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The pH-Dependence of Second-Order Rate Constants of Enzyme Modification May Provide Free-Reactant pK_a Values

By KEITH BROCKLEHURST
Department of Biochemistry, St. Bartholomew's Hospital Medical College, University of London, Charterhouse Square, London EC1M 6BQ, U.K.
and HENRY B. F. DIXON
Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, U.K.

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1. Reactions of enzymes with site-specific reagents may involve intermediate adsorptive complexes formed by parallel reactions in several protonic states. Accordingly, a profile of the apparent second-order rate constant for the modification reaction ($k_{obs.}$, the observed rate constant under conditions where the reagent concentration is low enough for the reaction to be first-order in reagent) against pH can, in general, reflect free-reactant-state molecular pK_a values only if a quasi-equilibrium condition exists around the reactive protonic state (EHR) of the adsorptive complex. 2. Usually the condition for quasi-equilibrium is expressed in terms of the rate constants around EHR:

$$EH + R \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EHR \xrightarrow{\tilde{k}_{mod.}} P$$

i.e. $\tilde{k}_{mod.} \ll k_{-2}$. This often cannot be assessed directly, particularly if it is not possible to determine $\tilde{k}_{mod.}$. 3. It is shown that $\tilde{k}_{mod.}$ must be much less than k_{-2}, however, if $\tilde{k}_{obs.}$ (the pH-independent value of $k_{obs.}$) $\ll k_{+2}$. 4. Since probable values of $k_{+2} \geq 10^6 M^{-1} \cdot s^{-1}$ and since values of $\tilde{k}_{obs.}$ for many modification reactions $\ll 10^6 M^{-1} \cdot s^{-1}$, the equilibrium assumption should be valid, and kinetic study of such reactions should provide reactant-state pK_a values. 5. This may not apply to catalyses, because for them the value of $\tilde{k}_{cat.}/\tilde{K}_m$ may exceed $5 \times 10^5 M^{-1} \cdot s^{-1}$. 6. The conditions under which the formation of an intermediate complex by parallel pathways may come to quasi-equilibrium are discussed in the Appendix.

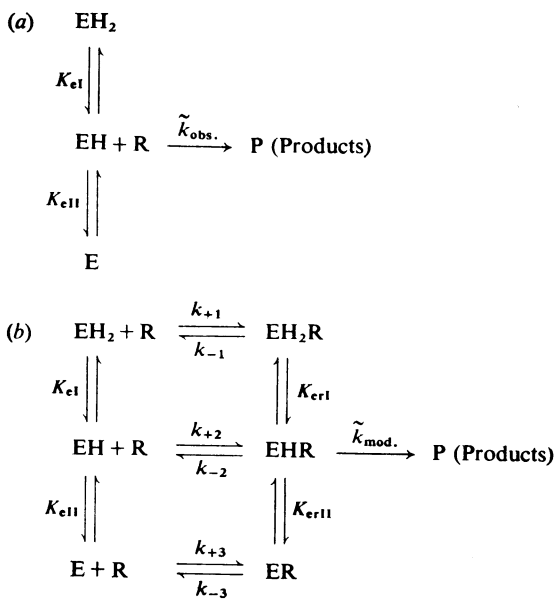
The validity of the common practice of deducing pK_a values characteristic of enzyme molecules from pH–rate data was rendered particularly doubtful by the realization that they are not necessarily provided either by profiles of $k_{cat.}/K_m$ against pH or by analogous profiles ($k_{obs.}$ against pH; see below) from modification studies (Schmidt & Westheimer, 1971; Knowles, 1976; Brocklehurst & Dixon, 1976). A major difficulty arises because it seems unrealistic to make the assumption (Peller & Alberty, 1959) that interconversion of enzyme and enzyme–substrate or enzyme–reagent complex occurs in only one ionization state rather than by parallel pathways. As a result, profiles of $k_{cat.}/K_m$ or $k_{obs.}$ against pH can, in general, provide free-reactant-state pK_a values only if the assumption of quasi-equilibrium is valid for the interconversion of free enzyme and enzyme–substrate or enzyme–reagent complex states as well as for protonation–deprotonation steps [see Ottolenghi (1971) and compare with Cornish-Bowden (1976)]. The usefulness of kinetics of modification as against kinetics of catalysis for determining enzyme pK_a values was suggested by Schmidt & Westheimer (1971). Even for modification reactions, however, it is necessary to assume a quasi-equilibrium condition (Knowles, 1976; Brocklehurst & Dixon, 1976), and often this assumption cannot be assessed by the conventional criterion. This involves making a direct comparison of the values of the two first-order rate constants that characterize respectively (1) the retrogression of the reactive protonic state of the intermediate complex to enzyme and substrate (or reagent), and (2) the formation of product.

