Vol. 96, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1980 Pages 991-996

BRUSH BORDER MEMBRANE HYDROLYSIS OF S-BENZYL-CYSTEINE-P-NITROANILIDE, AN ACTIVITY OF AMINOPEPTIDASE M

Barbara B. Rankin, Thomas M. McIntyre and Norman P. Curthoys

Department of Biochemistry, University of Pittsburgh School of Medicine
Pittsburgh, PA 15261 USA

Received July 2,1980

SUMMARY: A peptidase activity which can hydrolyze cysteinylglycine and S-benzyl-cysteine-p-nitroanilide was purified from rat renal brush border membranes. The purified peptidase exhibits an even greater specific activity when assayed with substrates for aminopeptidase M, leucine-p-nitroanilide and alanine-p-nitroanilide. All three activities copurify and coelectrophorese. In addition titration of the three activities in isolated brush border membrane vesicles with Fab antibodies prepared against the highly purified peptidase produced similar inactivation profiles. Therefore, the activity within brush border membrane towards S-benzyl-cysteine-p-nitroanilide is due to the action of aminopeptidase M (EC 3.4.11.2.).

Rat kidney contains a particulate peptidase activity which is capable of hydrolyzing cysteinylglycine (1,2) and its S-substituted derivatives (3). By using S-benzyl-cysteine-p-nitroanilide as the substrate, it was shown that this peptidase activity is localized along with γ-glutamyltranspeptidase in the brush border membrane of the proximal tubule (3,4). It was postulated that together these two activities could account for the renal hydrolysis of glutathione and of glutathione S-conjugates (mercapturic acid precursors). Characterization of the purified peptidase suggested that it could hydrolyze a variety of dipeptides which contained a free amino-terminus (3). The peptidase was most active with aminoacylglycines in which the amino-terminal amino acid contained a hydrophobic side chain, whereas peptides with an aspartyl- or prolyl-amino terminus were not hydrolyzed. This specificity is similar to that of the well characterized aminopeptidase M which has been isolated from the kidney (5-7) and intestine (8) of various mammals. The data presented in this communication establishes that the rat renal enzyme which hydrolyzes S-benzyl-cysteine-p-nitroanilide is aminopeptidase M.

0006-291X/80/190991-06$01.00/0

MATERIALS AND METHODS

White male Sprague-Dawley rats (200-400 g) were obtained from Zivic Miller and were maintained on Purina rat chow. S-Benzyl-cysteine-p-nitroanilide was a product of Aldrich. Alanine-p-nitroanilide was purchased from Vega Fox Biochemicals. All other biochemicals were obtained from Sigma.

The peptidase was purified and assayed with S-benzyl-cysteine-p-nitroanilide as described previously (3). Peptidase activity was also determined using a mixture containing 50 mM potassium phosphate pH 7.0, 0.4 mM $MnCl_2$ and either 2.0 mM leucine-p-nitroanilide or 2.5 mM alanine-p-nitroanilide. Cysteinylglycine hydrolysis was determined by using an organomercurial agarose column to separate [^{3}H]glycine generated from cysteinyl-[^{3}H]glycine (9). All enzyme activities are reported in units of $\mu moles.min^{-1}$. Protein was assayed by the procedure of Lowry *et al.* (10) using bovine serum albumin as a standard. Polyacrylamide gel electrophoresis of undenatured proteins were performed according to the procedure of Davis (11). Gels were stained for protein in 0.05% Coomassie Blue in methanol: acetic acid:water (5:1:5) and were destained in a solution of 7.5% acetic acid and 5% methanol.

Brush border membranes were purified from rat kidney cortex using the procedure of Booth and Kenny (12) except that $CaCl_2$ was used instead of $MgCl_2$. Monovalent Fab antibodies against the purified peptidase that was also subjected to preparative polyacrylamide gel electrophoresis were prepared as described previously (13).

RESULTS

The activity of the purified peptidase was determined using two of the substrates frequently employed to assay aminopeptidase M (5-7). It was found that the specific activity of the purified peptidase was 7-fold or 6-fold greater when assayed with leucine-p-nitroanilide or with alanine-p-nitroanilide, respectively than when assayed with S-benzyl-cysteine-p-nitroanilide (Table I). When the various chromatographic procedures used in the purification were assayed with the three synthetic substrates, three identical elution profiles were generated.

