Clarification of the pH-dependent kinetic behaviour of papain by using reactivity probes and analysis of alkylation and catalysed acylation reactions in terms of multihydronic state models: implications for electrostatics calculations and interpretation of the consequences of site-specific mutations such as Asp-158–Asn and Asp-158–Glu

Geoffrey W. MELLOR,*‡ Manij PATEL,*§ Emrys W. THOMAS† and Keith BROCKLEHURST*||

*Laboratory of Structural and Mechanistic Enzymology, Department of Biochemistry, Queen Mary and Westfield College, University of London, Mile End Road, London E1 4NS, U.K. and †Department of Biological Sciences, University of Salford, Salford M5 4WT, U.K.

1. The complex behaviour of papain (EC 3.4.22.2) in acidic media has been investigated by (a) stopped-flow reactivity probe kinetics using 4,4'-dipyrimidyl disulphide (I) and 2,2'-dipyridyl disulphide (II) as thiol-specific time-dependent inhibitors with markedly different susceptibilities to activation by hydronation (protonation) and (b) using the multitasking application program SKETCHER for the rapid evaluation of pH-dependent kinetic data by means of interactive manipulation of calculated curves. 2. The substantially lower basicity of (I) (pK_a 0.91) than that of (II) (pK_a 2.45) combined with retention of high reactivity permitted the pK_a for the formation of the (Cys-25)-S⁻/(His-159)-Im⁺H ion-pair state of papain to be determined kinetically as 3.4, a value close to that (3.3) deduced by potentiometric difference titration [Lewis, Johnson and Shafer (1976) *Biochemistry* **15**, 5009–5017] and lower than the value (approx. 4) often reported from pH-dependent kinetic studies. The higher values are now known to arise from inadequate data analysis that does not take account of other overlapping kinetically influential ionizations. 3. Re-evaluation of the extensive sets of pH- k_{cat}/K_m data for the hydrolysis of nine substrates by papain reported by Polgár and Halász (1978) (*Eur. J. Biochem.* **88**, 513–521) by making use of SKETCHER, the known pK_a value (3.4) from the reaction with compound (I) and two additional kinetically influential pK_a values deduced from the reaction with compound (II) now permits the identification of the pH-dependent events in reactions of papain with inhibitors and substrates. 4. A major conclusion is that, whereas in reactions of simple alkylating agents and compound (I) full nucleophilic character of (Cys-25)-S⁻/(His-159)-Im⁺H is provided by hydronic dissociation with pK_a 3.3–3.4, in catalysis relatively little catalytic competence is produced consequent upon ion-pair formation. Substantial catalytic competence requires further hydronic dissociation with pK_a approx. 4, and for cationic substrates further enhancement is produced

by hydronic dissociation with pK_a approx. 5. 5. The present work, together with the kinetic analysis of reactions of papain in alkaline media reported by Mellor, Thomas, Topham and Brocklehurst [*Biochem. J.* (1993) **290**, 289–296], defines the kinetically influential ionizations of papain as 3.4, 4.0, 5.0, 8.3 and 10.0 of which 3.4 and 8.3 relate to the formation and subsequent dehydronation of the ion-pair state. 6. The determination of the pK_a for the formation of the ion-pair state of papain as 3.4 rather than 4.0 and the ambiguities inherent in determining pK_a values of systems with overlapping ionizations compel the view that the analysis and interpretation of the putative pK_a shifts inferred from changes in the pH-dependence of k_{cat}/K_m for papain mutants such as Asp-158–Asn and Asp-158–Glu reported by Ménard, Khouri, Plouffe, Dupras, Ripoll, Vernet, Tessier, Laliberté, Thomas and Storer [*Biochemistry* (1990) **29**, 6706–6713] and Ménard, Khouri, Plouffe, Laflamme, Dupras, Vernet, Tessier, Thomas and Storer [(1991) *Biochemistry* **30**, 5531–5538] need to be reconsidered. Substantial shifts in pH-rate curves can be due to changes in pH-independent rate constants as well as or instead of changes in pK_a values. The view is compelled that the data in acidic media reported for the mutants by Ménard et al. (1990, 1991) may relate to perturbation of the two as-yet-unassigned ionizations with pK_a values close to 4 and 5 and provide no evidence for or against electrostatic perturbation of the (Cys-25)-S⁻/(His-159)-Im⁺H ion-pair. Some evidence for the lack of perturbation of the ion-pair in the Asp-158–Glu mutant is provided by the fact that the pK_a value for ion-pair dehydronation to -S⁻/-Im is essentially the same for the mutant (8.25) as for native papain (8.3). 7. Implications of the nature of the complex pH-dependent kinetic behaviour of papain for the characteristics of other cysteine proteinases, notably the calpain II 80 kDa/18 kDa heterodimer, are briefly discussed.

INTRODUCTION

Papain (EC 3.4.22.2) is the best-characterized member of the cysteine proteinase family, both structurally and mechanistically [see Brocklehurst et al. (1987b) for an in-depth review, Brockle-

hurst (1987), Baker and Drenth (1987) and Polgár (1990) for shorter reviews, and Patel et al. (1992) and Mellor et al. (1993b) for recent structure-related kinetic studies and for references to recent crystallographic, spectroscopic and molecular-biological studies]. Despite the extensive literature on this enzyme, im-

Abbreviations used: PPΩ, papaya proteinase Ω; MCA, methylcoumarylamide; Z, benzyloxycarbonyl.

‡ Present address: The Wellcome Research Laboratories, The Wellcome Foundation Ltd., Langley Court, South Eden Park Road, Beckenham, Kent BR3 3BS, U.K.

§ Present address: Department of Biochemical and Clinical Pharmacology, St. Jude Children's Research Hospital, 332 North Lauderdale, P.O. Box 318, Memphis, TN 38101-0318, U.S.A.

|| To whom correspondence should be addressed.

portant aspects of its pH-dependent behaviour and the relationship of this to the pH-dependent behaviour of other cysteine proteinases [see e.g. Topham et al. (1991)] still need to be clarified.

We recently introduced 4,4'-dipyrimidyl disulphide (I) as a thiol-specific time-dependent inhibitor and showed that by using both this compound and 2-pyridyl disulphides [such as 2,2'-dipyridyl disulphide (II), 2-carboxyethyl-2'-pyridyl disulphide *N*-methylamide (III) and 2-(*N*'-acetyl-L-phenylalanyl-amino)ethyl-2'-pyridyl disulphide (IV)] as reactivity probes it was possible to dissect the complex pH-dependent behaviour of papain in alkaline media and assign unequivocally the two kinetically influential ionizations (Mellor et al., 1993b). Thus the interconversion of the catalytically competent state of papain [of which the (Cys-25)-S⁻/(His-159)-Im⁺H ion-pair is a plausible component] and the non-catalytic (Cys-25)-S⁻/(His-159)-Im anion is characterized by a molecular pK_a value of 8.3, and another ionization of pK_a 10.0 modulates catalytic-site reactivity. The pK_a of 8.3 is revealed in the reaction of papain with compound (I) and the pK_a of 10.0 is revealed in reactions with 2-pyridyl disulphides devoid of substrate-like recognition sites [such as compound (III)]. In the latter reaction, the kinetic consequence of crossing pK_a 8.3 is suppressed by a compensating effect involving reaction via a transition state in which the 2-mercaptopyridine leaving group is activated by hydrogen-bonding with (His-159)-Im⁺H and thus the pK_a of 10.0 is seen particularly clearly. Both of the ionizations (pK_a 8.3 and 10.0) influence the rate of reaction of papain with the substrate-derived inhibitor, compound (IV), which is the situation also for the pH-dependence of k_{cat}/K_m for the papain-catalysed hydrolysis of substrates (C. M. Topham and K. Brocklehurst, unpublished results). Earlier indications that an ionization of pK_a approx. 10 might perturb the environment of the papain active centre include those reported by Brubaker and Bender (1966), Hinkle and Kirsch (1970, 1971) and Lewis and Shafer (1974).

