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THE THREE STATES CRITERION OF NUCLEOPHILIC REACTIVITY IN THE CONJUGATE ACID OF AN ANIONIC NUCLEOPHILE AND SOME PROBLEMS OF PROTON PARTITIONING: IMPLICATIONS FOR THE REACTIONS OF 2,2'-DIPYRIDYL DISULPHIDE WITH PAPAIN AND WITH L-ERGOTHIONEINE

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Abstract—The circumstances in which observation of three reactive protonic states of a reaction, involving an electrophile that increases its reactivity consequent on protonation, may be regarded as compelling evidence of nucleophilic character in the conjugate acid of an anionic nucleophile ("three states criterion") are delineated. Aspects of the reaction of papain with 2,2'-dipyridyl disulphide not previously reported suggest that this reaction at pH 4 is best described as an intracomplex thiol-disulphide interchange involving the unionized thiol group of the cysteine-25-histidine-159-asparagine-175 hydrogen bonded system of papain with 2,2'-dipyridyl disulphide hydrogen bonded at one nitrogen atom to the carboxyl group of aspartic acid-158. The reaction appears to involve pre-transition state proton transfer in the thiol-imidazole hydrogen bond; the protonation of the side chains of aspartate-158 and histidine-159 may be positively cooperative. Rate equations for reactions involving up to three reactive protonic states are presented in an appendix.

One of the problems that is often central to the delineation of the mechanisms of chemical reactions is the location of protons in transition states and this is of particular interest when considering mechanisms of enzyme catalysis.

The difficulty of locating protons in transition states derives from the fact that the main approach to the study of transition state structure is kinetic analysis. Due to the (usually) rapid equilibration of protons between the various electronegative atoms of a reacting system, formulation of the rate equation for a given reaction will provide information only about the stoichiometry of the transition state with respect to protons and not about their location within it.[1] The additional problem in the interpretation of pH-rate profiles that arises from change in rate-limiting step with pH is illustrated by the classic paper of Jencks[2] on the reactions of carbonyl compounds with nitrogen bases (see also Refs 1, 3–5).

A particular aspect of the general problem of proton partitioning[4,5] concerns the detection of nucleophilic reactivity in the proton-rich member (AH) of a conjugate neutral acid-anionic base pair (AH and A^-). Nucleophilicity in this type of conjugate acid might arise in several ways, viz: (i) a lone pair of electrons on A may provide nucleophilicity (ii) AH may be "activated" by hydrogen bonding to a basic centre as has been envisaged in certain assemblies of functional groups in enzyme active centres (see later), (iii) A^- may be a multi-atom resonance-stabilized anion and the nucleophilic form of the conjugate acid be one in which the proton is bonded to some atom other than the nucleophilic atom as seems to be the case in certain aminothiones (*vide infra*).

Nucleophilicity is less readily envisaged in cationic acids although presumably a mechanism of type (ii) might be considered to provide a nucleophile of type (i).

The present paper classifies proton partitioning into two main types (1) partitioning that provides reacting systems (nucleophile and electrophile) of different intrinsic reactivities (catalytic proton partitioning) and (2) partitioning that gives rise to no (appreciable) change in the reactivity of the system (non-catalytic proton partitioning). In addition, it shows how in certain cases it is possible to demonstrate the existence of nucleophilic reactivity in a conjugate acid. This is facilitated by use of electrophilic reagents that increase their electrophilicity by protonation. These concepts are illustrated by reference to reactions of papain and of aminothiones such as L-ergothioneine with al-

kylating agents and with pyridyl disulphides. These reactions have been the subject of recent work in this laboratory.[6-9]

Materials. A molecular model of papain (EC 3.4.22.2) (scale 1 cm = 1Å) based on the coordinate determinations of Drenth *et al.*[10] (see also Drenth *et al.*[11]) was purchased from Labquip, 18, Rosehill Park Estate, Caversham, Reading, RG4 8XE.

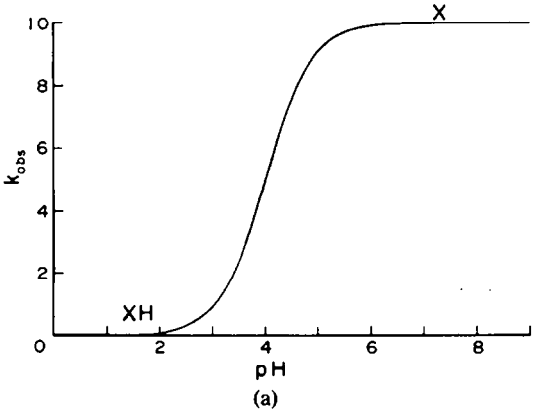
DISCUSSION

Catalytic and non-catalytic proton partitioning. Consider a simple, one-step bimolecular reaction in which an anionic nucleophilic centre A^- in a molecule M_1 reacts with an electrophilic centre e in another molecule M_2. The reaction under specified conditions is characterized by a pH-independent rate constant $\bar{k}$ (Eq 1) and at a stated pH value by a pH-dependent rate constant k (Eq 2). The subscript T denotes total concentrations and M_1 and M_2 represent those parts of the molecule not shown as functional groups.

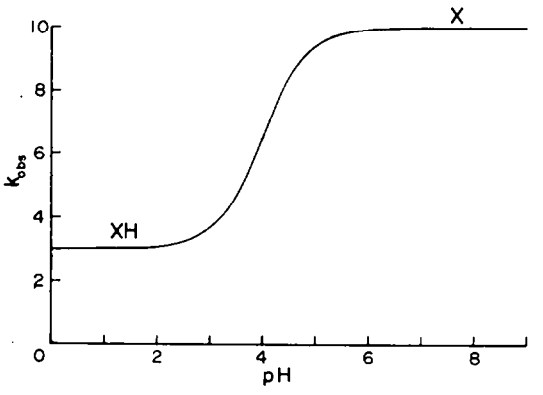
$$\text{Rate} = \bar{k}\,[M_1A^-]\,[M_2e] \qquad (1)$$

$$\text{Rate} = k\,[M_{1T}]\,[M_{2T}]. \qquad (2)$$

The simplest type of such a reaction would be that in which M_1 and M_2 contain no proton binding sites other than A^-. In such a case the pH-rate profile would reveal two protonic states designated X and XH to indicate their stoichiometries with respect to protons. The X state would correspond to reaction of M_1A^- with M_2e and the XH state to reaction of M_1AH with M_2e. If M_1AH is devoid of nucleophilic reactivity, the pH-k profile would be of simple sigmoid form and reveal only one reactive protonic state, that in the plateau region at high pH (the X state) (Fig 1a). Observation of a second plateau at low pH (Fig 1b), i.e. a reactive XH state would for such a system provide definitive evidence that M_1AH possessed nucleophilic reactivity. For such a system a third but unreactive protonic state XH_2 would be predicted in solutions of sufficiently high acidity. This is because if M_1AH is nucleophilic, the original stipulation of only one proton binding site (A^-) cannot strictly be held to



