Removal of *N*-terminal methionine from haemoglobin nascent peptides by a membrane-bound rat liver methionine aminopeptidase

Carlos TERMIGNONI,*† José O. FREITAS, Jr.* and Jorge A. GUIMARÃES†§

*Departamento de Bioquímica, Escola Paulista de Medicina, Rua Botucatu 862, 01000 São Paulo, Brazil, and

†Departamento de Bioquímica, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, C.P. 68041, 21910 Rio de Janeiro, R.J., Brazil

A membrane-bound aminopeptidase able to remove methionine from haemoglobin nascent peptides is described. The enzyme also hydrolyses methionine from methionyl-lysyl-bradykinin but not lysine from lysyl-bradykinin. The tripeptide Met-Ala-Ser is poorly hydrolysed. This aminopeptidase also splits amino acid 2-naphthylamides, being, however, less specific with respect to these synthetic substrates.

INTRODUCTION

Aminopeptidases (EC 3.4.11.–) are enzymes able to hydrolyse the peptide bond of *N*-terminal amino acids in peptides and proteins. These enzymes are also able to split amino acid 2-naphthylamides, the latter hydrolysis usually being named arylamidase activity. Arylamino-peptidases obtained from several sources and showing activity upon *N*-terminal leucine, alanine, arginine (or lysine) and aspartic acid (or glutamic acid) have been reported by several authors (for review see McDonald & Schwabe, 1977). A role for these enzymes in the catabolism of peptides and proteins is usually suggested, but only a few pieces of evidence for their physiological role in cells have been unequivocally shown. Possible exceptions are the inactivation of oxytocin (Tuppy & Nesvadba, 1957) and enkephalins (Hambrook *et al.*, 1976), conversion of endorphins (Burch *et al.*, 1980; Hersh *et al.*, 1980), dynorphins (Berg & Marks, 1984) and the less active peptides methionyl-lysyl-bradykinin and lysyl-bradykinin into the more potent hypotensive nonapeptide bradykinin (Guimarães *et al.*, 1973).

Another function of these enzymes could be related to the presence of an intracellular aminopeptidase, which is suggested to be responsible for the removal of *N*-terminal methionine from nascent peptides during protein synthesis (Yoshida & Lin, 1972). There are certain cases, however, as happens in the synthesis of human growth hormone by a DNA-recombinant technique, in which the methionine residue remains attached to the *N*-terminal end of the synthesized protein (Olson *et al.*, 1981). Thus clear evidence for the presence of such an enzyme and of its characterization have not been provided so far, although its existence is widely accepted. We have previously shown that rat liver subcellular fractions contain a typical methionine aminopeptidase distinct from previously described arylaminopeptidases (Freitas *et al.*, 1981, 1985). In the present work we have further studied the properties of this aminopeptidase, with special reference to its ability to hydrolyse substrates

containing *N*-terminal methionine in amino acid derivatives as well as peptides, including haemoglobin nascent peptides.

MATERIALS AND METHODS

Rat liver homogenate

The livers from adult Wistar rats were minced and homogenized in an Ultra-Turrax homogenizer (30 s at 20000 rev./min) with 4 vol. of 0.25 M-sucrose/10 mM-sodium phosphate buffer, pH 7.0, and filtered through four layers of tissue cloth. All steps were performed at 4 °C. The filtrate was centrifuged at 700 *g* for 10 min to give the crude liver homogenate.

Subcellular fractions

Subcellular fractionation of rat liver homogenate was performed essentially by the differential centrifugation method (Chambers & Rickwood, 1978). Subcellular fractions obtained included nuclei (N), heavy mitochondria (M), light mitochondria (L), microsomal fraction (P) and the soluble fraction (S). The integrity of the particles was checked and confirmed by electron microscopy as well as by using the enzyme markers glutamate dehydrogenase, glucose-6-phosphatase and tartrate-inhibitable acid phosphatase.

