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The inhibition of proinsulin-processing endopeptidase activities by active-site-directed peptides

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Inhibitor studies were performed on the two endopeptidase activities involved in proinsulin conversion in isolated insulin secretory granules [Davidson, Rhodes & Hutton (1988) Nature (London) **333**, 93–96]. The active-site-directed peptides L-alanyl-L-arginyl-L-arginylmethyldimethylsulphonium and L-alanyl-L-lysyl-L-arginylmethyldimethylsulphonium inhibited these activities in accordance with the observed cleavage pattern, suggesting that the primary amino acid sequence of the dibasic site was an important determinant of the endopeptidase substrate specificities.

INTRODUCTION

Many polypeptide hormones and secreted proteins are synthesized as inactive precursors and activated by limited proteolysis at sites usually marked by a dibasic amino acid sequence [for reviews, see Docherty & Steiner (1982) and Loh *et al.* (1984)]. In the case of proinsulin, two endopeptidase activities (Davidson *et al.*, 1988) and carboxypeptidase-H (Docherty & Hutton, 1983; Davidson & Hutton, 1987) are involved. One of the endopeptidase activities (type I) cleaves exclusively on the carboxylic side of amino acids Arg^{31}/Arg^{32} of the proinsulin sequence (the B-chain/C-peptide junction), and the other (type II) preferentially cleaves proinsulin on the carboxylic side of Lys^{64}/Arg^{65} (the C-peptide/A-chain junction), though this latter activity will also recognize the Arg^{31}/Arg^{32} site to a lesser extent. Both these endopeptidase activities have a common requirement for Ca^{2+} and an acidic pH (5–6) for activity and are localized principally in the insulin secretory granule (Davidson *et al.*, 1987, 1988), which is the major site of proinsulin conversion (Kemmler & Steiner, 1970; Sorenson *et al.*, 1970; Orci, 1985; Rhodes *et al.*, 1987).

Studies with active-site-directed inhibitors based on tripeptide chloromethanes have demonstrated that these enzymes have a preference for dibasic, as opposed to monobasic, sequences (Davidson *et al.*, 1987). On the basis of this assumption, [^{125}I]iodotyrosinylated derivaties have been used previously as radiolabelled affinity probes to attempt to identify the molecular forms of the processing enzymes (Docherty *et al.*, 1982, 1983, 1984). The results of such studies, however, have been equivocal.

Peptidylmethanesulphonium salts [R-CO-CH_2-$S^+(CH_3)_2$] have been suggested as useful affinity-labelling reagents for proteinases. They have diminished reactivity, for example, to thiol groups, and this is expected to involve fewer side reactions in biological systems than the corresponding chloromethanes. In addition they also have a narrower range of anti-proteolytic activity (Rauber *et al.*, 1988; Shaw, 1988; Zumbrunn *et al.*, 1988). In this particular study we have used the tripeptides L-alanyl-L-arginyl-L-arginylmethyldimethylsulphonium [Ala-Arg-Arg-CH_2-$S^+(CH_3)_2$] and L-alanyl-L-lysyl-L-arginylmethyldimethylsulphonium [Ala-Lys-Arg-CH_2-$S^+(CH_3)_2$] as active-site-directed reagents of the proinsulin-processing endopeptidases I and II (Davidson *et al.*, 1988) to investigate the different substrate specificities of these endopeptidases.

METHODS

Materials

Human mono[^{125}I]iodinated proinsulin was a gift from Dr. B. Frank (Eli Lilly, Indianapolis, IN, U.S.A.). Tripeptidyl sulphonium salts and chloromethane tripeptides were prepared as previously described (Kettner & Shaw, 1981; Zumbrunn *et al.*, 1988). Unless indicated, all other chemicals were purchased from either Sigma (London), Poole, Dorset, U.K., or BDH, Poole, Dorset, U.K., and were of the highest grade or purity available.

