Ionization characteristics of the Cys-25/His-159 interactive system and of the modulatory group of papain: resolution of ambiguity by electronic perturbation of the quasi-2-mercaptopyridine leaving group in a new pyrimidyl disulphide reactivity probe

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1. A new thiol-specific reactivity probe 4,4′-dipyrimidyl disulphide [compound (VII), m.p. 110 °C, pK_a of its monohydronated form 0.91] was synthesized and used to resolve the ambiguity of interpretation of the behaviour of papain (EC 3.4.22.2) in alkaline media known to depend to varying extents on two ionizations with pK_a values approx. 8.0–8.5 and $\geqslant$ 9.5 respectively. 2. A new extensive pH–second-order rate constant (k) data set for the reaction of papain with 2-(acetamido)-ethyl 2′-pyridyl disulphide (IV) demonstrated the existence of a striking rate maximum at pH approx. 4, the independence of k around pH 8 and the increase in k with increase in pH across a pK_a value of 10.0, behaviour similar to that of other 2-pyridyl disulphides (R-S-S-2-Py) that lack key substrate-like binding sites in R. 3. Although the simplest interpretation of the pK_a value of 10.0 assigns it to the formation of (Cys-25)-S^-/(His-159)-Im from the ion-pair state of the papain catalytic site, another interpretation may be conceived in which this pK_a value is assigned to another group remote from the catalytic site, the state of ionization of which modulates catalytic-site behaviour. This alternative assignment is shown to require compensating effects in the pH region around 8 such that the formation of (Cys-25)-S^-/(His-159)-Im across pK_a 8.0–8.5 is without net kinetic effect in the reactions of simple 2-pyridyl disulphides such as compound (IV) and 2,2′-dipyridyl disulphide (II). 4. The lower basicity of compound (VII) relative to that of compound (II) (pK_a 2.45) was predicted to diminish or abolish the compensation postulated as a possibility in reactions of 2-pyridyl disulphides because of the decreased effectiveness of reaction via a (His-159)-Im^+H-assisted transition state. The characteristics of the pH-dependence of the reaction of papain with compound (VII) which are quite different from those for its reaction with compound (II) support both this prediction and the alternative assignment with a value of 8.3 for the pK_a of the formation of (Cys-25)-S^-/(His-159)-Im. 5. Evidence that the behaviour of papain towards both substrates and some substrate-derived time-dependent inhibitors is determined not only by the loss of the (Cys-25)-S^-/(His-159)-Im^+H ion-pair state by dehydronation with pK_a 8.3 but also by another ionization of pK_a approx. 10.0 is 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 and Brocklehurst (1987) and Polgar (1990) for shorter reviews, and Varughese et al. (1989), Bjork and Ylinenjarvi (1990), Stubbs et al. (1990), Menard et al. (1990, 1991a,b,c), Khouri et al. (1991), Vernet et al. (1990), Harris et al. (1992) and Lindahl et al. (1992) for recent crystallographic, spectroscopic and molecular biological studies that further illuminate structure, ligand binding and catalytic mechanism]. A major feature of the catalytic site of papain is the interactive system comprising the side chains of Cys-25 and His-159 of which a thiolate/imidazolium ion pair is a plausible component. The S atom of Cys-25 becomes transiently acylated during catalysis and the side chain of His-159 is considered to play a number of roles involving maintenance of the thiolate anion in neutral and weakly acidic media, general acid catalysis of leaving-group departure from an anionic tetrahedral intermediate during acylation and general base catalysis of the hydrolysis of the acylenzyme intermediate. Recent calculations by Arad et al. (1990) suggest that the nucleophilic sulphur atom of papain can approach the carbonyl carbon atom of a substrate but the potential is repulsive until accompanied by (His-159)-Im^+H...amide N hydron transfer. The formation of the hydronic state that could contain the -S^-/Im^+H ion pair from that containing -SH/Im^+H is characterized by a macroscopic pK_a value of approx. 4, and this dehydronation is co-operatively linked to another, also with macroscopic pK_a approx. 4 (Brocklehurst and Little, 1972; Shipton et al, 1975; Shipton and Brocklehurst, 1978; Lewis et al., 1978; Salih et al., 1987; Brocklehurst et al., 1988). The identity of the group associated with the additional macroscopic pK_a approx. 4 remains to be discovered. A long-favoured hypothesis that it might be the carboxy group of Asp-158 is now discounted because two pK_a values each of approx. 4 are still observed in the pH–$k_{cat.}/K_m$ profile for the hydrolysis of Cbz-Phe-Arg-4-methylcoumaryl-7-amide catalysed by the Asp-158→Asn mutant as well as in that for the same hydrolysis catalysed by native papain (Menard et al., 1990).

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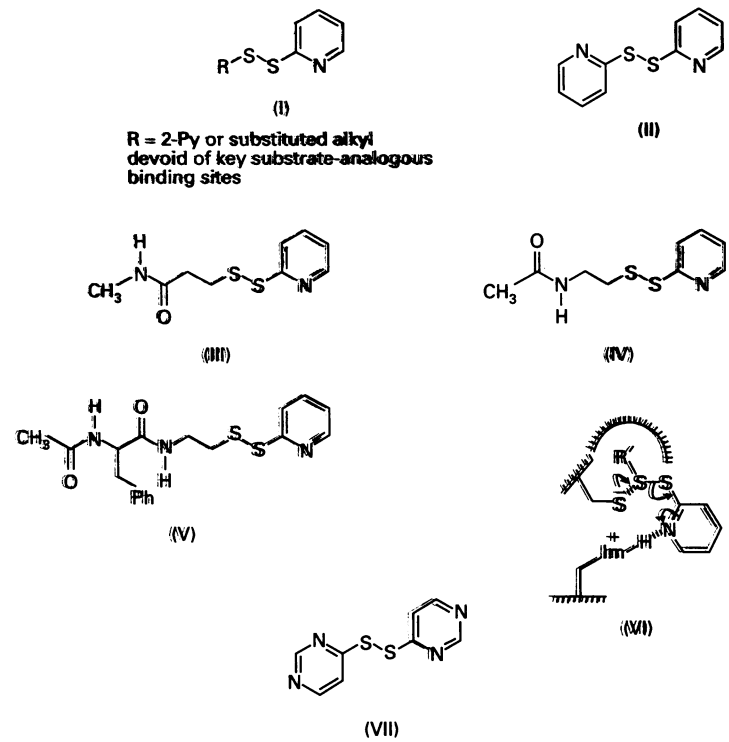


