Natural structural variation in enzymes as a tool in the study of mechanism exemplified by a comparison of the catalytic-site structure and characteristics of cathepsin B and papain

pH-dependent kinetics of the reactions of cathepsin B from bovine spleen and from rat liver with a thiol-specific two-protonic-state reactivity probe (2,2'-dipyridyl disulphide) and with a specific synthetic substrate (*N*- α -benzyloxycarbonyl-L-arginyl-L-arginine 2-naphthylamide)

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1. Cathepsin B (EC 3.4.22.1) from bovine spleen and the analogous enzyme from rat liver were investigated at 25°C at 10.1 in acidic media by kinetic study of (a) the reactions of their catalytic-site thiol groups towards the two-protonic-state reactivity probe 2,2'-dipyridyl disulphide and (b) their catalysis of the hydrolysis of *N*- α -benzyloxycarbonyl-L-arginyl-L-arginine 2-naphthylamide. 2. Reactivity-probe kinetics showed that nucleophilic character is generated in the sulphur atom of cathepsin B by protonic dissociation with pK_a 3.4, presumably to form an S^-/ImH^+ ion-pair. 3. Substrate-catalysis kinetics showed that ion-pair formation is not sufficient to generate catalytic competence in cathepsin B, because catalytic activity is not generated as the pH is raised across pK_a 3.4 but rather as it is raised across pK_a 5–6 (5.1 for k_{cat} ; 5.6 for k_{cat}/K_m for the bovine spleen enzyme and 5.8 for k_{cat}/K_m for the rat liver enzyme). 4. The implications of these results and of known structural differences between the catalytic sites of the rat liver enzyme and papain (EC 3.4.22.2) for the mechanism of cysteine-proteinase-catalysed hydrolysis are discussed.

Investigation of the chemical mechanisms of enzyme catalysis is constrained by the extent of structural variation that it is possible to introduce into the components of the reactant state while retaining some catalytic competence and by the ease with which such variation may be designed and achieved. For enzymes, such as proteinases, that process oligomeric substrates, tolerance of structural change in the substrate or analogous reactivity probe permits mechanism-targeted kinetic studies based on systematic structural change in the low- M_r half of the enzyme-ligand reactant state. Application of the technique of site-directed mutagenesis should increasingly permit specific structural changes to be made in the enzyme molecule to test hypotheses concerning the roles of particular amino acid residues in the mechanism. Another approach to the study of the effects of

structural change in the enzyme on its catalytic properties, which is more immediately available, is to make use of a series of analogous or homologous enzymes for which there is evidence of differences in catalytic-site characteristics. The cysteine proteinases constitute such a series (see Brocklehurst *et al.*, 1983, and references cited therein).

A particular example of the way in which study of structurally different analogous enzymes may contribute to the delineation of structure-reactivity relationships is provided by a comparison of the catalytic-site characteristics of cathepsin B (EC 3.4.22.1) and papain (EC 3.4.22.2). A long-standing uncertainty in the mechanism of cysteine-proteinase-catalysed hydrolysis is the role, if any, of the carboxy group in the side chain of the amino acid residue adjacent on the *N*-terminal side to the catalytic-site histidine residue. This has been discussed mainly with regard to aspartic acid-158 and histidine-159 of papain (see, e.g., Angelides & Fink, 1978; Brocklehurst & Malthouse, 1978;

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

Baines & Brocklehurst, 1982). It was of particular interest, therefore, that Takio *et al.* (1983) reported that in rat liver cathepsin B the residue adjacent on the *N*-terminal side to the catalytic-site histidine (residue 197) is glycine (residue 196), and not aspartic acid as it is in papain. In the present paper the catalytic-site characteristics of cathepsin B from rat liver, cathepsin B from bovine spleen and papain are compared by considering the results obtained from pH-dependent kinetic studies on (a) the reactions of the catalytic-site thiol groups towards the two-protonic-state electrophile 2,2'-dipyridyl disulphide (see Brocklehurst, 1982, for a review of this type of reactivity-probe technique) and (b) the catalytic action of the enzymes on a specific substrate. Sufficient results were obtained with rat liver cathepsin B to suggest close similarity in its catalytic-site characteristics with bovine spleen cathepsin B. It was possible to collect considerably more data for the latter because of the ready availability of the enzyme in larger quantity. The considerable differences detected between the catalytic-site characteristics of cathepsin B and papain are discussed.

