Human insulin production from a novel mini-proinsulin which has high receptor-binding activity

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To increase the folding efficiency of the insulin precursor and the production yield of insulin, we have designed a mini-proinsulin (M2PI) having the central C-peptide region replaced with a sequence forming a reverse turn. The mini-proinsulin was fused at the N-terminus to a 21-residue fusion partner containing a His₁₀ tag for affinity purification. The gene for the fusion protein was inserted downstream of the T7 promoter of the expression plasmid pET-3a, and the fusion proteins were produced as inclusion bodies in the *Escherichia coli* cytoplasm at levels up to 25% of the total cell protein. The protein was sulphonated, cleaved by CNBr and the M2PI mini-proinsulin was purified

using ion-exchange chromatography. The refolding yield of M2PI was 20–40% better than that of proinsulin studied at the same molar concentrations, indicating that the short turn-forming sequence is more effective in the refolding process than the much longer C-peptide. Native human insulin was successfully generated by subsequent enzymic conversion of mini-proinsulin. The mini-proinsulin exhibited high receptor-binding activity, about 50% as potent as insulin, suggesting that this single-chained mini-proinsulin may provide a foundation in understanding the receptor-bound structure of insulin as well as the role of C-peptide in the folding and activity of proinsulin.

INTRODUCTION

Insulin is a polypeptide hormone secreted by the β -cells of the pancreas and consists of two polypeptide chains, A and B, which are linked by two inter-chain and one intra-chain disulphide bridge. The hormone is synthesized as a single-chain precursor, proinsulin, and produced by the proteolytic processing of proinsulin in the pancreas.

Three major methods have been used for the production of human insulin in micro-organisms, two of which involve *Escherichia coli*, with either the expression of a large fusion protein in the cytoplasm [1,2] or the use of a signal peptide to enable secretion into the periplasmic space [3]. A third method utilizes *Saccharomyces cerevisiae* to secrete the insulin precursor into the medium [4].

One typical scheme for preparing human insulin utilized proinsulin that was produced in *E. coli* cytoplasm as an inclusion body of a fusion protein. After chemical cleavage of the fusion protein and sulphonation of the released proinsulin, the refolding reaction of S-sulphonated proinsulin was performed with relatively high yield [2]. The same procedure has been described in more detail in a recent report [5].

At present, the role of the C-peptide in the folding of proinsulin is not clearly understood. The length of the C-peptide varies between 26 and 38 residues in various animal species and shows more sequence variation than the insulin moiety [6]. The dibasic amino acid residues at the B-chain–C-peptide (B–C) and C-peptide–A-chain (C–A) junctions are conserved and considered to be a minimum requirement for conversion into insulin. The three-dimensional structure of insulin indicates that the A- and B- chains could be joined with a peptide linker much smaller than the 31-residue C-peptide. Whether shortening of the C-peptide

would still allow for efficient formation of native disulphide bridges is unclear.

In the present study, the 31-residue C-peptide was replaced with a short turn-forming pentapeptide sequence to facilitate the refolding process. The designed mini-proinsulin (see Figure 1 for the sequence) was fused at the N-terminus to a 21-residue fusion

A

FVNQHLGSHLVEALYLVCGERGFFYTPKTRR[YPGDV]KRGIVEQCCTSIICSLYQLENYCN

B

Figure 1 Amino acid sequence (A) and modelled structure (B) of M2PI mini-proinsulin

The single-letter code for amino acids is used in (A). The turn-forming pentapeptide residues between the two dibasic sites are boxed. (B) The modelled structure of M2PI mini-proinsulin (right) and insulin structure (left).

partner containing a His₁₀ tag for Ni²⁺-chelated affinity purification. The mini-proinsulin showed enhanced refolding yield, and human insulin was efficiently liberated from the mini-proinsulin by enzyme treatment. The high proportion of insulin moiety in the fusion protein and the increased refolding capacity of the mini-proinsulin suggest that this mini-proinsulin could be used as an intermediate in the production of recombinant human insulin in *E. coli*.

MATERIALS AND METHODS

Materials

The expression vector pET-3a and *E. coli* host strains were obtained from Novagen. Restriction enzymes, proteases and CNBr were purchased from Sigma. Human insulin, with an activity of 28 units per mg of protein, was also purchased from Sigma. ¹²⁵I-Labelled insulin (A-14) was obtained from Amersham.