More uncertainty surrounds the pK_a value characteristic of the further hydronic dissociation of the catalytic site to produce $-S^-/-Im$. Inspection of 33 papers on papain from other laboratories spanning approx. 30 years from the mid 1960s onwards reveals that its behaviour in alkaline media has been reported to depend on a range of pK_a values from 7.8 to 9.5, many of which lie in the range 8.0–8.6. These values were inferred from kinetic experiments with substrates and a variety of time-dependent inhibitors and from spectroscopic studies. Some of the uncertainty about this pK_a value may derive from the use of relatively unreactive time-dependent inhibitors and the consequent low rates of reaction coupled with the possibility of conformational instability of papain in alkaline media. Another possible origin of variation in observed free reactant state pK_a values is the existence of two or more kinetically influential ionizations with overlapping pK_a values (Brocklehurst et al., 1983).

A particularly clear way of revealing the existence and characteristics of a nucleophilic Cys/His interactive system is by kinetic analysis of its pH-dependent reactions with 2-pyridyl disulphides (I). These reagents are two-hydronic-state electrophiles (Brocklehurst, 1974, 1982) and reactions of papain with simple members of the series, e.g. 2,2′-dipyridyl disulphide [2-Py-S-S-2-Py (II) or the retroamide probe (III)] that do not contain key substrate-analogous binding sites (see later) are characterized by a striking rate maximum at pH approx. 4, a rate minimum at pH 6–7 and a plateau of higher reactivity at high pH. The high reactivity of these reagents the reactions of which (specifically with thiol groups) occur within the stopped-flow time scale permits reliable kinetic study of reactions of enzymes such as papain over a wide range of pH. The particularly high reactivity at pH values around 4 derives from the coexistence of significant concentrations of the activated (hydronated) inhibitor (2-Py-S-S-2-Py^+H) and the nucleophilic interactive Cys-25/His-159 system (hereafter designated $-S^-/-Im^+H$ ion pair). Analysis of the pH–second-order rate constant (k) profile provides pH-independent rate constants for each of the reactive ionization states and the macroscopic pK_a values characteristic of their formation and loss by further hydronic dissociation. Reaction of the (Cys-25)$-S^-$/(His-159)$-Im^+H$ ion pair of papain with non-hydronated inhibitor R-S-S-2-Py occurs as a rate minimum in the ionization state that predominates at pH values around 7, and k increases with increasing pH to approach a plateau rate at high pH where the reaction of R-S-S-2-Py with uncomplicated thiolate anion (Cys-25)S^-/(His-159)-Im occurs. In marked contrast with the shape of such pH–k profiles (with k maximal at pH 4), pH–k profiles for reactions of papain with 2-pyridyl disulphide inhibitors (R′-S-S-2-Py) containing key substrate-like binding sites in R′ (e.g. compounds IV and V) resemble profiles of pH versus $k_{cat.}/K_m$ for substrate hydrolysis with a rate maximum around pH 6–7 instead of a rate minimum (Brocklehurst et al., 1988). These rate maxima are considered to result from substantial reaction via a transition state (VI) in which reaction of (Cys-25)-S^- at electrophilic S is assisted by association of (His-159)-Im^+H with the pyridyl N atom of the leaving group.

Of particular interest in connection with the present paper is the observation that for the substrate-like reactions (via VI), the decrease in k with increase in pH from pH approx. 6 occurs mainly across a pK_a value of 8.1–8.3, whereas for the non-substrate-like reactions, the increase in k with increase in pH occurs across a pK_a value $\geqslant 9.5$ and k is essentially invariant with pH at pH values around 8 (see Salih et al., 1987; Brocklehurst et al., 1988). The simplest interpretation of this marked difference in pK_a values is that the value ($\geqslant 9.5$) relating to reaction of the non-substrate-like inhibitors might be assigned to $-S^-/-Im^+H \rightleftharpoons -S^-/-Im + H^+$ and the value (approx. 8.1–8.3) relating to reaction of the substrate-like inhibitors might be assigned to another group the state of ionization of which affects the disposition of the ion-pair components and controls the possibility for $-S^-$ and $-Im^+H$ to act in concerted fashion on electrophilic centre and leaving group respectively. Concerted attack by both ion-pair components is necessary to explain maximal reactivity at pH

approx. 6 via (VI). In terms of these assignments, the characteristics of the catalytic-site ion pairs of papain and papaya proteinase Ω and their ionization-dependent modulation would need to differ in important respects (Topham et al., 1991).

As was pointed out previously, however, the simplest interpretation is not necessarily the correct one, and it was not possible on the available evidence to rule out the possibility that the pK_a assignments might be reversed (Brocklehurst et al., 1988). With the assignments reversed, the decrease in the high reactivity towards the substrate-like molecules at pH values around 6 with increasing pH would be attributed to conversion of $-S^-/-Im^+H$ into $-S^-/-Im$. Then the residual problem is to account for the lack of increase in k with increase in pH across pK_a approx. 8 as $-S^-/-Im^+H$ becomes $-S^-/-Im$ in reactions with the disulphides that lack key specificity features (I) and for the increase instead across $pK_a \geqslant 9.5$. The present paper reports evidence in favour of the less obvious assignments with a pK_a value of 8.3 for the formation of $-S^-/-Im$ from the ion-pair state and a pK_a value of 10.0 for a group remote from the catalytic site, the ionization state of which modulates the reactivity of the thiol group of Cys-25. The ambiguity in the assignment was removed by using a new thiol-specific reactivity probe, 4,4′-dipyrimidyl disulphide (VII) of substantially lower basicity than 2-pyridyl disulphides (I). This electronic perturbation of the reactivity probe removes a compensatory reaction via a transition state of the type shown in (VI) and revealed the kinetic consequence of the formation of $-S^-/Im$ from the ion-pair state of papain as an increase in k with increase in pH across pK_a 8.3.

