Proteolytic conversion of proinsulin into insulin

Identification of a Ca^{2+} -dependent acidic endopeptidase in isolated insulin-secretory granules

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The nature of the endoproteolytic activity involved in the post-translational processing of proinsulin has been investigated in rat insulinoma tissue. ^{125}I -proinsulin was converted by lysed insulin-secretory granules into insulin via an intermediate form identified as des-dibasic-proinsulin. This activity co-localized with immunoreactive (endogenous) insulin and carboxypeptidase H upon subcellular fractionation of the tissue, indicating a secretory-granular location. Under optimized conditions, conversion was quantitative. Inhibitor studies demonstrated that processing occurred by a reaction sequence involving cleavage on the C-terminal side of the pairs of basic amino acids, with subsequent removal of the newly exposed basic residues by carboxypeptidase H. Endoproteolytic activity was abolished by EDTA and CDTA (1,2-cyclohexanediaminetetra-acetic acid), but not by 1,10-phenanthroline or by group-specific inhibitors of serine, thiol or acidic proteinases. Inhibition by EDTA and CDTA could be reversed by both Ca^{2+} and Zn^{2+} , although the former appeared to be the ion of physiological importance. Addition of Ca^{2+} in the absence of chelators stimulated endoproteinase activity, with a maximal effect at 5 mM, a concentration consistent with the intragranular environment. Similarly the pH optimum of 5.5 coincides with the prevailing intragranular pH. Together these properties suggest that the Ca^{2+} -dependent endopeptidase described here is involved *in vivo* in the proteolytic processing of proinsulin.

INTRODUCTION

The majority of polypeptide hormones and many secreted proteins are synthesized as precursors from which the active molecule is excised by limited proteolysis, generally at sites marked by pairs of basic amino acids (Docherty & Steiner, 1982; Loh *et al.*, 1984). For proinsulin it has been suggested that enzymes with trypsin-like and carboxypeptidase-B-like specificity might be responsible for the overall reaction (Nolan *et al.*, 1971). Indeed, trypsin and carboxypeptidase B derived from the exocrine pancreas can catalyse the conversion of proinsulin into insulin *in vitro* (Kemmler *et al.*, 1971). The endogenous endopeptidase, unlike trypsin, however, is specific to paired basic residues. This is illustrated by failure of proinsulin-A/C-chain junction cleavage in a case of familial proinsulinaemia, in which a point mutation results in the substitution of the sequence Lys⁶⁴-His⁶⁵ for Lys⁶⁴-Arg⁶⁵ at this site (Robbins *et al.*, 1981).

Several candidate enzymes have been proposed as a specific proinsulin endopeptidase, including cathepsin B-related thiol proteinases (Docherty *et al.*, 1983, 1984), glandular kallikrein (Ole-Moi Yoi *et al.*, 1979) and plasminogen activator (Virji *et al.*, 1980). However, there is as yet no instance where it has been established that the specificity of cleavage, reaction kinetics and subcellular localization of the enzyme are consistent with a physiological role, or whether the properties of the enzyme are compatible with the environment of the processing compartment.

The identity of the carboxypeptidase-B-like enzyme is far less controversial. Such an enzyme was first demonstrated in rat islets by Kemmler *et al.* (1973), has been localized to the secretory granule (Docherty & Hutton, 1983) and purified to homogeneity from a transplantable rat insulinoma (Davidson & Hutton, 1987). It is identifiable as carboxypeptidase H (EC 3.4.17.10), an enzyme widely distributed in tissue involved in the secretion of biologically active polypeptides (Fricker & Snyder, 1983).

We report here an investigation of the subcellular localization and initial characterization of the properties of a proinsulin-processing activity in a transplantable rat insulinoma. This tissue is available in gram quantities, has a high insulin content (Chick *et al.*, 1977) and processes proinsulin to insulin with kinetics similar to those observed in rat islets (Gold *et al.*, 1984).

