The renal degradation of myelin basic protein peptide 43–88 by two enzymes in different subcellular fractions

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Previous studies have demonstrated that the kidney is the major site for clearance and catabolism of a peptide (residues 43–88) of encephalitogenic or basic protein (BP) derived from central-nervous-system myelin. In the present investigation rat renal tissue was shown to be capable of degrading human BP peptide 43–88 over the pH range 4–11.5 with peaks of activity at pH 5 and pH 9. The enzymic activity at pH 5 was localized mainly to the 5900 g pellet (crude mitochondrial fraction) and, on the basis of its sulphhydryl features, was inferred to be cathepsin B. The enzyme activity at pH 9 was greatly enriched in the 100 000 g pellet (microsomal brush-border fraction), and its sensitivity to inhibitors suggested that it was a metalloproteinase. The activity at alkaline pH in the 100 000 g pellet was stimulated 3-fold by non-ionic detergents and 20-fold by ATP and polyphosphates. Through a series of experiments the ATP stimulation of the alkaline proteinase activity was concluded to be the result of a reversal of inhibition imposed by the presence of another cationic protein, methylated bovine serum albumin. Inhibition by certain bivalent cations, the irregular effects of chelators and the effects of poly-L-lysine supported this conclusion. These studies indicate the availability of renal enzymes of different types and in different cellular compartments that are capable of degrading BP peptide 43–88. In particular, the relative amounts of bivalent cations, anions and charged proteins and peptides are likely to be major influences on the activity of the alkaline proteinase *in vivo*. The control of this degradation as well as the features of the smaller fragments of the peptide formed may determine biological and immune events subsequent to the release of this potentially autoantigenic material.

Myelin encephalitogenic or basic protein appears in human cerebrospinal fluid as a result of damage to central-nervous-system myelin (Cohen *et al.*, 1976; Whitaker, 1977; Whitaker *et al.*, 1980a). The major antigenic form of the MBP-like material present in cerebrospinal fluid cross-reacts with MBP peptide 43–88 from the parent MBP molecule of 169 residues (Whitaker, 1977; Whitaker *et al.*, 1980b). MBP peptide 43–88 may be generated by the action of cathepsin D (Whitaker & Seyer, 1979), which is increased in the tissue lesions of multiple sclerosis (Einstein *et al.*, 1972). Little or no MBP-like material cross-reactive with MBP peptides 1–42 or 89–169 can be detected in cerebrospinal fluid after central-

nervous-system myelin damage (Whitaker *et al.*, 1980b). The metabolic fate of the released MBP-like material is not well understood but is of importance because of the autoantigenic potential that MBP and its peptides have for inducing inflammatory demyelination. The region of MBP peptide 43–88 encompassing residues 68–88 contains the encephalitogenic determinant for inducing experimental allergic encephalomyelitis in the Lewis rat (Chou *et al.*, 1977; Hashim *et al.*, 1978).

Previous studies of the metabolism *in vivo* of human MBP peptide 43–88 in the rabbit demonstrated its rapid disappearance from the intravascular space, and organ-exclusion experiments revealed that the kidney was the major organ for clearance and degradation of this peptide (Bashir & Whitaker, 1980). The present studies were undertaken to identify the enzymes participating in the

Abbreviations used: MBP, myelin basic protein; INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium; MBSA, methylated bovine serum albumin.

renal degradation of human MBP peptide 43–88. The results indicate that two renal enzymes in different cellular compartments are capable of digesting human MBP peptide 43–88.

Materials and methods

Materials

Antipain, chymostatin, leupeptin and pepstatin were obtained from the Protein Research Foundation (Osaka, Japan). Sodium iodoacetate, *N*-ethylmaleimide, iodoacetamide, MBSA, 7-amino-1-chloro-3-*L*-tosylamidoheptan-2-one (*N*- α -*p*-tosyl-*L*-lysine chloromethyl ketone; 'TLCK'), 1-chloro-4-phenyl-3-*L*-toluene-*p*-sulphonamidobutan-2-one (*N*- α -*p*-tosyl-*L*-phenylalanine chloromethyl ketone; 'TPCK'), Kunitz pancreatic (KP) trypsin inhibitor (T0256), phenylmethanesulphonyl fluoride, *p*-hydroxymercuribenzoate, INT, *p*-nitrophenyl phosphate, glucose 6-phosphate, β -glycerophosphate, 'Fiske and SubbaRow Reducer', 1,10-phenanthroline, EGTA, EDTA, dithiothreitol, ATP (A-5394), ADP (A-6521), AMP (A-1877), adenosine 3':5'-cyclic monophosphate (A-6885), adenosine 2':3'-cyclic monophosphate (A-9376), adenosine 5'-[α,β -methylene]triphosphate (M-4504), adenosine 5'-[β,γ -methylene]triphosphate (M-7510), CTP (C-1506), GTP (G-5881), GDP (G-6506), guanosine 5'-tetraphosphate (G-6256), UTP (U-6625), tripolyphosphate (T-5758), tetrapolyphosphate (T-5633) and phosphocreatine (P-6502) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ATP was also purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.) and crystalline bovine serum albumin from Miles Laboratories (Elkhart, IN, U.S.A.). All other chemicals were of reagent grade and were purchased commercially. [*Me*-¹⁴C]Haemoglobin was obtained from New England Nuclear (Boston, MA, U.S.A.).

