Two peptidases that convert ^{125}I-Lys-Arg-(Met)enkephalin and ^{125}I-(Met)enkephalin-Arg^6, respectively, to ^{125}I-(Met)enkephalin in bovine adrenal medullary chromaffin granules

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Two peptidases which convert ^{125}I-Lys-Arg-ME and ^{125}I-ME-Arg^6, respectively, to ^{125}I-ME, have been identified and characterized in bovine adrenomedullary chromaffin granules. The former is referred to as a secretory granule peptidase (SGP) and the latter as a carboxypeptidase B-like enzyme (CPB-like) [7] which is here further characterized. SGP cleaved ^{125}I-Lys-Arg-ME to produce only ^{125}I-ME and was localized in chromaffin granules which contained Co^{2+}-stimulated CPB-like activity, ME, and catecholamines. Both the SGP and the CPB-like enzymes appear to be thiol-metalloproteases. While the CPB-like enzyme seems likely to be involved in processing the enkephalin precursors [7], SGP may function as a trypsin-like or aminopeptidase enzyme in secretory granules.

Limited proteolysis *Secretory granule* *Proenkephalin*

1. INTRODUCTION

Secretory granules in peptidergic cells synthesize, store, and release small peptides and neurotransmitters as extracellular chemical messengers. These granules should contain a trypsin-like endopeptidase which cleaves on the carboxyl terminal side of basic amino acid residues and a carboxypeptidase B-like (CPB-like) enzyme which removes C-terminal lysine or arginine residues for processing hormone precursors into their mature forms. Indeed, secretory granule trypsin-like enzymes have been found that cleave proinsulin [1–3] and POMC (proopiomelanocortin) [4–6]. CPB-like enzymatic activities which may be involved in the processing of proenkephalin and POMC or provasopressin have been demonstrated in chromaffin granules [7,8] and pituitary secretory granules [9], respectively. Other peptidases may be important for secretory granule function. Here, we describe a peptidase in bovine adrenomedullary chromaffin granules which catalyzes the conversion of ^{125}I-Lys-Arg-ME to ^{125}I-ME and refer to it as a secretory granule peptidase or SGP. Its possible functions are discussed and the CPB-like activity described in [7] is further characterized.

Abbreviations: ME, methionine enkephalin; Bz1SA, benzylsuccinic acid; APMSA, aminomercaptosuccinic acid; GPSA, guanidinopropylsuccinic acid; PMSF, phenylmethylsulfonyl fluoride; PCMPSA, *p*-chloromercuriphenylsulfonic acid; TLCK, tosyllysine chloromethyl ketone; TLC, thin-layer chromatography

2. MATERIALS AND METHODS

2.1. *Preparation of bovine adrenal medulla chromaffin granules*

Purified chromaffin granules were prepared by a modification of the method in [10]. As described [7], purified granules were made by applying a crude granule preparation (10 ml in 0.32 M sucrose) on 1.6 M sucrose (25 ml) and centrifuging at 25000 rpm for 120 min in a Beckman SW-28 rotor; the pellet contains purified chromaffin

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granules. Centrifugation through an additional 1.6 M sucrose gradient results in a preparation virtually free from lysosomal contamination (this fraction contained 0.05% of total tissue acid phosphatase, a lysosomal enzyme marker). The granules were lysed in 0.015 M KCl at 4°C for 2 h. Membrane and soluble granule fractions were separated by centrifugation for 1 h at 10000 rpm in a Sorvall SS-34 rotor. For analysis of chromaffin granule and lysosomal content in the crude and purified granule fractions, each sample was layered on a multistep discontinuous sucrose gradient consisting of 4 ml each of 2.2, 2.0, 1.8, 1.6, 1.4, 1.2, and 1.0 M sucrose and centrifuged at 25000 rpm for 100 min in a Beckman SW-28 rotor. Each 1.0 ml fraction from the gradient was assayed for ME immunoreactivity [11], catecholamines [12], acid phosphatase activity [13], and protein [14].

2.2. *SGP assay for the conversion of ^{125}I-Lys-Arg-ME to ^{125}I-ME*

Lys-Arg-ME (Peninsula Laboratory, San Carlos, CA) was iodinated by the chloramine-T method [7]. The reaction mixture contained, in a total volume of 20 μl, 5–10 $\times$ 10^{-5} M ^{125}I-Lys-Arg-ME, 150 mM K-phosphate (pH 7.5) and 150 mM NaCl. Enzyme sample (10 μl) was added to the reaction mixture and incubated at 37°C for 60 min. The reaction was stopped by adding 10 μl of 95% EtOH. Substrate and product were separated by TLC as described for the CPB assay [7]. Radioactivity was scanned along each lane by a BID 100 radiogram imaging system (Bioscan Inc., Washington, DC) and total cpm ^{125}I-ME was determined by computing the integral of each peak with an HP-85 computer.