Materials and methods

Materials

Bovine spleen was obtained from the Municipal Abattoir, Woolwich, London SE18, U.K., stripped of its fasciae and larger blood vessels and stored frozen in 2 kg quantities. Rat livers donated by other research workers were stored frozen until the 500 g (approx. 60 livers) needed for a preparation of cathepsin B had been accumulated. Arg-NNap, the specific substrate for cathepsin H, which is a possible contaminant in preparations of cathepsin B, was obtained from Sigma Chemical Co. Stock solutions in dimethyl sulphoxide were stable at room temperature (approx. 22°C) for several weeks. 2,2'-Dipyridyl disulphide was the Aldrich product, twice recrystallized from light petroleum (b.p. 60–80°C), and as usual had m.p. 58°C. CM-cellulose (CM-32) was the Whatman product, analytical-grade anion-exchange resin AG1-X2 (100–200 mesh, Cl⁻ form) the product of Bio-Rad Laboratories and Sepharoses and Sephadexes were obtained from Pharmacia.

Preparation of cathepsin B and its substrate

Cathepsin B was prepared from bovine spleen and from rat liver essentially as described by Zvonar *et al.* (1979) and by Towatari *et al.* (1979) respectively. The enzymes were identified as cathepsin B by their ability to catalyse the hydrolysis of their specific substrate Z-Arg-Arg-NNap (see Barrett, 1980). The absence of cath-

epsin H was confirmed by the lack of catalytic activity towards its specific substrate Arg-NNap.

Z-Arg-Arg-NNap dipicrate was prepared by the method of Knight (1980). It had m.p. 226–227°C, which is the literature value. The dipicrate (0.5 g, 0.48 mmol) in 15 ml of aq. 67% (v/v) *NN*-dimethylformamide was passed through a column (2.5 cm × 10 cm) of Bio-Rad AG1-X2 resin. The yellow eluate was passed through a similar column of the same resin. The eluate from the second column was colourless. Removal of solvent left an oil, which was dissolved in water (20 ml). This stock solution (approx. 20 mM) was stored at 4°C and was stable for several months. Its concentration was determined by allowing a sample to undergo complete hydrolysis at pH 6.0 catalysed by cathepsin B (see below).

Continuous spectroscopic assay for cathepsin B

The hydrolysis of Z-Arg-Arg-NNap was monitored by following the release of 2-naphthylamine at 338 nm on the 0–0.02 and 0–0.05 ranges of a Cary 118 spectrophotometer. The substrate itself has negligible absorbance at this wavelength, and spontaneous hydrolysis was shown to be negligible in the pH range 4.0–8.3. Z-Arg-Arg-NNap could not be used in pyrophosphate or bicarbonate buffers, as a white precipitate formed. This was not due merely to a pH effect, because no precipitate formed on addition of the substrate to 0.1 M- or 0.01 M-NaOH. Routine assays were performed at 25°C in 0.1 M-sodium phosphate buffer, pH 6.0, containing 1 mM-EDTA and 2 mM-cysteine. The final substrate concentration was approx. 0.25 mM and the maximum active enzyme concentration was 0.02 μM. For kinetic analysis of the pH-dependence of the Michaelis parameters, substrate concentrations in the range 0.1–0.5 mM were used, other conditions being as stated for the routine assay.

pH-dependence of ϵ_{338} for 2-naphthylamine

At pH values above 7, ϵ_{338} was taken as the value reported by Lee *et al.*, (1971), i.e. $2.07 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. At pH values below 7, ϵ_{338} was predicted to decrease consequent on the protonation of 2-naphthylamine. The form of the pH-dependence was determined as follows. To a solution containing approx. 2 mM-Z-Arg-Arg-NNap in 0.1 M-phosphate buffer, pH 6.0, containing 1 mM-EDTA and 1 mM-cysteine was added bovine spleen cathepsin B to give a final concentration of approx. 1 μM, and the reaction was allowed to proceed to completion (approx. 4 h). Then 0.2 ml portions of the reaction mixture were added to a series of buffer solutions (pH 1.6–7) containing 1 ml of buffer (10.3) and 1.8 ml of water. A_{338} was recorded for each solution against a

reference cell containing a similar solution but devoid of substrate. The pH-dependence of ϵ_{338} is shown in Fig. 1 to follow eqn. (1):

$$\epsilon_{338} = \tilde{\epsilon}_{338} / (1 + [\text{H}^+]/K_a) \quad (1)$$

where $pK_a = 4.3$ and $\tilde{\epsilon}_{338} = 2.07 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. ϵ_{338} of the 2-naphthylammonium cation is negligible.

