Carboxypeptidase activity in the insulin secretory granule

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Carboxypeptidase activity was studied in subcellular fractions from a transplantable rat insulinoma and found to be localised principally in the insulin secretory granule. The activity, which was specific for peptide substrates with C-terminal basic amino acids, appeared to be a single enzyme with M_r 54000. This enzyme differed with respect to size and pH optimum from other basic amino acid-specific carboxypeptidases, such as carboxypeptidases B and N, and may be a secretory granule-specific enzyme involved in propolypeptide processing.

Proinsulin *Processing* *Carboxypeptidase* *Granule* *Insulinoma* *Prohormone*

1. INTRODUCTION

The conversion of polypeptide hormone and neurosecretory peptide precursors commences after the arrival of the propolypeptide in the Golgi apparatus and continues for several hours during maturation of the secretory or storage granules [1]. The site of cleavage of the peptide chain is usually marked by pairs of basic amino acids and seems to be brought about by two different enzymes. An endopeptidase cleaves on the carboxyl side of the basic amino acids, and an exopeptidase then trims off the remaining basic residues from the newly exposed C-terminus (review [2]).

The ability to produce insulin from proinsulin by the combined action of trypsin and carboxypeptidase B in vitro can be cited as evidence for such a mechanism. However, the enzymes operative in vivo have not been fully characterised both with respect to their subcellular localisation and nature. An endoprotease which is present in crude granule preparations from rat islets appears to be more closely related to lysosomal cysteine proteases than to trypsin [1,3,4]. Carboxypeptidase activity has also been demonstrated in crude granule preparations [5]. It was not clear however, whether the latter activity was associated with the insulin secretory granule or with other contaminating organelles. Because of the small amount of available starting material, it has not been possible to purify insulin secretory granules in sufficient yield from rat islets. This problem can be overcome by using a transplantable rat insulinoma. This tumour has been shown to consist almost exclusively of insulin secreting B cells [6], and to release insulin by a process which is similar to that in islets [7]. We describe here the distribution of carboxypeptidase activity in the various subcellular fractions of the insulinoma, and characterise the activity associated with the insulin granule fraction.

2. EXPERIMENTAL

Subcellular fractions designated A_1–G from the transplantable rat insulinoma were prepared as in [8]. These subcellular fractions were collected from the 27% Percoll gradient shown in fig.1 of [8] and represented the following gradient fractions: (1) A_1; (2–6) granules; (12,13) D; (14,15) E; (16,17) F; (20–24) G. Gradient fractions 2–6 which were enriched in granules were centrifuged on a 40% Percoll gradient (fig.2 of [8]) to generate fractions A_2 (1–5), B (6–16) and C (17–24), where the

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numbers in parentheses represent the gradient fractions. The purity of each subcellular fraction with respect to marker enzyme activity was determined as in [8].

The assay for carboxypeptidase activity was based on the fluorimetric measurement of carboxy-terminal arginine liberated from hippuryl-L-arginine (Sigma Chemical Co., Kingston Upon Thames, Surrey). Samples were diluted to about 1 mg protein/ml in 0.1% (w/v) Brij 35 and 5 or 10 μl aliquots were added to 245 or 240 μl of assay mixture containing 0.15 M sodium acetate (pH 5.5) and 5 mM hippuryl-L-arginine in microcentrifuge tubes. These were incubated at 37°C, and at certain time intervals, 25 μl triplicate aliquots were removed and added to 25 μl 0.1 M borate (pH 9) maintained at 100°C (in 5 cm × 9.4 cm glass tubes) in a dri-block (Tecam DB 3). After 5 min at 100°C the samples were cooled to room temperature, and the liberated arginine was determined using fluorescamine (Sigma Chemical Co.). To each tube was added 700 μl 0.1 M borate (pH 9) and, while mixing on a vortex mixer, 250 μl fluorescamine solution [30 mg% (w/v) in acetonitrile]. The fluorescence was measured on a Perkin Elmer LS 5 fluorimeter (excitation, 390; emission, 465). The assay was checked for linearity with respect to time (up to 30 min), protein concentration (up to 10 μg) and reaction product (up to 10 nmol). Protein was determined as in [9].

3. RESULTS

A typical analysis of marker proteins in subcellular fractions A_1 to G is shown in table 1. Fraction A_1 is a lysosome-enriched fraction, A_2 a granule fraction with lysosomal contaminants and C a granule fraction with mitochondrial contamination. Fraction B is the most enriched granule fraction. Fraction D contains the majority of mitochondrial enzyme activity, while fractions E and F represent fractions enriched with endoplasmic reticulum and plasma membrane markers, respectively. Fraction G, at the top of the gradient, contains principally cytosolic proteins. The Golgi apparatus marker enzyme galactosyl transferase was concentrated in fraction F (not shown), [8]. Carboxypeptidase activity in these fractions was concentrated in fractions B and C where it attained its highest specific activity (table 2). Activity in the lysosomal fraction A_1, and for that matter in fractions D–F, could be readily accounted for by contamination of those fractions with granules. This suggests that the activity in fractions B and C is specifically associated with the insulin secretory granule. All further characterisation of the enzyme activity was performed with fraction C.