Enzyme fractionation

MAP was prepared as described previously (Freitas *et al.*, 1981). Rat liver homogenate or its subcellular organelles were treated with Triton X-100 in a detergent/protein ratio of 5:1 (w/w). The mixture was stirred for 30 min and centrifuged at 10000 *g* for 30 min to remove debris. The supernatant was precipitated with (NH₄)₂SO₄ (0.35–0.60 saturation). The pellet obtained after centrifugation was dissolved in and dialysed against 20 mM-sodium phosphate buffer, pH 7.0, containing 0.05% (v/v) Triton X-100 and 1.0 mM-2-mercaptoethanol. The clear Triton-solubilized samples were

Abbreviation used: MAP, methionine aminopeptidase.

† Present address: Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, 90000 Porto Alegre, R.S., Brazil.

§ To whom correspondence should be addressed.

applied to the DEAE-cellulose columns previously equilibrated with the sample mixture buffer. After the column had been washed with the equilibrating buffer to remove the non-adsorbed protein peak, a linear gradient (20–200 mM-phosphate, pH 7.0) was then applied to the column. The active fractions obtained at the non-retained protein peak were combined and concentrated by pressure dialysis and gel-filtered on a 2.6 cm $\times$ 92 cm Bio-Gel A-0.5 m column equilibrated and percolated with 20 mM-sodium phosphate buffer, pH 7.0, containing 1.0 mM-2-mercaptoethanol. The active fractions were pooled and used as MAP (Freitas *et al.*, 1985).

Preparation of haemoglobin nascent peptides

Haemoglobin nascent peptides were prepared from rabbit reticulocytes as described by Yoshida *et al.* (1970). Reticulocytes were obtained from rabbits made anaemic by phenylhydrazine treatment. The cells were washed with cold saline solution (140 mM-NaCl/5 mM-KCl/1.5 mM-MgCl₂), suspended in 100 ml of this medium containing glucose, salts, labelled methionine (350 μ Ci of [¹⁴C]- or [³⁵S]-methionine) and a mixture of non-radioactive amino acids (Yoshida *et al.*, 1970) and incubated at 37 °C for 12 min. After incubation the reticulocytes were washed three times with cold saline and then haemolysed in water. The material was centrifuged and the ribosomes were precipitated from the supernatant at pH 5.1. Nascent peptides were dissociated from ribosomes by alkaline treatment (pH 10.5, 30 °C, 3 h). Ribosomes were removed by a new precipitation at pH 5.1, and the nascent peptides were obtained by freeze-drying the supernatant. Peptides were fractionated on a previously calibrated Sephadex G-25 column (1.80 cm $\times$ 190 cm) developed with 0.5% (v/v) formic acid. The fractions corresponding to peptides smaller than glucagon and larger than a single amino acid were pooled, freeze-dried and used as the preparation of haemoglobin nascent peptides. These labelled-methionine-containing peptides were then used for testing MAP peptidase activity.

Assay of enzyme activity

Hydrolysis of amino acid 2-naphthylamides (e.g. methionine 2-naphthylamide) was monitored by the method of Barrett (1972). Briefly, the incubation mixture (1.5 ml at 37 °C) contained 410 nmol of the L-forms of the amino acid derivatives, 5–100 μ l of enzyme samples and 1.0 mM-2-mercaptoethanol in 150 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.0. The reaction was interrupted by adding 1.10 ml of a freshly prepared reagent containing a 1:1 mixture of (a) Fast Garnet solution [0.5 mg/ml containing 4% (v/v) Brij-35] and (b) 10 mM-*p*-hydroxymercuribenzoate containing 50 mM-EDTA. Hydrolysis of methionyl-lysyl-bradykinin was monitored by amino acid analysis by the procedure of Spackman *et al.* (1958). The enzyme samples were incubated with 280 μ M substrate in the above buffer, for 30 min at 37 °C. One enzyme unit is defined as the amount of enzyme hydrolysing 1.0 μ mol of substrate/min under the described conditions.

RESULTS AND DISCUSSION

Rat liver homogenate contains several enzymes displaying arylamidase activity, the highest activity always being that towards methionine 2-naphthylamide (Mäkinen & Raekallio, 1968; Freitas *et al.*, 1981).

