Biochem. J. (1980) **189**, 447–453
Printed in Great Britain

Human cathepsin B

APPLICATION OF THE SUBSTRATE *N*-BENZYLOXYCARBONYL-L-ARGINYL-L-ARGININE 2-NAPHTHYLAMIDE TO A STUDY OF THE INHIBITION BY LEUPEPTIN

C. Graham KNIGHT
Tissue Physiology Department, Strangeways Laboratory, Cambridge CB1 4RN, U.K.

(*Received 28 January 1980*)

1. The kinetic parameters $k_{cat.}$ and K_m were determined for the hydrolysis of some arginine naphthylamides by human cathepsin B. 2. A new and efficient synthesis of Z-Arg-Arg-NNap (benzyloxycarbonyl-L-arginyl-L-arginine 2-naphthylamide) was developed. 3. Z-Arg-Arg-NNap was a specific and sensitive substrate for cathepsin B, and was used for kinetic studies. 4. Values of $k_{cat.}$ were maximal in the pH range 5.4–6.2, and depended on a single ionizing group pK_a 4.4. 5. Leupeptin was a purely competitive inhibitor of human cathepsin B. 6. The effect of pH on the apparent inhibitor constant, K_i (app.), was determined. K_i (app.) was pH-independent in the range pH 4.3–6.0, with the mean value 7×10^{-9} M.

Cathepsin B (EC 3.4.22.1) is a lysosomal proteinase containing an essential cysteine residue found in the tissues of many animals (Barrett, 1977). The normal physiological role of this enzyme appears to be the intralysosomal degradation of proteins (Huisman *et al.*, 1974; Dean, 1976), and there is evidence that cathepsin B may also be responsible for the cleavage of some precursor proteins to their active forms (Smith & Van Frank, 1975; Quinn & Judah, 1978; MacGregor *et al.*, 1979*b*). Cathepsin B may also be involved in pathological processes such as tumour growth (Poole *et al.*, 1978), muscle disease (Kar & Pearson, 1977) and cartilage breakdown (Bayliss & Ali, 1978).

It has been recognized recently that cathepsin B is only one of a number of thiol-dependent tissue proteinases (Katanuma, 1977). These enzymes have some common features, but they also display certain differences. For example, rat liver cathepsin H (Kirschke *et al.*, 1976, 1977*a*) hydrolyses Bz-Arg-NNap, the routine assay substrate for cathepsin B (Barrett, 1972), but it is less sensitive than cathepsin B to inhibition by leupeptin (a mixture of *N*-acetyl- and *N*-propyl-L-leucyl-L-leucyl-DL-1-amino-4-guanidinovaleraldehyde), a proteinase inhibitor produced by actinomycetes (Aoyagi *et al.*, 1969). By contrast, rat liver cathepsin L has very little activity towards synthetic substrates of cathepsin B, but it is strongly inhibited by leupeptin (Kirschke *et al.*, 1976, 1977*b*).

Recent studies in this laboratory (W. N. Schwartz & A. J. Barrett, unpublished work) have shown that human liver contains, in addition to cathepsin B, proteinases analogous to rat liver cathepsins H and L in their action on synthetic substrates and in their inhibition by leupeptin. These findings indicated the need to define the kinetic properties of these proteinases in more detail. The present paper reports studies of human cathepsin B using the substrate Z-Arg-Arg-NNap and describes the characteristics of the inhibition of this enzyme by leupeptin.

Abbreviations used: Bz-, benzoyl; Z-, benzyloxycarbonyl; -NNap, 2-naphthylamide; -NNapOMe, 2-(4-methoxy)naphthylamide; -ONp, 4-nitrophenyl ester.

Materials and methods

Cathepsin B from human liver was given by Dr. A. J. Barrett and Dr. W. Schwartz of this laboratory. Leupeptin was kindly given by the Banyu Pharmaceutical Co. Ltd., Chuo-ku, Tokyo, Japan. Z-Arg-Arg-NNapOMe,2HCl and Z-Ala-Arg-Arg-NNapOMe,2HCl were kindly given by Dr. R. E. Smith, Eli Lilly and Co., Indianapolis, IN, U.S.A. Z-Arg, Arg-NNap·HCl, 2-(4-methoxy)naphthylamine and Bz-DL-Arg-NNap were from Bachem Feinchemikalien, Bubendorf, Switzerland. Dicyclohexylcarbodi-imide and picric acid were from BDH Chemicals, Poole, Dorset, U.K. 1-Hydroxybenzotriazole hydrate was from Aldrich Chemical Co., Gillingham, Dorset, U.K. 2-Naphthylamine hydrochloride and dithiothreitol were from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Other

0306-3275/80/090447-07$01.50/1

reagents were obtained commercially and were of the highest purity available. Dimethylformamide was further purified by the method of Knight & Green (1976).