Tissue

Insulinoma tissue (Chick *et al.*, 1977; Hutton *et al.*, 1981) propagated in New England Deaconess Hospital (NEDH)-strain rats was used as a source of insulin secretory granules. In brief, approx. 5 g of rat insulinoma tissue was homogenized at 4 °C in 25 ml of 0.27 M-sucrose/10 mM-Mes/1 mM-EGTA, pH 6.5, with eight to ten strokes of a Potter–Elvehjem homogenizer. The homogenate was centrifuged (6 min; 4 °C; 1770 *g*; MSE Coolspin centrifuge) and the resulting supernatant (25–30 ml) layered on to a two-step density gradient composed of 5 ml of 20% (w/v) and 6 ml of 10% Nycodenz (Nyegaard and Co. AS, Oslo, Norway) in 0.27 M-sucrose. The Nycodenz gradients were then centrifuged (60 min;

Abbreviations used: Ala-Lys-Arg-CH_2Cl, L-alanyl-L-lysyl-L-arginylchloromethane; Ala-Nle-Arg-CH_2Cl, L-alanyl-L-norleucyl-L-arginylchloromethane; Ala-Lys-Arg-$CH_2S^+(CH_3)_2$, L-alanyl-L-lysyl-L-arginylmethyldimethylsulphonium; Ala-Arg-Arg-$CH_2S^+(CH_3)_2$, L-alanyl-L-arginyl-L-arginylmethyldimethylsulphonium; CDTA, 1,2-cyclohexanediaminetetra-acetic acid; Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane ('TPCK'); E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; iPr_2P-F, di-isopropyl phosphofluoridate; PMSF, phenylmethanesulphonyl fluoride; NEM, *N*-ethylmaleimide; IAA, iodoacetic acid; IAM, iodoacetamide; PCMB, *p*-chloromercuribenzoate.

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4 °C; 100000 *g*; Beckman L5-65 ultracentrifuge; SW28 rotor) and insulin secretory granules recovered at the 10/20 %-Nycodenz interface. This secretory-granule fraction was then washed twice in 0.27 M-sucrose/10 mM-Mes buffer, pH 6.5, before use.

Ion-exchange chromatography

Separation of type I and II proinsulin-processing endopeptidases was achieved by subjecting an insulin-secretory-granule fraction to DEAE-cellulose ion-exchange chromatography (Davidson *et al.*, 1988). Secretory granules (10–20 mg of protein) were sonicated (15 s; 4 °C; 25 W; MSE Soniprep-150) in 4–5 ml of 20 mM-Bistris/0.5 % Triton X-100/1 mM-EDTA/0.1 mM-Tos-Phe-CH_2Cl (tosylphenylalanylchloromethane; 'TPCK')/10 μM-*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64)/10 μM-pepstatin A, pH 5.5. The resultant suspension was then centrifuged (15 min; 4 °C; 35000 *g*; Sorvall RC-5B centrifuge; SS-34 rotor) and the supernatant applied at 0.5 ml/min to a column (8 mm × 40 mm) of DEAE-cellulose DE-52 (Whatman, Maidstone, Kent, U.K.) equilibrated in 20 mM-Bistris buffer, pH 5.5. The column was washed with 27.5 ml of the same equilibration buffer, then eluted with a 30 ml linear gradient of 0–0.4 M-NaCl gradient in the same buffer. Fractions (1 ml) were collected, and the buffer in each of these was immediately exchanged with 50 mM-sodium acetate, pH 5.5, over a 1 ml desalting Sephadex G-25 'spun' column. Each desalted fraction was then assayed for proinsulin-processing activity.

Proinsulin-processing assay

Samples of column fractions (20–30 μl) were incubated for 3 h at 30 °C in 100 μl (final volume) of 50 mM-sodium acetate/5 mM-$CaCl_2$/10 μM-pepstatin-A/0.1 mM-Tos-Phe-CH_2Cl/10 γM-E-64, pH 5.5, containing 30000 d.p.m. of ^{125}I-proinsulin (15–20 ng). The assay was either carried out in the presence of purified carboxypeptidase-H (10 ng/sample; Davidson & Hutton, 1987) or terminated at the end of 3 h by the addition of 12.5 μl of 1 M-Tris/HCl, pH 8.0, with a further 15 min incubation performed at 30 °C with di-isopropyl phosphofluoridate (iPr_2P-F)-treated carboxypeptidase B (125 ng/sample; 50 mM-Tris/HCl, pH 7.5), to remove newly generated *C*-terminal basic amino acids derived from endopeptidase activity. The ^{125}I-labelled products generated were analysed by alkaline-urea/polyacrylamide-gel electrophoresis and autoradiography as previously described (Davidson *et al.*, 1987, 1988).

Other procedures

Protein was determined by the method of Bradford (1976), with bovine serum albumin (Sigma) as standard. Statistical differences between experimental groups was determined by Student's *t* test.

