THE INTRINSIC pK_a-VALUES OF FUNCTIONAL GROUPS IN ENZYMES: IMPROPER DEDUCTIONS FROM THE pH-DEPENDENCE OF STEADY-STATE PARAMETERS

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INTRODUCTION

For many years, the ease with which the pH of enzyme reactions could be varied has led to innumerable reports of the pH-dependence of the kinetic parameters of such reactions. These studies have been provided with a veneer of respectability by suggestions that they allow deductions to be made about the nature of the ionizing groups upon which the catalytic activity of the enzyme depends. It is the purpose of this article to summarize some of the reasons why a large proportion of pH-dependence studies yields little information about the real pK_a-values of ionizing groups in enzymes, and still less about the identity of these groups.

In Table 1 is listed, in order of increasing meaningfulness and utility, the range of pH-dependence studies to which enzymes are or can be subjected. The categories listed will be discussed in order.

pH-"ACTIVITY" CURVES

When a new enzyme is isolated or a known enzyme is obtained from a hitherto unstudied source, it is natural to scan the pH range and obtain some idea of the pH-optimum so that future work can be done at a pH where the rate is near maximal and where small differences in the pH of buffers used will have the least effect upon the measured rates. Frequently, a standard assay may for simplicity rely on a single measurement of substrate consumed after a given time. While such determinations will yield the pH-optimum for the arbitrarily selected conditions of initial substrate concentration, enzyme concentration, temperature, ionic strength, and the rest, they serve no other useful purpose. If K_m is itself pH-dependent, it will be clear that the observed extent of the reaction after fixed time can be a sensitive function of $[E]_o$, $[S]_o$, the variation of K_m with pH, and the accumulation of (possibly inhibitory) product. Even for such a crude purpose as fol-

TABLE 1

pH-Dependence Studies in Order of Increasing Utility

pH-"Activity" curves at fixed $[S]_o$
pH-Dependences of k_{cat}, K_m, k_{cat}/K_m, and K_i
pH-Dependences of $k_{modification}$ (with specific reagents)
Competitive labeling (with nonspecific reagents)
pH-Dependence of an elementary step
Direct observation of a titrating group

lowing an enzyme through its purification, therefore, such pH-"activity" curves can be misleading. If a continuous assay is available, then true values of the initial rate of reaction, v_o, can be determined. No longer is one at the mercy of the arbitrary choice of $[E]_o$ and $[S]_o$, but the possible variation of K_m with pH still prevents any but pragmatic use of the observed pH-dependency. Certainly no deductions can be made about pK_a-values, apparent or otherwise, from such data. Finally, at the risk of being presumptuous, the presentation of such a pH-dependence without evidence as to the stability of the enzyme (and of any coupling enzymes and cofactors) as a function of pH is close to worthless. A number of published pH-"activity" curves can be interpreted in terms of the instability of the target enzyme at one of the pH extremes (this is quite often a cooperative process where the change in slope of the double logarithmic plot is greater than ±1 [see Reference 1]) or of the instability, for instance, of NADH at low pH or of NAD^+ at high pH.

The very first criterion for an acceptable pH-dependence must be that it fits a theoretical titration curve. Where it does not, the dependence is only useful as a guide to the protocol for an enzyme under the stated conditions, and no other deductions can or should be made.

pH-DEPENDENCE OF k_{cat}, K_m, k_{cat}/K_m, AND K_i

The determination of the pH-dependence of the operational kinetic parameters of the Michaelis-Menten equation has been, and is, a common pastime of enzymologists. Of all the categories listed in Table I, this group certainly contains the most examples and has led to the largest number of unwarrantedly definitive statements (including some from the present author[2]) about the nature of functional groups in enzymes. One reason for this would appear to be the acceptance of the elegant work of Alberty et al.,[3,4] coupled with a blithe disregard for the assumptions that these workers clearly stated. In summary, Alberty said that *if* there is only one state of ionization of the "active site" that is capable of catalyzing the interconversion of substrate and product, and *if* all prototropic equilibria involving the ionizing groups are fast with respect to all interconversion steps in the reaction, then a number of conclusions are possible from the variation of k_{cat}/K_m and of k_{cat}, with pH. Plots of k_{cat}/K_m versus pH will yield the pK_a-values for free enzyme and/or free substrate, and plots of k_{cat} versus pH will provide the pK_a-values of the enzyme-substrate complex whose decomposition is rate-limiting.

