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Preparation of cathepsins B and H by covalent chromatography and characterization of their catalytic sites by reaction with a thiol-specific two-protonic-state reactivity probe

Kinetic study of cathepsins B and H extending into alkaline media and a rapid spectroscopic titration of cathepsin H at pH3–4

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1. A procedure for the isolation of cathepsin B (EC 3.4.22.1) and of cathepsin H from bovine spleen involving covalent chromatography by thiol–disulphide interchange and ion-exchange chromatography was devised. 2. The stabilities of both cathepsins in alkaline media are markedly temperature-dependent, and reliable kinetic data can be obtained at pH values up to 8 by working at 25°C with a continuous spectrophotometric assay. 3. Both enzyme preparations contain only one type of thiol group as judged by reactivity characteristics towards 2,2′-dipyridyl disulphide at pH values up to 8; in each case this thiol group is essential for catalytic activity. 4. Cathepsin H was characterized by kinetic analysis of the reactions of its thiol group with 2,2′-dipyridyl disulphide in the pH range approx. 2–8 and the analogous study on cathepsin B [Willenbrock & Brocklehurst (1984) *Biochem. J.* **222**, 805–814] was extended to include reaction at pH values up to approx. 8. 5. Cathepsin H, like the other cysteine proteinases, was shown to contain an interactive catalytic-site system in which the nucleophilic character of the sulphur atom is maintained in acidic media. 6. The considerable differences in catalytic site characteristics detected by this two-protonic-state reactivity probe between cathepsin B, cathepsin H, papain (EC 3.4.22.2) and actinidin (EC 3.4.22.14) are discussed. 7. Reaction with 2,2′-dipyridyl disulphide in acidic media, which is known to provide a rapid spectrophotometric active centre titration for many cysteine proteinases, is applicable also to cathepsin H. This is useful because other active-centre titrations have proved unsuitable in view of the relatively low reactivity of the thiol group in cathepsin H.

In a recent paper (Willenbrock & Brocklehurst, 1984) we reported considerable differences between cathepsin B (EC 3.4.22.1) and papain (EC 3.4.22.2) deduced from kinetic studies in acidic media. It was shown by reactivity-probe kinetics that nucleophilic character is generated in the sulphur atom of cathepsin B by protonic dissociation with pK_a 3.4, to form an interactive system presumably containing some S^-/ImH^+ ion-pair. Substrate-catalysis kinetics showed that ion-pair formation is not sufficient to generate catalytic competence in cathepsin B, however, and that some other event that occurs as the pH is raised across pK_a 5–6 is necessary to endow this ion-pair with catalytic capability.

In the present paper, reactivity-probe kinetics with 2,2′-dipyridyl disulphide have been applied to cathepsin H over a wide range of pH up to pH8, and the studies on cathepsin B have been extended to pH8. This is not a trivial extension, because substantial changes in the kinetic constants occur in the pH range 7–8. It proved possible to carry out reliable kinetic studies on these enzymes up to pH8, despite their well-known instability outside acidic media, by using continuous spectroscopic assays and by working at a lower temperature (25°C) than usually had been used hitherto. The

Abbreviations used: Z, benzyloxycarbonyl; -NNap, 2-naphthylamide.

considerable differences in catalytic-site characteristics detected between cathepsin B, cathepsin H, papain and actinidin (EC 3.4.22.14) are discussed. In addition to the kinetic studies, a new procedure for the isolation of both cathepsin B and cathepsin H from bovine spleen by methods involving both covalent chromatography (Brocklehurst *et al.*, 1973, 1985) and ion-exchange chromatography is reported, and the 2,2′-dipyridyl disulphide monocation is shown to be a valuable spectroscopic titrant for cathepsin H, a cysteine proteinase that has proved difficult to titrate in the past because of the low reactivity of its thiol group.

In the accompanying paper (Willenbrock & Brocklehurst, 1985), pH-dependent kinetics of substrate hydrolysis catalysed by cathepsins B and H are presented. Those results, together with the results from the reactivity-probe studies reported in the present paper and the amino acid sequence data of Takio *et al.* (1983), are shown to provide a general framework within which cysteine-proteinase mechanism may be further understood.

Materials and methods

Materials and general methods

The materials and many of the methods have been described previously (see Willenbrock & Brocklehurst, 1984). These include buffers, kinetic methods for both substrate-hydrolysis and reactivity-probe studies, data processing and spectroscopic properties of cathepsin B, pyridine-2-thione and 2-naphthylamine. Protein concentrations were determined by using the value of $\varepsilon_{280} = 4.0 \times 10^4\,M^{-1}\cdot cm^{-1}$ reported by Bajkowski & Frankfater (1983) for bovine spleen cathepsin B and the value of $\varepsilon_{280} = 3.4 \times 10^4\,M^{-1}\cdot cm^{-1}$ for cathepsin H. This value was calculated by using the values of $M_r = 28000$ and $A^{1\%}_{1cm} = 12.2$ reported for the human liver enzyme by Schwartz & Barrett (1980). Catalytic activities were determined by the continuous spectroscopic assays with Z-Arg-Arg-NNap for cathepsin B and Arg-NNap for cathepsin H as described by Willenbrock & Brocklehurst (1984).

