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Differential rates of conversion of rat proinsulins I and II

Evidence for slow cleavage at the B-chain/C-peptide junction of proinsulin II

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Rat proinsulin I is converted into insulin more rapidly than is proinsulin II. To study this further, rat islets were labelled (10 min) and conversion kinetics of the labelled proinsulins were monitored during a 120 min chase. Proinsulins, conversion intermediates and both insulins were separated by h.p.l.c. The accumulation of des-64,65-(split proinsulin II) during the chase suggests that the B-chain/C-peptide junction of proinsulin II is cleaved more slowly than the equivalent site on proinsulin I. This accounts for the differential kinetics of conversion of proinsulins I and II, and is presumed to be caused by one (or more) of the amino acid replacements which distinguish the two proinsulins.

INTRODUCTION

Insulin production by the pancreatic B-cell consists of a series of intracellular events, including biosynthesis, packaging into secretory vesicles, processing of precursors and exocytosis of granule contents [1,2]. The bulk of the processing of proinsulin occurs after its delivery to immature clathrin-coated secretory granules [1,3] and involves cleavage at two pairs of basic residues [2]. Based upon the latest data, two endoproteases and a carboxypeptidase are believed to be implicated [4,5]. The endoprotease activities are directed against the two different sites of conversion (pairs of basic residues): (i) *C*-terminal of Arg^{31}–Arg^{32} (between the B-chain and the C-peptide) for type 1 endoprotease; and (ii) *C*-terminal of Arg^{64}–Arg^{65} (between the C-peptide and the A-chain) for type 2 endoprotease, leading to the production of split 32/33 proinsulin and split 65/66 proinsulin respectively (i.e. proinsulins cleaved between residues 32 and 33 and residues 65 and 66 respectively). These two intermediates are immediately processed by carboxypeptidase H [6], which trims off residual *C*-terminal basic amino acids. Des-31,32-(split proinsulin) and des-64,65-(split proinsulin) are thus the major proinsulin conversion intermediates encountered in the B-cells [2,4]. A second round of endoprotease/carboxypeptidase attack produces fully processed insulin. These enzymes display an acidic pH optimum which is satisfied during the maturation of the secretory granule by the action of the granule ATP-dependent proton pump [3,4,7,8].

The rat (and mouse) has two insulins (I and II), which are the products of non-allelic genes [9,10]. The processing of the two proinsulins follows differential kinetics in isolated islets [11]: in a pulse–chase experiment at early chase times (under 60 min), insulin I accounts for more than 85% of the radiolabelled insulin present. At later times, the steady-state ratio of insulin I/insulin II of 6:4 is reached [11]. Proinsulin I is thus converted into insulin more rapidly than is proinsulin II.

Following a brief pulse label of isolated rat islets, we have used reversed-phase h.p.l.c. to measure the proportions of both labelled proinsulins, their conversion intermediates and the two insulins during a subsequent chase incubation. We confirm the more rapid rate of conversion of proinsulin I and show a disproportionate accumulation of des-64,65-(split proinsulin II). Our data suggest that the B-chain/C-peptide junction of proinsulin II is more resistant to endoproteolytic attack than the same site on proinsulin I. This is presumed to be caused by one (or more) of the four amino acid replacements which distinguish the two proinsulins.

MATERIALS AND METHODS

Animals

Adult male Sprague–Dawley rats (200–250 g) were allowed free access to water and standard laboratory chow, except for the 12 h before islet isolation, when only water was available. For each experiment, four rats were used.

Isolation of pancreatic islets

Islets of Langerhans were isolated according to the method of Sutton *et al.* [12] as modified by Rouiller *et al.* [13]. In brief, the pancreas was distended by infusion via the pancreatic duct of 16 mg of collagenase (Type I; Sigma, St. Louis, MO, U.S.A.) in 6 ml of Hanks buffer (pH 7.2; 6 mM-$CaCl_2$) and digested by static incubation at 37 °C for 20 min. The digested tissue was washed, strained and then resuspended in 10 ml of Histopaque 1077 (Sigma), and the suspension was overlayered by 10 ml of Hanks/0.35% BSA. The gradient was spun at 1000 *g* for 20 min at 16 °C. The islets, which were present at the Histopaque/buffer interface, were collected and washed three times with cold Hanks/0.35% BSA, pH 7.4. Between 400 and 600 islets per rat pancreas were isolated.