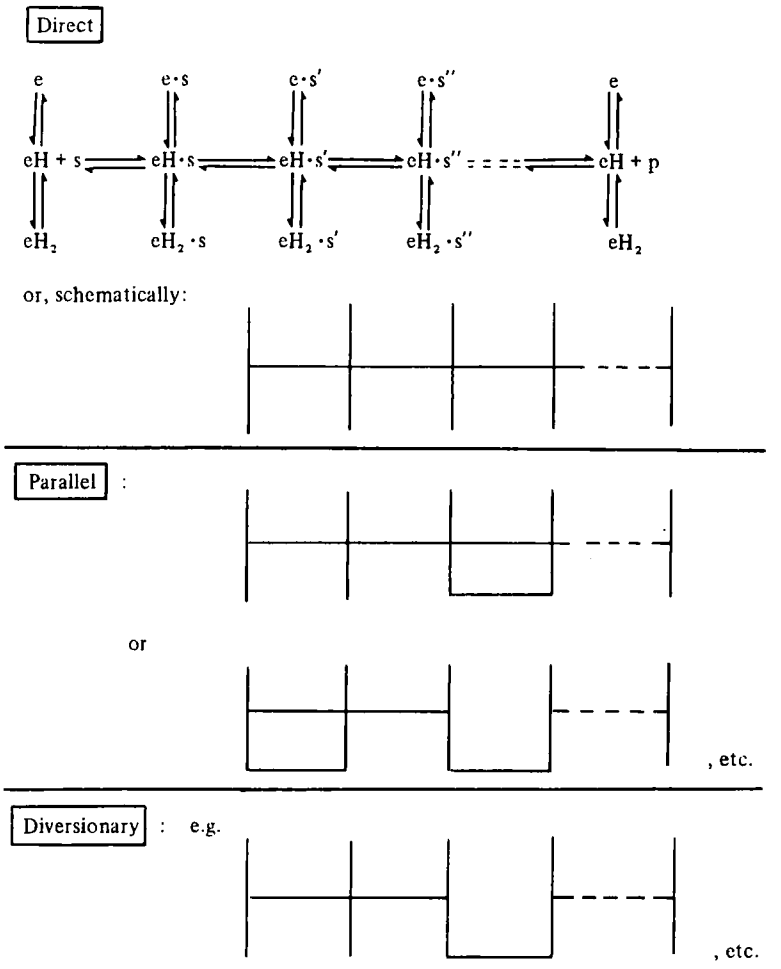
The first assumption of Alberty (some of the consequences of which have been considered in detail[5]) is illustrated in Scheme 1. It must be assumed that there are no parallel pathways and no diversionary pathways in the course of the interconversion of substrate and product. If such pathways do exist, then, as forcefully pointed out by Schmidt and Westheimer.

> ... the pK_a s derived from these profiles (k_{cat}/K_m, or k_{cat}) are not simple quantities, but contain, in addition to various ionization constants, rate constant ratios of completely unknown magnitudes.[6]