METHODS

Materials

Mono[^{125}I]iodinated proinsulin was a gift from Dr. B. Frank (Eli Lilly, Indianapolis, IN, U.S.A.). Ala-Lys-Arg-CH₂Cl and Ala-Nle-Arg-CH₂Cl were kindly provided by Dr. E. Shaw (Friedrich Miescher-Institut, Basel, Switzerland), and Ep-459 and Ep-475 by Dr. K. Hanada (Taisho Pharmaceutical Co., Saitama, Japan). E-64 was obtained from Cambridge Research Biochemicals, Cambridge, U.K., 3,4-dichloroisocoumarin from Novabiochem A.G., Laufelfingen,

Abbreviations used: Ala-Lys-Arg-CH₂Cl, alanyl-L-lysyl-L-arginylchloromethane ('ALACK'); Ala-Nle-Arg-CH₂Cl, alanyl-L-norleucyl-L-arginylchloromethane ('ANACK'); GEMSA, guanidinoethylmercaptosuccinic acid; T1C2, des-(31,32)-proinsulin and/or des-(64,65)-proinsulin; CDTA, 1,2-cyclohexanediaminetetra-acetic acid.

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Table 1. Distribution of marker proteins in insulinoma subcellular fractions

Subcellular fractions prepared from insulinoma tissue were assayed for marker proteins for lysosomes (arylsulphatase), secretory granules (insulin and carboxypeptidase H), mitochondria (cytochrome *c* oxidase), endoplasmic reticulum (NADPH-cytochrome *c* reductase), plasma membranes (5'-nucleotidase), Golgi (galactosyltransferase) and cytosol (lactate dehydrogenase) as described in the Methods section: ND, not detected.

	Homogenate	Fraction						
		Ia	Ib	Ic	II	III	IV	V
Protein (mg)	620	5.5	8.0	7.5	63	39	33	198
Arylsulphatase ($\mu\text{mol}/\text{min per mg}$)	0.2	6.3	3.3	1.0	0.2	0.3	0.2	< 0.01
Insulin (i.u./mg)	0.51	5.6	10.9	6.4	1.0	1.1	1.6	< 0.1
Carboxypeptidase H ($\mu\text{mol}/\text{min per mg}$)	20	< 100	402	306	52	58	49	< 5
Cytochrome <i>c</i> reductase ($\mu\text{mol}/\text{min per mg}$)	67	10	10	60	380	210	40	< 10
NADPH-cytochrome <i>c</i> reductase (nmol/min per mg)	4.1	ND	ND	ND	8.5	17.7	16.7	0.02
5'-Nucleotidase (nmol/min per mg)	51	80	30	60	130	120	180	10
Galactosyltransferase (nmol/min per mg)	ND	ND	ND	ND	0.42	0.61	0.56	0.05
Lactate dehydrogenase ($\mu\text{mol}/\text{min per mg}$)	144	ND	ND	ND	40	90	90	720

Switzerland, and GEMSA from Calbiochem, Cambridge Bioscience, Cambridge, U.K. All other enzyme inhibitors were purchased from Sigma (London), Poole, Dorset, U.K. Other chemicals were obtained from BDH, Poole, Dorset, U.K., except where indicated.

Tissue

Insulinoma tissue was propagated in New England Deaconess Hospital (NEDH) rats as described previously (Hutton *et al.*, 1981). Insulin-secretory granules were prepared by the method of Hutton *et al.* (1982).

Assay of proinsulin-processing activity

In general, purified insulin-secretory granules (25 μg) were incubated in 100 mM-sodium acetate adjusted to pH 5.5 with HCl and containing 0.1% (w/v) Brij 35, and 50000 c.p.m. (20 ng) of mono- ^{125}I -proinsulin (final vol. 100 μl) in 1.5 ml micro-centrifuge tubes (Alpha, Eastleigh, Hants., U.K.), for 90 min at 37 °C. The reaction was terminated by addition of trichloroacetic acid (final concn. 8.3%, w/v). After 30 min on ice, the precipitated proteins were collected by centrifugation for 5 min at 9000 *g* ($r_{\text{av.}}$ = 4.7 cm; MSE Microcentaur). Residual trichloroacetic acid was extracted with diethyl ether (500 μl), the pellet was air-dried, and the proteins were separated by urea/polyacrylamide-gel electrophoresis.

Alkaline-urea/polyacrylamide-gel electrophoresis was carried out by a modification of the continuous buffer system of Perrie & Perry (1970). Slab gels (15 cm $\times$ 12 cm $\times$ 0.15 cm) were polymerized from acrylamide (150 g/l) and *NN*-methylenebisacrylamide (0.9 g/l) containing 12.5 mM-Tris/80 mM-glycine, pH 8.6, and 6 M-urea. The gels were pre-run at 300 V for 3 h, and the upper tank buffer was replaced before samples resuspended in 40 μl of 2.5 mM-Tris/HCl, pH 8.6, containing 8 M-urea were loaded. After electrophoresis at 450 V for 4 h, the gels were fixed in 25% (v/v) propan-2-ol for 30 min, dried under vacuum, and

exposed to pre-flashed Cronex film [Du Pont (U.K.), Stevenage, Herts., U.K.] at -70 °C. For quantification, the resulting autoradiograms were analysed by scanning densitometry (Joyce-Loebl Chromoscan 3). In some experiments the dried gel was cut into small pieces and the radioactivity determined with a γ -radiation counter (NE 1600; Nuclear Enterprises, Edinburgh, U.K.). No qualitative differences between the distribution of radioactivity and the results of the scanning densitometry were observed. Results were expressed as the percentage of the total radiolabel recovered in a particular product.