Having successfully defined the behaviour of papain in alkaline media by the combined use of 2-pyridyl- and 4-pyrimidyl disulphides, we turned our attention to its at least equally complex behaviour in acidic media. Many kinetic studies have provided evidence that the formation of the (Cys-25)-SH/(His-159)-Im $\rightleftharpoons$ (Cys-25)-S⁻/(His-159)-Im⁺H ionization state of papain

appears to be characterized by a macroscopic pK_a value of approx. 4 and that this ionization is co-operatively linked to another also with macroscopic pK_a approx. 4 (Brocklehurst and Little, 1972; Shipton and Brocklehurst, 1978; Lewis et al., 1978; Salih et al., 1987; Brocklehurst et al., 1988a,b; Ménard et al., 1990). The fact that these two pK_a values appear to be so similar has hindered assessment of whether (Cys-25)-S⁻/(His-159)-Im⁺H is catalytically competent without prior or simultaneous ionization of another group in the enzyme. In addition to the two ionizations with kinetically determined pK_a values apparently of approx. 4, another ionization with pK_a approx. 5 influences the kinetics of some reactions of papain (Brocklehurst et al., 1988a,b).

A particular difficulty with the pH-dependent behaviour of papain in acidic media was noted by Lewis et al. (1976). They pointed out that the pK_a value for the formation of the ion-pair state of papain from -SH/-Im⁺H deduced by their potentiometric titration method is substantially lower than the value (pK_a approx. 4) obtained from pH-dependent kinetics. Interpolation using their values of this pK_a (3.09 at 15 °C, *I* 0.05 M; 3.29 at 29 °C, *I* 0.05 M; 3.34 at 29 °C, *I* 0.15 M) suggests a value of approx. 3.3 at 25 °C and *I* 0.1 M. One practical difficulty with many of the kinetic experiments is that they involve reactions with alkylating agents of relatively low reactivity and the reactions with papain at low pH are slow. Some of the data therefore may be prone to substantial error. Reactions of papain with 2-pyridyl disulphides such as (II) and (III) do not suffer from this difficulty. They occur on the stopped-flow time scale over a wide range of pH including acidic media and provide the clearest evidence for the influence of the second ionizing group of pK_a approx. 4. The first evidence for this second pK_a was obtained from kinetic study of the reaction of papain with compound (II) [Brocklehurst and Little (1972) and see also Shipton et al. (1975), Shipton and Brocklehurst (1978)]. The evidence is particularly clear because the two pK_a values close to 4 occur on opposite sides of a striking bell-shaped component at low pH in the pH-second-order rate constant (*k*) profile. This contrasts with the situation in many other reactions of papain including the pH-dependence of k_{cat}/K_m [see e.g. Salih et al. (1987), Ménard et al. (1990)] where the evidence for a second pK_a value of approx. 4 depends upon the observation and analysis of a small deviation at low pH from a single (sigmoidal) ionization curve. Although the evidence for the existence of the second kinetically influential pK_a in reactions of papain is most clearly revealed by the bell-shaped component of the pH-*k* profile for reactions of 2-pyridyl disulphides such as (II) and (III) (Mellor et al., 1993b), unambiguous accurate determination of the two pK_a values is rather difficult. Their close numerical proximity in the narrow bell-shaped component means that a unique fit to the data cannot be achieved and a number of solutions are possible, particularly when allowance is made for the kinetically influential pK_a (2.45–2.9) of the hydronated probe reagent [e.g. (V) pK_a 2.45].

In the present work we have made use of the same property of 4,4'-dipyrimidyl disulphide (I) [its substantially lower basicity, pK_a of (VI) = 0.91, than that of a 2-pyridyl disulphide, combined with retention of high reactivity] that permitted incisive analysis of the behaviour of papain in alkaline media (Mellor et al., 1993b) to demonstrate convincingly a kinetically influential pK_a of papain with a value (pK_a 3.4) close to that (pK_a 3.3) deduced by potentiometric titration (Lewis et al., 1976). In addition the ability of the computer program SKETCHER (Brocklehurst et al., 1990; Topham et al., 1991) to provide rapid evaluation of pH-*k* data in terms of multihydronic state models permitted evaluation of a new kinetic data set for the reaction of papain with 2-Py-S-S-Py (II) and re-evaluation of data in the literature [particularly the data for 13 reactions of papain reported by

Polgár and Halász (1978)]. The combination of the kinetic data obtained by using the pyrimidyl (I) and pyridyl (II) disulphide reactivity probes and the computer analysis using SKETCHER now permits identification of the pH-dependent events in reactions of papain with a range of inhibitors and substrates. A major conclusion is that, whereas in reactions of simple alkylating agents and compound (I) full nucleophilic character of (Cys-25)-S⁻/(His-159)-Im⁺H is provided by hydronic dissociation with pK_a 3.4, in catalysis relatively little catalytic competence is produced consequent upon ion-pair formation. Substantial catalytic competence requires further hydronic dissociation with pK_a approx. 4.0 and in some cases further enhancement is produced by additional hydronic dissociation with pK_a 5.0. The determination of the pK_a value characteristic of ion-pair formation as 3.4, rather than 4 as is commonly supposed, and the ambiguities inherent in assigning pK_a values to systems with overlapping hydronic dissociations, demonstrate that the analysis of the kinetic data for reactions catalysed by mutants of papain such as those with Asp-158-Asn and Asp-158-Glu and the consequent interpretation reported by Ménard et al. (1990, 1991) need to be reconsidered. Implications of the nature of the complex pH-dependent kinetic behaviour of papain for the characteristics of other cysteine proteinases, notably the calpain II 80 kDa/18 kDa heterodimer, are briefly discussed.

MATERIALS AND METHODS

Reactivity probes

The synthesis of 4,4'-dipyrimidyl disulphide (I) was described by Mellor et al. (1993b), the synthesis of 2-(*N'*-acetyl-L-phenylalanyl-amino)ethyl-2'-pyridyl disulphide (IV) by Brocklehurst et al. (1987a), and the purification of 2,2'-dipyridyl disulphide (II) (Aldrich) by Salih et al. (1987).

Papain

Papain was the twice-crystallized product supplied by Sigma (Poole, Dorset, U.K.) as a suspension in 0.05 M sodium acetate buffer, pH 4.5. In preparation for a set of kinetic experiments, 0.5 ml of the suspension was mixed with an equal volume of 40 mM cysteine solution in sodium pyrophosphate buffer, pH 8.0, 1.03 M and allowed to stand for 30 min at room temperature (approx. 22 °C) to convert any reversibly oxidized papain to active enzyme. Low- M_r material was then removed by gel filtration on a Sephadex G-25 column (15.0 cm × 2.5 cm). Elution with 0.1 M KCl containing 1 mM EDTA and collection of approx. 10 ml fractions produced activator-free papain in approx. 10 ml of eluate after approx. 30 ml had been collected and discarded; 10–12 ml of the post-30 ml eluate contains sufficient papain for approx. 30–40 stopped-flow kinetic runs. Papain thus prepared was shown to be free of contaminant chymopapains by both thiol-titration with 2,2'-dipyridyl disulphide (2-Py-S-S-2-Py) at pH 4 and 8 (Baines and Brocklehurst, 1978) and f.p.l.c. analysis with Pharmacia LKB equipment on a Mono S HR5/5 column. Papain is eluted very early from the ion-exchange column well separated from the position at which chymopapains would be eluted. Papaya proteinase Ω (PP Ω) is eluted even later at the end of the elution profile (see e.g. Dubois et al., 1988). In some batches of twice-crystallized papain supplied by Sigma, f.p.l.c. analysis demonstrated the presence of small amounts of contaminant chymopapains. In such cases, papain was purified by preparative f.p.l.c. Papain is readily identified and distinguished from the other cysteine proteinases of papaya latex by its unique reactivity characteristics towards 2-(*N'*-acetyl-

L-phenylalanyl-amino)-ethyl-2'-pyridyl disulphide (IV), a substrate-derived time-dependent inhibitor containing a P₁-P₂ amide bond, an L-phenylalanyl side chain as an occupant for the S₂-subsite and a 2-mercaptopyridine leaving group which provides for activation by its association with the imidazolium side chain of His-159 (Brocklehurst et al., 1988b). For example, whereas for the reaction of this inhibitor with papain, $k_{pH6}/k_{pH3.5} = 4.8$, for the analogous reaction with PP Ω , $k_{pH6}/k_{pH3.5} = 0.7$ (M. Thomas and K. Brocklehurst, unpublished work). Fully active papain containing 1 mol of thiol and 1 mol of intact catalytic-site Cys-25/His-159 interactive system per mol of enzyme is conveniently prepared as required by covalent chromatography (Brocklehurst et al., 1985).

