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THE ISOLATION AND CHARACTERIZATION OF THE METHYL ACCEPTOR PROTEIN FROM ADRENAL CHROMAFFIN GRANULES*

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SUMMARY: A methyl acceptor protein (MAP), which serves as a substrate for adrenal medullary protein carboxymethylase (PCM, E.C. 2.1.1.24), has been isolated from a hypotonic lysate of adrenal chromaffin granules. The isolated MAP was shown to be distinct from the adrenal chromaffin granule protein, dopamine β-hydroxylase(DBH). The properties of MAP, including its amino acid composition, were comparable to those reported for chromogranin A, a major acidic protein found in adrenal chromaffin granules.

Diliberto et al (1) recently reported that the cytosol of adrenal chromaffin cells contains a protein carboxymethylase (S-adenosyl-L-methionine: protein O-methyltransferase, PCM, E. C. 2.1.1.24), which catalyzes the transmethylation from S-adenosylmethionine (SAM) to a protein carboxyl group (glutamyl or aspartyl residue) forming the protein methyl ester. The endogenous substrate for PCM, a so-called methyl acceptor protein (MAP), is found to be associated with the chromaffin granules. Upon exposure of chromaffin granules to methylation conditions, including PCM and SAM, a carboxymethylated protein is formed and reportedly released from the granules (1). A likely candidate for MAP is the chromaffin granule protein, chromogranin A. Chromogranin A contains large amounts of glutamyl and aspartyl residues (2) suggesting that it would be a good substrate for the carboxymethylation catalyzed by PCM. Therefore we have isolated MAP from adrenal chromaffin granules and compared its properties to those reported for chromogranin A.

Materials and Methods

PCM was purified from calf thymus (Pel-Freez Biologicals) or fresh bovine

*The abbreviations used are: SAM, S-adenosyl-L-methionine; DBH, dopamine β-hydroxylase; MAP, methyl acceptor protein; PCM, protein carboxymethylase.

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adrenal medulla through a DEAE-Sephadex step according to the procedure of Kim (3). The calf thymus PCM was used for assaying MAP during its purification from adrenal chromaffin granules. The adrenal PCM was used for kinetic studies. The MAP was assayed by a variation of the method of Jamaluddin et al (4). The assay mixture (0.25 ml) contained 40 mM sodium phosphate-sodium citrate buffer pH 6.5, 2 mM dithiothreitol, 6 mM EDTA, 6.64 μM S-adenosyl-L-[methyl-^{14}C] methionine (specific activity = 60 uCi/umole), PCM and MAP. The reaction was incubated at 37°C for 30 minutes and then terminated with 0.5 ml of saturated sodium borate solution. The assay solution was then incubated at 37°C for 1 minute, to complete hydrolysis of the methyl esters, and placed on ice. The borate solution was extracted with 5 ml of toluene-isoamyl alcohol (3:2). Radioactivity in an aliquot (4 ml) of the organic supernatant was determined by liquid scintillation spectrometry.

Dopamine β-hydroxylase (DBH) was assayed according to the procedure of Nelson and Molinoff (5). The chromaffin granules were isolated from fresh adrenal medulla (10-15 gms) by sucrose gradient centrifugation according to the procedure of Smith and Winkler (6) and lysed using hypotonic conditions (0.015 M KCl) (7,8). Chromaffin granule membranes were prepared by hypotonic lysis of chromaffin granules in 0.015 M KCl (2 ml/gm medulla) followed by washing with the same medium. The membranes were dialyzed against 0.15 M KCl (4 liters) for 18 hrs at 4° and again washed twice with 0.15 M KCl. All washings were followed by sedimentation at 390,000 g-min (7,8). For gel electrophoresis experiments, 8% polyacrylamide gels were prepared with 0.4 M Tris buffer, pH 6.8 and the electrophoresis was conducted with 0.2 M Tris-glycine buffer, pH 7.2. The gels were fixed and stained by incubation in a solution of Coomassie Brilliant Blue and 12% trichloroacetic acid. The gels were destained in 7% trichloroacetic acid.

