APPENDIX

Analysis of pH-dependent kinetics in up to four reactive hydronic states

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Although it has long been conventional to consider that the reversible dependence of enzyme activity on pH will have the same form as the concentration of the monohydronated species of a dibasic acid (Tipton & Dixon, 1979; Dixon *et al.*, 1987), our work with a variety of cysteine proteinases (Brocklehurst *et al.*, 1988*b*) is continuing to reveal more complex pH-dependences. This is true for steady-state catalysis kinetics in some cases, such as $k_{cat.}/K_m$ for the chymopapain A-catalysed hydrolysis of some anilide substrates (M. O'Driscoll, E. Salih & K. Brocklehurst, unpublished work) (see Appendix Fig. 1*a*), but is encountered to greater extent for reactions of catalytic-site thiol groups with site-specific inhibitors, particularly 2-pyridyl disulphides, which have different electrophilic reactivities in hydronated and non-hydronated forms (Brocklehurst, 1987; Brocklehurst *et al.*, 1988*a*). Examples of shapes of pH–*k* profiles with four reactive hydronic states, characterized by four pK_a values and four pH-independent rate constants, $\tilde{k}$, are shown in Appendix Figs. 1(*b*) and 1(*c*). The profile shape in Appendix Fig. 1(*b*) is like the pH–*k* profiles for reactions of actinidin analysed and discussed in the

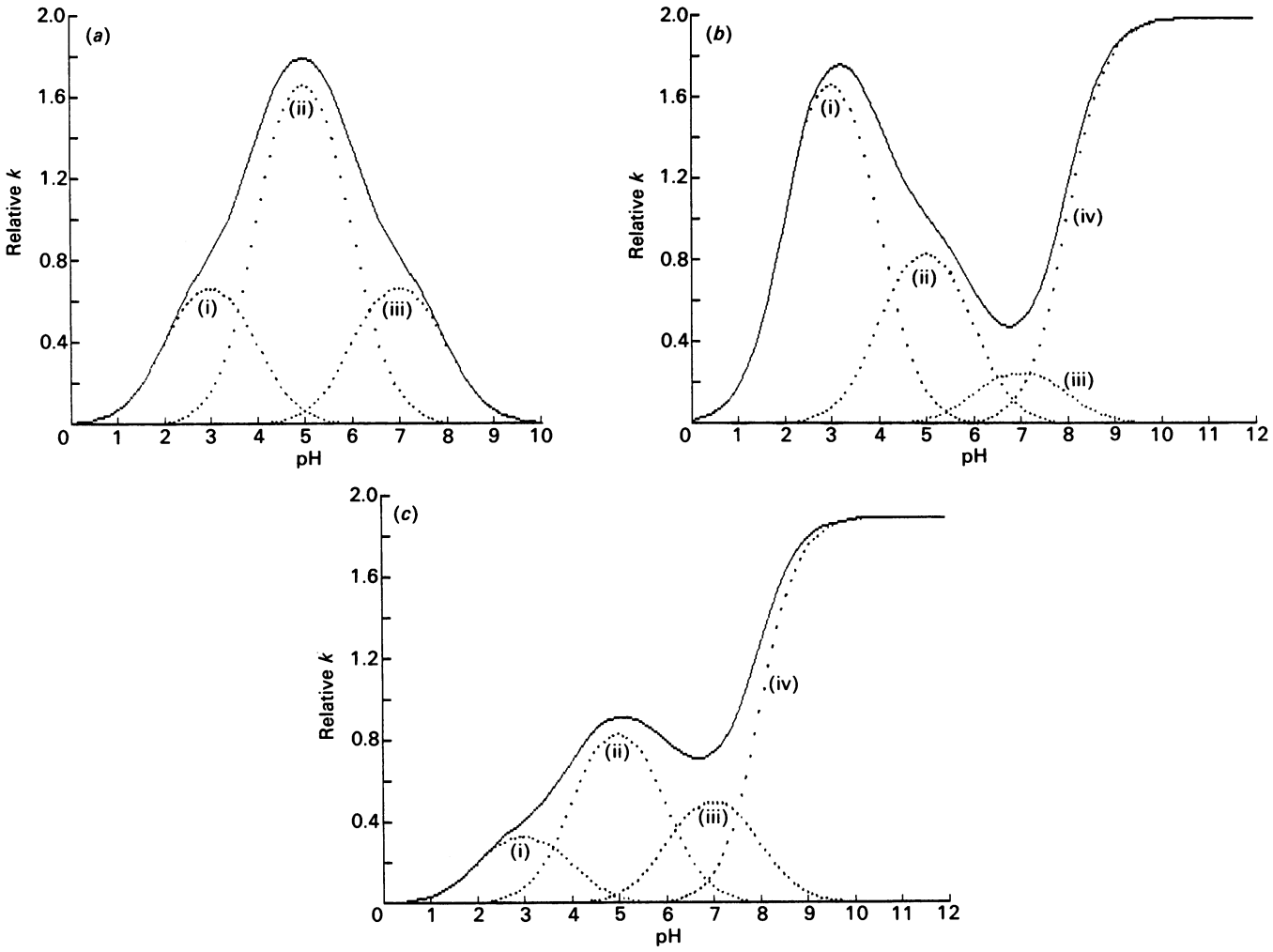


Fig. 1. Examples of forms of pH–*k* profiles with four ionizations, and their constituent components computed by using eqn. (5) and a BBC Microcomputer