Instrumentation and software

Kinetic studies were performed with an Applied Photophysics SF.17MV stopped-flow spectrophotometer, kinetics workstation and data-acquisition and analysis software. Monochromator entrance and exit slit widths were set at 1 mm. pH-dependence studies of the type reported in the present paper involving a multiplicity of reactive states require a rapid method of evaluating a series of kinetic models differing in the number and reactivity of the reactive states. These were evaluated by using a multi-tasking application program (SKETCHER) written in ANSI C running under RISCOS on an Acorn Archimedes microcomputer (Brocklehurst et al., 1990; Topham et al., 1991) which was developed from an earlier program (QUATRO) written in BBC BASIC for use with a BBC Micro Model B (Brocklehurst and Brocklehurst, 1988). SKETCHER permits estimation of characterizing parameters in the generic set of equations for the various models by means of interactive manipulation of calculated curves. Values of the parameters thus obtained are generally close to the final mean values obtained by weighted non-linear regression performed by using the AR computer program from the statistical software package BMDP (Dixon et al., 1988) and a Compaq Deskpro 386/20c computer, particularly when one or more of the parameters are known from an independent experiment which obviates problems deriving from multiple local minima. As demonstrated in the present paper, information from independent experiments is essential for a unique solution for systems with overlapping ionizations. Rate equations for reactions in a variety of hydronic states were written down and generated within the Archimedes by using the simple general expression, eqn. (1).

$$k = \sum_{i=1}^n \frac{A_i}{1 + \sum_{j=1}^n B_{i,j}} \quad (1)$$

Eqn. (1) relates an experimentally determined pH-dependent rate constant k , and macroscopic acid dissociation constants, K , where n is the number of reactive hydronic states; the numerator A_i is a generalized pH-independent rate constant ($\bar{k}_{xH_{i-1}}$) where i , which may take values from 1 to n , specifies the particular hydronic state. The denominator of eqn. (1) provides for the pH-dependent variation in each of the contributions to k from reaction in a particular hydronic state, i.e. reaction associated with an individual pH-independent rate constant ($\bar{k}$). Each term generated by j in the denominator comprises a product of [H⁺] and one or more acid dissociation constants each raised to a particular power. The construction of specific rate equations for particular kinetic models from eqn. (1) involves determining expressions for $B_{i,j}$ by using the two information matrices described by Brocklehurst et al. (1990) and Topham et al. (1991). pH- k data and the associated theoretical curves were displayed

by using the Sigmaplot 4.1 software package (Jandel Scientific), a Tandon MCS 486/33PC and a Hewlett-Packard Colour Pro Plotter.

Stopped-flow kinetics

All reactions were carried out at 25 °C and 1.0 M in solutions containing 1 mM EDTA under pseudo-first-order conditions with [disulphide] $\gg$ [papain]. In all cases the disulphide was in solution in the appropriate buffer and the enzyme was in solution in 0.1 M KCl. Reactions of 2-Py-S-S-2-Py (II) were monitored at 343 nm [$\Delta\epsilon_{343} = 8080 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Stuchbury et al., 1975)] with [papain] = 2–3 μM and [II] = 175 μM . Reactions of 4,4'-dipyrimidyl disulphide (I) were monitored at the isosbestic point of pyrimidine-4-thione, 312 nm, with [papain] = 2–3 μM and [I] = 80–100 μM . Although unnecessary for the determination of first-order rate constants, the absorbance changes at 312 nm can be used at pH values ≥ 2 to calculate the concentration of product formed by using $\Delta\epsilon_{312} = 7763\text{--}317$ (the value of ϵ_{312} for (I) at pH ≥ 2) = $7446 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Mellor et al., 1993b). First-order rate constants (k_{obs}) were obtained by fitting the absorbance (A)– t data collected by the Acorn Archimedes microcomputer of the SF-machine either to an equation for a single exponential process, i.e. $A = P_1 e^{-P_2 t} + P_3$, where $P_1 = A_\infty - A_0$, $P_2 = k_{\text{obs}}$, and $P_3 = A_\infty$, or to an equation for a single exponential with a zero-order component, i.e. $A = P_1 e^{-P_2 t} + P_3 t + P_4$, where $P_1 = A_\infty - A_0$ for the single-exponential component, $P_2 = k_{\text{obs}}$, P_3 is the slope of the zero-order component of the A versus t trace in s^{-1} , and P_4 is the extrapolated value of the ordinate intercept, i.e. A_∞ for the single exponential component. Reactions of compound (II) were evaluated by using the single-exponential equation. Reactions of compound (I) exhibited small but noticeable deviations from a single-exponential process, particularly at pH values > 7.5 (see Mellor et al., 1993b), and for these reactions the fit was improved by incorporation of a zero-order component. k values were calculated from $k = k_{\text{obs}}/[\text{disulphide}]$ [see Mellor et al. (1993b) for a demonstration of overall second-order kinetics].

RESULTS AND DISCUSSION

pH-dependence of the reactions of the catalytic-site thiol group of papain (Cys-25) with 4,4'-dipyrimidyl disulphide [(I) and (VI)] and 2,2'-dipyridyl disulphide [(II) and (V)] in acidic media: the five kinetically influential ionizations of the papain molecule on which catalytic-site reactivity depends

The pH-dependence of the second-order rate constant (k) for the reaction of papain with 4,4'-dipyrimidyl disulphide is shown in Figure 1(a). The good fit to the data corresponds to predominant reaction of the ion-pair state of the catalytic site with non-hydrated probe reagent (I) with a small contribution from reaction with the hydrated probe (VI) [pK_a 0.91, Mellor et al., 1993b]. Reaction of papain with 4,4'-dipyrimidyl disulphide provides reliable data in acidic media on the stopped-flow time scale and, because of the low pK_a value of compound (VI), is not complicated by substantial reaction of the hydrated form of the probe. Thus the data in Figure 1(a) compel the view that the macroscopic pK_a value characteristic of the formation of the ion-pair state of papain from (Cys-25)-SH/(His-159)-Im $^+$ H is close to 3.4, a value similar to that obtained by Lewis et al. (1976) using their potentiometric titration method (3.3, see the Introduction) and to that (3.25) reported by Polgár and Halász (1978) obtained by using simple alkylating agents (see below)

despite potential uncertainties deriving from slow reaction in acidic media.

Figure 1(b) shows a new data set for the reaction of papain with 2,2'-dipyridyl disulphide. In marked contrast with the approximately sigmoidal form of Figure 1(a), Figure 1(b) illustrates the striking bell-shaped component of the pH– k profile for this reaction in acidic media. The data shown in Figure 1(b) are similar to those reported previously [see particularly Shipton and Brocklehurst (1978)]. In that paper the data were fitted to the equation for a bell-shaped pH– k profile with $pK_a = 3.85$ and $pK_{II} = 3.90$, and it was noted that the data in the pH range 4.5–5.5 fell substantially above the fitted line, indicating the presence of an additional kinetically influential pK_a in that pH region. The availability of SKETCHER now readily permits the additional pK_a (5.0) to be used in calculating the theoretical line (see Figure 1b). The particularly important observation, however, is that it is possible to fit the data for the reaction of 2,2'-dipyridyl disulphide in Figure 1(b) with a pK_a value (3.45) essentially the same as that (3.4) deduced from the simpler form of pH-dependence that describes the reaction of 4,4'-dipyrimidyl disulphide (Figure 1a). With this value of the first pK_a characteristic of the enzyme molecule, the value of the second pK_a needs to be 4.0. The potentiometric titration data of Lewis et al. (1976) and the kinetic data for the reactions of papain with 4,4'-dipyrimidyl disulphide and 2,2'-dipyridyl disulphide here reported combine to provide the value for the macroscopic pK_a characteristic of the formation of the ion-pair state of papain as 3.4 at 25 °C and 1.0 M. The reaction of the ion-pair of papain with 2-Py-S-S-2-Py $^+$ H (V) is modulated by two additional ionizations of pK_a 4.0 and 5.0 respectively. The kinetic study of the reactions of papain with 4,4'-dipyrimidyl disulphide and 2,2'-dipyridyl disulphide in alkaline media (Mellor et al., 1993b) defined the value of the pK_a for dehydration of the ion-pair state of papain (to S^-/Im) as 8.3 and the value of a pK_a that modulates the reactivity of S^-/Im as 10.0. The major kinetically influential ionizations of the papain molecule are now defined therefore as 3.4, 4.0, 5.0, 8.3 and 10.0. The circumstances in which various combinations of these control the catalytic capability of papain are discussed below.

