Kinetic analysis of the type-1 proinsulin endopeptidase by a monoclonal antibody-based immunoadsorbent assay

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A simple, rapid and sensitive assay for the type-1 endopeptidase (Arg-Arg cleaving) was developed by using an anti-proinsulin monoclonal immunoadsorbent to separate reaction products from the substrate. The values obtained by this assay were identical with those obtained by an h.p.l.c.-based procedure and yielded similar values for the pH optimum (5.6) and Ca^{2+} activation ($K_{0.5} = 2 \text{ mM}$). It was shown that the type-1 endopeptidase was readily solubilized by Triton X-114 ($87 \pm 3\%$, $n = 12$) and partitioned principally into the aqueous phase at 30°C ($90.1 \pm 2.6\%$, $n = 12$). Activity was lost on gel filtration, but could be restored by adenosine 5'-[γ -thio]triphosphate ($K_{0.5} = 6 \mu\text{M}$), 50 μM -dithiothreitol or 50 μM - Ca^{2+} -*trans*-1,2-diaminocyclohexane-*NNN'*-tetra-acetic acid (CDTA), indicating that the enzyme was particularly sensitive to heavy metal ions. The K_m obtained with proinsulin as substrate ($13 \pm 1.7 \mu\text{M}$) indicated that the enzyme works at close to its $V_{\max}$ in the nascent secretory granule. The $V_{\max}$ of the enzyme prepared from insulin granules (0.6% proinsulin converted/min) corresponded closely to the rate measured *in vivo* in rat islets. The type-1 endopeptidase also appears to be capable of binding to proinsulin in the region of the C-peptide/A-chain junction, since a peptide spanning this region was found to inhibit the ^{125}I -proinsulin processing measured by this assay.

INTRODUCTION

Many polypeptide hormones, neuropeptides and secreted proteins are synthesized initially as larger precursor proteins from which the mature forms are excised by specific endoproteolytic cleavage on the C-terminal side of dibasic amino acid sequences (Docherty & Steiner, 1982; Sossin *et al.*, 1989; Lindberg & Hutton, 1991). Genetic and enzymological data suggest that the rate of initial cleavage is determined, at least in part, by the tissue content of a related family of Ca^{2+} -dependent enzymes which exhibit different specificities for the alternative combinations of lysine and arginine at the S-1, -2 and -4 positions relative to the cleavage site (Davidson *et al.*, 1988; Docherty *et al.*, 1989; Rhodes *et al.*, 1989; Hutton, 1990; Thorne & Thomas, 1990). The pancreatic β -cell contains an endopeptidase (designated type 1) which cleaves exclusively at the B-chain/C-peptide junction of proinsulin (Lys-Thr-Arg-Arg in man), and a second endopeptidase (type 2) which cleaves predominantly at the C-peptide/A-chain junction (Lys-Arg). Both enzymes require Ca^{2+} and an acidic pH for activity (Davidson *et al.*, 1988) and these factors are postulated to be of regulatory importance *in vivo*.

Most of the previous studies on the substrate specificity of these and other putative proprotein endopeptidases have been qualitative in nature, and kinetic studies need to be performed before their functional specificity can be defined and their physiological roles determined. One major difficulty in attaining this objective has been the nature of the assays themselves. These have generally used substrates either identical with or based on the precursor molecule, followed by separation of reaction products by electrophoresis (Loh *et al.*, 1985; Davidson *et al.*, 1987), ion-exchange chromatography (Gluschankof *et al.*, 1984), h.p.l.c. (Plevrakis *et al.*, 1989; Davidson *et al.*, 1988) or h.p.l.c. in combination with radioimmunoassay (Gluschankof *et al.*, 1985). Such assays are not only tedious to perform but difficult to quantify, especially in cases where the precursor contains multiple cleavage sites yielding multiple products. The quantities of the

prohormones needed for kinetic measurements have also posed a considerable problem except in the instances where the sequences have been expressed through recombinant DNA techniques. Alternative approaches based on the use of synthetic peptides, and especially of short peptides linked to fluorogenic reporter molecules (Shennan *et al.*, 1991), although potentially useful, require verification with the native substrate before such assays are interpretable.

The present paper describes the development of a rapid assay with high sample capacity for the type-1 proinsulin endopeptidase. The assay incorporates the desirable features of being site-specific, of using a native precursor as substrate and being easily quantifiable at a wide range of substrate concentrations. It is based on the use of a monoclonal antibody (A6) raised against human proinsulin, to which it binds with high affinity (K_d 4 nM) in the region of the B-chain/C-peptide junction (Sobey *et al.*, 1989). The antibody also binds 65,66-split proinsulin and des-64,65-proinsulin with high affinity, but not insulin, C-peptide, 32,33-split proinsulin or des-31,32-proinsulin. As a consequence, the addition of A6 coupled to a suitable solid phase at the end of an enzyme incubation with ^{125}I -proinsulin results in the binding of the unchanged substrates of the type-1 endopeptidase. Determination of the radioactivity remaining in the supernatant thus provides a direct measurement of the enzyme activity.