The isolated islets were cultured overnight in Dulbecco's modified Eagle's medium (Gibco, Irvine, Scotland, U.K.), containing 8.3 mM-glucose, 10% foetal calf serum (Gibco), penicillin (100 units/ml), streptomycin (0.1 mg/ml) and gentamycin (2 mg/ml) at 37 °C in a humidified atmosphere of CO_2/air (1:19).

Pulse–chase radiolabelling of isolated islets

The islets, after the night of tissue culture, were harvested under a binocular microscope and washed three times with F10 medium (Gibco) without leucine and methionine, but containing 16.7 mM-glucose, 0.25% BSA and 25 mM-Hepes, pH 7.4. The islets were then pulse-labelled in 100 μl of F10 (same as above) containing 0.2 mCi of [^{3}H]leucine and 0.2 mCi of [^{35}S]methionine (both from Amersham International) for 10 min at 37 °C.

Abbreviation used: KRB, Krebs–Ringer bicarbonate.

After the labelling period, the islets were washed three times in cold Krebs–Ringer bicarbonate buffer (KRB) containing 1.67 mM-glucose, 1 mM-leucine, 1 mM-methionine, 0.25% BSA and 10 mM-Hepes, pH 7.4. The islets were distributed in seven plastic microfuge tubes (final volume 1 ml of KRB; 200–250 islets). The tubes were incubated at 37 °C for different post-label (chase) times. In order to ensure that all labelled proinsulin had reached the secretory granules and that conversion had been initiated, a first tube was removed from the incubator after 30 min, placed on ice and then centrifuged at 80 *g* for 2 min. The supernatant was removed and the islet pellet was resuspended in 100 μl of 1 M-acetic acid/0.1% BSA and frozen. The same manipulation was performed on the other samples at 15 min intervals. After the last sample (120 min), all samples were freeze–thawed three times, sonicated for 1 min and centrifuged at 11000 *g* at 4 °C for 10 min, and the supernatant was collected for analysis.

H.p.l.c. analysis of the islet sonicates

The sonicated islet supernatants were analysed by h.p.l.c. by direct injection without any prior purification [14,15]. A Beckman Ultrasphere 5u ODS C_{18} column (4.6 mm × 250 mm) was eluted with two different buffer systems. For system 1 TEAP buffer contained 50 mM-phosphoric acid, 20 mM-triethylamine and 50 mM-sodium perchlorate, pH 3, and solution A contained acetonitrile/water (9:1, v/v). Rat insulins I and II were eluted isocratically (34.2% solution A) at 18 min and 22 min respectively after sample injection. At 25 min the percentage of solution A was increased linearly up to 36.5% over 70 min, allowing elution of the intermediates and proinsulins. System 2 was a modification of the method of Davidson *et al.* [5]. PPH buffer contained 50 mM-H_3PO_4, 100 mM-$NaClO_4$ and 10 mM-heptanesulphonic acid, pH 3; solution A was as in system 1. Insulins were eluted isocratically at 38% solution A. At 25 min the amount of solution A was increased linearly to 42% over 70 min, allowing elution of intermediates and proinsulins.

In both cases, the eluate was collected in 1 min fractions (1 ml/fraction), then 4 ml of scintillation cocktail (Luma Flow II; Lumac, Olen, Belgium) was added and radioactivity was measured in a liquid scintillation counter.

Oxidation of proinsulin II and its conversion products.

Proinsulin II and its conversion products are sensitive to oxidation of Met^{B29} [15]. Proinsulin I contains no methionine. Advantage can be taken of the decreased elution time of the oxidized products to facilitate their separation from proinsulin-I-related products [15]. Oxidation was achieved by adding 10 μl of 5% (v/v) H_2O_2 in 1 M-acetic acid to 40 μl of the sample and incubating for 1 h at room temperature [15]. Samples were stored frozen before analysis.