pH-dependence of k_{cat}/K_m for the papain-catalysed hydrolysis of substrates: evidence that the (Cys-25)-S $^-$ /(His-159)-Im $^+$ H ion-pair lacks substantial catalytic competence until additional dehydration of the papain molecule has occurred

Having established that the formation of the ion-pair state of papain occurs as the pH is raised across a pK_a value close to 3.4, it was useful to re-evaluate the pH-dependent kinetic data for the acylation of the papain catalytic-site thiol group by substrates during catalysis. A particularly extensive and interesting collection of data is that contained in the paper by Polgár and Halász (1978). They reported that, although the rate of alkylation of papain by the simple alkylating agents, methyl and ethyl bromoacetates, depends upon a pK_a value of 3.25 and alkylation by chloroacetate on a pK_a value of 3.6, acylation by six electrically neutral substrates depends on a pK_a value of 4.0 and acylation by three cationic substrates (with Arg at P $_1$) on a pK_a value of 4.30.

Polgár and Halász (1978) analysed each of their sets of pH-dependent kinetic data in terms of a single ionization curve and presented the data as plots of the log of the second-order rate constant (the log of k_{cat}/K_m for substrates) versus pH. They interpreted the marked variation that appears to occur in the kinetically influential pK_a value as the structure of the inhibitor or substrate is varied in terms of 'fluctuation fit'. This

Figure 1 pH-dependence of the second-order rate constant (k) for the reaction of papain with (a) 4,4'-dipyrimidyl disulphide (I) and (b) 2,2'-dipyridyl disulphide (II) at 25 °C and / 0.1 M in acidic aqueous media

The points are experimental. For (a) the continuous line is theoretical for the pH-dependent rate equation pertaining to two reactive hydronic states and a third, unreactive, state at low pH [eqn. (2)] and the following values of the characterizing parameters with values of $\tilde{k}_1$ and $\tilde{k}_2$ in $\text{M}^{-1} \cdot \text{s}^{-1}$: $\tilde{k}_1 = 1.0 \times 10^2$, $\tilde{k}_2 = 2.05 \times 10^3$, $\text{p}K_1 = 0.91$ [the $\text{p}K_a$ value of (VI), the conjugate acid of (I)] and $\text{p}K_{II} = 3.4$.

$$k = \frac{\tilde{k}_1}{1 + \frac{[\text{H}^+]}{K_1} + \frac{K_{II}}{[\text{H}^+]}} + \frac{\tilde{k}_2}{1 + \frac{[\text{H}^+]^2}{K_1 K_{II}} + \frac{[\text{H}^+]}{K_{II}}} \quad (2)$$

The broken lines (i) and (ii) correspond to contributions to k at pH values ≥ 2 of reactions in the individual hydronic states provided by the terms of eqn. (2) associated with $\tilde{k}_1$ and $\tilde{k}_2$ respectively. For (b) the continuous line is theoretical for the pH-dependent rate equation pertaining to four reactive hydronic states and a fifth, unreactive, state [eqn. (3)] and the following values of the characterizing parameters with values of $\tilde{k}_1$ – $\tilde{k}_4$ in $\text{M}^{-1} \cdot \text{s}^{-1}$: $\tilde{k}_1 = 1.0 \times 10^3$, $\tilde{k}_2 = 3.9 \times 10^4$, $\tilde{k}_3 = 5.5 \times 10^3$, $\tilde{k}_4 = 5.0 \times 10^2$, $\text{p}K_1 = 2.45$ [the $\text{p}K_a$ value of (V) the conjugate acid of (II)], $\text{p}K_{II} = 3.45$, $\text{p}K_{III} = 4.0$ and $\text{p}K_{IV} = 5.0$.

$$k = \frac{\tilde{k}_1}{1 + \frac{[\text{H}^+]}{K_1} + \frac{K_{II}}{[\text{H}^+]}} + \frac{\tilde{k}_2}{1 + \frac{[\text{H}^+]^2}{K_1 K_{II}} + \frac{[\text{H}^+]}{K_{II}}} + \frac{\tilde{k}_3}{1 + \frac{[\text{H}^+]^3}{K_1 K_{II} K_{III}} + \frac{[\text{H}^+]^2}{K_1 K_{III}} + \frac{[\text{H}^+]}{K_{III}} + \frac{K_{IV}}{[\text{H}^+]}} + \frac{\tilde{k}_4}{1 + \frac{[\text{H}^+]^4}{K_1 K_{II} K_{III} K_{IV}} + \frac{[\text{H}^+]^3}{K_1 K_{III} K_{IV}} + \frac{[\text{H}^+]^2}{K_{II} K_{IV}} + \frac{[\text{H}^+]}{K_{IV}}} \quad (3)$$

The broken lines (i)–(iv) correspond to contributions to k of reactions in the individual hydronic states provided by the terms in eqn. (3) associated with $\tilde{k}_1$ – $\tilde{k}_4$ severally.

hypothesis allows substrates or inhibitors to select different conformations of the enzyme from an equilibrium stereopopulation and thereby, supposedly, provides for different $\text{p}K_a$ values to be exhibited. This hypothesis did not survive analysis of a kinetic model of conformational selectivity, however, which demonstrated that the same $\text{p}K_a$ value will always be obtained whether or not several rapidly fluctuating conformations react and whether or not different substrates or inhibitors react selectively with different conformations of the enzyme (Brocklehurst et al., 1983). A computer simulation study (Brocklehurst et al., 1983) suggested a kinetically valid explanation of the interesting observations of Polgár and Halász (1978) in terms of ionizations with overlapping $\text{p}K_a$ values.

In the present work we have used SKETCHER to re-evaluate the data of Polgár and Halász (1978), making use of the now known $\text{p}K_a$ value (3.4) for the formation of the ion-pair state of papain. The pH–($k_{\text{cat.}}/K_m$) profiles for the various papain-catalysed hydrolyses are shown in Figure 2. The $\text{p}K_a$ values needed in addition to $\text{p}K_a$ 3.4 to fit the data and the associated pH-independent rate constants ($\tilde{k}_{\text{cat.}}/\tilde{K}_m$) are collected in Table 1.

Polgár and Halász (1978) had noticed that the nine substrates listed in Table 1 of the present paper could be classified into two groups: the electrically neutral substrates [(a)–(f) in Figure 2 and Table 1] and the cationic substrates [(g)–(i) in Figure 2 and Table 1]. In terms of their analysis, the pH-dependence of $k_{\text{cat.}}/K_m$ in acidic media was characterized by a single $\text{p}K_a$ of 4.0 for the former group of substrates and 4.3 for the latter, both values being substantially greater than the value ($\text{p}K_a$ 3.25) characteristic

of the reactions with uncharged alkylating agents, methyl- and ethyl-bromoacetate. In terms of the present analysis the rate of acylation of papain ($k_{\text{cat.}}/K_m$) by the electrically neutral substrates Figures 2a–2f increases with pH in acidic media across two $\text{p}K_a$ values, the $\text{p}K_a$ of 3.4 characteristic of the formation of the ion-pair state of papain and another $\text{p}K_a$ (4.0). For all six substrates, the value of $\tilde{k}_{\text{cat.}}/\tilde{K}_m$ when both dehydrations have occurred ($\tilde{k}_2 \equiv \tilde{k}_{\text{cat.}}/\tilde{K}_m$ of Table 1) is 6–9 times larger than the value of $\tilde{k}_{\text{cat.}}/\tilde{K}_m$ when only the dehydration ($\text{p}K_a$ 3.4) that provides the ion-pair state has occurred ($\tilde{k}_1 \equiv \tilde{k}_{\text{cat.}}/\tilde{K}_m$ of Table 1). The new analysis of these data therefore demonstrates that little catalytic capability (which relies upon the rate-determining acylation process reflected in $k_{\text{cat.}}/K_m$) develops in the papain molecule solely as a result of formation of the (Cys-25)-S[−]/(His-159)-Im⁺H state. Substantial enhancement (6–9-fold) requires a second dehydration of $\text{p}K_a$ 4.0. The apparent shift in the papain $\text{p}K_a$ reflected in the kinetics of alkylation reactions from 3.25 when methyl and ethyl bromoacetates are used to 3.6 when chloroacetate is used (Polgár and Halász, 1978) is accounted for by the kinetic influence of the ionization with $\text{p}K_a$ 4.0 in the reaction with chloroacetate.