EXPERIMENTAL

Materials

Human proinsulin and ^{125}I -proinsulin (monoiodinated at Tyr-14 of the A-chain; sp. radioactivity 233 $\mu\text{Ci}/\mu\text{g}$ of proinsulin) were supplied by Lilly Research Laboratories (Indianapolis, IN, U.S.A.). The human B-chain/C-peptide junction peptide (residues 26–37, YTPKTRREAEDL) was a gift from Dr. Ole Madsen, Hagedorn Research Laboratory, DK-2820, Gentofte, Denmark. The rat C-peptide/A-chain junction peptide analogue, containing an added N-terminal tyrosine (Tyr-residues 58–70,

Abbreviations used: ATP[S], adenosine 5'-[γ -thio]triphosphate; ADP[S], adenosine 5'-[β -thio]diphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; PMSF, phenylmethanesulphonyl fluoride; CDTA, *trans*-1,2-diaminocyclohexane-*NNN'*-tetra-acetic acid.

YEVARQKRGIVDQ) and the human proinsulin B-chain/C-peptide junction peptide analogue containing a D-arginine [residues 26–37, YTPKTR(D-Arg)EAEDL] were supplied by Multiple Peptide Systems (San Diego, CA, U.S.A.). Adenosine 5'-[γ -thio]triphosphate (ATP[S]), adenosine 5'-[β -thio]diphosphate (ADP[S]) and guanosine 5'-[γ -thio]triphosphate (GTP[S]) were obtained from Boehringer-Mannheim, U.K. Ultrogel AcA34 was obtained from IBF Biotechnics, Villeneuve-la-Garenne, France.

Preparation of solubilized endopeptidases

Insulin secretory granules were prepared from a transplantable rat insulinoma by Nycodenz gradient centrifugation, and stored under liquid nitrogen until required, at 10–20 mg of protein/ml (Hutton *et al.*, 1990). The proinsulin endopeptidases were solubilized by incubating the granules with an equal volume of extraction buffer [50 mM-sodium acetate, pH 5.5, 20 μ M-E64, 20 μ M-pepstatin A, 0.2 mM-Tos-Phe-CH₂Cl (TPCK), 2 mM-phenylmethanesulphonyl fluoride (PMSF)] containing either 2% (w/v) Triton X-114 or Triton X-100 at 4 °C for 20 min, followed by centrifugation at 48000 g_{max} for 30 min. The supernatants from the Triton X-114 extractions were warmed to 30 °C for 3 min, and centrifuged at 11000 g for 3 min to separate the detergent-enriched and aqueous phases. In 12 separate extractions with Triton X-114, $87 \pm 3\%$ (S.E.M.) of the enzyme activity was recovered in the supernatant. After phase separation, $90.1 \pm 2.6\%$ (S.E.M.) of the solubilized activity was recovered in the two phases, of which $93 \pm 1\%$ (S.E.M.) was recovered in the aqueous phase.

Chromatographic separations of solubilized granule endopeptidases

Gel filtration. The Triton X-114 aqueous extract (1 ml) was applied to an Ultrogel AcA34 column (0.9 cm $\times$ 54 cm) equilibrated in 50 mM-sodium acetate (pH 5.5)/5 mM-CaCl₂/1 mM-MgCl₂/0.02% Triton X-100. Fractions (1.5 ml) were collected every 10 min and assayed for enzyme activity in the presence and absence of ATP[S]. Peak fractions of enzyme activity were pooled and stored at –70 °C.

Ion-exchange chromatography. Solubilized granules were applied to a DEAE-cellulose column as described in Davidson *et al.* (1988). Fractions were assayed for enzyme activity in the presence and absence of ATP[S].

Assay of enzyme activity

Preparation of A6 immunoadsorbent. Monoclonal antibody A6 IgG was partially purified from ascites fluid by (NH₄)₂SO₄ precipitation (Sobey *et al.*, 1989) and coupled to aminocellulose by diazotization to form a high-capacity immunoadsorbent containing approx. 0.2 mg of protein/mg of cellulose (binding capacity 200 pmol of proinsulin/mg of cellulose) (Bailyes *et al.*, 1987; Sobey *et al.*, 1989). This was stored at 5 mg of cellulose/ml at 4 °C in veronal buffer (50 mM-sodium barbitone/85 mM-NaCl/0.5% BSA/0.1% NaN₃, pH 8) and washed by centrifugation and resuspension immediately before use.