Acid-urea/polyacrylamide-gel electrophoresis was carried out by the method of Panyim & Chalkley (1969), modified to utilize the polymerization method of Amory *et al.* (1980). Samples were resuspended in 0.9 M-acetic acid containing 8 M-urea and 0.001% Methyl Green, and loaded on to slab gels (12 cm $\times$ 12 cm $\times$ 0.15 cm). Electrophoresis was carried out at 500 V for 5 h (Methyl Green eluted after 2.5 h). Autoradiography was carried out as described above.

The products of processing were identified by reference to the electrophoretic mobility of standards generated from proinsulin by using a combination of trypsin and carboxypeptidase B (Kemmler *et al.*, 1971), and by comparison with unlabelled intermediates prepared by h.p.l.c. (Given *et al.*, 1985), whose identities had been confirmed by amino acid analysis.

Other assays

Protein was determined by the method of Bradford (1976), with bovine serum albumin (Sigma) as standard.

Insulin-secretory-granule carboxypeptidase H activity was determined by the method of Fricker & Snyder (1983). Other insulinoma marker proteins were assayed as described previously (Hutton *et al.*, 1982).

Free metal ion concentrations were calculated from reference stability constants (Stability Constants of Metal-Ion Complexes, Spec. Chem. Soc. Publ. 25, 1973)

by using an iterative computer program (Storer & Cornish-Bowden, 1976) modified to run on the University of Cambridge I.B.M. 3081D MVS instrument.

RESULTS

Intracellular localization

Subcellular fractions designated Ia, Ib, Ic, II, III, IV and V were prepared from insulinoma tissue by the method of Hutton *et al.* (1982). Analysis of selected marker proteins revealed that lysosomes were concentrated in fraction Ia, whereas fractions Ib and Ic contained insulin-secretory granules (Table 1). Fraction II contained mitochondria, III endoplasmic reticulum and Golgi, IV plasma membrane, and V cytosolic proteins.

Investigations of the ability of these fractions to convert exogenous ^{125}I -proinsulin into insulin demonstrated that the enzymes responsible were located primarily in fractions Ib and Ic (Fig. 1). Scanning densitometry revealed that these fractions together contained approx. 85% of the total processing activity detected in all fractions. Under conditions where non-specific proteolysis was prevented by the inclusion of selected proteinase inhibitors (see legend to Fig. 1), conversion of proinsulin into insulin plus intermediate forms was essentially quantitative. Fraction Ib, which exhibited the highest specific activity of the endopeptidase, converted 30% of the radiolabelled proinsulin into insulin in 90 min (Fig. 2).

Inclusion of GEMSA, a potent inhibitor of carboxypeptidase H (Fricker & Snyder, 1983), in the incubation

Fig. 1. Subcellular distribution of proinsulin-processing activity

Various subcellular fractions were prepared from the rat insulinoma by the method of Hutton *et al.* (1982) and suspended in 100 μl of 100 mM-sodium acetate, pH 5.5, containing 0.1% Brij 35, 100 μM -tosylphenylalanyl-chloromethane, 10 μM -E-475, 10 μM -pepstatin A and 5 mM- CaCl_2 . These were either heated at 100 $^\circ\text{C}$ for 10 min (a) or kept on ice (b). ^{125}I -proinsulin (50000 c.p.m.) was then added, and the samples were incubated at 37 $^\circ\text{C}$ for 90 min. Reactions were terminated by the addition of trichloroacetic acid, and the precipitated proteins were analysed by alkaline-urea/polyacrylamide-gel electrophoresis as described in the text.