When Lys-Arg-ME and its products were to be separated by high-voltage electrophoresis (HVE), the SGP reaction mixture (in a volume of 20 μl) contained 20 μg Lys-Arg-ME (non-radioactive), 150 mM K-phosphate (pH 7.5) and 150 mM NaCl. The soluble fraction of chromaffin granules was dialyzed to remove catecholamines, which produce interfering fluorescence. Enzyme sample (10 μl) was added to the reaction mixture (20 μl), and incubated at 37°C for 15 min to 6 h. After addition of 25 μl of 90% EtOH–10% acetic acid, samples were vortex mixed, kept on ice for 30 min, and spotted on cellulose TLC plates (Avicel, 250 μm, Analtech, Newark, DE). HVE and visualization of peptides by fluorescamine were performed as in [9].

2.3. *Carboxypeptidase assay*

The conversion of ^{125}I-ME-Arg^6 to ^{125}I-ME was measured at pH 6.0 as in [7].

2.4. *Gel filtration column chromatography*

The soluble fraction of chromaffin granules (purified through one 1.6 M sucrose gradient) was concentrated and applied onto a Sephadex G-75 (Pharmacia, 1.2 $\times$ 71 cm) column equilibrated in 50 mM K-phosphate buffer (pH 6.0). Fractions of 3 ml were collected with a flow rate of 20 ml/h, and aliquots of 10 μl were assayed for peptidase activities.

3. RESULTS

3.1. *SGP activity in chromaffin granules*

SGP activity for the conversion of ^{125}I-Lys-Arg-ME to ^{125}I-ME was present in the soluble component of chromaffin granules, as shown by the production of a single radioiodinated product which comigrated with ^{125}I-ME (fig.1). The activity was dependent on enzyme concentration and incubation time, and no activity was seen at 0°C or with a boiled sample (not shown). Purified pancreatic trypsin also converted ^{125}I-Lys-Arg-ME to a single product comigrating with ^{125}I-ME (not shown).

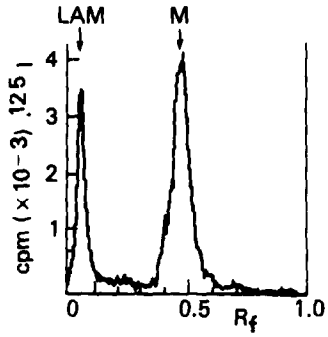

Fig.1. SGP activity for the conversion of ^{125}I-Lys-Arg-ME to ^{125}I-ME in the soluble fraction of bovine adrenomedullary chromaffin granules. Activity was measured at pH 7.4. Substrate and products were separated by TLC. Arrows indicate the migration positions of standard ^{125}I-Lys-Arg-ME (LAM) and ^{125}I-ME (M).

SGP had optimal activity at pH 7.4–8.5, with 30% of optimum activity present at pH 6.0. No activity was found in the membrane fraction (not shown).

To determine whether the cleavage occurred between the Arg-Tyr residues or if removal of Lys is followed rapidly by removal of Arg, substrate and products were separated on the basis of their differential charge by HVE (fig.2). Standard migration positions of Lys-Arg, Lys, Arg, Lys-Arg-ME, and ME show that the majority of these species could be separated by HVE. The migration positions of standard Lys and Arg overlapped with one another; therefore, fluorescamine-labelled spots comigrating with Lys or Arg were referred to as a mixture of Lys/Arg. Incubation of Lys-Arg-ME with the soluble granule fraction for 4 h followed by HVE resulted in the visualization of 3 fluorescamine-positive spots (fig.3): one migrating with Lys/Arg, one with ME, and one, presumably

