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Certain mouse strains are deficient in a kidney brush-border metallo-endopeptidase activity

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Preparations of microvilli from kidneys of BALB/c mice contain an alkaline metallo-endopeptidase, meprin (**me**tallo-endo**p**eptidase from **r**enal tissue). Certain genealogically related inbred mice are markedly deficient in meprin activity. The meprin-deficient strains (CBA/J and C3H/HeJ) exhibit normal levels of other brush-border enzymes: alkaline phosphatase, aminopeptidase M and another proteinase, a phosphoramidon-sensitive neutral endopeptidase. Meprin deficiency cannot be attributed to a shift in pH optimum and is unlikely to be due to the presence of endogenous inhibitors.

Of mammalian tissues the kidney is a major source of metallo-endopeptidase activity (Erdos & Yang, 1967; Aswanikumar & Radhakrishnan, 1975; Varandani & Shroyer, 1977; Sogawa *et al.*, 1981). One of the best characterized of these enzymes is the rabbit kidney brush-border neutral endopeptidase (NEP; EC 3.4.24.11) described by Kenny and co-workers (Kerr & Kenny, 1974*a*,*b*; Kenny, 1977); this enzyme is optimally active at neutral pH values, is sensitive to inhibition by phosphoramidon (the thermolysin inhibitor found in *Streptomyces* spp. culture filtrates) and has little ability to digest large proteins. Fairly recently we purified a metallo-endopeptidase from kidney of the BALB/c mouse that differs from NEPO in a number of respects (Beynon *et al.*, 1981). This proteinase, termed meprin (Shannon *et al.*, 1981) (**me**tallo-endo**p**eptidase from **r**enal tissue) is optimally active at pH 9.5, has considerable ability to digest large proteins such as casein in addition to polypeptides and synthetic substrates, and is totally insensitive to inhibition by phosphoramidon. By several criteria, it would appear that meprin is present in addition to an NEP-like enzyme in mouse kidney (Mulligan *et al.*, 1982).

Abbreviations used: NEP, rabbit kidney brush-border neutral endopeptidase; Mops, 4-morpholinepropanesulphonic acid; Bz-Arg-Nap, benzoylarginine 2-naphthylamide; Z-Phe-Arg-NMec, benzyloxycarbonylphenylalanylarginine 4-methyl-7-coumarylamide; Leu-Nap, L-leucine 2-naphthylamide; Aces, 2-[2-amino-2-oxoethyl)-amino]ethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Bicine, *NN*-bis-(2-hydroxyethyl)glycine.

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We have found recently that although ten mouse strains (including BALB/cJ, C57BL/6J, C57Br/cdJ, A/J, DBA/1J) possess meprin, three related inbred strains (CBA/J, CBA/CaJ and C3H/HeJ) are markedly deficient in this activity (R. J. Beynon & J. S. Bond, unpublished work). We report here that meprin is a brush-border enzyme and that meprin deficiency is not associated with a widespread alteration of the enzymic complement of kidney microvilli.

Experimental

Animals

Adult male mice (20–25 g) were maintained at 23°C on a 12 h-dark/12 h-light cycle and were allowed free access to water and Purina Lab Chow. All strains were inbred and obtained from Jackson Laboratories, Bar Harbor, ME, U.S.A.

Preparation of tissue samples

Animals were killed by cervical dislocation and the vascular system was immediately perfused through the left ventricle with 0.9% NaCl to remove blood from the kidneys. The kidneys were excised, dissected free of fat, adrenals and ureters, and were homogenized in 5 vol. of ice-cold double-distilled water. The homogenate was centrifuged at 600 ***g*** for 10 min and the supernatant fluid was decanted and retained. The sedimented material was homogenized in 1.5 vol. of water and was centrifuged at 600 ***g*** for 10 min. The supernatant fractions from both centrifugations were pooled and centrifuged at 100 000 ***g*** for 1 h. The resulting sedimented material was

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suspended in 5vol. (per g of original tissue) of ice-cold water and is referred to subsequently as 'membrane preparation'. This preparation contained over 85% of the total meprin activity in kidney. Microvilli were prepared as described by Booth & Kenny (1974), but whole mouse kidney was used as starting material.