RESULTS

At the completion of the 3 h incubation *in vitro*, between 30 and 40 % of the initial ^{125}I-proinsulin radioactivity was converted into ^{125}I-des-Arg^{31}-Arg^{32}-proinsulin (type-I proinsulin-processing endopeptidase activity), between 25 and 30 % ^{125}I-des-Lys^{64}-Arg^{65}-proinsulin (type-II proinsulin-processing activity) and between 4 and 5 % ^{125}I-insulin (combined activities). Initial experiments showed that pepstatin A, Tos-Phe-CH_2Cl and E-64 did not significantly affect proinsulin processing *in vitro* (Davidson *et al.*, 1987), and these reagents were subsequently used in all incubations.

Table 1. Effect of proteinase inhibitors on the proinsulin-processing endopeptidases types I and II

Inhibitors were dissolved in water or ethanol as appropriate, and added as 10-fold-concentrated solutions to the proinsulin-processing-endopeptidase assay mixture as previously described (see the Methods section). After incubating the mixtures at 30 °C for 3 h, the reactions were terminated by the addition of trichloroacetic acid, and the precipitated proteins analysed by alkaline-urea/polyacrylamide-gel electrophoresis as described previously (see the Methods section). The values shown are expressed as a percentage of the control activity (no added inhibitor) and are means for at least two separate experiments; the two (or more) determinations did not differ from the mean by more than 5 %. Pepstatin A, Tos-Phe-CH_2Cl and E-64 did not inhibit the endopeptidases. The lack of inhibition by E-64 establishes that they are not papain-like proteinases. Abbreviation: PHEN, 1,10-phenanthroline.

Inhibitor	Concn. (mM)	Endopeptidase...	Activity (% of control) Type I	Type II
PMSF	1		88.4	92.6
iPr_2P-F	1		98.2	103.7
NEM	1		98.9	106.4
IAA	1		87.6	97.1
IAM	1		93.2	98.0
Hg^{2+}	1		2.6	2.7
PCMB	1		5.1	2.5
PHEN	6		89.3	90.4
EDTA	6		2.7	4.5
CDTA	6		1.7	0

The group-specific inhibitors of serine proteinases [phenylmethanesulphonyl fluoride (PMSF) and iPr_2P-F; Table 1] had little effect on either type-I or type-II proinsulin-processing endopeptidases, as did the cysteine-proteinase inhibitors [*N*-ethylmaleimide (NEM), iodoacetic acid (IAA) and iodoacetamide (IAM); Table 1]. However, *p*-chloromercuribenzoate (PCMB) and Hg^{2+}, also cysteine-proteinase inhibitors, abolished type-I and -II endopeptidase activities (Table 1). EDTA and CDTA inhibited both type-I and type-II endopeptidases (Table 1) at a concentration which would chelate the 5 mM-Ca^{2+} in the incubation assay (Davidson *et al.*, 1987, 1988). However, the heavy-metal-ion chelator 1,10-phenanthroline had no effect on either of the endopeptidases (Table 1).

The effect of the dibasic tripeptidyl sulphonium salts Ala-Arg-Arg-$CH_2S^+(CH_3)_2$ and Ala-Lys-Arg-$CH_2S^+(CH_3)_2$ on the type-I and type-II proinsulin-processing endopeptidases is illustrated in Fig. 1. The type-I endopeptidase was more markedly inhibited by the Arg-Arg tripeptide ($K_{0.5} = 6.1 \pm 0.6\,\mu$M; $n = 3$) than by the Lys-Arg tripeptide ($K_{0.5} = 106.0 \pm 11.6\,\mu$M; $n = 3$); $P < 0.01$, whereas the converse was true for the type-II endopeptidase, which was more susceptible to the Lys-Arg tripeptide ($K_{0.5} = 65.0 \pm 10.2$ nM; $n = 3$) than to the Arg-Arg tripeptide ($K_{0.5} = 9.6 \pm 1.86\,\mu$M; $n = 3$); $P <$