Methods

Subcellular fractionation. The animals used were male Holtzman rats (350–450 g body wt.). Kidney homogenates and subcellular fractions were prepared by a modification (Duckworth, 1976) of the method of Austin *et al.* (1969).

Assay of reference enzymes. Succinic:INT reductase was measured by the method of Pennington (1961), with INT as acceptor. Acid phosphatase activity (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was assayed in the presence of 0.2% Triton X-100 to give total activity. Sodium β -glycerophosphate was used as a substrate as described by Goldstone *et al.* (1973). Glucose 6-phosphatase (EC 3.1.3.9) was measured by the method of Hubscher & West (1965). P_i released was measured by the method of Fiske & SubbaRow (1925). Alkaline *p*-nitrophenyl phosphatase (EC 3.1.3.1) was measured by the method of Ray (1970).

Antigens and antibodies. Human MBP peptide 43–88 was prepared as previously described (Whitaker *et al.*, 1980a). Briefly, human MBP, isolated from normal post-mortem brain tissue, was digested with bovine brain cathepsin D and the resulting peptides were purified by carboxymethyl-cellulose chromatography and gel filtration through Sephadex G-50 (Superfine). Results of amino acid analysis and *N*-terminal sequencing indicated the peptide contained residues 43–88 (Whitaker *et al.*, 1980a). Peptide 43–88 was radiolabelled with ¹²⁵I using lactoperoxidase-catalysed iodination (Whitaker, 1977). Sheep (S104) anti-(human MBP peptide 43–88) serum and burro anti-(sheep immunoglobulin G) serum were prepared as described previously (Whitaker *et al.*, 1980b). Immunochemical analysis using small fragments from MBP peptide 43–88 showed that S104 antiserum recognizes primarily intact peptide 43–88 (Whitaker *et al.*, 1980b).

Assay of human MBP peptide 43–88-degrading activities. Degrading activities were measured by the increase in non-immunoprecipitable materials from ¹²⁵I-labelled MBP peptide 43–88. The typical enzyme reaction mixture consisted of 450 μ l of enzyme diluted in the appropriate buffer and 50 μ l of ¹²⁵I-labelled peptide 43–88 (approx. 50000 c.p.m.) in 0.2 M-Tris/acetate, pH 7.2, containing 0.2% (w/v) MBSA. MBSA was included to prevent the radioiodinated peptide from adhering to test-tube walls during the enzyme reaction period or during the subsequent immunoassay (Whitaker, 1977). Unless stated otherwise, the standard amount of MBSA present during the enzyme reaction was 200 μ g/ml. Samples were incubated for various periods of time at 37°C. Preliminary studies indicated that the digestion was best terminated by changing the pH or adding an inhibitor and then heating the reaction mixture. When the enzyme source was the 5900 g pellet, the enzyme was diluted in 0.1 M-sodium citrate, pH 5.0, and the reaction was terminated by adding 50 μ l of 10 mM-sodium iodoacetate and heating for 15 min at 70°C. When the enzyme source was the 100000 g pellet, the enzyme was diluted in 0.1 M-sodium borate, pH 9.0, and the reaction was terminated by adding 50 μ l of 0.25 M-citric acid and heating for 15 min at 70°C. For pH curves the enzymes were diluted in a universal buffer solution of the Britton and Robinson type (Dawson *et al.*, 1969). The buffer was titrated to the desired pH by the addition of NaOH pellets or 2 M-NaOH to minimize the change in volume. After cooling samples in ice, 100 μ l portions were removed for measurement of immunoprecipitability by a double-antibody radioimmunoassay.

The incubation mixture for the double-antibody radioimmunoassay consisted of a 100 μ l portion of enzyme reaction solution, 500 μ l of Tris/acetate/MBSA, and 200 μ l of S104 serum, diluted 1:200 in

Tris/acetate buffer. After 18 h at 4°C, 200 µl of burro anti-(sheep immunoglobulin G) serum, diluted 1:4 with Tris/acetate, was added, and the samples were incubated for an additional 6 h at 4°C. The pellets were then washed and counted for radioactivity as described previously (Whitaker, 1977). Results were expressed as specific activity (peptide solubilized per mg of protein), velocity (peptide solubilized per mg of protein per minute of incubation) or, for subcellular fractions, as relative specific activity (percentage recovered activity/percentage recovered protein) (Duckworth, 1976). Under the conditions described 90% or more of the radiolabelled peptide was precipitated when no degradation occurred. Substitution of S104 [anti-(human MBP peptide 43–88)] with normal sheep serum resulted in precipitation of less than 2% of radiolabelled peptide. In experiments in which additional substances were tested for their effect on degradation, a control was included to determine if any of these materials had any effect on the immunoprecipitation. No alteration in immunoprecipitation was detected in any instance.