Since this is true, is the determination of such pH-dependences a pointless activity, or can we reasonably assume – in a real case – that only the direct path exists? Unfortunately this assumption is at best dangerous, and at worst, wrong. For a number of systems, there are good chemical reasons for proposing that diversionary pathways will be followed, on the grounds that the direct pathway would involve reaction intermediates of very high relative free energy. Thus in the case of acetoacetate decarboxylase,[6] chemical precedent strongly suggests that the lysyl residue at the active site will be deprotonated to form the Schiff base with the substrate, but that the nitrogen atom must subsequently reprotonate to catalyze the decarboxylation reaction itself. The hydrolysis of the acetone Schiff base will then (initially, at least) liberate the enzyme with its essential lysyl residue unprotonated. In such cases, it is evidently dangerous to assume that a direct pathway is followed. Further, in any enzyme-catalyzed reaction involving an unspecified number of elementary steps, there is no experimental test that allows one to rule out the incursion of a diversionary or a parallel pathway. While it is clear that no statements about the meaning of observed pK_a-values are possible *without* making the assumption that only the direct pathway is followed, that fact does not provide either justification or excuse for the assumption. For the early steps in an enzyme-catalyzed reaction, which are in general more accessible experimentally, the existence of parallel pathways is well known. Neutral substrates and competitive inhibitors of α-chymotrypsin are

SCHEME 1

Direct, Parallel, and Diversionary Pathways for Enzyme-Catalyzed Reactions[a]



[a]The change in charge consequent upon protonation and deprotonation has been omitted for clarity. s is substrate, p is product, eH·s, eH·s′, eH·s″, etc. denote various reaction intermediates.

known to bind to the enzyme without significant perturbation between pH 3 and 9, during which there are undoubted changes in the state of ionization of the residues at and near the active site. The evident complications even in the early steps of this extremely well-studied system[7,8] should curtail attempts to interpret the pH-dependence of the steady-state parameters of any enzyme whose course is less well charted.

Finally, even if we do assume that the direct pathway is followed, an observed pK_a need not relate to the intrinsic pK_a of the ionizing group. As has been pointed out by Jencks, a "mirage" pK_a may be seen if the elementary step affected by the ionization is not rate-determining at all pH-values.[9] Suppose that there is a single step in the reaction requiring an enzymic base of high pK_a to be unprotonated, and that the rate of this step at pH 8 is 100 times faster than a subsequent pH-independent step. As the pH is lowered, the rate of the pH-dependent step is slowed by tenfold for each pH unit, so only a little above pH 6 will the rate of the pH-dependent step begin to be felt in k_{cat}, and a very wrong view of the intrinsic pK_a would be obtained. A number of these and other problems involved, including specific interactions

between protonation sites, pre-equilibria,[10] and the consequences of pathways other than the direct one, have been treated in rigorous algebraic detail.[11]