Synthesis of Z-Arg-Arg-NNap dipicrate

Z-Arg,HCl (1.14g, 3.3mmol), 1-hydroxybenzotriazole hydrate (0.61g, 4.0mmol) and dicyclohexylcarbodi-imide (0.73g, 3.5mmol) were dissolved in dry dimethylformamide (10ml) at 0°C. Arg-NNap,HCl (1.00g, 3.0mmol) was added and the mixture was stirred overnight, during which time the temperature rose to 21°C. Acetic acid (0.25ml, 4.4mmol) was added and after 30min the mixture was filtered. Dimethylformamide was removed by rotary evaporation at 40°C *in vacuo* and the oil remaining was dispersed in water (150ml) at 21°C. The mixture was filtered and water was removed by rotary evaporation at 40°C *in vacuo*. The oily residue was dissolved in ethanol (25ml) and ethanolic picric acid (25ml, 0.35M) was added. After the mixture was boiled for 5min it was allowed to cool to 21°C. Crystals of Z-Arg-Arg-NNap dipicrate formed on standing at 4°C overnight. The supernatant was removed and the crystals were re-dissolved in refluxing ethanol/water/dimethylformamide (10:3:1, by vol.; 140ml). Crystallization commenced at 21°C overnight. After a further 4 days at 4°C the crystals were filtered, washed with ethanol/water (7:3, v/v), ethanol and diethyl ether. After the crystals were dried *in vacuo* over P_2O_5 the yield was 2.4g (m.p. 226–227°C). A further 0.4g of product (m.p. 228–229°C) was recovered from the mother liquors. The total yield of Z-Arg-Arg-NNap dipicrate (2.8g) was 90% based on Arg-NNap,HCl (Found: C, 48.0; H, 4.3; N, 20.0. Calc. for $C_{48}H_{48}N_{18}O_{25}$: C, 48.1; H, 4.3; N, 20.1%).

Z-Arg-Arg-NNap,2HCl

Z-Arg-Arg-NNap dipicrate (0.50g, 0.48mmol) was dissolved in dimethylformamide (10ml). This solution was diluted with water (5ml) and applied to a column (1cm × 10cm) of Bio-Rad AG1-X2 resin (Cl^- form) in dimethylformamide/water (2:1, v/v). The eluant was collected under gravity as a single pool during the application of the sample and during subsequent elution with dimethylformamide/water (2:1, v/v; 10ml). After removal of the solvent at 40°C *in vacuo* the residual oil was dissolved in water (20ml). This solution was passed through a membrane filter (0.45μm) and stored at 4°C.

The concentration of Z-Arg-Arg-NNap in this stock solution (approx. 20mM) was determined by measuring the amount of 2-naphthylamine released during digestion with papain (0.1mg/ml) for 16h at 37°C and pH6.0 (Barrett, 1972). The 2-naphthylamine was diazo-coupled with Fast Garnet/Mersalyl/Brij reagent (Barrett, 1976). The concentration of substrate was calculated by using $\Delta\varepsilon_{520} = 27900 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for the azo-dye product (see below).

Determination of molar absorption coefficients

Solutions were prepared in volumetric glassware and dispensed with calibrated micropipettes. 2-Naphthylamine hydrochloride (28.8mg), previously dried at 60°C *in vacuo* over P_2O_5, was dissolved in ethanol (10ml). A portion of this solution (0.1ml) was diluted with water (2.4ml), and portions (0.05–0.25ml) were diazo-coupled with Fast Garnet GBC, as described by Barrett (1972). The concentrations of azo-dye were calculated by assuming complete reaction of 2-naphthylamine, and $\Delta\varepsilon_{520}$, the increase in molar absorption coefficient on forming the azo dye, was determined by using a least-squares linear-regression program. The same method was used to determine the value of $\Delta\varepsilon_{520}$ for the azo dye formed by Fast Garnet GBC and 2-(4-methoxy)naphthylamine.