Isolation of cathepsins B and H from bovine spleen by a procedure involving covalent chromatography

Frozen bovine spleen (2kg) was allowed to thaw overnight at 4°C and was then homogenized in 3 litres of a solution containing 5g of NaCl/l and 1mM-EDTA. The solution was adjusted to pH4 by addition of 1M-HCl, stirred overnight at room temperature (approx. 22°C) and centrifuged at 5000*g* for 30min at 4°C. The supernatant was adjusted to 40% saturation with solid $(NH_4)_2SO_4$ and centrifuged at 15000*g* for 30min at 4°C. Solid $(NH_4)_2SO_4$ was added to the supernatant to give a final concentration of 70% saturation, and the solution was centrifuged at 15000*g* for 30min at 4°C. The precipitate was resuspended in 50–100ml of water and was dialysed overnight against 2×5 litres of 1mM-EDTA at 4°C. The supernatant was concentrated by using an Amicon ultrafiltration cell with a Diaflo PM10 membrane to approx. 10ml.

The concentrated protein solution was applied to a column of Sephadex G-75 (90cm × 1cm) that had been equilibrated at room temperature (approx. 22°C) with 0.02M-sodium acetate buffer, pH4.0, containing 0.2M-NaCl and 1mM-EDTA, and was eluted with the same buffer at a flow rate of 16ml/h. This step and all subsequent chromatographic steps were carried out at room temperature. Cathepsins B and H were eluted together, and active fractions were pooled, concentrated to a volume of 10ml and adjusted to pH6.5 with 0.1M-NaOH. Solid cysteine was added to give a final concentration of 20mM, and the solution was stirred at room temperature for 30min, after which time an appropriate volume of a 50mM solution of dithiothreitol was added to give a final concentration of 1mM. After a further 5min the enzymes were separated from the low-M_r thiol by application to a column (30cm × 3cm) of Sephadex G-25 equilibrated in 0.1M-sodium acetate buffer, pH4.0, containing 0.3M-NaCl and 1mM-EDTA, and elution with the same buffer at a flow rate of 60ml/h; 8ml fractions were collected and those with A_{280} values greater than 0.2 were pooled and mixed with 50ml of the Pharmacia product 'activated thiopropyl-Sepharose-6B' (i.e. 2-pyridyl disulphide hydroxypropylether–Sepharose) that had been equilibrated in the acetate buffer. The mixture was stirred at room temperature for 1h, after which the gel was isolated on a sintered-glass funnel and washed with the acetate buffer (approx. 2 litres) until the values of A_{280} and A_{343} had fallen below 0.03. The gel was then allowed to equilibrate in 2 litres of 0.1M-KH_2PO_4/NaOH buffer, pH6.8, containing 0.3M-NaCl and 1mM-EDTA.

The covalently bound cathepsins B and H were released from the gel by stirring it with 100ml of 20mM-cysteine in the phosphate buffer for 45min, and the enzyme solution was collected on a sintered-glass funnel. This process was repeated with another 100ml of the cysteine solution, and the two enzyme solutions were pooled and concentrated to approx. 20ml. This solution was dialysed for 24h against 3×5 litres of 0.02M-sodium acetate buffer, pH5.0, containing 1mM-EDTA and was then applied to a column (13cm × 1cm) of CM-cellulose, pre-equilibrated in the same buffer. The column was washed with the acetate buffer until A_{280} fell below 0.02 (approx. 250ml), and the cathepsin B was eluted with a gradient of 200ml of acetate buffer and 200ml of the acetate buffer

containing 0.02M-NaCl. The column was then washed with the acetate buffer containing 0.05M-NaCl, and the cathepsin H was eluted with a gradient of 200ml of buffer containing 0.05M-NaCl and 200ml of buffer containing 0.15M-NaCl.

Active fractions of cathepsin B and of cathepsin H were each pooled, concentrated to a final protein concentration of 1–5mg/ml, and stored at 4°C. When stored in this state for periods of up to 1 month cathepsin H showed no loss of catalytic activity. Over the same length of time, however, approx. 50% of the cathepsin B active-centre thiol underwent irreversible oxidation. Residual, fully active, enzyme may be isolated at any time by an additional covalent chromatography step.

When cathepsin B was required for kinetic study it was activated by incubation for 20min with an appropriate volume of 60mM-L-cysteine in KH_2PO_4/NaOH buffer, pH6.8, containing 1mM-EDTA to give a final concentration of 5mM. Cathepsin H was incubated for 40min in an appropriate volume of 240mM-L-cysteine in the same buffer to give a final concentration of 20mM. The solutions were then made 1mM in dithiothreitol and the protein was separated from low-M_r thiol on a column (15cm × 2.5cm) of Sephadex G-25. The enzymes were eluted with 0.1M-KCl containing 1mM-EDTA at a flow rate of 60ml/h.

