The prohormone processing activity is enriched in a low-density subpopulation of chromaffin granules

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Bovine adrenomedullary chromaffin granules can be separated into two subpopulations by differential centrifugation. The subpopulation which sediments at the interface of two sucrose layers, 1.6 and 1.8 M respectively, is found to be enriched about 10-times in prohormone processing activity, as measured by in vitro degradation of synthetic peptide substrates. The enhanced proteolytic activity is not due to lysosomal contaminations which are very low and only slightly increased in the more active fraction. The low density of the enriched subpopulation suggests that we are dealing with immature granules. The physiological implications of this finding are discussed. Furthermore, the enriched fraction can be used as the starting material for the isolation of proenkephalin processing enzymes.

Chromaffin granule *Prohormone processing*

1. INTRODUCTION

Evidence has accumulated in recent years that peptide hormones are cleaved from larger protein precursors in which paired basic residues are recognized as signals for limited proteolysis [1]. Proprotein processing is believed to begin shortly after synthesis in the rough endoplasmic reticulum and transfer of the peptides to the Golgi apparatus where they are packaged into secretory granules. Processing then continues for many hours within newly formed granules as they mature [2]. The converting proteases involved in processing seem to be required in very low levels [2] and only limited progress has been reported in their isolation and characterization. Carboxypeptidases acting in the second step are better characterized [3,4] than the basic pair recognizing enzymes acting in the first converting step [5,6].

In the course of our attempts to measure proenkephalin processing activity by non-immunological methods [7], we developed a new assay using convenient synthetic peptide substrates and HPLC for detection of the degradation fragments. By this method, we observed that lysed chromaffin granules from bovine adrenal medulla contained more processing activity when they had sedimented through a 1.6 M than through a 1.8 M sucrose bed. The purpose of this work was therefore to isolate and characterize the subpopulation of granules which would sediment at the interface of 2 layers of sucrose, 1.6 and 1.8 M, respectively, during ultracentrifugation. We were able to show that this fraction is indeed enriched in prohormone processing activity without a concomitant increase in lysosomal contaminants.

2. MATERIALS AND METHODS

Chromaffin granules from bovine adrenal medulla were prepared by the method of Bartlett and Smith [8] modified so as to allow the separation of 2 populations of granules. Briefly, the medulla of 25 adrenal glands was separated from the cortex and homogenized (glass/glass clearance 0.12 mm) in about 5 vols of 0.3 M sucrose. After centrifugation at 400 × *g* at 2°C of this suspension, and at 9000 × *g* at 2°C of the supernatant, the new sediment was resuspended in a small volume (2 ml in each of the 4 tubes) of 0.3 M sucrose. This crude

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preparation of granules was then pelleted through 1.6 M sucrose for 1 h at 82000 × *g* and 2°C. A subpopulation of granules could be further isolated when the last ultracentrifugation was performed in tubes containing 10 ml of 1.8 M and 10 ml of 1.6 M sucrose. The band forming between the 2 sucrose layers (15–20% of the total pellet) was then cautiously gathered, diluted to 1.5 M sucrose and sedimented under the same conditions through 1.6 M sucrose. Lysis of the granules was then obtained when each pellet was suspended in 5 mM Tris-5 mM succinate (pH 5.7), frozen, thawed, sonicated and centrifuged at 61000 × *g* for 1 h at 2°C. The supernatant was dialyzed against Tris-succinate buffer and then used for incubation with the synthetic peptide substrates.

The tetrapeptide Ala-Lys-Arg-Tyr (AKRY) was prepared in our laboratory by classical methods of peptide synthesis in solution and characterized by elemental and amino acid analysis. The dodecapeptide Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu (BAM 12P) was purchased from Bachem, Bubendorf, Switzerland.