(a)



(b)

Fig 1. Typical pH-rate profiles for reactions exhibiting two protonic states. The curves are theoretical for: $k_{obs} = k_2/\{1 + K_{III}/[H^+]\} + k_3/\{1 + [H^+]/K_{III}\}$ (see the appendix) in which $pK_{III} = 4$ and for (a) $k_2 = 0$, $k_3 = 10$ units and for (b) $k_2 = 3$ units, $k_3 = 10$ units.

apply. If M_1AH reacts with the electrophilic centre e, it must be presumed that M_1AH will bind another proton (i.e. $M_1A^+H_2$) thus losing its nucleophilic character.

Even reactions of relatively simple organic molecules, let alone enzymes, can only rarely be held to conform to this simple model. This is because even if the same step in a more complex reaction is rate limiting at all pH values[4] M_1 and/or M_2 often contain other proton binding sites and proton partitioning between these sites may provide ambiguities in the interpretation of pH-rate profiles.

Consider the next most complex situation, i.e. when the reacting system ($M_1 + M_2$) contains two proton binding sites (other than AH), i.e. A^- and one other (B). Proton partitioning between these two sites may be classified as catalytic (positive or negative) or non-catalytic according to whether or not protonation of B alters (detectably) the intrinsic reactivity of the M_1M_2 system. For simplicity it is assumed that for all model systems containing more than one proton binding site, group pK_a values are well separated from each other.

The most widely recognized examples of kinetically significant proton partitioning are of the catalytic type Protonation of a site B_2 in M_2 (e.g. a leaving group bonded to e, i.e. $M_2e\text{-}B_2$) that results in an increase in its reactivity towards M_1A^- would provide an example of positive catalytic proton partitioning. Protonation of a site B_1 in M_1 or B_2 in M_2 that results in a decrease in the reactivity of M_1A^- towards M_2 would constitute an example of negative catalytic proton partitioning. If M_1 is an enzyme, one way in which this might be brought about is a conformational change consequent on protonation of B_1.

The practical problem with which this paper is concerned is that of trying to ascertain the circumstances in which observation of a reactive XHn

($n \geqslant 1$) state of a reaction provides compelling evidence for nucleophilic reactivity in M_1AH. The intrinsic reactivity of M_1A^- towards M_2e can often be obtained experimentally as the pH-independent rate constant of the X state. By contrast the intrinsic reactivities of those components of the XH (and higher) protonic states that arise from (positive) catalytic proton partitioning are frequently unknown. Consequently rejection of one or more of the chemically reasonable mechanisms that can be written for a given protonic state usually has to rely on the criterion of the diffusion-controlled limit. Thus if a mechanism would be characterized by a pH-independent rate constant greater than that of hydroxide ion with a proton (approx. 10^{11} $M^{-1}s^{-1}$ see Jencks[1]; Bender[12]) it is conventional to reject it. Similarly, if the rate constant would approach this limiting value, the mechanism is viewed with considerable scepticism.

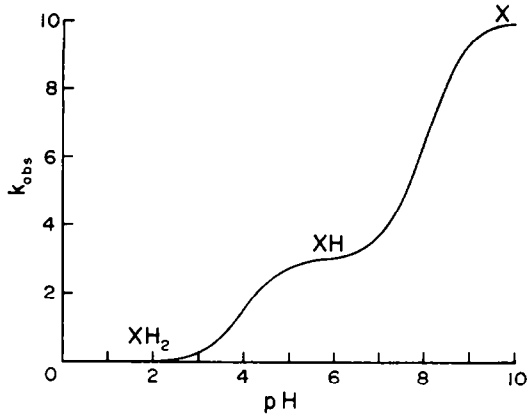
Unfortunately, the criterion of the diffusion limit is of only limited value. In many cases, both of catalytic and non-catalytic proton partitioning, the reactivity of a particular component of a protonic state is found to be less by several orders of magnitude than the diffusion limit. Thus, although such a mechanism may be regarded with reservation, it cannot strictly be ruled out by this criterion. In the case of an XH state produced by non-catalytic proton partitioning, however, the extra piece of information available, i.e. that the intrinsic reactivities of the X and XH states are (essentially) the same, can permit the elimination of such a mechanism. A clear example of this is provided by the alkylation of L-ergothioneine by iodoacetamide.[9] This reaction is characterized in the pH range approx. 2·5–11·5 by two reactive protonic states (X and XH). The X state is reasonably interpreted as reaction of the resonance stabilized thiolate ion of the L-ergothioneine dianion **1** with neutral iodoacetamide. One candidate for the reactive XH state is reaction of the thiolate ion of the L-ergothioneine monoanion **2** with neutral iodoacetamide. The molecular pK_a values (1·3 and 10·8) of L-ergothioneine should be good approximations to the intrinsic pK_a values of the L-ergothioneine carboxyl and aminothione moieties respectively.[9] Thus at pH 3 only 5×10^{-10} of the L-ergothioneine will be present such that the carboxylate ion is protonated and the aminothione moiety is ionized, i.e. as **2**. The XH state of this reaction is characterized by a second order rate constant, calculated using total concentrations of the reactants, of $3 \times 10^{-2} M^{-1}s^{-1}$ (Carlsson *et al.*[9]). Thus the rate constant that would characterize the reaction of the L-ergothioneine monoanion **2** with neutral iodoacetamide would have to be $0{\cdot}03/5 \times 10^{-10} = 6 \times 10^7 M^{-1}s^{-1}$. This value is more than 3 orders of magnitude below the diffusion-controlled limit and thus cannot be eliminated by this criterion. This approach takes no account of the relationship of the intrinsic reactivities of the X state and the component of the XH state under consideration. If the assumption is made, however, that this is an example of non-catalytic proton partitioning, i.e. the reactivity of **2** towards iodoacetamide is essentially the same as that of **1** ($4 M^{-1}s^{-1}$, Carlsson *et al.*[9]) then reaction of **2** may be rejected as an interpretation of the reactive XH state. This is because the calculation given above shows that its reaction would be characterized by a rate constant that is 7 orders of magnitude too large. Alternatively, it may be said that reaction of **2** with neutral iodoacetamide would contribute an observed rate constant at pH 3 of only $2 \times 10^{-9} M^{-1}s^{-1}$, whereas in fact it is $3 \times 10^{-2} M^{-1}s^{-1}$.