Continuous spectroscopic assay for cathepsin H

Cathepsin H (isolated from bovine spleen; F. Willenbrock & K. Brocklehurst, unpublished work) was assayed by monitoring the release of 2-naphthylamine from Arg-NNap by spectral analysis at 338 nm to demonstrate that any cathepsin H present in preparations of cathepsin B would be detectable. Assays were performed at pH 6.8 in 0.1 M-sodium phosphate buffer containing 1 mM-EDTA. Arg-NNap (33.6 mg) was dissolved in dimethyl sulphoxide (1 ml), and this was added to 99 ml of buffer to give a final concentration of 1 mM. Activation of cathepsin H by low- M_r thiol

was found to be slow. For evaluation of samples of cathepsin B for the presence of cathepsin H, therefore, the enzyme was preincubated with 20 mM-cysteine in the pH 6.8 buffer for 20 min. The activated enzyme solution (0.2 ml) was then added to the substrate solution (2.8 ml) to start the reaction.

Enzyme concentrations

Protein concentrations were determined by using the value for $\epsilon_{280} = 4.03 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ reported by Bajkowski & Frankfater (1983) for bovine spleen cathepsin B. Concentrations of enzyme active sites were determined by reaction with 2,2'-dipyridyl disulphide at pH 4 with the use of $\epsilon_{338} = 8.08 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Stuchbury *et al.*, 1975) to quantify the amount of 2-thiopyridone released.

Kinetics of catalysis

The adherence to Briggs-Haldane (Michaelis-Menten) kinetics was established at selected pH values by the linearity of plots of $[S]/v$ against $[S]$, and values of V and K_m were obtained by using the computer program described by Cleland (1963). In the study of the pH-dependence of k_{cat} , and of k_{cat}/K_m , most values of the parameters here reported were obtained by the half-time analysis described by Wharton & Szawelski (1982). The hydrolysis of the substrate was first followed to completion in order to obtain an accurate infinity reading.

Other methods

Most of the other methods and materials, notably buffers, determination of second-order rate constants (k) for reactions with 2,2'-dipyridyl disulphide and determination of the characterizing parameters in pH- k profiles by an optimization procedure, have been described previously (see Baines & Brocklehurst, 1982, and references cited therein). All kinetic studies were performed at 25°C at I 0.1. At pH values below 3, where the stoichiometry of the reactions of the enzyme thiol group with 2,2'-dipyridyl disulphide begins to decrease because of acid-promoted catalytic-site distortion within the time scale of a stopped-flow experiment, the rate constants were calculated by treating the progress curve as representing two parallel first-order reactions (Frost & Pearson, 1963), only the intact catalytic site reacting rapidly to produce 2-thiopyridone with consequent increase in A_{343} .

Results and discussion

Purification of Z-Arg-Arg-NNap

The final stage of the preparation of this substrate in the procedure described by Knight (1980)

Fig. 1. pH-dependence of ϵ_{338} of 2-naphthylamine at 25°C at I 0.1

The points are experimental and the continuous line is theoretical for:

$$\epsilon_{338} = \tilde{\epsilon}_{338} / (1 + [\text{H}^+]/K_a)$$

where $\tilde{\epsilon}_{338} = 2.07 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $pK_a = 4.31$.

involves conversion of the dipicrate into the dihydrochloride by passage through a single column of Bio-Rad AG1-X2 resin. When this procedure was followed, the aqueous solution that is the end product was always yellow. It liberated 2-naphthylamine on admixture with cathepsin B, but unless cysteine (5 mM) was incorporated into the assay mixture the progress curves were markedly curved from $t = 0$ and the enzyme activity was rapidly lost, presumably owing to inhibition by picric acid. When the ion-exchange step was repeated, however, the end product was colourless and the progress curves for the cathepsin B-catalysed hydrolysis were linear and identical with or without the inclusion of 5 mM-cysteine in the assay mixture. Thereafter, the inclusion of two ion-exchange treatments was instituted as a routine.