Endopeptidase assays

Meprin activity was determined by monitoring the digestion of azocasein at pH9.5 as described previously (Beynon *et al.*, 1981). One unit of activity is approximately equal to the solubilization of 1.3μg of azocasein/min. The assay using iodinated B-chain of insulin was as described by Kenny (1977), but the performic acid-oxidized peptide replaced the reduced carboxymethylated peptide. The peptide was radiolabelled, using chloramine-T, to a specific radioactivity of 7.2 MBq/μg. The assay mixture contained 10μg of substrate in 0.5ml of 0.02M-Mops buffer, pH7.0, and the reaction was initiated by the addition of approx. 10–40μg of protein. Phosphoramidon was included in certain assays at 50μM; this concentration was sufficient to inhibit completely all phosphoramidon-sensitive activity (Mulligan *et al.*, 1982). Samples (0.1 ml) were removed at suitable time intervals and added to 0.1 ml of 2% (w/v) casein as a co-precipitant. Immediately, 0.2ml of 25% (w/v) trichloroacetic acid was added and after approx. 10min the mixture was centrifuged at 10000g for 2min in a Beckman microcentrifuge. A portion of the supernatant fluid (0.2ml) was removed and the trichloroacetic acid-soluble radioactivity was determined. A sample of the original reaction mixture was used to determine the specific radioactivity of the substrate at the time of each assay. It should be noted that the solubilization of this substrate may be enhanced by the presence of exopeptidases in impure proteinase preparations (Kenny, 1977).

Cathepsin B (EC 3.4.22.1) was assayed with Bz-Arg-Nap as substrate at pH6.0 (Barrett, 1977). The hydrolysis of Z-Phe-Arg-NMec was monitored by a continuous fluorimetric assay (excitation wavelength 350nm, emission wavelength 460nm) at 25°C using 5.0μM-substrate in 0.02M-ethanolamine/HCl (pH9.5)/0.14M-NaCl.

Other enzyme assays

Alkaline phosphatase (EC 3.1.3.1) was assayed at 25°C with 1mM-4-nitrophenyl phosphate in 1M-Tris/HCl buffer, pH9.0, containing 10mM-$MgCl_2$; the absorbance change at 410nm was monitored. Aminopeptidase M (EC 3.4.11.2) was assayed at 37°C wtih 0.3mM-Leu-Nap in 0.05M-Tris/HCl, pH7.1; the product was determined as described by Barrett (1977). NADPH-cytochrome reductase (EC 1.6.2.4) was measured as described by Masters *et al.* (1967). Succinate dehdyrogenase activity (EC 1.3.99.1) was determined as described by Veeger *et al.* (1969). Malate dehydrogenase (EC 1.1.1.37) was assayed as described by Place & Beynon (1982). Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) was assayed as described by Johnson & Velick (1972).

pH optima studies

The buffers used for this study were as follows: for pH6.0, 6.8 and 7.6, Aces/NaOH; for pH6.8, 7.6 and 8.4, Hepes/NaOH; for pH7.8, 8.4 and 9.0, Bicine/NaOH; for pH8.8, 9.5 and 10.2, ethanolamine/HCl. All buffers were at a final concentration of 0.02M and contained NaCl to maintain the ionic strength at I 0.15.

Protein assays

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Statistical calculations

The rates of digestion of proteinase substrates were calculated by linear-regression analysis of four-point time courses. In all instances the correlation coefficients were greater than 0.98. Comparisons between the different mouse strains were analysed by Student's t test for small samples.

Materials

Azocasein was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and the oxidized B-chain of insulin was purchased from Boehringer-Mannheim, Indianapolis, IN, U.S.A. Carrier-free sodium [^{125}I]iodide (IMS-30) was purchased from Amersham International (Arlington Heights, IL, U.S.A), and phosphoramidon [N-(L-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan] was obtained from the Peptide Research Foundation, Osaka, Japan. Z-Phe-Arg-NMec was purchased from Peninsula Laboratories, San Carlos, CA, U.S.A. All other chemicals were of analytical grade.

Results and discussion

Brush-border localization of meprin

Azocasein is a particularly suitable substrate for the determination of meprin as it is not hydrolysed by NEP (Kenny, 1977; Mulligan *et al.*, 1982). In kidney homogenates from BALB/c mice, over 95% of the azocasein-digesting activity at pH9.5 is inhibitable by 10mM-EDTA or 1,10-phenanthroline, demonstrating that the hydrolysis of this substrate is almost entirely due to metallo-endopeptidases (Beynon *et al.*, 1981).

Meprin is a membrane-bound proteinase (Beynon *et al.*, 1981). To determine the subcellular