Variations in the assay. All experiments were performed with duplicate or triplicate specimens, whose values were averaged for the described result, and repeated at least twice. Results displayed in the Tables are from representative experiments but in all repeat determinations a similar pattern and magnitude of change from control was observed. Between-assay variation was approx. $\pm 10\%$, but statistical assessment was not routinely performed because usually only two determinations were made. As an example of the variation, the inhibitory effect of 10 mM-EDTA on the degradation of MBP peptide 43–88 by the alkaline proteinase (see below) recorded in five separate experiments was $48 \pm 10\%$ (mean $\pm$ S.E.M.).

Degradation of [Me-¹⁴C]haemoglobin. Renal homogenate, the 5900 g pellet and the 100000 g pellet were tested for degradation effect on [Me-¹⁴C]haemoglobin by a modification of the method of Hatcher *et al.* (1978). For the digestions at pH 5 and pH 9, 0.1 M-citrate and 0.1 M-borate were used respectively. Homogenate and subfractions were diluted 1:5 or 1:10 in the appropriate buffer, and 450 µl of this solution was mixed with 50 µl of the same-pH buffer containing 6000 c.p.m. of [Me-¹⁴C]-haemoglobin and 100 µg of denatured haemoglobin. In some of the assays with the 100000 g pellet, ATP (1 mM) was also included in the reaction mixture. After 30–120 min at 37°C, 500 µl of 10% trichloroacetic acid was added, the mixture was centrifuged at 15000 rev./min for 1 min and the radioactivity in the supernatant was counted in a liquid-scintillation counter.

Electron microscopy of subfractions. The 800 g pellet, 5900 g pellet, 13000 g pellet and the 100000 g

pellet were fixed in 2% glutaraldehyde in cacodylate buffer, embedded in Araldite, sectioned and viewed with a Hitachi HU-12 electron microscope.

Other methods. Protein concentration was quantified by the Folin-phenol method (Lowry *et al.*, 1951) with crystalline bovine serum albumin as standard.

Results

Studies with renal homogenate

Rat renal homogenate degraded MBP peptide 43–88 over the pH range 4–11.5 with two optima at pH 5 and pH 9 (Fig. 1). The degradation was linear for 30 min at both pH 5 and 9 and linear up to 0.3 mg of protein and 0.15 mg of protein at pH 5 and 9 respectively. These limiting conditions for linearity were not exceeded for the remainder of the comparative studies. The possibility of a buffer effect (George & Kenny, 1973) was considered. When the Britton–Robinson buffer was compared at pH 5 with acetate or citrate buffer and at pH 9 with borate or veronal buffer, it was shown to promote equal or better degradation than the other buffers tested. At pH 5 citrate gave nearly equal results, and at pH 8–9

Fig. 1. The pH range over which rat renal homogenate degrades human MBP peptide 43–88 in the presence (●) and absence (○) of MBSA (200 µg/ml)

Degradative effect is expressed as the percentage increase of soluble c.p.m. (loss of immunoprecipitable radioiodinated peptide) per mg of protein in renal homogenate per min of incubation at 37°C. Values represent duplicates in one assay.

the borate buffer produced similar results. The pH5 enzyme was inhibited by *N*-ethylmaleimide (5 mM) but not by EDTA (10 mM) or phenylmethanesulphonyl fluoride (1 mM); the pH9 enzyme was inhibited by EDTA but not by *N*-ethylmaleimide or phenylmethanesulphonyl fluoride (results not shown). The biphasic pH optimum and the effects of inhibitors indicated the existence of at least two different degradative enzymes.

Subfractionation of renal homogenate

Homogenized rat renal tissue was subfractionated into an 800 g pellet, 5900 g pellet, a 13000 g pellet, a 100000 g pellet and a 100000 g supernatant to yield fractions with the characteristic enzymic markers of mitochondria (succinic:INT reductase), lysosomes (acid phosphatase), microsomes (glucose 6-phosphatase) and brush border (alkaline phosphatase) (Duckworth, 1976) (Fig. 2). Electron-microscopic studies confirmed the enrichment of these organelles in the designated subfractions (Plate 1). Specifically, the 5900 g pellet contained mitochondria and abundant lysosomes, whereas vesicles with the typical morphology and 6 nm-thick membranes of brush border dominated the 100000 g pellet. The pH5 enzyme occurred primarily in the 5900 g pellet (Fig. 2a), which had twice the activity of the homogenate and the pH9 enzyme in the 100000 g pellet (Fig. 2b), which had three times the activity of the homogenate. The 5900 g pellet showed its major activity at pH4–6 with a small amount of activity at pH9–10, whereas the 100000 g pellet showed activity only in the pH7–11 range. More extensive washing of the 5900 g pellet failed to remove the remaining activity in the alkaline pH range, which amounted to approx. 10% of that of the 100000 g pellet. Enzyme assay conditions for the 5900 g and 100000 g fractions were restudied and shown to be identical with those found with the unfractionated homogenate. Both the pH5 and pH9 enzymes were relatively stable to heat treatment. Exposure of the 5900 g pellet or the 100000 g pellet for 15 min at 0°C, 25°C or 37°C resulted in minimal or no change in MBP-degrading activity at pH5 or pH9.