The relative positions of the carboxyl and aminothione groups in the L-ergothioneine molecule makes it probable that the partitioning of their protonation is of the non-catalytic type. If in other systems the partitioning cannot be specified as non-catalytic, this means of obviating the difficulties of definitive use of the diffusion-limit cannot normally be used. It may in some cases, however, by analogy with other reactions, be possible to estimate the expected reactivity of the particular component of the protonic state under consideration sufficiently accurately to be able to assess whether it represents a probable interpretation.

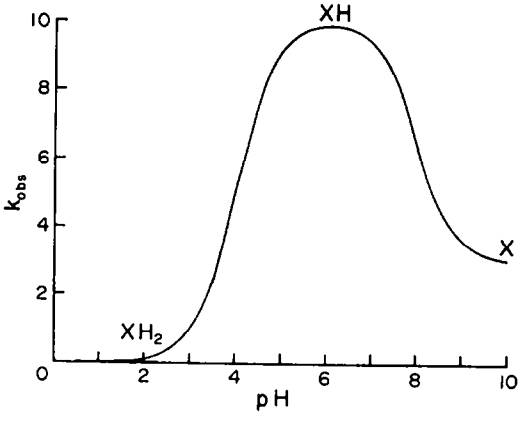
The three-states criterion. The main purpose of this paper is to present a way to demonstrate that nucleophilic reactivity resides in M_1AH as well as in M_1A^- if M_1A^- shows such reactivity. The method involves study of the reaction of M_1 with an electrophilic reagent that increases its electrophilicity by protonation. In suitable cases such a reacting system will be characterized by 3 reactive protonic states (X, XH and XH_2).

Consider a one-step bimolecular reaction between M_1 and M_2e-B_2 in which A^- (in M_1) and B_2 are the only proton binding sites in the system. The site B_2 is bonded to or conjugated with the electrophilic centre e such that M_2 increases its reactivity when B_2 is protonated. Two general cases are considered.

(a) If M_1AH does not possess nucleophilic character, the reaction of M_1 with M_2 will be characterized by two reactive protonic states: the X state, reaction of M_1A^- with M_2e-B_2 and the XH state, reaction of M_1A^- with M_2e-$B_2{}^+H$. The third protonic state in which M_1 exists as M_1AH will be unreactive. The relative reactivities observed in the X and XH states (see Figs 2a–c) will depend on the separation of the pK_a values of M_1AH and $M_2eB_2{}^+H$ and the difference in the pH-independent rate constants that characterize the reactions of M_1A^- with M_2eB_2 and with $M_2eB_2{}^+H$. Thus the observed reactivity of the XH state may be less than (Fig 2a) or greater than (Fig 2b) that of the X state depending on the relative magnitudes of the products of the relevant pH-independent rate constants and the relevant concentration terms. There remains of course the (perhaps somewhat unlikely) possibility that the increase in electrophilicity of M_2 that



(a)



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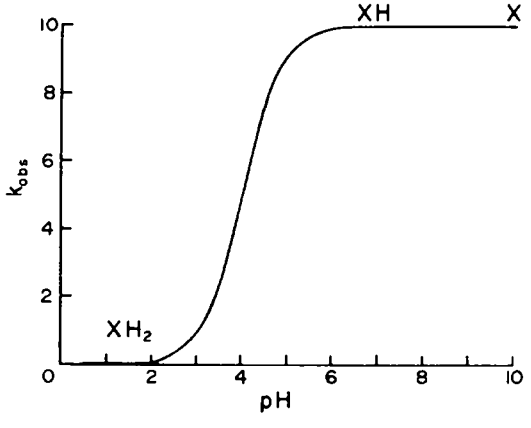
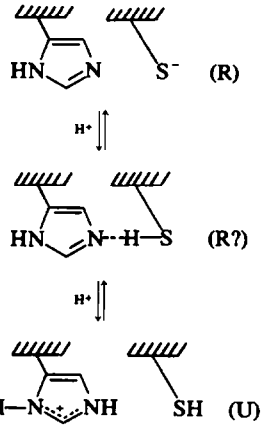


(c)

Fig 2. Typical pH-rate profiles for reactions exhibiting two reactive protonic states and one unreactive protonic state. The curves are theoretical for: $k_{obs} = k_2/\{1 + [H^+]/K_{II} + K_{III}/[H^+]\} + k_3/\{1 + [H^+]/K_{III}\}$ (see the appendix) in which $pK_{II} = 4$, $pK_{III} = 8$ and for (a) $k_2 = 3$ units, $k_3 = 10$ units, for (b) $k_2 = 10$ units, $k_3 = 3$ units and for (c) $k_2 = k_3 = 10$ units.

results from protonation of B_2 is exactly compensated for by the decrease in the concentration of A^-. In such a case only the protonation of B_2 would be reflected in the pH-k profile as in Fig 2c. All three of these situations are possible if pK_a $M_1AH \gg pK_a$ $M_2eB_2^+H$. If pK_a $M_1AH \ll pK_a$ $M_2eB_2^+H$, however, only profiles of the type shown in Fig 2b are possible because the first protonation of the X state provides an increasing concentration of the system of higher intrinsic reactivity.



SCHEME 1. Postulated activation of cysteinyl thiol groups by hydrogen bonding to histidinyl imidazole groups in enzyme active centres. R = reactive as a nucleophile and U = unreactive as a nucleophile.

(b) For simplicity the (probably) reasonable assumption is made that the intrinsic reactivity of M_1AH is less than that of M_1A^-. For any situations in which this is not so, the discussion given below is readily extended to cover such cases.