Molecular modelling of mini-proinsulin

The 9-residue sequence comprising an Arg-Arg, the mini-sequence and a Lys-Arg, was inserted into the structure of human insulin (protein databank file 1hiu) and was fully optimized by energy minimization and molecular dynamics using the QUANTA/CHARMm program (Molecular Simulations Inc.) with 8 Å spherically hydrated water solvents. Thereafter, the whole mini-proinsulin was fully energy minimized and was further modelled by 50 ps molecular dynamics simulations. The root-mean-square deviation of the backbone atoms between the structures of 1hiu and of modelled mini-proinsulin was 1.21 Å.

Bacterial strains and plasmids

The *E. coli* host strain used in this study was BL21 (DE3) (*F[−]ompT rB[−]mB[−]*) whose chromosome carries the T7 RNA polymerase gene under the control of the lacUV5 promoter. A DNA fragment encoding amino acids 8–12 of tumour necrosis factor-α (TNF-α) and a His₁₀ tag were synthesized chemically. After digestion with restriction endonucleases *Nde*I and *Bam*HI, the DNA fragment was inserted downstream of the T7 promoter of the expression plasmid pET-3a [7], which was linearized with the same restriction endonucleases, and the resulting plasmid was named pT2. The gene for human proinsulin was prepared by PCR using five overlapping DNA fragments, each 65–68 bases in length, as template. These DNA fragments were synthesized by an Applied Biosystems Inc. DNA synthesizer (model 392). The gene encoding the human proinsulin was digested with *Bam*HI and *Hind*III and inserted into the *Bam*HI and *Hind*III enzyme restriction sites of plasmid pT2. The resulting plasmid was named pT2-hPI. The gene encoding mini-proinsulin (M2PI) was prepared by PCR using the human proinsulin gene as template DNA. The gene for M2PI was digested with *Bam*HI and *Hind*III and inserted into plasmid pT2. The resulting plasmid was named pT2M2PI (Figure 2).

Purification of mini-proinsulin

Cultured *E. coli* cells were collected by low-speed centrifugation, and the cell pellet was resuspended in 20 mM Tris/HCl, pH 8.0/1 mM EDTA/1 mM PMSF. After cell disruption by sonication, the cell lysate was centrifuged at 6000 *g* for 10 min. The pellet was solubilized in 50 mM Tris/HCl, pH 9.0/6 M guanidine-HCl at a concentration of 10 mg/ml. Oxidative sulphytolysis was performed by adding sodium sulphite and sodium tetrathionate to final concentrations of 300 mM and

Figure 2 Diagram representing the mini-proinsulin expression plasmid

The 21-residue T2 fusion partner comprises amino acids 8–12 of the N-terminus of TNF-α, ten histidine residues and a five residue spacer.

60 mM respectively and incubating for 12 h at room temperature. The proteins were precipitated by adding 2 vol of 0.1 M ZnCl₂ solution and white precipitates were collected by centrifugation for 15 min at 6000 *g*. The precipitates were dissolved in 8 M urea/0.3 M HCl at a concentration of 10 mg/ml, and cleaved by CNBr at room temperature for 16 h in the dark. Proteins were precipitated again by adding 2 vol of 0.5 M ZnCl₂ solution.

Precipitates of CNBr-cleaved product were solubilized in 7 M urea/20 mM formic acid buffer (pH 4.0) and loaded onto an S-Sepharose cation-exchange column (Pharmacia Biotechnology) equilibrated with the same buffer. The column was eluted at a rate of 3 ml/min using a linear gradient of 0–0.5 M NaCl in 7 M urea/20 mM formic acid buffer (pH 4.0) for 50 min. Eluted fractions were analysed by tricine SDS/PAGE and the fractions containing S-sulphonated mini-proinsulin were pooled. Distilled water (2 vol) was added and, after 30 min at room temperature, precipitates were collected by centrifuge at 6000 *g* for 15 min. After washing with the same volume of water again, the protein was lyophilized and stored at −20 °C.