To gain information on how restricted the substrate was for the enzyme activities at pH5 and pH9, studies were performed with [*Me*-¹⁴C]haemo-

globin as substrate. Although the pH5 enzyme actively degraded this substrate (0.18% soluble/mg per min), the pH9 enzyme showed only about 10% (0.012% soluble/mg per min) as much activity, even with large amounts of enzyme in the presence of 1 mM-ATP (see below) over incubation times for up to 2 h. These findings indicated a measure of specificity of the pH9 but not the pH5 enzyme for the degradation of MBP peptide 43–88. Similar to the results with MBP peptide 43–88, the degrading activity for [*Me*-¹⁴C]haemoglobin was greater at pH5 in the 5900 g pellet or at

Fig. 2. Distribution among subfractions of rat renal homogenate of degrading activity toward MBP peptide 43–88 at pH5 (a) and pH9 (b), succinic:INT reductase activity (c), alkaline phosphatase activity (d), acid phosphatase activity (e) and glucose 6-phosphatase activity (f).

Nuclear (N) (800 g pellet), mitochondrial (M) (5900 g pellet), lysosomal (L) (13000 g pellet), microsomal brush-border (R) (100000 g pellet) and cytosol (C) (100000 g supernatant) subfractions were tested and are shown as relative specific activity (percentage recovered activity/percentage recovered protein).

EXPLANATION OF PLATE 1

Electron microscopic characterization of subfractions of rat renal homogenate

(a) 800 g pellet (magnification $\times 27500$) shows nuclear remnants (N), basement membrane (BM) and collections of other large subcellular structures. (b) 5900 g pellet (magnification $\times 8250$) contains predominance of mitochondria (M) but also other organelles, especially lysosomes (L). (c) 13000 g pellet (magnification $\times 8250$) contains assortment of membrane fragments, lysosomes (L) and dense mitochondria (M). (d) 100000 g pellet (magnification $\times 55000$; inset magnification $\times 8250$) contains vesicles, many having the appearance of brush-border fragments. The calibration bar represents 1 μ m.

pH 9 in the 100000 *g* pellet than the homogenate. Although this increased activity likely reflected enrichment of the enzyme by subfractionation, it may also have been due to the removal of an inhibitor.

pH 5 enzyme in the 5900 g pellet

The degrading activity in the 5900 *g* pellet at pH 5 was inhibited more than 60% by *N*-ethylmaleimide, antipain, chymostatin, leupeptin, iodoacetamide and iodoacetate (Table 1). The activity was stimulated by cysteine and dithiothreitol. After the ATP stimulation of the pH 9 enzyme was demonstrated (see below), the effect of ATP (1 mM) on the pH 5 enzyme in the 5900 *g* pellet was examined. A maximum of 14% stimulation was detected at pH 5 (results not shown). On the basis of its pH optimum and its sulphhydryl features the enzyme in the 5900 *g* pellet of rat renal homogenate that degraded human MBP peptide 43–88 was tentatively identified as cathepsin B (Barrett, 1977).

pH 9 enzyme in the 100000 g pellet

Similar studies with potential inhibitors and stimulators were conducted at pH 9 with the 100000 *g* pellet. This degrading activity was inhibited by EDTA, 1,10-phenanthroline, chymostatin (20 µg/ml) and slightly by EGTA but was not affected by a variety of other materials (Table 2). The enzyme was totally inhibited by chymostatin at a concentration of 100 µg/ml (Table 3). Neither cysteine nor dithiothreitol at concentrations of 1 mM altered the extent of degradation at pH 9. In contrast with the mild stimulation produced by *N*-ethylmaleimide (Table 1), *p*-hydroxymercuribenzoate

(1 mM) caused an 80% reduction in activity. Although the pH 9 enzyme primarily showed the features of a zinc metalloproteinase, the inhibition by *p*-hydroxymercuribenzoate raised the possibility that a thiol proteinase or a thiol group on the metalloproteinase was important in the peptide degradation.

Because of the description of an ATP-stimulated alkaline proteinase in liver (Rose *et al.*, 1979), reticulocytes (Murakami *et al.*, 1979) and bacteria (Etlinger & Goldberg, 1977), the effects of this nucleotide on the pH 9 enzyme were examined. The degradation of peptide by the 100000 *g* pellet at pH 9 was markedly enhanced by ATP (Table 3), which showed a maximal influence at 0.5–1.0 mM-ATP (Fig. 3). In the presence of ATP the reaction velocity was much greater (Fig. 4) and the pH optimum was not altered (results not shown).