Immunoadsorbent-based assay. Samples were incubated at 30 °C for 1–4 h in micro-centrifuge tubes (1.5 ml capacity) in a total volume of 100 μ l of cocktail containing (final concn.) ¹²⁵I-proinsulin (250000 d.p.m./ml), 50 mM-sodium acetate, pH 5.5, 5 mM-CaCl₂, 1 mM-MgCl₂, 0.1% Triton X-100 plus proteinase inhibitors (10 μ M-E64, 10 μ M-pepstatin A, 0.1 mM-Tos-Phe-CH₂Cl, 1 mM-PMSF) (Davidson *et al.*, 1988). Fractions obtained by ion-exchange chromatography or gel filtration were also assayed in the presence of 250 μ M-ATP[S]. Reactions were terminated by addition of 12.5 μ l of 1 M-Tris/HCl, pH 8. The samples were immunoprecipitated (either immediately or after

storage at –20 °C) by addition of 50 μ l of A6 immunoadsorbent suspension to the reaction products followed by incubation at room temperature, without further mixing, for at least 1 h. The immunoadsorbent was then pelleted by centrifugation at 11000 g for 3 min, and 100 μ l of the supernatant was removed for determination of radioactivity in a Nuclear Enterprises 1600 gamma counter (¹²⁵I counting efficiency approx. 75%). Each determination was corrected for the radioactivity that remained unbound in the absence of enzyme activity (approx. 3–7% of the total radioactivity, depending on the batch and storage time of the ¹²⁵I-labelled proinsulin) and the activity was expressed as the percentage conversion of the initial radioactivity.

The effect of pH on catalysis over the range 5–8 was determined with 50 mM-sodium acetate (pH 5 and 5.5), Mes/NaOH (pH 5.5, 6, 6.5 and 7) and Tes/NaOH (pH 7.5 and 8). Assays that determined the effect of Ca²⁺-concentration on activity contained 2 mM-EDTA and 0.5–7 mM-CaCl₂. Ionized calcium was computed from reference stability constants by using an iterative calculation method (Storer & Cornish-Bowden, 1976).

K_m and V_{max} determinations were performed by addition of unlabelled proinsulin (13.4 nM–42 μ M final concn.) to the cocktail from a stock solution of 25 mM. At higher concentrations of proinsulin (> 0.5 μ M) only a proportion of each total assay volume was incubated with the immunoadsorbent, so that the binding capacity of the immunoadsorbent for proinsulin was not exceeded. To compensate for this, an increased amount of ¹²⁵I-proinsulin was added to these samples. The kinetic constants were determined by a Lineweaver–Burk transformation of the data (Fersht, 1985).

The effect of the truncated proinsulin peptides on ¹²⁵I-proinsulin conversion was determined by addition of the indicated amounts of each peptide to the assay cocktail. The effect of the peptides on the binding of the immunoadsorbent to proinsulin, in the absence of endopeptidase, was determined at each concentration used. Both the B-chain/C-peptide junction peptides competed with proinsulin for binding to the immunoadsorbent, and accordingly only a proportion of each total assay volume was incubated with the immunoadsorbent, so that the binding capacity of the immunoadsorbent was not exceeded. The C-peptide/A-chain junction peptide did not affect the binding of ¹²⁵I-proinsulin.

H.p.l.c. analysis of reaction products. The ¹²⁵I-labelled proinsulin conversion products generated under the standard incubation conditions were analysed by reversed-phase h.p.l.c. as described in Davidson *et al.* (1988).

Statistical analysis

All straight-line graph plots were calculated by least-squares linear regression by using the Apple Macintosh Cricket Graph program.

Other assays

Protein was measured by the method of Lowry *et al.* (1951), with BSA as standard. Rat insulin was measured as described in Guest *et al.* (1989).

RESULTS

Characterization of the proinsulin proteolysis measured by the immunoadsorbent

The principle of the immunoadsorbent assay that was developed relies on the ability of the monoclonal antibody A6 to deplete the reaction mixture of the initial substrate, ¹²⁵I-proinsulin, together with any products of the cleavage of proinsulin which leave the B-chain/C-peptide junction intact.