MATERIALS AND METHODS

Materials

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, I 0.3 mol/l and allowed to stand for 30 min at room temperature (approx. 22 °C) to convert any reversibly oxidized papain into 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 HR 5/5 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 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. A new method by which papain is readily identified and distinguished from the other cysteine proteinases of papaya latex derives from its unique reactivity characteristics towards 2-(*N*′-acetyl-L-phenylalanylamino)ethyl 2′-pyridyl disulphide, a substrate-derived time-dependent inhibitor containing a P_1–P_2 amide bond, an L-phenylalanyl side chain as an occupant for the S_2-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., 1988). For example, whereas for the reaction of this inhibitor with papain $k_{pH6}/k_{pH3.5} = 4.8$, for the analogous reaction with papaya proteinase 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 is conveniently prepared as required by covalent chromatography (Brocklehurst et al., 1985).

4,4′-Dipyrimidyl disulphide [compound (VII)]

4(3*H*)-Pyrimidone (VIII) (10 g) and phosphorus pentasulphide (10 g) in dry pyridine (50 ml) were heated under reflux for 1 h. The reaction mixture was then diluted with water (100 ml) and the solvent was removed by rotary evaporation *in vacuo* at 40 °C. The residue was washed with water, dried over P_2O_5 *in vacuo* and crystallized from ethanol to give pyrimidine-4-thione (IX) (5.9 g, 50% yield) m.p. 187 °C [Boarland and McOmie (1952) give 188 °C and Armarego (1965) gives 186–188 °C]. To a stirred suspension of the thione (IX) (0.9 g) in water (15 ml) was added H_2O_2 (2 ml of 20 vol.) dropwise at room temperature (approx. 22 °C) over 5 min, and stirring was continued for 1 h. The solid product was removed by filtration, washed with iced water, air-dried and twice crystallized from ethanol (yield 60%). It had m.p. 110 °C and was a single compound, as judged by t.l.c. (SiO_2, ethyl acetate). (Found: C, 43.61; H, 2.62; N, 25.04; S, 28.47%; $C_8H_6N_4S_2$ requires C, 43.23; H, 2.72; N, 25.20; S, 28.85%). The mass spectrum gave m/z 222 [(M^+); $C_8H_6N_4S_2$ requires M^+ 222]. A sample produced the predicted yield of pyrimidine-4-thione (IX) consequent on thiolysis with 2-mercaptoethanol at pH 4.5 deduced by spectral analysis at 327 nm (see below). The thiolysis analysis, the m.s. and the following n.m.r. spectroscopic data established the identity of the product as the disulphide (VII): n.m.r. data, δ (p.p.m.) ([^{2}H]chloroform) 9.02 (2H, d, J 1.2 Hz), 8.58 (2H, H_A of AB quartet, J 4.6 Hz), 7.57 (2H, H_B of AB quartet, J 4.6 Hz with further fine splitting, J 1.2 Hz).

2-Carboxyethyl 2′-pyridyl disulphide *N*-methylamide (III)

This compound, which is the retroamide of 2-(acetamido) ethyl 2′-pyridyl disulphide (IV), was synthesized by reaction of methylamine with 3-(2′-pyridyldithio)propanoic acid *N*-hydroxysuccinimide ester as described previously (Brocklehurst et al., 1988).

Instrumentation and software

M.s. was carried out by using a Kratos MS 50RF ultra-high-resolution m.s., n.m.r. spectroscopy by using a Bruker AM 250 spectrometer and electronic absorption spectroscopy by using a Uvikon 810 spectrophotomer. 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.

Kinetic models differing in the number and characteristics of reactive hydronic states were evaluated by using a multitasking application program (SKETCHER) written in ANSI C (Kernigham and Ritchie, 1988) running under RISCOS on an Acorn Archimedes microcomputer (Brocklehurst et al., 1990; Topham et al., 1991). Rate equations for reactions in a variety of hydronic states were written down by using the simple general expression

and two information matrices described by Brocklehurst et al. (1990) and Topham et al. (1991). pH–k and pH–ϵ data and the associated theoretical curves were displayed by using the Sigmaplot 4.1 software package (Jandel Scientific), a Tandon MCS 486/33 PC and a Hewlett–Packard Colour Pro Plotter.

Elemental analysis

This was performed by the University of London Intercollegiate Research Service at University College, London (Department of Chemistry).

Analysis of 4,4′-dipyrimidyl disulphide (VII) by thiolysis and determination of the isosbestic point and associated absorption coefficient of pyrimidone-4-thione (IX)

Pyrimidine-4-thione (nominally 66.67 μM) was generated *in situ* by thiolysis of 4,4′-dipyrimidyl disulphide (33.33 μM by weighing) by reaction with an excess of 2-mercaptoethanol at various pH values in the range 1.6–10.5, and the electronic absorption spectrum was recorded between 240 nm and 440 nm. The spectrum at pH 4.5 is characterized by $\lambda_{max.}$ = 285 nm, ϵ_{285} = 10470 $M^{-1}\cdot cm^{-1}$ (log ϵ_{285} = 4.02) and $\lambda_{max.}$ = 327 nm, ϵ_{327} = 7943 $M^{-1}\cdot cm^{-1}$ (log ϵ_{327} = 3.90). These values of the absorption coefficients are in good agreement with those reported by Boarland and McOmie (1952) for an aqueous solution of compound (IX) at pH 4.5 (log ϵ_{285} = 4.03; log ϵ_{327} = 3.91) which confirms the identity and purity of compound (VII). In the present work the set of pH-dependent spectra was shown to be characterized by an isosbestic point at 312 nm (ϵ_{312} = 7763 $M^{-1}\cdot cm^{-1}$). Boarland and McOmie (1952) report the pK_a value for hydron loss from neutral pyrimidine-4-thione as 6.7 and that for hydron loss from the corresponding cation as < 1.