Activity-loss experiments

Cathepsins B and H (1–2μM) were each allowed to react at 25°C with various concentrations of 2,2′-dipyridyl disulphide that were lower than the active enzyme concentration. The reactions of cathepsin B were carried out at pH6.0 and those of cathepsin H at pH6.8. The reactions were followed to completion by measuring the release of 2-thiopyridone at 343nm, and the total absorbance change was therefore a direct measure of the concentration of blocked enzyme. A 0.2ml sample of each reaction mixture was then removed and assayed for activity with the appropriate substrate, Z-Arg-Arg-NNap for cathepsin B and Arg-NNap for cathepsin H at the same pH as that used for the thiol-modification reaction. The results were plotted as percentage activity against the concentration of blocked enzyme. The total active enzyme concentration was determined independently by using a large excess of 2,2′-dipyridyl disulphide.

Determination of the stabilities of cathepsin B and H at 25°C and at 37°C

Samples of activated enzyme (1.5ml of 10–20μM) were mixed with equal volumes of buffer containing 2mM-cysteine, and the mixtures were left to stand in a thermostat bath (25°C or 37°C). Samples were measured at $t = 0$ and at 5–10min intervals for up to about 1h and assayed for catalytic activity towards the appropriate substrate (see above) at the pH of the incubation and 25°C. Results were plotted as percentage of activity at $t = 0$ against time.

Results and discussion

Isolation of cathepsins B and H from bovine spleen by a procedure involving covalent chromatography

It seemed appropriate to apply covalent chromatography by thiol–disulphide interchange in the isolation of these two cysteine proteinases. This method, which was developed originally for the isolation of papain (Brocklehurst *et al.*, 1973), has been used subsequently for the isolation of a large number of thiol-containing proteins (for reviews see Lozinskii & Rogozhin, 1980; Brocklehurst *et al.*, 1985). We preferred our general and chromogenic method of covalent chromatography by thiol–disulphide interchange, which makes use of the remarkable properties of mixed disulphides containing the 2-pyridyl group (Brocklehurst, 1982) to the covalent affinity method reported by Evans & Shaw (1983) for the purification of cathepsin B, particularly as our aim was to isolate both cathepsins B and H (for a discussion of the differences in specificity of cathepsins B and H see Green & Shaw, 1981). By carrying out covalent chromatography with a Sepharose–2-pyridyl disulphide gel at low pH(3–4) it is possible to produce considerable selectivity for cysteine proteinases without any knowledge of important enzyme–ligand contacts in the extended binding site of each enzyme.

Zvonar *et al.* (1979) had reported a procedure for the isolation of cathepsins B and H from bovine lymph nodes that makes use of the Sepharose–glutathione–2-pyridyl disulphide gel. In the isolation of cathepsin B from bovine spleen (Willenbrock & Brocklehurst, 1984) it was not necessary to make major changes in this procedure. It was helpful, however, to introduce the following minor changes, which have been retained in the method here reported for the simultaneous isolation of cathepsins B and H: (i) application of the crude mixture containing the enzymes to the covalent-chromatography gel at pH4.0 rather than at pH5.0 to increase opportunity for selective attachment of cysteine proteinases, (ii) facilitation of the release of cysteine proteinases from the gel by reaction with cysteine at pH6.5 rather than at pH5.0, and (iii) replacement of dialysis for removal of low-M_r material after reductive activation of the enzymes by gel filtration to minimize re-oxidation of the enzyme thiol group. This procedure, however, was found to provide only a very low yield of cathepsin H, and a large fraction of this enzyme did not react readily with the Sepharose–glutathione–2-pyridyl

disulphide gel even at pH 5.0. A procedure that was successful in providing both cathepsins B and H from bovine spleen and that left no catalytic activity towards either Z-Arg-Arg-NNap or Arg-NNap in the unbound fraction of the covalent chromatography procedure was developed by including the following additional changes: (i) use of the Pharmacia product 'thiopropyl-Sepharose 6B' (2-pyridyl disulphide hydroxypropylether–Sepharose) developed by Axén *et al.* (1975) instead of the Sepharose–glutathione–2-pyridyl disulphide gel, (ii) use of a batchwise process of covalent chromatography similar to that used by Stuchbury *et al.* (1975) for the preparation of papain, and (iii) use of more vigorous conditions of reductive activation of the enzymes before their application to the covalent-chromatography gel (20mM-cysteine at pH6.5 instead of 5mM-cysteine at pH5.0). The last two changes are important because reduction of mixed disulphides involving the catalytic-site thiol group of cathepsin H appears to be relatively difficult.