Assay of the hydrolysis of Z-Arg-Arg-NNap

The cathepsin B-catalysed hydrolysis of Z-Arg-Arg-NNap has usually been monitored (as, e.g., by Knight, 1980) by using procedures based on the stopped assay designed by Barrett (1972, 1976). In this assay the 2-naphthylamine produced is allowed to undergo a coupling reaction with the diazonium salt Fast Garnet GBC in the presence of a mercurial and the detergent Brij 35, and the absorbance of the azo dye produced is measured. This type of assay is not convenient for extensive kinetic analysis, and in the present work the formation of 2-naphthylamine was recorded directly and continuously as an increase in A_{338} . The release of product was quantified by making use of the pH-dependent value of ϵ_{338} [see Fig. 1 and eqn. (1)].

Kinetics of the cathepsin B-catalysed hydrolysis of Z-Arg-Arg-NNap

(i) *Determination of k_{cat} and K_m .* The objective of the study here reported was the characterization of the behaviour of cathepsin B in acid media by both substrate-hydrolysis kinetics and reactivity-probe kinetics. Cathepsin B has been reported to be unstable in alkaline media (see, e.g., Knight, 1980), and, in any case, study of the postulated interactive catalytic-site system was expected to be most informative when carried out in acidic media. The substrate-hydrolysis kinetics were studied mainly by using the half-time analysis of the integrated Michaelis equation described by Wharton & Szawelski (1982), which provides considerable economies in both time and materials. A prerequisite to the study of the pH-dependence of the Michaelis parameters by this method was a conventional kinetic study by measuring initial rates at various values of $[S]$ at pH 4.4 and at pH 7.4 to establish the adherence to the Michaelis equation

and to provide the K_m values needed to design the experiments for the half-time method.

At both of these pH values (which are close to the limits of pH in the pH-rate profiles here reported) the catalysis was shown to obey the Michaelis equation by the linearity of plots of $[S]/v$ against $[S]$ (Fig. 2). The values of k_{cat} and of k_{cat}/K_m provided by the initial-rate studies are in good agreement with those obtained at similar pH values by the half-time method (see Figs. 3 and 5).

The half-time analysis makes use of eqn. (3), which is derived from the integrated Michaelis equation eqn. (2) by substitution of $[S] = [S]_0/2$ when $t = t_1$:

$$V \cdot t = ([S]_0 - [S]) + K_m \cdot \ln([S]_0/[S]) \quad (2)$$

$$V \cdot t_1 = K_m \cdot \ln 2 + [S]_0/2 \quad (3)$$

Progress curves for the reaction followed to completion are analysed by using a series of local $[S]_0$ values calculated at various times along the progress curve and the corresponding t_1 values. A plot of t_1 against local $[S]_0$ gives a straight line (see Fig. 3 for an example) with slope $1/2V$ and ordinate intercept $\ln 2 \cdot K_m/V$. It should be noted that in the paper by Wharton & Szawelski (1982, p. 353, column 1, line 9) the value of the slope is printed incorrectly as $\frac{1}{2}V$.

(ii) *pH-dependence of the Michaelis parameters.* The pH-dependences of k_{cat}/K_m and of k_{cat} for the cathepsin B-catalysed hydrolysis of Z-Arg-Arg-NNap are shown in Figs. 4 and 5 respectively. More data were collected for the bovine spleen enzyme than for the rat liver enzyme, but it is clear that the reactions catalysed by both enzymes have similar pH-dependences. In acidic media both k_{cat}/K_m and k_{cat} increase with increasing pH across pK_a values 5–6. The pH-independent values of k_{cat} and k_{cat}/K_m ($\bar{k}_{cat}$ and $\bar{k}_{cat}/\bar{K}_m$) and the pK_a values are shown in Table 1, together with those for the corresponding catalysis by the human liver enzyme (reported by Knight, 1980, and refined by Barrett & Kirschke, 1981). The forms of the pH-dependences of the characterizing parameters for all three enzymes are very similar and, allowing for the different conditions used in the study of the human liver enzyme, the values of $\bar{k}_{cat}$ and of $\bar{k}_{cat}/\bar{K}_m$ are similar. The more limited data collected for the rat liver enzyme suggest that the pK_a that controls the pH-dependence of k_{cat}/K_m might be somewhat higher (approx. 5.8) than the corresponding value (5.6) obtained for the bovine spleen enzyme.