distribution of the enzyme, perfused kidneys were homogenized in 0.25 M-sucrose and fractionated by differential centrifugation (de Duve *et al.*, 1955). The relative specific activities (percentage of total activity associated with a cellular fraction divided by the percentage of total protein associated with that fraction) of meprin were: nuclear fraction, 0.34; lysosomal/mitochondrial fraction, 4.90; microsomal fraction, 6.33; and supernatant fraction, 0.22. For a microsomal marker enzyme, NADPH-cytochrome reductase, the relative specific activities were: 1.2, 2.1, 3.0 and <0.1 respectively. For a lysosomal enzyme marker, cathepsin B, relative specific activities were: 0.36, 5.5, <0.1 and <0.1. These data indicated that meprin was associated with the microsomal fraction; however, this fraction contains several types of membranes. A significant component of the preparation from kidney would be derived from the brush border of the microvilli of the proximal tubule (Kerr & Kenny, 1974*a*) and, therefore, microvilli were prepared (Table 1). Microvillus preparations showed no enrichment in a microsomal marker enzyme (NADPH-cytochrome reductase), a mitochondrial enzyme (succinate dehydrogenase), a lysosomal enzyme (cathepsin B) or a cytosolic enzyme (fructose-1,6-bisphosphate aldolase). By contrast, an 8–11-fold enrichment was observed for known brush-border enzymes: alkaline phosphatase, aminopeptidase M and a phosphoramidon-sensitive neutral endopeptidase (an NEP-like enzyme). Meprin was enriched 10-fold, indicating that it too is a brush-border enzyme. Phosphoramidon-insensitive proteolytic activity, using ^{125}I-labelled insulin B-chain at pH 7.0, was also enriched in the microvillus preparation; specific activity (units as in Table 1): for homogenates, 1.2; and for microvilli, 19.4. Inasmuch as meprin is capable of digesting this substrate (Mulligan *et al.*, 1982), at least part of the activity is due to this enzyme.

Table 1. *Enzyme activities in mouse microvilli*
Homogenates and microvillus preparations from kidneys of BALB/cJ mice were prepared as described in the Experimental section. Specific activities are the average values for two preparations. The percentage recovery of the enzymes in the microvillus preparations were (in the order shown): 9.7, 7.5, 5.5 and 9.5. Non-brush-border enzymes present in homogenates but not detectable in microvilli were: fructose-1,6-bisphosphate aldolase, succinate dehydrogenase, NADPH-cytochrome reductase. Two other non-brush-border enzymes, cathepsin B and malate dehydrogenase, were present at low levels in microvillus preparations and had enrichments of 0.25 and 0.08 respectively (enrichment is defined as specific activity in microvillus preparations/specific activity in homogenates). Units for meprin are expressed as increase in A_{340} of 0.001/min per mg; for alkaline phosphatase and aminopeptidase M, nmol of substrate cleaved/min per mg; for neutral endopeptidase, μg of ^{125}I-labelled insulin B-chain solubilized/min per mg of protein.

Enzyme	Specific activity of enzyme in: Homogenate	Microvilli	Enrichment in microvilli
Meprin	4.5	47.5	10.6
Alkaline phosphatase	250	2610	10.4
Aminopeptidase M	32.7	260	8.0
Neutral endopeptidase (phosphoramidon-sensitive)	0.9	8.7	9.6

Meprin deficiency

The specific activity (units/mg of protein) of meprin in kidney homogenates from BALB/cJ mice was (mean ± S.D., $n = 4$) 3.8 ± 0.35. Two other inbred strains, CBA/J and C3H/HeJ, had specific activities of approx. 2% of those of the BALB/c mice: 0.096 ± 0.30 and 0.049 ± 0.018 respectively. The deficiency was also apparent in membrane preparations (Table 2). In contrast with the observed differences in meprin activity, two other brush-border enzymes, alkaline phosphatase and aminopeptidase M, were not significantly different in the three strains. Meprin deficiency, therefore, is not associated with a widespread defect in brush-border enzymes.

The phosphoramidon-sensitive ^{125}I-labelled-insulin-B-chain-degrading activity [probably similar to NEP described by Kerr & Kenny (1974*a*,*b*) in rabbit kidney] was decreased approx. 25%. Phosphoramidon-insensitive ^{125}I-labelled-insulin-B-chain-degrading activity, probably due to a mixture of proteinases, including meprin, was decreased by approx. 75% in meprin-deficient strains (R. J. Beynon, unpublished work). Because the phosphoramidon-insensitive activity was not as dramatically decreased as was meprin activity against azocasein, we propose either (*a*) that another proteinase, in addition to meprin and NEP, capable of hydrolysing insulin B-chain, is present in membrane preparations, or (*b*) that the deficient mice have an altered form of meprin that exhibits a different substrate specificity. Further studies are required to resolve these possibilities.

The hydrolysis of Z-Phe-Arg-NMec at pH 9.5 was decreased to undetectable levels in preparations from meprin-deficient animals, confirming our previous suggestion (Beynon *et al.*, 1981) that meprin hydrolyses this substrate.

Azocasein digestion by meprin-deficient preparations was suppressed in the pH range 6.0–10.2 (Fig. 1). This precludes the possibility of an altered form of meprin active optimally at a pH other than 9.5. No indication of an endogenous inhibitor in

Table 2. *Activities of brush-border enzymes in three mouse strains*
Values are means ± S.D. of specific activities of enzymes in kidney membrane preparations from groups of four mice. Units of enzyme activities are as in Table 1; Z-Phe-Arg-NMec hydrolysis is expressed in nmol/min per mg of protein. (*$P < 0.05$ versus the BALB/c-mouse value.)