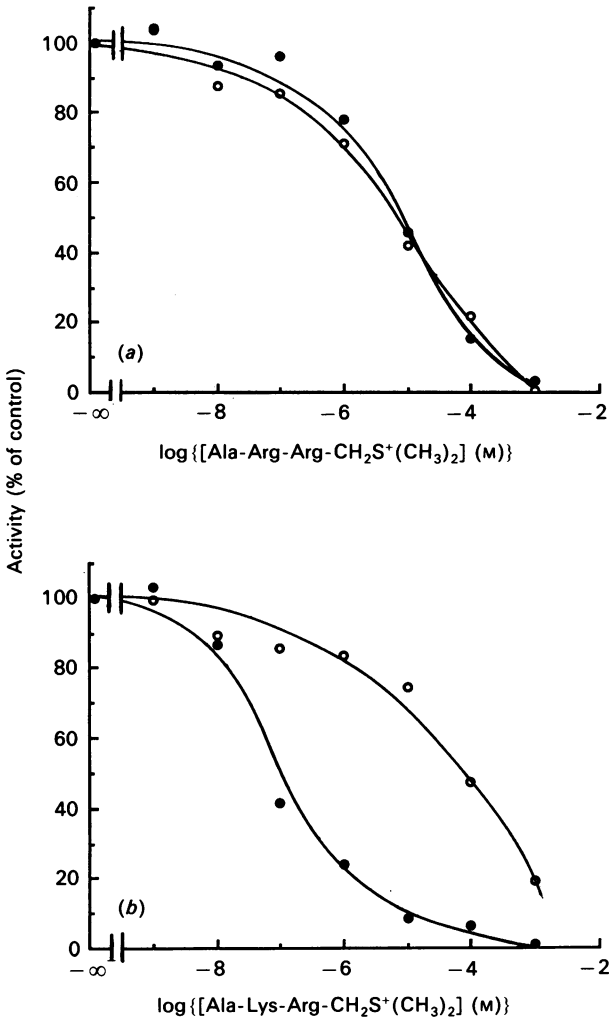

Fig. 1. Dose-dependent inhibition of proinsulin-processing endopeptidases by Ala-Arg-Arg-$CH_2S^+(CH_3)_2$ and Ala-Lys-Arg-$CH_2S^+(CH_3)_2$

^{125}I-proinsulin-processing activity by either type-I or type-II insulin-secretory-granule endopeptidase *in vitro* was assayed at 30 °C in the presence of various concentrations of Ala-Arg-Arg-$CH_2S^+(CH_3)_2$ (*a*) or Ala-Lys-Arg-$CH_2S^+(CH_3)_2$ (*b*) as described in the Methods section. Products of this activity were analysed by alkaline-urea/polyacrylamide-gel electrophoresis and fluorography. Activity is expressed as a percentage of control activity (no inhibitor) of the type-I (○) or type-II (●) endopeptidase.

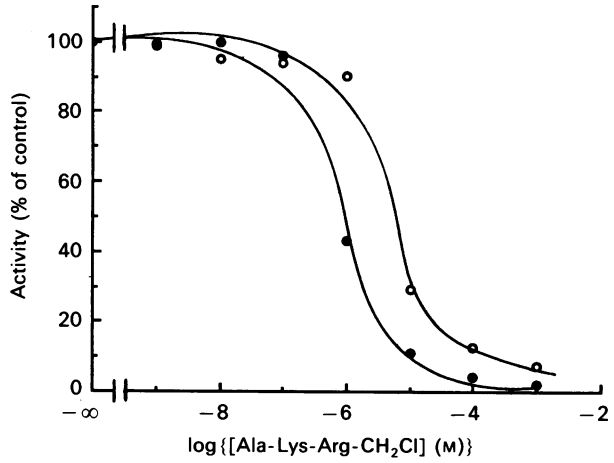

Fig. 2. Dose-dependent inhibition of proinsulin-processing endopeptidases by Ala-Lys-Arg-CH_2Cl

^{125}I-proinsulin-processing activity by either type-I or type-II insulin-secretory-granule endopeptidase *in vitro* was assayed at 30 °C in the presence of various concentrations of Ala-Lys-Arg-CH_2Cl as described in the Methods section. Products of this activity were analysed by alkaline-urea/polyacrylamide-gel electrophoresis and fluorography. Activity is expressed as a percentage of control activity (no inhibitor) of the type-I (○) or type-II (●) endopeptidase.

0.01. It was noteworthy that the concentration of Arg-Arg analogue required for half-maximal inhibition was similar in the case of both enzymes, whereas the half-maximally effective concentration of the Lys-Arg analogue was three orders of magnitude lower with the type-II than with the type-I activity ($P < 0.001$).

The peptide chloromethane Ala-Lys-Arg-CH_2Cl, examined under the same conditions, inhibited the type-II activity ($K_{0.5} = 0.8\ \mu$M) more readily than type-I ($K_{0.5} = 5.0\ \mu$M) (Fig. 2). An analogue with a single basic residue, Ala-Nle-Arg-CH_2Cl, was considerably less effective, and at 100 μM decreased both activities by 40%. Leupeptin, a tripeptide with a *C*-terminal arginine residue that is an inhibitor of both serine and cysteine proteinases (Soyagi & Umezawa, 1975), also inhibited the endopeptidases at high concentrations (for type-I, $K_{0.5} =$ 2.0 mM; for type-II, $K_{0.5} = 0.1$ mM).