Let us now consider the second assumption required by the Alberty treatment, namely that all protonation-deprotonation steps are fast relative to the substrate-product interconversion steps. (In the terms of Scheme 1, this says that all vertical processes are at equilibrium and much faster than any horizontal step.) Since the acids of interest in proteins are those of oxygen, nitrogen, and sulphur, this assumption may not appear either improbable or restrictive. Yet for many systems, the rates may come rather close. For *associative* steps involving the proton with oxygen, nitrogen, and sulphur bases, Eigen has shown that for small molecules in free solution, $10^{10}\ M^{-1}\ sec^{-1}$ is a reasonably common value.[12] This number may, however, be perturbed for bases in the active sites of enzymes. If the accepted Grotthus chain pathway for proton migration is upset, and it may easily be in the restricted environment of the active site, then reprotonation rates for enzymic bases may fall below the level of $10^{10}\ M^{-1}\ sec^{-1}$. (Analogous arguments may be used in support of a more ordered structure for water near active sites, of course, which could *increase* the rate of proton transfer to the levels seen in ice.) Since the bimolecular "on" rate constants for enzymes and their substrates are between 10^6 and $5 \times 10^8\ M^{-1}\ sec^{-1}$ (see Reference 13) and may be higher[14] it does not seem safe to assume that protons will axiomatically associate with enzyme bases faster than substrates with enzyme active sites. For *dissociative* steps, an acid having an intrinsic pK_a of ~6 will (assuming an association rate of $\sim 10^{10}\ M^{-1}\ sec^{-1}$, dissociate with a rate constant of $\sim 10^4\ sec^{-1}$. (It has been pointed out however [A. J. Cornish-Bowden, private communication] that this assumes that H_2O is the only important Brønsted base; but the contribution of OH^- which reacts with common conjugate acids with a rate constant around $10^{10}\ M^{-1}\ sec^{-1}$ will become important at pH values above 7 and will accelerate dissociative steps.) Yet an enzyme with a turnover number of 600,000 min^{-1} (and there are several) will have *as its slowest catalytic step*, a rate constant of $10^4\ sec^{-1}$. For carbonic anhydrase, it is known that the rate of proton transfer limits the overall rate of reaction, since increasing the buffer concentration from 0 to 10 m*M* enhances the rate of the *catalyzed* reaction by 80% at pH 8.3 (see Reference 15). Further, it is known that the rate of ionization of an active-site acid in an enzyme: substrate reaction intermediate (of triose phosphate isomerase) is only eightfold faster than the substrate conversion step leading to this intermediate and twofold *slower* than the step leading from it to products.[16] Nor can this be a rare phenomenon, as the number of examples of "sticky protons"[17] in enzyme-catalyzed reactions grows. There is thus a real danger that in many enzyme-catalyzed reactions, the protonation-deprotonation reactions (the vertical steps of Scheme 1) are not maintained at equilibrium. The consequences of this breakdown have been explored and discussed previously by Ottolenghi.[18] The message must therefore be that one does not know, and in many cases *cannot* know, whether either of the assumptions necessary to apply the Alberty treatment may be validly made. Worse, for many enzyme reactions that have been studied in enough detail, it is known that these assumptions do *not* hold.

One area of usefulness in the determination of the pH-dependence of k_{cat} and K_m is that under appropriate circumstances, the invariance of K_m with pH while k_{cat} changes indicates that $K_m = K_s$ (the enzyme + substrate binding equilibrium constant). Since this has been elegantly discussed elsewhere,[19] it will not be elaborated here.

Finally, what of the pH-dependence of K_i? The inhibition constant for an enzyme-catalyzed reaction (assuming the simplest case of linear competitive inhibition) is a true thermodynamic equilibrium constant, and some authors have suggested that the pH-dependence of K_i is therefore more amenable to mechanistic interpretation than are the catalytic parameters, k_{cat} and K_m, or any combination of them. But K_i is a dissociation constant and is equal to $[E][I]/[EI]$. When the pH is varied, therefore, K_i will change in a way that is determined by the product of the pH functions for free enzyme and free inhibitor, divided by the pH function for the EI complex. As has been fully discussed elsewhere,[1,11] it is unlikely that the experimental data will allow these three pH functions to be disentangled, even though it is possible in theory.

pH-DEPENDENCE OF $k_{m\,modification}$

In an important paper, Schmidt and

Westheimer[6] suggested that while none of the pK_a-values that might be derived from the pH-dependence of k_{cat}/K_m or of k_{cat} has a simple interpretation, the reaction of a reagent specifically with an ionizing group at the active site offers a way of determining the pK_a of that group. The argument runs as follows. If the reaction scheme is as shown in Scheme 2(a), then (assuming the prototropic equilibrium is fast, again) the variation of k_{obs} with pH will yield K_1, the true ionization constant for the modified group. If we now allow a binding step to occur as in Scheme 2(b), then the equation is more complex, but can be simplified if we ensure that the experimental conditions are such that the second denominator term disappears. Schmidt and Westheimer[6] subjected this to experimental test and worked at low reagent concentrations such that the pH-dependence of k_{obs} gives K_1, the dissociation constant of the free enzyme. Allowing now a more complex scheme where the e·r complex can ionize independently (Scheme 2[c]), an equation results that yields K_1, like Scheme 2(b), if the reaction is strictly first order in r. However, consider the fuller Scheme 2(d), which is not improbable if r is a neutral species. Immediately it can be seen that even if one works at very low reagent concentrations so that the second denominator term is negligible, the pH-dependence of inactivation no longer gives K_1. The expression for Scheme 2(d) only reduces to a form that yields K_1 if one assumes that all the prototropic equilibria are fast *and* that $k_2 \ll k_{-1}$. These assumptions will be valid in particular cases, of course, but it is clearly improper to presume that they hold universally.