Digestion *in vitro* and h.p.l.c. analysis of conversion intermediates and proinsulin

In order to prepare useful amounts of all products of interest for subsequent characterization, isolated islets were labelled for 1 h, followed by a 1 h chase. The different products of labelled proinsulin processing were then separated by h.p.l.c. using the TEAP/acetonitrile system as described above (system 1). Fractions of 1 min (1 ml) were collected into tubes containing 100 μl of a 1% solution of BSA, 10 μl of which was counted for radioactivity in a liquid scintillation counter in order to determine the time of elution of the different labelled products, and the fractions corresponding to each product were pooled. Acetonitrile was removed by evaporation and the products were loaded on C_{18} Sep-Pack cartridges (Waters, Millipore Corp., Milford, MA, U.S.A.). After washing the cartridge [16], products were eluted in 45% acetonitrile/0.1% trifluoroacetic acid [16]. The acetonitrile was again evaporated and the samples were then lyophilized and resuspended in 200 μl of water.

For their unambigous characterization (see the results section), samples were digested according to Davidson *et al.* [5]. An amount of the sample corresponding to 20000 c.p.m. was added to 20 μl of 1 M-Tris/formate, pH 7.4, containing 20 μl of trypsin (1 μg/ml; specific activity 12000 BAEE units/mg; Type XIII from Sigma) and 20 μl of non-radioactive human proinsulin (10 mg/ml in 10 mM-HCl) and made up to a final volume of 200 μl with water and incubated at 30 °C for 30 min. The mixture was then heated at 80 °C for 10 min. Half (100 μl) was removed and frozen at −80 °C; the other half was placed on ice, and 5 μl of trypsin inhibitors [200 μM-Tos-Phe-CH_2Cl ('TPCK')/2 mM-phenylmethanesulphonyl fluoride] and 5 μl of carboxypeptidase B (0.5 mg/ml; specific activity 150 units/mg; Boehringer Mannheim, Rotkreuz, Switzerland) were added. This mixture was incubated at 30 °C for 30 min and then frozen at −80 °C. Before injection on h.p.l.c., the samples were acidified by adding 5 μl of 1 M-HCl. They were analysed with both buffer systems as described earlier. Absorbance at 213 nm was used to determine the elution position of the human proinsulin and intermediates.

RESULTS

Characterization of conversion intermediates by limited digestion with trypsin and carboxypeptidase

It has been known for many years that the limited digestion of proinsulin by trypsin leads to the generation of conversion intermediates (cleaved either between the B-chain and the C-peptide, or between the C-peptide and the A-chain), and C-peptide or insulin which still carry one or two residual *C*-terminal basic amino acids [17]. Within the B-cell, by contrast, such residual arginine or lysine residues are rapidly removed by carboxypeptidase H [4,6]. Advantage can be taken of this difference to aid the identification of conversion intermediates extracted from islets [5]. The digestion by trypsin of des-31,32-(split proinsulin) will thus produce fully processed native insulin and lysyl- or lysyl-arginyl-C-peptide, whereas digestion of des-64,65-(split proinsulin) will produce mono- or di-arginylinsulin and native C-peptide. In keeping with the known separation of human insulin and mono- or di-arginylinsulin [5], arginyl rat insulins were both eluted approx. 15 min later than the native rat insulins using the PPH buffer system.

In order to prepare useful amounts of all conversion intermediates, rat islets were labelled with [^{3}H]leucine and [^{35}S]methionine for 1 h, followed by a 1 h chase period. The identification of products derived from rat proinsulin II is facilitated by the presence of a unique methionine residue at position B29. Rat proinsulin I contains no methionine. This allows for the selective radiolabelling of rat proinsulin II with [^{35}S]methionine. Labelled conversion intermediates were purified by h.p.l.c. using both the TEAP and PPH buffer systems. Each product was then digested with trypsin under conditions known to favour cleavage only at the basic residues linking the insulin chains to the C-peptide. Half of each digested sample was then further exposed to carboxypeptidase B to remove *C*-terminal basic amino acids. All products were then analysed by h.p.l.c. using the PPH buffer system (to separate those with *C*-terminal basic amino acids from their trimmed counterparts) or the TEAP system (to separate insulins I and II). When material in peak 7 or 8 (Fig. 1*a*) was digested with trypsin alone and subsequently analysed using the PPH buffer system, a new radioactive peak was generated which was eluted at 35 min (i.e. later than native insulin). Further digestion with carboxypeptidase accelerated the elution of this material such that it was now co-eluted with native insulin.