Refolding reactions

Purified S-sulphonated mini-proinsulin was dissolved in 50 mM glycine buffer at a concentration of 50 μM (0.375 mg/ml) and the refolding reaction was started by adding 2 equivalents of 2-mercaptoethanol. After 18 h of gentle agitation at 4 °C, the reaction was stopped by acidification with H₃PO₄ to pH 2.5 and analysed by analytical reverse-phase HPLC. The refolding yields of the reaction products were determined by comparing the peak size of the correctly folded mini-proinsulin in the HPLC profile with the standard.

Fingerprint analysis

Refolded mini-proinsulin was purified by a Vydac C₈ semi-preparative HPLC column with a linear gradient of 20–30 %

CH_3CN in 0.1% trifluoroacetic acid (TFA) for 60 min. The major peak was collected, lyophilized and enzymically processed with trypsin and carboxypeptidase B, as previously reported for proinsulin [8]. The reaction was stopped by addition of HCl to pH 2.5 and an aliquot was injected into a Vydac C_{18} HPLC column. Elution was carried out with a linear gradient of 20–50% CH_3CN in 0.1% TFA for 30 min, with UV monitoring at 215 nm. The major peak was pooled and lyophilized. The correct disulphide connections were verified by fingerprint analysis using Glu-C endoprotease (*Staphylococcus aureus* strain V8 protease) [9]. Insulin (0.2 mg/ml) in 0.2 M Tris/HCl (pH 7.5) was incubated with a 1:10 (w/w) enzyme:substrate ratio at 25 °C for 14 h and eluted on a Vydac C_{18} column with a linear gradient of acetonitrile in TFA as described above. The eluted peptides were collected and analysed by MS and N-terminal peptide sequencing.

Receptor binding assay

An insulin receptor binding assay was performed using IM-9 lymphocytes as described previously [10,11].

RESULTS

Design of mini-proinsulin

Figure 1(A) shows the amino acid sequence of M2PI mini-proinsulin, which was designed to have a short turn-forming sequence and enzyme-processing sites between the two chains of insulin. A molecular modelling study on M2PI showed that the distance between the α -carbons of the C-terminal B-chain and the N-terminal A-chain was not altered and the insulin structure was well maintained (Figure 1B). This indicates that the insertion of the nonapeptide (RRYPGDV KR) between the A- and B-chains places no significant structural constraints on the remaining parts of the mini-proinsulin. The modelling results also showed that the turn structure could be stabilized by the charge-interaction between Arg-32 and Asp-36.

Cloning and expression of mini-proinsulin

The gene encoding the proinsulin sequence was prepared by PCR using five overlapping oligonucleotides of 65–68 bases in length as templates. The proinsulin gene was constructed with consideration of the codon preference of *E. coli* in order to increase the expression rate of the DNA in the bacterial hosts. The gene for the mini-proinsulin was prepared by PCR using the proinsulin gene as a template.

In our previous study [12], the 57 N-terminal residues of human TNF- α were used as a fusion partner to produce glucagon with high-level expression in *E. coli*. The property of high-level expression along with the characteristic β -sheet structure of TNF- α has been utilized in the production of peptide hormones as fusion proteins [13]. In order to increase the proportion of mini-proinsulin relative to the TNF- α moiety in the fusion protein, the N-terminal pentapeptide sequence (PSDKP) of TNF- α was used in the present study. For convenience in the purification process, ten histidine residues and a methionine residue for chemical cleavage were inserted between the PSDKP sequence and the target protein.

The expression plasmid pT2M2PI (Figure 2) was used to transform *E. coli* BL21 (DE3) cells harbouring a chromosomal copy of the bacteriophage T7 RNA polymerase. Recombinant *E. coli* cells were grown in Luria–Bertani medium containing 200 mg/l ampicillin. After induction of the T7 promoter by isopropyl β -D-thiogalactopyranoside, cells were harvested 10 h post-induction. Cells were disrupted by sonication, and the

Figure 3 SDS/PAGE analysis of the fermentation and purification of sulphonated mini-proinsulin

Lanes: M, molecular-mass markers (values indicated on the left); 1, total cell lysate; 2, soluble fraction after cell disruption; 3, insoluble fraction after cell disruption; 4, CNBr-cleaved fusion protein; 5, sulphonated M2PI after cation-exchange chromatography.

fusion protein produced was analysed by SDS/PAGE (Figure 3). The expression level of the fusion protein, T2M2PI, was about 20–25% of the total cell protein, indicating that this expression system is highly efficient, considering the relatively small size of the fusion protein (81 residues).