Fig. 1. Characterization of proinsulin processing by using the immunoadsorbent assay

(a) and (b) Granule endopeptidases were solubilized with Triton X-100 and the supernatant was incubated with assay cocktail. Percentage conversion of proinsulin was determined by the immunoadsorbent assay, as described in the Experimental section. (a) 15 μ l of supernatant was incubated for the times indicated. (b) 5–20 μ l of supernatant was incubated for 150 min. (c) A Triton X-114 aqueous extract of insulin granules was assayed for endopeptidase activity over the pH range 5–8 as described in the Experimental section. (d) Type-1 endopeptidase activity (free of type-2 endopeptidase activity) was prepared by ion-exchange chromatography (Davidson *et al.*, 1988) and used to determine the Ca^{2+} requirements of the enzyme. The results shown in (a) and (b) are means $\pm$ S.E.M. of three determinations and the results shown in (c) and (d) are means and ranges of two determinations.

The supernatant would be expected to contain split 32,33-proinsulin (the immediate product of type-1 activity), des-31,32-proinsulin (produced by type-1 enzyme in the presence of endogenous carboxypeptidase H), diarginyl-insulin (produced by type-1 and -2 cleavage in concert) and insulin (the product of type-1 and -2 enzymes in concert with carboxypeptidase H). The other expected product, C-peptide, is unlabelled. The radioactivity remaining in the supernatant is therefore a measure of the extent of cleavage at the Arg-Arg sequence, independently of any cleavage at the Lys-Arg site.

Under the standard assay conditions, with a soluble extract of insulin granules, the percentage conversion of proinsulin increased linearly with the time of the enzyme incubation (Fig. 1a) and with the amount of enzyme added (Fig. 1b). The optimum temperature for the reaction was 30 $^{\circ}\text{C}$. At 37 $^{\circ}\text{C}$ there was a time-dependent loss of activity, presumably due to denaturation or inactivation of the enzyme (Fig. 2a). The reaction exhibited a sharp acidic pH optimum of 5.6 (Fig. 1c) and a requirement for millimolar concentrations of Ca^{2+} , with a $K_{0.5}$ of 2 mM (Fig. 1d). The last two features are characteristic of the insulin granule type-1 endopeptidase which cleaves exclusively at the B-chain/C-peptide junction of proinsulin (Davidson *et al.*, 1988).

Further evidence for the specificity of the assay for the type-1 endopeptidase was obtained by comparing the results of the immunoadsorbent-based assay of the reaction products with those obtained by the h.p.l.c.-based assay. Analysis of the proinsulin conversion reaction products by h.p.l.c. revealed a profile of reaction products that is typical of the combined activities of the type-1 and the type-2 endopeptidase in the presence of carboxypeptidase H (Fig. 3a). In total, 58 samples, containing various proportions of the type-1 and type-2 endopeptidases, were analysed by both h.p.l.c. and the immunoadsorbent method. The results derived from the immunoadsorbent method showed a high positive correlation ($r = 0.947$) with cleavage at the B-chain/C-peptide junction (sum of des-

31,32-proinsulin plus insulin radioactivity), as measured by h.p.l.c. (Fig. 3b). The line intersected the origin at zero and had a slope of 0.88. In contrast, there was no significant correlation ($r = 0.663$) with cleavage at the C-peptide/A-chain junction, as measured by h.p.l.c. (sum of des-64,65-proinsulin plus insulin radioactivity) (Fig. 3c).

During the course of initial experiments investigating the effect of nucleotides on proinsulin conversion, an effect of ATP[S] on the enzyme activity was observed. This was particularly striking in the case of enzyme preparations that had been subjected to gel filtration, where there was an 85–95% ($n = 4$) loss of activity which was completely restored by the addition of 30 μM -ATP[S] (Fig. 2b). A similar re-activation was achieved with two other thionucleotides (ADP[S] and GTP[S]) (results not shown). ATP[S] has been shown to react with the thiol reagent 2,4-dinitrophenylglutathionyl disulphide, and is thus capable of acting as a reducing agent (Stournaras *et al.*, 1988). Moreover, dithiothreitol (25–100 μM) restored enzyme activity to 92–93% of that obtained in the presence of ATP[S]. Higher concentrations of dithiothreitol did not increase the enzyme activity further. The heavy-metal chelator *trans*-1,2-diaminocyclohexane-*NNN'*-tetra-acetic acid (CDTA), at 50 μM in the presence of 5 mM- Ca^{2+} , restored enzyme activity to 40–70% of that obtained by ATP[S]. Both CDTA and dithiothreitol had little or no effect on the endopeptidase before chromatography, and ATP[S] produced a small increase in activity of $22 \pm 2.6\%$ (S.E.M., $n = 14$). Re-activation of the type-1 endopeptidase by the thionucleotides, dithiothreitol and CDTA suggests that the decrease in activity may be due to oxidative damage to the enzyme, catalysed by trace contamination with heavy metals.