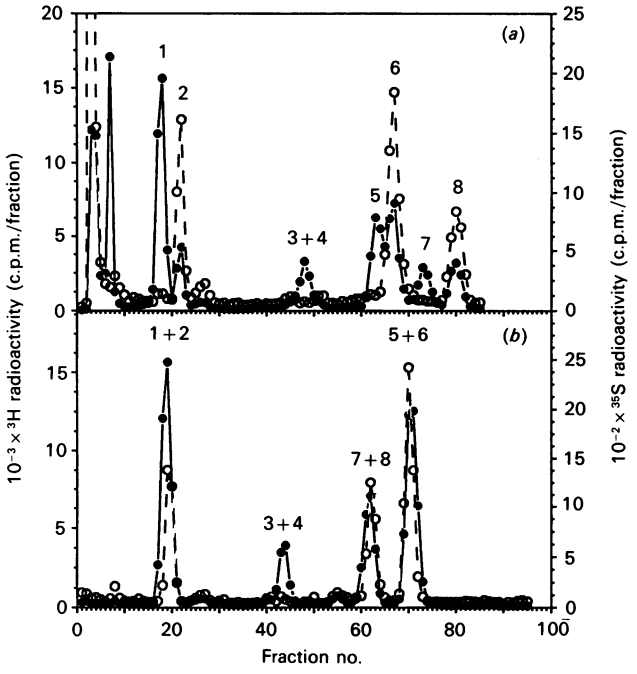

Fig. 1. H.p.l.c. elution profiles using (*a*) TEAP/acetonitrile and (*b*) PPH/acetonitrile buffer systems

●, ^{3}H radioactivity; ○, ^{35}S radioactivity. Islets were labelled with [^{3}H]leucine and [^{35}S]methionine for 10 min. After a subsequent 60 min chase period, the islets were sonicated and the extract was analysed by h.p.l.c. Numbers above each identified peak correspond to: 1, insulin I; 2, insulin II; 3, des-31,32-(split proinsulin I); 4, des-31,32-(split proinsulin II); 5, proinsulin I; 6, proinsulin II; 7, des-64,65-(split proinsulin I); 8, des-64,65-(split proinsulin II).

Taking these data and the presence of [^{35}S]methionine only in peak 8 into account, peaks 7 and 8 could be identified as des-64,65-(split proinsulin I) and des-64,65-(split proinsulin II) respectively. By contrast, the simple digestion of peaks 3 and 4 by trypsin alone generated radioactive material which was co-eluted with the native insulins. Its elution time was not affected by subsequent incubation with carboxypeptidase B. Des-31,32-(split proinsulin I) and des-31,32-(split proinsulin II) were therefore assigned to peaks 3 and 4 respectively.

Separation and quantification of labelled products

Representative h.p.l.c. elution profiles of radioactive products extracted from islets after 10 min of labelling and a 60 min chase using the TEAP and PPH buffer systems are shown in Fig. 1. It can be seen that the TEAP system affords good separation of insulins I and II and of the des-64,65-(split proinsulins) (Fig. 1*a*). Des-31,32-(proinsulins I and II) are not resolved from each other but are well separated from other products, and the same applies to the two proinsulins. It has been shown previously that the methionine residue at position 29 of the B-chain of proinsulin II, its conversion intermediates and insulin II are readily oxidized under acidic conditions [15]. The oxidized products are eluted some 10–15 min sooner than their native counterparts using the TEAP h.p.l.c. buffer system, and can thus be resolved from proinsulin-I-related products, which are not oxidized under such conditions [15].

The PPH system (Fig. 1*b*) allows for an excellent separation of insulin, of both conversion intermediates and of proinsulin, but fails to revolve the individual products of the two insulin genes. It is intriguing to note that des-64,65-(split proinsulin) (I or II) elutes before proinsulin using the PPH system, but after it using the TEAP system (compare peaks 7 and 8 in Figs. 1*a* and 1*b*).