The separations obtained in two of the three chromatographic steps are illustrated in Fig. 1. Chromatography of Sephadex G-75 (results not shown) provided a mixture containing cathepsins B and H, which were eluted together. Covalent chromatography was used to refine this mixture of enzymes and to ensure that only thiol-containing forms were collected. Fig. 1(*a*) illustrates the large amount of cathepsin H that is lost in the breakthrough fraction when the Sepharose–glutathione–2-pyridyl disulphide gel is used. This loss does not occur when the hydroxypropylether gel is used. The separation of the two cathepsins by ion-exchange chromatography is illustrated in Fig. 1(*b*). Most of the cathepsin H activity is eluted only after application of the 0.05–0.15M-NaCl gradient, whereas cathepsin B is eluted before this with a 0–0.02M-NaCl gradient. Cathepsin B activity is eluted in two peaks, with 60–70% of the enzyme in the first peak and 30–40% in the second. This is in accord with the report by Etherington (1974) but contrasts with that by Zvonar *et al.* (1979), who found only one peak of cathepsin B activity. The two fractions of cathepsin B produced in the present work were initially investigated separately by kinetics of hydrolysis of Z-Arg-Arg-NNap and by kinetics of the reactions of their essential thiol groups towards 2,2′-dipyridyl disulphide. In these respects they were found to be indistinguishable from each other and from recombined mixtures of the two fractions. Thereafter the two fractions were recombined and used for kinetic studies in

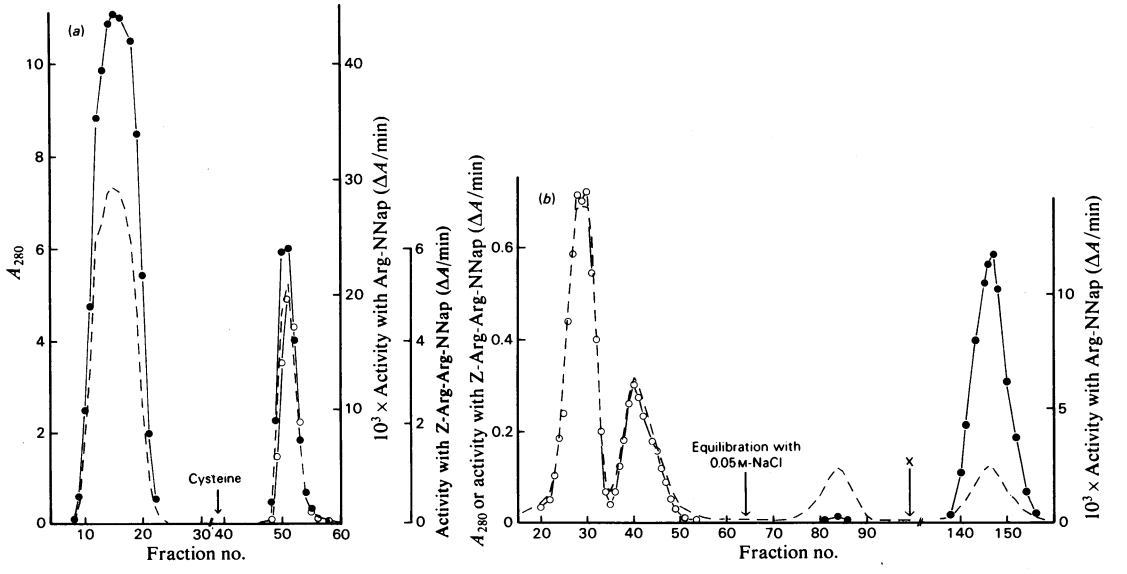

Fig. 1. *Some chromatographic steps in the isolation of cathepsins B and H from bovine spleen*

----, A_{280}; ○, activity towards Z-Arg-Arg-NNap; ●, activity towards Arg-NNap. (*a*) Covalent chromatography of the active fractions (38–45) from the Sephadex G-75 step by the method of Zvonar *et al.* (1979). Fractions 49–55 were pooled. When the procedure reported in the text was used, no catalytic activity towards either substrate appeared in the unbound fraction. (*b*) Separation of cathepsin H from cathepsin B by ion-exchange chromatography on CM-cellulose (CM-32). Flow rate, 60ml/h; fraction volume, 4ml. Cathepsin B was eluted with a gradient of 0–0.02M-NaCl in 0.02M-sodium acetate buffer, pH5.0, containing 1mM-EDTA, which was applied at fraction 0. Cathepsin H was eluted with a gradient of 0.05–0.15M-NaCl in the same buffer, which was applied at X.

order to conserve material. The fractions presumably contain closely related froms of cathepsins B differing sufficiently in charge to permit separation by ion-exchange chromatography but without any structural differences that determine catalytic-site reactivity characteristics or binding-site characteristics, at least towards the substrate and reactivity probe used in the present work. The cathepsin B fractions were devoid of activity towards Arg-NNap, and cathepsin H was devoid of activity towards Z-Arg-Arg-NNap, which confirms the lack of cross-contamination of the two enzymes (see Barrett, 1980).

Stabilities of cathepsins B and H

Cathepsin B from a variety of sources has been shown to be conformationally unstable in alkaline media. Studies of the pH-dependence of its catalytic activity have produced a rate optimum at pH approx. 6.5 with a very sharp decline at pH values above 7 and complete loss of activity at pH8, presumably due to denaturation (see, e.g., Barrett, 1972, 1973; Suhar & Marks, 1979). These studies have involved preincubation of the enzyme at 37°C or 40°C and analysis based on the stopped assay designed by Barrett (1972). In the present work it has been shown that the instability of cathepsin B from bovine spleen in weakly alkaline media is markedly temperature-dependent (Fig. 2), and that by carrying out kinetic studies at 25°C and using a continuous spectrophotometric assay it is possible to obtain reliable kinetic data up to pH8. The marked increase in $k_{cat.}/K_m$ for the cathepsin B-catalysed hydrolysis of Z-Arg-Arg-NNap in the pH range 7–8, reported in the accompanying paper (Willenbrock & Brocklehurst, 1985), which involves a decrease in K_m, supports the view that the pH-dependent changes in this pH range are not due to denaturation.