Kinetic analysis of the type-1 endopeptidase

K_m determinations. The variation of reaction rate with proinsulin concentration was determined in four separate experiments, two using the Triton X-114 aqueous extract as the enzyme

Fig. 2. Effect of temperature and ATP[S] on proinsulin processing determined by the immunoadsorbent assay

(a) The Triton X-114 aqueous extract of insulin granules was incubated in assay cocktail for 1–3 h at 30 °C (■) and 37 °C (□) in the presence of 950 μM-ATP[S]. The results shown are means and ranges of two determinations. (b) Samples of endopeptidase activity, recovered after gel filtration as described in the Experimental section, were incubated in assay cocktail for 2.5 h in the presence of 0–750 μM-ATP[S]. The results shown are means $\pm$ S.E.M. ($n = 3$).

source and two using type-1 endopeptidase separated from the type-2 endopeptidase by ion-exchange chromatography after Triton X-100 extraction (Davidson *et al.*, 1988). A plot of substrate concentration against velocity (not shown) demonstrated classic Michaelis–Menten kinetics with the velocity being directly proportional to substrate concentration in the range 13.4–340 nM, and becoming increasingly non-linear at the higher substrate concentrations (1–42 μM). Lineweaver–Burk transformation of the data obtained in two separate experiments yielded K_m values of 10.3 μM (shown in Fig. 4a) and 13.2 μM. The same analysis of the type-1 endopeptidase activity after its chromatographic separation from the type-2 endopeptidase gave similar K_m values of 17.7 μM (shown in Fig. 4b) and 10.6 μM. These results are consistent with the conclusions drawn from the Ca^{2+} and pH-optima data, that the type-1 enzyme was the predominant activity measured by the immunoadsorbent-based assay.

$V_{\max}$ determinations. The $V_{\max}$ of the enzyme in the granule homogenate was calculated from the $V_{\max}$ value of the Triton X-114 aqueous extract (Fig. 4a). Given a recovery of 70% of the enzyme in the extract relative to the granules, the $V_{\max}$ of the activity in the granules was 177 pmol/min per mg of protein. Assuming that the proinsulin content of the nascent granules was equivalent to the insulin content of the isolated mature granules

Fig. 3. Comparison of proinsulin processing measured by h.p.l.c. with the immunoadsorbent assay

Samples containing type-1 and type-2 endopeptidase activities were assayed by h.p.l.c. and the immunoadsorbent assay. (a) Typical h.p.l.c. separation of proinsulin conversion assay products: insulin (A), des-31,32-proinsulin (B), des-64,65-proinsulin (C) and proinsulin (D). (b) and (c) Percentage conversion of proinsulin obtained by the immunoadsorbent assay was plotted against percentage cleavage at the B-chain/C-peptide junction of proinsulin (b) and the C-peptide/A-chain junction (c) as determined by h.p.l.c.

(29 nmol of insulin/mg of protein in this preparation), this $V_{\max}$ value corresponds to a conversion rate at the B-chain/C-peptide junction *in vivo* of 6 pmol/min per nmol of proinsulin or 0.6% of the initial proinsulin/min.

Effect of truncated proinsulin peptides on proinsulin conversion

The immunoadsorbent assay provided a simple means of examining truncated peptides as potential substrates or inhibitors of the enzyme. A peptide corresponding to the B-chain/C-peptide junction of human proinsulin (YTPKTRREAEDL) and a peptide analogue of the rat C-peptide/A-chain junction (YEVARQKRGIVDQ) inhibited the conversion of ^{125}I -proinsulin by a solubilized granule extract. The C-peptide/A-chain junction peptide produced 50% inhibition at 6 μM and the

Fig. 4. Kinetic analysis of type-1 endopeptidase

(a) The Triton X-114 aqueous extract of insulin granules (5 μ l) was incubated with 1.25–40 μ M-proinsulin for 70 min as described in the Experimental section. The values shown are duplicate determinations. (b) Samples (20 μ l) of type-1 endopeptidase activity, free of type-2 endopeptidase activity (see Fig. 1 legend) were incubated with 1–40 μ M-proinsulin for 120 min. The results shown are means $\pm$ S.E.M. ($n = 4$). A Lineweaver-Burk reciprocal plot of the data was used to determine the K_m and V_{max} of the endopeptidase in both (a) and (b).

B-chain/C-peptide junction peptide produced 50 % inhibition at 53 μ M (Fig. 5). The human proinsulin B-chain/C-peptide junction peptide modified to contain a D-Arg residue [YTPKTR(D-Arg)EAEDL] had only a slight effect on conversion (Fig. 5).

