Catalytic-site characteristics of the porcine calpain II 80 kDa/18 kDa heterodimer revealed by selective reaction of its essential thiol group with two-hydronic-state time-dependent inhibitors: evidence for a catalytic site Cys/His interactive system and an ionizing modulatory group

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1. Four calpain II heterodimers (80 kDa/30 kDa, 80 kDa/29 kDa, 80 kDa/26 kDa and 80 kDa/18 kDa) were isolated from fresh porcine kidney by $(\text{NH}_4)_2\text{SO}_4$ precipitation, chromatography on DEAE-Sepharose CL-6B and subsequently on Reactive Red 120/agarose followed by f.p.l.c. on a Q-Sepharose Hi-Load 16/10 column. 2. The major component (80 kDa/30 kDa) was used to provide the catalytically active calpain II 80 kDa/18 kDa heterodimer by treatment with CaCl_2 ; titration with *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64) in the presence of monothio glycerol showed the preparation to have 1.0 ± 0.05 catalytic sites per molecule of heterodimer. 3. The 80 kDa/30 kDa heterodimer was separated from monothio glycerol and other low-molecular-mass material by gel filtration on Sephadex G-25 without loss of catalytic activity towards sulphanilic acid/azocasein in the presence of added Ca^{2+} . On storage overnight at a concentration of $3 \mu\text{M}$ in KCl at 4°C in the absence of Ca^{2+} the activator-free preparation still produced fully active 80 kDa/18 kDa heterodimer on addition of Ca^{2+} . 4. Activator-free 80 kDa/30 kDa heterodimer (in the absence of Ca^{2+}) reacts relatively slowly with ethyl 2-pyridyl disulphide at pH 5.9; over 5000 s five thiol groups per molecule react, all at similar rates. In the presence of 8 mM CaCl_2 under otherwise identical conditions (and also in the pH range 3.8–10.4) an initial faster phase of reaction corresponding to approx. one thiol

group per molecule of heterodimer is generated, but it is not cleanly separated from the subsequent slower reactions on the stopped-flow trace. This fast phase of reaction does not occur when E64-inactivated calpain II is substituted for active 80 kDa/18 kDa heterodimer. 5. Greatly improved resolution of the fast phase of reaction involving the catalytic-site thiol group was achieved by using 2,2'-dipyridyl disulphide (2-Py-S-S-2-Py) instead of ethyl 2-pyridyl disulphide. 6. The pH-dependence of the second-order rate constant (k) for the reaction of the catalytically active activator-free 80 kDa/18 kDa calpain II heterodimer with 2-Py-S-S-2-Py was studied by stopped-flow spectral analysis in the pH range approx. 3–8 without interference from reactions of other thiol groups. 7. The form of the pH- k profile establishes for the first time the existence of an interactive catalytic site system [probably containing a (Cys)-S⁻/(His)-Im⁺H ion pair] analogous to those present in monomeric non- Ca^{2+} -activated cysteine proteinases. 8. The reactivity and kinetically influential ionization characteristics of the calpain II 80 kDa/18 kDa heterodimer, which are strikingly different from those of other cysteine proteinases, are compared with those of papain, actinidin, ficin and papaya proteinase Ω . 9. The possibility that in the calpain II 80 kDa/18 kDa heterodimer, movement of the ion-pair components might be controlled by the electrostatic influence of a carboxylate anion is discussed.

INTRODUCTION

Calpains (EC 3.4.22.17), cysteine proteinases with an absolute requirement for Ca^{2+} , are widely distributed in mammalian, avian, crustacean and piscine tissues, as well as in insects such as *Drosophila* (Murachi, 1989). Although calpain has been associated with a wide variety of physiological processes including turnover of the constituent proteins of cell membranes, the cytoskeleton, neurofilaments and intermediate filaments and microtubules, protection of erythrocytes against the nucleation of damaged proteins, roles in the nervous system and transmembrane signalling (Melloni and Pontremoli, 1989; Croall and DeMartino, 1991) and involvement in mitosis (Schollmeyer, 1988), the exact functions of Ca^{2+} -dependent proteolysis remain to be discovered. Calpain has been implicated also in the pathophysiological conditions of Duchenne muscular dystrophy (Nagy and Samaha, 1986), Montreal platelet syndrome (Okita et al., 1989), Alzheimer's disease (Nixon et al., 1990; Croall and DeMartino, 1991; Iwamoto et al., 1991) and essential hyper-

tension (Melloni and Pontremoli, 1989). Various aspects of the study of calpain and its behaviour have been reviewed: structural and genetic determinants (Suzuki, 1987), physiological functions, mechanism of activation and the role of the endogenous inhibitor calpastatin (Murachi, 1989; Mellgren, 1987; Melloni and Pontremoli, 1989; Croall and DeMartino, 1991), and the development of inhibitors (Wang, 1990; Mehdi, 1991). Detailed reviews of many aspects of calpain appear in the book edited by Mellgren and Murachi (1990).

There are two calpain isoenzymes, calpain I and calpain II, which differ in sensitivity to Ca^{2+} activation. Both are heterodimers composed of a large (80 kDa) and a small (30 kDa) subunit before proteolytic activation. The domain structure of calpain has been described, by making use of sequence identities with other proteins and cDNA methodology, and the functions of the enzyme have been related to these domains (Suzuki, 1990). The large subunit contains four domains designated I, II, III and IV and the small subunit contains two domains designated V and IV'. Domains IV and IV' are the Ca^{2+} -binding regions.

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They show significant sequence similarities to calmodulin and troponin-C and each possess four EF hand structures. Domain V of the small subunit contains a high proportion of glycine residues interspersed with hydrophobic residues and is thought to be involved in membrane binding. This domain is completely removed by autolytic activation. Domain I is also partially cleaved by this process. The functions of the remaining parts of domain I and domain III are not yet known and these regions are not homologous with other known proteins. Domain II has high sequence similarity with cysteine proteinases such as cathepsins B, H and L and papain. Catalytically competent porcine calpain II produced by Ca^{2+} -promoted autolysis consists of two subunits, one of mass 80 kDa (composed of domains II, III, IV and part of domain I) and one of mass 18 kDa (domain IV') (Crawford et al., 1990).

