Volume 244, number 2, 477-480 FEB 06867 February 1989

A peptidyl α-amidation activity in chromaffin granules of bovine adrenal medulla

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Received 22 December 1988

Peptidyl α-amidation activity in bovine adrenal medulla has been localized in chromaffin granules by density gradient centrifugation. The activity was found to be both soluble and membrane-associated. Both enzymatic activities were stimulated by the addition of Cu^{2+} and ascorbate. The pH maximum for α-amidation in the chromaffin granules in pH 8.0–8.5. By gel filtration, the soluble enzyme activity appeared as a protein of approx. 40 kDa. It is suggested that this enzyme is involved in the carboxyl-terminal amidation of metorphamide, amidorphin and neuropeptide Y.

Chromaffin granule; Peptidyl glycine α-amidating monooxygenase

1. INTRODUCTION

Carboxyl-terminal α-amidation is a frequent feature of bioactive peptides. This essential post-translational modification is catalysed by an enzyme known as peptidyl α-amidating monooxygenase (PAM). It was originally identified in the soluble fraction of pituitary secretory granules [1], has been shown to be stimulated by copper and ascorbate and to be dependent on the presence of molecular oxygen [2].

Since several neuropeptides have been found in chromaffin granules of adrenal medulla [3], including the amidated peptides neuropeptide Y [4], metorphamide [5] and amidorphin [6], we investigated whether an α-amidation activity is present in the adrenal medulla.

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Abbreviations: DBM, dopamine-β-monooxygenase; HPLC, high-performance liquid chromatography; PAM, peptidyl glycine α-amidating monooxygenase; Tes, *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid

2. MATERIALS AND METHODS

2.1. *Preparation of soluble and membrane proteins from chromaffin granules*

Chromaffin granules were isolated over 1.8 M sucrose according to [7]. The granules were lysed in 20 mM Na-Tes (pH 8.0) at 4°C for 1 h. After freeze-thawing, the membranes were sedimented at 100000 × g_{max} for 30 min and the supernatant was used as a source for soluble proteins. Membranes were further purified by three washes with lysis buffer and cycles of freeze-thawing.

2.2. *Subcellular fractionation of bovine adrenal medulla*

A crude granule fraction was obtained essentially as in [8]. This fraction was resuspended in 0.3 M sucrose and layered onto a linear sucrose gradient of 1.1–2.2 M sucrose. This gradient was centrifuged for 2 h at 272000 × g_{max} and 12 fractions were collected afterwards.

2.3. *PAM assay*

A PAM enzyme assay was adapted from [2] which tests enzyme sources according to their ability to convert ^{125}I-D-Tyr-Val-Gly into ^{125}I-D-Tyr-Val-NH_2. Assays were generally performed in a volume of 100 μl for 5 h with 10–40 μg protein, 0.5 μM D-Tyr-Val-Gly, 15000–30000 cpm ^{125}I-D-Tyr-Val-Gly, 100 mM Na-Tes (pH 8.0), catalase (100 μg/ml) and various amounts of $CuSO_4$ and/or ascorbate. After incubation, substrate and product were separated by cation-exchange chromatography. Data are expressed as pmol D-Tyr-Val-NH_2 formed/mg protein per h.

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00145793/89/$3.50

2.2. *Other assays*

Catecholamines were assayed by the method of Von Euler and Hamberg [9]. Protein was estimated by the method of Bradford [10]. Acid phosphatase and succinate hydrogenase were assayed as in [11,12], respectively. Dopamine-β-monooxygenase (DBM) was assayed as in [13].

2.5. *Gel-filtration chromatography*

The soluble proteins of chromaffin granules were concentrated by precipitation with 50% saturated ammonium sulfate and applied to a Superose-12 HR 10/30 column (Pharmacia). The column was equilibrated with 20 mM Na-Tes (pH 8.0) and 1-min fractions were collected at a flow rate of 0.5 ml/min.