Determination of the pK_a of monohydronated 4,4′-dipyrimidyl disulphide

The electronic absorption spectrum (240–440 nm) of the disulphide (VII) (80.2 μM in various buffers) was recorded at various pH values in the range 0.23–7.04. The absorption band with $\lambda_{max.}$ 287 nm observed at the low pH values was replaced by a new band with $\lambda_{max.}$ 268 nm as the pH was increased to 7.0. The decrease in A_{287} with increase in pH was used to determine the pK_a value of the hydronated monocation of compound (VII) as 0.91 and the values of ϵ_{287} as 20920 $M^{-1}\cdot cm^{-1}$ for the monohydronated form of compound (VII) and 5910 $M^{-1}\cdot cm^{-1}$ for the neutral disulphide (VII) ($\lambda_{max.}$ 268 nm, ϵ_{268} = 13890 $M^{-1}\cdot cm^{-1}$).

Stopped-flow kinetics

All reactions were carried out at 25 °C and I 0.1 mol/l in solutions containing 1 mM EDTA under pseudo-first-order conditions with [disulphide] ≫ [papain]. Reactions of 2-(acetamido)ethyl 2′-pyridyl disulphide (IV) were monitored at 343 nm ($\Delta\epsilon_{343}$ = 8080 $M^{-1}\cdot cm^{-1}$) with [papain] = 1.9 or 3.3 μM and compound (IV) = 81.5 or 126.5 μM. Reactions of 4,4′-dipyrimidyl disulphide (VII) were monitored at the isosbestic point of pyrimidine-4-thione (IX), 312 nm with [papain] = 1.1–1.3 μM and compound [VII] = 14.0–320 μM (14.0–15.0 μM for the pH-dependence study). 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 − 317 (the value of ϵ_{312} for compound VII at pH ≥ 2) = 7446 $M^{-1}\cdot cm^{-1}$. First-order rate constants ($k_{obs.}$) were obtained by fitting the absorbance (A)–t data collected by the Acorn Archimedes microcomputer of the stopped-flow machine either to an equation for a single exponential process, i.e. $A = P_1e^{-P_2t} + P_3$, where $P_1 = A_\infty - A_0$, $P_2 = k_{obs.}$ and $P_3 = A_\infty$, or to an equation for a single exponential with a zero-order component, i.e. $A = P_1e^{-P_2t} + P_3t + P_4$, where $P_1 = A_\infty - A_0$ for the single exponential component, $P_2 = k_{obs.}$, P_3 = the slope of the zero-order component in s^{-1} and P_4 = the extrapolated value of the ordinate intercept, i.e. A_∞ for the single exponential component. Reactions of the 2-pyridyl disulphide (IV) were evaluated by using the single exponential equation. Reactions of compound (VII) exhibited small but significant deviations from a single exponential process (see Figure 4) presumably due to hydrolysis, and for these reactions, particularly at pH values > 7.5, the fit was improved by incorporation of a zero-order component (see Figure 4b of the Results and discussion section for an example). Values k were calculated from $k = k_{obs.}$/[disulphide] (see Figure 5 for a demonstration of overall second-order kinetics).

RESULTS AND DISCUSSION

pH-dependence of the behaviour of the papain catalytic site in alkaline media: the uncertainty

The range (7.8–9.5) of the pK_a values characteristic of the behaviour of the papain catalytic site in alkaline media reported by other laboratories was discussed in the Introduction. Many of these values lie in the range 8.0–8.6 and values in this range include those for the reactions of the Cys-25 with relatively simple electrophilic reagents. By contrast, reactions of Cys-25 with 2-pyridyl disulphides that lack substrate-like-binding sites [R-S-S-2-Py, (I)] and for which k increases with increase in pH in alkaline media are characterized by a pK_a ≥ 9.5 (see e.g. Brocklehurst et al., 1988). The advantages of using this type of reagent for the characterization of cysteine proteinases (see Brocklehurst et al., 1987a) include the fact that the reactions occur on a stopped-flow time scale and thus can be studied over a wide range of pH without the need to expose the enzyme to potentially denaturating media for more than a short time. The fact that k for the reactions of papain with such reagents is essentially constant at pH values around 8 is confirmed by the data shown in Figure 1. This new extensive data set for the reaction of papain with the retroamide (III) (a 2-pyridyl disulphide the reaction of which with papain provides a relatively large increase in k with increase in pH in alkaline media) was collected by using improved stopped-flow technology and demonstrates that the behaviour of the enzyme in alkaline media depends on a pK_a value of 10.0 and not on a pK_a value around 8.

The ambiguity

By contrast with the reactions of papain with non-substrate-like 2-pyridyl disulphides [R-S-S-2-Py (I)], reactions with substrate-derived 2-pyridyl disulphides [R′-S-S-2-Py, such as compounds (IV) and (V)] do exhibit a pK_a value of 8.1–8.3 in their pH–k profiles. Unlike the profile in Figure 1, these profiles are characterized by a rate maximum around pH 6–7, and the pK_a of 8.1–8.3 characterizes the decrease in k from this maximum with increase in pH (see Brocklehurst et al., 1988). It is important to emphasize that all of these reactions obey overall second-order kinetics and thus the pK_a values determined from these studies are free reactant-state values (free enzyme pK_a values in these

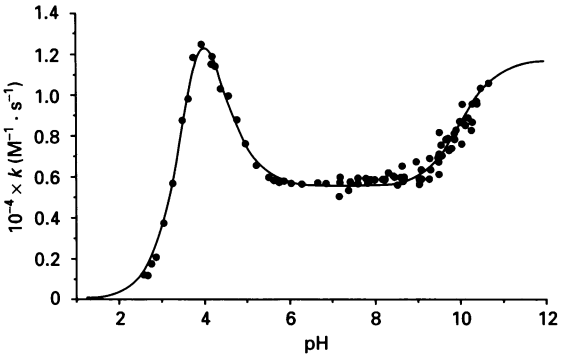


Figure 1 pH-dependence of k for the reaction of papain with 2-(acetamido)ethyl 2′-pyridyl disulphide (IV) at 25 °C in aqueous buffers

The points are experimental and the continuous line is the pH-dependent rate equation pertaining to four reactive hydronic states (XH_3–X) and a fifth unreactive state (XH_4) [eqn. (1)] (see the Materials and methods section) and the following values of the characterizing parameters with values of $\tilde{k}$ in $M^{-1} \cdot s^{-1}$.