When both members of the conjugate pair possess nucleophilic character, the reaction will be characterized by three protonic states, all of which will be reactive: the X state, reaction of M_1A^- with M_2e-B_2; the XH state made up of reaction of M_1A^- with M_2e-B_2^+H and reaction of M_1AH with M_2e-B_2 and the XH_2 state, reaction of M_1AH with M_2e-B_2^+H. Thus neglecting the probably rather rare compensatory situation relating the X and XH states of Fig 2c, the pH-rate profile revealing the three reactive protonic states would be of the form shown in Fig 3a (which derives from Fig 2a) or in Fig 3b (which derives from Fig 2b). Figs 3a and 3b arbitrarily show the reactivities of the related X and XH_2 states to be equal. This may be so in some systems but in general, one of the reactivities would probably be less than the other.

Observation of three reactive protonic states for such a reacting system containing only the two proton binding sites provides definitive evidence for nucleophilic reactivity in M_1AH. This is of course the more striking when the reactivity of the XH_2 state is greater than that of the XH state as in Fig 3a. This appears to be the situation that obtains

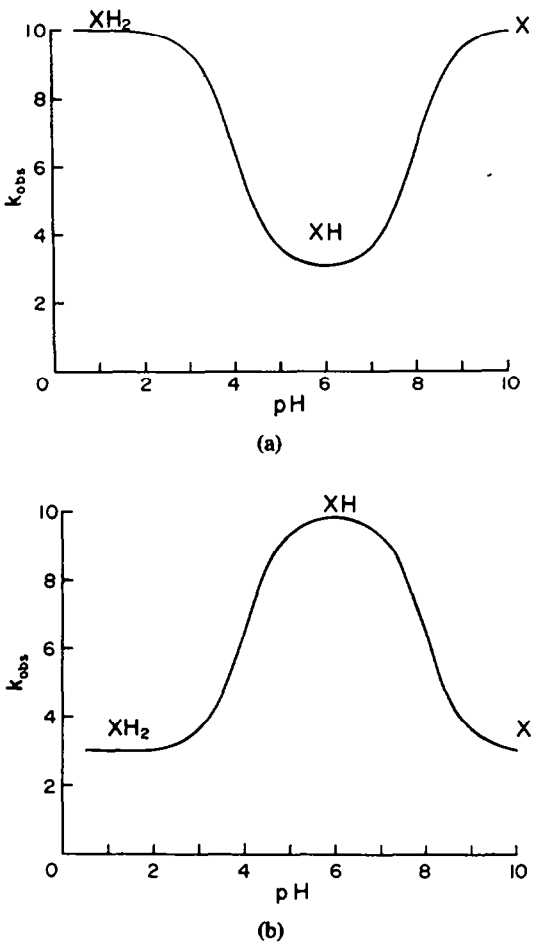
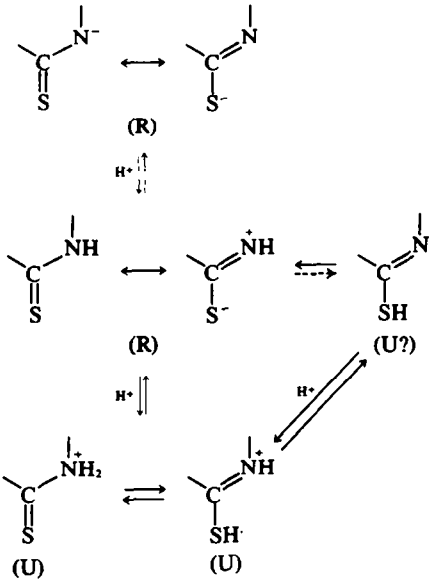


Fig 3. Typical pH-rate profiles for reactions exhibiting three reactive protonic states in which the reactivities of the X and XH_2 states are arbitrarily made equal. The curves are theoretical for: $k_{obs} = k_I/\{1 + K_{II}/[H^+] + k_2/\{1 + [H^+]/K_{II} + K_{III}/[H^+]\} + k_3/\{1 + [H^+]/K_{III}\}$ (see the appendix) in which $pK_{II} = 4$, $pK_{III} = 8$ and for (a) $k_1 = k_3 = 10$ units and $k_2 = 3$ units and for (b) $k_1 = k_3 = 3$ units and $k_2 = 10$ units. Variations in the shapes of these profiles that result from inequality of the X and XH_2 state reactivities are readily envisaged.

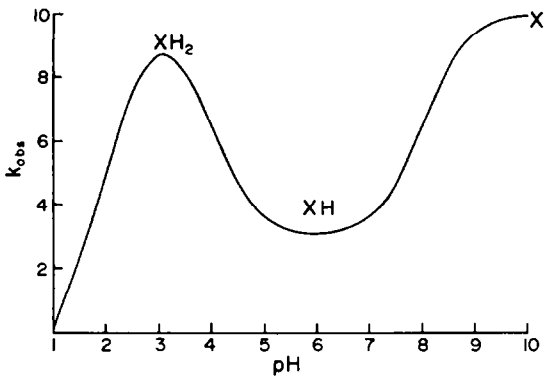
in the reaction of L-ergothioneine with 2,2'-dipyridyl disulphide (2-Py-S-S-2-Py) and the "three states criterion" has been used as evidence of nucleophilic character in the unionized mercaptoimidazole moiety of L-ergothioneine.[9] In many systems, however, there will be other proton binding sites. Indeed, two of the ways in which it was considered nucleophilicity in AH might originate (see above) involve a third proton binding site, protonation of which would be predicted to destroy the nucleophilicity in AH. Thus if "activation" of AH is brought about by hydrogen bonding to a basic centre B_1 [see 3] protonation of B_1 4 would prevent this activation process. This is the type of activation that has been envisaged in the active centres of the thiol proteases (Scheme 1) (see, e.g. Lowe,[13] Brocklehurst & Little,[7] Polgar,[14] but compare Sluyterman & Wolthers[15]). Similarly activation by electron donation from a basic centre conjugated to the nucleophilic atom as envisaged in the case of L-ergothioneine and some other aminothiones would be destroyed by protonation (Scheme 2).



SCHEME 2. Nucleophilic and non-nucleophilic forms of aminothiones such as L-ergothioneine. R = reactive as a nucleophile and U = unreactive as a nucleophile.