The above exercise demonstrates some of the difficulties. While the use of active-site modification reagents is less fraught with dangers than is the use of the kinetic parameters of substrate reaction, it is clear that the probable pathway of Scheme 2(d) is only amenable to interpretation if the following criteria are met: (1) that the reaction be cleanly first-order in reagent (experimentally testable), (2) that the value of $k_2 \ll k_{-1}$, and (3) that the prototropic equilibria are fast compared with the reaction steps. As with any kinetic measurement, of course, consistency with a particular pathway cannot prove it, and it needs little imagination to conceive of more tortuous pathways for the formation of e - r whose pH-dependence behavior would be less helpful.

One extension of the use of chemical modification reactions that is frequently used in the literature is the measurement of the inactivation rate of the enzyme, v_i, as a function of reagent concentration, [r]. Since k_{obs} in Scheme 2 is $v_i/[E]_o$, the equations of Schemes 2(b), (c), and (d) have the form of the Michaelis-Menten equation, and one can present the data as a double-reciprocal plot. Now, there are two extreme cases, one where $V_{i(max)}$ is measured as a function of pH, which gives the apparent ionization constant K_2, and the other where k_2/K_m is measured as a function of pH (this is equivalent to the procedure of Schmidt and Westheimer,[6] using very low [r]), which – as discussed above – might or might not give K_1. Neither procedure necessarily yields K_1, the dissociation constant of the free enzyme. And since each procedure may give a different K, it should not be a surprise that quite different apparent pK_a-values have been suggested even for the same reagent modifying the same enzyme.

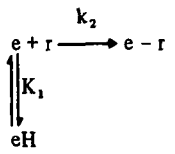
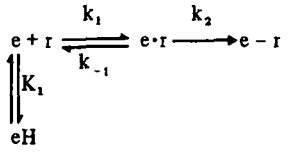
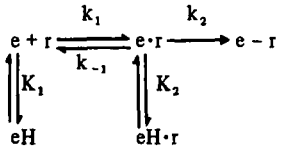
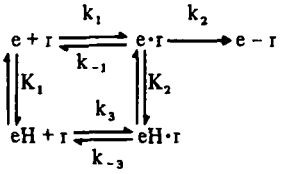
COMPETITIVE LABELING

From the form of the pathways illustrated in Scheme 2 and the equations derived from them, it will be clear that the major complication in chemical modification studies arises from the pre-reaction binding of reagent to enzyme. The exploitation of enzyme active-site specificity is just what is required for specific modification at a unique site, yet it is just this that causes trouble in the variation of the inactivation rate with pH. One way to circumvent this problem has been used by Kaplan et al.,[20] whose method involves the simultaneous modification of all amino groups in a protein with a nonspecific reagent, acetic anhydride. The principle of this method is that the various amino groups compete for a small amount of radioactive reagent, and the amount incorporated onto any one amino group is determined by its intrinsic nucleophilicity and pK_a. After labeling with the trace quantity of radioactive reagent, the protein is made chemically homogeneous by treatment with excess of nonradioactive reagent. The protein is then digested enzymically, and the specific radioactivity of each of the peptides containing a labeled amino group is determined.

This procedure is repeated at a number of pH values, and the intrinsic reactivities and pK_a-values of each of the amino groups are determined, assuming a simple bimolecular reaction between reagent and amino group.