For the papain-catalysed hydrolysis of the three cationic substrates (with Arg at P₁) the analogous enhancement of catalytic activity ($\tilde{k}_2/\tilde{k}_1$) is 12–28-fold and this activity is further enhanced by a third dehydration (of $\text{p}K_a$ 5.0) such that $\tilde{k}_2/\tilde{k}_1 = 19$ –53 (see Table 1). It may be noteworthy that a $\text{p}K_a$ of 5.0 (as well as that of 4.0) modulates the reactivity of the thiol group of Cys-25 towards the cationic probe molecule 2-Py-S-S-2-Py⁺H (V) (see Figure 1b).

Figure 2 pH-dependence of k (i.e. k_{cat}/K_m) for the hydrolysis of nine substrates catalysed by papain at 25 °C and / 0.09 M in acidic media

The points, which are experimental for (a) 4-Nph-OAc, (b) Bz-Gly-OEt, (c) Bz-L-Ala-OMe, (d) Z-Gly-OEt, (e) Bz-L-Cit-OMe, (f) Bz-Gly-OPh, (g) Bz-L-Arg-NH₂, (h) Bz-L-Arg-OEt and (i) Z-L-Arg-OMe, were taken from the plots of $\log k$ versus pH reported by Polgár and Halász (1978). For (a)–(f) the continuous lines are theoretical for the pH-dependent rate equation pertaining to two active hydronic states and a third, inactive, hydronic state at low pH [eqn. (2), see Figure 1(a) legend] and the following values of the parameters, with values of $\tilde{k}_1$ ($\tilde{k}_{\text{cat}_1}/K_m$) and $\tilde{k}_2$ ($\tilde{k}_{\text{cat}_2}/K_m$) in $\text{M}^{-1} \cdot \text{s}^{-1}$: $pK_1 = 3.4$ and $pK_{\text{III}} = 4.0$ in all cases and, for (a) $\tilde{k}_1 = 1.0$, $\tilde{k}_2 = 6.6$; for (b) $\tilde{k}_1 = 40$, $\tilde{k}_2 = 2.75 \times 10^2$; for (c) $\tilde{k}_1 = 75$, $\tilde{k}_2 = 4.3 \times 10^2$; for (d) $\tilde{k}_1 = 1.0 \times 10^2$, $\tilde{k}_2 = 8.7 \times 10^2$; for (e) $\tilde{k}_1 = 3.5 \times 10^2$, $\tilde{k}_2 = 2.29 \times 10^3$; for (f) $\tilde{k}_1 = 4.0 \times 10^3$, $\tilde{k}_2 = 2.57 \times 10^4$. With $pK_1 = 3.4$ [the value characteristic of the formation of the (Cys-25)-S[−]/(His-159)-Im⁺H ion-pair state of papain] the additional pK_2 ($pK_{\text{II}} = 4.0$) is necessary to obtain a good fit to the data. For (g)–(i) the continuous lines are theoretical for the pH-dependent rate equation pertaining to three active hydronic states and a fourth, inactive, hydronic state at low pH, eqn. (4) and the following values of the parameters with values of $\tilde{k}_1$ – $\tilde{k}_3$ in $\text{M}^{-1} \cdot \text{s}^{-1}$: $pK_1 = 3.4$, $pK_{\text{II}} = 4.0$ and $pK_{\text{III}} = 5.0$ in all cases and, for (g) $\tilde{k}_1 = 15$, $\tilde{k}_2 = 2.0 \times 10^2$, $\tilde{k}_3 = 3.7 \times 10^2$; for (h) $\tilde{k}_1 = 1.0 \times 10^2$, $\tilde{k}_2 = 1.2 \times 10^3$, $\tilde{k}_3 = 1.9 \times 10^3$; for (i) $\tilde{k}_1 = 5.0 \times 10^2$, $\tilde{k}_2 = 1.4 \times 10^4$, $\tilde{k}_3 = 2.65 \times 10^4$. With $pK_1 = 3.4$ (see above) two additional pK_2 values [i.e. $pK_{\text{II}} = 5.0$ in addition to the $pK_{\text{II}} = 4.0$ needed to fit the data for (a)–(f)] are required to obtain a good fit to the data for (g)–(i).

$$k = \frac{\tilde{k}_1}{1 + \frac{[\text{H}^+]}{K_1} + \frac{K_{\text{II}}}{[\text{H}^+]} + \frac{K_{\text{II}}K_{\text{III}}}{[\text{H}^+]^2}} + \frac{\tilde{k}_2}{1 + \frac{[\text{H}^+]^2}{K_1K_{\text{II}}} + \frac{[\text{H}^+]}{K_{\text{II}}} + \frac{K_{\text{III}}}{[\text{H}^+]}} + \frac{\tilde{k}_3}{1 + \frac{[\text{H}^+]^3}{K_1K_{\text{II}}K_{\text{III}}} + \frac{[\text{H}^+]^2}{K_{\text{II}}K_{\text{III}}} + \frac{[\text{H}^+]}{K_{\text{III}}}} \quad (4)$$

Consequences of a pK_a value close to 3.4 characteristic of the formation of the ion-pair state of papain and multiple overlapping ionizations for detailed studies on electrostatic perturbations within the native enzyme and mutants produced by site-directed mutagenesis

In recent years attempts have been made to provide detailed interpretation of electrostatic influences that affect the papain catalytic site by pH-dependent kinetic studies on various papain mutants and calculations involving apparent shifts in pK_a values derived from $\text{pH}-k_{\text{cat}}/K_m$ profiles (e.g. Ménard et al., 1990, 1991). All of these studies have assigned one of the two pK_a

values close to 4 to the formation of the ion-pair state and with two pK_a values assumed close to 4, there appears to be no need to consider a pK_a value close to 5, even when using a cationic substrate such as benzyloxycarbonyl (Z)-L-Phe-L-Arg methylcoumarylamide (MEC). The evidence discussed above, however, compels the view that the formation of the ion-pair state is close to 3.4 rather than 4 and, for Z-L-Phe-L-Arg-MCA, the substrate used by Ménard et al. (e.g. Ménard et al., 1990), this pK_a is essentially outside the pH range in which catalytic activity is substantial (see Figure 3). There are two consequences of this. One is that electrostatics calculations based on a pK_a value close to 4 for ion-pair formation in native papain need to be re-

Table 1 Variation in the effect of ionizations additional to the production of the (Cys-25)-S⁻/(His-159)-Im⁺H ion-pair state of papain ($pK_i = 3.4$) on the effectiveness of the acylation process of the catalytic act (k_{cat}/K_m) with change in substrate structure

The parameters pK_{II} , pK_{III} , $\tilde{k}_1 (= \tilde{k}_{cat}/\tilde{K}_{m1})$, $\tilde{k}_2 (= \tilde{k}_{cat}/\tilde{K}_{m2})$, $\tilde{k}_3 (= \tilde{k}_{cat}/\tilde{K}_{m3})$ are those characteristic of the theoretical lines shown in Figure 2; the data are those of Polgár and Halász (1978) collected at 25 °C and / 0.09 M; $\tilde{k}_1$ represents the value of $\tilde{k}_{cat}/\tilde{K}_{m1}$ for the ion-pair state of papain without additional dehydration; $\tilde{k}_2$ represents the value after dehydration with $pK_a = 4.0$ and $\tilde{k}_3$ the value after dehydration with pK_a 5.0; NA (not applicable) indicates that no further increase in k_{cat}/K_m occurs as pH is increased above $pK_a = 5.0$.

