PEPTIDES IN THE ADRENAL MEDULLA CHROMAFFIN GRANULE

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1. Introduction

Of the many soluble proteins contained within the adrenal medulla chromaffin granule, only dopamine β-monooxygenase has a known function [1,2]. Much attention has been focused on the other soluble proteins, collectively called chromogranins [1]. Of these proteins, only two have been extensively studied: chromogranin A, an acidic protein with a reported minimum mol. wt $3.5–4 \times 10^4$ or 7×10^4 and a leucine amino terminus [3–5]; and chromolipin, a high molecular weight soluble lipid–protein complex containing a single, 2×10^3 mol. wt polypeptide species [6]. Although the presence of active peptides in the chromaffin granule has been predicted on the basis of its embryological origin, the neural crest [7], the methods used for purifying the chromogranins discriminate against the isolation of small peptides and proteins. The appearance of fast-moving components in sodium dodecyl sulfate (SDS)-containing polyacrylamide gels suggests the presence of such peptides. Therefore, we have examined the soluble lysate of bovine chromaffin granules for peptides, focusing on those of $10^3–10^4$ mol. wt. The data below describe a series of peptides which are of potential biological interest.

2. Experimental

Bovine chromaffin granules were prepared from freshly dissected adrenal medulla (300 g) using standard differential centrifugation techniques described [8]. The purified granules were subjected to hypotonic lysis in 20 vol. of 10 mM potassium phosphate (pH 6.5) for 45 min at 4°C. After centrifugation at 20 000 × *g* for 1 h, the pellet was resuspended in the same buffer for the same amount of time, and then recentrifuged.

The combined supernatants were concentrated in an Amicon pressure cell using a PM10 membrane (10^4 mol. wt cutoff). The filtrate from this step, our peptide starting material, was in turn concentrated using a UM2 membrane (10^3 mol. wt cutoff). The material concentrated by this procedure was dialyzed against 4 changes of 10 l each of 1 M NaCl using a Spectrapore 6 dialysis tubing (10^3 mol. wt cutoff) to remove the remaining salts, nucleotides and catecholamines. The dialyzed material was then applied to a Sephadex G-50 superfine column, 1.0 cm × 2 m, in 25 mM potassium phosphate buffer (pH 7.2) containing 1 M NaCl and eluted at 5 ml/h flowrate. A_{220} was used to detect the presence of peptides.

The filtrate from the UM2 concentration step was concentrated with a UM05 membrane (500 mol. wt cutoff) and repeatedly washed with water to diminish the ionic strength. The salt-free concentrate was lyophilized, redissolved in 1 ml 0.05 M pyridine acetate (pH 2.5) and applied to a 0.6 × 20 cm column of AA-15 resin (Beckman Instr.) equilibrated in the same buffer at 55°C. The column was washed with the starting buffer for 1 h at 10 ml/h and eluted with a gradient consisting of 50 ml each of: 0.05 M pyridine acetate (pH 2.5), 0.2 M pyridine acetate (pH 3.2) and 0.5 M pyridine acetate (pH 3.75). The column was stripped with 1 M pyridine acetate (pH 5.0) [9]. Peptides were detected using 150 μl aliquots of each tube

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which had been pre-hydrolyzed [10].

Amino acid analyses were performed using a Dionex fluorescent amino acid analyzer with an M.D. Robin laboratory mount. Secondary amine conversion for proline detection was used only on some samples because of the decreased sensitivity of the method. Dionex Femtobuffers stored under nitrogen were used for the analyses. Samples were prepared by hydrolysis under reduced pressure at 110°C in redistilled, constant boiling HCl for 24–72 h. Performic acid oxidized samples were used for the detection of $Cys_{1/2}$ residues [11]. Tryptophan was determined spectrophotometrically [12]. Electrophoresis on polyacrylamide gels in 0.1% SDS and 8 M urea was performed according to [13].

3. Results

3.1. *Electrophoresis of soluble chromaffin granule lysate*

When the total soluble material from chromaffin granule lysates is analyzed by electrophoresis in 12.5% gels, we detect $\geqslant 4$ bands which have mobilities greater than myoglobin (fig.1). These polypeptides of app. mol. wt $< 10^4$ migrate with the ion front in gels of $\leqslant 8\%$, frequently used for these systems. In order to determine the nature of this material, we processed the lysate by differential concentration through ultrafiltration membranes of 10^4 and 10^3 mol. wt cutoff as in section 2. During preliminary experiments we have found that much of the starting material concentrated by the UM2 membrane, that is of mol. wt 10^3–10^4, aggregates at low ionic strength in the absence of the other lysate components. Therefore, this higher molecular weight pool was dialyzed in 1 M NaCl and then applied to the gel filtration column described below.