Detection and characterization of the interactive catalytic-site system of cathepsin B by two-protonic-state-reactivity-probe kinetics

Most of the cysteine proteinases studied to date each contain an interactive catalytic-site system

Fig. 2. Demonstration of the adherence to the Michaelis equation of the hydrolysis of Z-Arg-Arg-NNap catalysed by bovine spleen cathepsin B at 25°C at 10.1, (a) at pH 4.40 and (b) at pH 7.42

The points are $[S], v$ data pairs and the continuous lines are linear-regression lines corresponding to: for (a) $V = 1.22 \mu\text{M} \cdot \text{s}^{-1}$ (i.e. for $[E] = 0.081 \mu\text{M}$, $k_{\text{cat}} = 15.1 \text{ s}^{-1}$) and $K_m = 0.34 \text{ mM}$; for (b) $V = 0.40 \mu\text{M} \cdot \text{s}^{-1}$ (i.e. for $[E] = 0.0081 \mu\text{M}$, $k_{\text{cat}} = 49.6 \text{ s}^{-1}$) and $K_m = 0.045 \text{ mM}$.

Table 1. Characterizing parameters for the hydrolysis of Z-Arg-Arg-NNap catalysed by cathepsin B from three sources at 10.1 In the pH range approx. 4–7 both k_{cat} and k_{cat}/K_m vary according to $k = \tilde{k}/(1 + [\text{H}^+]/K_a)$ (see Figs. 4 and 5), where k represents the experimentally determined value of k_{cat} , or k_{cat}/K_m and $\tilde{k}$ represents the appropriate pH-independent parameter $\tilde{k}_{\text{cat}}$ or $\tilde{k}_{\text{cat}}/\tilde{K}_m$.

Source of enzyme	Temperature (°C)	$\tilde{k}_{\text{cat}}$ (s^{-1})		$10^{-5} \tilde{k}_{\text{cat}}/\tilde{K}_m$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)		Reference
		$\text{p}K_a$		$\text{p}K_a$		
Bovine spleen	25	5.1	76.8	5.6	7.1	Present work
Rat liver	25	5.0	68.7	5.8	5.0	Present work
Human	40	4.4	65.5	5.4	3.8	Knight (1980)
			178*		9.4*	Barrett & Kirschke (1981)

* In the present work the values were calculated by using catalytic-site concentrations determined by titration with 2,2'-dipyridyl disulphide at pH 3–4; the value for the human liver enzyme was calculated first on a protein basis (Knight, 1980) and subsequently by using catalytic-site concentrations determined by titration with L-3-carboxy-trans-2,3-epoxypropanoyl-leucylamido-(4-guanidino)butane (Barrett & Kirschke, 1981).

that produces nucleophilic character in the sulphur atom of the catalytic-site thiol group by protonic dissociation with $\text{p}K_a$ 3–4. This interactive system is commonly considered to contain S^-/ImH^+ ion-pair that allows both for nucleophilic attack of S^- on the substrate and for general acid catalysis by ImH^+ of leaving-group departure (see, e.g., Polgár

& Halász, 1982). Its existence is readily demonstrated by kinetic analysis of the reaction of the enzyme with a thiol-specific two-protonic-state reactivity probe such as 2,2'-dipyridyl disulphide (see Brocklehurst, 1982). The pH-dependence of the second-order rate constant for the reaction reveals a striking maximum in acidic media in the

Fig. 3. Example of the determination of Michaelis parameters from a single kinetic run by the half-time method

The hydrolysis of Z-Arg-Arg-NNap by bovine spleen cathepsin B was carried out at 25°C at pH 5.23 at 10.1 as described in the Materials and methods section. The points are values of t_1 plotted against the local values of $[S]_0$ along the progress curve. The continuous line is a linear-regression line corresponding to $V = 2.09 \mu\text{M} \cdot \text{s}^{-1}$ (for $[E] = 0.0053 \mu\text{M}$, $k_{\text{cat}} = 39.4 \text{ s}^{-1}$) and $K_m = 0.22 \text{ mM}$.

Fig. 4. pH-dependence of k_{cat}/K_m for the hydrolysis of Z-Arg-Arg-NNap catalysed by cathepsin B at 25°C at 10.1

(a) Bovine spleen enzyme: ●, points determined by the half-time method; ○, points determined from initial-rate measurements; the continuous line corresponds to $k = \bar{k}/(1 + [H^+]/K_a)$, where $k = k_{\text{cat}}/K_m$; $\bar{k} = \bar{k}_{\text{cat}}/\bar{K}_m = 7.09 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $pK_a = 5.6$. (b) Rat liver enzyme: ■, points determined by the half-time method; the broken line corresponds to the equation given in (a), where $\bar{k}_{\text{cat}}/\bar{K}_m = 4.97 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $pK_a = 5.8$.

pH- k profile where the reaction of the interactive system with the 2,2'-dipyridyl disulphide monocation predominates.