However, at least four distinct arylamidase activities could be separated after detergent solubilization and DEAE-cellulose chromatography (Freitas *et al.*, 1981). It is now shown that two of these enzymes are active on splitting methionine 2-naphthylamide (Fig. 1a). A similar chromatographic pattern was obtained when the particulate preparations from microsomal fraction, light mitochondria and heavy mitochondria were fractionated (Figs. 1b–1d).

The results also show that in rat liver homogenate and particles the methionine 2-naphthylamidase activity is associated with two distinct enzymes. A comparison between these two activities showed that only the enzyme from the first peak (Fig. 1) is a typical aminopeptidase, the second enzyme being active only upon amino acid 2-naphthylamides (Table 1). Other comparative studies also showed (Table 1) that the true aminopeptidase has a marked preference for the hydrolysis of *N*-terminal methionine from peptides and synthetic chromogenic substrates. These results confirm our previous finding (Freitas *et al.*, 1981) suggesting the existence of these two distinct enzymes with methionine 2-naphthylamidase activity, one of them being a methionine aminopeptidase (MAP), which is defined as the one not retained on the DEAE-cellulose column.

Further purification of the detergent-solubilized MAP was obtained after sequential fractionation with (NH₄)₂SO₄ (0.35–0.60 saturation), DEAE-cellulose chromatography and gel filtration on a Bio-Gel A-0.5 m column. A high degree of instability of the fractionated enzyme was always observed, even at –20 °C. The enzyme activity could, however, be preserved in the organelles if they were kept frozen (–20 °C), but not at 4 °C. No protection of the enzyme could be obtained with phosphatidylcholine, aminophospholipids or 2-mercaptoethanol. The results suggest that on solubilization the enzyme loses a membrane stabilizing factor, probably as the result of conformational changes occurring during membrane solubilization and further purification. Thus purification beyond this step could not be performed. However, polyacrylamide-disc-gel electrophoresis of this purified preparation showed only one active band when unstained gels prepared in non-denaturing conditions were sliced, extracted with buffer and assayed with methionine 2-naphthylamide. Stained gels showed, in addition, the presence of two contaminant though enzymically inactive protein bands.

The purified MAP preparation was next incubated with haemoglobin nascent peptides having radioactive *N*-terminal methionine, obtained from rabbit reticulocytes (see the Materials and methods section). It can be seen (Fig. 2) that the amount of [¹⁴C]methionyl-peptides present in the peptides from the control sample decreased, whereas in the MAP-treated sample the peak corresponding to free [¹⁴C]methionine increased proportionally. The time-course release of [³⁵S]methionine from [³⁵S]methionine-labelled peptides is shown in another series of experiments (Fig. 2 inset). These results clearly show that MAP is able to remove *N*-terminal methionine from the nascent peptides, thus confirming its typical aminopeptidase activity.

The properties of MAP clearly indicate its distinction from all other well-characterized arylaminopeptidases. It displays the unique property of being more active upon methionine 2-naphthylamide, whereas others markedly prefer alanine 2-naphthylamide (Freitas *et al.*, 1979;

Fig. 1. DEAE-cellulose chromatographic fractionation of rat liver methionine 2-naphthylamidase activities

(a) Crude liver homogenate. The livers (110 g) from ten adult Wistar rats were homogenized as described (see the Materials and methods section). The homogenate or the liver organelles were treated with Triton X-100 at a 5:1 detergent/protein ratio. After centrifugation (10000 *g* for 30 min at 4 °C) the supernatant was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (0.35–0.60 saturation). The pellet was dissolved and dialysed against the column equilibrating buffer (see the Materials and methods section). The sample, 1.270 ml containing 3.2 g of protein, was then applied to the column (2.25 cm $\times$ 26.2 cm) of DEAE-cellulose at 4 °C. After the column had been washed with the equilibrating buffer, adsorbed proteins were eluted in a 20–200 mM-phosphate gradient. Fractions (17 ml) were collected at a flow rate of 130 ml/h. $\circ$, Protein; $\bullet$, methionine 2-naphthylamidase activity; —, phosphate gradient. (b)–(d) Rat liver subcellular fractions: (b) heavy mitochondria (622 mg of protein); (c) light mitochondria (441 mg of protein); (d) microsomal fraction (104 mg of protein). The chromatographic procedure was similar to that of (a) except for column sizes (3.4 cm $\times$ 11.0 cm each). Fractions (2.0 ml) were collected at a flow rate of 40 ml/h in each case.