Fig. 2. Time-dependent conversion of proinsulin (Δ) into T1C2 ($\bullet$) and insulin ($\circ$) by lysed insulin-secretory granules

Purified insulin-secretory granules (25 μg of fraction Ib) were incubated at 37 $^\circ\text{C}$ in 100 mM-sodium acetate, pH 5.5, containing 0.1% Brij 35, inhibitor mixture, 5 mM- CaCl_2 and ^{125}I -proinsulin (50000 c.p.m.) (final vol. 100 μl), for the times indicated. The reactions were then terminated by the addition of trichloroacetic acid, and the precipitated proteins analysed by alkaline-urea/polyacrylamide-gel electrophoresis as described in the text.

medium did not affect the apparent subcellular distribution of processing activity. However, in this case proinsulin was cleaved at one or other of the two dibasic sites to generate seco[32/33] or seco[65/66]proinsulin and diarginyl-insulin. Although the former products could not be separated from intact proinsulin by the alkaline-urea/polyacrylamide-gel-electrophoretic method, they were resolved in the acid-urea system. The products produced in reactions containing GEMSA could be subsequently converted into des-dibasic proinsulin (T1C2) and insulin by treatment with carboxypeptidase B (results not shown).

Analysis of the time course of the reaction showed that the appearance of the product identified as T1C2 preceded that of insulin, reached a maximum at 20 min, and declined thereafter. Insulin appeared after a lag phase of 5–10 min and increased progressively for at least 3 h (Fig. 2). Such results are consistent with T1C2 being an intermediate in the conversion of proinsulin into insulin. The order of the appearance of products and their relative proportions are comparable with the kinetics observed *in vivo* with biosynthetically radiolabelled (pro)insulin in intact cell preparations (Hutton *et al.*, 1987a).

Freshly prepared granules incubated in an iso-osmotic medium did not process exogenous proinsulin unless lysed by hypo-osmotic shock, or by the addition of non-ionic detergent (results not shown), indicating that the proinsulin-processing endopeptidase is associated with the interior of the secretory granule.

Effects of inhibitors on proinsulin processing

Secretory-granule fraction Ic was chosen for the investigation of the effects of various inhibitors on the processing of proinsulin *in vitro*, as it contained the lowest lysosomal contamination (Table 1). These studies

Table 2. Effect of proteinase inhibitors on proinsulin conversion

Inhibitors were dissolved in water or ethanol as appropriate, and added as 10-fold-concentrated solutions to 20 mM-Mes/NaOH, pH 6.5, containing 0.1% (w/v) Brij 35 and 50 μ g of purified insulin-secretory granules (fraction Ic) (final vol. 100 μ l). The reaction was initiated by the addition of 125 I-proinsulin, and products were separated, after 3 h incubation at 37 °C, by alkaline-urea/polyacrylamide-gel electrophoresis as described in the text. The tabulated values are means of duplicate analyses performed on three separate occasions.

Inhibitor	Concn. (mM)	Inhibition (%)
Phenylmethanesulphonyl fluoride	1.0	0
3,4-Dichloroisocoumarin	0.4	0
2,2'-Dipyridyl disulphide	1.0	0
Iodoacetic acid	1.0	0
Iodoacetamide	1.0	0
Dithiothreitol	1.0	0
<i>p</i> -Hydroxymercuribenzoate	0.5	100
EDTA	0.2	100
EGTA	1.0	100
CDTA	1.0	100
1,10-Phenanthroline	1.0	0*
GEMSA	1.0	0*
Pepstatin A	1.0	14
Tosyl-L-phenylalanylchloromethane	1.0	0
Tosyl-L-lysylchloromethane	1.0	0
Ala-Nle-Arg-CH ₂ Cl	0.075	12
	0.1	50
Ala-Lys-Arg-CH ₂ Cl	0.075	80
	0.1	95
Leupeptin	1.0	20
Antipain	1.0	35
E-64	1.0	0
Ep-459	1.0	0
Ep-475	1.0	0

* Inhibition of carboxypeptidase H only.

were also carried out at pH 6.5, to minimize the effect of residual lysosomal acidic proteinases. Despite these precautions, 10–20% of the radiolabel was not recovered as substrate or defined products. Such non-specific degradation could be prevented by inhibitors of cathepsin B. Under the conditions used, up to 20% of the proinsulin was converted into insulin and insulin intermediates. This reaction was essentially linear with time for up to 3 h.