Section of Figure 2 showing the pH- k_{cat}/K_m profile	Substrate	pK_{II}	pK_{III}	$\tilde{k}_1$ ($M^{-1} \cdot s^{-1}$)	$\tilde{k}_2$ ($M^{-1} \cdot s^{-1}$)	$\tilde{k}_3$ ($M^{-1} \cdot s^{-1}$)	$\tilde{k}_2/\tilde{k}_1$	$\tilde{k}_3/\tilde{k}_1$
(a)	4-Nph-OAc	4.0	NA	1.0	6.6	NA	6.6	NA
(b)	Bz-Gly-OEt	4.0	NA	40	275	NA	6.9	NA
(c)	Bz-L-Ala-OMe	4.0	NA	75	430	NA	5.7	NA
(d)	Z-Gly-OEt	4.0	NA	100	870	NA	8.7	NA
(e)	Bz-L-Cit-OMe	4.0	NA	350	2290	NA	6.5	NA
(f)	Bz-Gly-OPh	4.0	NA	4000	25700	NA	6.4	NA
(g)	Bz-L-Arg-NH ₂	4.0	5.0	15	200	370	13.3	24.7
(h)	Bz-L-Arg-OEt	4.0	5.0	100	1200	1900	12	19
(i)	Z-L-Arg-OMe	4.0	5.0	500	14000	26500	28	53

Figure 3 Demonstration that the Asp-158-Asn mutation in papain may not result in perturbation of the kinetically influential pK_a values

The pH- k_{cat}/K_m data for the hydrolysis of Z-L-Phe-L-Arg-MCA catalysed by (a) native papain and (b) the Asp-158-Asn mutant are taken from Ménard et al. (1990). The two continuous lines and the two broken lines are all theoretical for the pH-dependent rate equation pertaining to three active and one inactive hydronic states [eqn. (4), Figure 2 legend] and the following values of the parameters with values of $\tilde{k}_1-\tilde{k}_3$ (i.e. $\tilde{k}_{cat}/\tilde{K}_{m1}-\tilde{k}_{cat}/\tilde{K}_{m3}$) in $M^{-1} \cdot s^{-1}$: for all four lines $pK_{II} = 4.2$ and $pK_{III} = 4.8$; for both broken lines, $pK_i = 3.4$; for both continuous lines, $pK_a = 3.6$; for (a) $\tilde{k}_1 = 1.0 \times 10^2$, $\tilde{k}_2 = 4.5 \times 10^4$, $\tilde{k}_3 = 1.15 \times 10^5$; for (b) $\tilde{k}_1 = 1.0 \times 10^2$, $\tilde{k}_2 = 1.5 \times 10^4$, $\tilde{k}_3 = 1.93 \times 10^4$.

considered making use of a pK_a value close to 3.4. The other is that because the fit of a theoretical line to the data around pH 3.4 will be exceedingly insensitive to the actual value ascribed to the pK_a around 3.4, perturbation in mutant papains will be difficult to quantify with confidence, at least when using a cationic substrate such as Z-L-Phe-L-Arg-MCA. Thus the data in acidic media reported and discussed by Ménard et al. for papain mutants such as the Asp-158-Asn mutant (Ménard et al., 1990) relate to possible perturbation of the two as yet unassigned ionizations with pK_a values close to 4 and 5 and provide no evidence for or against electrostatic perturbation of the (Cys-25)-S⁻/(His-159)-Im⁺H ion-pair.

In addition to the need to re-evaluate the pH-dependent kinetic data for papain and its mutants in terms of the pK_a of 3.4 discussed above, there is another problem. This relates to the ambiguity involved in fitting the data to appropriate pH-

dependent rate equations and in consequent interpretation of the parameters in situations where two or more overlapping kinetically influential ionizations occur. Thus for sets of curves of pH-dependence of kinetic parameters that are displaced along the pH axis and appear to involve even large pK_a shifts, displacement can result instead from variation in the associated pH-independent rate constants, as was demonstrated by the simulation study reported by Brocklehurst et al. (1983) (see also Bashford and Karplus, 1991). Figure 3 provides an illustration of the consequences of the assigned value of the pK_a for ion-pair formation as 3.4 for the fit to the data of Ménard et al. (1990) for the hydrolysis of Z-L-Phe-L-Arg-MCA catalysed by native papain and of the uncertainty of fit with overlapping ionizations in the absence of independent evidence that permits the value of at least one of the characterizing parameters to be known. Thus, although the experimental data shown in Figure 3 have been reported to demonstrate pK_a values of the Asp-158-Asn mutant [$pK_I = 3.6$, $pK_{II} = 4.22$ (Ménard et al., 1990); $pK_I = 3.76$, $pK_{II} = 4.04$ (Ménard et al., 1991)] different from those of native papain (reported to be $pK_I = 3.9$, $pK_{II} = 4.44$), Figure 3 shows that it is possible to provide a good fit to the data with no change in the pK_a values (3.4–3.6, 4.2 and 4.8 for both native papain and the Asp-158-Asn mutant). The lack of change in the pK_a values arises when a pK_a value of 3.4–3.6 for ion-pair formation is used and a pK_a value close to 5 (4.8 in the case of the data in Figure 3) predicted for a cationic substrate and the data of Polgár and Halász (1978) (see Figure 2) is acknowledged together with a permitted change in the ratio of the pH-independent rate constants ($\tilde{k}_2/\tilde{k}_1$) consequent upon the Asp-158-Asn mutation.

The uncertainty of assigning changes in pK_a values postulated as a result of site-specific mutations is illustrated more markedly in Figure 4 where a range of good fits to the data for the Asp-158-Glu mutant of papain is shown. The theoretical lines shown in Figure 4(a)–4(d) are essentially identical with each other even though pK_i varies from 4.0 to 5.5. This uncertainty in the value of pK_i arises from uncertainty also in the value of $\tilde{k}_{cat,1}/\tilde{K}_{m1}$, which is allowed to vary from $10 M^{-1} \cdot s^{-1}$ to $8.0 \times 10^3 M^{-1} \cdot s^{-1}$. The variation in the parameters that characterize the good fits to the data in Figure 4(a)–4(d) is shown in Table 2. Strictly it is not known whether one of the kinetically influential pK_a values of Figure 4(a)–4(d) is characteristic of the formation of the ion-pair state of Asp-158-Glu papain. It seems at least possible, however,

Figure 4 Illustration of permitted variation of the overlapping kinetically influential ionizations of the Asp-158-Glu mutant of papain deduced from the pH-dependence of k_{cat}/K_m for the catalysed hydrolysis of Z-L-Phe-L-Arg-MCA

The data, which are taken from Ménard et al. (1991), are identical in (a)–(d). The continuous lines are theoretical for the pH-dependent rate equation pertaining to two active hydronic states bounded by two inactive states [eqn. (4) of Figure 2 legend with $k_3 = 0$, i.e. the first two terms of eqn. (4)] and the following values of the parameters with values of $\tilde{k}_1$ – $\tilde{k}_3$ (i.e. $\tilde{k}_{\text{cat}}/\tilde{K}_m$ – $\tilde{k}_{\text{cat}_2}/\tilde{K}_m$) in $\text{M}^{-1} \cdot \text{s}^{-1}$: in all cases $\text{p}K_{\text{III}} = 8.25$ [a value closely similar to that, 8.3, characteristic of the dehydronation of the catalytically competent state of papain to provide S^-/Im (Mellor et al. 1993b)] and for (a) $\text{p}K_I = 4.0$, $\text{p}K_{\text{II}} = 5.45$, $\tilde{k}_1 = 10$, $\tilde{k}_2 = 9.2 \times 10^3$; for (b) $\text{p}K_I = 4.5$, $\text{p}K_{\text{II}} = 5.45$, $\tilde{k}_1 = 5.0 \times 10^2$, $\tilde{k}_2 = 9.2 \times 10^3$; for (c) $\text{p}K_I = 5.0$, $\text{p}K_{\text{II}} = 5.45$, $\tilde{k}_1 = 2.0 \times 10^3$, $\tilde{k}_2 = 9.2 \times 10^3$; for (d) $\text{p}K_I = 5.5$, $\text{p}K_{\text{II}} = 5.75$, $\tilde{k}_1 = 8.0 \times 10^3$, $\tilde{k}_2 = 9.0 \times 10^3$. The data do not extend to pH values below approx. 4.7, i.e. to the pH region in which $\text{S}^-/\text{Im}^+\text{H}$ ion-pair formation is occurring across $\text{p}K_a$ 3.4 in native papain. Although the assignment of $\text{p}K_I$ or $\text{p}K_{\text{II}}$ (whatever their actual values) to ion-pair formation in the Asp-158-Glu mutant strictly cannot be ruled out, it seems more likely that $\text{p}K_I$ and $\text{p}K_{\text{II}}$ characterize the kinetically influential ionizations with $\text{p}K_a$ values close to 4 and 5 in native papain, the structural assignments of which are as yet unknown.