In the present paper it is shown that a comparison of the pH-independent value of $k_{cat.}/K_m$ or $k_{obs.}$ with the probable value of the second-order rate constant for the initial encounter of enzyme and substrate or reagent permits an assessment of the validity of the equilibrium assumption. This type of comparison suggests that, for many enzyme-modification reactions, an assumption of quasi-equilibrium should be valid, and thus study of such reactions can provide free-reactant-state pK_a values.

Argument

1. *The problem*

The problem may be simply discussed in terms of a stoicheiometric reaction of one protonic state of a site-specific reagent, R, with a protein functional group that is rendered unreactive by either loss or addition of one proton. The covalency change in the



Scheme 1. *Kinetic models for the reaction of an enzyme with a site-specific reagent R (a) without and (b) with the intermediacy of adsorptive complexes*

$\tilde{k}_{obs.}$, $\tilde{k}_{mod.}$, k_{+1}–k_{+3} and k_{-1}–k_{-3} are pH-independent rate constants and K_{eI}, K_{eII}, K_{erI} and K_{erII} are molecular acid-dissociation constants; 'free' protons and formal charge differences between the various ionization states are omitted for clarity; in the catalysis model analogous to (*b*), R is replaced by substrate (S), $\tilde{k}_{mod.}$ by $\tilde{k}_{cat.}$ and P includes regenerated EH.

reaction is assumed to be a single-step process as in an S_N2 reaction. Conditions of reagent concentration are chosen such that the reaction is first-order with respect to concentration in R, as suggested by Schmidt & Westheimer (1971). The simplest representation of such a reaction is shown in Scheme 1(*a*), and in terms of this model the pH-dependence of the apparent second-order rate constant, $k_{obs.}$, provides the two molecular (see Dixon, 1976) pK_a values of the free enzyme (eqn. 1):

$$k_{obs.} = \frac{\tilde{k}_{obs.}}{1+\frac{[H^+]}{K_{eI}}+\frac{K_{eII}}{[H^+]}} \quad (1)$$

The model of Scheme 1(*a*), however, ignores the possibility of adsorptive-complex-formation before the covalency change. In some cases evidence for such intermediates may be provided by a decrease in the order with respect to concentration in R at higher concentrations of R. Even if such a saturation effect cannot be demonstrated at experimentally accessible concentrations of R, it would be dangerous to ignore the possibility of adsorptive-complex-formation in view of the well-known propensity of proteins to bind small molecules, and it seems more reasonable to take, as a general model, that in Scheme 1(*b*). In terms of this model the pH-dependence of $k_{obs.}$ is given by eqn. (2) (see Brocklehurst & Dixon, 1976) in which $\tilde{K}_{m_{mod.}} = (k_{-2}+\tilde{k}_{mod.})/k_{+2}$, $\tilde{k}_{mod.}$ is the pH-independent value of the first-order rate constant for the covalency change of the modification within the enzyme–reagent complex (analogous to $\tilde{k}_{cat.}$ for a catalysis) and the factors B, C, and D are defined by eqns. (3)–(5):

$$k_{obs.} = \frac{\tilde{k}_{mod.}}{\tilde{K}_{m_{mod.}}} \cdot \frac{D}{B \cdot C} \quad (2)$$

where

$$B = \left(1+\frac{[H^+]}{K_{eI}}+\frac{K_{eII}}{[H^+]}\right) \quad (3)$$

$$C = \left[1+\frac{[H^+]}{K_{erI}}\left(\frac{k_{-1}}{k_{-2}+\tilde{k}_{mod.}}\right)+\frac{K_{erII}}{[H^+]}\left(\frac{k_{-3}}{k_{-2}+\tilde{k}_{mod.}}\right)\right] \quad (4)$$

$$D = \left(1+\frac{[H^+]}{K_{erI}}\cdot\frac{k_{-1}}{k_{-2}}+\frac{K_{erII}}{[H^+]}\cdot\frac{k_{-3}}{k_{-2}}\right) \quad (5)$$

Eqn. (2) predicts that a pH–$k_{obs.}$ profile should contain extra sigmoidal waves in addition to the commonly encountered bell-shaped component (see Brocklehurst & Dixon, 1976). These extra waves may be of too small an amplitude to be observed experimentally, however, and the apparently simple bell-shaped profile may be deceptive in that it will not necessarily provide values of K_{eI} and K_{eII}.