When activation of AH is occasioned by its interaction with another basic centre B_1 the pH-k profile for the reaction with M_2 will differ from those in Figs 3a and 3b by the presence of another ionization leading to an unreactive XH_3 state. If it is assumed for simplicity that the pK_a of $B_1{}^+H$ is significantly lower than either of the other two pK_a values of the system, profiles of the type presented in Figs 4a and 4b (derived from Figs 3a and 3b respectively) will be obtained. Strictly, Figs 3a and 3b, which relate to the systems lacking B_1, will also contain the extra ionization shown in Figs 4a and 4b. In these cases, this characterizes the protonation of M_1AH to provide unreactive $M_1A^+H_2$. In many such cases, however, e.g. when AH is an aminothione group, protonation of AH will occur only in solutions of very much higher acidity than the pH regions in which the other protonations occur and may be neglected for all practical purposes.

When the basic centre B_1 (in M_1) is not conjugated electronically with A, observation of three reactive protonic states in the reaction of B_1M_1 with $M_2c\text{-}B_2$ is not necessarily definitive of nucleophilic



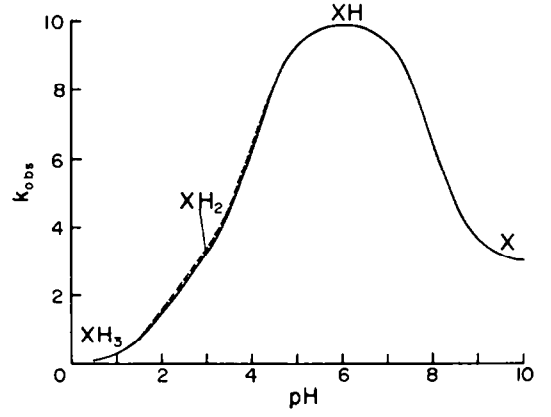


Fig 4. Typical pH-rate profiles for reactions exhibiting three reactive protonic states and one unreactive protonic state in which the reactivities of the X and XH_2 states are arbitrarily made equal. The (solid) curves are theoretical for: $k_{obs} = k_I/\{1 + [H^+]/K_I + K_{II}/[H^+]\} + k_2/\{1 + [H^+]^2/K_IK_{II} + [H^+]/K_{II} + K_{III}/[H^+] + k_3/\{1 + [H^+]/K_{III}\}$ (see appendix) in which $pK_I = 2$, $pK_{II} = 4$, $pK_{III} = 8$ and for (a) $k_1 = k_3 = 10$ units and $k_2 = 3$ units and for (b) $k_1 = k_3 = 3$ units and $k_2 = 10$ units. The effect on profile (a) of omitting the squared term in the rate equation is negligible. The effect of omitting the square term on profile (b) which has a high XH state reactivity is just appreciable and is shown by a dotted line. Variations in the shapes of these profiles that result from the inequality of the X and XH_2 state reactivities are readily envisaged.

reactivity in B_1M_1AH. The uncertainty arises because proton partitioning between A^- and B_1 (without interaction) may provide sufficiently high concentrations of the zwitterion HB_1^+-M_1-A^- which may have sufficiently high intrinsic reactivity to account for the observed reactivities of the XH and XH_2 states of profiles of the types given in Figs 4a and 4b. It may be possible to obviate this difficulty if there is reason to suppose that proton partitioning between A^- and B_1 is of the non-catalytic or negative catalytic type. The reactivity of $B_1M_1A^-$ towards M_2eB_2 can often be determined experimentally. A rate enhancement factor to predict the reactivity of $B_1M_1A^-$ (and the maximum reactivity of $HB_1^+M_1A^-$) towards $M_2eB_2^+H$ may be estimated from measurements on related systems, e.g. reaction of $M_2eB_2^+H$ with some nucleophile related to $B_1M_1A^-$ that lacks B_1 (M_3A^- e.g. M_1A^- without B_1). Thus it would be possible to calculate the reactivities (or maximum reactivities) of the XH and XH_2 states assuming that they arise from non-catalytic (or negative catalytic) proton partitioning and thus ascertain whether this phenomenon could account for the measured reactivities.

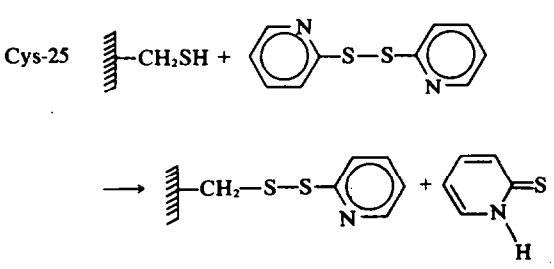
A more serious problem exists if chemical experience suggests that proton partitioning between A^- and B_1 might be of the positive catalytic type. Unless analogous systems provide some indication of the enhancement of the reactivity of A^- that would be expected consequent on protonation of B_1 it may not be possible to decide whether XH and XH_2 states of profiles such as those in Figs 4a and 4b result from this type of proton partitioning or from nucleophilic reactivity in AH.

The concepts discussed above and the difficulties encountered in applying them to more complex systems are illustrated below by consideration of the reaction of papain with 2-Py-S-S-2-Py. Examples of reactions that exhibit some of the types of pH-rate profiles shown in Figs 1–4 are presented in Table 1.

The reaction of papain with 2-Py-S-S-2-Py

We have recently reported that the reaction of papain with 2-Py-S-S-2-Py (Scheme 3) is characterized by a pH-rate profile of the general type presented in Fig 4a. This is so irrespective of whether the papain preparation is a commercial partially active one,[6,7] papain-BK activated by incubation with L-cysteine,[16] or fully active papain prepared by covalent chromatography.[17]

The reactive protonic states of the reaction are characterized by $k^X \simeq 1{\cdot}5 \times 10^3 M^{-1}s^{-1}$, $k^{XH} \simeq 7 \times 10^2 M^{-1}s^{-1}$, $k^{XH_2} \simeq 5 \times 10^4 M^{-1}s^{-1}$, $pKa_I \simeq pKa_{II} \simeq 3{\cdot}8$



SCHEME 3. The reaction of papain with 2-Py-S-S-2-Py. The products are the papain-2-pyridyl mixed disulphide and 2-thiopyridone.