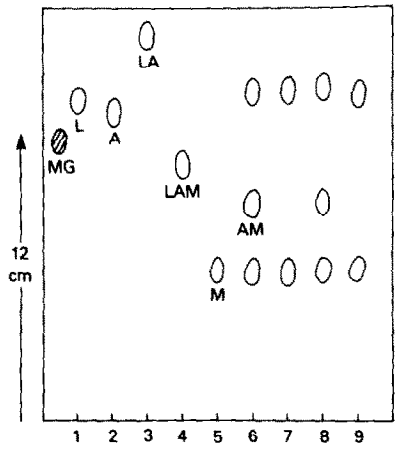

Fig.2. Cleavage of Lys-Arg-ME by soluble fraction of bovine adrenomedullary chromaffin granules. Substrate and cleavage products were separated by HVE on cellulose TLC plates. Electrophoresis was run at 800 V until the methyl green (MG) marker dye migrated 12 cm from the origin. Migration position of standards: lane 1, lysine (L); lane 2, arginine (A); lane 3, Lys-Arg (LA); lane 4, Lys-Arg-ME (LAM); lane 5, ME (M). LAM was incubated with 1 μg pancreatic trypsin for 15 min (lane 6) and 1 h (lane 7), and incubated with the soluble fraction of purified chromaffin granules for 4 h (lane 8) and for 6 h (lane 9). In lane 6, since free Lys was liberated, the other expected product would be Arg-ME, indicated by AM, which would be predicted to migrate to a smaller R_f than LAM based on their differential charge.

Arg-ME, which migrated between ME and Lys-Arg-ME. Further incubation for 6 h resulted in complete conversion of Lys-Arg-ME to ME and Lys/Arg; no spot corresponding to the dipeptide Lys-Arg was visualized. An identical pattern was seen with purified pancreatic trypsin. The formation of Arg-ME and free Lys/Arg suggests that the soluble fraction of chromaffin granules, like the purified pancreatic trypsin, removed the N-terminal basic residues sequentially.

3.2. *Sucrose density gradient fractionation of SGP and CPB-like activities*

To determine whether the SGP and CPB-like activities reside uniquely in chromaffin granules, a preparation of crude chromaffin granules and purified granules (crude granules purified through one 1.6 M discontinuous sucrose gradient) was fractionated on a multistep sucrose gradient of 2.2–1.0 M sucrose. Chromaffin granules were found to make up the major part of the crude granule preparation, but there is a small lysosomal contamination (fig.3). SGP- and CPB-like activities were present in ME and catecholamine-containing granule fractions (fig.4). Co^{2+}-stimulated CPB-like activity was associated with the granular but not the lysosomal fractions, as reported in [8]. Protease(s) in the lysosomal fractions cleaved ^{125}I-Lys-Arg-ME (not shown) and ^{125}I-ME-Arg^6 [7] substrates to many unidentified radiolabelled substances in addition to ^{125}I-ME. Only the granule enzymes cleaved the substrates to produce ME exclusively.

Sucrose gradient analysis of purified granules (fig.3) shows that lysosomal contamination has been removed, and SGP and Co^{2+}-stimulated CPB-like activities remained with the granule fractions. The large amount of enzyme activities, ME, catecholamines, and protein in fractions 31–34 was probably due to the presence of some lysed granules.

3.3. *Properties of SGP and CPB-like activities*

The effect of several protease inhibitors on SGP and CPB-like activities was studied (table 1). The soluble fraction of granules purified through two 1.6 M discontinuous sucrose gradients was used here since this fraction contained large amounts of both enzymes. Thiol reagents such as Cu^{2+} and PCMPSA [15], inhibited both enzyme activities.

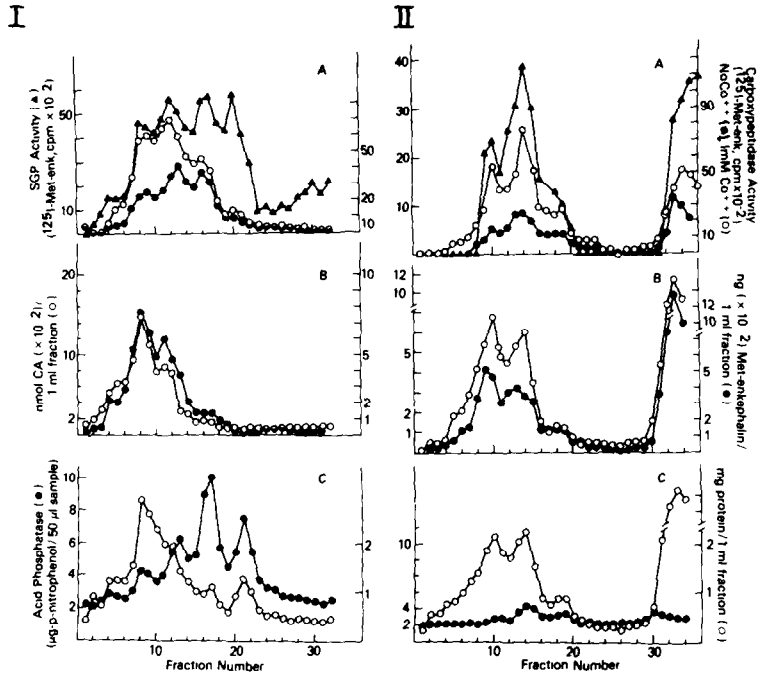

Fig.3. Sucrose density gradient fractionation of crude and purified chromaffin granules from bovine adrenal medulla. Crude (panel I) or purified (panel II) chromaffin granules were fractionated on a multi-step sucrose gradient (2.2–1.0 M sucrose). Each fraction was measured for: (A) SGP and CPB-like activities; (B) catecholamine and ME content; (C) acid phosphatase activity and protein content.