In all cases the values pK_I–pK_{IV} were set at 2.0, 4.0, 6.0 and 8.0 respectively and values of the (relative) pH-independent rate constants, $\tilde{k}_{XH_3}$–$\tilde{k}_X$, are as follows: (*a*) 0.8, 2.0, 0.8 and 0; (*b*) 2.0, 1.0, 0.3 and 2.0; (*c*) 0.4, 1.0, 0.6 and 1.9. The continuous lines correspond to eqn. (5) and the broken lines (i)–(iv) to its constituent terms associated with $\tilde{k}_{XH_3}$–$\tilde{k}_X$ respectively. Examples of enzyme reactions that correspond to each of these profile shapes are given in the text, and examples of (*b*) are discussed more fully in the associated main paper (Brocklehurst *et al.*, 1988*c*).

associated main paper (Brocklehurst *et al.*, 1988*c*); that in Appendix Fig. 1(*c*) is characteristic both of the reaction of the catalytic-site thiol group of cathepsin B with 2-(*N*'-acetyl-L-phenylalanylamino)ethyl 2'-pyridyl disulphide and of $k_{cat.}/K_m$ for its catalysis of the hydrolysis of an analogous anilide substrate (C. M. Topham, F. Willenbrock, E. Thomas & K. Brocklehurst, unpublished work). Like Appendix Figs. 1(*b*) and 1(*c*), Appendix Fig. 1(*a*) has four pK_a values but in this case only three $\tilde{k}$ values because catalytic activity would be expected to fall to zero at high pH. The same is true for the catalytic activity of cathepsin B, and so, when Appendix Fig. 1(*c*) is taken to represent this rather than thiol-group reactivity, the profile shown is only a partial one.

To determine the characterizing parameters (pK_a and $\tilde{k}$ values) for profiles like those shown as continuous lines in Appendix Fig. 1, it is necessary to make use of the appropriate pH-dependent rate equation. This is derived in its simplest form by using a model (Appendix Scheme 1) in which ionizations in the enzyme and in the substrate or reactivity probe reagent are not distinguished, are considered to be at quasi-equilibrium (see Brocklehurst & Dixon, 1976, 1977; Brocklehurst, 1979) and are defined by macroscopic acid dissociation constants. When kinetically significant ionizations occur in both enzyme and substrate or reagent, both the macroscopic pK_a values and values of $\tilde{k}$, as determined by fitting the experimental data to a rate equation like that presented below, may contain pK_a values of both enzyme and reagent, and the $\tilde{k}$ values characteristic of some of the hydronic states may need to be corrected if the reactivity derives from a minor form of the particular hydronic state [see Malthouse & Brocklehurst (1976) for a discussion of this type of correction for a single bell-shaped component].

Conventional analysis of the model in Appendix Scheme 1 makes use of the conservation equation, eqn. (1), where $[X]_T$ represents the total concentration of the reactant state in all hydronic states, four equations like eqn. (2) that define the macroscopic acid dissociation constants, and the rate equation, eqn. (3), expressed in terms of $[X]_T$, in which $k_{obs.}$ is the experimentally determined rate constant, shown here as a first-order rate constant characteristic of the composite reactant state used in the model of Appendix Scheme 1:

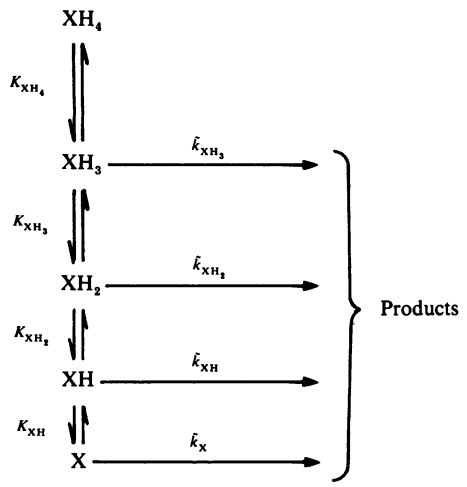
$$[X]_T = [XH_4]+[XH_3]+[XH_2]+[XH]+[X] \quad (1)$$

$$K_{XH_4} = [XH_3][H^+]/[XH_4] \quad (2)$$

$$\text{Rate} = k_{obs.}[X]_T \quad (3)$$

The rate equation may be written in terms of pH-independent rate constants, $\tilde{k}_{XH_3}$–$\tilde{k}_X$, as eqn. (4), and the relationship between $k_{obs.}$ and $\tilde{k}_{XH_3}$–$\tilde{k}_X$, eqn. (5), is obtained by combining eqns. (1), (3) and (4) and the four equations like eqn. (2):

$$\text{Rate} = \tilde{k}_{XH_3}[XH_3]+\tilde{k}_{XH_2}[XH_2]+\tilde{k}_{XH}[XH]+\tilde{k}_X[X] \quad (4)$$



Scheme 1. Kinetic model for a reaction involving four reactive hydronic states

The reactive hydronic states of a generalized reactant state are designated X–XH_3 to denote relative stoichiometries in hydrons and XH_4 is unreactive. Relative ionic charges are not shown. The model is characterized by four macroscopic acid dissociation constants K_{XH_4}–K_{XH} and by four pH-independent rate constants, $\tilde{k}_{XH_3}$–$\tilde{k}_X$.