Chicken calpain shows high sequence similarities to other cysteine proteinases around Cys-108 and His-265 and on this basis these have been suggested as the principal catalytic-site residues. Chemical evidence for Cys-108 as the residue containing the catalytically essential thiol group was provided by specific labelling with iodoacetate (Suzuki et al., 1983) and inhibition experiments with this reagent and with the epoxysuccinyl derivative E64c (Suzuki, 1983). Although the evidence from sequence analysis and thiol labelling/inhibition data is highly suggestive of the cysteine proteinase nature of calpain, the existence of the key feature common to cysteine proteinase catalytic sites, a nucleophilically competent interactive Cys-His system probably containing an $\text{S}^-/\text{Im}^+\text{H}$ ion pair (Brocklehurst, 1986, 1987; Brocklehurst et al., 1987) has not yet been reported. Suzuki (1990) has pointed out that clear evidence for the involvement of His-265 in catalysis has not yet been obtained and indeed evidence for any catalytic-site functionality in calpain other than a cysteinyl thiol group has not been reported.

In the present work the technique of using stopped-flow kinetic analysis of reactions of 2-pyridyl disulphides as two-hydronic-state reactivity probes (Brocklehurst, 1982) to identify and characterize cysteine proteinase catalytic sites has been applied to porcine calpain II. This technique has been used extensively with monomeric non- Ca^{2+} -activated cysteine proteinases, e.g. with ficin (Malthouse and Brocklehurst, 1976), papain (Shipton and Brocklehurst, 1978), actinidin (Brocklehurst et al., 1981), cathepsins B and H (Willenbrock and Brocklehurst, 1985), gingivain (Shah et al., 1991) and papaya proteinase Ω (Topham et al., 1991). Characterization of calpain by using disulphide reactivity probes necessitated the establishment of an experimental protocol that takes account of the multiplicity of thiol groups in calpain, the reported demand for the presence of low-molecular-mass mercaptan for catalytic activity, the need for Ca^{2+} activation (Murachi, 1983), and the effect of autolysis on catalytic-site integrity.

The present paper reports (a) that it is possible to study activated activator-free calpain II by both substrate- and time-dependent inhibitor kinetics, (b) that reaction of the essential thiol group with 2,2'-dipyridyl disulphide can be studied by stopped-flow spectral analysis at pH values between about 3 and 8 without interference from reactions of other thiol groups, and (c) that catalytic-site reactivity of calpain II is controlled by two molecular pK_a values of 4.4 and 5.6, one of which presumably characterizes the formation of an ionization state containing the essential (Cys)- $\text{S}^-/(\text{His})\text{-Im}^+\text{H}$ ion pair predicted from earlier work on other cysteine proteinases. The reactivity and kinetically influential ionization characteristics of the calpain II 80 kDa/18 kDa heterodimer, which are strikingly different from those of other cysteine proteinases, are compared with those of papain, actinidin, ficin and papaya proteinase Ω .

MATERIALS AND METHODS

Substrate and inhibitors

Sulphanilic acid/azocasein was synthesized from casein and diazotized sulphanilic acid as described by Barrett and Kirschke (1981). In our hands, sulphanilamide/azocasein (Sigma) failed to act as a substrate for calpain II. *trans*-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64) was the Sigma product. The purification and characterization of 2,2'-dipyridyl disulphide (2-Py-S-S-2-Py) (Aldrich) has been described previously (Salih et al., 1987) as has the synthesis and characterization of ethyl 2-pyridyl disulphide (Et-S-S-2-Py) (Brocklehurst et al., 1988). Concentrations of the 2-pyridyl disulphides were determined by thiolysis with excess of 2-mercaptoethanol at pH 7 and spectral analysis of the resulting pyridine 2-thione ($\epsilon_{343} = 8.08 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$; Stuchbury et al., 1975); under these conditions 1 mol of 2-Py-S-S-2-Py provides 2 mol of pyridine 2-thione.

Calpain II

The enzyme was purified from fresh porcine kidney (Polyclonal Antibodies Ltd., Blaenwaun Farm, Llandysul, Dyfed, Wales, U.K.). Freshly excised kidneys were transported refrigerated and used immediately on arrival. In our hands frozen or old tissue did not yield active enzyme. The fresh tissue (approx. 1.4 kg; approx. 1.1 kg after removal of fat and outer membrane) was cut into small pieces (approx. 2 cm^3) before homogenization. The steps of the purification procedure before f.p.l.c., involving homogenization, $(\text{NH}_4)_2\text{SO}_4$ precipitation, chromatography first on DEAE-Sepharose CL-6B and subsequently on Reactive Red 120/agarose were carried out at 4 °C as described by Crawford et al. (1990). Addition of phenylmethanesulphonyl fluoride to the various buffers to give concentrations of 0.5–1.0 mM (particularly to the homogenization buffer to give 1 mM) should be made, as a solution in approx. 5 ml of acetone, immediately before their use to minimize loss by hydrolysis. F.p.l.c. was carried out at room temperature (approx. 22 °C), essentially as described by Parkes et al. (1985) for the preparation from chicken gizzard except that a Q-Sepharose Hi-Load 16/10 column was used instead of a Mono Q 10/10 column. A linear gradient (0.26–0.365 M NaCl) separates intact potentially active calpain II (yield 5–15 mg) from partially degraded but still potentially active calpain (see the Results and discussion section).

The concentration of calpain II protein was determined by using $A_{280} = 1.4$ for a solution of concentration 1 mg/ml in a 1 cm cell (Parkes et al., 1985) which corresponds to $\epsilon_{280} = 1.54 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for a molecular mass of 110 kDa. Active-site titration was carried out by using E64 as described by Parkes et al. (1985).

Activator-free calpain II

The f.l.p.c. fractions containing intact calpain II were concentrated to 2 ml by using an Amicon 8010 concentrator with a PM10 membrane. The monothioglycerol present during the purification procedure together with other low-molecular-mass material was then removed by gel filtration on a Sephadex G-25 column (25 cm $\times$ 2.5 cm). Elution with 0.1 M KCl and collection of approx. 10 ml fractions produced activator-free enzyme in approx. 20 ml of eluate after approx. 40 ml had been collected and discarded. The catalytic activity of this preparation towards sulphanilic acid/azocasein (see below) was not increased by addition of low-molecular-mass mercaptan and did not decrease on storage at 4 °C overnight.