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

$\tilde{k}_{XH_3} = 2.8 \times 10^4$, $\tilde{k}_{XH_2} = 8.0 \times 10^3$, $\tilde{k}_{XH} = 5.5 \times 10^3$, $\tilde{k}_X = 1.2 \times 10^4$, $pK_{XH_4} = pK_{XH_3} = 3.85$, $pK_{XH_2} = 5.0$, $pK_{XH} = 10.0$.

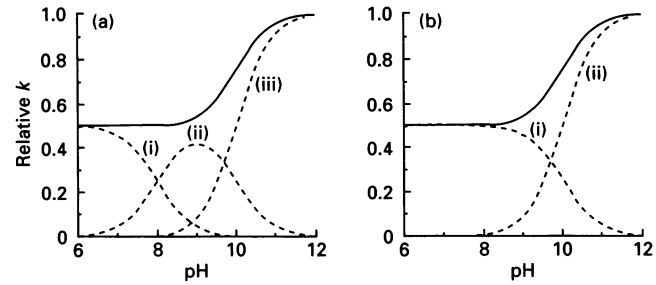


Figure 2 Computed pH–k profiles for a reaction in two observed reactive hydronic states connected by pK_a 10.0 (a) with and (b) without an additional pK_a of 8.0 obscured by compensating kinetic effects

In both (**a**) and (**b**) the continuous line represents the same observed pH-dependence of k which increases from the (relative) value (0.5) associated with the hydronic state that predominates at pH 6.0 to that (1.0) approached at high pH across $pK_a = 10.0$. (**a**) The continuous line is theoretical for the pH-dependent rate equation pertaining to three reactive hydronic states (XH_2–X) (see the Materials and methods section) [eqn. (2)] and the following values of the characterizing parameters.

$$k = \frac{\tilde{k}_{XH_2}}{1+\frac{K_{XH_2}}{[H^+]}+\frac{K_{XH_2}K_{XH}}{[H^+]^2}} + \frac{\tilde{k}_{XH}}{1+\frac{[H^+]}{K_{XH_2}}+\frac{K_{XH}}{[H^+]}} + \frac{\tilde{k}}{1+\frac{[H^+]^2}{K_{XH_2}K_{XH}}+\frac{[H^+]}{K_{XH}}} \quad (2)$$

$\tilde{k}_{XH_2} = \tilde{k}_{XH} = 0.5$, $\tilde{k}_X = 1.0$, $pK_{XH_2} = 8.0$, $pK_{XH} = 10.0$.

The broken lines (i)–(iii) correspond to contributions to k of the individual hydronic states provided by the terms of eqn. (2) associated with $\tilde{k}_{XH_2}$–$\tilde{k}_X$ severally. (**b**) The continuous line is theoretical for the pH-dependent rate equation pertaining to two reactive hydronic states (XH and X) [eqn. (3)] and the following values of the characterizing parameters.

$$k = \frac{\tilde{k}_{XH}}{1+\frac{K_{XH}}{[H^+]}} + \frac{\tilde{k}_X}{1+\frac{[H^+]}{K_{XH}}} \quad (3)$$

$\tilde{k}_{XH} = 0.5$, $\tilde{k}_X = 1.0$, $pK_{XH} = 10.0$.

The broken lines (i) and (ii) correspond to contributions to k of the individual hydronic states provided by the terms of eqn. (3) associated with $\tilde{k}_{XH}$ and $\tilde{k}_X$ respectively.

reactions) (Brocklehurst, 1979). The rate maximum at pH 6–7 is considered to result from substantial reaction via transition-state (VI) (Brocklehurst et al., 1987a, 1988). If the decrease in k with increase in pH across pK_a 8.1–8.3 results from loss of the (His-159)-imidazolium cation rather than from another hydronic dissociation that controls ion-pair geometry or substrate and inhibitor binding (see the Introduction), the different pH-dependence (with pK_a 10.0 instead of approx. 8) typified by the data shown in Figure 1 needs to be accounted for.

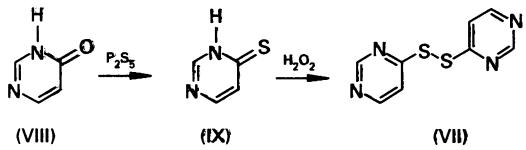
A possible explanation is illustrated by the computed pH-dependence curves shown in Figure 2. In both Figures 2(a) and 2(b) the continuous line represents the same observed pH-dependence of k which increases from the value associated with the hydronic state that predominates at pH 6 to that approached at high pH across pK_a 10.0. The simplest situation is that in Figure 2(b), which could represent hydron loss from the -S^-/-Im^+H ion-pair state to provide -S^-/-Im characterized by pK_a 10.0. The alternative (Figure 2a) could represent hydron loss from the -S^-/-Im^+H ion-pair state across pK_a 8.0 [broken line (i)] to provide -S^-/-Im [acid limb of broken line (iii)] without change in the observed value of k. This could arise if reaction at pH values around 6, even for non-substrate-like 2-pyridyl disulphides, contains a contribution from an -Im^+H-assisted transition state (VI) and increased nucleophilicity of -S^-/-Im is balanced by loss of the concerted reaction of -S^-/-Im^+H. The additional increase in k across pK_a 10.0 [broken line (iii)] would then be assigned to another hydronic dissociation in papain, the state of ionization of which affects the reactivity of the catalytic site.