Table 1. Examples of reactions that exhibit some of the types of pH-rate profiles shown in Figs 1–4

Figure	Features of the profile observed	Reaction	Ref
1a	1 reactive protonic state and 1 unreactive protonic state	reactions of simple thiols with reagents that cannot increase their electrophilicity by protonation, e.g. 5,5'-dithio-bis-(2-nitrobenzoate) dianion	19
1b	2 reactive protonic states: X state reactivity > XH state reactivity; a third (unreactive) protonic state would be predicted in solutions of high acidity	reaction of L-ergothioneine with iodoacetamide	9
2a	2 reactive protonic states and 1 unreactive protonic state: X state reactivity > XH state reactivity	reaction of papain with chloroacetamide	14
2b	2 reactive protonic states and 1 unreactive protonic state: XH state reactivity > X state reactivity	reactions of papain with L(−)α-iodopropionate and chloroacetate	20, 21
3a	3 reactive protonic states: XH_2 state reactivity > XH state reactivity and X state reactivity > XH state reactivity; a fourth (unreactive) protonic state would be predicted in solutions of high acidity	reaction of L-ergothioneine with 2-Py-S-S-2-Py	9
4a	3 reactive protonic states and 1 unreactive protonic state; state reactivities: $XH_3 \simeq 0$;		
	(i) $XH_2 > X > XH$	reaction of papain with 2-Py-S-S-2-Py	7
	(ii) $X > XH_2 > XH$	reaction of 2-(mercapto-methyl) 4,5-benzimidazole with 2-Py-S-S-2-Py	T. Stuchbury, E. Ager, G. V. Garner, P. Duke, K. Brocklehurst and H. Suschitzky, unpublished work

and $pKa_{III} \simeq 9$. The molecular pK_a values of 2-H^+Py-S-S-2-PyH^+ are $pKa_I < 1$, $pKa_{II} = 2{\cdot}45$; this corresponds to an intrinsic (group) pKa_2 for monoprotonated 2-Py-S-S-2-Py of 2·15.[8]

The outline interpretation of this profile[7] effectively in terms of the "three states criterion" discussed above is attractive because it appears to provide strong evidence for the existence of nucleophilic reactivity in the unionized thiol group of the cysteine - 25 - histidine - 159 - asparagine - 175 interacting system of the papain catalytic site which contains the enzyme's only thiol group. Some unusual features of the profile not discussed previously and the general problems of proton partitioning referred to above demand more detailed consideration of this remarkable reaction.

The X state. The X state of the reaction is reasonably interpreted as reaction of the papain thiolate ion with unprotonated 2-Py-S-S-2-Py. The low reactivity of this X state ($1{\cdot}5 \times 10^3 M^{-1}s^{-1}$) compared with those of the X states of the analogous reactions of low molecular weight thiols ($5 \times 10^4 M^{-1}s^{-1}$, Stuchbury and Brocklehurst, unpublished work; see also Ref 7) could reflect the inaccessibility of the thiol group of cysteine-25. The thiol group of propapain which is probably that of cysteine-63 (or possibly cysteine-22) of the papain sequence is similarly unreactive towards 2-Py-S-S-2-Py ($k^X = 2{\cdot}3 \times 10^2 M^{-1}s^{-1}$, $pK_a = 7{\cdot}6$, Brocklehurst and Kierstan[16]).

These low reactivities may arise in part from poor binding of the reagent to the protein. The rapidity of these reactions necessitated their study (using conventional spectrophotometry) at approximately equimolar concentrations (of enzyme and reagent). Under these conditions the reactions obeyed second order kinetics. If, however, the reactions proceed through the intermediacy of a

protein-reagent adsorptive complex, the observed second order rate constant will represent the ratio of the first order rate constant of the intracomplex thiol-disulphide interchange and the dissociation constant of the protein-reagent complex. In the case of papain it seems possible that in the adsorptive complex of the XH_2 state, the reagent may be hydrogen bonded to the carboxyl group of aspartate-158 (see later). If this is so, binding of the reagent in the same location in the X state would present the lone pair of electrons of one of the pyridyl nitrogen atoms to the carboxylate ion of aspartate-158. This could contribute to a poor binding constant.

The XH state. The most obvious component of this state is the reaction of the papain thiolate ion with 2-Py-S-S-2-Py monoprotonated on nitrogen (2-Py-S-S-2-PyH$^+$). Studies on reactions of 2-Py-S-S-2-Py with simple low molecular weight thiols (Stuchbury and Brocklehurst, unpublished work; see also Ref 7) show that the electrophilicity of 2-Py-S-S-2-Py increases by a factor of 4×10^3 when it becomes monoprotonated in free solution.

Using this rate enhancement factor the reaction of the papain thiolate ion with 2-Py-S-S-2-Py$^+$H is calculated to provide an XH state reactivity of approximately 20 $M^{-1}s^{-1}$ which is considerably less than the observed value ($7 \times 10^2 M^{-1}s^{-1}$).

To account for the high reactivity of the XH state it is necessary to invoke proton partitioning which may or may not involve significant contributions from binding effects.

If one nitrogen atom of the neutral 2-Py-S-S-2-Py molecule and a basic site in the enzyme share a proton in a hydrogen bond, this could increase the reactivity of the XH state by proximity, orientation and microsolvation effects. If binding effects of this nature are invoked, the reactivity of the XH state could be accounted for in terms of reaction involving the enzyme's thiolate ion as the only nucleophilic species. Consideration of the highly reactive XH_2 state of the reaction (see later), however, makes it probable that the unionized thiol group of papain also possesses considerable nucleophilic reactivity. If this is so, the remaining reactivity of the XH state is readily accounted for in terms of reaction of the unionized thiol group with unprotonated 2-Py-S-S-2-Py.

The XH_2 state. The reactivity of the XH_2 state is approximately $5 \times 10^4 M^{-1}s^{-1}$. Its formation from a (probably) completely unreactive XH_3 state and its decomposition to the XH state, are both characterized by molecular pK_a values of 3·8 which are considerably higher than the pK_a of 2-Py-S-S-2-Py$^+$H. Under the conditions of concentration in which the reactions were carried out, it is probable that saturation is not approached. Thus if proton transfer is not kinetically significant, the kinetically determined pK_a values should reflect ionizations in free enzyme and/or free reagent unperturbed by adsorptive complex formation. Since the reagent does not have a pK_a value as high as 3·8, the two pK_a values must characterize free enzyme ionizations.