Fig. 3 shows that the stability in weakly alkaline media of cathepsin H also is considerably greater at 25°C than at 37°C.

Relationship between thiol modification and loss of catalytic activity

Fig. 4 shows that reaction of the accessible thiol group in both cathepsins B and H of bovine spleen with 2,2′-dipyridyl disulphide results in loss of catalytic activity. Thus these thiol groups are phenomenologically 'essential' for catalytic activity, and it seems reasonable to identify them as those of the catalytic-site cysteine residues [residues 29 and 26 in the case of the rat liver cathepsins B and H respectively (Takio *et al.* 1983)]. This conclusion is strongly supported by the nature of their pH-dependent reactivity characteristics (see below). If there are additional free thiol groups in these enzymes (see Takio *et al.*, 1983), either they react much more slowly with 2,2′-dipyridyl disulphide in the pH range 4–8 than do the catalytic-site

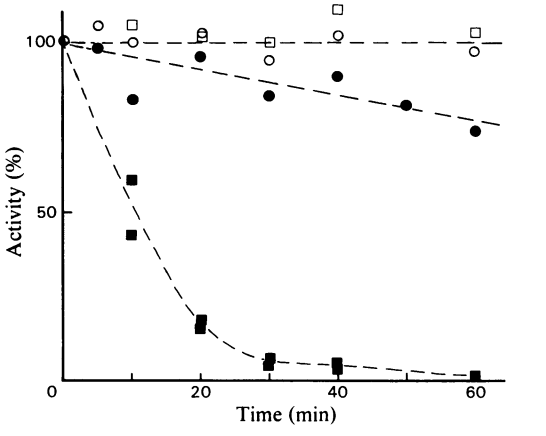

Fig. 2. *Comparison of the stability of cathepsin B at 25°C with that at 37°C at pH5.4 and at pH7.6*
The enzyme was incubated at the appropriate temperature in sodium acetate buffer, pH5.4 and *I*0.1, containing 2mM-cysteine or in KH_2PO_4/NaOH buffer, pH7.6 and *I*0.1, containing 2mM-cysteine. Samples (0.2ml) were withdrawn at intervals of time and assayed for catalytic activity towards Z-Arg-Arg-NNap in the presence of 2mM-cysteine in the same buffer used for the incubation but in all cases at 25°C. Activity is expressed as a percentage of that at zero time. ○, 25°C/pH5.4; □ 25°C/pH7.6; ●, 37°C/pH5.4; ■, 37°C/pH7.6.

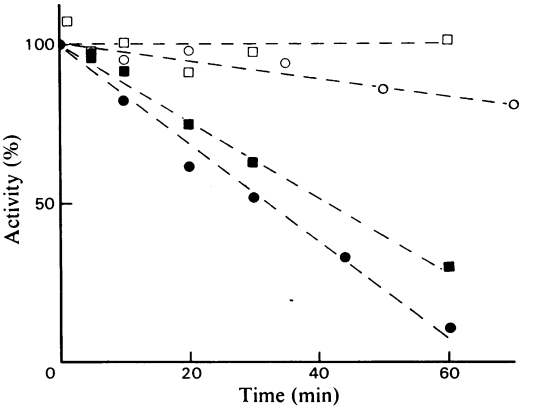

Fig. 3. *Comparison of the stability of cathepsin H at 25°C with that at 37°C at pH7.6 and at pH8.2*
The experiment was carried out as described in Fig. 2 legend, except that the substrate was Arg-NNap and the buffers were KH_2PO_4/NaOH, pH7.6 and *I*0.1, and Tris/HCl, pH8.2 and *I*0.1. □, 25°C/pH7.6; ○, 25°C/pH8.2; ■, 37°C/pH7.6; ●, 37°C/pH8.2.