3. RESULTS

3.1. *Peptidyl α-amidation activity in chromaffin granules*

Low, but detectable amidation activity was found in the homogenate of bovine adrenal medulla. In the soluble fraction of chromaffin granules, however, significantly higher activity was present.

Reversed-phase HPLC analysis of the reaction products formed by the granule-associated activity indicated that more than 95% of the labeled reaction product co-eluted with synthetic ^{125}I-D-Tyr-Val-NH_2.

The pH optimum for the granule-associated α-amidation activity was found to be pH 8.0, with a rapid decline in activity below pH 6.5 (fig.1). The reaction was linear both in amount of granule protein, up to 50 μg protein/assay tube, and with time to about 6 h. A boiled enzyme blank showed no activity. Of all metal ions tested, only copper was capable of stimulating the amidation activity and of reversing the inhibitory effect of EDTA. Maximal stimulation of PAM activity was obtained at 1–5 μM, and varied slightly with each preparation of granules. This copper concentration resulted in 5–6-fold stimulation of amidation activity (table 1). Addition of optimal levels of ascorbate (0.5 mM) stimulated PAM activity 1.5–2-fold (table 1). Higher levels of ascorbate had an inhibitory effect on the activity. When the soluble fraction of chromaffin granules was chromatographed on a Superose-12 gel-filtration column, the major activity emerged at an elution position corresponding to approx. 40 kDa (fig.2).

There was also a significant amount of PAM activity in the membranes of chromaffin granules. This activity was solubilised by non-ionic detergents such as Triton X-100, but not by high salt concentrations. Membraneous PAM activity was also stimulated by copper and ascorbate (table 1), and showed a pH optimum at pH 8.0 (fig.1).

Table 1

Comparison between the membraneous and soluble α-amidation activities from chromaffin granules

	Soluble	Membraneous
pH optimum	8.0–8.5	8.0
$CuSO_4$ stimulation (-fold)	5.5	3
$CuSO_4$ optimum (μM)	3	5
Ascorbate stimulation (-fold)	2	1.5
Ascorbate optimum (mM)	0.5	0.5

3.2. *Sucrose density gradient centrifugation*

Even highly purified granules are not completely

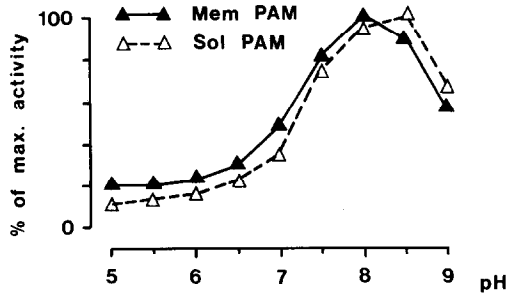

Fig.1. pH dependence of soluble and membraneous chromaffin granule PAM activity. All assays contained 5 μM $CuSO_4$ and 0.5 mM ascorbate at the pH indicated. Maximal activity was 25 pmol/mg protein per h.

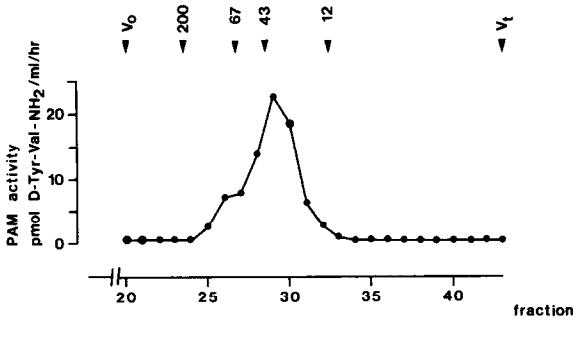

Fig.2. Gel filtration on a Superose-12 column of a soluble fraction from chromaffin granules. PAM activity was determined in the presence of 5 μM $CuSO_4$ and 0.5 mM ascorbate. Numbers above profile in kDa.