SCHEME 2

Possible Pathways for a Reagent, r, to React with an Enzyme, e[a]

Scheme	Equation[b]
(a) 	$k_{obs} = \frac{k_2[r]}{1 + H/K_1}$
(b) 	$k_{obs} = \frac{k_2[r]}{K_m(1 + H/K_1) + r}$
(c) 	$k_{obs} = \frac{k_2[r]}{K_m(1 + H/K_1) + r(1 + H/K_2)}$
(d) 	$k_{obs} = \frac{k_2[r]}{\frac{(K_m + k_{-3}H/k_1K_2)(1 + H/K_1) + r(1 + H/K_2)}{(1 + k_3H/k_1K_1)}}$

[a] It is assumed (and can be verified experimentally) that r does not react with eH and that r reacts stoichiometrically with an ionizing group at the active site.
[b] k_{obs} is the observed inactivation rate constant, H is the proton concentration, and $K_m = (k_{-1} + k_2)/k_1$.

This approach will yield proper pK_a-values for systems amenable to the somewhat laborious peptide chemistry, as long as (1) all the labeling sites can be identified, (2) no protein structural change occurs at the pH values required to define the pK_a-values, and (3) no pre-reaction binding occurs. Certainly the use of acetic anhydride as a relatively small, uncharged, reagent seems attractive, even though enzymes are well able to recognize organic molecules much smaller than this. Strictly, therefore, unless the absence of pre-reaction binding can be demonstrated (and this is usually difficult), even this method can yield suspect pK_a-values. For instance, the approach has been extended to the use of dinitrofluorobenzene as a reagent for histidine residues in α-chymotrypsin.[21] Here, both His_{57} *and* His_{40} react with "unusual" ease compared with free histidine, and the known predilection of chymotrypsin for amino acids with aromatic side-chains makes it probable that the apparent pK_a-values that have been determined by this method for two residues so close to the reagent binding site are substantially different from their actual values in the free enzyme.

pH-DEPENDENCE OF AN ELEMENTARY STEP

Much of the above discussion has hinged on the difficulty of making deductions from the pH-dependence of steady-state parameters, whether those relate to catalytic turnover or to covalent modification of a functional group. If, however, a single elementary step in an enzyme-catalyzed reaction can be cleanly isolated over the whole pH range of interest, then the pK_a-value of a group on which this step depends will be properly determin-

ed. The isolation of an elementary step means, of course, that the concentration of the reaction intermediate undergoing the step in question can be monitored directly, and this often limits the approach to systems showing a sequence of marked spectrophotometric changes that can be followed by rapid reaction techniques.

DIRECT OBSERVATION OF A TITRATING GROUP

It is obvious that if an ionizing group in a protein can be observed directly, e.g., by direct titration, then its intrinsic pK_a can be determined, provided that the protein survives exposure to the pH range necessary to span the pK_a-value. The identity of the ionizing group and its relationship to the function of the protein may not be so clear, but at least this method yields reliable information about the pK_a. Careful chemical modification may allow the assignment of the pK_a-values to particular protein groups, though experiments of the necessary subtlety[22] are not often done. Recently an ingenious method for the determination and assignment of the intrinsic pK_a-values of histidine residues has been suggested, in which the exchange of the C-2 proton of the imidazole ring with solvent tritium is followed as a function of pH.[23,24] This method is a splendid example of the competitive labeling technique (see above) where the reagent (H_3O^+) is about as small and nonspecific as it could possibly be. Using D_2O, this approach has resulted in the reassignment of the four histidine NMR signals in ribonuclease A.[25] The use of NMR for the determination of pK_a-values of histidine residues has become widespread, though the ribonuclease saga indicates that considerable caution must be exercised in resonance assignment, even when the crystal structure of the protein is known. This problem has also been faced by Robillard and Shulman,[26] who have observed a single proton absorption in the proton magnetic resonance spectrum of δ-chymotrypsin, at very low field. This proton shows a pK_a at 7.2, the resonance titrating between -18 and -15 ppm. Again, the question here is in the assignment of the proton – the authors have suggested that it is the proton in the His_{57}-Asp_{102} active site "charge-relay" system of this enzyme. Using an analogous enzyme, α-lytic protease, Hunkapiller et al.[27] have overcome the assignment problem by the enrichment of one carbon atom with ^{13}C, in the C-2 position of the only histidine residue in the protein. While there are still some questions about the relative importance of chemical shift and coupling constant in this experiment, the perturbation of just this one residue can be neatly followed as a function of pH.