Calpain II derivatized by reaction with E64

Calpain II 80 kDa/30 kDa heterodimer (4 ml of 1.4 μM in 0.1 M Tris/HCl buffer, pH 7.5) was mixed with aqueous solutions of E64 (4 ml of 3 μM) and CaCl_2 (1 ml of 100 mM) and stirred for 1 h at 25 °C. The solution was concentrated to 1.5 ml by ultrafiltration and subjected to gel filtration on Sephadex G-25 as described above. The protein-containing fractions were combined (20 ml) and concentrated to give approx. 4 ml of approx. 2 μM of derivatized calpain II on the basis of A_{280} and $\epsilon_{280} = 1.54 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

SDS/PAGE

SDS/PAGE was performed with gels of 10% (w/v) polyacrylamide, the discontinuous buffer system of Laemmli (1970) and Coomassie Blue staining.

Assay of calpain II activity

Calpain II activity was determined by using sulphanilic acid/azocasein essentially as described by Parkes et al. (1985) except that the assay mixtures were incubated at 37 °C instead of 30 °C.

Kinetics of the reactions of calpain II 80 kDa/18 kDa heterodimer with the 2-pyridyl disulphide reactivity probes

These were studied by using an Applied Photophysics SF.17MV stopped-flow spectrophotometer, kinetics work station and data acquisition and analysis software running on an Acorn Archimedes 410/1 microcomputer. Monochromator entrance and exit slit widths were set at 1 mm. Release of pyridine-2-thione was monitored at 343 nm ($\epsilon_{343} = 8.08 \times 10^3 / (1 + K/[\text{H}^+]) \text{ M}^{-1} \cdot \text{cm}^{-1}$, where $\text{p}K = 9.8$ (Brocklehurst and Little, 1973; Stuchbury et al. (1975)). All reactions were carried out under pseudo-first-order conditions with the following concentrations in the mixing chamber: [calpain II] = 1.5 μM , and either [Et-S-S-2-Py] = 50–70 μM or [2-Py-S-S-2-Py] = 20 μM . In some experiments CaCl_2 (16 mM) was present in the enzyme syringe (see the Results and Discussion section).

For the reaction of calpain II 80 kDa/18 kDa heterodimer with 2-Py-S-S-2-Py in the pH range 3.59–7.75, the reaction of the catalytic-site thiol group was sufficiently faster than those of other thiol groups in calpain that its reaction provided a simple first-order increase in A_{343} . For these reactions the 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 to an equation for a single exponential process:

$$A = (A_{\infty} - A_0) e^{-k_{\text{obs}} \cdot t} + A_{\infty}$$

At pH 9.38, reaction of at least one other thiol group of calpain occurred at a noticeable (though still relatively low) rate and the progress curve for this reaction was fitted to an equation for a single exponential with a zero-order component:

$$A = (A_{\infty} - A_0) e^{-k_{\text{obs}} \cdot t} + A_{\infty} + St$$

where A_{∞} and k_{obs} relate to the exponential component and S is the slope of the zero-order component. Second-order rate constants (k) were obtained from $k = k_{\text{obs}} / [\text{disulphide}]$.

pH-dependent kinetic data for the reaction of calpain II with 2-Py-S-S-2-Py were evaluated by using a multitasking application

program (SKETCHER) written in ANSI C (Kernighan 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 given number of hydronic states are readily written down as described by Brocklehurst et al. (1990) and Topham et al. (1991). In the present work the data required three-parameter and six-parameter rate equations (see the Results and discussion section). pH– k 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.

RESULTS AND DISCUSSION

Preparation of fully active activator-free calpain II

Properties common to catalytically active calpains from various sources have been reported to include the need for 'Ca²⁺ and an SH-reducing agent' (Murachi, 1983). The need for Ca²⁺ to be present is beyond dispute and it appears to be common practice to include also a low-molecular-mass mercaptan during purification and in reaction mixtures when catalytic activity is assayed. Surprisingly, low-molecular-mass mercaptan is present also when chemical modification of calpain thiol groups is performed by reaction with electrophilic reagents such as *N*-ethylmaleimide (see e.g. Wang and Jiang, 1991). The inclusion of low-molecular-mass mercaptan in thiol-targeted calpain inhibition reactions appears to be a general practice, although this is not made clear in all cases [see e.g. Anglikier et al. (1992)]. It was necessary, therefore, to ascertain whether there really is a requirement for low-molecular-mass mercaptan as well as for Ca²⁺ in order to design experiments for the kinetic investigation of the catalytic-site thiol group of calpain II with disulphide reactivity probes.

The f.p.l.c. elution profile (Figure 1) contains four peaks of protein with proteolytic activity in the presence of added Ca²⁺. A

Figure 1 Isolation of the 80 kDa/30 kDa heterodimer of porcine calpain II by f.p.l.c.

Catalytically active fractions from the Reactive Red 120/agarose column were combined and dialysed against 2 litres of buffer 1 [20 mM Bistris propane/HCl, pH 7.0, containing 0.2 M NaCl, 5 mM EDTA, 0.1% (v/v) monothioglycerol and 0.01% (w/v) NaN₃]. The dialysate containing approx. 18 mg of protein was concentrated to 40 ml by using a PM10 membrane. A 50 ml superloop was prepared in buffer 1 and loaded with the protein sample to a volume of 45.5 ml. A Q-Sepharose Hi-Load 16/10 column was equilibrated sequentially with filtered and degassed buffer 1 and buffer 2 (buffer 1 in which 0.2 M NaCl is replaced by 0.5 M NaCl) and the f.p.l.c. apparatus (Pharmacia LKB) was operated by using a program kindly supplied by Dr. C. Crawford, Laboratory of Molecular Biophysics, University of Oxford; 10 ml fractions were collected. The eluate was monitored for A_{280} (—) and for proteolytic activity towards sulphanilic acid/azocasein in the presence of 5 mM CaCl_2 (◇).

Figure 2 Identification of the component of peak 1 from Figure 1 as the 80 kDa/30 kDa calpain II heterodimer by SDS/PAGE

Markers: 1, β -galactosidase; 2, phosphorylase *b*; 3, BSA; 4, glutamate dehydrogenase; 5, chicken egg albumin; 6, lactate dehydrogenase; 7, carbonic anhydrase; 8, trypsin inhibitor; 9, lysozyme.

