Proinsulin Endopeptidase Substrate Specificities Defined by Site-directed Mutagenesis of Proinsulin*

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Two endopeptidases are involved in the conversion of proinsulin; a type I activity directed at the B chain, Arg31,Arg32, C-peptide junction, and type II which cleaves exclusively on the C-terminal side of Arsl,Arg8 (B chain/C-peptide junction), whereas the type II endopeptidase cleaves preferentially on the C-terminal side of Lys84,Arg85 (C-peptide/A chain junction) (Davidson et al., 1988).

Both endopeptidases display an acidic pH optimum and are activated by calcium. Studies with group-specific proteinase inhibitors have failed to define the mechanism of catalysis and the possibility remains that they constitute a new class of proteinase. Active site-directed reagents have shown that the primary amino acid sequence of the dibasic site is an important determinant of endopeptidase substrate specificity. Thus, the type I activity was more susceptible to inhibition by an Arg-Arg containing sulfonium salt than by a Lys-Arg analogue, and the converse was true for the type II activity (Rhodes et al., 1989).

Cleavage of peptides at sites marked by pairs of basic amino acids is a common feature of prohormone processing (Docherty and Steiner, 1982). In many cases multiple copies of a bioactive peptide (e.g. met-enkephalin) or different bioactive peptides (e.g. proopiomelanocortin) may reside in the same precursor sequence, permitting tissue-specific processing to generate different peptides from the same parent molecule (Loh et al., 1984). Not all paired basic sequences are cleaved within a propolypeptide, suggesting that factors in addition to the primary sequence are involved in determining the specificity of processing endoproteases.

By site-directed mutagenesis of the encoding DNA, it is now possible to change specific amino acid residues in a protein (for reviews, see Knowles (1987) and Shaw (1987)) and thereby examine the importance of a particular amino acid, sequence, or structural motif on catalytic function of enzymes or the susceptibility of protein substrate to enzymatic attack. To examine further the substrate specificity of the proinsulin processing type I and type II endoproteases, we have used oligodeoxynucleotide site-directed mutagenesis to change the paired basic sites within proinsulin. Mutant radiolabeled proinsulins for use as substrates were synthesized by microinjecting appropriate cRNAs into Xenopus oocytes.

MATERIALS AND METHODS

Animals—Xenopus oocytes were purchased from Xenopus Ltd., Redhill, Surrey, United Kingdom.

Chemicals and Reagents—L-[4,5-3H]Leucine (120-190 Ci/mmol) and [α-35S]dATPαS (1000 Ci/mmol) were obtained from Amersham International, Little Chalfont, Bucks, United Kingdom; plasmid pT7 and Sequenase were purchased from United States Biochemical Corporation, through Cambridge Bioscience, Cambridge, Cambs, United Kingdom; T7 RNA polymerase, nucleotide triphosphates, mGTP/ampppG cap structure analogue, and RNaseguard were from Pharmacia, Milton Keynes, United Kingdom; and the human proinsulin cDNA (Bell et al., 1979) was kindly provided by Dr. G. I. Bell, Howard Hughes Medical Institute, University of Chicago. Oligodeoxynucleotides were purchased from Alta Bioscience, University of Birmingham.

Oligodeoxynucleotide Site-directed Mutagenesis—Oligodeoxynucleotide site-directed mutagenesis was performed as described by Carter

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1 The abbreviations used are: dATPαS, deoxyadenosine 5'-α-[35S]thiotriphosphate; SDS, sodium dodecyl sulfate; BuTris, 2,6-bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; HPLC, high performance liquid chromatography.
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et al. (1985). The human preproinsulin cDNA was subcloned into the EcoRI site of M13K19 replicative form DNA and transfected into competent Escherichia coli strain TG1. Single stranded template was prepared, and mutant second strand synthesized using the Klenow fragment of DNA polymerase in the presence of T, DNA ligase. Two primers were used simultaneously: one a mutagenic oligodeoxynucleotide, purified and used to generate desired nucleotide change, and a second selection oligodeoxynucleotide (Sel 2) which converted an EcoB site to an EcoB site. The double stranded DNA was purified from a low melting point agarose gel and used to transfect a nonpermissive E. coli strain HB2154. To mutate an additional site in the same cDNA, the mutagenesis reaction was performed on the single stranded mutant template with a second mutagenic primer and a selection primer (Sel 3) which converted the EcoB site to an EcoK site. The double stranded DNA was annealed and used to transfect E. coli HB2154.