Enzyme assays and analysis of reaction-rate data

The activity of cathepsin B solutions was assayed at pH6.0 with Bz-DL-Arg-NNap,HCl as described by Barrett (1972, 1976). One unit of enzyme activity is defined as that releasing 1μmol of 2-naphthylamine/min (16.7nkat) under the assay conditions (Barrett, 1973).

Assays to determine the kinetic constants of the diarginine naphthylamide substrates were made as follows in the pH range 4.8–6.2. To 2.0ml of assay buffer, containing 1.33mM-EDTA (sodium salt) and 2mM-cysteine, was added cathepsin B (50μl, approx. 50nM) in 0.05M-acetate buffer, pH4.5, containing 2mM-EDTA (sodium salt) and bovine serum albumin (100μg/ml). Since the volume of added substrate solution varied up to 150μl, the assay volume was kept constant at 2.20ml by the addition of water. After incubation of the diluted enzyme solution for 5min at 40°C, portions of the substrate solution were added at intervals with vortex-mixing. Below pH4.8 the assays were started by the addition of preactivated cathepsin B (50μl, approx. 150nM) to the buffered substrate solutions (2.15ml). Cathepsin B was activated for 10min at 40°C in 0.05M-acetate buffer, pH4.5, containing 2.5mM-dithiothreitol, 2mM-EDTA (sodium salt) and bovine serum albumin (100μg/ml). Each assay was terminated after 10min by the addition of 2ml of Fast Garnet/Mersalyl/Brij reagent (Barrett, 1976). At least 10min were allowed for colour development before measurement of A_{520}. The assays were made with 0.05M-acetate buffers pH3.8–5.4 and with 0.05M-phosphate buffers pH5.6–6.2. All buffer solutions contained 0.05M-NaCl. Rates of naphthylamine release were measured in triplicate at each substrate concentration, with a blank tube without

enzyme. The individual values of ΔA_{520} were fitted to the Michaelis–Menten equation directly (Wilkinson, 1961), by using an iterative computer program, to give the best estimates of K_m and $V_{max.}$ with their standard errors. Values of $k_{cat.}$ were calculated by assuming that pure cathepsin B had a specific activity of 125 units/μmol (Barrett, 1972; 1973).

Inhibition experiments with leupeptin were made at fixed concentrations of enzyme and Z-Arg-Arg-NNap by increasing the concentration of inhibitor. The assay conditions were similar to those used to determine the values of K_m and $V_{max.}$ at that pH value. Briefly, cathepsin B (approx. 1.6nM) was assayed in triplicate with Z-Arg-Arg-NNap (0.09 or 0.35mM) in the presence of DL-leupeptin (0–73nM). Leupeptin was a mixture of the monohydrates of *N*-propionyl-L-leucyl-L-leucyl-DL-1-amino-4-guanidinovaleraldehyde hydrochloride (mol.wt. 495.02) and the *N*-acetyl analogue (mol.wt. 481.00) in the ratio 3:1 (w/w) (Aoyagi *et al.*, 1969). Leupeptin concentrations were calculated by using the weight-average mol.wt. ($\bar{M}_w$) 491.5. The estimated uncertainty in molar concentrations of inhibitor was about 2%. The mechanism of inhibition and the value of the apparent inhibitor constant K_i (app.) at pH6.0 were determined from these dose–response curves by the graphical methods of Cornish-Bowden (1974) and Dixon (1953). In subsequent experiments, value of K_i (app.) were determined by the method of Bosshard & Berger (1974), with 0.35mM-substrate.

Results

Hydrolysis of arginine naphthylamides by human cathepsin B

Bz-DL-Arg-NNap, the routine assay substrate for cathepsin B, was not suitable for kinetic studies since the value of K_m was greater than the substrate solubility (Barrett, 1972). However, naphthylamide substrates with two adjacent arginine residues were found to be more water-soluble and to be rapidly hydrolysed by the enzyme, with greatly decreased values of K_m (Table 1). The values of $k_{cat.}/K_m$ showed that human cathepsin B hydrolysed Z-Arg-Arg-NNapOMe about 110 times more efficiently than Bz-Arg-NNap, and it was decided to adopt Z-Arg-Arg-NNapOMe, or the 2-naphthylamide analogue, for kinetic studies.