The carboxypeptidase activity had a pH optimum of 5.6 to 5.8 (fig.1), a value which is similar to the predicted internal pH of the insulin secretory

Table 1

Marker protein content of the subcellular fractions of the transplantable rat insulinoma

Subcellular fraction	Total protein (mg)	Insulin (units/mg)	Aryl sulphatase[a]	Cytochrome oxidase[b]	Catalase[a]	NADPH–cytochrome *c* reductase[b]	Lactate dehydrogenase[b]	5′-Nucleotidase[b]
A_1	3.2	1.62	3.34	4.3	16.0	<0.1	<1	123.0
A_2	8.8	5.17	3.07	4.8	12.4	0.2	62.0	90.4
B	17.0	8.64	1.41	11.4	9.0	1.4	60.0	65.9
C	37.7	2.70	1.17	123.0	33.1	32.2	227.0	214.6
D	118.6	1.97	0.65	195.0	11.2	26.9	30.0	116.4
E	93.6	1.17	0.17	113.0	24.4	9.1	46.0	105.7
F	16.5	1.55	0.12	16.4	28.1	14.6	139.0	220.5
G	396.0	0.01	0.02	<0.1	2.8	0.7	554.0	6.2

[a] μmol.min^{-1}.mg^{-1}; [b] nmol.min^{-1}.mg^{-1}. Fractions A_1–G were as described in section 2. The assays were performed as described [8]

Table 2

Distribution of carboxypeptidase activity in subcellular fractions of a transplantable rat insulinoma

Subcellular fraction	Carboxypeptidase (μmol.min^{-1}.mg^{-1})
A_1	0.153 (1)
A_2	0.162 ± 0.02 (5)
B	0.741 ± 0.08 (6)
C	0.647 ± 0.07 (7)
D	0.222 ± 0.06 (4)
E	0.176 ± 0.02 (4)
F	0.162 ± 0.01 (3)

Fractions A_1–F were prepared and stored at −70°C. Each fraction was diluted in 0.1% Brij 35, and 10 μl (~10 μg) was assayed for carboxypeptidase activity

granule [10]. Activity could be readily solubilised by freezing and thawing in hypotonic buffer, with <3% being associated with the granule membrane (not shown).

The activity was stimulated about 3-fold by $CoCl_2$ and less markedly by higher concentrations of $NiCl_2$ (table 3). The activity was inhibited by $CuCl_2$, $HgCl_2$ (K_i 4×10^{-5}) and *p*-chloromercuriphenyl sulphonate (K_i 2×10^{-5}), and by the metal ion chelators EDTA and 1,10-phenanthroline. Citrate at high concentrations inhibited the enzyme

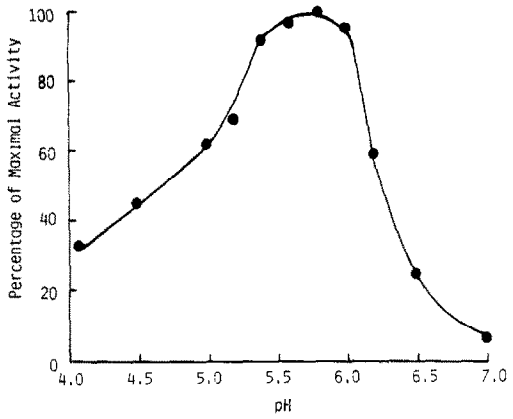

Fig.1. Effect of pH on the carboxypeptidase activity of the insulin secretory granule. Fraction C was diluted into 0.1% (w/v) Brij 35, and 10 μl (~10 μg) was assayed for carboxypeptidase activity in 0.15 M cacodylate buffer adjusted to the indicated pH-value with NaOH. The assay mixture contained 5 mM hippuryl-L-arginine.

Table 3

Effect of various additions on the carboxypeptidase activity of the insulin secretory granule

Addition	Conc. (mM)	% Control activity
$NiCl_2$	10	133
$CoCl_2$	1	309
$CuCl_2$	1	17
$ZnSO_4$	1	93
$CaCl_2$	1	96
$HgCl_2$	0.1	3
p-Chloromercuriphenylsulphonate	0.1	6
N-ethylmaleimide	10	60
Iodoacetic acid	10	99
Dithiothreitol	10	103
Antipain	10	98
Leupeptin	10	104
Phenylmethylsulphonylfluoride	1	97
Citrate	10	88
EDTA	0.1	15
1,10-Phenanthroline	1	11

Granule samples (5 μg in 0.15 M acetate, pH 5.5) were incubated in 10 μl for 2 h at 4°C with the indicated concentrations of effector substances; 240 μl 0.15 M acetate (pH 5.5) containing 5 mM hippuryl-L-arginine was then added, and the samples incubated at 37°C for 30 min. The liberated arginine was measured as in section 2. Each compound was tested in each experiment at: 10 μM, 100 μM, 1 mM and 10 mM. The values given are the average from 2 expt and represent the lowest or highest concentrations at which effects were observed

activity. $ZnSO_4$, $CaCl_2$, *N*-ethylmaleimide, iodoacetate and dithiothreitol had no effect, nor did the protease inhibitors antipain, leupeptin and phenylmethylsulphonyl fluoride.