In Vitro Transcription—The normal and mutant cDNAs were subcloned into the EcoRI site of plasmid pBluescript and capped RNA synthesized from HindIII linearized plasmid in the presence of T4 RNA polymerase. Two selection oligodeoxynucleotides were designed (position shown in Fig. 1) for converting Arg31,Arg32 to Arg31,Arg32, and D35 (5' ATGCCACGCG*TCTGCAGG 3') for converting LysM,Arg6s processing site of the wild-type preproinsulin and 60 GTP, 0.5 mM GTP, 0.1 mM dATP, 0.5 mM dTTP, 0.5 mM dCTP, 0.25 mM dGTP, bovine serum albumin (RNase- and DNase-free), 10 units RNase A, 70 units of T, RNA polymerase, and 1 mg of plasmid DNA. The mixture was incubated at 37 °C for 80 min. After treatment with DNase (RNase-resistant) at 37 °C for 10 min, the cRNA was extracted once with phenol-chloroform-isomyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). The cRNA was then precipitated using 0.1 volumes 7 M ammonium acetate and 2.5 volumes ethanol. The pellet was collected following centrifugation for 10 min at 12,000 × g and the cRNA resuspended in water at a concentration of 1–2 µg/ml.

Xenopus Oocyte Microinjections—Oocytes were isolated from female Xenopus laevis as described by Coleman (1984) and maintained at 20 °C in modified Barth’s saline consisting of 7.5 mM Tris-HCl (pH 7.6), 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.3 mM CaCO3, 0.41 mM MgCl2, 0.82 mM MgSO4, 10 mg/ml sodium penicillin, 10 mg/ml streptomycin sulphate, 100 mg/ml gentamicin, 20 units/ml nystatin, and 125 µg/ml fungizone. Oocytes were microinjected with 50 nl of wild-type and mutant preproinsulin cRNAs and incubated at 20 °C for 6 h. Healthy oocytes (10) were then incubated in 30 µl of modified Barth’s saline containing 5% (v/v) dialyzed fetal calf serum, 0.45 kallikrein inactivator unit/ml Trasylol, and [3H]leucine (1 mCi/ml) for 18 h. Medium was removed and centrifuged at 12,000 × g for 5 min.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed as described previously (Shennan and Docherty, 1988).

Assay of Preproinsulin Conversion—Insulin secretory granules were prepared from rat transplantable insulinomas as described previously (Dawson et al., 1985) and lyed in a buffer comprised of 20 mM BisTris, 0.5% (v/v) Triton X-100, 0.5 mM EDTA, 10 µM phenylmethylsulfonyl fluoride, 10 µM pepstatin A, 10 µM trans-epoxyoxycinnyl-α-leucylamido-(4-guanidino)butane (E-64), and 50 µM tosylphenylalanyl-nylchloromethane (TPCK) (pH 5.5). Insoluble material was removed from the lysate by centrifugation and subjected to anion exchange chromatography on a DE-52 column, as described previously (Dawson et al., 1988), to separate the type I and type II endopeptidase activities. In the standard assay 20 µl of the incubation medium derived from five radiolabeled oocytes (10,000–25,000 dpm of trichloroacetic acid-precipitable material) was added to a 1.5-ml capacity microcentrifuge tube to form a total volume in a buffer containing 100 mM NaCl, 50 mM Tris-HCl, 10 µM phenylmethylsulfonyl fluoride, 10 µM pepstatin A, 10 µM E-64, and 50 µM TPCK, carboxypeptidase B (50 µg/ml), and 10–30 µg of protein containing the enzymic activities. The incubation was terminated after 3 h at 30 °C by addition of 300 µl of ice-cold 5% (w/v) trichloroacetic acid. A mixture of human proinsulin, des 31,32-proinsulin, des 64,65-proinsulin, and insulin (2–5 µg each in 10 µl 10 mM HCl) was added at this stage as carrier and to serve as calibration standards for HPLC. The mixture was centrifuged for 5 min at 12,000 × g, the pellet washed further with 1 ml of 5% (w/v) trichloroacetic acid and then extracted twice with 500 µl of diethyl ether. After air-drying at room temperature, the precipitated material was reconstituted in 120 µl of 10 mM HCl, a 10-µl sample removed for determination of total radioactivity and 100 µl then loaded onto a 30 × 0.8× cm column packed with Lichrosorb C-18 silica resin (Merek, Darmstadt, West Germany). The column was developed at 1 ml/min as described previously (Dawson et al., 1988), and 0.6-ml fractions collected. To each of these was added 0.5 ml of water and 4 ml of Optiphase Hisafe II scintillation mixture (LKB, Milton Keynes, United Kingdom) and their radioactivity determined by liquid scintillation counting.