Table 1. Specificity of the membrane-bound rat liver methionine 2-naphthylamidase activities on some 2-naphthylamides and peptides

Peaks 1 and 2 refer to the enzyme peaks in the DEAE-cellulose column chromatography (Fig. 1). Abbreviations: -Nap, 2-naphthylamide; N.D., not determined.

Substrate	Enzyme ...	Relative activity	
		Peak 1 (MAP)	Peak 2
Met-Nap		1.00	1.00
Leu-Nap		0.57	0.81
Ala-Nap		0.15	1.08
Lys-Nap		0.04	0.90
Arg-Nap		0.07	1.10
Met-Ala-Ser		0.01	N.D.
Met-Lys-bradykinin		2.31	0
Lys-bradykinin		0.01	0

Sidorowicz *et al.*, 1981), arginine 2-naphthylamide (Hopsu *et al.*, 1966; Freitas *et al.*, 1979) or leucine 2-naphthylamide (Delange & Smith, 1971). This particular feature of MAP has been shown to be responsible for its higher catalytic efficiency ($V_{\text{max.}}/K_m$) for hydrolysis of methionine 2-naphthylamide compared with other amino acid 2-naphthylamides (Freitas *et al.*, 1985). Furthermore, we found that MAP, in contrast with other well-characterized aminopeptidases, is not inhibited by EDTA, puromycin or bestatin (Freitas *et al.*, 1985). In addition, the lack of EDTA inhibition indicates that MAP is also distinct from all other known metallo-aminopeptidases (Barrett, 1977). It is now shown that MAP displays preferential hydrolytic activity for the cleavage of *N*-terminal methionine from oligopeptides such as methionyl-lysyl-bradykinin (Table 1) and haemoglobin nascent peptides (Fig. 2).

These properties of MAP may connect it with two other enzymes. The existence of two distinct aminopeptidases present in rabbit reticulocytes and able to hydrolyse off

Fig. 2. Removal of *N*-terminal methionine from rabbit haemoglobin nascent peptides by MAP: identification of released [14 C]methionine

[14 C]Methionine-labelled haemoglobin nascent peptides obtained as described in the Materials and methods section, and corresponding to 25000 c.p.m., were incubated for 12 h at 37 °C in 500 μ l of 20 mM-sodium phosphate, pH 7.0, containing 1 mM-2-mercaptoethanol in the absence (control, $\triangle$) or in the presence of 14 μ g of MAP ($\blacktriangle$). Samples from the incubation mixtures as well as non-radioactive methionine (added to the above buffer containing MAP) were applied to a Whatman 3MM chromatographic paper. After development (butanol/acetic acid/water, 4:1:1, by vol.) the chromatograms were dried; that corresponding to the control run (unlabelled methionine) was stained with ninhydrin, and the others were further cut into 1.5 cm pieces and the radioactivity of each piece was measured by liquid-scintillation counting. The inset shows a time-course curve for the hydrolysis of *N*-terminal methionine from haemoglobin nascent peptides. The enzyme (27.6 μ g) was incubated with [35 S]methionine-labelled haemoglobin nascent peptides (20000 c.p.m.) in 500 μ l of the above buffer. At different incubation times (30–180 min) 100 μ l portions were removed and applied to the chromatographic paper, and handled as indicated for (a). Results represent means $\pm$ S.E.M. obtained in four experiments.