Table 2 Exemplification of permitted variation in the parameters that provides indistinguishable fits to the data for the pH-dependence of k_{cat}/K_m for the hydrolysis of Z-L-Phe-L-Arg-MCA by the Asp-158-Glu mutant of papain

The sets of values of the characterizing parameters $\text{p}K_I$, $\text{p}K_{\text{II}}$, $\text{p}K_{\text{III}}$, $\tilde{k}_1$ ($= \tilde{k}_{\text{cat}}/\tilde{K}_m$) and $\tilde{k}_2$ ($= \tilde{k}_{\text{cat}_2}/\tilde{K}_m$) [(a)–(d)] are those corresponding to the equivalent fits to the data shown in Figure 4(a)–(d). Considerable variation in both $\text{p}K_I$ and $\text{p}K_{\text{II}}$ is permitted by compensating variation in $\tilde{k}_1$.

	$\text{p}K_I$	$\text{p}K_{\text{II}}$	$\text{p}K_{\text{III}}$	$10^{-3} \times \tilde{k}_1$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$10^{-3} \times \tilde{k}_2$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
(a)	4.0	5.45	8.25	0.01	9.2
(b)	4.5	5.45	8.25	0.5	9.2
(c)	5.0	5.45	8.25	2.0	9.2
(d)	5.5	5.75	8.25	8.0	9.0

that $\text{p}K_I$ (4.0–5.5) and $\text{p}K_{\text{II}}$ (5.45–5.75) in the Asp-158-Glu mutant correspond to the same two as yet structurally unidentified ionizations that are characterized by $\text{p}K_a$ values close to 4.0 and 5.0 in the native papain molecule. It would appear that the dehydronation of (Cys-25)- $\text{S}^-/(\text{His-159})\text{Im}^+\text{H}$ to produce catalytically inactive (Cys-25)- $\text{S}^-/(\text{His-159})\text{Im}$ may not be perturbed in the Asp-158-Glu mutant in view of the fact that the value of $\text{p}K_{\text{III}}$ (8.25) is essentially the same as the value for native papain (8.3; see Mellor et al., 1993b).

It is important to emphasize the substantial uncertainty in assigning $\text{p}K_a$ values in situations like those illustrated in Figure 4 which arise because it is possible to obtain quite marked shifts in the position of pH–rate curves along the pH axis by changes in the ratios of the pH-independent rate constants as well as or instead of changes in $\text{p}K_a$ values (Brocklehurst et al., 1983). Valid interpretations of such data require the value of at least one of the relevant parameters to be known from an independent experiment in which the other overlapping ionization has negligible effect on the kinetics. This type of experiment, described above for native papain, has not yet been reported for papain mutants. For the mutants therefore the putative $\text{p}K_a$ shifts and the consequent evaluation of electrostatic effects must be regarded as uncertain.

The pH-dependent kinetic behaviour of some other cysteine proteinases

The kinetic behaviour of actinidin (EC 3.4.22.14), papaya proteinase Ω (PP Ω) and the 80 kDa/18 kDa heterodimer of porcine calpain II (EC 3.4.22.17) also is known to depend upon more ionizations than the two required to account for the pH-dependent formation and loss by further dehydronation of the (Cys)- $\text{S}^-/(\text{His})\text{Im}^+\text{H}$ ion-pair state. Both catalytic competence and thiol reactivity develop in actinidin (Salih et al., 1987) and in PP Ω (Topham et al., 1991) as the pH is increased between 3 and 4 to a much greater extent, however, than is the case for papain. Reactivity-probe data from reactions of these two enzymes

with both 4,4'-dipyrimidyl disulphide (I) (G. W. Mellor and K. Brocklehurst, unpublished work) and 2-pyridyl disulphides provide the pK_a values characteristic of the formation of the ion-pair states as $pK_a = 3.1$ – 3.2 for actinidin (Salih et al., 1987; Topham et al., 1991) and $pK_a = 3.4$ for PPQ. Catalytic activity (k_{cat}/K_m) towards L-Bz-Arg nitroanilide is further enhanced substantially as the pH is increased across pK_a 5.5 in the case of actinidin (Salih et al., 1987) and pK_a 5.8 in the case of PPQ (Topham et al., 1991). The enhancements are similar to those deduced above for the papain-catalysed hydrolysis of cationic substrates.

The pH- k profile in acidic media for the reaction of 2,2'-dipyridyl disulphide (II) with the catalytic-site thiol group of the calpain II 80 kDa/18 kDa heterodimer is strikingly different from those of the corresponding reactions with papain, PPQ and ficin (Mellor et al., 1993a). The bell-shaped component of the profile is shifted markedly to higher pH values and the data were fitted with pK_a values of 4.4 and 5.6. Although one of these pK_a values could be characteristic of the formation of the (Cys)-S⁻/(His)-Im⁺H ion-pair state of calpain II, the work on papain and its mutants discussed above suggests that they might both be characteristic instead of other ionizations that modulate the reactivity of the thiolate anion component of the ion-pair and that the pK_a value characteristic of the ion-pair state might be ≤ 3.5 . Indeed the fit of the pH- k data reported by Mellor et al. (1993a) is improved by the inclusion of an additional ionization with pK_a 3.4 (S. K. Sreedharan and K. Brocklehurst, unpublished work). It seems possible therefore that the pK_a value characteristic of ion-pair formation in calpain II might be less than 3.5 as are the analogous values for papain, actinidin and PPQ. In terms of this new fit, reactivity of calpain II towards 2,2'-dipyridyl disulphide develops across pK_a 3.4 ($k = 1.0 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) and is then further enhanced to $k = 4.0 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ as the pH is increased across pK_a 4.4 before decreasing again across pK_a 5.6 as reported by Mellor et al. (1993a).

CONCLUDING COMMENTS

Results from reactivity-probe kinetics using 4,4'-dipyrimidyl disulphide and 2,2'-dipyridyl disulphide compel the view that the formation of the (Cys-25)-S⁻/(His-159)-Im⁺H ion-pair state of papain is characterized by a pK_a value close to 3.4 in accord with the value (3.3) deduced from the potentiometric titration method of Lewis et al. (1976). Formation of the ion-pair state of papain (pK_a approx. 3.4) appears to be sufficient for the full development of nucleophilic reactivity of the thiolate anion component of the ion-pair in reactions with simple alkylating agents (Polgár and Halász, 1978) and with 4,4'-dipyrimidyl disulphide. In other reactions, including reaction with 2,2'-dipyridyl disulphide and in the acylation process of the catalytic act, at least one other ionization (pK_a approx. 4) and in some cases two (pK_a 4 and 5) are necessary to provide full expression of nucleophilic capability or catalytic competence. These additional requirements may be connected with the need for particular dispositions of -S⁻ and -Im⁺H ion-pair components for particular reactions [see Brocklehurst et al. (1988b) and Kowlessur et al. (1989) for discussion of changes in transition-state geometry in reactions of papain]. Perturbation of ion-pair reactivity by other ionizations occurs also in at least some other cysteine proteinases and may be particularly marked in reactions of the catalytic-site thiol group of the calpain II 80 kDa/18 kDa heterodimer.