The assay for enzymatic processing activity was performed by incubation at 37°C and pH 5.7 of 180 μl of the lysate with 20 μl aqueous substrate solution. The concentrations of AKRY and BAM 12P were 10 and 1 mg/ml, respectively, while the protein concentration varied between 1.7 and 7.2 mg/ml. Samples were removed at several times between 0 and 24 h for AKRY, and 0 and 6 h for BAM 12P. Quantitative detection of the degradation products was obtained by HPLC on reversed phase (Spherisorb S5 ODS2 from Phase Sep, Waddinxveen, The Netherlands) at λ = 220 nm using shallow aqueous/acetonitrile gradients for elution. The conditions were: (i) for tetrapeptide and degradation products: gradient from 0 to 7% acetonitrile in aqueous 5 mM sodium phosphate/100 mM sodium perchlorate (pH 7.4) over 15 min; (ii) for dodecapeptide and degradation fragments: 20–22% acetonitrile in aqueous 0.1% trifluoroacetic acid over 30 min. A whole spectrum of the eluted peaks was obtained which allowed us to follow, at the 0.1 nmol level for AKRY and at the pmol level for BAM 12P, the time-dependent disappearance of the substrate and the formation of the degradation fragments. When using AKRY as substrate, the degradation rate of the tetrapeptide was chosen as a measure of the processing activity, 1 unit (U) being defined as the enzyme quantity which degraded 1 pmol AKRY per min at 37°C and pH 5.7. When using BAM 12P, the formation of methionine-enkephalin was observed, 1 unit being defined as the enzyme quantity which produced 1 pmol methionine-enkephalin per min under the same conditions. Controls indicated that the substrates were stable when the lysate was boiled for 5 min immediately after mixing, and that the lysate did not contain interfering amounts of methionine-enkephalin or of other degradation fragments used in calibration.

Protein was determined according to Bradford [9] since this method was shown to be insensitive to catecholamines and to give reliable results for adrenal tissue extracts [10]. β-Glucuronidase activity was estimated according to Gianetto and De Duve [11].

3. RESULTS

The 2 peptide substrates were found to be degraded by the lysed fractions of granules. Proteolytic activities of the 2 subpopulations from 3 independent preparations are displayed in table 1. There is an about 10-fold increase of the specific activity in the low-density fraction and this ratio is seen to be approximately the same for the 2 substrates. Also included in table 1 is the β-glucuronidase activity as a marker for lysosomal

Table 1

Prohormone processing and β-glucuronidase activities in the two subpopulations of chromaffin granules

Substrate	Activity (± SD[a])		Ratio
	Dense fraction (5.7 mg/ml)[b]	Light fraction (2.7 mg/ml)[b]	
AKRY	20 ± 8 U/mg[c]	180 ± 22 U/mg	9.0
BAM 12P	2.1 ± 0.3 U/mg	21.7 ± 1.7 U/mg	10.3
Glucuronide	192 ± 13 U′/mg[d]	355 ± 26 U′/mg	1.87

[a] SD, standard deviation (9–15 measurements)
[b] Mean protein concentration in lysates
[c] For definition of unit (U), see section 2
[d] One unit (U′) is the glucuronidase quantity which hydrolyzes 1 pmol phenolphthalein glucuronide per min at 37°C and pH 5

contaminants in the granule lysates. The low glucuronidase activity is increased less than 2-fold in the light fraction.

4. DISCUSSION

Our experiments demonstrate that the 2 peptide substrates AKRY and BAM 12P can be used to assay proenkephalin processing activity in vitro. The assay, although cumbersome since an extract of each fraction has to be run on HPLC, is operative and reproducible. However, the measured activity is likely to originate from several proteases with different cleavage mechanisms which are not easily distinguished at this stage. Also, due to the shortness of the peptide substrates, 3-dimensional factors which are probably effective for the choice of the cleavage site in vivo are not taken into account in this assay.

The main result reported here is the comparatively high processing activity in the lighter subpopulation of granules. Interestingly enough, the same activity increase by approximately a factor 10 was observed with the 2 substrates. The enhanced activity cannot be due to a concomitant increase of the lysosomal contaminants, since the specific β-glucuronidase activity, used as a marker, only increased 2-fold in the enriched fraction.

Since it is well known from earlier work [12] that immature granules are less dense than mature ones, the result suggests that prohormone, in particular proenkephalin processing might predominantly occur in the early stages of granule maturation. This observation agrees well with at least one of the current views [1] according to which fusion of the processing enzymes containing vesicles with the budded off area of the Golgi would occur just prior to the complete formation of the secretory granules, thus initiating intragranular processing. Our results also fit to the findings of Chaminade et al. [13] who classify chromaffin granules into mature granules in which proenkephalin is fully processed and immature ones in which high-M_r fragments of the precursors are found. Our result also probably implies an inactivation mechanism of the processing enzymes during granule maturation.

From a practical point of view, the enriched fraction of granules is a convenient starting material for the isolation of prohormone processing enzymes.

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