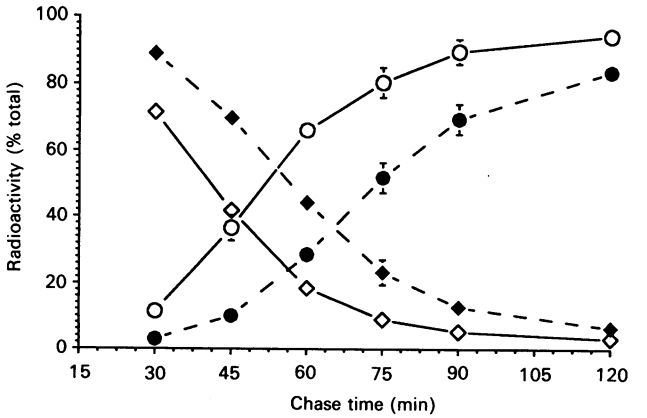

Fig. 2. Percentage of proinsulin-I and -II-related radioactivity in the form of unprocessed proinsulin and fully processed insulin

◇, Proinsulin I; ○, insulin I; ◆, proinsulin II; ●, insulin II. The data are presented as the means ± S.E.M. for three independent experiments.

In order to quantify the percentage of radioactivity in all products of interest, portions of islets extracted after the various chase times were analysed using the TEAP system, either with or without prior oxidation, as well as with the PPH system, thus allowing for the unambiguous identification of all products. It remained possible, however, than an apparently symmetrical peak presumed to contain only one product may in fact mask the presence of a contaminant (either related or not to proinsulin). To exclude this possilibity, the ratio of $^{35}S/^{3}H$ radioactivity was calculated for insulin II and for des 64,65-(split proinsulin II) (the two products best resolved by TEAP from their insulin I gene product counterparts). Taking the number of leucine residues in proinsulin and insulin (11 and 6 respectively) into account, it was found that the ratios for the two products matched perfectly. These ratios were then applied to products not well separated by h.p.l.c. in order to estimate the relative amounts of insulin I and II gene products. In no case was there evidence for significant contamination by non-proinsulin-related labelled material.

Percentages of radioactivity in proinsulin, conversion intermediates and insulin during the chase

After the 10 min pulse label at 16.7 mM-glucose (to maximize the incorporation of labelled amino acids), the islets were incubated for a 120 min chase period at 1.67 mM-glucose. This low concentration of glucose limited the release of labelled products during the chase. Thus only 2.1% and 2.5% of proinsulin-I or -II-related radioactivity respectively was released during the entire 120 min chase period. The relative proportions of proinsulin, intermediates and insulin in the secreted material essentially reflected the proportions encountered in the islet extracts (results not shown). Only radioactive products retained within the islets have therefore been considered for the purposes of this study. The data for [^{3}H]insulin I have been corrected for the loss of leucine residues associated with the conversion of proinsulin into insulin. Fig. 2 shows the percentages of proinsulin-I- and -II-related radioactivity in the form of unprocessed proinsulin or fully processed insulin. At 40 min, approx. 50% of proinsulin I was already processed, whereas 50% of proinsulin II was processed after 56 min. A similar difference was observed for the two insulins, with 50% of insulin I appearing after 52 min; for insulin II this time was 73 min. The times elapsed between the moment at which 50% of proinsulin I or II was processed and the moment at which 50% of insulin I or II appeared was 11 min

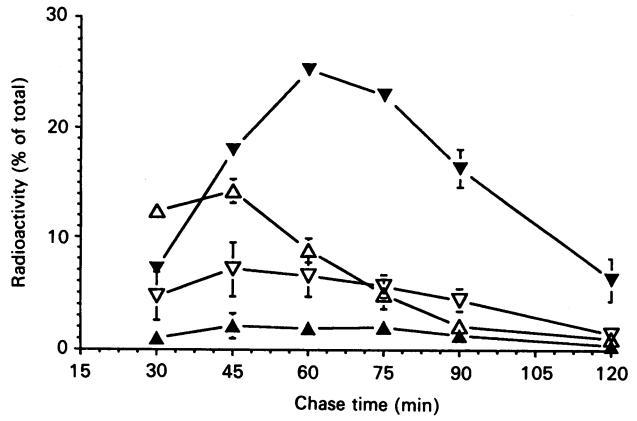

Fig. 3. Percentage of radioactivity related to proinsulins I and II recovered as conversion intermediates during chase

△, Des-31,32-(split proinsulin I); ▲, des-31,32-(split proinsulin II); ▽, des-64,65-(split proinsulin I); ▼, des-64,65-(split proinsulin II). The data are presented as the means ± S.E.M. for three independent experiments.