The specific mechanism by which ATP stimulates the degradation at pH 9 was investigated further. By using related chemicals, it was demonstrated that the molecular portion of the ATP molecule that stimulated the peptide degradation at pH 9 resided in the phosphate (Table 3). CTP, GTP, UTP, adenosine 5'-[α,β-methylene]triphosphate and adenosine 5'-[β,γ-methylene]triphosphate also stimulated the degradation. Because of the reported differences among sources of ATP for stimulating alkaline proteinase (Rose *et al.*, 1979), ATP from two commercial firms was examined. Both exerted the same stimulatory effect. The possibility that the stimulation was actually a result of a proteinase contaminating the nucleotide preparations was excluded by demonstrating that these stimulators in the same concentration but without the 100000 *g*

Table 1. Effects of materials on the degradation of MBP peptide 43–88 at pH 5 by the 5900 *g* pellet

Addition	Proteolytic activity (% soluble/mg per min)	Change (%)
Expt. 1		
None	5.95	0
EDTA (10 mM)	6.55	+10
<i>N</i> -Ethylmaleimide (5 mM)	2.14	–64
KP trypsin inhibitor (1 mg/ml)	4.88	–20
Expt. 2		
None	4.52	0
Antipain (20 µg/ml)	0.48	–89
Chymostatin (20 µg/ml)	0.48	–89
Leupeptin (20 µg/ml)	0.36	–92
Pepstatin (20 µg/ml)	4.40	–3
Iodoacetamide (1 mM)	1.43	–68
Iodoacetate (1 mM)	0.12	–97
Expt. 3		
None	2.86	0
Cysteine (1 mM)	19.50	+582
Dithiothreitol (1 mM)	22.50	+687

Table 2. *Effects of materials on the degradation of MBP peptide 43–88 at pH 9 by the 100 000 g pellet*

	Addition	Proteolytic activity (% soluble/mg per min)	Change (%)
Expt. 1			
	None	15.8	0
	<i>N</i> -Ethylmaleimide (5 mM)	21.4	+35
	KP trypsin inhibitor (1 mg/ml)	17.9	+13
Expt. 2			
	None	18.4	0
	Antipain (20 µg/ml)	17.9	–3
	Chymostatin (20 µg/ml)	12.4	–33
	Leupeptin (20 µg/ml)	17.5	–5
	Pepstatin (20 µg/ml)	20.5	+11
	Iodoacetamide (1 mM)	18.4	0
Expt. 3			
	None	25.3	0
	EDTA (10 mM)	16.1	–36
	EDTA (30 mM)	13.8	–54
	EGTA (1 mM)	23.0	–9
	EGTA (3 mM)	20.7	–18
	1,10-Phenanthroline (1 mM)	10.3	–59
	1,10-Phenanthroline (3 mM)	8.0	–68
	1-Chloro-4-phenyl-3-L-toluene- <i>p</i> -sulphonamidobutan-2-one (0.2 mM)	27.6	+9
	7-Amino-1-chloro-3-L-tosylamidoheptan-2-one (1 mM)	29.9	+18

Table 3. *Effects of nucleotides and related compounds on the degradation of MBP peptide 43–88 at pH 9 by the 100 000 g pellet*

The material added was at a concentration of 1 mM during the digestion period of 30 min at 37°C. The greater stimulatory effect measured in Expt. 2 is a result of decreasing the amount of enzyme tested so that the control, i.e., the mixture without additives, resulted in near-zero degradation.

	Addition	Proteolytic activity (% soluble/mg per min)	Change (%)
Expt. 1			
	None	13.9	0
	ATP	127.8	+819
	ADP	44.4	+219
	AMP	41.7	+200
	Cyclic AMP	22.2	+60
	Adenosine 5'-[α,β -methylene]triphosphate	97.2	+599
	Adenosine 5'-[β,γ -methylene]triphosphate	83.3	+499
	CTP	83.3	+499
	GTP	119.4	+759
	GDP	80.6	+480
	UTP	105.6	+660
	Phosphocreatine	22.2	+60
	PP _i	27.8	+100
Expt. 2			
	None	12.8	0
	ATP	282.0	+2103
	Triphosphate	333.3	+2504
	Tetraphosphate	384.6	+2905
	Guanosine 5'-tetraphosphate	359.0	+2705

pellet produced no degradation of peptide. Addition of compounds with fewer phosphate groups such as in ADP, GDP, AMP, cyclic AMP, PP_i or phosphocreatine led to a decrease or complete loss of

stimulation. Adenine, adenosine and caffeine at a concentration of 1 mM caused no stimulation (results not shown). Stimulation comparable with that produced by ATP occurred with polyphosphates

Fig. 3. Concentration-dependent stimulation by ATP of the degradation of human MBP peptide 43–88 at pH 9 by the renal 100 000 g pellet

Points represent duplicates from the same assay performed in the presence of MBSA.