Another physical method allowing the determination of intrinsic pK_a-values is the use of differential infrared spectra[28] to monitor carboxyl ionizations. This approach has been used with β-lactoglobulin (by direct observation at the carbonyl stretching frequency in the D_2O "window") and has allowed the detection of two carboxyl groups with unusually high pK_a-values.

Finally, it is hoped that X-ray crystallographic work will be extended to the study of the ionizations of groups in proteins. Where the crystal is rugged enough to allow difference maps to be calculated at a number of different pH-values, very detailed information about the intrinsic pK_a-values of individual amino acids will be forthcoming.

THE THERMODYNAMICS OF IONIZATION

One further practice that is commonly indulged in is the determination of the apparent pK_a at a number of temperatures, in order to obtain values of $\Delta H_{ionization}$ and $\Delta S_{ionization}$. This is often done in order to define more closely the nature of the ionizing group, since for small molecules in aqueous solution, the enthalpies of ionization are rather characteristic of the group that ionizes. Amino groups, for instance, have fairly large positive values for $\Delta H_{ionization}$, and carboxyl groups have ionization enthalpies close to zero. Does the determination of these thermodynamic quantities provide independent information about the nature of an ionizing group on a protein? The answer is unfortunately no; once again, the ease of obtaining the data provides no rationale for its validity or utility. A pK_a can be expressed as a ΔG_o for the thermodynamic change in state. All that one achieves in the determination of ΔH_o and $T\Delta S_o$ is a division of ΔG_o into two parts, and no new information is derived from the division. Consider a group of unknown identity, of apparent pK_a ~5. Let us assume that this is either an unperturbed carboxyl group, or a perturbed amino group of extraordinarily low pK_a. Now a pK_a of 5 corresponds to a ΔG_o of 6.8 kcal/mol at

25°C, and if we determine the ΔH_o and $T\Delta S_o$ do we obtain new information? If we find that ΔH_o is near zero and that the $T\Delta S_o$ contribution dominates ΔG_o, then *either* the group is a carboxyl group showing the normal ΔH_o *or* it is an amino group, and the change in ΔG_o from the normal value (say, of 13.6 kcal/mol corresponding to a pK_a of ~10) is due to effects on the microenvironment of the amino group that alters ΔH_o. *Only* if we were to assume that when a pK_a is changed the $T\Delta S_o$ contribution to ΔG_o was unaffected, could any new information be gleaned from the value of ΔH_o. Since the values of ΔH_o and $T\Delta S_o$ for any process in aqueous solution are usually dominated by solvation effects which themselves involve large and often compensating changes in enthalpy and entropy, it is manifestly wrong to assume that the contribution of $T\Delta S_o$ to the overall Gibbs free energy change remains constant as ΔG_o (i.e., the pK_a) is perturbed from the value common for small molecules in free solution. The determination of ΔH_o and $T\Delta S_o$ for the ionization of groups attached to proteins does not, therefore, provide any *new* information about the identity of the ionizing group.

CONCLUSION

The main burden of this paper has been to summarize some of the assumptions implicit in deductions that are made from the pH-dependence of rate measurements of enzyme-catalyzed reactions. The biochemical journals continue to contain conclusions that are based on hair-raising logic from the pH-dependences of k_{cat} (or V_{max}) and k_{cat}/K_m (or V_{max}/K_m). The fact that so many enzymes show bell-shaped pH-dependences with pH optima near neutrality does not necessarily mean that they all depend for catalytic activity on a histidine residue being in its basic form. The deductive jump from an observed apparent pK_a of 6.5 to the statement that histidine is in the active site is, on the basis of pH-dependence data alone, unwarranted and improper.

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