DISCUSSION

Features of the immunoabsorbent-based assay

The immunoabsorbent assay developed in this study provided a rapid and quantitative measurement of the endopeptidase activity directed towards the B-chain/C-peptide junction of proinsulin. In solubilized extracts of insulin granules it measured principally the activity of the type-1 enzyme since the type-2 enzyme shows little activity towards this site in the molecule. Accordingly the pH profile and Ca $^{2+}$ -dependence of the activity recorded by using the assay were indistinguishable from those of the type-1 enzyme determined by an electrophoresis-based assay after its chromatographic separation from the type-2 activity (Davidson *et al.*, 1988). In contrast with analyses by h.p.l.c. or alkaline-urea gel electrophoresis, autoradiography and densitometry, which take several days, an assay of around 60 samples by the immunoabsorbent technique can be completed within 2 h of

Fig. 5. Effect of truncated proinsulin peptides on proinsulin processing

Proinsulin processing by a Triton X-114 aqueous extract of insulin granules was determined as described in the Experimental section, in the presence of the following truncated proinsulin peptides (0–50 μ M): $\square$, human proinsulin residues 26–37, YTPKTRREAEDL; $\blacksquare$, rat Tyr-(proinsulin residues 58–70), YEVARQKRGIVDQ; $\bullet$, human proinsulin residues 26–37, YTPKTR(D-Arg)EAEDL. The values shown are means and ranges of two determinations.

the termination of the incubation of enzyme with substrate. Enzyme activity of as little as 1 % conversion of substrate/h (approx. 50 amol/h) can be readily and reproducibly measured. Such low levels are difficult to determine accurately by electrophoretic or chromatographic separation of the proinsulin-conversion reaction products.

A number of factors are important in the practical application of the assay. Proinsulin is used at tracer concentrations (approx. 50 pM) and so the reaction rate is effectively first order. Therefore the extent of substrate conversion by the end of the enzyme incubation should be limited to less than 25 % to provide acceptable assay linearity. The presence of endogenous prohormone in samples for assays constitutes a potential source of interference if it reaches concentrations of 1 μ M or more. This was not a factor in the present study, since detergent solubilization of the enzyme activity was achieved at a pH value where the prohormone has low solubility. Since the assay depends on the loss of an epitope in the region of the B-chain/C-peptide junction, it is likely that cleavage of proinsulin at sites in this region other than at the correct site (between Arg-32 and Glu-33) would also produce a signal in the assay. This applies particularly to cleavage within the dodecapeptide representing residues 26–37 of human proinsulin, which includes the linear epitope recognized by the antibody (see the Experimental section). There also remains the possibility that even a cleavage outside this region could produce an anomalously folded intermediate which would not be recognized by the antibody. It is therefore essential that the assay is validated by initial characterization of the products of the reaction by h.p.l.c. Non-specific proteolysis was avoided in the present study by inclusion of proteinase inhibitors in the assay cocktail. Similarly, deterioration of the radiolabel with time could result in the formation of damaged proinsulin that was still antigenic and a good enzyme substrate, but which would migrate anomalously on h.p.l.c. The tendency of the A6 immunoabsorbent assay to produce rates some 12 % higher than those obtained by h.p.l.c. assay could be related to such a phenomenon.

The approach used in the present study should be generally

applicable to the assay of other proprotein-processing enzymes and site-specific proteinases, provided that cleavage-site-specific monoclonal antibodies are available. The monoclonal antibody used in this assay was produced by immunization with proinsulin, followed by selecting clones which recognized proinsulin but not insulin (Sobey *et al.*, 1989). Production of anti-peptide antibodies to sequences spanning the cleavage site would appear to be a good alternative approach, since polyclonal antibodies which distinguish between proinsulin and des-64,65-proinsulin have been raised by immunizing with haptenized C-peptide/A-chain junction peptide (Steiner *et al.*, 1987).

Kinetic properties of the type-1 endopeptidase

The derivation of kinetic constants for the type-1 endopeptidase using the immunoadsorbent-based assay provides a number of insights into proinsulin conversion *in vivo*. The K_m of the endopeptidase (13 μM) is three orders of magnitude lower than the estimated initial content of proinsulin in the nascent secretory granule (Hutton *et al.*, 1982). The type-1 endopeptidase should therefore be working close to its V_{max} even at the point where proinsulin conversion reaches 97–98% completion. This would account for the essentially linear kinetics of B-chain/C-peptide junction cleavage observed in pulse-chase experiments in isolated rat islets 20–120 min after synthesis (Davidson *et al.*, 1988). Moreover, the rate observed *in vivo* of 0.64% cleavage/min closely approximates to the estimated V_{max} of the type-1 endopeptidase in the secretory granule (0.6% at 30 °C), which suggests that the intragranular content of the processing enzyme is a rate-limiting factor in the overall prohormone conversion process. It furthermore indicates that there is no need to postulate factors beyond the already known ones of Ca^{2+} and pH in the intracellular activation of the type-1 endopeptidase.