Molar absorption coefficients

In order to calculate values of $k_{cat.}$ it was necessary to determine accurate values of $\Delta\varepsilon_{520}$ for the azo dyes derived from 2-naphthylamine, or 2-(4-methoxy)naphthylamine, and Fast Garnet GBC. Two independent experiments with 2-naphthylamine gave $\Delta\varepsilon_{520} = 27800 \pm 100$ and 28000 ± 100 litre·mol^{-1}·cm^{-1}. Similarly, 2-(4-methoxy)naphthylamine gave $\Delta\varepsilon_{520} = 31100 \pm 100$ and 31500 ± 200 litre·mol^{-1}·cm^{-1}. For diazo-coupling assays the modest increase in sensitivity obtained by using the 4-methoxy derivative was outweighed by the greatly increased cost, and Z-Arg-Arg-NNap was synthesized for the kinetic studies.

Synthesis of Z-Arg-Arg-NNap

The method used to synthesize and purify Z-Arg-Arg-NNap was found to be more convenient than the procedure of Plapinger *et al.* (1965). The isolation of the product as a picric acid salt gave a good yield after recrystallization, and conversion into the water-soluble dihydrochloride salt was readily achieved by the use of the anion-exchange resin Bio-Rad AG1-X2. It was found that aqueous solutions of Z-Arg-Arg-NNap,2HCl (approx. 20mM) were stable during storage for many months at 4°C.

Kinetics of the hydrolysis of Z-Arg-Arg-NNap at pH6.0

Z-Arg-Arg-NNap had similar kinetic constants at pH6.0 and 40°C to those of the 2-(4-methoxy)-naphthylamide analogue. The value of K_m was much lower than that for Bz-Arg-NNap and the value of $k_{cat.}$ was about 7-fold greater (Table 1). In assays

Table 1. *Hydrolysis of arginine naphthylamide substrates by human cathepsin B*

Assays were made at 40°C in 0.1M-phosphate buffer, pH6.0, containing 1.33mM-EDTA (sodium salt) and 2mM-cysteine. Release of 2-naphthylamines was measured by diazo-coupling (Barrett, 1972). Values of K_m and $k_{cat.}$, and their standard errors, were calculated by the method of Wilkinson (1961).

Substrate	[S] range (mM)	$10^3 \times K_m$ (M)	$k_{cat.}$ (s^{-1})	$10^{-3} \times (k_{cat.}/K_m)$ ($M^{-1} \cdot s^{-1}$)
Bz-DL-Arg-NNap*	0.45–1.80	4.3 ± 0.5	9 ± 1	2.1
Z-Arg-Arg-NNapOMe	0.09–0.47	0.23 ± 0.01	54 ± 1	233
Z-Ala-Arg-Arg-NNapOMe	0.09–0.47	0.38 ± 0.01	63 ± 1	166
Z-Arg-Arg-NNap	0.22–1.09	0.191 ± 0.002	61.3 ± 0.3	321

* Values for L-isomer only.

using 0.33 mM-Z-Arg-Arg-NNap the sensitivity was approx. 20 times greater than that of the standard assay with Bz-Arg-NNap (Barrett, 1972).

Variation of kinetic constants with pH

Values of the kinetic constants for the hydrolysis of Z-Arg-Arg-NNap by human cathepsin B were determined between pH 3.8 and pH 6.2. It was found that the values of K_m increased markedly as the pH was lowered, whereas the values of $k_{cat.}$ were maximal the range pH 5.4–6.2 (Fig. 1). The pH-corrected kinetic constants $k_{cat.}$(lim.) and $(k_{cat.}/K_m)_{lim.}$, and their apparent pK_a values, were estimated from these data (Cornish-Bowden, 1976). A plot of $1/k_{cat.}$ against $[H^+]$ was a straight line (linear regression coefficient 0.987) and gave $k_{cat.}(lim.) = 65.5 \pm 3.7\,s^{-1}$ and pK_a (app.) = 4.4 for the enzyme–substrate complex. A graph of $\log(k_{cat.}/K_m)$ against pH (Fig. 2) showed a linear portion of slope $+1.06 \pm 0.09$ at pH values below 4.4. A plot of $(K_m/k_{cat.})$ against $[H^+]$ in the pH range 4.5–6.2 (linear regression coefficient 0.972) gave $(k_{cat.}/K_m)_{lim.} = 3.81(\pm 0.84) \times 10^5\,M^{-1}\cdot s^{-1}$ and pK_a (app.) = 5.4 for the free enzyme.