Both enzymes were inhibited by the metal chelators 1,10-phenanthroline and EDTA. SGP activity was inhibited by Ni^{2+}, Cd^{2+}, and Co^{2+}, while the CPB-like activity was stimulated by Co^{2+} [8] and inhibited by the Zn-metallocarboxypeptidase inhibitors Bz1SA, APMSA, GPSA, and the potato inhibitor [16–18]. The effect of the Zn-metalloprotease inhibitors was not originally apparent [7]; this may be due to the present use of ^{125}I-ME-Arg^6 with higher specific activity and a Bioscan 100 imaging system with greater resolution, resulting in a more sensitive enzyme assay. Lack of inhibition by PMSF and TLCK suggests that neither enzyme is a serine protease.

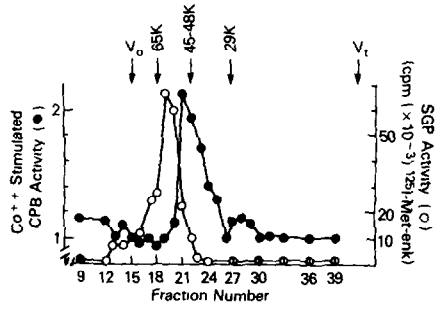

Fig.4. Sephadex G-75 column chromatography of soluble fraction from chromaffin granules: SGP and Co^{2+}-stimulated CPB-like activities.

SGP- and CPB-like activities in adrenal medulla chromaffin granules and lysosomes were compared. The lysosomal SGP-like activity was inhibited by antipain but the granule enzyme was only slightly inhibited. Chloroquine had no effect on the granule CPB-like activity but somewhat inhibited lysosomal carboxypeptidase. Co^{2+} stimulated the granule CPB but had no effect on the lysosomal activity.

The soluble fraction of chromaffin granules was chromatographed on a Sephadex G-75 gel filtration column (fig.4). SGP and Co^{2+}-stimulated CPB-like activities eluted from the column as separate peaks with apparent M_r values of approx. 55000–60000 and 40000–50000, respectively.

Table 1

Effect of inhibitors and activators on SGP and CPB activities in chromaffin granules and lysosomes from bovine adrenal medulla

Inhibitor	Concentration	Enzyme activity (% control, $x \pm$ SE)			
		Chromaffin granules		Lysosomes	
		SGP	CPB	SGP-like	CPB-like
None	–	100	100	100	100
PCMPSA	4.1×10^{-4}	23 ± 5[a]	10 ± 38[a]	44 ± 20[a]	29 ± 8[a]
$CuCl_2$	1.4×10^{-4}	11 ± 3[a]	13 ± 1[a]	25 ± 23[a]	28 ± 10[a]
1,10-Phenanthroline	1.4×10^{-3}	16 ± 8[a]	57 ± 18[a]	17 ± 17[a]	82 ± 11
EDTA	1.4×10^{-4}	67 ± 6[a]	71 ± 13[a]	51 ± 48	88 ± 3
Potato inhibitor	4.6×10^{-5}	92 ± 7	82 ± 3[a]	82 ± 21	99 ± 8
BzlSA	1.4×10^{-4}	88 ± 3	69 ± 10[a]	99 ± 3	36 ± 6[a]
APMSA	2.8×10^{-4}	97 ± 4	57 ± 13[a]	96 ± 2	94 ± 10
GPSA	6.4×10^{-4}	96 ± 3	40 ± 8[a]	102 ± 13	54 ± 1[a]
$CoCl_2$	1.4×10^{-4}	41 ± 6[a]	205 ± 43[a]	67 ± 2[a]	94 ± 9
$CdCl_2$	1.4×10^{-4}	58 ± 10[a]	81 ± 5	36 ± 19[a]	85 ± 10
$PbCl_2$	1.4×10^{-4}	93 ± 2	95 ± 1	93 ± 7	94 ± 11
$NiCl_2$	1.4×10^{-4}	34 ± 10[a]	91 ± 9	42 ± 24[a]	92 ± 7
$ZnSO_4$	1.4×10^{-3}	100 ± 5	96 ± 6	38 ± 50	61 ± 16[a]
PMSF	1.4×10^{-4}	101 ± 6	100 ± 10	72 ± 21	59 ± 13[a]
TLCK	1.4×10^{-4}	106 ± 3			
Soybean trypsin inhibitor	0.1 mg/ml	96 ± 2		79 ± 31	
Ovoinhibitor	14 g/ml	100 ± 1			
Aprotinin	0.35 mg/ml	106 ± 6		83 ± 20	
Leupeptin	3.7×10^{-3}	102 ± 2		76 ± 39	
Antipain	2.1×10^{-3}	90 ± 7		32 ± 4[a]	
Chloroquine	1.4×10^{-4}	101 ± 6	120 ± 40	70 ± 28	69 ± 11[a]