Figure 3 pH-dependence of the second-order rate constant (k) for the reaction of the calpain II 80 kDa/18 kDa heterodimer with 2-Py-S-S-2-Py at 25 °C and / 0.1 mol/l in aqueous buffers

The points are experimental; each point was obtained from at least three essentially identical progress curves which were overlaid on the visual display unit of the stopped-flow machine before signal averaging and kinetic analysis. The continuous line is theoretical for $k =$ the first term of eqn. (1) of the text, with $pK_I = 4.4$, $pK_{II} = 5.6$ and $\bar{k}_{XH_2} = 4.0 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. The broken line is theoretical for the complete eqn. (1) of the text with the above values of the parameters and $pK_{III} = 9.5$, $\bar{k}_{XH} = 5.0 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $\bar{k}_X = 4.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$. The broken line is intended only to illustrate the possibility that the data points at pH 7.8 and 9.3 are consistent with eqn. (1). The values chosen for $\bar{k}_X$ and pK_{III} are arbitrary but the value for pK_{III} is similar to values of this parameter found for analogous reactions of papain and actinidin (Brocklehurst et al., 1988).

linear gradient (0.26–0.36 M NaCl) separates the 80 kDa/30 kDa heterodimer from three other potentially active components which probably contain 80 kDa/29 kDa, 80 kDa/26 kDa and 80 kDa/18 kDa heterodimers respectively (see Crawford et al. 1990). Peak 1 contained intact calpain II comprising one large subunit of mass approx. 80 kDa and one small unit of mass approx. 30 kDa as demonstrated by SDS/PAGE (Figure 2). This electrophoretogram is closely similar to that reported by Parkes et al. (1985) for chicken gizzard calpain II. Peaks 2–4 contained material with degraded small subunits of lower mass as

demonstrated by SDS/PAGE (not shown) analogous to those reported by Parkes et al. (1985) and Crawford et al. (1990). Titration with E64 (Sugita et al., 1980; Parkes et al., 1985) showed the enzyme contained in peak 1 of Figure 1 (which also contains monothioglycerol) to possess 1.0 ± 0.05 catalytic sites per molecule of calpain II heterodimer after autolysis promoted by added CaCl_2 (5 mM). The enzyme in peak 1 was shown to exhibit catalytic activity towards sulphanilic acid/azocasein but, as expected, only after addition of CaCl_2 (to give 5 mM).

To compare this enzyme solution with that devoid of low-molecular-mass mercaptan, the solution from peak 1 was concentrated, subjected to gel filtration on Sephadex G-25, and concentrated again as described in the Materials and methods section to approx. 6 μM . The concentration was then adjusted to be the same (approx. 3 μM) as that obtained directly from the f.p.l.c. column in peak 1. As before, the enzyme was devoid of catalytic activity in the absence of Ca^{2+} but exhibited the same activity towards sulphanilic acid/azocasein when devoid of low-molecular-mass mercaptan as it had originally in the presence of the monothioglycerol. Addition of a further quantity of monothioglycerol (to provide 20 mM of the newly added mercaptan) did not result in any change in activity. The solution of potentially fully active activator-free calpain II (3 μM) in KCl stored overnight at 4 °C in the absence of Ca^{2+} produced the same activity as before on addition of CaCl_2 , and was used on that day for kinetic experiments with the disulphide inhibitors.

Reactions of fully active activator-free calpain II with 2-pyridyl disulphides

Initially the reaction with Et-S-S-2-Py was studied to provide a basis for subsequent studies with reactivity probes containing a variety of potential binding sites in R [$\text{R}-(\text{CH}_2)_n$ -S-S-2-Py]. These experiments demonstrated that (i) in the absence of Ca^{2+} , Et-S-S-2-Py (50–70 μM) reacts relatively slowly with at least five thiol groups of the calpain II 80 K/30 K heterodimer over 5000 s at pH 5.9 all at similar rates, (ii) in the presence of 8 mM CaCl_2 under the same conditions (and also in the pH range 3.8–10.4), an initial faster phase of reaction corresponding to approx. one thiol group per molecule of heterodimer is generated but is not clearly separated from the subsequent slower reactions, and (iii) this more rapid phase of reaction is not present when E64-inactivated calpain II is allowed to react with Et-S-S-2-Py in the presence of 8 mM CaCl_2 at pH 7.4. These results suggest that the catalytic-site thiol group of calpain II becomes accessible in the presence of Ca^{2+} and reacts more rapidly with Et-S-S-2-Py than the other thiol groups of calpain II. The difference in reactivity towards this reagent, however, is not sufficiently great to permit clean separation in time of the reaction of the catalytic-site thiol group which is desirable for reliable kinetic analysis. Kinetic study of the pH-dependence of the reactivity of the catalytic-site thiol group of the active activator-free calpain II 80 kDa/18 kDa heterodimer produced from the inactive 80 kDa/30 kDa precursor in the presence of Ca^{2+} (Crawford et al., 1990) was carried out therefore by using 2-Py-S-S-2-Py. The reaction of the symmetrical disulphide with the catalytic-site thiol group proved to be considerably faster than the reaction of Et-S-S-2-Py and permitted kinetic study by stopped-flow spectral analysis without interference from reactions of other calpain II thiol groups.

The nucleophilic interactive system of the calpain II catalytic site revealed by kinetic analysis of its thiol group with 2-Py-S-S-2-Py

The major catalytic-site feature of all of the monomeric cysteine proteinases studied to date is an interactive system comprising

Figure 4 Comparison of the major bell-shaped components in acidic media of the pH- k profiles for the reactions of 2-Py-S-S-2-Py with (a) ficin, (b) papaya proteinase Ω , (c) actinidin, (d) papain and (e) the calpain II 80 kDa/18 kDa heterodimer

The lines are theoretical for k = the first term of eqn. (1) of the text and the following values of the characterizing parameters given in the sequence pK_1 , pK_{II} and k_{cat} ($\text{M}^{-1} \cdot \text{s}^{-1}$): (a) 2.4, 3.8, 1.1×10^5 (Malthouse and Brocklehurst, 1976); (b) 2.45, 3.4, 2.8×10^4 (Topham et al., 1991); (c) 2.5, 3.0, 2.4×10^4 (Salih et al., 1987); (d) 3.85, 3.9, 4.2×10^4 (Shipton and Brocklehurst, 1978); (e) 4.4, 5.6, 4.0×10^5 , present work, Figure 3).

the side chains of a cysteine residue and a histidine residue of which a thiolate/imidazolium ion pair is a plausible component (Brocklehurst, 1987; Brocklehurst et al., 1987). The S atom of the cysteine residue becomes transiently acylated by substrate during catalysis and the side chain of the histidine residue 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.