It is possible to propose two types of mechanism for the XH_2 state: those in which the only reactive nucleophile in both this and the XH state is the enzyme's thiolate ion and those in which the undissociated thiol group also has nucleophilic character. Inspection of a molecular model of papain strongly suggests the imidazole group of histidine-159 as the origin of the nucleophilic character of the undissociated thiol group if such character exists. This interaction has of course been postulated by several authors on many occasions. The pK_a value of the imidazolium ion of histidine-159 is probably approximately 4, the low value probably resulting from its hydrophobic environment.[18]

Inspection of models of papain and of 2-Py-S-S-2-Py led us to propose that binding in which one of the pyridyl nitrogen atoms of the reagent and either the carboxylate ion of aspartate-158[6,8] or the oxygen atom of the backbone carbonyl group of this residue[8] share a proton could present one or other of the electrophilic sulphur atoms of the reagent to the nucleophilic sulphur atom of cysteine-25. Allen and Lowe[18] have suggested that binding of 7 - chloro - 4 - nitrobenzo - 2 - oxa - 1,3 - diazole to papain involves a group of pK_a 3·7 (35°C) which they suggest could be the carboxyl group of aspartic acid-158.

It seems reasonable to suppose that the two ionizations of pK_a 3·8 that influence the reaction of papain with 2-Py-S-S-2-Py could characterize the side chain groups of aspartic acid-158 and histidine-159 and further consideration of the possible mechanisms is given in terms of these residues.

Whatever the nature of the nucleophile, the most obvious interpretation of the increase in reactivity on passing from the XH to the XH_2 state is that the acidic form of the side chain of one of the active centre residues (158 or 159) partially donates a proton to one of the pyridyl nitrogen atoms of 2-Py-S-S-2-Py. This would certainly increase its electrophilicity (though perhaps not as effectively as full protonation) and may also provide proximity and orientation effects.

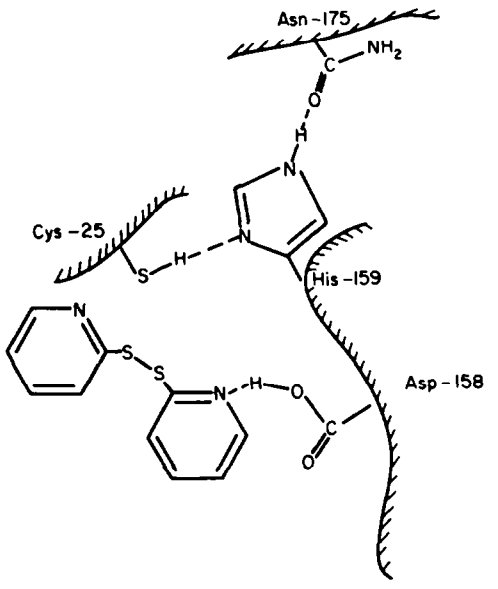
The effect of protonation of the second nitrogen atom of the 2-Py-S-S-2-Py molecule ($pk_a < 1$) on its electrophilicity is not known but experiments with 4,4'-dipyridyl disulphide (Stuchbury and Brocklehurst, unpublished work, see also Ref 7) suggest that the difference in the electrophilicities of the mono- and di-protonated dipyridyl disulphides is not large (for the 4,4'-isomer the two intrinsic pK_a values are only approximately 0·5 unit apart, Brocklehurst and Little[8]). Thus the low concentrations of 2-H$^+$Py-S-S-2-PyH$^+$ present at pH values > 2 will probably not be compensated for by an

inordinately high electrophilicity and reactions of the diprotonated reagent are unlikely to contribute significantly to the XH_2 state unless the rate enhancement results from the specific binding of the diprotonated form. That this is not the case is demonstrated by the close similarity in most respects of the pH-k profile of the reaction of papain with 2-pyridyl-n-propyl disulphide 5 (Shipton and Brocklehurst, unpublished work) to that of the papain-2-Py-S-S-2-Py reaction.

Mechanisms in which the thiolate ion is the only reactive nucleophile in both the XH and XH_2 states. Any mechanism of this type would have to depend on a second act of positive catalytic proton partitioning in addition to that which increases the electrophilicity of 2-Py-S-S-2-Py by its partial monoprotonation. Without it the XH_2 state reactivity would be predicted to be approximately 10^3 times less than its observed value. Thus with only non-catalytic proton partitioning between the thiol group of pK_a 9 and one other group pK_a 4 only approximately 5×10^{-6} of the enzyme will possess a thiolate ion at pH 4. If the reactivity of this thiolate ion towards 2-Py-S-S-2-Py ($1{\cdot}5 \times 10^3 M^{-1}s^{-1}$) is increased by 4×10^3 consequent on protonation of the reagent, as it is in the case of simple thiols, the XH_2 state reactivity would be $5 \times 10^{-6} \times 1{\cdot}5 \times 10^3 \times 4 \times 10^3 = 30\ M^{-1}s^{-1}$.

It is of course possible to postulate that protonation of one of the two implicated proton binding sites (other than the thiolate ion) increases the reactivity of the papain thiolate ion towards 2-Py-S-S-2-Py (either unprotonated or hydrogen bonded) by some unknown mechanism. If binding of 2-Py-S-S-2-Py to the enzyme protonated at one of the implicated sites (say the carboxyl group of aspartate-158, see later) not only increases the electrophilicity of the reagent by 4×10^3 (see above) but also increases the intrinsic nucleophilic reactivity of the enzyme's thiolate ion by a factor of 20 (i.e. the X state reactivity from $1{\cdot}5 \times 10^3\ M^{-1}s^{-1}$ to $3 \times 10^4\ M^{-1}s^{-1}$, the value characteristic of the reaction of simple thiols) the reactivity of the XH state would be calculated to be $20 \times 20 = 400\ M^{-1}s^{-1}$. This is within a factor of 2 of the observed value. Binding of 2-Py-S-S-2-Py to aspartic acid-158 might be expected to be doubly effective in this way if the low reactivity of the X state derives from poor binding as discussed above. Even if this is so, however, the unlikely intervention of positive catalytic proton partitioning would be required to account for the increase in reactivity on passing from the XH to the XH_2 state. In the absence of any evidence for this type of rate enhancement, it seems reasonable to consider nucleophilic character in the unionized thiol group of papain a more reasonable alternative.