Purification and refolding of mini-proinsulin

The inclusion bodies of the fusion protein were sulphonated at their cysteine residues and chemically cleaved by CNBr treatment. S-Sulphonated mini-proinsulin was purified by cation-exchange column chromatography at pH 4.0. As the histidine tag was rich in positive charges at this pH, it was convenient to separate the mini-proinsulin from the fusion partner and the uncleaved form. The purity and molecular mass of mini-proinsulin was analysed using tricine SDS/PAGE (Figure 3).

Refolding reactions of mini-proinsulin were performed according to the previously described method [2]. 78% of S-sulphonated mini-proinsulin was converted into correctly refolding species at a protein concentration of 50 μM at pH 11.5 (Figure 4). Proinsulin which was prepared by the same procedure showed a maximum refolding yield of 57% at the same molar concentration at pH 10.5. The refolding of mini-proinsulin at a higher concentration of 200 μM (1.5 mg/ml), which could be accomplished with 51% yield at pH 11.5, still exceeds the

Figure 4 Refolding yields of M2PI (■) and proinsulin (●)

Refolding yields were determined by comparing the peak size of the correctly folded species in the HPLC profile with the standard.

Figure 5 HPLC elution profiles

(A) Proinsulin; (B) enzyme-digested proinsulin; (C) M2PI; (D) enzyme-digested M2PI; (E) standard human insulin (Sigma) (left chromatograms). Right chromatograms indicate the HPLC profiles of (D) and (E) which were cleaved with *S. aureus* V8 protease.

proinsulin yield of 37 % at the same molar concentration. These results indicate that the short turn-forming sequence is more effective in the refolding process than the much longer native C-peptide.

Production of insulin and its identification

Refolded mini-proinsulin was purified by a Vydac C_{18} semi-preparative reverse-phase HPLC column. Conversion of mini-proinsulin into insulin was accomplished by incubating mini-proinsulin with trypsin and carboxypeptidase B. HPLC analysis showed that mini-proinsulin was almost completely converted into insulin (Figure 5). The major peak, which was eluted at the same position as that of standard insulin, was collected and lyophilized. When analysed by MS it gave the expected molecular mass of human insulin (expected, 5807.8; found, 5807.0). Amino acid composition and N-terminal protein sequencing also agreed well with the expected values. The correct disulphide formation was verified by subsequent fingerprint analysis using *S. aureus* strain V8 protease (Glu-C endoproteinase) that specifically cleaves the peptide bond on the carboxyl side of the glutamic acid residue [9]. The HPLC profile of the proteolytic digest was identical with that of standard human insulin (Figure 5).

Receptor binding activity

The biological activities *in vitro* of insulin, proinsulin and mini-proinsulin were examined by a competitive receptor binding assay. The insulin prepared from enzymic cleavage of M2PI displayed identical receptor binding affinity with standard insulin within the error range. M2PI yielded an ED_{50} value of 1.48 nM, while the ED_{50} values of standard insulin and proinsulin were 0.71 nM and 36.1 nM respectively (Figure 6). (ED_{50} values represent the concentration of insulin or insulin derivatives giving half-maximal inhibition of ^{125}I -labelled insulin receptor binding.) This indicates that the relative potency of M2PI is 48 %

Figure 6 Receptor binding assay on cultured human IM-9 lymphocytes

Competition with ^{125}I -labelled insulin of human insulin (●), M2PI (■) and proinsulin (▲) for binding to the insulin receptor. Values are means $\pm$ S.D. of quadruplicate determinations. The ED_{50} values were calculated to be 0.71 nM (insulin, 100%), 1.48 nM (M2PI, 48%) and 36.1 nM (proinsulin, 2%) respectively. Values in parentheses reflect potency of the analogue relative to insulin.

of that of insulin, in marked contrast to proinsulin which is about 2 % as potent as insulin.