Fig. 4. Degradation of human MBP peptide 43–88 for various periods of time by the 100 000 g pellet of rat renal homogenate in the presence of MBSA at pH 9 in the absence (●) and presence (○) of 1 mM-ATP

with or without an associated base (Table 3). Triphosphates, tetraphosphates and guanosine 5'-tetraphosphate effected a stimulation equal to or greater than that of ATP. The

Table 4. Effects of cations and inhibitors in the presence and absence of ATP on the degradation of MBP peptide 43–88 at pH 9 by the 100 000 g pellet

Each cation was added in a concentration of 1 mM during the digestion period. Some dissolved poorly in the reaction mixtures, all of which were agitated vigorously when incubation was begun. The results shown were obtained in two separate assays in which the degradation of radiolabel was 28.5 and 34.2% soluble/mg of protein per min in the absence of ATP.

Addition	Change in proteolytic activity (%)	
	–ATP	+ATP
None	0	+800
Ca ²⁺	–4.9	+675
Co ²⁺	–4.9	+300
Cu ²⁺	–60	–50
Mg ²⁺	–10	+75
Mn ²⁺	–65	+100
Zn ²⁺	–100	–75
EDTA (10 mM)	–50	+250
1,10-Phenanthroline (1 mM)	–50	+500
Chymostatin (100 µg/ml)	–100	+75

non-ionic detergents Triton X-100, Tween-80 and Nonidet P40 at a concentration of 0.1% stimulated the pH 9 enzyme greater than 3-fold (results not shown). ATP (1 mM) did not stimulate the degradation of [Me-¹⁴C]haemoglobin by the pH 9 enzyme.

The peptide-degrading activity at pH 9 was sensitive to bivalent cations. Cu²⁺, Mn²⁺ and Zn²⁺ produced the greatest inhibition in the presence or absence of ATP (Table 4). Ca²⁺, Co²⁺ and Mg²⁺ produced only marginal inhibition in the absence of ATP, but all six cations decreased the stimulation produced by ATP. Except for Cu²⁺ and Zn²⁺, the cations permitted various degrees of stimulation by ATP. Ca²⁺ showed the least inhibition of the ATP-induced stimulation. Because of the large differences produced by Mg²⁺ and Ca²⁺, the effects of these two cations on the ATP stimulation were examined in more detail. The inhibition by Mg²⁺ of the ATP-induced stimulation was shown to be concentration-dependent (Fig. 5). The possibility was considered that the Mg²⁺ inhibition might occur through the activation of an ATPase in the brush-border membranes in the 100 000 g pellet, which in turn might decrease the effective concentration of the added ATP. When ouabain (1 mM) was included in the reaction mixture, the inhibitory effect of Mg²⁺ was not altered (results not shown). Mg²⁺ and, to a lesser extent, Ca²⁺ reduced the stimulation by triphosphates in a concentration-dependent manner (Fig. 6).

Fig. 5. Inhibitory effect of various concentrations of Ca^{2+} (○) and Mg^{2+} (●) on the degradation of human MBP peptide 43–88 by the 100 000 g pellet of rat renal homogenate at pH 9 in the presence of MBSA and 1 mM-ATP

The reaction mixture was incubated at 37°C for 30 min.

Fig. 6. Inhibitory effect of various concentrations of Ca^{2+} (○) and Mg^{2+} (●) on the degradation of human MBP peptide 43–88 by the 100 000 g pellet of rat renal homogenate at pH 9 in the presence of MBSA and 1 mM-tripolyphosphate

The reaction mixture was incubated at 37°C for 30 min.

Effects of MBSA

Because of the unusual stimulatory and inhibitory profile of the pH9 enzyme, the role of components of the enzyme reaction solution was

Fig. 7. Effect of ATP on the inhibitory effect of MBSA on the degradation of human MBP peptide 43–88 by the 100 000 g pellet of rat renal homogenate at pH 9

Degradation was allowed to proceed in various concentrations of MBSA in the presence (○) or absence (●) of 1 mM-ATP.

assessed. MBSA was added to the solution containing the radiolabelled antigen so that 200 μg of MBSA/ml was present in the enzyme reaction solution. As was shown previously in a double-antibody radioimmunoassay (Whitaker, 1977), the removal of MBSA from the buffer increased the adherence of the radiolabelled antigen to the test-tube walls. In the absence of MBSA approx. 50% of the radioactivity attached to test-tube walls with each transfer. However, the elimination of MBSA also led to an enhanced degradation of the peptide at pH 9 and slightly at pH 5 (Fig. 1). Increasing amounts of MBSA inhibited the enzyme, but this inhibition could be reversed by ATP (Fig. 7). In the absence of MBSA, 0.1–1 mM-ATP had no stimulatory effect on the degradation at pH 9. The effect of poly-L-lysine (200 μg/ml) was similar to that of MBSA. MBSA did not shift the two pH optima in the homogenate (Fig. 1) or separated subfractions of the 5900 g pellet and the 100 000 g pellet (results not shown). BSA (200 μg/ml) allowed 8–10% of the radioactivity to attach to the test-tube walls but did not inhibit the degradation of MBP peptide 43–88 by the pH 9 enzyme.