The K_m of the type-1 endopeptidase is similar to the K_m of purified carboxypeptidase H, the enzyme that removes the C-terminal basic amino acids generated by endopeptidase action (17.5 μM with dansyl-Phe-Leu-Arg: Davidson & Hutton, 1987). The activity of carboxypeptidase H (Docherty & Hutton, 1983) in insulin granules (V_{max} of 12.6 nmol of hippuryl-L-arginine hydrolysed/min per nmol of insulin) is, however, some 2000-fold higher than the activity of the type-1 endopeptidase, a finding that is consistent with the observation that the circulating forms of the intermediates in the conversion of proinsulin into insulin are the des-dibasic rather than the split forms (Given *et al.*, 1985).

Studies on the bivalent-cation dependence and inhibition profile of the proinsulin convertases have suggested that they are related to a eukaryotic class of subtilisin-like proteins, which includes the yeast KEX-2 gene product, and, in mammals, furin, PC1(3) and PC2 (Fuller *et al.*, 1989; Smeekens & Steiner, 1990; Seidah *et al.*, 1990; Van den Ouweland *et al.*, 1990; Smeekens *et al.*, 1991). Experiments in which these gene products have been co-transfected with prohormone-encoding sequences indicate that the enzymes have proteolytic activity directed at sequences containing multiple basic amino acids (Bresnahan *et al.*, 1990; Van de Ven *et al.*, 1990; Hosaka *et al.*, 1991; Korner *et al.*, 1991; Thomas *et al.*, 1991). In addition, PC2 expressed in *Xenopus* oocytes has been shown to have activity *in vitro* towards small fluorogenic peptides containing Arg-Arg or Lys-Arg sequences (Shennan *et al.*, 1991). It is difficult, however, to draw firm conclusions regarding the substrate specificity *in vivo* under these conditions, particularly as the enzymes are expressed in near-stoichiometric amounts with their substrates. This problem is illustrated by the finding that differences in the inhibitory effects of tripeptide sulphonium salts bearing Lys-Arg or Arg-Arg sequences on the type-1 and -2 proinsulin endopeptidases relate to differences in the affinities of the enzymes for different dibasic

sequences rather than an absolute difference in substrate recognition (Rhodes *et al.*, 1989). The finding that the rat proinsulin C-peptide/A-chain junction peptide (Lys-Arg sequence) is an effective inhibitor of processing at the B-chain/C-peptide junction (Arg-Arg sequence) shows that the type-1 endopeptidase has a significant affinity for this site, even though it does not cleave at the C-peptide/A-chain junction in proinsulin itself. An additional feature of the rat proinsulin C-peptide/A-chain junction peptide which may favour it acting as a competitive inhibitor of processing at the B-chain/C-peptide junction is the basic amino acid (arginine) at the S-4 position, which in the human B-chain/C-peptide junction is occupied by lysine. The difference in inhibitor capability between the two peptides may be explained by assuming that the B-chain/C-peptide junction peptide is acting as an alternative substrate whereas the C-peptide/A-chain junction peptide is acting as an inhibitor.

Future attempts to characterize the prohormone-processing enzymes should be facilitated by using enzyme assays of the type described in this paper, especially as the assays can be extended to situations where the enzyme in question is expressed at low levels in cultured cells. The rapid assay technique should also facilitate evaluation of synthetic oligopeptides as substrates or inhibitors, and aid the search for natural inhibitors of this group of enzymes.