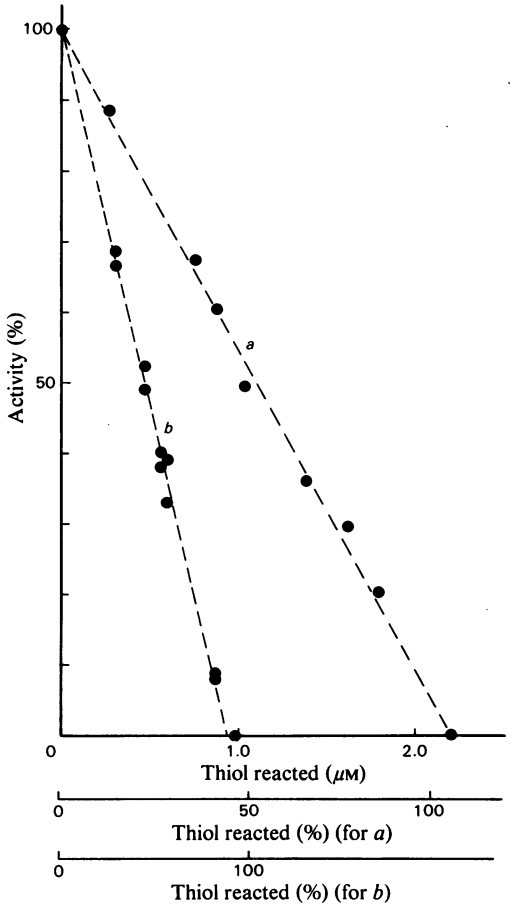

Fig. 4. *Demonstration of the dependence of catalytic activity (a) in cathepsin B and (b) in cathepsin H on the thiol group in each enzyme that reacts rapidly with 2,2′-dipyridyl disulphide in weakly acidic media*

The concentration of thiol that had reacted with a given small quantity of 2,2′-dipyridyl disulphide was determined by measuring the release of pyridine-2-thione at 343nm. The catalytic activity remaining was then measured by using Z-Arg-Arg-NNap as substrate, in the absence of cysteine, for cathepsin B, and by using Arg-NNap as substrate, in the absence of cysteine, for cathepsin H. Thiol-group modification and measurement of catalytic activity was carried out at pH6.0 for cathepsin B and at pH6.8 for cathepsin H. In each case the concentration of active enzyme present initially, i.e. before the addition of any 2,2′-dipyridyl disulphide, was determined by reaction with a large excess of 2,2′-dipyridyl disulphide in a separate experiment. These values (2.1 μM for cathepsin B and 1.0 μM for cathepsin H) corresponded closely (±10%) with the values obtained in the stepwise-addition experiments when catalytic activity had been abolished.

thiol groups or they exist in a modified, non-nucleophilic, state (such as sulphinic acid) in these preparations.

Detection and characterization of the interactive catalytic-site system of cathepsin H by two-protonic-state reactivity probe kinetics: comparison with the analogous systems in cathepsin B, papain and actinidin

The presence of an interactive system, presumably containing the S^-/ImH^+ ion-pair, which appears to be a common feature of cysteine proteinases including cathepsin B (see Willenbrock & Brocklehurst, 1984), is demonstrated in cathepsin H by the data in Fig. 5. The shape of this pH–second-order rate constant (k) profile in the pH range 2–8 with a rate maximum at low pH is characteristic of the reactions of such catalytic-site systems with 2,2′-dipyridyl disulphide, a reagent that increases its reactivity consequent on protonation (Brocklehurst, 1982). The pH–k profiles for the reactions of this reagent with cathepsin B, cathepsin H, actinidin and papain are compared in Fig. 6, which contains the new data collected for the cathepsin B reaction in the pH range 7.0–8.0 and a new theoretical line for the papain reaction that takes account of the relatively high values of k at pH values around 4.8–5.5 reported by Shipton & Brocklehurst (1978). The characterizing parameters for the reactions of 2,2′-dipyridyl disulphide with the cysteine proteinases each in the four reactive protonic states that are revealed by the pH–k profiles are shown in Table 1.

The following features of Fig. 6 and the data in Table 1 are particularly worthy of comment. (i) Only in the case of papain is the value of pK_I sufficiently different from the value of the pK_a of the 2,2′-dipyridyl disulphide monocation to provide convincing evidence for a kinetically influential group of pK_a 3–4 additional to that (pK_{II}) which characterizes the formation of the interactive nucleophilic state probably containing the S^-/ImH^+ ion-pair; pK_{II} is approx. 4 in papain and approx. 3 in the other three enzymes. (ii) The pK_a that appears to control the reactivity of S^-/ImH^+ towards the cationic form of the reagent (pK_{II}) is in the range 5–6 for the three enzymes in which its presence is manifest; the reactivity in the state in which this group is proton-rich ($\tilde{k}_2$) is lower than that where it is proton-deficient ($\tilde{k}_3$) for cathepsins B and H, but the reverse of this ($\tilde{k}_2 > \tilde{k}_3$) applies to papain. (iii) There is no evidence from these data for such a group in actinidin and, if it exists, presumably $\tilde{k}_2 \simeq \tilde{k}_3$. A group with pK_a 5.5 does appear to control the reactivity of the ion-pair of actinidin towards another electrophilic reactivity probe, benzofuroxan (Salih & Brocklehurst, 1983), whereas no such effect is seen in the corresponding reaction of papain (Shipton & Brocklehurst, 1977). If the state of ionization of the groups with pK_a 5–6 controls the mutual disposition of the two compo-