Group-specific inhibitors of serine, thiol and acidic proteases all failed to inhibit processing, whereas the metalloproteinase inhibitors EDTA, CDTA and EGTA all abolished conversion (Table 2). This effect was not shared by 1,10-phenanthroline, which did not affect the extent of proinsulin conversion, although the products of the reaction did not include insulin (Fig. 3). In this case, three major products could be identified; T1C2, monoarginyl-insulin and diarginyl-insulin. Such products would be expected in situations where the endopeptidase was unaffected but where there was incomplete inhibition of carboxypeptidase H. Previous studies have shown 89% inhibition of carboxypeptidase H by 1,10-phenanthroline under conditions similar to those used in the present study (Docherty & Hutton, 1983).

Fig. 3. Effects of selected inhibitors on proinsulin processing

Purified insulin-secretory granules (50 μ g; fraction Ic) were incubated in 20 mM-Mes adjusted to pH 6.5 with NaOH and containing 0.1% Brij 35 (lane 1) or buffer containing 75 μ M-Ala-Lys-Arg-CH₂Cl (lane 2), 75 μ M-Ala-Nle-Arg-CH₂Cl (lane 3), 1 mM-CDTA (lane 4) or 1 mM-1,10-phenanthroline (lane 5). The reaction was initiated by the addition of 125 I-proinsulin and terminated after 3 h incubation at 37 °C by the addition of trichloroacetic acid. The precipitated proteins were separated by alkaline-urea/polyacrylamide-gel electrophoresis as described in the text.

The active-site-directed inhibitors tosyl-L-phenylalanyl chloromethane and tosyl-L-lysylchloromethane, which are both potent inhibitors of lysosomal thiol proteinases, showed no effect on the conversion of proinsulin to insulin and intermediates at millimolar concentrations, whereas the tripeptide chloromethanes Ala-Lys-Arg-CH₂Cl and Ala-Nle-Arg-CH₂Cl were both inhibitory at 100 μ M (Table 2). Considerable inhibitory specificity was expressed by the dibasic inhibitor compared with the monobasic analogue (Fig. 3). The requirement for dibasic residues was further illustrated by the partial inhibition shown by leupeptin and antipain (Table 2), which do not contain dibasic sequences. No effect of the epoxide inhibitors E-64, Ep-459 or Ep-475, which are potent inhibitors of lysosomal cathepsins B and L (Hanada *et al.*, 1983), on specific proinsulin processing was observed.

Effects of metal ions on processing

Under basal conditions the calculated ionic concentrations in incubation media containing purified secretory granules (25 μ g) at pH 5.5 were 60 μ M-Ca²⁺, 40 μ M-Mg²⁺ and 10 μ M-Zn²⁺ respectively (Hutton *et al.*, 1983). EDTA (2 mM), which abolished conversion, decreased the calculated ion concentrations to 0.25 μ M-Ca²⁺, 10 μ M-Mg²⁺ and 90 fM-Zn²⁺; however, the endopeptidase

Fig. 4. Effect of increasing $[Ca^{2+}]$ on proinsulin processing

Purified insulin-secretory granules ($25 \mu\text{g}$; fraction Ib) were incubated in 100 mM-sodium acetate, pH 5.5, containing 0.1% Brij 35, inhibitor mixture, 2 mM-EDTA and ^{125}I -proinsulin (50000 c.p.m.). CaCl_2 was added to give calculated free ion concentrations as indicated (final vol. 100 μl), and the fractions were incubated at 37°C for 3 h. The reactions were then terminated by the addition of trichloroacetic acid, and the precipitated proteins analysed by acid-urea/polyacrylamide-gel electrophoresis as described in the text.

activity was restored by the addition of Ca^{2+} , with half-maximal processing obtained at $150 \mu\text{M-Ca}^{2+}$ (Fig. 4). Under these conditions the concentration of Zn^{2+} was in the picomolar range.

CDTA and EDTA inhibition could also be relieved by addition of Zn^{2+} , but not with Mg^{2+} (Table 3). However, in these cases the effects were attributable to the ability

Fig. 5. Effect of added Ca^{2+} on the conversion of proinsulin into insulin

Purified insulin-secretory granules ($25 \mu\text{g}$; fraction Ib) were incubated in 100 mM-sodium acetate, pH 5.5, containing 0.1% Brij 35, inhibitor mixture, CaCl_2 as indicated and ^{125}I -proinsulin (50000 c.p.m.) (final vol. 100 μl). After incubation at 37°C for 90 min, the reactions were terminated by the addition of trichloroacetic acid, and the precipitated proteins analysed by acid-urea/polyacrylamide-gel electrophoresis as described in the text.

of these ions to displace Ca^{2+} . Thus significant proinsulin processing was only observed in those incubations in which there was over $10 \mu\text{M-Ca}^{2+}$, but the extent of the processing appeared unrelated to the concentration of Zn^{2+} .