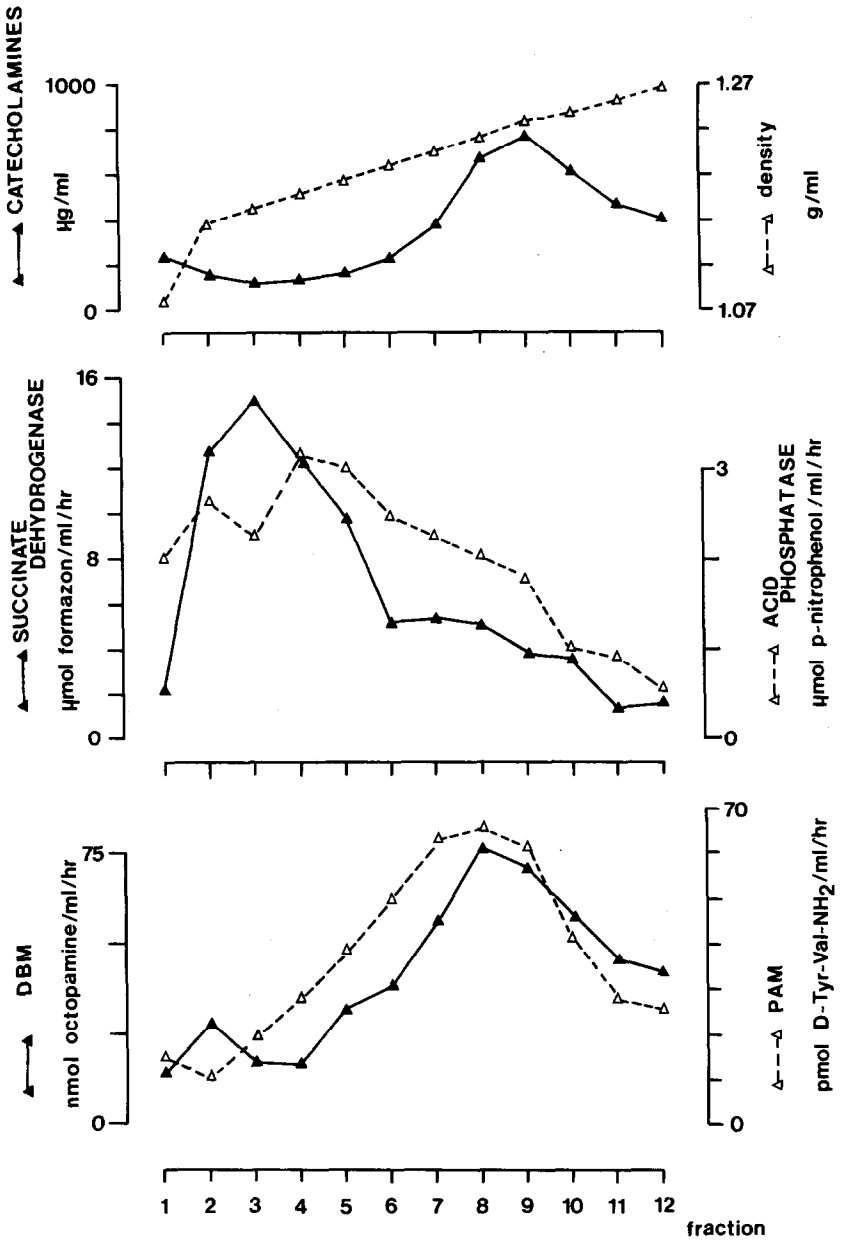

Fig.3. Sucrose density gradient fractionation of a large granule fraction. Each fraction was measured for catecholamines and density; succinate dehydrogenase and acid phosphatase, and DBM and PAM.

free from contamination with other cell particles. Therefore, we compared the distribution of PAM with that of markers for various cell particles in fractions obtained by density gradient centrifugation. In this gradient (fig.3) the distribution of PAM closely matched that of catecholamines and DBM, i.e. markers for chromaffin granules.