Inhibition of human cathepsin B by leupeptin

The addition of increasing concentrations of leupeptin to a constant concentration of cathepsin B (1.6 nM) at substrate concentrations of 0.09 and 0.35 mM gave in each case dose-related decreases in enzyme activity. Less inhibition was observed at the higher substrate concentration. These data, when plotted as $[S]/\Delta A_{520}$ against leupeptin concentration (Cornish-Bowden, 1974), gave parallel straight lines (Fig. 3) showing that leupeptin acted as a purely competitive inhibitor of cathepsin B.

The 1-amino-4-guanidinovaleraldehyde residue

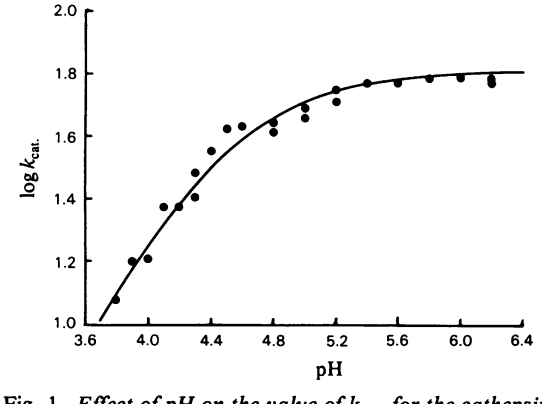

Fig. 1. *Effect of pH on the value of $k_{cat.}$ for the cathepsin B-catalysed hydrolysis of Z-Arg-Arg-NNap*
Assays were carried out at 40°C as described in the Materials and methods section. Values of $k_{cat.}$ and K_m were calculated by the method of Wilkinson (1961), by assuming that pure cathepsin B had a specific activity of 125 units/μmol. The line was drawn by assuming that $pK_a = 4.4$ for the enzyme–substrate complex and that the pH-corrected rate constant $k_{cat.}$ (lim.) = $65.5\,s^{-1}$.

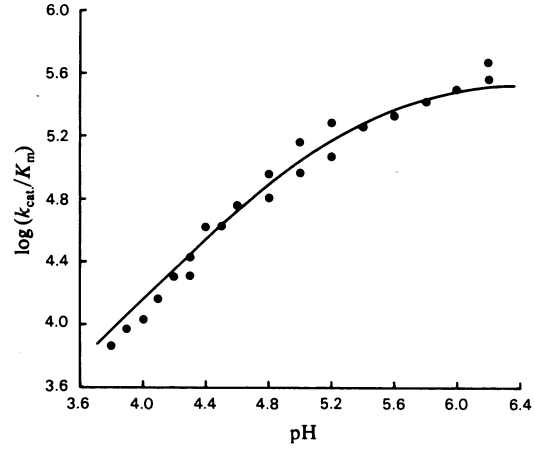

Fig. 2. *Effect of pH on the value of $k_{cat.}/K_m$ for the cathepsin B-catalysed hydrolysis of Z-Arg-Arg-NNap*
Assays were made, and values of $k_{cat.}$ and K_m were calculated, as in Fig. 1. The line was drawn by assuming that $pK_a = 5.4$ for the free enzyme and that the pH-corrected rate constant $(k_{cat.}/K_m)_{lim.} = 3.81 \times 10^5\,M^{-1}\cdot s^{-1}$.