The existence of these catalytic-site systems may be demonstrated and their reactivity and ionization characteristics obtained by using time-dependent inhibitors of the 2-pyridyl disulphide series as reactivity probes [see Topham et al. (1991) for an example involving the characterization of papaya proteinase Ω]. These reagents are two-hydronic (protonic)-state electrophiles (Brocklehurst, 1974, 1982), and reaction of cysteine proteinase catalytic sites with simple members of the series, i.e. 2-Py-S-S-2-Py and alkyl 2-pyridyl disulphides, devoid of key substrate-analogous-binding sites is characterized by a striking rate maximum at low pH, a rate minimum in approximately neutral media and a plateau of higher reactivity at high pH. 2-Py-S-S-2-Py contains a chromophoric 2-mercaptopyridine leaving group, the activation of which by hydronation (pK_a 2.45) permits the detection and characterization of the $-\text{Im}^+\text{H}/\text{S}^-$ ion pairs and of other ionizations that affect reactivity characteristics of the ion pairs and their components.

To study the kinetics of the calpain II 80 kDa/18 kDa heterodimer with 2-Py-S-S-2-Py, one syringe of the stopped-flow spectrophotometer was filled with a solution of $40 \mu\text{M}$ 2-Py-S-S-2-Py in buffer and the other with a solution of activator-free calpain II 80 kDa/30 kDa heterodimer in 0.1 M KCl which had been made 16 mM in CaCl_2 to convert the calpain into active 80 kDa/18 kDa heterodimer immediately before the solution was transferred to the syringe. At least three essentially identical progress curves were overlaid on the visual display unit at each of the pH values in Figure 3 before signal averaging and kinetic analysis were performed with the computer.

The second-order rate constants (k) used to construct the pH- k profile (Figure 3) were obtained from such stopped-flow records, as described in the Materials and methods section, recorded within 2 min of the addition of CaCl_2 to the calpain II 80 kDa/30 kDa heterodimer. The fact that identical progress curves were overlaid before analysis demonstrates that further progressive change in the 80 kDa/18 kDa heterodimer either did not occur on this time scale or was without kinetic consequence. The reliability of the kinetic data is further supported by the results of the following experiment carried out with $1.5 \mu\text{M}$ calpain II 80 kDa/18 kDa heterodimer and $20 \mu\text{M}$ 2-Py-S-S-2-Py in the mixing chamber at pH 4.79. Progress curves were recorded (a) within 2 min of the addition of CaCl_2 to transform the 80 kDa/30 kDa heterodimer to the active 80 kDa/18 kDa heterodimer, (b) after 30 min, (c) after 1 h and (d) after 24 h. The extent of the rapid reaction (ΔA_{343}) decreased to 66% of its initial value after 30 min, to 38% of its initial value after 1 h and to zero after 24 h. This demonstrates the loss of catalytic sites presumably due to further proteolytic processing. The important observation, however, is that the rate constants of the residual rapid increases in A_{343} after 30 min and after 1 h were essentially unchanged, being 94% of the original value after 30 min and 98% of the original value after 1 h. Thus although catalytic sites characteristic of the intact 80 kDa/18 kDa heterodimer remain, their reactivity is unchanged. Further proteolytic processing destroys the high thiol reactivity completely and thus does not interfere with the kinetic characterization of the remaining intact catalytic sites.

The pH-dependence of k for the reaction of the catalytic site thiol group of the calpain II 80 kDa/18 kDa heterodimer with 2-Py-S-S-2-Py is shown in Figure 3. The data demonstrate the existence of a bell-shaped component of high reactivity maximal at pH approx. 5. In the pH range approx. 3.5–8.0 the reaction of the catalytic-site thiol group was clearly separated from reactions of other thiol groups and the progress curves, of appropriate amplitude, obeyed good (pseudo) first-order kinetics. In alkaline media reactions of other thiol groups became progressively faster and clean separation of the reaction of the catalytic-site thiol group as a single exponential could not be achieved. The data point shown at pH 9.3 was obtained by fitting the stopped-flow $A-t$ record to a single exponential with a zero-order component.

The general shape of the pH- k profile in Figure 3 is similar to those shown to be characteristic of other well-characterized monomeric non- Ca^{2+} -activated cysteine proteinases (see e.g. Brocklehurst, 1987; Topham et al., 1991). This shape provides a convincing demonstration of the existence of a thiol-containing catalytic-site interactive system in the calpain II 80 kDa/18 kDa heterodimer with nucleophilic character in acidic media, presumably the $(\text{Cys-108})\text{S}^-/(\text{His-265})\text{Im}^+\text{H}$ ion pair (chicken calpain numbering).

Although the general shape of the pH- k profile in Figure 3 is similar to those of analogous profiles for the reactions of many other cysteine proteinases with 2-Py-S-S-2-Py, there are two marked differences which are illustrated in Figure 4. These are (i) that k is maximal at a significantly higher pH (approx. 5) than is the case for the reactions of other cysteine proteinases (e.g. papain, actinidin, ficin and papaya proteinase Ω) where k is maximal in the pH range approx. 3–4 and (ii) the reactivity of the calpain II 80 kDa/18 kDa heterodimer in the bell-shaped component of the profile at low pH is substantially greater than the reactivities of the other cysteine proteinases. Indeed the reactivity of the calpain II 80 kDa/18 kDa heterodimer towards 2-Py-S-S-2-Py at low pH is the highest of any cysteine proteinase found to date. The next highest reactivity so far recorded is that of ficin (see Figure 4). The occurrence of the acid bell component

Table 1 Comparison of the characterizing parameters of the main feature of the reactions of the calpain II 80 kDa/18 kDa heterodimer and some other cysteine proteinases with 2-Py-S-S-2-Py in acidic media at 25 °C and / 0.1 mol/l

The characterizing parameters are related to the experimentally determined pH-dependent second-order rate constant (k) by $k = \tilde{k} / \{1 + ([H^+]/K_i) + (K_{eI}/[H^+])\}$.