for proinsulin I/insulin I and 18 min for proinsulin II/insulin II. These data confirm previous studies showing that labelled insulin I is generated more rapidly than insulin II during a chase incubation [15,18]. Careful inspection of the curves presented in Fig. 2 further shows that the conversion of proinsulins I and II displays non-parallel kinetics, and that for both proinsulin I and proinsulin II the rate of disappearance of proinsulin is not perfectly matched by the appearance of the corresponding insulin. This discrepancy is accounted for by accumulation of radioactivity in the form of proinsulin conversion intermediates (Fig. 3). For proinsulin-I-related radioactivity, des-31,32-(split proinsulin) is the major intermediate, reaching a maximum of $14.3 \pm 1.1\%$ at 45 min and declining thereafter. The combined radioactivity in the two proinsulin I conversion intermediates never exceeded 21.4% (at 45 min). The pattern was strikingly different from proinsulin II. Thus des-64,65-(split proinsulin) was the major proinsulin-II-related intermediate throughout the chase, accounting for $25.3 \pm 0.5\%$ of the total proinsulin-II-related radioactivity at 60 min, with significant levels ($6.2 \pm 1.9\%$) even after 120 min of chase. Des-31,32-(split proinsulin II) was, by contrast, only a minor component. The total percentage of proinsulin-II-related radioactivity in the form of a conversion intermediate was higher than that for proinsulin I at all times as from 60 min of chase.

DISCUSSION

The conversion of proinsulin into insulin [2], as for the majority of prohormones [19–21], involves cleavage at pairs of basic residues linking the C-peptide to the insulin A- and B-chains [2], and occurs in secretory granules [1,3]. Two endoproteases appear to be implicated [5]. They have different pH and Ca^{2+} requirements, and it has been suggested that each enzyme has a preference for just one of the two conversion sites [5]. The proinsulin conversion machinery displays an absolute requirement for the naturally occuring pairs of basic residues. If these are replaced by analogues of arginine or lysine [22,23], or by non-basic amino acids by site-directed mutagenesis [24], proinsulin conversion is seriously impaired. Naturally occurring mutations resulting in the substitition of just one of the two basic amino acids at the C-peptide/A-chain junction have further been shown to account for several cases of familial hyperproinsulinaemia [25]. As for other prohormones [26–28], however, the mere presence of the appropriate pair of basic residues exposed to the surface of the proinsulin molecules is not enough in itself to ensure conversion; proinsulin secondary/tertiary structure appears to be an additional feature of the substrate specificity of the conversion endoproteases. Proinsulin conversion is thus completely blocked by deletion of the first four residues only [29] or of a major fragment [24] of the C-peptide, even though both pairs of basic residues were still present in these mutant proinsulin molecules.

Rat proinsulins I and II provide an interesting model for studying the substrate specificity of the conversion endoproteases. The two molecules differ by four residues (two in the B-chain and two in the C-peptide) [10]. Proinsulin I is known to be converted more rapidly into insulin than is proinsulin II [11,18], but no explanation has been provided for this. In the present study, advantage has been taken of the ability to separate the two proinsulins, both conversion intermediates of each proinsulin and the two insulins by reversed-phase h.p.l.c. When all the data are considered together it can be concluded that: (*a*) the initial cleavage of proinsulin II to a conversion intermediate is slower than that for proinsulin I; (*b*) whereas for proinsulin I the preferred site for this initial cleavage event is the B-chain/C-peptide junction [to form des-31,32-(split proinsulin I)], for proinsulin II it is the C-peptide/A-chain junction (to form des-64,65-(split proinsulin II)]; (*c*) the second cycle of cleavage, which produces native insulin, is a rate-limiting event at later times of chase for the processing of des-64,65-(split proinsulin II) to insulin II. The cleavage of proinsulin II at the B-chain/C-peptide junction thus appears to be very slow compared with that of the same site on proinsulin I. Davidson *et al.* [5] have used a similar approach to measure the conversion of rat proinsulin II only. They also found accumulation of des-64,65-(split proinsulin II) at early times of chase. The kinetics of conversion in their study were, however, somewhat different to those presented here, presumably due to differences in the handling of the islets.