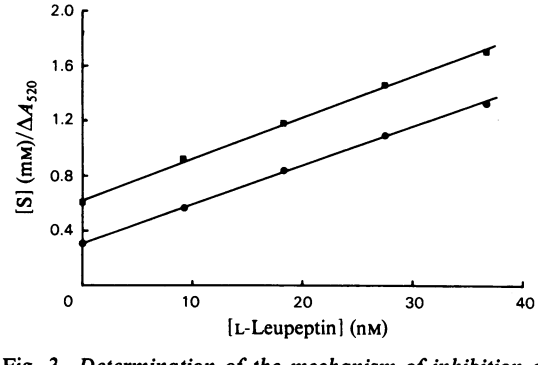

Fig. 3. *Determination of the mechanism of inhibition of human cathepsin B by leupeptin*
The activity of cathepsin B (approx. 1.6 nM) was assayed at pH 6.0 with Z-Arg-Arg-NNap (●, 0.09 mM; ■, 0.35 mM) in the presence of leupeptin (0–36.5 nM in L-isomer). The dose–response data were analysed graphically by the method of Cornish–Bowden (1974) to give parallel straight lines indicating purely competitive inhibition.

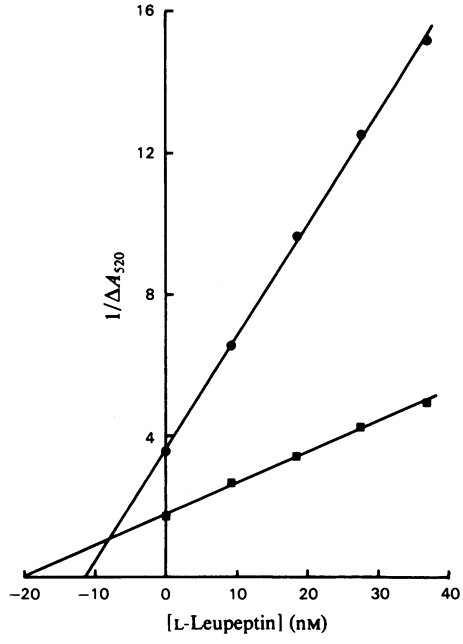

Fig. 4. *Determination of the apparent inhibitor constant for the inhibition of human cathepsin B by leupeptin*
Inhibition experiments were carried out as described in Fig. 3. The dose–response data were analysed graphically by the method of Dixon (1953). The intersection point of the lines gave K_i (app.) = 8 nM.

Table 2. *Apparent inhibitor constants for human cathepsin B and* L-*leupeptin*
Experiments were carried out as described in the Materials and methods section. Values of the inhibitor constant [K_i (app.)] refer to the L-isomer of leupeptin and were determined, with their standard errors, as described by Bosshard & Berger (1974).

pH	K_i (app.) (nM)
4.3	8.2 ± 0.5
4.8	7.3 ± 0.1
5.2	6.5 ± 0.2
5.6	6.7 ± 0.1
5.8	6.1 ± 0.1
6.0	7.1 ± 0.1

in our leupeptin was racemic, and Umezawa (1972) has reported that only the L-isomer inhibits plasmin, trypsin and papain. The inhibition of cathepsin B by leupeptin is also probably similarly stereospecific. On this assumption the inhibitor constant K_i (app.) for L-leupeptin and cathepsin B determined by the method of Dixon (1953) was about 8 nM (Fig. 4).

pH-dependence of inhibition by L-*leupeptin*

Inhibition experiments were carried out in the range pH 4.3–6.0 and the values of K_i (app.) are given in Table 2. The apparent inhibitor constant did not change appreciably with pH and remained in the range 6–8 nM.

Discussion

The present study confirms the observations of Smith & Van Frank (1975) that human liver cathepsin B, in common with cathepsin B isolated from bovine spleen (McDonald & Ellis, 1975), rat liver (Davidson & Poole, 1975), rat preputial gland (Szego *et al.*, 1976) and porcine parathyroid tissue (MacGregor *et al.*, 1979*a*), shows enhanced activity towards diarginine naphthylamides. When compared with Bz-Arg-NNap the substrates with the arginylarginine sequence showed at pH 6.0 both increased values of $k_{cat.}$ and decreased values of K_m, resulting in an increase of up to 160-fold in the specificity constant ($k_{cat.}/K_m$) (Table 1). Assays of human cathepsin B made with Z-Arg-Arg-NNap were approx. 20 times more sensitive than those made with Bz-Arg-NNap (Barrett, 1972), this increase in sensitivity being determined by the greater value of $k_{cat.}$ and the more favourable ratio of K_m to [S] (Knight, 1977).