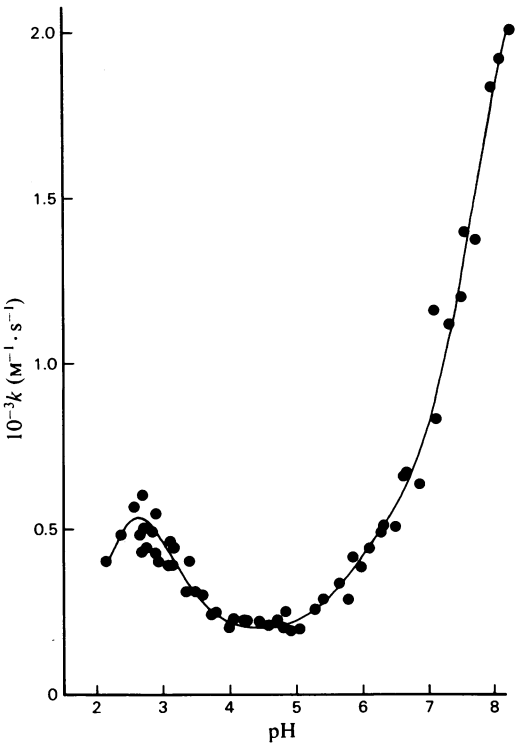

Fig. 5. *pH-dependence of the second-order rate constant (k) for the reaction of the catalytic-site thiol group of cathepsin H with 2,2'-dipyridyl disulphide at 25°C at 10.1*

The points are experimental and the line is theoretical for:

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

in which $\tilde{k}_1 = 1.15\times10^3\,M^{-1}\cdot s^{-1}$, $\tilde{k}_2 = 170\,M^{-1}\cdot s^{-1}$, $\tilde{k}_3 = 500\,M^{-1}\cdot s^{-1}$, $\tilde{k}_4 = 2.5\times10^3\,M^{-1}\cdot s^{-1}$, $pK_I = 2.45$, $pK_{II} = 2.7$, $pK_{III} = 5.8$ and $pK_{IV} = 7.7$. This equation relates to reaction of a system with four reactive protonic states produced sequentially from a fifth state of zero reactivity, which predominates at low pH, i.e.:

$$EH_4 \overset{pK_I}{\rightleftharpoons} EH_3 \overset{pK_{II}}{\rightleftharpoons} EH_2 \overset{pK_{III}}{\rightleftharpoons} EH \overset{pK_{IV}}{\rightleftharpoons} E$$
$$\downarrow \tilde{k}_1 \qquad \downarrow \tilde{k}_2 \qquad \downarrow \tilde{k}_3 \qquad \downarrow \tilde{k}_4$$

in which only pK_I and pK_{II} are sufficiently close together to necessitate the inclusion of a squared term in $[H^+]$; terms with higher multiple powers of $[H^+]$ also may be neglected (see Brocklehurst, 1974); $\tilde{k}_1$–$\tilde{k}_4$ are pH-independent (pH-corrected) rate constants and pK_I–pK_{IV} are system (macroscopic) pK_a values.

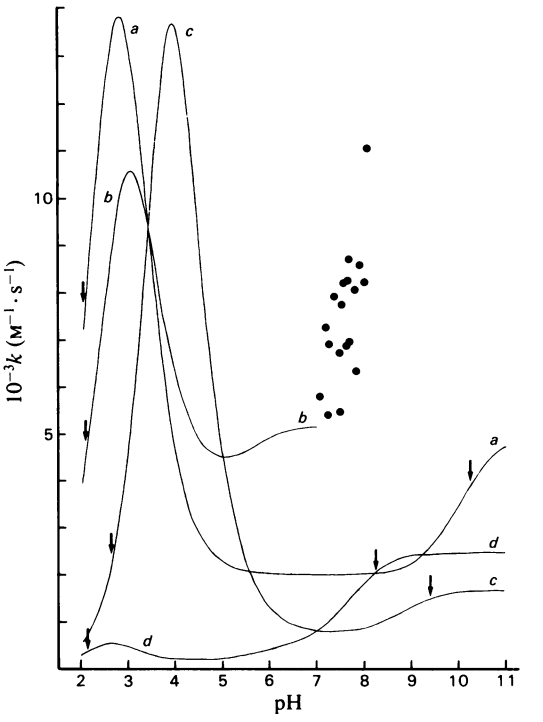

Fig. 6. *pH-dependence of the second-order rate constant (k) for the reaction at 25°C at 10.1 of 2,2'-dipyridyl disulphide with (a) actinidin, (b) cathepsin B, (c) papain and (d) cathepsin H*

The new data for the cathepsin B reaction in the pH range 7–8 are shown as ●. The lines are theoretical for eqn. (1) of Fig. 5 legend and the values of the characterizing parameters are given in Table 1. The line for the cathepsin H reaction (*d*) is taken from Fig. 5, that for the actinidin reaction (*a*) from unpublished work by E. Salih & K. Brocklehurst, that for the cathepsin B reaction (*b*) from Willenbrock & Brocklehurst (1984) and that for the papain reaction (*c*) by fitting the data of Shipton & Brocklehurst (1978) to eqn. (1) of Fig. 5 legend with a PDP11/10 computer. The arrows indicate the extent of the data used to obtain the theoretical lines.

