apparent equilibrium constants of other reactions involving that protein, provided the same site in the protein is involved. The catalyzed reaction may involve oxidation or reduction of a site in the reactant protein or some other type of site.

It is important to connect apparent equilibrium constants K' with $\Delta_r G'^o$ for reactions and $\Delta_f G'^o$ for reactants because this opens up the possibility of interpreting these values with $\Delta_r H'^o$, $\Delta_f H'^o$, $\Delta_r S'^o$, and $\Delta_f S'^o$, which in turn can be related to $\Delta_f H^\circ$ and $\Delta_f S^\circ$ for species. These values can be calculated if the effect of temperature on K' is determined. The standard transformed enthalpy of reaction and the standard transformed enthalpies of formation are calculated using the Gibbs−Helmholtz equation:

$$\Delta_r H'^o = -T^2 \left[\frac{\partial(\Delta_r G'^o/T)}{\partial T} \right]_{P,\text{pH}} = \sum \nu_i' \Delta_f H_i'^o \quad (42)$$

The standard transformed entropy of reaction and the standard transformed entropies of formation are calculated using

$$\Delta_r S'^o = -\left(\frac{\partial \Delta_r G'^o}{\partial T} \right)_{P,\text{pH}} = \sum \nu_i' \Delta_f S_i'^o \quad (43)$$

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