Enzyme	Molecular pK values		pH-independent rate constant ($10^{-4} \times \tilde{k}$) ($M^{-1} \cdot s^{-1}$)	Reference
	pK _i	pK _{II}		
Calpain II 80 kDa/18 kDa heterodimer	4.4	5.6	40.0	Present work
Papain	3.85	3.9	4.2	Shipton and Brocklehurst (1978)
Ficin	2.4	3.8	11.0	Malthouse and Brocklehurst (1976)
Papaya proteinase Ω	2.45	3.4	2.8	Topham et al. (1991)
Actinidin	2.5	3.0	2.4	Salih et al. (1987)

(Figures 3 and 4) at higher pH values for the reaction of the calpain II 80 kDa/18 kDa heterodimer than found for reactions of the other cysteine proteinases derives from its pK_a values, particularly the higher value of pK_{iI}. Thus whereas for the reactions of the other cysteine proteinases shown in Figure 4 the values of pK_{iI} are in the range 3.0–3.9, pK_{iI} for the reaction of the calpain II 80 kDa/18 kDa heterodimer is substantially higher (approx. 5.6). The pK_a values and pH-independent rate constants (k) characteristic of the main feature of the reactions of the five cysteine proteinases with 2-Py-S-S-2-Py in acidic media are compared in Table 1. It is particularly noteworthy that, as is the case for the papain reaction, the reaction of the calpain II 80 kDa/18 kDa heterodimer is characterized by two molecular pK_a values associated with the enzyme molecule, i.e. in each case the value of pK_i is substantially greater than 2.45 (the pK_a value of 2-Py-S-S-2-Py⁺H). This contrasts with the reactions of the three other cysteine proteinases where the value of pK_i is closely similar to the pK_a of the monohydrated probe reagent.

The most obvious interpretation of the rapid reactions associated with the bell-shaped components of Figures 3 and 4 is that they are reactions of the activated monohydrated form of the inhibitor, 2-Py-S-S-2-Py⁺H, with enzyme forms containing a catalytic site -S⁻/-Im⁺H ion pair. In all cases the pH-dependence of the experimentally determined value of k is described by eqn. (1) which relates to the generalized reactant state of Scheme 1(a):

$$k = \frac{\tilde{k}_{xH_2}}{1 + \frac{[H^+]}{K_i} + \frac{K_{iI}}{[H^+]}} + \frac{\tilde{k}_{xH}}{1 + \frac{[H^+]^2}{K_i K_{iI}} + \frac{[H^+]}{K_{iI}}} + \frac{\tilde{k}_x}{1 + \frac{[H^+]}{K_{iI}}} \quad (1)$$

For the reactions of ficin, actinidin and papaya proteinases Ω , where the value of pK_i corresponds to the pK_a value of 2-Py-S-S-2-Py⁺H (pK_r), the model shown in Scheme 1(c) is appropriate and more informative than the generalized-reactant state model

of Scheme 1(a). The pH-dependence of k in the belt-shaped component at low pH may be described therefore by eqn (2).

$$k = \frac{\tilde{k}'_{xH_2} \left(\frac{K_e}{K_e + K_r} \right)}{\frac{[H^+]}{K_e + K_r} + \frac{K_e K_r / (K_e + K_r)}{[H^+]}} \quad (2)$$

The value of the numerator of eqn. (2) corresponds to the experimentally determined value of the pH-independent rate constant, $\tilde{k}_{xH_2}$ of Scheme 1(a) and eqn. (1). This value needs to be multiplied by the assembly of acid dissociation constants $[(K_e + K_r)/K_e]$, which approximates to K_r/K_e when $K_r \gg K_e$ to provide the value of the rate constant $\tilde{k}'_{xH_2}$, characteristic of the reaction of the ion-pair state of the enzyme (EH₂ in Scheme 1c) with the activated probe cation, 2-Py-S-S-2-Py⁺H. Eqn. (2) allows for the possibility that, as is the case in the reactions considered here, the limbs of the bell-shaped curves correspond to 'crossed-over' ionizations [see Dixon and Webb (1964) and Malthouse and Brocklehurst (1976)]. Thus chemical logic insists that the increase in k with decrease in pH must result from hydronation of 2-Py-S-S-2-Py even though the pK_a observed to be associated with the increase in k is that associated with the conversion of reactive -S⁻/-Im⁺H ion pair to unreactive -SH/Im⁺H. Reaction in the bell-shaped components of Figure 4 (curves a–c), therefore corresponds to reaction of minor ionic forms of the reactant state and thus the 'true' rate constant $\tilde{k}'_{xH_2}$ has values considerably larger than the experimentally determined values of $\tilde{k}_{xH_2}$ (see Table 2).

In marked contrast with the reactions of ficin, actinidin and papaya proteinases Ω , reactions of both papain (Figure 4, curve d) and the calpain 80 kDa/18 kDa heterodimer (Figure 3 and Figure 4, curve e) with 2-Py-S-S-2-Py are characterized by pH– k profiles in which the value of pK_i is considerably larger than that of pK_r (2.45). The simplest model for these two reactions therefore is that of Scheme 1(b) in which all ionizations are associated with the enzyme molecule. In terms of this model, for the reaction of papain pK_{eI} $\approx$ pK_{eII} $\approx$ 3.9 and for the reaction of the calpain II 80 kDa/18 kDa heterodimer pK_{eI} = 4.4 and pK_{eII} = 5.6. As was pointed out above, however, chemical logic compels the view that these reactions in acidic media involve the catalytic site -S⁻/-Im⁺H ion pair and the hydronated (activated) probe cation (2-Py-S-S-2-Py⁺H). A more appropriate model therefore is that shown in Scheme 1(d) in which reactions with both hydronated and non-hydronated forms of the probe molecule are explicitly recognized. Reaction in acidic media in terms of this model is described by eqn. (3) when $K_r \gg K_{eI}$.

$$k \approx \frac{\tilde{k}'_{xH_2} \left(\frac{K_{eI}}{K_r} \right)}{1 + \frac{[H^+]}{K_r} + \frac{K_{eI}}{[H^+]} + \frac{K_{eI} K_{eII}}{[H^+]^2}} + \frac{\tilde{k}'_{xH_2} + \tilde{k}^*_{xH_2} \left(\frac{K_{eII}}{K_r} \right)}{1 + \frac{[H^+]}{K_{eI}} + \frac{K_{eII}}{[H^+]} + \frac{[H^+]^2}{K_{eI} K_r}} + \frac{\tilde{k}_{xH}}{1 + \frac{[H^+]}{K_{eII}} + \frac{[H^+]^2}{K_{eI} K_{eII}} + \frac{[H^+]^3}{K_{eI} K_{eII} K_r}} \quad (3)$$