Results and Discussion

The supernatant and membrane fractions obtained after centrifugation (390,000 g-min) of hypotonically lysed chromaffin granules were used as sources of MAP. The supernatant of the hypotonic lysate, which contained MAP activity (21.2 pmoles of ^{14}C-methyl/mg protein/min), was dialyzed against 5 mM sodium phosphate buffer, pH 6.5 and then lyophilized. The lyophilized protein was resuspended in 1-2 ml of distilled H_2O and applied to a Biogel A-15 (Biorad) column and eluted with 5 mM sodium phosphate buffer, pH 6.5. As shown in Figure 1 the MAP activity has an elution profile similar to that for DBH. A similar elution profile for DBH and chromogranin A has been reported when attempts were made to separate these proteins on Sephadex G-200 (6). The fractions containing MAP and DBH activities, which are obtained from the Biogel A-15 chromatography, exhibited two major proteins bands by polyacrylamide gel electrophoresis. The band with the lowest R_f co-electrophoresed with authentic DBH. The fractions (#55-80) containing DBH-MAP activity were pooled and concentrated using either an Aminco Protein Concentrator (PM-10 membrane) or lyophilization. The MAP present

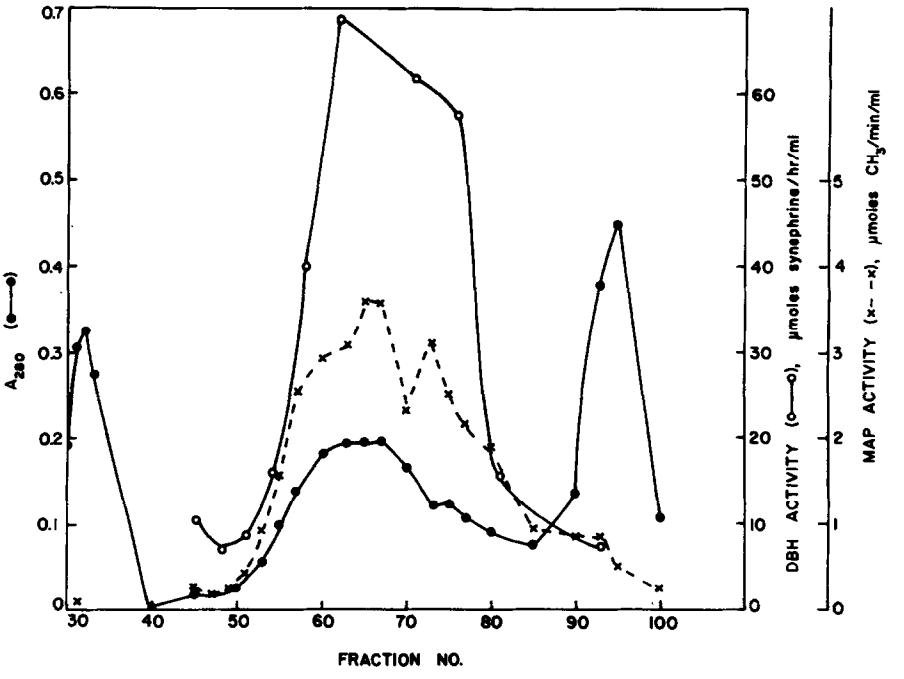

Figure 1. Elution profile for adrenal chromaffin granular MAP and DBH on Biogel A-15. An aliquot of the adrenal chromaffin granule lysate (7 mg protein) (7,8) was applied to a Biogel A-15 column (1.5 x 90 cm) eluting with 5 mM sodium phosphate buffer pH 6.5. 0.93 ml fractions were collected and assayed for DBH activity according to the method of Nelson and Molinoff (5) and MAP according to the method of Janaluddin et al (4) as described in the Material and Methods Section. MAP and DBH activity are expressed in terms of per ml of eluate. Protein concentration was estimated by monitoring absorbance at 280 nm.

in this pooled fraction had a specific activity of 25-30 nmoles of ^{14}C-methyl/mg protein/min.

The DBH activity was separated from MAP activity by chromatography on immobilized concanavalin A-agarose (Sigma), eluting with 0.1 M sodium acetate buffer, pH 6.5. The MAP activity eluted in the void volume, whereas the majority of the DBH activity could only be eluted with 0.2 mM α-D-methyl mannoside in the same buffer (Table 1). This chromatography step yielded a 6-fold purification of MAP, when compared to the lysed supernatant. Aunis et al (10) and Rush et al (11) have previously reported the separation of DBH from chromogranin proteins using a similar immobilized-concanavalin A-agarose column.

Table 1

Elution Pattern of MAP and DBH From an Immobilized Concanavalin A-Agarose Column[a]

Pooled Fractions No.	Elution Buffer[b]	MAP Activity[c] (pmoles methyl/mg protein/min)	DBH Activity[c] (pmoles synephrine/ mg protein/hr)
1-2	0.1 M sodium acetate, pH 6.5	124	83
3-19	0.1 M sodium acetate, pH 6.5 + 0.2 mM α-D-methyl mannoside	nd[d]	1000

[a] To an immobilized concanavalin A-agarose (Sigma) column (1.5 x 7 cm) was applied ∿1 mg of the concentrated DBH-MAP peak from the Biogel A-15 column (Figure 1). 10 ml fractions were collected and MAP and DBH activities monitored.