RESULTS

Site-directed Mutagenesis—Oligodeoxynucleotides site-directed mutagenesis was used to change the coding sequence for the paired basic residue processing sites within the follod oligodeoxynucleotides were designed (position of mismatch is indicated by asterisk after corresponding nucleotide): D34 (5' TCTGCCCTCCCC*GCCGGTTC 3') for converting Arg31, Arg32 to Arg31,Gly32, and D35 (5' ATGCCACCGG*TCTGGAGG 3') for converting Lys31,Arg65 to Thr31,Arg65. The changes were confirmed by DNA sequencing as shown in Fig. 1. The double mutant Arg31,Gly32 combined with Thr31,Arg65 was also generated using oligodeoxynucleotides D34 and D35.

In addition to the above, a mutant cDNA containing a deletion of the major part of the C-peptide coding region (des-38–62-proinsulin) was generated by using strategically situated restriction enzyme sites (Shennan and Docherty, 1988).

Expression of Wild-type and Mutant CDNAS in Xenopus Oocytes

FIG. 1. Sequence of wild-type and mutant preproinsulin cDNAS. A, DNA sequence encoding the Arg31,Arg32 processing site of the wild-type preproinsulin (WT) and Arg31,Gly32 site of the mutant preproinsulin (D34) generated using oligodeoxynucleotide D34. The normal cDNA has the sequence CCCGCG and the mutant the sequence CCCCGG as indicated by the arrow. B, DNA sequence encoding the Lys31,Arg65 processing site of the wild-type proinsulin (WT) and Thr31,Arg65 site of the mutant proinsulin (D35) generated using oligodeoxynucleotide D35. The normal cDNA has the sequence CGGCTT and the mutant the sequence CGCGTG as indicated by the arrow.
In Vitro Processing of Mutant Proinsulins—Xenopus oocytes microinjected with cRNA corresponding to the wild-type proinsulin cDNA secreted a radiolabeled peptide with a retention on HPLC identical to a human recombinant proinsulin secreted by Xenopus oocytes (Shennan and Docherty, 1988) and on the rate of secretion of the mutant proinsulin (Shakur et al., 1989).

The double mutant, Arg3',Art2 sequence. The mutant des-31,32-proinsulin also elutes slightly before the wild-type proinsulin, it is concluded that the molecule has been cleaved by the type I activity in the expected manner after the unmodified C-terminal side of the LysB4,Arg6 sequence. Processing in the presence of the type I and II endopeptidases was equivalent to the processing observed in the presence of type I activity alone.