The influence of selected inhibitors on the degradation of MBP peptide 43–88 at pH 9 by the 100 000 g pellet was redetermined in the absence of MBSA. Similar to the studies where MBSA was included, PHMB, EDTA, chymostatin and Zn^{2+} inhibited the pH 9 activity and phenylmethanesulphonyl fluoride, leupeptin, Ca^{2+} and Mg^{2+} showed little or no effect (Table 5). Unexpectedly and reproducibly, 1,10-phenanthroline failed to inhibit the pH 9 enzyme in the absence of MBSA. In assays of the pH 9 enzyme in the presence of bovine

Table 5. *Effects of materials on the degradation of MBP peptide 43–88 at pH 9 by the 100000 g pellet in the absence of MBSA*

Addition	Change in proteolytic activity (%)
None	0
Phenylmethanesulphonyl fluoride (0.1 mM)	–4
<i>p</i> -Hydroxymercuribenzoate (1 mM)	–48
EDTA (10 mM)	–36
1,10-Phenanthroline (1 mM)	–4
Leupeptin (100 µg/ml)	0
Chymostatin (100 µg/ml)	–56
Ca ²⁺ (1 mM)	+4
Mg ²⁺ (1 mM)	0
Zn ²⁺ (1 mM)	–94

serum albumin (200 µg/ml) 1 mM-1,10-phenanthroline also failed to inhibit the enzyme.

Discussion

MBP is readily degraded by a number of purified enzymes (Hashim & Eylar, 1969) and tissue extracts (Buletza & Smith, 1976) over a broad pH range. In spite of this susceptibility to degradation, the roles of selective endopeptidases and less restricted enzymes in the normal turnover or pathological destruction of MBP remain to be clarified. For example, cathepsin D degrades bovine MBP in a selective limited manner to yield MBP peptides 1–36, 43–88, 89–169 and 92–169 (Whitaker & Seyer, 1979). The more terminal and rapid destruction of the larger MBP peptides such as peptide 43–88 requires additional degradation by enzymes that may be critically placed in neural or extraneural locations to remove this potentially autoantigenic material.

Results of the present investigation reveal that human MBP peptide 43–88, which is rapidly cleared *in vivo* predominantly by a renal mechanism (Bashir & Whitaker, 1980), can be degraded *in vitro* by two different enzymes in different subfractions of rat renal homogenate. One of these two enzymic activities has the features of cathepsin B (Barrett, 1977) in that it has a pH 5 optimum and shows the inhibitory and stimulatory responses of a thiol proteinase. It was located predominantly in the renal 5900 g pellet, which showed the enzymic profile and morphological characteristics of a crude mitochondrial fraction but also contained lysosomes and other cellular organelles. The distribution of cathepsin B outside its expected lysosomal subfraction may be a result of the variable density of renal lysosomes (Beaufay, 1972; Duckworth, 1976). The second enzyme had a pH optimum of 9 and was located mainly in the 100000 g pellet, which contained the microsomal brush-border subfraction, and to a lesser extent in the lysosomal subfraction (13000 g pellet).

The pH 9 enzyme displayed the features of a metalloproteinase in that it was inhibited by chelators. Certain bivalent cations, in particular Cu²⁺ and Zn²⁺, were potent inhibitors. In addition to being stimulated by non-ionic detergents, activity of the membrane-bound alkaline metalloproteinase was markedly enhanced by ATP. A selectivity, possibly related to the size or charge of the substrate, is suggested by the limited and slow rate of digestion of [*Me*-¹⁴C]haemoglobin by the same renal fractions and the lack of stimulation by ATP of the degradation of haemoglobin by the pH 9 enzyme. The relative resistance of haemoglobin to degradation by reticulocyte alkaline proteinase has been observed (Etlinger & Goldberg, 1977). Also, the 100000 g pellet used in the present investigation contains a mixture of enzymes so that it is possible that the enzymes degrading haemoglobin and MBP peptide 43–88 are not the same.

The mechanism of the effect of ATP was explored in a series of experiments. Although Mg²⁺ inhibited the ATP stimulation of the enzyme, it did not work through a ouabain-sensitive ATPase. The inhibition by Mg²⁺ precluded the use of a hexokinase-containing ATP 'trap' (Hershko *et al.*, 1979) to determine if ATP is required at the initiation or throughout the digestion process. Even though chymostatin, which is reported to inhibit energy-dependent non-lysosomal protein degradation (Grinde & Seglen, 1980), decreased enzyme activity, there was no evidence for an energy-generating step involving high-energy phosphate bonds.