The pH- k profiles for the reactions of 2,2'-dipyridyl disulphide with the catalytic-site thiol groups of bovine spleen cathepsin B, rat liver cathepsin B and papain, in acidic media, are shown in Fig. 6, and the characterizing parameters are compared with those for the corresponding reactions of papain and actinidin (EC 3.4.22.14) in Table 2. The increase in k from the minimum value around pH 5 as the pH is decreased from 5 to 3 demonstrates clearly the existence of a nucleophilic interactive system in both bovine spleen cathepsin B and rat liver cathepsin B. The more extensive data for the bovine spleen enzyme suggest that the bell-shaped component in Fig. 6 is characterized by pK_a values of approx. 2.6 (the pK_a value of the 2,2'-dipyridyl disulphide monocation is 2.45; see Brocklehurst, 1982) and 3.4. The more limited data for the rat liver enzyme suggest that the thiol group of this enzyme has very similar

reactivity characteristics to those of the bovine spleen enzyme. The simplest interpretation of the data in Fig. 6 is that the pK_a of approx. 2.6 is the reagent pK_a and thus that the nucleophilic state of the interactive system (say S^-/ImH^+) is produced by protonic dissociation associated with molecular pK_a 3.4. This is similar to the situation that obtains for actinidin (see Table 2 and Brocklehurst *et al.*, 1981), except that for actinidin the first catalytic-site pK_a is even lower (3.1). The characteristics of the papain catalytic site remain uniquely different from those found for other cysteine proteinases. The pH- k profile for the reaction of papain with 2,2'-dipyridyl disulphide (see Fig. 6) contains a narrower bell-shaped component optimal at pH 3.9 in which the reactivity is controlled by two positively co-operative pK_a values of 3.85 and 3.9 rather than one enzyme pK_a value and the reagent

Table 2. Characterizing parameters of the reactions in acidic media at 25°C and 10.1 of 2,2'-dipyridyl disulphide with the catalytic-site thiol groups of cathepsin B, papain and actinidin

The pK_a values and the pH-independent rate constants ($\tilde{k}$) relate to the expression for the pH-dependence of the second-order rate constant (k) given in Fig. 6 legend. For the papain reaction the experimental values of k were found (by Shipton & Brocklehurst, 1978) to lie above the theoretical curve shown in Fig. 6 at pH values around 5, indicating the possibility of another small sigmoid wave in the profile with pK_a approx. 5. Thus the values of pK_{III} and $\tilde{k}_3$ for the cathepsin B reaction might relate to the parameters for this minor component of the papain reaction rather than to the values shown, which relate to the papain reaction in alkaline media. The parameters given for the cathepsin B reaction were obtained for the bovine spleen enzyme, but the reaction of the rat liver enzyme appears to be characterized by similar values of the parameters (see Fig. 6).

Enzyme	pK_I	pK_{II}	pK_{III}	$\tilde{k}_1$ ($M^{-1} \cdot s^{-1}$)	$\tilde{k}_2$ ($M^{-1} \cdot s^{-1}$)	$\tilde{k}_3$ ($M^{-1} \cdot s^{-1}$)
Cathepsin B	2.6	3.3	5.5	18000	3900	5200*
Papain	3.85	3.9	8.8	42000	850	1700†
Actinidin	2.45	3.1	10.0	25900	2000	5000‡

* Present work.
† Shipton & Brocklehurst (1978).
‡ E. Salih & K. Brocklehurst (unpublished work).

Fig. 5. pH-dependence of k_{cat} for the hydrolysis of Z-Arg-Arg-NNap catalysed by cathepsin B at 25°C at 10.1 (a) Bovine spleen enzyme: ●, points determined by the half-time method; ○, points determined from initial-rate measurements; the continuous line corresponds to the equation given in Fig. 4(a), where $k \equiv k_{cat}$; $\tilde{k}_{cat} = 76.8 s^{-1}$ and $pK_a = 5.12$. (b) Rat liver enzyme: ■, points determined by the half-time method; the broken line corresponds to the equation determined in (a), where $\tilde{k}_{cat} = 68.7 s^{-1}$ and $pK_a = 5.01$.

pK_a value (see Brocklehurst & Malthouse, 1980). One marked difference between the reactivity characteristics of the cathepsin B thiol group and those of the other two enzymes is the relatively large values of k in the pH range 5–7 for the cathepsin B reaction (see Fig. 6 and Table 2). At these pH values the enzyme modification involves mainly reaction of the nucleophilic interactive system with the neutral 2,2'-dipyridyl disulphide molecule. The reaction of cathepsin B in this pH region could be modulated by a protonic dissociation with pK_a approx. 5.5 (see Fig. 6).