The model of Scheme 1(d) and eqn. (3) describe the reactions of both papain (Figure 4, curve d) and the calpain II

Scheme 1 Kinetic models for the reaction of an enzyme, E-EH₃, with a two-hydronic-state reactivity probe, R + R⁺H

K_I – K_{III} , K_e , K_r and K_{eI} – K_{eIII} are macroscopic acid dissociation constants and $\tilde{k}_{\text{XH}_2}$ – $\tilde{k}_{\text{X}}$ etc. are pH-independent rate constants. Free hydrons (H⁺) and formal charges of the various ionization states, except for R⁺H, are omitted for clarity. Model (a) uses different hydronic states of a generalized reactant state XH₃–X in which enzyme and reactivity probe (or substrate in other contexts) are not distinguished (Brocklehurst and Brocklehurst, 1988). In model (b) all of the hydronic dissociations are assigned to the enzyme molecule. Model (c) involves reaction of a particular hydronic state of an enzyme (EH₂) with the hydronated form of the probe (R⁺H). Model (d) (Brocklehurst and Malthouse, 1980) explicitly acknowledges reaction of two reactive hydronic states of an enzyme (EH₂ and EH) with both forms of the reactivity probe (R⁺H and R). In the context of the present paper reactions via the enzyme states EH₂ and EH are considered to involve reactions of the catalytic site (Cys)-S[−]/(His)-Im⁺H ion pair in two enzyme forms one of which contains a hydron-rich form of another ionizing group and one a hydron-poor form of this modulatory group.

80 kDa/18 kDa heterodimer (Figures 3 and 4, curve e) provided that reaction of 2-Py-S-S-2-Py⁺H with EH (rate constant $\tilde{k}_{\text{XH}_2}^*$) is the overwhelmingly dominant pathway of product formation. For the reaction of papain $\tilde{k}_{\text{XH}_2}^*$ was calculated to be approx. $1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Brocklehurst and Malthouse, 1980) and, in the present work, for the reaction of the calpain II 80 kDa/18 kDa heterodimer, $\tilde{k}_{\text{XH}_2}^*$ is calculated to be $5.7 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$. Table 2 presents a comparison of the rate constants for the reactions of 2-Py-S-S-2-Py⁺H with the reactive -S[−]/Im⁺H ion-pair states of all five cysteine proteinases. The intrinsic reactivity of the calpain II 80 kDa/18 kDa heterodimer is almost 700 times greater than that of actinidin (the least reactive enzyme in Table 2). This reactivity ratio is considerably larger than those for ficin (30), papain (14) and papaya proteinase Ω (3). A possible explanation for the large variation in reactivity towards 2-Py-S-S-2-Py⁺H may derive from variations in the electrostatic field effects to which the various catalytic-site regions are exposed [see Kowlessur et al. (1989b) and Topham et al. (1991) and references therein for a comparison of the active-centre regions of papain, actinidin and papaya proteinase Ω].

The values of the macroscopic acid dissociation constants $\text{p}K_{eI} = 4.4$ and $\text{p}K_{eII} = 5.6$ are characteristic of the calpain 80 kDa/18 kDa heterodimer. One of these values is presumably

associated with the formation of the (Cys)-S[−]/(His)-Im⁺H ion pair and the other with another hydronic dissociation that must be postulated to be necessary for the high reactivity of the thiolate anion component with 2-Py-S-S-2-Py⁺H. The $\text{p}K_a$ value associated with the formation of the Cys-S[−]/His-Im⁺H state of many other cysteine proteinases is in the range 3.0–4.0 (see e.g. Table 1), and another dehydronation with $\text{p}K_a$ value approx. 5.0–6.0 has been shown to affect the active-centre behaviour of some cysteine proteinases to various extents and in various situations. For example, ionizations with $\text{p}K_a$ values of 5.5 and 5.8 are major features of the pH-dependence of $k_{\text{cat.}}/K_m$ for the catalysis of the hydrolysis of L-Bz-Arg-Nan by actinidin and papaya proteinase Ω respectively and an ionization with $\text{p}K_a$ value of 5.2 is a noticeable feature of the reactions of some 2-pyridyl disulphide inhibitors with the catalytic-site thiol group of actinidin (see Figure 5). Also, although a $\text{p}K_a$ value of 5.0–6.0 does not influence the catalytic behaviour of native papain (see e.g. Figure 5), an ionization with $\text{p}K_a$ 5.14 is a major feature of the pH-dependence of $k_{\text{cat.}}/K_m$ for the hydrolysis of Z-Phe-Arg-MCA catalysed by the Ser-176-Ala papain mutant (Ménard et al. 1991b). Ser-176 is situated at the interface of the two domains of papain close to the catalytic site and forms an interdomain hydrogen bond with Gln-19. Its replacement with Ala removes

Table 2 Comparison of the values of the rate constant ($\bar{k}'$) for the reactions of the 2-Py-S-S-2-Py⁺H cation with the (Cys)-S⁻/(His)-Im⁺H ion-pair states of the calpain II 80 kDa/18 kDa heterodimer and some monomeric non-Ca²⁺-activated cysteine proteinases

For the reactions of the calpain II 80 kDa/18 kDa heterodimer and papain, the values of $\bar{k}'$ are calculated as values of $\bar{k}'_{\text{XH}_2}$ from eqn. (3) of the text and values of $\bar{k}$ (Table 1), $pK_i = 2.45$ and the values of pK_{cat} given as pK_{II} in Table 1. For the reactions of the other three cysteine proteinases, the values of $\bar{k}'$ are calculated as values of $\bar{k}'_{\text{XH}_2}$ from eqn. (2) of the text, and values of $\bar{k}$ (Table 1), $pK_i = 2.45$ and the values of pK_{cat} given as pK_{II} in Table 1; [1] denotes unity by definition.

Enzyme	$10^{-4} \times \bar{k}'$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$\frac{\bar{k}'_{\text{enzyme}}}{\bar{k}'_{\text{Act}}}$
Calpain II 80 kDa/18 kDa heterodimer	5700	679
Ficin	250	30
Papain	120	14
Papaya proteinase Ω	25	3
Actinidin (Act)	8.4	[1]

this hydrogen bond and could modulate the interaction within the Cys-25/His-159 interactive system.