Table I

Comparison of peptidase activity using various substrates

	S-benzyl-cysteine-p-nitroanilide	Leucine-p-nitroanilide	Alanine-p-nitroanilide
		$\mu mol \cdot min^{-1} \cdot mg^{-1}$	
Crude Homogenate	0.0088	0.057	0.060
Brush Border Membrane	0.056 (6.4)	0.34 (6.0)	0.40 (6.7)
Purified Peptidase	1.6 (180)	11.9 (210)	9.4 (160)

Numbers in parentheses represent fold purification relative to specific activity of crude homogenate.

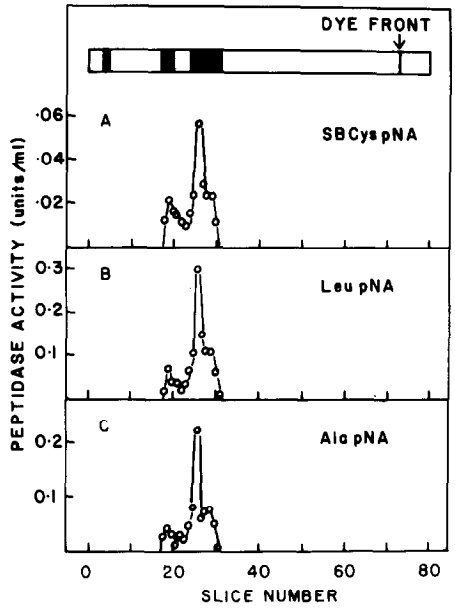

Fig. 1. Polyacrylamide gel electrophoresis of purified peptidase. Samples containing 33 µg of purified peptidase were applied to 5% polyacrylamide gels, electrophoresed for 6 h at 3 mA per tube and either stained for protein or cut into slices. The slices were indubated overnight with buffer and assayed for activity with A. S-benzyl-cysteine-p-nitroanilide (SBCyspNA), B. leucine-p-nitroanilide (LeupNA), and C. alanine-p-nitroanilide (AlapNA). For each substrate approximately 50% of the applied activity was recovered.

The increase in specific activities observed for the three substrates during the isolation of brush border membrane vesicles or the purification of the peptidase were nearly identical.

The purified peptidase could be separated into two forms by polyacrylamide gel electrophoresis (Fig. 1). However, the enzymatic activity towards all three substrates comigrated with both forms of the enzyme. These properties are similar to those reported by Kim et al. (8) for the aminopeptidase purified from rat intestinal brush border membrane. The authors showed that the electrophoretically distinguishable forms of aminopeptidase have identical enzymatic activities but differ in their carbohydrate and sialic acid content.

The purified peptidase catalyzed the hydrolysis of cysteinylglycine (Fig. 2A). Incubation of the purified peptidase with Fab antibody fragments prepared against the pure peptidase inactivated the hydrolysis of cysteinylglycine and the hydrolysis of leucine-p-nitroanilide in parallel. The maximum extent of