Table 3. Effect of Ca^{2+} , Mg^{2+} and Zn^{2+} on proinsulin processing after inhibition with EDTA and CDTA

Purified insulin-secretory granules ($25 \mu\text{g}$ of fraction Ib) were incubated in 100 mM-sodium acetate, pH 5.5, containing 0.1% Brij 35, inhibitor mixture, metal ions and chelators as indicated, and ^{125}I -proinsulin (50000 c.p.m.) (final vol. 100 μl). After incubation at 37°C for 3 h, the reactions were terminated by addition of trichloroacetic acid, and the precipitated proteins were analysed by acid-urea/polyacrylamide-gel electrophoresis as described in the text. Metal ion concentrations were calculated as described in the text. The tabulated values are means of duplicate analyses performed on at least three different occasions.

Addition	Proinsulin processing (%)	Product formed (%)			$[\text{Ca}^{2+}]$ (μM)	$[\text{Mg}^{2+}]$ (μM)	$[\text{Zn}^{2+}]$ (μM)
		TIC2	Insulin	Diarginyl-insulin			
None	42	17	21	0	60	40	10
1.2 mM- Ca^{2+}	52	6	38	0	1260	40	10
1.2 mM- Mg^{2+}	30	16	8	0	60	1240	10
0.2 mM- Zn^{2+}	28	14	8	0	60	40	210
1.0 mM-EDTA	0	0	0	0	0.5	16	2×10^{-7}
1.0 mM-EDTA + 1.2 mM- Ca^{2+}	58	5	3	26	240	38	6×10^{-6}
1.0 mM-EDTA + 1.2 mM- Mg^{2+}	3	2	0	0	1	700	4×10^{-7}
1.0 mM-EDTA + 1.2 mM- Zn^{2+}	32	15	11	0	60	40	210
1.0 mM-CDTA	0	0	0	0	0.2	12	2×10^{-8}
1.0 mM-CDTA + 1.2 mM- Ca^{2+}	53	4	9	18	220	39	2×10^{-6}
1.0 mM-CDTA + 1.2 mM- Mg^{2+}	4	3	0	0	0.4	630	5×10^{-8}
1.0 mM-CDTA + 1.2 mM- Zn^{2+}	34	17	15	0	60	40	210
1.0 mM-1,10-Phenanthroline	54	0	0	18	60	39	2×10^{-7}
5.0 mM-1,10-Phenanthroline	48	0	0	16	59	36	2×10^{-9}

Table 4. Effect of metal ions on proinsulin processing

Purified insulin-secretory granules (25 μ g of fraction Ib) were incubated in 100 mM-sodium acetate, pH 5.5, containing 0.1% Brij 35, inhibitor mixture, 125 I-proinsulin (50000 c.p.m.) and salts (1 mM) as indicated (final vol. 100 μ l). After incubation at 37 °C for 90 min, the reactions were terminated by the addition of trichloroacetic acid and the precipitated proteins analysed by alkaline-urea/polyacrylamide-gel electrophoresis as described above. The tabulated values are the results of duplicate analyses performed on three separate occasions.

Ion	Processing (% of control)
None	100
CaCl ₂	320
MgCl ₂	75
SrCl ₂	81
BaCl ₂	100
MnCl ₂	50
ZnCl ₂	25
CoCl ₂	6
HgCl ₂	0
LaCl ₃	10

Addition of millimolar concentrations of Ca²⁺ to the incubation mixture in the absence of chelating agents potentiated conversion of proinsulin into insulin. Examination of the concentration-dependency of this effect showed a maximum degree of stimulation at 5 mM-Ca²⁺ (Fig. 5). Equimolar (1 mM) concentrations of Mg²⁺, Sr²⁺ or Ba²⁺ had little effect on processing, whereas millimolar concentrations of La³⁺, Co²⁺ or Hg²⁺ abolished endoprotease activity. Zn²⁺ and Mn²⁺ (1 mM) caused partial inhibition of processing (Table 4).