Considerable simplification of eqn. (2) is effected by the assumption of quasi-equilibrium around EHR, the reactive form of the adsorptive complex. The equilibrium condition is usually expressed as $\tilde{k}_{mod.} \ll k_{-2}$, which is a sufficient but not a necessary condition for the reversibly connected components of Scheme 1(*b*) to be at quasi-equilibrium (see the Appendix). When the condition $\tilde{k}_{mod.} \ll k_{-2}$ is applied, the factors D and C of eqn. (2) become essentially identical. The pH-dependence of $k_{obs.}$ is then given by eqn. (6), which differs from eqn. (1) only in the significance assigned in the models to the pH-independent apparent second-order rate constant:

$$k_{obs.} = \frac{\tilde{k}_{mod.}/\tilde{K}_{m_{mod.}}}{1+\frac{[H^+]}{K_{eI}}+\frac{K_{eII}}{[H^+]}} \quad (6)$$

To summarize the problem: the necessity to take account of adsorptive complexes and parallel pathways in protein-modification reactions (Scheme 1*b*) means that the pH-dependence of $k_{obs.}$ will not necessarily provide K_{eI} and K_{eII} unless a quasi-equilibrium condition exists around EHR, and it is often difficult or impossible to assess this possibility by direct comparison of k_{-2} and $\tilde{k}_{mod.}$, especially when $\tilde{k}_{mod.}$ cannot be separately determined.

2. *Towards a solution*

Consider a reaction that can reasonably be held to conform to the model of Scheme 1(*b*), and suppose that a profile of $k_{obs.}$ versus pH appears to be a symmetrical bell-shaped curve. As was pointed out above, this bell-shaped component may be only a part of a more complex profile and its apparent pK_a values could arise either both from factor B (eqn. 3) or one from factor B and one from factor C (eqn. 4). When both pK_a values arise from factor B, the pH-independent rate constant that characterizes the bell-shaped component, $\tilde{k}_{obs.}$, is equal to $\tilde{k}_{mod.}/\tilde{K}_{m_{mod.}}$ (see eqns. 1 and 6). In other circumstances the relative values of factors B, C, and D (note especially that D$\geqslant$C) discussed by Brocklehurst & Dixon (1976) provide that $\tilde{k}_{obs.} > \tilde{k}_{mod.}/\tilde{K}_{m_{mod.}}$. For example, for a profile like that shown by Brocklehurst & Dixon (1976, p. 68, column 1, Fig. 4) the two pK_a values of the bell-shaped component arise from factors C (pK_I = pK_{1C}) and B (pK_{II} = pK_{1B}) respectively. For such a profile, the relative positions of pK_a values provide that $K_{1B} > K_{1D}$ (the first pK_a value of factor D, eqn. 5) and eqn. (28) given by Brocklehurst & Dixon (1976, p. 68, column 2) may be used to show that $\tilde{k}_{obs.}$ (which approximates closely to $\frac{\tilde{k}_{mod.}}{\tilde{K}_{m_{mod.}}} \cdot \frac{K_{1B}}{K_{1D}}$) $> \tilde{k}_{mod.}/\tilde{K}_m$. Thus relationship (7) applies generally:

$$\tilde{k}_{obs.} \geqslant \frac{k_{+2} \cdot \tilde{k}_{mod.}}{k_{-2} + \tilde{k}_{mod.}} \qquad (7)$$

A useful transform of relationship (7) is relationship (8), in which $y = \tilde{k}_{mod.}/k_{-2}$ and $x = \tilde{k}_{obs.}/k_{+2}$:

$$y \leqslant \frac{x}{1-x} \qquad (8)$$

It is clear from relationship (8) that the conventional condition for quasi-equilibrium ($y \ll 1$, i.e. $\tilde{k}_{mod.} \ll k_{-2}$) is a necessary consequence of a value of $x \ll 1$, i.e. $k_{obs.} \ll k_{+2}$. Thus, if there are circumstances that allow the magnitude of k_{+2} to be inferred, an experimentally determined value of $\tilde{k}_{obs.}$ that is much less than the inferred value of k_{+2} should constitute good evidence that $\tilde{k}_{mod.} \ll k_{-2}$ and that the bell-shaped pH–$k_{obs.}$ profile should provide free-reactant-state pK_a values.