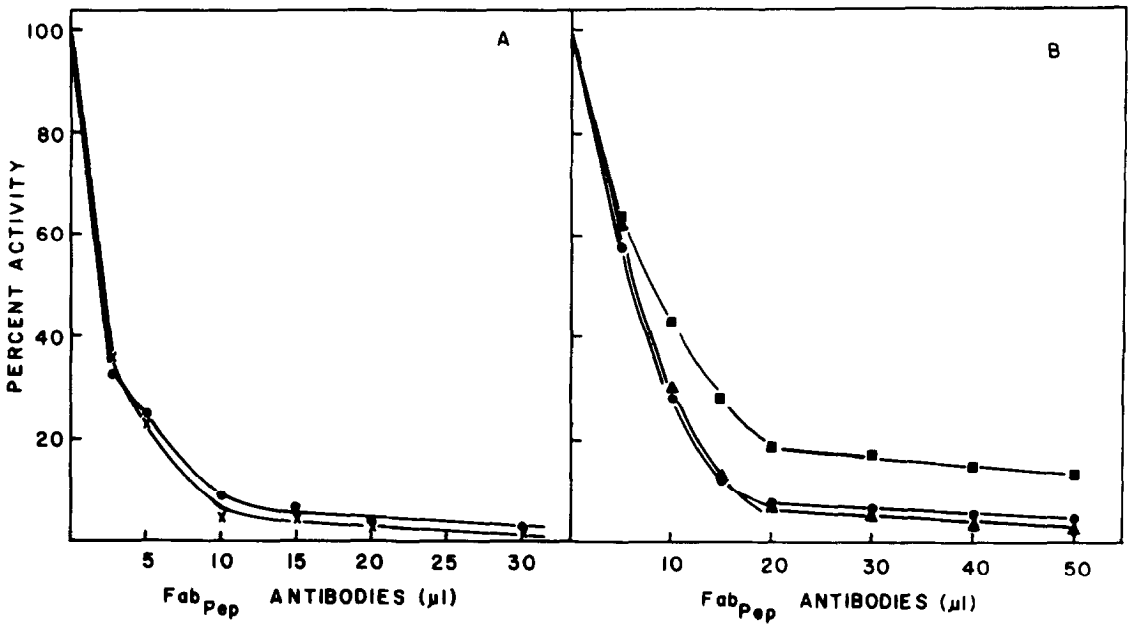

Fig. 2. Inactivation of various peptidase activities by Fab antibodies prepared against purified peptidase (Fab_{Pep}). A series of 50 µl aliquots containing peptidase activity were added to increasing amounts (0 to 50 µl) of Fab_{Pep}. The final volumes of all samples were adjusted to 100 µl. Following an incubation at 37°C for 4 h, the residual peptidase activities were determined. A. Aliquots of purified peptidase (2.5 µg) were incubated and then assayed for ability to hydrolyze cysteinylglycine (x-x) or leucine-p-nitroanilide (●-●). B. Aliquots of brush border membranes (90 µg protein) were incubated and then assayed using S-benzylcysteine-p-nitroanilide (▲-▲), leucine-p-nitroanilide (●-●) and alanine-p-nitroanilde (■-■).

inactivation was 99% with cysteinylglycine and 97% with the aminopeptidase M substrate. The incubation of isolated brush border membrane vesicles with increasing amounts of the Fab antibody preparation resulted in a coincident inactivation of approximately 95% of the total activity observed with either leucine- or S-benzyl-cysteine-p-nitroanilide (Fig. 2B). The titration of the alanine-p-nitroanilide activity plateaued at 15% residual activity suggesting that at least 85% of the total brush border membrane activity towards this substrate is attributable to aminopeptidase M.

DISCUSSION

The plasma concentration of glutathione is maintained at a low steady level as a result of its rapid extraction and hydrolysis which occurs primarily within the kidney (14). Experiments with single renal nephrons perfused in situ have

demonstrated that this hydrolysis occurs within the lumen of the proximal tubule (15). When renal metabolism of glutathione is blocked by either genetic mutation (16) or selective inhibition (17) of γ-glutamyltranspeptidase, a pronounced glutathionemia and glutathionuria occurs. In addition to γ-glutamyltranspeptidase, the complete hydrolysis of glutathione within the kidney requires the presence of a peptidase activity which can cleave cysteinylglycine. Isolated kidney cells rapidly hydrolyze glutathione and convert glutathione S-conjugates to mercapturic acids (18). The processing of various xenobiotic compounds by this pathway is thought to utilize the same enzymes involved in the hydrolysis of glutathione (19). Therefore, a renal peptidase activity which is involved in this process should also be able to hydrolyze various S-substituted derivatives of cysteinylglycine.