Enzyme	Specific activity of enzyme preparations		
Mouse strain ...	BALB/cJ	CBA/J	C3H/HeJ
Meprin	8.78 ± 0.74	0.25 ± 0.04*	0.02 ± 0.01*
Alkaline phosphatase	274 ± 48	296 ± 31	323 ± 31
Aminopeptidase M	36.4 ± 7.3	35.0 ± 2.4	37.4 ± 2.5
Neutral endopeptidase (phosphoramidon-sensitive)	2.0 ± 0.37	1.5 ± 0.62	1.5 ± 0.19*
Z-Phe-Arg-NMec hydrolase	0.28 ± 0.06	<0.01*	<0.01*

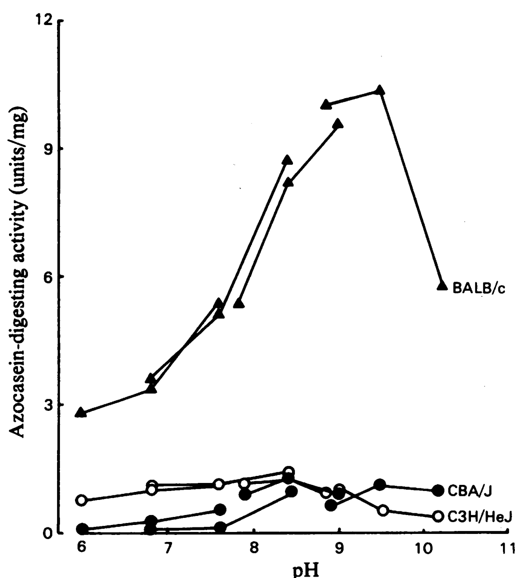

Fig. 1. *Effect of pH on azocasein-digesting activity of membrane preparations from three inbred strains of mice*
Samples of the membrane preparations from BALB/cJ (▲), CBA/J (●) and C3H/HeJ (○) mice were incubated with azocasein at the pH values indicated as described in the Experimental section. Results are expressed in units of azocaseinase activity/mg of protein. Values obtained with individual buffers are connected by lines.

meprin-deficient strains has been found. Mixing of homogenates or combinations of membrane preparations with supernatant fractions from meprin-deficient or -sufficient strains caused no suppression or activation of azocasein digestion. Also, high salt concentrations (1.0M-NaCl) or detergents [such as 1% (w/v) Triton X-100] had no effect on meprin activities (results not shown). Thus we propose that CBA-J and C3H/HeJ mice are deficient in active meprin.

It is not yet known whether an inactive form of meprin is present in deficient mouse kidney, nor are the consequences of the deficiency known. The deficient strains, however, provide a valuable experimental system in which to assess the function of meprin in mouse kidney and to determine the molecular basis of this unique cellular proteinase deficiency.

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References

Aswanikumar, S. & Radhakrishnan, A. N. (1975) *Biochim. Biophys. Acta* **384**, 194–202

Barrett, A. J. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 181–208, Elsevier/North-Holland, Amsterdam

Beynon, R. J., Shannon, J. D. & Bond, J. S. (1981) *Biochem. J.* **199**, 591–598

Booth, A. G. & Kenny, A. J. (1974) *Biochem. J.* **142**, 575–581

de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617

Erdos, E. G. & Yang, H. Y. T. (1967) *Life Sci.* **6**, 569–574

Johnson, L. W. & Velick, S. F. (1972) *J. Biol. Chem.* **247**, 4138–4143

Kenny, A. J. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 393–444, Elsevier/North-Holland, Amsterdam

Kerr, M. A. & Kenny, A. J. (1974*a*) *Biochem. J.* **137**, 477–488

Kerr, M. A. & Kenny, A. J. (1974*b*) *Biochem. J.* **137**, 489–495

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Masters, B. S. S., Williams, C. H. & Kamin, H. (1967) *Methods Enzymol.* **10**, 565–573

Mulligan, M. T., Bond, J. S. & Beynon, R. J. (1982) *Biochem. Int.* **5**, 337–343

Place, G. A. & Beynon, R. J. (1982) *Int. J. Biochem.* **14**, 305–309

Shannon, J. D., Beynon, R. J. & Bond, J. S. (1981) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40**, 1718

Sogawa, K., Ichihara, Y. & Takahashi, K. (1981) *J. Biochem. (Tokyo)* **90**, 1243–1248

Varandani, P. T. & Shroyer, L. A. (1977) *Arch. Biochem. Biophys.* **181**, 82–93

Veeger, C., DeVartarian, D. V. & Zeylemaker, W. P. (1969) *Methods Enzymol.* **13**, 81–90