Data obtained with rapid-reaction techniques suggest that the second-order rate constants that characterize the formation of enzyme–substrate complexes may generally be in the range 10^6–10^8 $M^{-1} \cdot s^{-1}$ (Hammes & Schimmel, 1970), and in some cases even larger (Chou & Jiang, 1974; Li & Chou, 1976) than the traditional value (10^9 $M^{-1} \cdot s^{-1}$; Debye, 1942; Alberty & Hammes, 1958) for a diffusion-limited encounter. The range 10^6–10^8 $M^{-1} \cdot s^{-1}$ encompasses values for the binding of both physiological and analogue substrates and even includes values for reactions involving covalent-bond formation, e.g. oxime formation by reaction of the aldehydic form of aspartate aminotransferase with hydroxylamine (3.7×10^6 $M^{-1} \cdot s^{-1}$; Hammes & Fasella, 1963). It would seem reasonable to suppose, therefore, that the rate constants for the adsorptive binding of many site-specific reagents to enzymes will be $\geqslant 10^6$ $M^{-1} \cdot s^{-1}$. If this is the case, then, since for many protein-modification reations $\tilde{k}_{obs.} \ll 10^6$ $M^{-1} \cdot s^{-1}$, the equilibrium assumption should be valid, and the pH–$k_{obs.}$ profile should provide the free-enzyme molecular acid dissociation constants.

Ficin (EC 3.4.22.3) can be used as an example. The rate constant of its reaction with the thiol-specific reagent 2,2′-dipyridyl disulphide tends to a pH-independent value, $\tilde{k}_{obs.}$, of 2.2×10^3 $M^{-1} \cdot s^{-1}$ at high pH (Malthouse & Brocklehurst, 1976). Since (*a*) this rate constant is much less than the limit of 10^6 $M^{-1} \cdot s^{-1}$ proposed above, and (*b*) the upper pK_a of the reagent is 2.45 (Brocklehurst & Little, 1973), it seems safe to conclude that the pK_a value of 8.6 that characterizes the increase in $k_{obs.}$ towards its plateau value at high pH is a molecular pK_a of the enzyme. A pK_a close to 8.6 also characterizes (1) the reaction of ficin with chloroacetamide ($k_{obs.}$ rising towards 7.5 $M^{-1} \cdot s^{-1}$ as the pH is raised with a pK_a of 8.55; Hollaway *et al.*, 1964), and (2) the pH-dependence of $k_{cat.}/K_m$ for ficin-catalysed hydrolyses, where, however, the rate rises to the plateau value as the pH is lowered across this pK_a (see Hollaway *et al.*, 1971). For the hydrolysis of N^2-benzoyl-L-arginine ethyl ester, $k_{cat.}/K_m < 10^3$ $M^{-1} \cdot s^{-1}$ (see Malthouse & Brocklehurst, 1976), so it is probably safe to conclude that the kinetics of catalysis also provide this free-enzyme pK_a value.

The value of $k_{cat.}/K_m$ for hydrolyses catalysed by ficin and by the similar proteinase papain (EC 3.4.22.2) falls from its plateau, not only as the pH is raised with a pK_a of 8–9, but also as it is lowered with a pK_a of 3–4 (see Lowe, 1976). That these pK_a values characterize two stages of ionization of the enzyme active centres, the first to produce, and the second to destroy, the interactive thiol–imidazole–thiolate–imidazolium systems, is effectively demonstrated by using as reactivity probe an electrophilic reagent that is reactive in two protonic states, such as 2,2′-dipyridyl disulphide (Brocklehurst, 1974; Malthouse & Brocklehurst, 1976) (or better still the unsymmetrical reagent *n*-propyl-2-pyridyl disulphide; M. Shipton & K. Brocklehurst, unpublished work). In reaction with ficin, 2,2′-dipyridyl disulphide shows not only the plateau at high pH already mentioned, but a much higher rate with a bell-shaped optimum at pH 3, characterized by pK_a values of 2.4 and 3.8 (Malthouse & Brocklehurst, 1976). The $\tilde{k}_{obs.}$ is 1.1×10^5 $M^{-1} \cdot s^{-1}$ (unusually high for protein-modification reactions), and, if corrected for the fact that it is presumably due to reaction of the monocation of the reagent with the deprotonated form of the enzyme, it gives a 'true' value for the rate constant of 2.9×10^6 $M^{-1} \cdot s^{-1}$. Although these values are close to the limit suggested

for the 'safe' designation of free-reactant pK_a values, the correspondence of the value of 3.8 from one side of the bell with values derived from hydrolysis kinetics, and the close correspondence (Malthouse & Brocklehurst, 1976) of the pK_a derived from the other limb (2.42) with the pK_a of the monocation of the reagent (2.45), support their being pK_a values for free reactants.