After the polyphosphate structure was shown to be the part of the ATP molecule needed for enzyme stimulation and the irregular effects of chelators and bivalent cations demonstrated, the balance between cations and anions in the enzyme reaction mixture was identified as an essential factor in determining enzyme activity at pH 9. MBSA was used in the enzyme assay and the double-antibody radioimmunoassay to prevent the attachment of the

cationic MBP peptide 43–88 to the test-tube wall. In the absence of MBSA the reaction was difficult to quantify because of the loss of substrate peptide to tube surface; however, MBSA or poly-L-lysine also partially inhibited the enzyme and this inhibition could be overcome by adding anionic molecules such as ATP. Although MBSA obscured some of the features of the enzyme activity at pH 9, it made the enzyme assay feasible. The features of an alkaline metalloproteinase remained in the presence or absence of MBSA, but the influence of MBSA to allow the inhibitory action of 1,10-phenanthroline cannot currently be explained. Substitution of bovine serum albumin for MBSA allowed more of the radiolabelled peptide to adhere to surfaces, and bovine serum albumin did not inhibit the pH 9 enzyme. 1,10-Phenanthroline also failed to inhibit the enzyme in the presence of bovine serum albumin. Because of the binding characteristics of 1,10-phenanthroline (Yielding & Tomkins, 1962; Dickinson & Berrieman, 1977) it could act in an additive manner with MBSA or it might stick to the test-tube wall in the absence of MBSA. The failure of bovine serum albumin to permit inhibition by 1,10-phenanthroline suggests that a synergistic action with MBSA occurs. The inhibition by *p*-hydroxymercuribenzoate is postulated to result from the reaction with a thiol group away from, but still affecting, the active site; however, the presence of an alkaline thiol proteinase remains to be excluded.

ATP has been reported to stimulate proteinases that are active in an alkaline pH range and that are present in *Escherichia coli* (Goldberg *et al.*, 1978; Murakami *et al.*, 1979) and cell-free preparations of rabbit reticulocytes (Etlinger & Goldberg, 1977; Ciehanover *et al.*, 1978; Hershko *et al.*, 1978, 1979) and of rat (DeMartino & Goldberg, 1979) and mouse (Rose *et al.*, 1979) liver. None of these enzyme activities has been chemically purified, and some of the data on characterization suggest that a mixture of enzymes (Etlinger & Goldberg, 1977; Murakami *et al.*, 1979) is present. The enzymes reported (Etlinger & Goldberg, 1977; Ciehanover *et al.*, 1978; Goldberg *et al.*, 1978; Hershko *et al.*, 1978, 1979; DeMartino & Goldberg, 1979; Murakami *et al.*, 1979; Rose *et al.*, 1979) share the alkaline-range pH condition and stimulation by ATP with the renal enzyme but are in the 100 000 *g* supernatant (Etlinger & Goldberg, 1977; DeMartino & Goldberg, 1979; Murakami *et al.*, 1979; Rose *et al.*, 1979) rather than in the 100 000 *g* pellet. The rat liver enzyme is also enhanced by non-ionic detergents but must be partially purified before the stimulation by ATP can be demonstrated (DeMartino & Goldberg, 1979). This requirement has raised the possibility that an inhibitor or high ATPase activity is present and is removed during purification. The enzyme in rabbit reticulocytes (Et-

linger & Goldberg, 1977; Hershko *et al.*, 1978) and *E. coli* (Goldberg *et al.*, 1978) but not rat liver (DeMartino & Goldberg, 1979) shares with the renal enzyme a selectivity for substrates. The renal brush border contains endopeptidases that can degrade a number of proteins and peptides. Selectivity of brush-border enzymes for digestion has been demonstrated for a neutral proteinase that degraded glucagon and insulin B chain but not insulin, albumin and casein (George & Kenny, 1973; Kerr & Kenny, 1974).

Similar to our findings, the phosphate portion has been implicated as the responsible molecular portion for stimulating the rat liver alkaline proteinase (DeMartino & Goldberg, 1979). The difference in stimulatory effects of the different nucleotide triphosphates reported (Hershko *et al.*, 1979; Murakami *et al.*, 1979) was only minimal for the renal enzyme. The mechanism of the ATP effect on the previously described alkaline proteinases is unknown, but energy production (Etlinger & Goldberg, 1977, thermostabilization (Hershko *et al.*, 1979) and allosteric effects may be related (DeMartino & Goldberg, 1979). It is possible that they, too, may be affected by the net charges present. Polyphosphate stimulation of enzymes is not restricted to alkaline metalloproteinases. Polyphosphates have been reported to increase the digestion of bovine serum albumin by cathepsin D at pH 4–6 (Watabe *et al.*, 1979).

The effects of bivalent cations, cationic proteins and chelators indicate renal handling of these substances will influence the rate of degradation of MBP peptide 43–88. The demonstration that other ATP-stimulated alkaline proteinases act to rapidly degrade abnormal proteins in a non-lysosomal compartment (Etlinger & Goldberg, 1977; Goldberg *et al.*, 1978; Hershko *et al.*, 1978) suggests that the renal enzyme may also serve as a ready mechanism for disposal of proteins and peptides from extrarenal sources. Elucidation of the structure of the fragments of MBP peptide 43–88 formed by the degradative processes at both pH 5 and pH 9 should prove helpful in identifying the small MBP peptides entering the urine or re-entering the blood.

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