Resolution of the ambiguity

It was considered that it should be possible to distinguish between the possibilities indicated in Figures 2(a) and 2(b) by electronic perturbation of the transition state (VI). A change in the structure of the leaving group that weakens its ability to become activated by hydronation or hydrogen-bonding would be expected to perturb the postulated balance between the higher nucleophilicity of -S^-/-Im and the loss of the concerted reaction by the S^-/-Im^+H ion pair. The pK_a around 8 postulated to be characteristic of the formation of -S^-/-Im from the ion-pair state in accord with Figure 2(a) with consequent increase in reactivity might thus be revealed, if only as an additional sigmoid wave in the pH–k profile.

Probe design, synthesis and characteristics

The lower value of the pK_a of the pyrimidine (X) [2.48, Albert and Barlin (1962)] relative to that of the corresponding pyridine



(VIII) (IX) (VII)

Scheme 1 Synthesis of 4,4′-dipyrimidyl disulphide [compound (VII)]

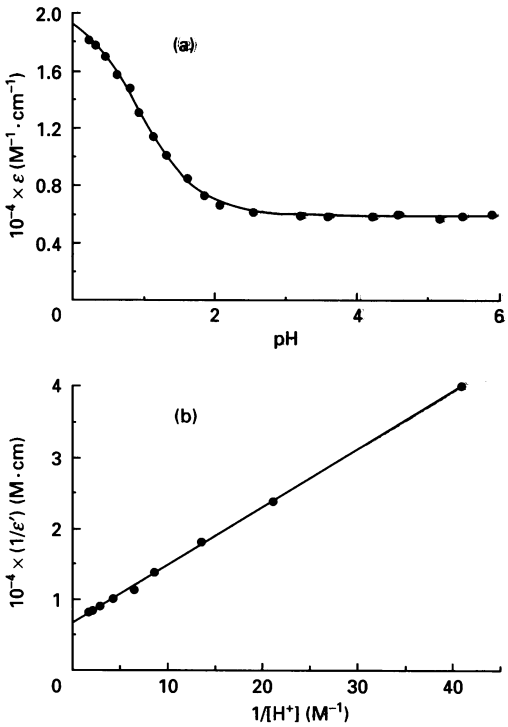


Figure 3 Determination of the pK_a value of monohydronated 4,4′-dipyrimidyl disulphide by spectral analysis at 287 nm

(**a**) pH-dependence of ϵ_{287}; the points are experimental and the continuous line is theoretical for eqn. (4) with $\tilde{\epsilon}^+ = 20920\ M^{-1}\cdot cm^{-1}$, $\tilde{\epsilon}^\circ = 5910\ M^{-1}\cdot cm^{-1}$ and p$K_a = 0.91$.

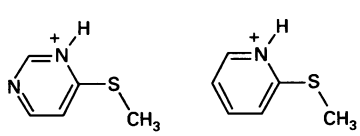
$$\epsilon = \frac{\tilde{\epsilon}^+ - \tilde{\epsilon}^\circ}{1 + \frac{K_a}{[H^+]}} + \tilde{\epsilon}^\circ \qquad (4)$$

(**b**) Determination of $\tilde{\epsilon}^+$ and pK_a by linear regression of $1/\epsilon'$ on $1/[H^+]$ [see eqn. (5)] where $\epsilon' = \epsilon - \tilde{\epsilon}^\circ$; the value of $\tilde{\epsilon}^\circ$ was obtained by inspection of ϵ in the pH range 4–6.

$$\frac{1}{\epsilon'} = \frac{1}{\epsilon - \tilde{\epsilon}^\circ} = \frac{1}{\tilde{\epsilon}^+ - \tilde{\epsilon}^\circ} + \frac{K_a}{(\tilde{\epsilon}^+ - \tilde{\epsilon}^\circ)}\frac{1}{[H^+]} \qquad (5)$$

The values of $\tilde{\epsilon}^+$ and K_a thus obtained together with the value of $\tilde{\epsilon}^\circ$ were used with eqn. (4) to construct the continuous line in (**a**).

(XI) [3.6, Albert and Barlin (1959)] suggested that 4,4′-dipyrimidyl disulphide (VII) might be a suitable reactivity probe with



(X) (XI)

which to attempt to reveal the kinetically influential pK_a around 8 not seen in the pH-dependent kinetics of the reaction of papain with 2,2′-dipyridyl disulphide (II) or with other non-substrate-like 2-pyridyl disulphides.

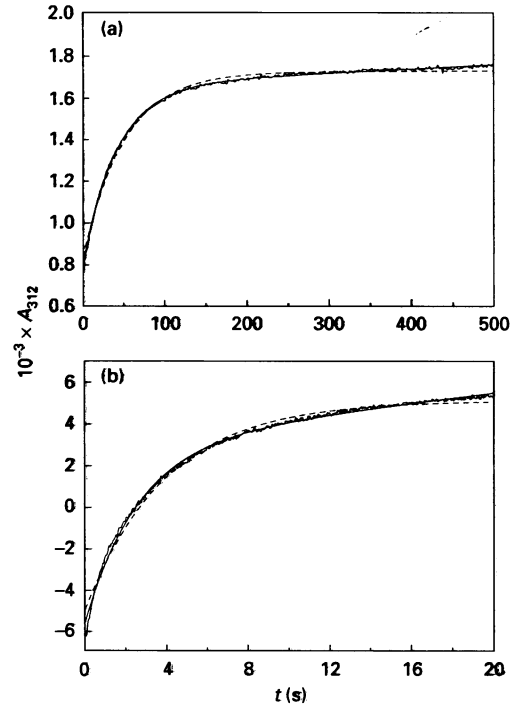


Figure 4 Typical stopped-flow records of the progress curves of reactions of papain with 4,4′-dipyrimidyl disulphide (VII)