3.2. *Gel filtration of UM2 concentrate*

The starting material concentrated by the UM2 membrane, and thus containing higher molecular weight peptides, was applied to a Sephadex G-50 superfine column as in section 2 (fig.2). Because these polypeptides tended to aggregate rapidly at the conditions of low ionic strength required for electrophoresis, even in the presence of SDS and urea, we ascertained the purity of fractionated material by perform-

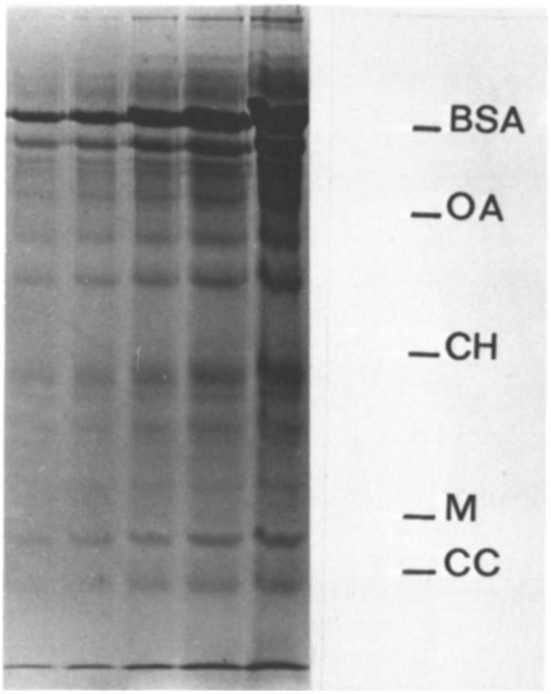

Fig.1. Electrophoresis of soluble chromaffin granule proteins. Varying concentrations of the soluble lysate were subjected to polyacrylamide gel electrophoresis in the presence of 0.1% SDS and 8 M urea. The bars at right indicate the positions of the protein standards: BSA, bovine serum albumin; OA, ovalbumin; CH, chymotrypsinogen; M, myoglobin; CC, cytochrome *c*.

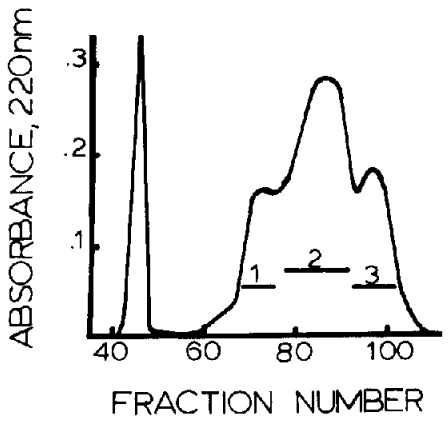

Fig.2. Gel filtration of larger chromaffin granule peptides. A Sephadex G-50 superfine column, 0.9 cm × 2 m in 1 M NaCl was eluted at 5 ml/h flowrate. Bars and numerals indicate fractions made.

Table 1
Amino acid compositions of chromaffin granule peptides (residues/mol)

Amino acid	G-1	G-2	G-3	A-1	A-2	A-3	A-4	A-5	A-6	A-7	A-8	A-9	A-10	A-11	A-12	UM05 filtrate
Cystine$_{1/2}$[a]	–	–	–													
Aspartic acid	4	1	2	1	1	1	1	1	1	1	1	1	1	1	1	5.0[d]
Threonine	1	–	1	1	1	1	1		1	1						2.0
Serine	4	1	1	1	2	1	1	1	1	1	1	1	1	1	2	3.4
Glutamic acid	14	1	2	3	1	2	3	2	2	2	2	2	2	2	2	10.8
Proline	4	n.d[c].	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glycine	5	4	2	1	2	2	2	2	2	2	2	3	1	2	1	42.8
Alanine	2	1	2	1	1	1	1	1	1	1	1	1	1	1	1	6.8
Valine	–	–	1		1											3.0
Methionine	2	–	–													1.4
Isoleucine	–	–	1													1.5
Leucine	2	–	2	1	1	1		1	1		1		1	1	1	5.3
Tyrosine	–	2	1													3.0
Phenylalanine	1	3	1													3.0
Histidine	–	–	–													2.1
Lysine	4	1	1						1		1	1	1	1	1	4.0
Arginine	2	3	1										1	1		5.9
Tryptophan[b]	–	–	–													
Total residues	45	17	18	9	10	9	9	8	10	8	9	9	9	10	9	
Mol. wt	5013	2014	1985													
Yield (nmol peptide)	190	600	430	10.5	23.2	7.5	7.9	21.4	33.5	5.7	34.9	7.6	39.0	17.6	61.8	