Mechanisms involving two nucleophilic states of papain. If the unionized thiol group of papain does possess nucleophilic character the pH-rate profile for its reaction with 2-Py-S-S-2-Py is readily accounted for. The XH_2 state is envisaged as reaction of the "unionized" thiol group of the cysteine - 25 - histidine - 159 - asparagine - 175 hydrogen bonded system with 2-Py-S-S-2-Py hydrogen bonded to the carboxyl group of aspartic acid-158 as in Scheme 4.



SCHEME 4. Schematic drawing of the postulated XH_2 state of the papain-2-Py-S-S-2-Py adsorptive complex.

The absence of a kinetic deuterium isotope effect in the XH state (Shipton and Brocklehurst, unpublished work) suggests that pretransition state proton transfer occurs in this reaction as it seems to do in the XH state of the reaction of papain with some alkylating agents.[14] These observations of course do not imply that the best description of the ground state of papain's catalytic site is a cysteine-histidine ion pair, but merely that in reactions at the supphur atom its proton is transferred to the nitrogen atom of histidine-159 before the transition state. Ion pair formation in the ground state is one extreme of a range of possible structures of the thiol-imidazole hydrogen bond.

One feature of the papain-2-Py-S-S-2-Py reaction deserves special mention. It derives from the narrowness of the pH-k profile (width at half height = 1·36 units, $pK_I - pK_{II} = 0$). Dixon[4] has recently demonstrated that such a profile is indicative of positively cooperative proton binding. This phenomenon would be particularly attractive in this reaction because the two proton binding sites may well be in the side chains of adjacent residues in the papain sequence.

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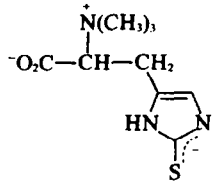

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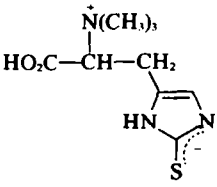

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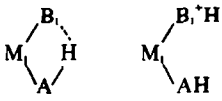

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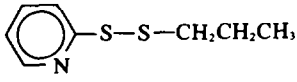

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E. M. Crook, Dr. H. B. F. Dixon, Dr. A. J. Kirby, Dr. G. Lowe, Dr. T. Stuchbury, Dr. C. W. Wharton, Dr. M. P. J. Kierstan and Mr. M. Shipton. I thank also the Science Research Council for financial support and Miss Angela Duffy for computing and drawing the theoretical curves.

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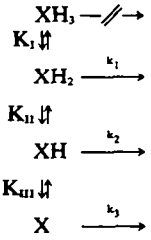
APPENDIX

Rate equations for reactions involving up to three reactive protonic states

Consider a reaction involving three reactive protonic states XH_2, XH and X characterized by pH-independent rate constants k_1, k_2, k_3 respectively (Scheme A). The fourth protonic state, XH_3 is unreactive and K_I, K_{II}, K_{III} are molecular (macroscopic) dissociation constants. All protonation and deprotonation steps are considered fast and not rate limiting.



SCHEME A. A kinetic model for a reaction involving three reactive protonic states.

From the conservation equation (Eq 1) and the expressions for the dissociation constants (Eqs 2–4) expressions (Eqs 5–7) may be obtained relating the concentrations of the three reactive protonic states to the total concentration of the system (X_T) and the molecular dissociation constants.

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

$$K_I = [XH_2][H^+]/[XH_3] \quad (2)$$

$$K_{II} = [XH][H^+]/[XH_2] \quad (3)$$

$$K_{III} = [X][H^+]/[XH] \quad (4)$$

$$[X] = [X_T]/\{1 + [H^+]^3/K_IK_{II}K_{III} + [H^+]^2/K_{II}K_{III} + [H^+]/K_{III}\} \quad (5)$$

$$[XH] = [X_T]/\{1 + [H^+]^2/K_IK_{II} + [H^+]/K_{II} + K_{III}/[H^+]\} \quad (6)$$

$$[XH_2] = [X_T]/\{1 + [H^+]/K_I + K_{II}/[H^+] + K_{II}K_{III}/[H^+]^2\}. \quad (7)$$

The rate equation (Eq 8) in which k_{obs} in the experimentally observed pH-dependent rate constant may be rewritten in terms of pH-independent rate constants as Eq 9 by combining Eqs 1 and 8. The relationship between k_{obs} and the pH-independent rate constants (Eq 10) is obtained by combining Eq 1 and Eqs 5–9.

$$\text{Rate} = k_{obs}[X_T] \quad (8)$$

$$\text{Rate} = k_1[XH_2] + k_2[XH] + k_3[X] \quad (9)$$

$$k_{obs} = k_1/\{1 + [H^+]/K_I + K_{II}/[H^+] + K_{II}K_{III}/[H^+]^2\} + k_2/\{1 + [H^+]^2/K_IK_{II} + [H^+]/K_{II} + K_{III}/[H^+]\} + k_3/\{1 + [H^+]^3/K_IK_{II}K_{III} + [H^+]^2/K_{II}K_{III} + [H^+]/K_{III}\}. \quad (10)$$

If all three dissociations are well separated from each other, the contributions of the terms containing multiple powers of $[H^+]$ may be neglected in which case Eq 10 becomes Eq 11.

$$k_{obs} = k_1/\{1 + [H^+]/K_I + K_{II}/[H^+]\} + k_2/\{1 + [H^+]/K_{II} + K_{III}/[H^+]\} + k_3/\{1 + [H^+]/K_{III}\}. \quad (11)$$

If only K_{II} and K_{III} are well separated and K_I and K_{II} are comparable, as is the case in the reaction of papain with 2-Py-S-S-2-Py, the term $[H^+]^2/K_IK_{II}$ in Eq (10) will, in the general case, not be negligible. The omission of this term will not have serious consequences for the shape of the pH-k_{obs} profile, however, if as in the papain-2-Py-S-2-Py reaction $k_1 \gg k_2$.

If the reacting system contains fewer than three reactive protonic states the relevant rate equation may be obtained from Eqs 10 or 11 by setting the superflous dissociation constants equal to infinity and the superfluous rate constants equal to zero.