Increase in A_{312} occurs consequent upon thiolysis of (VII) by papain to produce pyrimidine-4-thione (IX). In both (**a**) (pH 4.17 [VII] = 14.0 μM) and (**b**) (pH 8.39) [VII] = 15.0 μM) the record of the increase in A_{312} is shown together with the fit to the data according to (i) a single exponential (broken line) and (ii) a combination of a single exponential and a zero-order component (continuous line) with the following values of the parameters: (i) single exponential fits according to $A = P_1e^{-P_2t} + P_3$, where $P_1 = A_\infty - A_0$, $P_2 = k_{obs.}$ (the first-order rate constant) and $P_3 = A_\infty$, with for (**a**) $P_1 = 0.0091$, $P_2 = 0.01994\ s^{-1}$ and $P_3 = 0.01726$ and for (**b**) $P_1 = 0.0101$, $P_2 = 0.2574\ s^{-1}$ and $P_3 = 0.00503$; (ii) fits to a single exponential with a zero-order component according to $A = P_1e^{-P_2t} + P_3t + P_4$, where $P_1 = A_\infty - A_0$ for the single exponential component, $P_2 = k_{obs.}$, P_3 = the slope of the zero-order component of the A-versus-t curve and P_4 = the extrapolated value of the ordinate intercept, i.e. A_∞ for the single exponential component with for (**a**) $P_1 = 0.0088$, $P_2 = 0.02424\ s^{-1}$, $P_3 = 1.91 \times 10^{-6}\ s^{-1}$, and $P_4 = 0.0166$ and for (**b**) $P_1 = 0.0087$, $P_2 = 0.3872\ s^{-1}$, $P_3 = 1.21 \times 10^{-4}\ s^{-1}$, and $P_4 = 0.003$.

4,4′-Dipyrimidyl disulphide (VII) does not appear to have been described in the literature. It was synthesized by a simple two-step procedure (Scheme 1) involving thionation by P_2S_5 (Armarego, 1965) followed by oxidation with H_2O_2, and its identity and purity were established as described in the Materials and methods section. As expected from the pK_a values of compounds (X) and (XI), the pK_a value of the monocation of 4,4′-dipyrimidyl disulphide (VII), determined as 0.91 by spectroscopic titration (Figure 3), was found to be substantially smaller than that of the monocation of 2,2′-dipyridyl disulphide (II) [2.45, Brocklehurst and Little (1973)].

Kinetics of the reaction of papain with 4,4′-dipyrimidyl disulphide

4,4′-Dipyrimidyl disulphide (VII) reacts with papain on a stopped-flow time scale to produce pyrimidine-4-thione (IX) which is readily monitored over a wide range of pH by recording the increase in absorbance at the isosbestic point, 312 nm. Typical stopped-flow records for reactions carried out under pseudo-first-order conditions with the disulphide in excess are shown in Figure 4, and the adherence to overall second-order kinetics was established by the linear dependence on the observed first-order

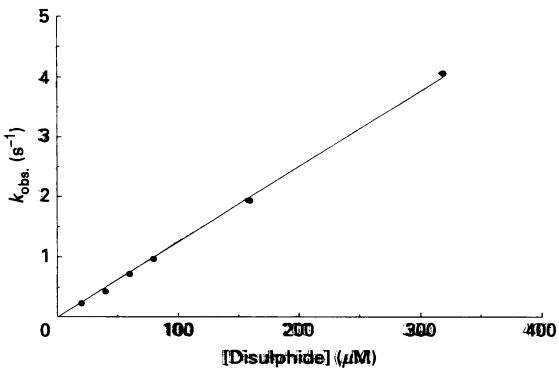


Figure 5 Demonstration of overall second-order kinetics for the reaction of papain with 4,4′-dipyrimidyl disulphide (VII) at 25 °C, pH 6.79 and I 0.1

Reactions were carried out under pseudo-first-order conditions with [papain] = 1.13–1.35 μM and [VII] = 20–320 μM. All reactions obeyed good first-order kinetics with respect to time after correction for a small zero-order component (see Figure 4), and the linear dependence of $k_{obs.}$ on [VII] establishes that the reaction is also first-order with respect to concentration in [VII]. The linear dependence of $k_{obs.}$ on [VII] was established also at pH 3.8 (up to 307 μM) and at pH 8.8 (up to 100 μM).

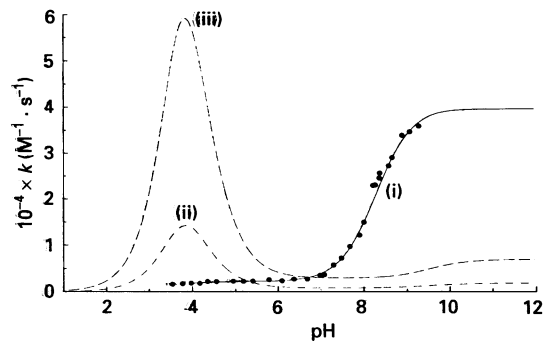


Figure 6 Comparison of the pH-dependences of k for the reactions of papain with (i) 4,4′-dipyrimidyl disulphide (VII) and (ii) and (iii) 2,2′-dipyridyl disulphide (II)

(i) The points are experimental for the reaction with 4,4′-dipyrimidyl disulphide, and the continuous line is theoretical for the pH-dependent rate equation pertaining to two reactive hydronic states (XH and X) [eqn. (3) of the legend to Figure 2] with $\tilde{k}_{XH} = 2.1 \times 10^3$ M^{-1} s^{-1}, $\tilde{k}_X = 3.95 \times 10^4$ $M^{-1} \cdot s^{-1}$ and $pK_{XH} = 8.3$; (ii) and (iii) [the main features of the pH–k profile for the reaction of papain with 2,2′-dipyridyl disulphide, see Shipton and Brocklehurst (1978) and Salih et al. (1987) for the experimental data] the broken line (ii) is theoretical for the pH-dependent rate equation pertaining to three reactive hydronic states (XH_2–X) and one unreactive hydronic state (XH_3) [eqn. (6)] with $\tilde{k}_{XH_2} = 4.2 \times 10^4$ $M^{-1} \cdot s^{-1}$, $\tilde{k}_{XH} = 7.0 \times 10^2$ $M^{-1} \cdot s^{-1}$, $\tilde{k}_X = 1.7 \times 10^3$ $M^{-1} \cdot s^{-1}$, $pK_{XH_3} = 3.85$, $pK_{XH_2} = 3.9$, $pK_{XH} = 9.5$.