Aspects of mechanism deduced from the combination of substrate-catalysis kinetics and reactivity-probe kinetics

Cysteine-proteinase-catalysed hydrolysis is usually considered to occur by the minimal three-step acyl-enzyme mechanism in which the catalytic-site thiol group is transiently acylated during catalysis (see, e.g., Polgár & Halász, 1982). For amide and anilide (and presumably for naphthylamide) substrates the minimum catalytic-site requirement for the successful acylation of the enzyme thiol group is the existence in acidic media of the S^-/ImH^+ ion-pair. For papain-catalysed hydrolysis the formation of S^-/ImH^+ , detected by reactivity-probe studies, and the development of catalytic competence, as measured by k_{cat}/K_m , both depend on protonic dissociation with pK_a 3–4. The picture is obscured somewhat, however, by the existence in papain of another (co-operatively linked) ionization with a pK_a in the same region (3–4) whose state of ionization affects both catalytic activity (k_{cat}/K_m) and reactivity of the ion pair.

The studies on cathepsin B reported in the present paper show that its catalytic-site characteris-

Fig. 6. pH-dependence of the second-order rate constant (k) for the reactions of 2,2-dipyridyl disulphide at 25°C at 10.1 with the catalytic-site thiol group of (a) bovine spleen cathepsin B (●), (b) rat liver cathepsin B (○) and (c) papain (theoretical line only)

The continuous lines are theoretical for:

$$k = \frac{\tilde{k}_1}{1 + \frac{[\text{H}^+]}{K_I} + \frac{K_{II}}{[\text{H}^+]}} + \frac{\tilde{k}_2}{1 + \frac{[\text{H}^+]^2}{K_I K_{II}} + \frac{[\text{H}^+]}{K_{II}} + \frac{K_{III}}{[\text{H}^+]}} + \frac{\tilde{k}_3}{1 + \frac{[\text{H}^+]}{K_{III}}}$$

For (a) $\tilde{k}_1 = 18000 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\tilde{k}_2 = 3900 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\tilde{k}_3 = 5200 \text{ M}^{-1} \cdot \text{s}^{-1}$; $\text{p}K_I = 2.6$, $\text{p}K_{II} = 3.3$ and $\text{p}K_{III} = 5.5$. For (b), the experimental points suggest that the values of the parameters might be similar to those for (a). For (c) $\tilde{k}_1 = 42000 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\tilde{k}_2 = 8500 \text{ M}^{-1} \cdot \text{s}^{-1}$, $\tilde{k}_3$ (not shown) $= 1700 \text{ M}^{-1} \cdot \text{s}^{-1}$; $\text{p}K_I = 3.85$, $\text{p}K_{II} = 3.9$ and $\text{p}K_{III}$ (not shown) $= 8.8$ (Shipton & Brocklehurst, 1978). For (c) the theoretical line describes the main features of the pH- k profile in this pH region. The experimental points, however, lie above the curve at pH values around 5. This suggests the existence of another small sigmoidal wave in the profile with $\text{p}K_a$ approx. 5 for the papain reaction.

tics are somewhat different from those of papain in a number of respects. Of particular importance is the observation that the two catalytic-site $\text{p}K_a$ values in the range below 7 are well separated in cathepsin B (3.4 and 5.5). This separation permits their functions to be more clearly distinguished.