specifically either *N*-terminal methionine or *N*-terminal *N*-formylmethionine was reported by Yoshida & Lin (1972). The authors suggested that these enzymes could be related to the removal of methionine and formylmethionine from nascent peptides. They also found a similar activity in the supernatant of a 10000 *g* rabbit liver homogenate as well as of other tissue homogenates. Suda *et al.* (1980) isolated from rat liver an arylamidase able to hydrolyse *N*-formylmethionine 2-naphthylamide that is distinct from a methionine 2-naphthylamide activity also present in the liver homogenate. The latter enzyme seems to be similar to the enzymic activity found by Yoshida & Lin (1972). Neither the former nor the latter authors further characterized these enzymes. It is, then, possible that MAP could be similar to the enzyme described by Yoshida & Lin (1972), since both mitochondria and microsomal fraction could be expected to be present in their 10000 *g* supernatant. Additional work is required in order to characterize the relationship between MAP and any other related aminopeptidase.

This work was supported in part by grants from FINEP and CNPq (Brazil). C. T. was recipient of a CAPES/PICD (Brazil) fellowship.

REFERENCES

- Barrett, A. J. (1972) *Anal. Biochem.* **47**, 280–297
 Barrett, A. J. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 1–53, Elsevier, Amsterdam
- Berg, M. J. & Marks, N. (1984) *J. Neurosci. Res.* **11**, 313–321
 Burch, J. P. H., Loeber, J. G., Verhoef, J., Wiegant, V. M., de Kloet, E. R. & de Wield, D. (1980) *Nature (London)* **283**, 96–97
 Chambers, J. A. A. & Rickwood, D. (1978) *Centrifugation: A Practical Approach*, pp. 33–46, Information Retrieval, London
 Delange, R. J. & Smith, E.-L. (1971) *Enzymes* 3rd Ed. **3**, 81–89
 Freitas, J. O., Jr., Guimarães, J. A., Borges, D. R. & Prado, J. L. (1979) *Int. J. Biochem.* **10**, 81–89
 Freitas, J. O., Jr., Termignoni, C., Borges, D. R., Sampaio, C. A. M., Prado, J. L. & Guimarães, J. A. (1981) *Int. J. Biochem.* **13**, 991–997
 Freitas, J. O., Jr., Termignoni, C. & Guimarães, J. A. (1985) *Int. J. Biochem.* **17**, in the press
 Guimarães, J. A., Borges, D. R., Prado, E. S. & Prado, J. L. (1973) *Biochem. Pharmacol.* **22**, 3157–3172
 Hambrook, J. M., Morgan, B. A., Rance, M. J. & Smith, C. F. C. (1976) *Nature (London)* **262**, 782–783
 Hersh, L. B., Smith, T. E. & McKelvy, J. F. (1980) *Nature (London)* **286**, 160–162
 Hopsu, V. K., Mäkinen, K. K. & Glenner, G. G. (1966) *Arch. Biochem. Biophys.* **114**, 567–575
 Mäkinen, P. L. & Raekallio, J. (1968) *Acta Chem. Scand.* **22**, 3111–3119
 McDonald, J. K. & Schwabe, C. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 311–391, Elsevier, Amsterdam
 Olson, K. C., Fenno, J., Lin, N., Harkins, R. N., Snider, C., Kohr, W. H., Ross, M. J., Fodge, D., Prender, G. & Stebbing, N. (1981) *Nature (London)* **293**, 408–411

- Sidorowicz, W., Zownir, O. & Behal, F. J. (1981) Clin. Chim. Acta **111**, 69–79
- Spackman, D. H., Stein, W. R. & Moore, S. (1958) Anal. Chem. **30**, 1190–1206
- Suda, H., Yamamot, K., Aoyagi, T. & Umezawa, H. (1980) Biochim. Biophys. Acta **616**, 60–67
- Tuppy, H. C. & Nesvadba, H. (1957) Monatsh. Chem. **88**, 977–988
- Yoshida, A. & Lin, M. (1972) J. Biol. Chem. **247**, 952–957
- Yoshida, A., Watanabe, S. & Morris, J. (1970) Proc. Natl. Acad. Sci. U.S.A. **67**, 1600–1607

Received 23 July 1985/18 November 1985; accepted 2 December 1985