Mutation of the Arg31,Arg32 sequence to Arg31,Gly32 prevented cleavage by the type I endopeptidase activity which is directed at this site (Fig. 3, panel 4; Table I). However, surprisingly, this mutant was a poor substrate for the type II endopeptidase in spite of there being no alteration in the normal Lys31,Arg32 sequence in this mutant. Little processing was likewise observed in the combined presence of type I and II activities or with unfractionated secretory granule lysates. The double mutant, Arg31,Gly32 combined with Thr24,Arg65 was also poorly processed by either enzyme (data not shown), an observation which was expected on the basis of the above findings with the singly mutated molecules.
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**DISCUSSION**

The use of mutant proinsulins generated by site-directed mutagenesis shows that the proinsulin processing type I and type II endopeptidases are highly specific for sequences containing pairs of basic amino acids. The type I enzyme activity, which is specific for Arg^{33}.Arg^{65} did not cleave at this site when the sequence was changed to Arg^{31}.Gly^{32}, whereas the type II enzyme activity, which is directed principally at the Lys^{34}.Arg^{65} site did not cleave at this site when the sequence was changed to Thr^{44}.Arg^{65}.

In addition, the results also demonstrated that the secondary structure of proinsulin was important in defining the activity of the two endopeptidases. When the Lys^{34}.Arg^{65} cleavage site was changed to Thr^{44}.Arg^{65}, both the type I and the Arg^{31}.Arg^{65} specific component of the type II enzyme cleaved at the Arg^{31}.Arg^{65} site. However, when the Arg^{31}.Arg^{65} cleavage site was changed to Arg^{31}.Gly^{32}, the type II enzyme did not cleave efficiently at the Lys^{34}.Arg^{65} site. One would expect this result if the processing of proinsulin were sequential, i.e. that the Lys-Arg site is susceptible to cleavage only after initial cleavage at the Arg-Arg site. However, the fact that the type II activity can produce split 65,66-proinsulin from wild-type proinsulin argues against this. There is similarly no indication that the split 32,33-proinsulin is preferred to proinsulin by the type I activity as a substrate, since processing in the combined presence of type I and II activities would have exceeded the sum of the activities where incubated alone. It is more likely that the activity of the enzymes may be dependent on the secondary structure around the cleavage site and that this structural requirement was disrupted by changing the Arg^{31}.Arg^{65} site to Arg^{31}.Gly^{32}. The conclusion that the structure around the dibasic cleavage sites is important is supported by the observed inability of the type I and type II enzymes to cleave at the dibasic sites in des-38-62-proinsulin.

It is difficult to predict what effect specific changes introduced into the proinsulin molecule would have on the overall secondary structure, since no three-dimensional x-ray crystallographic data on proinsulin (or any other prehormone) is available. Secondary structure predictions using the algorithms of Chou and Fasman (1978) indicated that the point
mutations at the dibasic cleavage sites do not have any effect on the $\alpha$ helical or $\beta$ sheet structure of proinsulin. The cleavage sites, it is predicted, are located in or close to $\beta$ turns and are flanked by an $\alpha$ helix or $\beta$ sheet. The same structure is present at the dibasic sites of des-38–62-proinsulin except that a predominant $\beta$ turn in the central portion of the C-peptide is no longer present, and the mini C-peptide is in a $\beta$ sheet.

Rholam and Cohen (1986) have made a survey using the Chou and Fasman rules of dibasic sites within a number of prohormones, and have concluded that dibasic processing sites may be located within $\beta$ turns, whereas those dibasic sites which are not cleaved are located in $\alpha$ helices or $\beta$ sheets. However, in our mutants where the dibasic site is not cleaved, i.e. the Lys$^{64}$,Arg$^{65}$ site in Gly$^{39}$ proinsulin, and the two dibasic sites in des-38–62-proinsulin, there is no indication that these sites are present in $\alpha$ helix or $\beta$ sheet structures.

Our results showing that the type I or type II endopeptidases were unable to cleave des-38–62-proinsulin contrasts with the findings of Thim et al. (1986), who found that des-38–62-proinsulin and proinsulins with C-peptides only two amino acids long were cleaved at one or other of the dibasic sites when expressed in yeast. This could be due to differences in specificity between the proinsulin processing endopeptidases and the yeast endopeptidase (presumably KEX2 (Julius et al., 1983)) responsible for processing expressed prohormones.

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