A renal peptidase activity which can hydrolyze S-benzyl-cysteinylglycine was previously shown to be localized along with γ-glutamyltranspeptidase in the brush border membrane of the rat renal proximal tubule (3,4). The homogeneous preparation of the peptidase obtained following preparative electrophoresis exhibited a 300-fold increase in specific activity compared to a crude kidney homogenate. This suggests that the peptidase comprises approximately 5% of the protein found in the renal brush border membrane. Aminopeptidase M has been shown to be present to such a large extent within porcine brush border membrane (20). The abundance of the peptidase activity that hydrolyzes S-benzyl-cysteine-p-nitroanilide and the previous determination that it exhibited a broad substrate specificity (3) led us to suspect that this activity was a partial reaction of aminopeptidase M.

Aminopeptidase M is frequently assayed using either leucine-p-nitroanilide (5,6) or alanine-p-nitroanilide (7). The activities towards either of these substrates was observed to copurify and to coelectrophoreses with the ability to hydrolyze S-benzyl-cysteine-p-nitroanilide. The titration of isolated brush border membranes with antibodies prepared against the purified peptidase causes the simultaneous inactivation of greater than 95% of the total activity towards

the leucine or cysteine containing synthetic substrates. In addition, both of these activities can be completely inhibited by treatment of isolated brush border membrane vesicles with o-phenanthroline[1], a known inhibitor of aminopeptidase M (8,20). Therefore, the peptidase activity observed in rat kidney brush border membranes towards S-benzyl-cysteine-p-nitroanilide is an activity of aminopeptidase M. This enzyme hydrolyzes cysteinylglycine and has a sufficiently broad specificity that it may participate along with γ-glutamyltranspeptidase in the renal degradation of glutathione or its various S-conjugates.

ACKNOWLEDGEMENTS

This investigation was supported in part by National Science Foundation Research grant PCM 7681534 and National Institutes of Health Research Career Development Award AM 00068 (NPC).

REFERENCES

1. Robinson, D.S., Birnbaum, S.M. and Greenstein, J.L. (1953) J. Biol. Chem. 201, 1-26
2. Semenza, G. (1957) Biochim. Biophys. Acta. 24, 401-413
3. Hughey, R.P., Rankin, B.B., Elce, J.S. and Curthoys, N.P. (1978) Arch. Biochem. Biophys. 186, 211-217
4. Grau, E.M., Marathe, G.V. and Tate, S.S. (1979) FEBS Lett. 98, 91-95
5. Thomas, L. and Kinne, R. (1972) Biochim. Biophys. Acta 255, 114-125
6. Wacker, H., Lehky, P., Vanderhaeghe, F. and Stein, E.A. (1976) Biochim. Biophys. Acta 429, 546-554
7. Maroux, S., Louvard, D. and Baratti, J. (1973) Biochim. Biophys. Acta 321, 282-295
8. Kim, Y.S., Brophy, E.J. and Nicholson, J.A. (1976) J. Biol. Chem. 251, 3199-3205
9. McIntyre, T.M. and Curthoys, N.P., submitted for publication.
10. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
11. Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404-427
12. Booth, A.G. and Kenny, A.J. (1974) Biochem. J. 142, 575-581
13. Tsao, B. and Curthoys, N.P. (1980) J. Biol. Chem., in press
14. Hahn, R., Wendel, A. and Flohé, L. (1978) Biochim. Biophys. Acta 539, 324-337
15. Wendel, A., Heinle, H. and Silbernagl, S. (1978) in Biochemical Nephrology (Guder, W.G. and Schmidt, U., eds.) pp. 73-80, Hans Huber Publisher, Bern.
16. Shulman, J.D., Goodman, S.I., Mace, J.W., Patrick, A.D., Tietze, F. and Butler, E.J. (1975) Biochem. Biophys. Res. Comm. 65, 68-74
17. Griffith, O.W. and Meister, A. (1979) Proc. Natl. Acad. Sci. USA 76, 268-272
18. Jones, D.P., Moldeus, P., Stead, A.H., Ormstad, K., Jörnvall, H. and Orrenius, S. (1979) J. Biol. Chem. 254, 2787-2792.
19. Boyland, E. and Chasseaud, L.F. (1969) Advan. Enzymol. Relat. Areas Biol. 32, 173-219.
20. Kenny, A.J. and Booth, A.G. (1978) Essays Biochem. 14, 1-44

[1] Unpublished observation of T.M. McIntyre and N.P. Curthoys.