The M_r of the granule-associated carboxypeptidase activity was 54000 as determined by gel permeation chromatography on Ultragel AcA44 (fig.2).

The presence of other carboxypeptidases in the insulin granule was examined using the substrate hippuryl-L-phenylalanine under the same assay conditions. Activity, while detectable, was ~50-fold less than that measured using the basic amino acid containing substrate at the same concentration (not shown).

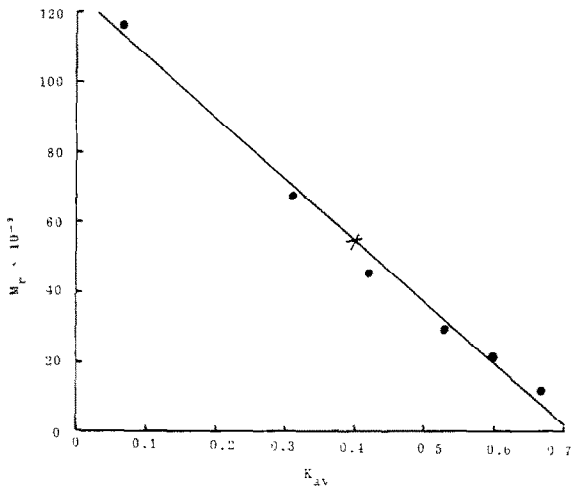

Fig.2. Determination of the relative molecular mass of the insulin granule associated carboxypeptidase. About 1 mg of fraction C was applied to a column (1.6 cm × 7.5 cm) of Ultrogel AcA44, equilibrated in 0.05 M phosphate, 0.1 M NaCl (pH 7.5). The flow rate was 3 cm/h; 2 ml fractions were collected and 50 μl samples assayed for carboxypeptidase activity. Blue dextran (V_o), β-galactosidase (M_r 116000), bovine serum albumin (M_r 66500), ovalbumin (M_r 45000), carbonic anhydrase (M_r 29000), soybean trypsin inhibitor (M_r 21000), cytochrome c (M_r 12300) and tryptophan (V_t) were used to calibrate the column. The K_{av} value for the granule carboxypeptidase is indicated (×).

4. DISCUSSION

The results are consistent with the presence of carboxypeptidase activity in the insulin secretory granule. The fact that the activity elutes as a single sharp peak on gel permeation chromatography (fig.2), and on DEAE-cellulose ion exchange chromatography (not shown) suggests that it is associated with a single enzyme, which has app. M_r 54000.

The insulin granule-associated carboxypeptidase differs from other carboxypeptidases involved in the removal of carboxy-terminal basic amino acids from proteins. Carboxypeptidase B (EC 3.4.12.3) is an exocrine pancreatic enzyme with M_r 34300, and a pH optimum in the range pH 7–9 [11], while carboxypeptidase N is a plasma enzyme involved in the inactivation of bradykinin and kallidin [12]. The latter is a multi-subunit protease with M_r 270000–280000 and a pH optimum around 7.7 [13]. However, similarities do exist between the carboxypeptidase of the insulin granule and carboxypeptidase N in so far as they are both stimulated by Co^{2+} [14], and that the active subunit of carboxypeptidase N also has M_r ~54000 [15].

Carboxypeptidase activities have been identified in chromaffin granules from bovine adrenal medullae, where they are purported to be involved in the processing of proenkephalin [16,17]. Named, enkephalin convertase, one such activity resembles that of the insulin granule in that it is activated by Co^{2+}, inhibited by EDTA and 1,10-phenanthroline, and unaffected by leupeptin and antipain. It differs, however, in that it is not inhibited by $HgCl_2$ nor by p-chloromercuriphenylsulphonate [16]. Inhibitor studies have distinguished enkephalin convertase from carboxypeptidase N [18]. Tissue distribution studies have demonstrated its presence in the anterior pituitary, where it may be involved in the processing of pro-opiomelanocortin [19]. Another chromaffin granule associated carboxypeptidase activity resembles that of the insulin granule enzyme, in being inhibited by $HgCl_2$ and p-chloromercuriphenylsulphonate, but differs in that EDTA and 1,10-phenanthroline have little effect, while leupeptin and antipain inhibit the activity [17].

The differences reported for the chromaffin granule carboxypeptidases may reflect the purity of the granule preparations, since substantial carboxypeptidase activity was observed in the lysosomal fraction of the adrenal medulla [16]. On the other hand it is possible that distinct enzymes are present in the insulin and chromaffin granules. It could be that a family of carboxypeptidase genes may exist and be expressed differently in different tissues, each enzyme being tailored for a specific peptide substrate.

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