The form of the pH-dependence of $k_{obs.}$ applies equally well to that of a second-order rate constant, k, which may be $k_{cat.}/K_m$ for a catalysis, or the rate constant characteristic of a reaction of enzyme with a site-specific reagent that obeys second-order kinetics under particular conditions of concentration. Then, $k_{obs.}$ is replaced by k, and the pH-independent rate constants $\tilde{k}_{XH_3}$–$\tilde{k}_X$, which are first-order rate constants in eqns. (4) and (5), become second-order rate constants. Clearly, when non-ionizing substrates or reagents are used, the macroscopic pK_a values will necessarily be characteristic of the enzyme molecule, unless a pH-dependent change in rate-determining step occurs, leading to a 'mirage pK_a' (see Dixon *et al.*, 1987, and references cited therein), and may in some circumstances approximate closely to intrinsic pK_a values characteristic of single ionizing groups in the enzyme molecule. When the substrate or reagent undergoes hydronic dissociation, it may be possible to associate one of the pK_a values in a pH–k profile with this ionization on the basis of its known pK_a value.

In circumstances where some of the macroscopic pK_a values are well separated, some of the terms involving multiple powers of $[H^+]$ may be neglected, although the validity of this simplification sometimes depends also on relative values of the pH-independent rate constants. In the work presented in the associated main paper (Brocklehurst *et al.*, 1988*c*), however, the full equation and its analogues for models with fewer than four reactive

$$k_{obs.} = \frac{\tilde{k}_{XH_3}}{1+\frac{[H^+]}{K_{XH_4}}+\frac{K_{XH_3}}{[H^+]}+\frac{K_{XH_3}K_{XH_2}}{[H^+]^2}+\frac{K_{XH_3}K_{XH_2}K_{XH}}{[H^+]^3}} + \frac{\tilde{k}_{XH_2}}{1+\frac{[H^+]^2}{K_{XH_4}K_{XH_3}}+\frac{[H^+]}{K_{XH_3}}+\frac{K_{XH_2}}{[H^+]}+\frac{K_{XH_2}K_{XH}}{[H^+]^2}}$$

$$+\frac{\tilde{k}_{XH}}{1+\frac{[H^+]^3}{K_{XH_4}K_{XH_3}K_{XH_2}}+\frac{[H^+]^2}{K_{XH_3}K_{XH_2}}+\frac{[H^+]}{K_{XH_2}}+\frac{K_{XH}}{[H^+]}} + \frac{\tilde{k}_X}{1+\frac{[H^+]^4}{K_{XH_4}K_{XH_3}K_{XH_2}K_{XH}}+\frac{[H^+]^3}{K_{XH_3}K_{XH_2}K_{XH}}+\frac{[H^+]^2}{K_{XH_2}K_{XH}}+\frac{[H^+]}{K_{XH}}} \quad (5)$$

hydronic states have been used in view of the overlap of at least some of the ionizations. Relatively complex equations such as eqn. (5) are readily applied when computers are used to evaluate experimental data in terms of chosen models. Examples of pH–k profiles generated by using eqn. (5) (with $k_{obs.} = k$) and a program written in BASIC for the BBC Microcomputer are shown in Appendix Fig. 1. The program allows the display of the contributions of the individual terms involving $\tilde{k}_{XH_3}$–$\tilde{k}_X$ of eqn. (5) as well as the complete pH–k profile. When profile components are well separated, parameters are readily determined also by optimization procedures (see, e.g., Shipton & Brocklehurst, 1978). Examples of enzyme reactions that correspond to profile shapes like those shown in Appendix Fig. 1 were mentioned above, and the analysis of experimental data in terms of a profile shape like that in Appendix Fig. 1(*b*) has a central role in the associated main paper (Brocklehurst *et al.*, 1988*c*). Some pH–k profiles that are described by eqn. (5) do not clearly exhibit four sigmoid waves because of closely overlapping ionizations. An example is provided by the profile for the reaction of papain with 2-(acetamido)ethyl 2′-pyridyl disulphide, discussed in the associated main paper (Brocklehurst *et al.*, 1988*c*), in which the acid limb has three overlapping pK_a values.

When models with fewer than four reactive hydronic states need to be evaluated, the appropriate equation is readily obtained from eqn. (5) by elimination of some $\tilde{k}$ terms and modification of others so as to conform to the particular truncated form of the model of Appendix Scheme 1.

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