Interpretation of the data shown in Figure 3 in terms of Scheme 1(d) and eqn. (3) suggests that the form of the calpain II 80 kDa/18 kDa heterodimer that exhibits nucleophilic character in its catalytic-site thiol group in acidic media is doubly dehydrated (macroscopic pK_a values 4.4 and 5.6) and contains not only the -S⁻/Im⁺H state but also a hydron-poor form of another ionizing group. If the microenvironment of the catalytic site of the calpain II 80 kDa/18 kDa heterodimer is not markedly dissimilar to those of the other cysteine proteinases discussed above, the assignment of the pK_a value of 4.4 to the formation of the ion-pair state would seem the more likely of the two possibilities. The value of the other hydronic dissociation (pK_a 5.6) that is needed to produce nucleophilic character in the catalytic-site ion pair suggests that it might be associated with a carboxy group (Asp or Glu) in a relatively hydrophobic environment or possibly with an imidazolium ion (i.e. another His) also in a relatively hydrophobic environment. Development of high nucleophilic character in a -S⁻/Im⁺H ion pair might be dependent on some separation of the ion-pair components. In reactions of papain, this process appears to be linked with the possibility of making particular combinations of specific binding interactions in enzyme-inhibitor complexes (Brocklehurst et al., 1988; Kowlessur et al., 1989a,b). Another mechanism by which movement of ion-pair components could be promoted is by the electrostatic influence of a third ionic component. The data discussed above suggest that this type of ionic perturbation mechanism could be operative in the calpain II 80 kDa/18 kDa heterodimer. One version of such a mechanism involves adjustment of the position of the (His)-Im⁺H component of the ion pair directly or indirectly by the influence of a carboxylate anion generated by hydronic dissociation across pK_a 5.6. This type of mechanism involving a major and direct role for Asp-158 has been considered in the past for papain (e.g. Angelides and Fink, 1979a,b; Brocklehurst and Malthouse, 1980) but more recently a major role for Asp-158 has been discounted and evidence and argument for more subtle roles for this residue have been presented (Pickersgill et al., 1988a,b; Brocklehurst et al., 1988; Kowlessur et al., 1989b; Menard et al., 1990, 1991a). The kinetically influential pK_a approx. 4 in papain (additional to that characteristic of the formation of the ion-pair state) observed in

Figure 5 Examples of reactions of some cysteine proteinases in which pH-dependent kinetic behaviour includes a kinetically influential ionization with pK_a 5.0–6.0

(a) pH-dependence of k_{cat}/K_m for the hydrolysis of α -Bz-L-Arg-Nan catalysed by (i) papain, (ii) actinidin and (iii) papaya proteinase Ω . The lines are theoretical for six- or eight-parameter pH-dependent kinetic equations [eqn. (1) of the text and eqn. (5) of Brocklehurst and Brocklehurst (1988) respectively] and the following values of the characterizing parameters (with values of $\bar{k}_{\text{cat}}/\bar{K}_m$ in $\text{M}^{-1} \cdot \text{s}^{-1}$: for (i) ordinate scaling factor, $n = 1$, $pK_i = 3.7$, $pK_{\text{II}} = 4.2$, $pK_{\text{III}} = 8.0$, $pK_{\text{IV}} = 9.6$, $(\bar{k}_{\text{cat}}/\bar{K}_m)_1 = 50$, $(\bar{k}_{\text{cat}}/\bar{K}_m)_2 = 220$, $(\bar{k}_{\text{cat}}/\bar{K}_m)_3 = 8$ (C. M. Topham, E. Salih and K. Brocklehurst, unpublished work); for (ii) $n = 25$, $pK_i = 3.1$, $pK_{\text{II}} = 5.5$, $pK_{\text{III}} = 9.7$, $25 \times (\bar{k}_{\text{cat}}/\bar{K}_m)_1 = 68.75$, $25 \times (\bar{k}_{\text{cat}}/\bar{K}_m)_2 = 212.5$ (Salih et al., 1987); for (iii) $n = 10$, $pK_i = 3.4$, $pK_{\text{II}} = 5.8$, $pK_{\text{III}} = 8.0$, $pK_{\text{IV}} = 9.4$, $10 \times (\bar{k}_{\text{cat}}/\bar{K}_m)_1 = 84$, $10 \times (\bar{k}_{\text{cat}}/\bar{K}_m)_2 = 220$, $10 \times (\bar{k}_{\text{cat}}/\bar{K}_m)_3 = 180$ [Topham et al., 1991; in Figure 7(b) of that paper, p. 87, column 2, the labels (ii) and (iii) were printed wrongly and need to be interchanged]. The existence of kinetically influential ionizations with pK_a values of 5.5 and 5.8 respectively for the pH versus k_{cat}/K_m profiles for actinidin [curve (ii)] and papaya proteinase Ω [curve (iii)] is noteworthy and contrasts with the lack of an analogous ionization with pK_a 5.0–6.0 in the profile for papain [curve (i)]. (b) pH-dependence of k for the reaction of actinidin with 2-(acetamido)ethyl 2'-pyridyl disulphide. The continuous line is theoretical for an eight-parameter pH-dependent kinetic equation with the following values of the characterizing parameters (with values of $\bar{k}$ in $\text{M}^{-1} \cdot \text{s}^{-1}$): $pK_i = 2.48$, $pK_{\text{II}} = 3.15$, $pK_{\text{III}} = 5.2$, $pK_{\text{IV}} = 9.5$, $\bar{k}_{\text{XH}_2} = 6.0 \times 10^3$, $\bar{k}_{\text{XH}_2} = 1.25 \times 10^3$, $\bar{k}_{\text{XH}_2} = 4.2 \times 10^2$, $\bar{k}_x = 3.4 \times 10^3$ (Brocklehurst et al., 1988). The broken lines [(i)–(iv)] correspond to the contributions of the four terms of the eight-parameter kinetic equation (Brocklehurst and Brocklehurst, 1988). Component (ii) is bounded by pK_a values 3.15 and 5.2.

the pH-dependence of both k_{cat}/K_m and k for reactions of 2-pyridyl disulphide reactivity probes therefore needs to be assigned to a group (possibly another carboxy group) remote from the catalytic site. The nature and extent of a catalytic role for the putative kinetically influential carboxy group of the calpain II 80 kDa/18 kDa heterodimer detected as a major feature of the reactivity probe data in Figure 3 remain to be discovered.

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