Z-Arg-Arg-NNap, rather than Z-Arg-Arg-NNapOMe, was selected for the kinetic studies since the addition of the 4-methoxy group to 2-naphthylamine gave only a small increase in $\Delta\varepsilon_{520}$ on diazo-coupling with Fast Garnet. This result was in marked contrast with that of Smith & Van Frank (1975), who reported that the 4-methoxy derivative gave 2.9 times the colour yield of 2-naphthylamine. The reasons for this discrepancy are not known.

Bajkowski & Frankfater (1975) have proposed the use of Z-Lys-ONp as a specific substrate for cathepsin B. Under their assay conditions, at pH 5.1, Z-Lys-ONp was hydrolysed 60 times more rapidly than Bz-Arg-NNap. A major disadvantage of the use of 4-nitrophenyl ester substrates is that enzymic hydrolysis is accompanied by spontaneous hydrolysis, especially in the presence of thiol compounds (Kirsch & Igelström, 1966). By contrast, Z-Arg-Arg-NNap showed no spontaneous release of 2-naphthylamine under the assay conditions used in the present study.

Assays with Z-Arg-Arg-NNap have enabled the pH-dependence of $k_{cat.}$ and K_m to be determined for human cathepsin B, although enzyme inactivation limited these studies to pH values below 6.2. The values of $k_{cat.}$ showed a plateau between pH 5.4 and pH 6.2 (Fig. 1), whereas the values of $k_{cat.}/K_m$ continued to increase over the range of pH studied (Fig. 2). Barrett (1972) reported that human cathepsin B hydrolysed Bz-Arg-NNap at the maxi-

mum rate close to pH6.2. At higher pH values a rather steep decline in activity occurred, attributable to irreversible inactivation of the enzyme during the assays (Barrett, 1973). The assays with Bz-Arg-NNap (Barrett, 1972) were made at substrate concentrations much less than K_m and so reflect the variation of $k_{cat.}/K_m$, rather than $k_{cat.}$, with pH.

Previous studies have shown that leupeptin is an effective inhibitor of human liver cathepsin B (Barrett, 1973) and rat liver cathepsin B (Kirschke *et al.*, 1976, 1977*c*). The present results show that leupeptin is a purely competitive inhibitor of human cathepsin B and so may be presumed to interact directly with the active site. Similarly, competitive inhibition has been observed with trypsin (Umezawa, 1972) and with rat liver cathepsins B and H (Kirschke *et al.*, 1976).

The inhibition of human cathepsin B by leupeptin has been characterized by apparent inhibitor constants K_i (app.), because the chemical nature of the inhibitory species is unknown. N.m.r. studies have shown the presence of three forms of leupeptin in aqueous solutions (Maeda *et al.*, 1971). Trace amounts of the free aldehyde existed in equilibrium with almost equal quantities of the covalent aldehyde hydrate and an intramolecular cyclic addition product. The apparent dissociation constant for the binding of leupeptin by cathepsin B (Table 2) was, however, several orders of magnitude smaller than that for the structurally related substrate Bz-Arg-NH_2 (Barrett, 1977). The similarly tight binding of peptide aldehydes by papain (Westerick & Wolfenden, 1972) was attributed to the covalent addition of the free aldehyde to the active-site thiol group to form a thiohemiacetal. Such adducts, whose presence in papain complexes with aldehyde inhibitors has been demonstrated by n.m.r. (Bendall *et al.*, 1977; Lewis & Wolfenden, 1977), have a tetrahedral structure resembling that of the transient intermediates occurring on the reaction pathway of papain-catalysed hydrolyses (Lowe, 1976). At present it is not known whether leupeptin inhibits cathepsin B by a similar mechanism. However, the formation of tetrahedral intermediates during catalysis is implied by the demonstration that cathepsin B cleaves Z-Lys-ONp by an acyl-enzyme mechanism (Bajkowski & Frankfater, 1976).

I thank Dr. Alan Barrett and Dr. Bill Schwartz for generously giving cathepsin B and for helpful advice and discussion. I also thank the Arthritis and Rheumatism Council for financial support.