Knowledge of the pK_a value for ion-pair formation permits the complex pH-dependence of k_{cat}/K_m for the hydrolysis of substrates by native papain and to some extent by its site-specific

mutants to be clarified. Ion-pair formation alone does not produce substantial catalytic capability in papain. For electrically neutral substrates, dehydration of another group associated with a molecular pK_a value close to 4 is required for development of high catalytic activity. For cationic substrates, catalytic activity is further enhanced by another hydronic dissociation associated with a pK_a value close to 5. The treatment of the data on the pH-dependence of k_{cat}/K_m for the hydrolysis of the cationic substrate Z-L-Phe-L-Arg-MCA by papain mutants [e.g. the Asp-158-Asn, Asp-158-Gly and Asp-158-Glu mutants (Ménard et al., 1991)] needs to be reconsidered. Their extensive calculations and interpretation of electrostatic effects in the papain active centre need to take into account that (a) the pK_a of ion-pair formation is approx. 3.4 rather than approx. 4, (b) the effects of the various mutations that they analyse and interpret probably relate to ionizations of two unknown groups in native papain, one with pK_a approx. 4 and the other with pK_a approx. 5 rather than to the formation of the (Cys-25)-S⁻/(His-159)-Im⁺H ion-pair and the unknown group of pK_a 4, (c) the magnitude of the apparent shifts in pK_a values produced by the site-specific mutations on which the electrostatic calculations depend are uncertain (and may in some cases be non-existent) because the effects of the mutations on the pH-independent values of k_{cat}/K_m for the different reactive ionization states are unknown. The ambiguities inherent in the analysis of pH-dependent kinetic data relating to multiple overlapping ionizations present considerable difficulties to interpretation (Brocklehurst et al., 1983; Bashford and Karplus, 1991) in the absence of additional information that permits assignments of values to particular parameters. The present work suggests that kinetic evaluation of papain mutants should be somewhat facilitated by using electrically neutral rather than cationic substrates. Such experiments may at least permit effects of particular mutations on ion-pair formation to be observed experimentally because shifts of the pH- k_{cat}/K_m curves along the pH axis may not be as large, although quantification may still present substantial difficulties with potential shifts in two pK_a values and two pH-independent kinetic parameters to be considered. Some of the ambiguities would be removed by additional kinetic studies using suitably designed time-dependent inhibitors as reactivity probes, as has been demonstrated in the present work. This serves to emphasize the need to carry out not only structural studies on mutants but also structure-related kinetic studies in addition to steady-state kinetic studies if soundly based mechanistic conclusions are to be drawn.

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REFERENCES

- Baines, B. S. and Brocklehurst, K. (1978) *Biochem. J.* **173**, 345–347
- Baker, E. N. and Drenth, J. (1987) in *Biological Macromolecules and Assemblies* (Jurnak, F. and McPherson, A., eds.), vol. 3, pp. 313–368, John Wiley and Sons, New York
- Bashford, D. and Karplus, M. (1991) *J. Phys. Chem.* **95**, 9556–9561
- Brocklehurst, K. (1987) in *Enzyme Mechanisms* (Page, M. I. and Williams, A., eds.), pp. 140–158, Royal Society of Chemistry, London
- Brocklehurst, S. M. and Brocklehurst, K. (1988) *Biochem. J.* **256**, 556–558
- Brocklehurst, K. and Little, G. (1972) *Biochem. J.* **128**, 471–474
- Brocklehurst, K., Willenbrock, S. J. F. and Salih, E. (1983) *Biochem. J.* **211**, 701–708
- Brocklehurst, K., Carlsson, J. and Kierstan, M. P. J. (1985) *Top. Enzyme Ferment. Biotechnol.* **10**, 146–188
- Brocklehurst, K., Kowlessur, D., O'Driscoll, M., Patel, G., Quenby, S., Salih, E., Templeton, W., Thomas, E. W. and Willenbrock, F. (1987a) *Biochem. J.* **244**, 173–181
- Brocklehurst, K., Willenbrock, F. and Salih, E. (1987b) *New Compr. Biochem.* **16**, 39–158

- Brocklehurst, K., Kowlessur, D., Patel, G., Templeton, W., Quigley, K., Thomas, E. W., Wharton, C. W., Willenbrock, F. and Szawelski, R. J. (1988a) *Biochem. J.* **250**, 761–772
- Brocklehurst, K., Brocklehurst, S. M., Kowlessur, D., O'Driscoll, D., Patel, G., Salih, E., Templeton, W., Thomas, E., Topham, C. M., and Willenbrock, F. (1988b) *Biochem. J.* **256**, 543–555
- Brocklehurst, S. M., Topham, C. M. and Brocklehurst, K. (1990) *Biochem. Soc. Trans.* **18**, 598–599
- Brubaker, L. J. and Bender, M. L. (1966) *J. Am. Chem. Soc.* **88**, 5871–5880
- Dixon, W. J. (Chief ed.), Brown, M. B., Engelman, L., Hill, M. A. and Jennrich, R. I. (1988) *BMDP Statistical Software*, pp. 389–417, University of California Press, Guildford, U.K.
- Dubois, T., Jacquet, A., Schnek, A. G. and Looze, Y. (1988) *Biol. Chem. Hoppe-Seyler* **369**, 733–740
- Hinkle, P. M. and Kirsch, J. F. (1970) *Biochemistry* **9**, 4633–4644
- Hinkle, P. M. and Kirsch, J. F. (1971) *Biochemistry* **10**, 3700–3707
- Kowlessur, D., Topham, C. M., Thomas, E. W., O'Driscoll, M., Templeton, W. and Brocklehurst, K. (1989) *Biochem. J.* **258**, 755–764
- Lewis, S. D. and Shafer, J. A. (1974) *Biochemistry* **13**, 690–698
- Lewis, S. D., Johnson, F. A. and Shafer, J. A. (1976) *Biochemistry* **15**, 5009–5017
- Lewis, S. D., Johnson, F. A., Ohno, A. K. and Shafer, J. A. (1978) *J. Biol. Chem.* **253**, 5080–5086
- Mellor, G. W., Sreedharan, S. K., Kowlessur, D., Thomas, E. W. and Brocklehurst, K. (1993a) *Biochem. J.* **290**, 75–83
- Mellor, G. W., Thomas, E. W., Topham, C. M. and Brocklehurst, K. (1993b) *Biochem. J.* **290**, 289–296
- Ménard, R., Khouri, H. E., Plouffe, C., Dupras, R., Ripoll, D., Vernet, T., Tessier, D. C., Laliberté, F., Thomas, D. Y. and Storer, A. C. (1990) *Biochemistry* **29**, 6706–6713
- Ménard, R., Khouri, H. E., Plouffe, C., Laflamme, P., Dupras, R., Vernet, T., Tessier, D. C., Thomas, D. Y. and Storer, A. C. (1991) *Biochemistry* **30**, 5531–5538
- Patel, M., Kayani, S., Templeton, W., Mellor, G. W., Thomas, E. W. and Brocklehurst, K. (1992) *Biochem. J.* **287**, 881–889
- Polgár, L. (1990) *Mechanisms of Protease Action*, pp. 123–155, CRC Press, Boca Raton, FL
- Polgár, L. and Halász, P. (1978) *Eur. J. Biochem.* **88**, 513–521
- Salih, E., Malthouse, J. P. G., Kowlessur, D., Jarvis, M., O'Driscoll, M. and Brocklehurst, K. (1987) *Biochem. J.* **247**, 181–193
- Shipton, M. and Brocklehurst, K. (1978) *Biochem. J.* **171**, 385–401
- Shipton, M., Kierstan, M. P. J., Malthouse, J. P. G., Stuchbury, T. and Brocklehurst, K. (1975) *FEBS Lett.* **50**, 365–368
- Stuchbury, T., Shipton, M., Norris, R., Malthouse, J. P. G., Brocklehurst, K., Herbert, J. A. L. and Suschitzky, H. (1975) *Biochem. J.* **151**, 417–432
- Topham, C. M., Salih, E., Frazao, C., Kowlessur, D., Overington, J. P., Thomas, M., Brocklehurst, S. M., Patel, M., Thomas, E. W. and Brocklehurst, K. (1991) *Biochem. J.* **280**, 79–92