These results are consistent with the endopeptidase being dependent on Ca²⁺, whereas the carboxypeptidase requires Zn²⁺ for activity.

pH optimum of processing

The pH optimum of the conversion of proinsulin into insulin and insulin intermediates by lysed secretory granules was 5.0–6.0 (Fig. 6a). Specific inhibition of carboxypeptidase H by the addition of GEMSA to the reaction medium did not significantly alter the pH profile (Fig. 6b), suggesting that the optimum of the overall reaction reflects that of the endopeptidase. Furthermore the pH/activity profiles for the generation of seco[32/33]proinsulin, seco[65/66]proinsulin and diarginyl-insulin were indistinguishable, suggesting that the pH optimum for hydrolysis at both of the available cleavage sites was similar.

The pH optimum observed was not affected by changing the Ca²⁺ concentration in the medium within the range 60 μ M–5 mM.

DISCUSSION

Previous studies of the biosynthesis of insulin in pancreatic β -cells have suggested that the insulin-secretory granule is the major, if not the sole, site of conversion of proinsulin into insulin (Kemmler & Steiner, 1970; Sorenson *et al.*, 1970; Orci *et al.*, 1985). Attempts to prepare purified secretory granules from rat islets of

Fig. 6. pH optima of proinsulin processing

(a) Purified insulin-secretory granules (25 μ g; fraction Ib) were incubated in 30 mM-sodium acetate/30 mM-Mes/30 mM-*N*-ethylmorpholine adjusted to pH 3.5–7 with HCl. The incubation mixture also contained 0.1% Brij 35, inhibitor mixture, 5 mM-CaCl₂ and 125 I-proinsulin (50000 c.p.m.) (final vol. 100 μ l). After incubation at 37 °C for 30 min, the reactions were terminated by the addition of trichloroacetic acid, and the precipitated proteins analysed by alkaline-urea/polyacrylamide-gel electrophoresis as described in the text. (b) Incubations were performed as described above, but with the inclusion of 1 mM-GEMSA in the medium. The precipitated proteins were separated by acid-urea/polyacrylamide-gel electrophoresis, and the percentage of radiolabel present as seco[32/33]proinsulin and seco[65/66]proinsulin (●) and diarginyl-insulin (○) was determined by scanning densitometry of the resultant autoradiograph.

Langerhans, however, have been hampered by the small amounts of starting material available, and many previous studies of the enzymes involved in the processing of proinsulin have used partially purified fractions, which contain lysosomes as a major contaminant. Lysosomal contamination, however, may lead to artefactual production of insulin-like products, or mask the low activity of granular converting enzymes.

The present study used well-characterized subcellular fractions prepared from a transplantable rat insulinoma, which converted mono-iodinated human proinsulin into insulin in high yield. The activity was localized to the insulin-secretory granule.

An intermediate in the conversion, identified as des-Arg³¹-Arg³²- and/or des-Lys⁶⁴-Arg⁶⁵-proinsulin, corresponded to the intermediate observed in pulse-chase

experiments of insulinoma cells labelled with [³⁵S]methionine (Hutton *et al.*, 1987a). The apparent pH optimum of the conversion is in agreement with previously reported optimal values for the processing of biosynthetically radiolabelled proinsulin by intact secretory granules (Sorenson *et al.*, 1972). It thus appears that the assay system developed is a suitable model for events occurring *in vivo* and can be used to characterize the nature of the individual enzymes involved in the process.

When inhibitors of carboxypeptidase H were added to secretory granules processing ¹²⁵I-proinsulin, a different set of peptides were produced. These peptides could subsequently be converted into peptides identified as des-Arg³¹-Arg³²- and/or des-Lys⁶⁴-Arg⁶⁵-proinsulin and insulin by the addition of carboxypeptidase B. This suggests that the endopeptidase responsible for initial cleavage cleaves on the C-terminal side of pairs of basic residues in proinsulin and that the C-terminal basic residues are subsequently removed by carboxypeptidase H, the mechanism originally proposed by Nolan *et al.* (1971). The finding that a peptidyl chloromethane containing a dibasic sequence was a potent inhibitor of processing, but that a monobasic analogue was less effective, also suggested the specificity of this enzyme for the dibasic sequence.

The endopeptidase involved appeared to be a Ca²⁺-dependent acidic proteinase with properties distinct from enzyme activities which previously have been associated with proinsulin processing. It is of interest therefore to consider the evidence supporting the involvement of these other candidate enzymes. On the basis of binding studies of radiolabelled site-specific affinity probes and inhibitor studies, an M_r-31 500 thiol proteinase immunologically indistinguishable from lysosomal cathepsin B was implicated in processing in rat islets (Docherty *et al.*, 1982, 1983). The subcellular localization was not established in these studies. It should be noted that, under conditions favouring limited proteolysis, purified rat liver cathepsins B and L can both convert proinsulin into insulin (Ansorge *et al.*, 1977). However, several other peptides, including the degradation product des-octapeptide-insulin, are also produced, and there is no apparent requirement for carboxypeptidase H.