References

Aoyagi, T., Miyata, S., Nanbo, M., Kojima, F., Matsuzaki, M., Ishizuka, M., Takeuchi, T. & Umezawa, H. (1969) *J. Antibiot.* **22**, 558–568

Bajkowski, A. S. & Frankfater, A. (1975) *Anal. Biochem.* **68**, 119–127

Bajkowski, A. S. & Frankfater, A. (1976) in *Proteolysis and Physiological Regulation* (Ribbons, D. W. & Brew, K., eds.), p. 132, Academic Press, New York

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

Barrett, A. J. (1973) *Biochem. J.* **131**, 809–822

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

Barrett, A. J. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 181–207, North-Holland Publishing Co., Amsterdam

Bayliss, M. T. & Ali, S. Y. (1978) *Biochem. J.* **171**, 149–154

Bendall, M. R., Cartwright, I. L., Clark, P. I., Lowe, G. & Nurse, D. (1977) *Eur. J. Biochem.* **78**, 201–209

Bosshard, H. R. & Berger, A. (1974) *Biochemistry* **13**, 266–277

Cornish-Bowden, A. (1974) *Biochem. J.* **137**, 143–144

Cornish-Bowden, A. (1976) *Principles of Enzyme Kinetics*, pp. 101–111, Butterworths, London

Davidson, E. & Poole, B. (1975) *Biochim. Biophys. Acta* **397**, 437–442

Dean, R. T. (1976) *Biochem. Biophys. Res. Commun.* **68**, 518–523

Dixon, M. (1953) *Biochem. J.* **55**, 170–171

Huisman, W., Lanting, L., Doddema, H. J., Bouma, J. M. & Gruber, M. (1974) *Biochim. Biophys. Acta* **370**, 297–307

Kar, N. C. & Pearson, C. M. (1977) *Biochem. Med.* **18**, 126–129

Katanuma, N. (1977) *Trends Biochem. Sci.* **2**, 122–125

Kirsch, J. F. & Igelström, M. (1966) *Biochemistry* **5**, 783–791

Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S., Bohley, P. & Broghammer, U. (1976) *Acta Biol. Med. Germ.* **35**, 285–299

Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S., Bohley, P. & Hanson, H. (1977*a*) *Acta Biol. Med. Germ.* **36**, 185–199

Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S. & Bohley, P. (1977*b*) *Eur. J. Biochem.* **74**, 293–301

Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S., Bohley, P. & Hanson, P. (1977*c*) in *Intracellular Protein Catabolism II* (Turk, V. & Marks, N., eds.), pp. 299–303, Plenum Press, New York

Knight, C. G. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 603–607, North-Holland Publishing Co., Amsterdam

Knight, C. G. & Green, N. M. (1976) *Biochem. J.* **159**, 323–333

Lewis, C. A., Jr. & Wolfenden, R. (1977) *Biochemistry* **16**, 4890–4895

Lowe, G. (1976) *Tetrahedron* **32**, 291–302

MacGregor, R. R., Hamilton, J. W., Shofstall, R. E. & Cohn, D. V. (1979*a*) *J. Biol. Chem.* **254**, 4423–4427

MacGregor, R. R., Hamilton, J. W., Kent, G. N., Shofstall, R. E. & Cohn, D. V. (1979*b*) *J. Biol. Chem.* **254**, 4428–4433

Maeda, K., Kawamura, K., Kondo, S., Aoyagi, T., Takeuchi, T. & Umezawa, H. (1971) *J. Antibiot.* **24**, 402–404

McDonald, J. K. & Ellis, S. (1975) *Life Sci.* **17**, 1269–1276
Plapinger, R. E., Nachlas, M. M., Seligman, M. L. & Seligman, A. M. (1965) *J. Org. Chem.* **30**, 1781–1785
Poole, A. R., Tiltman, K. J., Recklies, A. D. & Stoker, T. A. M. (1978) *Nature (London)* **273**, 545–547
Quinn, P. S. & Judah, J. D. (1978) *Biochem. J.* **172**, 301–309
Smith, R. E. & Van Frank, R. M. (1975) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Dean, R., eds.), vol. 4, pp. 193–249, North-Holland Publishing Co., Amsterdam
Szego, C. M., Seeler, B. J. & Smith, R. E. (1976) *Eur. J. Biochem.* **69**, 463–474
Umezawa, H. (1972) *Enzyme Inhibitors of Microbial Origin*, pp. 15–29, University Park Press, Baltimore, London and Tokyo
Westerick, J. O. & Wolfenden, R. (1972) *J. Biol. Chem.* **247**, 8195–8197
Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332