DISCUSSION

The present studies describe the design, production and biological assay of a novel mini-proinsulin, M2PI, and its proteolytic conversion into human insulin. In the design of M2PI, the turn-forming sequence was adopted for the following two reasons: firstly, turns can be formed in the early stages of protein folding [14] and may be instrumental in the later stages of protein folding. Secondly, most dibasic processing sites were predicted to occur in a β -turn adjacent to a region of helix or β -sheet [15], thus insertion of a β -turn between the two dibasic sites could aid the enzyme processing required to release the native insulin.

β -Turns are discrete elements of surface structure with backbone conformations determined primarily by short-range interactions. Owing to the characteristic nature of the turn structure, turns have been found in aqueous solutions of short peptides without difficulty [16–20]. In an extensive study using model peptides, Pro and Gly in the second and third positions respectively, were found to give the highest β -turn population, and the nature of the amino acid at position 4 influences the β -turn stability in the *trans* position, having a preference for Asp residues with deprotonated side-chains [18]. A YPGDV sequence was selected in this study since this peptide fulfilled the above criteria and has displayed highly populated turn conformations in isolation [18].

The refolding yield of M2PI was found to be 20–40 % better than that of proinsulin studied at the same molar concentrations. The difference in the refolding yield between M2PI and proinsulin becomes larger at higher concentrations, suggesting that this mini-proinsulin approach could be used as an alternative way to produce recombinant human insulin. The successful generation of human insulin by subsequent enzyme treatment, along with the high expression level of the fusion mini-proinsulin, substantiates the above prospect. The mini-proinsulin approach has been previously attempted with a joining sequence of Arg-Arg-Gly-Ser-Lys-Arg, but the purification yield of this mini-type proinsulin was very low, and whether the isolated form has the correct disulphide bonds was not determined [21]. M2PI showed its maximum refolding yield at pH 11–11.5, which was more

basic than the optimum condition for the refolding of proinsulin. This indicates that the repulsive force between the two dibasic sites, RR and KR, is more influential in mini-proinsulin than in proinsulin. The pH tolerance of proinsulin could be due to the neutralizing effect of the highly acidic EAED sequence adjacent to the RR site, as the refolding yield of an EAED-deleted proinsulin derivative showed a similar trend of pH dependence to M2PI mini-proinsulin (K.-D. Choi, S.-G. Chang and H.-C. Shin, unpublished work).

The receptor-binding activity found in our M2PI mini-proinsulin far exceeds those of proinsulin derivatives reported up to recently. Proinsulin is a weak insulin agonist with about 2% binding activity to the insulin receptor, relative to insulin. Increased activity was reported with proinsulin derivatives which have a free A-chain N-terminus, obtained by inverting the order of the natural B-C-A sequence to A-C-B (14%), or by specific cleavage at the Arg⁶⁵-Gly⁶⁶ peptide bond of proinsulin (22%) [22,23]. A free N-terminus at the Gly¹ residue of the A-chain was suggested to be important for the biological activity of insulin [24]. Our data indicate that the free N-terminus of the Gly¹ residue of the A-chain is not essential for receptor binding.

The superior receptor binding activity of M2PI also compares well with other types of mini-proinsulin reported. Mini-proinsulin composed of B-chain(1–29)-AAK-A-chain(1–21) was only 0.5% as potent as insulin [25]. Insulin cross-linked between A1 and B29 by diaminosuberic acid showed a reduced potency, about the same as that of proinsulin [26], and a direct connection between B29 and A1 by a peptide bond was reported to produce a completely inactive analogue [27]. Therefore it is rather surprising that the single-chained M2PI mini-proinsulin exhibits high receptor-binding activity, about 50% as potent as insulin. M2PI contains RR and KR enzyme processing sites, as in the case of proinsulin. Therefore it is likely that these basic residues have an effect on the mode of receptor binding, probably either by directly interacting with the receptor or by inducing a conformational change in the receptor-binding region of M2PI. In the case of proinsulin, the bulk of the C-peptide may interfere with the contact between some active surface in the insulin molecule and the receptor.

The results of the present study indicate that the 31-residue C-peptide in proinsulin can be replaced with a short pentapeptide sequence without disturbing the refolding process. The high expression level, enhanced refolding yield and successful generation of native human insulin makes this approach a favourable alternative to the currently used proinsulin process. In addition, the high receptor-binding activity displayed by M2PI mini-proinsulin suggests that this single-chained mini-proinsulin may provide a foundation in understanding the receptor-bound structure of insulin in the active form.

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