The criterion by which the probable validity of an assumption of quasi-equilibrium for a protein modification reaction may be assessed could, in principle, be applied generally to a simple catalytic mechanism (i.e. Scheme 1*b* with $\tilde{k}_{mod.}$ replaced by $\tilde{k}_{cat.}$ and P assumed to include regenerated enzyme). Many catalyses, however, are characterized by values of $k_{cat.}/K_m \geqslant 5\times10^5 M^{-1}\cdot s^{-1}$, which may be similar to probable values of k_{+2} ($\geqslant 10^6 M^{-1}\cdot s^{-1}$) and therefore the validity of the equilibrium assumption for catalysed transformations of specific substrates will often be in doubt. The extent to which information obtained by using reactivity probes can usefully be applied to an evaluation of probable interactions of enzyme and substrate needs to be assessed, of course, in each individual case, taking account of known structural features of the enzyme and similarities in the structure of the substrate and the probe reagent.

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APPENDIX

Conditions for Quasi-equilibrium

In our discussion (Brocklehurst & Dixon, 1976, p. 69) of the conditions under which the formation of a central complex like EHR of Scheme 1(*b*) (main paper) may come to quasi-equilibrium, two equations containing the ratio D/C (eqn. 29 and the unlabelled steady-state equation) were inadvertently written with terms omitted. Fortunately, these omissions introduced compensating errors and the main conclusion is therefore unchanged. It is necessary, however, that $\tilde{k}_{mod.}$ (or $\tilde{k}_{cat.}$) be much less than k_{-2} to make C and D (factors containing apparent ionization constants) identical, although it is not necessary for C and D to be identical to achieve the quasi-equilibrium condition. The argument may be correctly expressed as follows (to facilitate comparison with our previous discussion (Brocklehurst & Dixon, 1976, p. 69), $\tilde{k}_{cat.}$, K_{esI}, K_{esII} and S are used instead of $\tilde{k}_{mod.}$, K_{erI}, K_{erII} and R of Scheme 1(*b*) (main paper), i.e. the following should replace the last two paragraphs of column 1 of page 69 of Brocklehurst & Dixon (1976), one of which finishes on column 2).

It can be shown that the formation of EHS from reactants comes into quasi-equilibrium even if $\tilde{k}_{cat.} \geqslant k_{-2}$. The ratio D/C may be written as eqn. (1) (previously eqn. 29):

$$\frac{D}{C}=\left(\frac{\tilde{k}_{cat.}+k_{-2}}{k_{-2}}\right)\left(\frac{k_{-2}+k_{-1}\dfrac{[H^+]}{K_{esI}}+k_{-3}\dfrac{K_{esII}}{[H^+]}}{\tilde{k}_{cat.}+k_{-2}+k_{-1}\dfrac{[H^+]}{K_{esI}}+k_{-3}\dfrac{K_{esII}}{[H^+]}}\right) \quad (1)$$

The first-order rate constants $k_{-1}[H^+]/K_{esI}$, k_{-2} and $k_{-3}K_{esII}/[H^+]$, when multiplied by [EHS], provide the rates of dissociation of S from EHS by the three routes via EH_2S, directly and via ES respectively. Only one of these rate constants needs to be much larger than $\tilde{k}_{cat.}$ to make the second term in parentheses in the expression for D/C (eqn. 1) equal to unity, and thus D/C equal to $(\tilde{k}_{cat.}+k_{-2})/k_{-2}$. This condition brings substrate binding into quasi-equilibrium even if $\tilde{k}_{cat.} > k_{-2}$, since from the steady-state equations:

$$\frac{[EHS]}{[EH][S]_0}=\left(\frac{k_{+2}}{k_{-2}+\tilde{k}_{cat.}}\right)\frac{D}{C}$$

and when $D/C=(k_{cat.}+k_{-2})/k_{-2}$:

$$\frac{[EHS]}{[EH][S]_0}=\frac{k_{+2}}{k_{-2}}$$

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