Thus it can be established that the formation of thiolate anion (presumably as S^-/ImH^+ ion-pair) across $\text{p}K_a 3.4$ detected by the reactivity probe (Fig. 6) is not a sufficient condition for the production of catalytic activity towards Z-Arg-Arg-NNap. Fig. 4 shows that $k_{\text{cat.}}/K_m$ decreases across $\text{p}K_a 5.6$ as the pH is decreased for the bovine spleen enzyme ($\text{p}K_a 5.8$ for the rat liver enzyme) to very low values at pH values much higher than $\text{p}K_I$ of the SH/ImH^+ system. One possible explanation for this lack of correspondence in $\text{p}K_a$ values for production of ion-pair and catalytic competence is that the disposition of the S^- and ImH^+ components of the ion-pair formed across $\text{p}K_a 3.4$ is not suitable for provision of both nucleophilic attack, by S^- , at the electrophilic centre of the substrate and general acid catalysis, by ImH^+ , of leaving-group departure. Protonic dissociation of another group (XH) associated with $\text{p}K_a 5.6$ (or 5.8) might result in reorganization of the catalytic-site geometry to permit ImH^+ to function as a general acid catalyst. This postulate is illustrated schematically in Scheme 1. There is no evidence to suggest whether XH is close to ImH^+ . If it is, it might fulfill its role directly by causing ImH^+ to move towards the generated X^- . This type of modulatory mechanism could be similar to the UP-DOWN mechanism suggested for papain (Angelides & Fink, 1978) but with one important change. In the UP-DOWN mechanism it was envisaged that the side chain of the histidine-159 moved from its crystallographically determined position to form an $\text{S}^- \cdots \text{ImH}^+ \cdots \text{O}_2\text{C}$ interactive system involving not only the thiolate anion of cysteine-25 but also the carboxylate group of the aspartic acid residue (158) adjacent on the N-terminal side in the sequence to the histidine residue. In rat liver cathepsin B this residue is glycine rather than aspartic acid, and thus an aspartyl-histidine dipeptide residue is not an essential component of cysteine-proteinase catalytic sites. This structural difference between the catalytic sites of cathepsin B and papain might be responsible for the differences in their behaviour.

If the carboxy group of aspartic acid-158 does have a role in papain, the $\text{p}K_a$ of 5.6 (5.8) in cathepsin B may characterize an acidic group with a similar role even though the catalytic-site geometry must necessarily be somewhat different. The numerical value of this $\text{p}K_a$ could characterize a carboxy group in a relatively hydrophobic environment. The other possibility, of course, is that the

Angelides, K. J. & Fink, A. L. (1978) *Biochemistry* **17**, 2659-2668

Baines, B. S. & Brocklehurst, K. (1982) *Biochem. J.* **205**, 205-211

Barrett, A. J. (1972) *Anal. Biochem.* **47**, 280-293

Barrett, A. J. (1976) *Anal. Biochem.* **76**, 374-376

Barrett, A. J. (1980) in *Enzyme Regulation and Mechanism of Action* (Mildner, P. & Ries, B., eds.), pp. 307-315, Pergamon Press, Oxford

Barrett, A. J. & Kirschke, M. (1981) *Methods Enzymol.* **80C**, 535-561

Bajkowski, A. S. & Frankfater, A. (1983) *J. Biol. Chem.* **258**, 1645-1649

Brocklehurst, K. (1982) *Methods Enzymol.* **87C**, 427-469

Brocklehurst, K. & Malthouse, J. P. G. (1978) *Biochem. J.* **175**, 761-764

Brocklehurst, K. & Malthouse, J. P. G. (1980) *Biochem. J.* **191**, 707-718

Brocklehurst, K., Baines, B. S. & Malthouse, J. P. G. (1981) *Biochem. J.* **197**, 739-746

- Brocklehurst, K., Mushiri, S. M., Patel, G. & Willenbrock, S. J. F. (1983) *Biochem. J.* **209**, 873–879
- Cleland, W. W. (1963) *Nature (London)* **198**, 463–465
- Frost, A. A. & Pearson, R. B. (1963) *Kinetics and Mechanism*, 2nd edn., pp. 160–162, John Wiley and Sons, London
- Knight, C. G. (1980) *Biochem. J.* **189**, 447–453
- Lee, H. J., La Rue, J. N. & Wilson, I. B. (1971) *Anal. Biochem.* **41**, 397–401
- Polgár, L. & Halász, P. (1982) *Biochem. J.* **207**, 1–10
- Shipton, M. & Brocklehurst, K. (1978) *Biochem. J.* **171**, 385–401
- Stuchbury, T., Shipton, M., Norris, R., Malthouse, J. P. G., Brocklehurst, K., Herbert, J. A. L. & Suschitzky, H. (1975) *Biochem. J.* **151**, 417–432
- Takio, K., Towatari, T., Katunuma, N., Teller, D. C. & Titani, K. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3666–3670
- Towatari, T., Kawabata, Y. & Katunuma, N. (1979) *Eur. J. Biochem.* **102**, 279–289
- Wharton, C. W. & Szawelski, R. J. (1982) *Biochem. J.* **203**, 351–360
- Zvonar, T., Kregar, I. & Turk, V. (1979) *Croat. Chem. Acta* **52**, 411–416