Protease inhibitors (5 μl) and enzyme sample (10 μl) sat on ice for 30 min before the addition of the reaction mixture. Purified potato carboxypeptidase inhibitor was a gift from Dr M. Hass, University of Idaho. GPSA, APMSA, and BzlSA were gifts from Dr T.H. Plummer, New York Department of Public Health. Chromaffin granules, $n = 3$, [a] statistically significant, $p < 0.05$ (t-test). Lysosomes, $n = 2$, [a] statistically significant, $p < 0.10$ (t-test). Concentrations of inhibitors given as M unless stated otherwise

4. DISCUSSION

Purified bovine adrenal medullary chromaffin granules contain a peptidase (SGP) that converts ^{125}I-Lys-Arg-ME to ^{125}I-ME. These granules contain ME, catecholamines, and the CPB-like processing enzyme that we described in [7]. Unlike the enzyme(s) found in lysosomes which break down ^{125}I-Lys-Arg-ME into many products, the granule SGP produces ^{125}I-ME exclusively. In this regard, SGP resembles in its specificity chromaffin granule CPB-like activity which generates only ^{125}I-ME from ^{125}I-ME-Arg^6. SGP and CPB-like activities are clearly not associated with lysosomes. Thus, the suggestion in [8] that the carboxypeptidase we have observed was due to lysosomal contamination of our granule preparation is incorrect.

Inhibition by metal ion chelators and thiol reagents suggests that both SGP and CPB-like activities may be thiol-metalloproteases. The CPB-like activity was also inhibited by zinc metalloprotease inhibitors (BzlSA, APMSA, and GPSA) and was stimulated by Co^{2+} [8]. However, SGP was inhibited by Co^{2+}. The enzymes do not seem to be tightly bound to one another, as they

are readily separated on Sephadex G-75 in a non-denaturing buffer.

While the CPB-like enzyme seems likely to be involved in processing the enkephalin precursors [7,8,18], the function of SGP is presently unclear. SGP may be a trypsin-like enzyme, since purified pancreatic trypsin also converts ^{125}I-Lys-Arg-ME to ^{125}I-ME. Demonstration that SGP may be a trypsin-like enzyme must be based on the use of purified proenkephalin [19–21].

SGP may be an aminopeptidase since it appears to remove sequentially the two N-terminal amino acid residues from ^{125}I-Lys-Arg-ME. An aminopeptidase could be involved in enkephalin precursor processing if the trypsin-like enzyme cleaves between -Lys-Arg- residues to generate enkephalins extended at their N-termini with a basic amino acid residue. Although trypsin-like processing enzymes are generally thought to cleave at the C-terminal side of Arg at Lys-Arg pairs, no ME-Lys^6-Arg^7 has been found in adrenal medulla or striatum, regions rich in enkephalin peptides. The absence of Arg-ME in such regions might be accounted for by the rapid removal of the N-terminal Arg by an aminopeptidase.

The properties of SGP differ from those of previously studied trypsin [22,23] and aminopeptidase [24] enzymes. Unlike pancreatic trypsin, SGP is not a serine protease. In addition, SGP differs in its M_r (55000–60000) from aminopeptidase B (95000) which cleaves NH_2-terminal basic amino acid residues.

It is interesting that the chromaffin granule CPB-like enzyme, thought to be involved in enkephalin precursor processing, also differs from other studied carboxypeptidases such as pancreatic CPB and plasma CPN [16,17] in its thiol dependence, acidic pH optimum and apparent M_r. Other secretory granule peptidases such as the trypsin-like enzymes involved in processing proinsulin [1–3] and POMC [4–6], and carboxypeptidases thought to be involved in processing POMC and provasopressin [9] differ from serine pancreatic trypsin and Zn-metallocarboxypeptidases, respectively. Perhaps, secretory granules contain a set of peptidases with properties different from those found in other cellular compartments.

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