[a] Determined as performic acid [11]
[b] Determined spectrophotometrically [12]
[c] n.d., not determined
[d] As mol%

ing amino acid analyses and ultraviolet spectra across each peak. Of the 4 peaks eluted from the column, the first appeared to contain oxidized residual catecholamines, due to its spectral characteristics and the lack of amino acid material in acid hydrolyzates. The remaining 3 major peaks, G-1 to G-3, are not completely resolved by this fractionation step, but can be separated by a further gel filtration step on the same column. Each of the purified pools possesses a distinctive amino acid composition as shown in table 1. The first peak, G-1, is very acidic, and completely lacks Val, Ilu, Tyr and Cys½. Spectrophotometric analysis also revealed the absence of tryptophan. The integral number of residues total 45, for mol. wt ~5000. G-2, the second peak eluted from the gel filtration column, is particularly rich in glycine and in the aromatic amino acids. When the values from amino acid analysis of duplicate samples of timed acid hydrolysates are normalized, 17 residues are found to be present, yielding calculated mol. wt 2000. Peptide G-3 is distinct in amino acid composition from both G-1 and G-2 and contains 18 amino acid residues with mol. wt ~2000.

3.3. *Ion-exchange chromatography of UM05 concentrate*

The lower molecular weight material concentrate by the UM05 membrane was desalted, lyophilized, and dissolved in 0.05 M pyridine acetate (pH 2.5) before applying to a column of AA-15 resin. When the column was eluted as described in section 2, the profile shown in fig.2 was obtained. The pools marked by bars and numbers, A-1 to A-12, were those found to contain significant peptide material by amino acid analysis of acid hydrolysates of aliquots. Other fractions were pooled and analyzed before discarding. Although these peaks are chromatographically distinct, their amino acid compositions are not (table 1). The earlier pools are richer in more acidic amino acids, and the later pools are relatively rich in the basic amino acids, as expected from their elution characteristics. However, these analyses are strikingly similar to each other and different from the analyses of G-1, G-2 and G-3. This similarity found in such small peptides raises the possibility that they are structurally related.

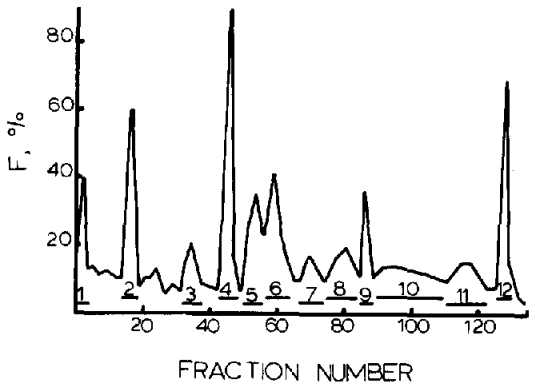

Fig.3. Chromatography of smaller chromaffin granule peptides. Cation exchange chromatography using a 0.6 × 20 cm column of AA-15 resin at 55°C in pyridine acetate buffers. Bars and numerals indicate pools made. F, relative fluorescence.

4. Discussion

For many years, only the higher molecular weight proteins in the chromaffin granule have been studied, ignoring the lower molecular weight material. Recently, the presence of enkephalin, the opiate peptide, has been reported within the granules [14]. The above results provide compelling evidence for the existence of a third distinct class of peptides of intermediate molecular weight. These peptides were detected only after treatment of granule preparations by hypotonic shock, and thus appear to be inherent to the granule, but not attached to its membrane. In experiments to be reported elsewhere, we have found no detectable proteolytic activity in our chromaffin granule preparations. These data would preclude the possibility that the peptides are generated during our experimental manipulations. In addition, we are now purifying the UM05 filtrate material from the above experiments. Although the amino acid composition shown in table 1 indicates the presence of the Tyr-rich enkephalin in this pool, the striking feature of the analysis is the abundance of Gly, 2700 nmol. The absence of free glycine in unhydrolyzed samples indicates that this material does not represent free glycine. Furthermore, it is partially retained in the crude UM05 concentrate, indicating mol. wt 500–600. This low molecular weight material is now being examined to determine the nature of the Gly-containing compound, and to

elucidate the enkephalin and other small peptides clearly present.

It has been predicted that adrenal medulla chromaffin cells contain biologically active peptides in addition to catecholamines, as representatives of the class of neuroendocrine cells derived embryologically from specialized ectoderm [7]. Our observations confirm the existence of peptides in the chromaffin granules. It will be important to define both their structures and their potential biological activities. Do these peptides perform some role within the granule, such as the sequestration of catecholamines, nucleotides or ions, or the modification of intragranular enzymatic activities? On the other hand, could these peptides act after being secreted, by somehow modulating the catecholamine responses? Experiments will be undertaken to determine whether these peptides are secreted, whether they possess some known pharmacological activity, or bind to a specific compound or subcellular component.

Acknowledgements

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