$$k = \frac{\tilde{k}_{XH_2}}{1 + \frac{[H^+]}{K_{XH_3}} + \frac{K_{XH_2}}{[H^+]} + \frac{K_{XH_2}K_{XH}}{[H^+]^2}} + \frac{\tilde{k}_{XH}}{1 + \frac{[H^+]^2}{K_{XH_3}K_{XH_2}} + \frac{[H^+]}{K_{XH_2}} + \frac{K_{XH}}{[H^+]}} + \frac{\tilde{k}_X}{1 + \frac{[H^+]^3}{K_{XH_3}K_{XH_2}K_{XH}} + \frac{[H^+]^2}{K_{XH_2}K_{XH}} + \frac{[H^+]}{K_{XH}}} \quad (6)$$

The broken line (iii) is an expanded version of (ii) in which the values of $\tilde{k}_{XH_2}$–$\tilde{k}_X$ have been multiplied by 4 to reveal the features of the profile more clearly; in view of the small amplitude of the sigmoid wave in which k increases from $\tilde{k}_{XH}$ to $\tilde{k}_X$ the value of pK_{XH} shown (9.5) should be regarded as a lower limit.

rate constant ($k_{obs.}$) on the concentration of compound (VII) (Figure 5).

The pH-dependence of k for the reaction of papain with 4,4′-dipyrimidyl disulphide (VII) is shown in Figure 6 [profile (i)] where it is compared with the pH-dependence of k for the reaction of papain with 2,2′-dipyridyl disulphide (II) on the same scale as profile (i) [profile (ii)] and with the values of k increased 4-fold [profile (iii)] to reveal the shape of the profile in alkaline media more clearly. There are two important differences between the profile for the reaction with 4,4′-dipyrimidyl disulphide and that for the reaction with 2,2′-pyridyl disulphide: (a) the increase in k in acidic media with a maximum around pH 4 that is characteristic of the reaction of papain with simple 2-pyridyl disulphides such as 2,2′-dipyridyl disulphide (see also Figure 1) does not occur in the reaction of papain with 4,4′-dipyrimidyl disulphide and (b) the increase in k in alkaline media for the reaction with 4,4′-dipyrimidyl disulphide depends on a pK_a value of 8.3 whereas the pK_a value is much higher (≥ 9.5, see also Figure 1) for the reaction with 2,2′-dipyridyl disulphide. These striking differences between the characteristics of the reactions of papain with the two disulphide reactivity probes [(II) and (VII)] support the suggestion discussed above that the pK_a value characteristic of hydron loss from the ion-pair state of papain to provide $-S^-/-Im$ is 8.3. The marked decrease in the basicity of the leaving group on changing from 2,2′-dipyridyl disulphide to 4,4′-dipyrimidyl disulphide clearly removes the possibility of significant hydronation at pH values around 4 needed for the high reactivity in this pH region found with non-substrate-like 2-pyridyl disulphides. This loss of sensitivity to activation by hydronation suggests also a loss of sensitivity to activation in a transition state analogous to (VI) involving concerted reaction of both partners of the $-S^-/-Im^+H$ ion pair. By rendering reaction via an $-Im^+H$-assisted transition state much less (or non) effective, the compensation postulated in Figure 3(a) would be much decreased or eliminated and the pK_a for hydron loss from the $-S^-/-Im^+H$ ion-pair state (8.3) revealed.

Concluding comments

The value of the pK_a characteristic of the formation of (Cys-25)-S^-/(His-159)-Im from the ion-pair state of papain is 8.3. Another dehydronation characterized by $pK_a \geq 9.5$ (probable approx. 10.0) modulates the reactivity of the papain catalytic site. This modulation, revealed particularly clearly by using 2-pyridyl disulphides as reactivity probes (which mask the pK_a of 8.3 by the compensatory effects discussed above), affects the catalytic behaviour of papain as well as its thiol reactivity towards time-dependent inhibitors. Thus k_{+2}/K_s for the papain-catalysed hydrolysis of *N*-benzoylglycine methyl thionoester was reported to decrease in alkaline media across pK_a 9.5 (Storer and Carey, 1985). They reported also that the analogous pK_a for the papain-catalysed hydrolysis of *N*-(β-phenylpropionyl)glycine methyl thionoester is 9.15. In both cases, the albeit rather sparse data show clearly that the pK_a value is certainly greater than 9. Variation in the apparent value of the pK_a obtained by fitting pH–k_{+2}/K_s (or $k_{cat.}/K_m$) data to a single ionization (e.g. 9.5 and 9.15) consequent on changing the structure of the substrate suggests the existence of two (or more) overlapping kinetically influential ionizations. The origin of this phenomenon of apparent variation in a free reactant state pK_a value was demonstrated by the simulation study reported by Brocklehurst et al. (1983) (see also Bashford and Karplus, 1991). In the present work, two pK_a values characteristic of the loss of the catalytically essential ion-pair state of papain (8.3) and of the modulation of

catalytic-site reactivity (approx. 10) have been identified by reactivity probe kinetics.

A comparison of the behaviour of other cysteine proteinases, e.g. papaya proteinase Ω (Topham et al., 1991), towards pyridyl and pyrimidyl disulphides should resolve ambiguities in pK_a assignments as the present study has done for papain. The pK_a of 10 still needs to be assigned. The structure of papain shows that a group with pK_a of this magnitude must be remote from the catalytic site and a possible candidate is the Tyr-61/67 hydrogen-bonded pair (see Topham et al., 1991).

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