Relatively little is known about the tertiary structure of proinsulin. A recent n.m.r. study by Weiss *et al.* [30] reports that the C-peptide in proinsulin is largely unstructured and that the insulin moiety of the prohormone is structurally reminiscent of the insulin monomer itself. They were able to deduce a critical structural domain (referred to as the 'CA knuckle'), which they suggest may be critical for conversion at the C-peptide/A-chain junction. Others have compared the structures of several different prohormones in an attempt to discover consensus structures at sites of conversion [31]. This comparative study suggested that cleavage sites arise typically in regions with high β-turn formation probability, flanked by regions displaying highly ordered tertiary structure. Neither study allows one to predict the precise impact on prohormone conversion of a single amino acid substitution outside of a cleavage site itself.

The C-peptide has been proposed by several authors to be implicated in the presentation of the proinsulin cleavage sites to the conversion endoproteases [24,29,32], although it should be stressed that, in contrast to the present study, none of these earlier studies concerned proinsulin processing within B-cell secretory granules, but rather in transfected non-B-cells [32], in yeast [29] or *in vitro* by semipurified endoproteases [24]. No other physiological role for C-peptide has yet been been convincingly demonstrated. It is quite possible that the two residues in the C-peptide which differ between rat proinsulins I and II could affect presentation of the cleavage sites. Both changes would have serious structural implications (proline for alanine at residue 10 of the C-peptide, and glutamate for glycine at residue 19). Neither of these residues, however, lies in a region of notable interspecies sequence similarity [29]. Furthermore, either or both of these changes would have to be responsible for the rather selective perturbation of cleavage at the B-chain/C-peptide

junction of proinsulin II shown in the present study. Although possible, this is certainly not self-evident.

Of the two amino acid substitutions in the B-chain, it is that at residue 29 which seems to be the most logical candidate for a selective perturbation of cleavage at the neighbouring B-chain/C-peptide junction. Rat (and mouse) proinsulin II are exceptional in presenting a methionine at this site, rather than a basic residue, lysine as found in rat (or mouse) proinsulin I and the majority of other mammalian insulins. The presence of a basic amino acid which is only one residue removed from the pair of basic residues constituting a cleavage site is a typical (albeit certainly not obligatory) motif [20]. Indeed, in both rat proinsulins, the penultimate residue at the *C*-terminus of the C-peptide (i.e. just before the C-peptide/A-chain cleavage site) is basic. It is therefore tempting to speculate that Met^{B29} disturbs the usual presentation of the B-chain/C-peptide junction to the responsible conversion endoprotease, thereby leading to slow conversion at this site and the observed accumulation of des-64,65-(split proinsulin II).

It is clearly impossible to draw firm conclusions concerning substrate specificity of the conversion endoproteases based upon the simple inspection of the primary sequence of the two proinsulins. It does, however, seem valid to suggest that one or more of the four amino acid substitutions which distinguish rat proinsulins I and II are implicated. Alternatively, one must postulate that proinsulins I and II are not packaged into the same granules or that different B-cells synthesize uniquely proinsulin I or II, with proinsulin-I-containing granules (or cells) being more efficient at conversion *per se*. Although our current understanding of how prohormones are introduced into granules [33–35] makes the concept of selective packaging of the two proinsulins into separate granules very unlikely, their co-packaging has yet to be demonstrated experimentally. Similarly, although functional heterogeneity between B-cells in the same islet has been documented [36], there has been no attempt to see whether this heterogeneity extends to the limited expression of one or the other of the insulin genes. It is perhaps relevant to note that the rat insulin II (but not insulin I) gene has recently been shown to be expressed outside the pancreas (yolk sac and liver) during foetal development [37], and that in some transformed pancreatic B-cell lines (RIN cells) only the insulin I gene is effectively expressed [38]. There are thus precedents for cell-specific expression of just one of the two rat insulin genes.

In conclusion, the present study provides evidence for a striking difference in the kinetics of conversion of the two rat proinsulins. It is proposed that one (or more) of the amino acid substitutions which distiguish the two proinsulin molecules is responsible. This must now be confirmed by introducing the proinsulin II substitutions into the proinsulin I gene by site-directed mutagenesis and then comparing the kinetics of conversion of the native and mutant proinsulins following their expression in an appropriate secretory cell.

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