[b] The buffer also contained 1 M NaCl, 1 mM $MgCl_2$, 1 mM $MnSO_4$ and 1 mM $CaCl_2$.

[c] MAP and DBH activities were measured as described in the Material and Methods Section. DBH was measured at optimal Cu^{2+} concentration (5).

[d] nd = not detectable.

The MAP obtained from this immobilized concanavalin A-agarose column was dialyzed against 5 mM sodium phosphate, pH 6.5 and used for gel electrophoresis, amino acid analysis, and determination of methyl acceptor properties. The results of the acidic amino acid analysis of the MAP are shown in Table 2, with a comparison of the literature values reported for chromogranin A (6, 12-14). As can be seen there is good agreement between the acidic amino acid composition of the isolated MAP and that reported for chromogranin A. This is especially true for the large number of glutamyl and aspartyl residues.

When the MAP, which was obtained from the immobilized concanavalin A-agarose column, was subjected to gel electrophoresis one major protein band and one minor band were observed. This major band migrated with a higher R_f value than authentic DBH. When one gel was stained with Coomassie blue and another gel was sliced, extracted and assayed for MAP activity, it could be shown that this major protein band contained the MAP activity (Figure 2). When an amino acid analysis

Table 2

Amino Acid Analysis of MAP and Chromogranin A[a]

	Chromogranin A[b]	MAP[c,d]
Asp	72-75	75
Thr	25	24
Ser	67-71	63
Glu	197-212	225
Gly	63-84	71
Ala	71-84	80
Val	32-42	35
Met	12-18	10
Ile	9-18	9
Leu	58-71	50
Tyr	8-13	10
Phe	13-16	10
Lys	75-82	68
His	15-17	16

[a] Expressed as moles of amino acid/10^5g of protein

[b] Values taken from ref. 6, 12, 13 and 14

[c] MAP was purified from chromaffin granule lysate by chromatography on Biogel A-15 and immobilized concanavalin A-agrose as described in the Materials and Methods section

[d] MAP was hydrolyzed for 20 hr at 110°C in 6 M HCl and analysis was carried out on a Beckman 120C Amino Analyzer.

was conducted on the major protein band (9), a value of 223 moles of glutamic acid/10^5g of protein was observed. This value is similar to that observed for chromogranin A (Table 2) (6, 12-14).

Well-washed chromaffin granule membranes also exhibited MAP activity. When these membranes were subjected to polyacrylamide gel electrophoresis, bands were observed which exhibited R_f values identical to DBH and MAP found in the chromaffin granule lysate. Apparent Km values for the MAP in the lysate and the

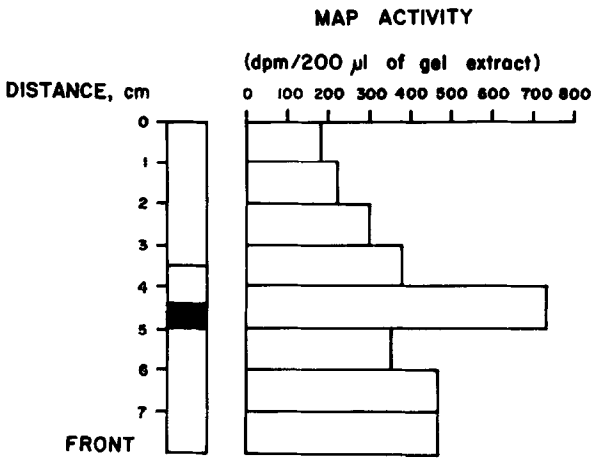

Figure 2. The polyacrylamide gel electrophoretic pattern vs methyl acceptor activity of MAP isolated from chromaffin granule lysate. MAP was purified through the immobilized concanavalin A-agarose column step. Duplicate gels were run. One gel was stained with Coomassie blue and the R_f values calculated. The other gel was sliced into 1 cm slices, extracted into 1 ml of sodium phosphate-sodium citrate buffer, pH 6.5 at 4°C for 20 hr. Aliquots (200 µl) of the gel extract were used to measure methyl acceptor activity.

membranes were 0.12 ± 0.03 and 0.45 ± 0.04 mg/ml, respectively. These values are somewhat lower but similar to the apparent Km of 0.96 ± 0.13 mg/ml reported by Diliberto et al (1) for chromaffin vesicle membranes.

The results of these studies indicate that the MAP found in the chromaffin granule lysate and chromaffin granule membrane is probably chromogranin A. Both the MAP and chromogranin A can be purified using similar procedures and they exhibit similar acidic amino acid analysis. We are currently continuing our investigation of the nature and physiological role of the MAP present in chromaffin granule membranes.

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