4. DISCUSSION

Since the soluble α-amidating enzyme characterized in the chromaffin granule is stimulated by Cu^{2+} and ascorbate, it probably belongs to the same family as other PAM enzymes [14,15].

The relatively broad peak of activity with a maximum of 40 kDa observed on gel filtration may reflect different forms of PAM in chromaffin granules. Previous reports have indeed reported heterogeneity in the apparent molecular mass of the enzyme in the pituitary, which was separated into two components of 54 and 38 kDa [16].

The subcellular fractionation experiments prove the presence of PAM in chromaffin granules. In accordance with two recent reports of PAM in the heart [17] and pituitary [18], membrane-associated PAM activity was also found in chromaffin granules. The membraneous and soluble forms of chromaffin granule PAM have nearly identical characteristics with respect to pH optimum and copper and ascorbate requirements. It is not yet clear whether the membrane form of PAM is similar to the putative precursor of PAM, for the sequence of this precursor [19] contains a membrane-spanning domain.

The finding that PAM is present in chromaffin granules is intriguing. There is a striking similarity of the enzyme to DBM [2], an important component of chromaffin granules. The relationship between these two enzymatic reactions occurring in the same granule seems an interesting topic for further research.

Acknowledgements: E.B. has a grant from IWONL. This investigation was supported by grants from the Queen Elisabeth Medical Foundation, 1987.

REFERENCES

[1] Bradbury, A.F., Finnie, M.D.A. and Smyth, D.G. (1982) Nature 298, 686–688.
[2] Eipper, B., Mains, R. and Glembotski, C. (1983) Proc. Natl. Acad. Sci. USA 80, 5144–5148.
[3] Winkler, H., Apps, D. and Fischer-Colbrie, R. (1986) Neuroscience 18, 261–290.
[4] Allen, J., Adrian, T., Polak, J. and Bloom, S. (1983) J. Autonom. Nerv. Syst. 9, 559–563.
[5] Weber, E., Esch, F., Bohlen, P., Paterson, S., Corbett, A., McKnight, A., Kosterlitz, H., Barchas, J. and Evans, C. (1983) Proc. Natl. Acad. Sci. USA 80, 7362–7366.
[6] Seizinger, B., Liebisch, D., Gramsch, C., Hersch, A., Evans, C., Esch, F. and Bohlen, P. (1985) Nature 313, 57–59.
[7] Smith, A.D. and Winkler, H. (1967) Biochem. J. 103, 480–482.
[8] Bastiaensen, E., De Block, J. and De Potter, W. (1988) Neuroscience 25, 674.
[9] Von Euler, U. and Hamberg, U. (1949) Acta Physiol. Scand. 19, 74–84.
[10] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[11] Li, H.C., Hsiao, K. and Sampathkumar, H. (1979) J. Biol. Chem. 254, 3368–3374.
[12] Porteous, J. and Clark, B. (1965) Biochem. J. 96, 159–171.
[13] Wallace, E., Krantz, M. and Lovenberg, J. (1973) Proc. Natl. Acad. Sci. USA 70, 2253–2255.
[14] Eipper, B., Myers, A. and Mains, R. (1985) Endocrinology 116, 2497–2504.
[15] Sakata, J., Mizuno, K. and Matsuo, H. (1986) Biochem. Biophys. Res. Commun. 140, 230–236.
[16] Murthy, A.S., Mains, R. and Eipper, B. (1986) J. Biol. Chem. 261, 1815–1822.
[17] Eipper, B., May, V. and Braas, Y. (1988) J. Biol. Chem. 263, 8371–8379.
[18] May, V., Cullen, E., Braas, K. and Eipper, B. (1988) J. Biol. Chem. 263, 7550–7554.
[19] Eipper, B., Park, L., Dickerson, I., Keutmann, H., Thiele, E., Rodriguez, H., Schafield, P. and Mains, R. (1987) Mol. Endocrinol. 1, 777–790.