DISCUSSION

A review of the patterns of cleavage of a number of propolypeptide hormones and neurotransmitters (Docherty & Steiner, 1982; Loh *et al.*, 1984) indicates that, within any given precursor, not all dibasic sites are cleaved and, in many instances, e.g. pro-opiomelanocortin, proenkephalin, proglucagon, the precursor can undergo tissue-specific processing to generate different active peptides from the same parent molecule. Differential processing may occur under different physiological conditions within the same tissue. We have postulated that this could be achieved quite simply by the participation of two sequence-specific endopeptidases of broad specificity which are regulated differentially by Ca^{2+} and pH (Davidson *et al.*, 1988) The proinsulin-gene product expressed in yeast (Thim *et al.*, 1986) and in mouse pituitary AtT20 cells (Moore *et al.*, 1983) is correctly processed further, indicating that the endopeptidase activity might be conserved both between species and between different mammalian tissues.

The results of the present study are consistent with this view, inasmuch as they indicate that the primary amino acid sequence of the dibasic site is an important determinant of the specificity of the type-I (Arg^{31},Arg^{32}-directed) and type-II (Lys^{64},Arg^{65}-directed) endopeptidase activities which process proinsulin (Davidson *et al.*, 1988). Thus the type-I activity was more susceptible to inhibition by the Arg-Arg sulphonium salt than by the Lys-Arg analogue, and the converse was true for the type-II activity. In terms of the actual concentrations of

the inhibitors which were required to achieve half-maximal inhibition, the ability of the Lys-Arg analogue to distinguish between the type-II and type-I activity was remarkable (1500-fold difference) compared with the Arg-Arg sulphonium salt, which inhibited both activities at approximately the same concentration. This was consistent with the observation that the type-I activity does not recognize the Lys-Arg sequence, whereas the specificity of the type-II activity is not absolute, since it cleaves at the Arg-Arg site in the standard assay at approx. 10–20 % of the rate at the Lys-Arg site (Davidson *et al.*, 1988). Whether, in the case of type-II activity, this is the reflection of the substrate specificity of a single catalytic activity remains to be resolved.

The specificity of the proinsulin endopeptidases for dibasic as opposed to monobasic sequences, which was apparent in previous studies performed with unfractionated granule preparations (Davidson *et al.*, 1987), was confirmed in this study for both type-I and -II activities using the chloromethane analogues Ala-Lys-Arg-CH_2Cl and Ala-Nle-Arg-CH_2Cl. The inference that the primary sequence was an important determinant of type-II-enzyme specificity could again be made from the observed difference in the half-maximally inhibitory concentration on both activities (Fig. 2).

A notable difference between peptidyl chloromethanes and the arginine-containing sulphonium salts is that the latter have not been found to have an inhibitory effect on serine proteinases, but do inactivate a number of cysteine proteinases (Zumbrunn *et al.*, 1988). This might suggest that the type-I and -II endopeptidases are cysteine proteinases; however, other inhibitory reagents of this particular enzymic class (NEM, IAA, IAM; Table 1) had no effect on type-I and -II activities. From studies of other inhibitors (Table 1) it was clear that, although both type-I and -II activities had similar properties, they did not fit into any readily recognizable proteinase class. The catalytic nature of these particular endopeptidases therefore remains undetermined, and further characterization will be necessary before their enzymic mechanisms can be established.

Recently, a Ca^{2+}-dependent proteinase activity has been demonstrated in rat hepatic microsomes that correctly processes proalbumin to albumin *in vitro* at pH 6.0 (Brennan & Peach, 1988). The characteristics for this proalbumin-processing activity are similar to the type-I and type-II endopeptidases for proinsulin processing (Davidson *et al.*, 1988) and to chromogranin-A-processing activity in the insulin secretory granule (Hutton *et al.*, 1987). Preliminary studies in angler-fish islets have also implicated a Ca^{2+}-dependent proteinase involved in islet prosomatostatin-I conversion into somatostatin-14 (Mackin *et al.*, 1988). The dibasic tripeptidyl sulphonium salts employed in the present study may prove to be useful tools in characterizing the dibasic substrate specificities of these and other important precursor protein-converting endopeptidases. In addition, these reagents also have important potential use as affinity ligands or probes for the further isolation and characterization of these endopeptidases.

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