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REFERENCES

- Bailyes, E. M., Richardson, P. J. & Luzio, J. P. (1987) in *Biological Membranes, A Practical Approach* (Findlay, J. B. C. & Evans, W. H., eds.), pp. 73–101, IRL Press, Oxford and Washington DC
- Bresnahan, P. A., Leduc, R., Thomas, L., Thorner, J., Gibson, H. L., Brake, A. J., Barr, P. J. & Thomas, G. (1990) *J. Cell Biol.* **111**, 2851–2859
- Davidson, H. W. & Hutton, J. C. (1987) *Biochem. J.* **245**, 575–582
- Davidson, H. W., Peshavaria, M. & Hutton, J. C. (1987) *Biochem. J.* **246**, 279–286
- Davidson, H. W., Rhodes, C. J. & Hutton, J. C. (1988) *Nature (London)* **333**, 93–96
- Docherty, K. & Hutton, J. C. (1983) *FEBS Lett.* **162**, 137–141
- Docherty, K. & Steiner, D. F. (1982) *Annu. Rev. Physiol.* **44**, 625–638
- Docherty, K., Rhodes, C. J., Shennan, K. I. J., Taylor, N. A. & Hutton, J. C. (1989) *J. Biol. Chem.* **264**, 18335–18339
- Fersht, A. (1985) *Enzyme Structure and Mechanism*, pp. 98–120, Freeman and Co., New York
- Fuller, R. S., Brake, A. & Thorner, J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1434–1438
- Given, B. D., Cohen, R. M., Shoelson, S. E., Frank, B. H., Rubenstein, A. H. & Tager, H. S. (1985) *J. Clin. Invest.* **76**, 1398–1405
- Gluschkoff, P., Morel, A., Gomez, G., Nicolas, P., Fahy, C. & Cohen, P. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6662–6666
- Gluschkoff, P., Morel, A., Benoit, R. & Cohen, P. (1985) *Biochem. Biophys. Res. Commun.* **128**, 1051–1057
- Guest, P. C., Lowing, C., Arden, S. D., Gray, I. P. & Hutton, J. C. (1989) *Mol. Cell. Endocrinol.* **67**, 173–178
- Hosaka, M., Nagahama, M., Kim, S., Watanabe, T., Hatsuzawa, K., Ikemizu, J., Murakami, K. & Nakayama, K. (1991) *J. Biol. Chem.* **266**, 12127–12130
- Hutton, J. C. (1990) *Curr. Opin. Cell Biol.* **2**, 1131–1142
- Hutton, J. C., Penn, E. J. & Peshavaria, M. (1982) *Diabetologia* **23**, 365–373
- Hutton, J. C., Bailyes, E. M., Rhodes, C. J. & Guest, P. C. (1990) in *Peptide Hormone Secretion, A Practical Approach* (Hutton, J. C. & Siddle, K., eds.), pp. 309–336, IRL Press, Oxford and Washington DC

- Korner, J., Chun, J., Harter, D. & Axel, R. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6834–6838
- Lindberg, I. & Hutton, J. C. (1991) in *Peptide Biosynthesis and Processing* (Fricker, L. D., ed.), pp. 141–173, CRC Press, Boston and London
- Loh, Y. P., Parish, D. C. & Tuteja, R. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **260**, 7194–7205
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Plevrakis, I., Clamagirand, C., Creminon, C., Brakch, N., Rholam, M. & Cohen, P. (1989) *Biochemistry* **28**, 2705–2710
- Rhodes, C. J., Bailyes, E. M., Zumbunn, A., Shaw, E. & Hutton, J. C. (1989) *Biochem. J.* **258**, 305–308
- Seidah, N. G., Gaspar, L., Mion, P., Marcinkiewicz, M., Mbikay, M. & Chretien, M. (1990) *DNA Cell Biol.* **9**, 415–424
- Shennan, K. I. J., Smeekens, S. P., Steiner, D. F. & Docherty, K. (1991) *FEBS Lett.* **284**, 277–280
- Smeekens, S. & Steiner, D. F. (1990) *J. Biol. Chem.* **265**, 2997–3000
- Smeekens, S., Avruch, A. S., La Mendola, J., Chan, S. J. & Steiner, D. F. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 340–344
- Sobey, W. J., Beer, S. F., Carrington, C. A., Clark, P. M. S., Frank, B. H., Gray, I. P., Luzio, S. D., Owens, D. R., Schneider, A. E., Siddle, K., Temple, R. C. & Hales, C. N. (1989) *Biochem. J.* **260**, 535–541
- Sossin, W. S., Fisher, J. M. & Scheller, R. H. (1989) *Neuron* **2**, 1407–1417
- Steiner, D. F., Michael, J., Houghten, R., Mathieu, M., Gardner, P. R., Ravazzola, M. & Orci, L. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6184–6188
- Storer, A. C. & Cornish-Bowden, A. (1976) *Biochem. J.* **159**, 1–5
- Stournaras, C., Merkler, I. & Faulstich, H. (1988) *Biochem. Biophys. Res. Commun.* **155**, 962–970
- Thomas, L., Leduc, R., Thorne, B. A., Smeekens, S. P., Steiner, D. F. & Thomas, G. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5297–5301
- Thorne, B. A. & Thomas, G. (1990) *J. Biol. Chem.* **265**, 8436–8443
- Van den Ouweland, A. M. V., Duijnhoven, H. L. P., Keizer, G. D., Dorsser, L. C. J. & Van De Ven, W. J. M. (1990) *Nucleic Acids Res.* **18**, 664
- Van de Ven, W. J. M., Voorberg, J., Fontijn, R., Pannekoek, H., Ans, M. W., Van den Ouweland, A. M. V., Duijnhoven, H. L. P., Roebroek, A. J. M. & Seizen, R. J. (1990) *Mol. Biol. Rep.* **14**, 265–275

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