Subsequent studies pointed to the possible involvement of an M_r-39 000 protein immunologically related to cathepsin B. This protein, which is present in subcellular fractions containing insulin granules and lysosomes, has yet to be characterized with respect to its catalytic action (Docherty *et al.*, 1984). A thiol enzyme may be involved in proinsulin processing in anglerfish β -cells (Fletcher *et al.*, 1981), although this may represent a special case, since the B-chain of anglerfish insulin has C-terminal lysine (Yameji *et al.*, 1972).

Several serine proteinases, including an M_r-70 000 protein isolated from bovine pancreas (Yip, 1971), human glandular kallikrein (Ole-Moi Yoi *et al.*, 1979) and plasminogen activator (Virji *et al.*, 1980), have been implicated in the conversion of proinsulin into insulin. However, these enzymes all have alkaline pH optima, whereas proinsulin processing occurs in an acidic environment (Sorenson *et al.*, 1972; Orci *et al.*, 1986). None has been localized to insulin-secretory granules.

Recently aspartyl proteinases, which process pro-opiomelanocortin *in vitro*, have been isolated from

bovine pituitary-posterior and neurointermediate-lobe secretory vesicles (Loh *et al.*, 1985; Parish *et al.*, 1986). These enzymes could also process ¹²⁵I-proinsulin *in vitro*, although the products of the reaction were not fully characterized. At present there is no evidence that such enzymes are present in cells other than the pituitary.

Processing enzymes which, like the present endopeptidase activity, can be classified as metalloproteinases have previously been implicated in the processing of parathyroid hormone (Habener *et al.*, 1977) and of yeast pro-pheromones (Julius *et al.*, 1984; Achstetter & Wolf, 1985). Moreover Ca²⁺ ions are also involved in both cases. These enzymes, however, each have pH optima in the range 7–9. Similarly the Ca²⁺-dependent neutral proteinase (calpain I), which has been localized in human pancreatic β -cells (Kitahara *et al.*, 1985), has an alkaline pH optimum, and, unlike the activity described here, is inhibited by the epoxide inhibitor E-64. In any case it is probably a cytosolic protein.

More recently, a Ca²⁺-dependent endopeptidase which processes chromogranin A to betagranin has been demonstrated in this laboratory in insulinoma tissue (Hutton *et al.*, 1987b). Its pH optimum (pH 4.8) is somewhat more acidic than for the proinsulin-processing enzyme, and half-maximal activation occurred at lower Ca²⁺ concentrations. Its sensitivity to various proteinase inhibitors, however, was identical.

The fact that the insulin-granule endoproteinase activity is dependent on Ca²⁺ may be important in determining its site of action in the cell. Ultrastructural studies have indicated that the insulin-secretory granule selectively accumulates Ca²⁺ in a glucose-responsive fashion (Andersson *et al.*, 1982). The free Ca²⁺ concentration in the granule, moreover, lies within a range (1–10 mM) where modulatory effects on the activity may be expected (Hutton *et al.*, 1983).

The second major factor determining activity of the endopeptidase appears to be the intragranular pH. This appears to be controlled by a proton-translocating ATPase in the insulin-granule membrane (Hutton & Peshavaria, 1982; Hutton, 1982). On the basis of present studies, the transition from a neutral environment in the endoplasmic reticulum/Golgi to one of around pH 5.5 in the granule may be expected to enhance the endopeptidase by over 10-fold. Moreover, pH may also affect the intragranular Ca²⁺ concentration through promoting the dissociation of Ca²⁺ complexes within the granule (Hutton *et al.*, 1983). These factors would account for the observation that conversion of proinsulin into insulin *in vivo* occurs co-ordinately with the acidification of maturing secretory vesicles (Orci *et al.*, 1986).

We conclude that the endopeptidase that we have identified is a novel enzyme and that it has features consistent with a physiological role in proinsulin processing. Its proteolytic action is both limited and selective, and the products of the reaction are equivalent to those found *in vivo*. Its sensitivity to pH and Ca²⁺ may ensure that it would be active in the secretory pathway only once the prohormone had been segregated into the secretory-granule compartment. Further studies aimed at the purification and characterization of the enzyme(s) appear warranted.

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