nents (S^- and ImH^+) of the ion-pairs, it might be expected that its kinetic effect for a given enzyme might vary from one type of electrophilic probe to another if the probes are bound differently. The fact that, for a given probe reagent or set of different probe reagents, the kinetic effect of this group varies from one enzyme to another suggests differences between the enzymes in ion-pair geometry or in the pH-induced change in ion-pair geometry. (iv) The value of the pK_a that characterizes formation of uncomplicated thiolate anion by deprotonation of ImH^+ (pK_{IV}) varies from enzyme to enzyme, between 7.7 (in cathepsin H) and 10 (in

Table 1. *Characterizing parameters of the reactions at 25°C and I0.1 of 2,2′-dipyridyl disulphide with the catalytic-site thiol groups of cathepsins B and H, actinidin and papain*

The pK_a values and the pH-independent rate constant ($\tilde{k}$) relate to the expression for the pH-dependence of the second-order rate constant (k) given in Fig. 5 legend. For the actinidin reaction (*) the additional sigmoidal wave seen in the other pH–k profiles with pK_a 5–6 was not convincingly demonstrated, although some evidence for such a wave was obtained for the analogous reaction with n-propyl 2-pyridyl disulphide (Brocklehurst *et al.*, 1981). The apparent absence of this sigmoid wave from the pH–k profile for the reaction of actinidin with 2,2′-dipyridyl disulphide could be due to closely similar values of $\tilde{k}_2$ and $\tilde{k}_3$, which has been assumed in this Table. Abbreviation used: N.D., not determined.

Enzyme	pK_I	pK_{II}	pK_{III}	pK_{IV}	$\tilde{k}_1$ ($M^{-1}\cdot s^{-1}$)	$\tilde{k}_2$ ($M^{-1}\cdot s^{-1}$)	$\tilde{k}_3$ ($M^{-1}\cdot s^{-1}$)	$\tilde{k}_4$ ($M^{-1}\cdot s^{-1}$)	Reference
Cathepsin B	2.6	3.3	5.5	>8	18000	3900	5200	>20000	Present work and Willenbrock & Brocklehurst (1984)
Cathepsin H	2.45	2.7	5.8	7.7	1150	170	500	2500	Present work
Actinidin	2.45	3.1	N.D.	10.0	25900	2000*	2000	5000	E. Salih & K. Brocklehurst (unpublished work)
Papain	3.85	3.9	5.3	8.8	37000	2500	750	1700	Present work and Shipton & Brocklehurst (1978)

actinidin), which adds support to the striking evidence from probes of different charge status (Brocklehurst *et al.*, 1983) and from a spectroscopic reporter group (Brocklehurst *et al.*, 1984) for differences in the electric fields experienced by the catalytic-site thiol groups in different cysteine proteinases.

(v) Although the values of $\tilde{k}_1$ and $\tilde{k}_2$, which probably characterize the reactions of ion-pair forms of the enzyme with the cationic form of the reactivity probe, are considerably lower for the cathepsin H reaction than for those of the other cysteine proteinases, the values of $\tilde{k}_3$ and $\tilde{k}_4$ are similar to those for the papain reaction. The reactions of the uncomplicated thiolate anions of the enzymes each with the neutral form of the probe reagent (characterized by $\tilde{k}_4$) reveal a striking difference between the catalytic site of cathepsin B and those of the other three enzymes. When the catalytic sites are fully deprotonated, only cathepsin B reacts at a rate that is probably similar to those of the reactions of low-M_r thiols with 2,2′-dipyridyl disulphide.

These reactivity-probe data are discussed in relation to kinetics of catalysis and the known structural features of the various enzymes in the accompanying paper (Willenbrock & Brocklehurst, 1985).

2,2′-Dipyridyl disulphide as an active-centre titrant for cathepsin H

Two types of active-centre titrant for cysteine proteinases that have proved particularly useful are (*a*) disulphides containing the 2-pyridyl moiety such as 2,2′-dipyridyl disulphide (Brocklehurst & Little, 1970, 1973; Brocklehurst, 1982) and certain epoxides, notably L-*trans*-3-carboxy-2,3-epoxypropionyl-leucinamido-(4-guanidino)butane (E-64) (Barrett & Kirschke, 1981; Barrett *et al.*, 1981). The former provides a very quick and convenient spectrophotometric titration for studies on isolated enzymes in which the specificity for catalytic sites, even in the presence of other thiol groups, is achieved by the consequences of reagent protonation. The latter, though more laborious, is useful for studies of cysteine proteinases in tissue extracts because of the sensitivity that can be achieved when the epoxide inhibitor is used in conjunction with a fluorogenic substrate. Unfortunately, cathepsin H does not react sufficiently rapidly with E-64 to permit accurate active-centre titration (Barrett & Kirschke, 1981). By contrast, the reaction of the catalytic-site thiol group of cathepsin H with 2,2′-dipyridyl disulphide, although slower in acidic media than those of other cysteine proteinases, is still very much faster (k = approx. 250–450 $M^{-1}\cdot s^{-1}$ at pH 3–4) than the analogous reactions of low-M_r thiols and provides a convenient and accurate active-centre titration for this enzyme.

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