Mini-proinsulins with a beta-turn motif

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ABSTRACT

To increase the folding efficiency of proinsulin, we have designed a series of mini-proinsulins having the central C-peptide region replaced with sequences forming reverse turns. These proteins were produced as fusion proteins in *E. coli* in the form of inclusion bodies. After isolation process of the sulfonated mini-proinsulins, the subsequent refolding experiments indicate that the mini-proinsulins, with non-native penta-peptide sequences inserted between two of the enzyme processing sites, show substantially increased folding yields compared with the proinsulin. The correct disulfide connections were verified by fingerprint analysis using Glu-C endoproteinase. These novel mini-proinsulins could be used for the study of folding mechanism of proinsulin.

INTRODUCTION

Insulin is produced via proinsulin process, in the order of firstly correct proinsulin folding and subsequent enzymatic cleavage to release the active hormone. The role of C-peptide in the folding of proinsulin was speculated to bring the two distant parts of the polypeptide, A and B chains, into proximity for efficient formation of disulfide bridges between the two chains (Steiner, 1984; Steiner, 1989), but the exact role is not known yet. Since the cross-linked insulins can be folded with similar yield compared to the proinsulin (Wollmer et al., 1974) and the native insulin structure is recovered with somewhat less yield from the oxidation of reduced A and B chains (Katsoyannis et al. 1967; Chance et al., 1981), we reasoned that folding yield depends on the rate of bringing the both ends of the proinsulin chain into proximity. To test this hypothesis and to increase the folding efficiency of proinsulin, we have designed several mini-proinsulins having the central C-peptide region replaced with sequences forming reverse turns. Reverse turns are ideal candidates for several reasons; they are likely sites for initiation of protein folding, since they are determined by short-range interactions, they limit the conformational space available to the polypeptide chain, and by bringing more distance parts of

the polypeptide chain together, they may be instrumental in directing subsequent folding events (Zimmerman & Scheraga, 1977; Wright et al., 1988). We describe in this paper the design of mini-proinsulins and the results of refolding experiments.

MATERIALS AND METHODS

Preparation of sulfonated mini-proinsulin The proinsulin and mini-proinsulins were produced as fusion proteins which form inclusion bodies in *E. coli* cells. The fusion proteins were solubilized in 6M GdmCl and sulfitolysis reactions were carried out. The sulfonated fusion proteins were precipitated using $ZnCl_2$, the precipitates were solubilized in urea and cleaved by CNBr reaction. The proinsulin and mini-proinsulins as sulfonated forms were purified by cation-exchange chromatography and Zorbax C8 reverse-phase HPLC.

Refolding Typical refolding reaction of the sulfonated mini-proinsulins was performed in 50mM glycine buffer (pH 11.0) at the protein concentration of 53μM (0.5mg/ml for proinsulin and 0.35mg/ml for mini-proinsulins). The reaction was started with the addition of 2 equivalents of β-mercaptoethanol (0.636mM) and after gentle agitation at 4°C for 20 hrs, the reaction was stopped by adding HCl. 17.5μg of protein solution was filtered and loaded onto a reverse-phase POROS-R1H perfusion column. The refolding yields of the reaction products were determined by comparing the peak size of the correctly folded species in the HPLC profile with the standard.

Enzymatic conversion of mini-proinsulin to insulin Refolded mini-proinsulins were enzymatically processed into insulin by 1/12500 (w/w) of trypsin and 1/2500 (w/w) of carboxypeptidase B in 50mM glycine buffer (pH 8.0) at 16°C for 20 hrs and eluted on reverse-phase Zorbax C8 column with linear acetonitrile gradient in 0.1% TFA. Insulin was identified by amino acid analysis and mass spectrometry.

Glu-C endoproteinase peptide mapping of insulin derived from mini-proinsulins The correct disulfide connections were verified by fingerprint analysis using Glu-C endoproteinase (*S. aureus* protease V8)(Johnson, 1982; Grau, 1985). 0.2mg/ml of insulin in 0.2M Tris (pH 7.3) buffer was incubated with 0.05mg/ml of the enzyme at 37°C for 4 hrs and eluted on Zorbax C8 column with linear gradient of 50mM H_3PO_4 and acetonitrile.

RESULTS AND DISCUSSION

According to the known three-dimensional structures of insulins in the Brookhaven Data Bank, the distance between C-terminal α-carbon of B-chain (Thr-30) and N-terminal α-carbon

of A-chain (Gly-1) is roughly 5~11Å apart, depending on the conditions studied. It has long been recognized that certain amino acid sequences have a high probability of being part of a turn conformation in proteins (Chou & Fasman, 1977), and this has more recently been shown to be true also for peptides in aqueous solution (Dyson et al. 1988; Shin, et al. 1993). Proline in the second position and Glycine in the third position was found to give the highest β-turn population, and extensive study revealed that the nature of the amino acid at position 4 influences the β-turn stability in trans position, and there is a preference for a deprotonated Asp 4 side chain (Wright et al. 1988; Dyson et al. 1988). Therefore we have designed beta-turn forming sequences as follows (M1~M4).

B-chain	RR-APGDV-KR	A-chain	----- (M1)
	RR-YPGDV-KR		----- (M2)
	RR-HPGDV-KR		----- (M3)
	RR-GPG-KR		----- (M4)
	RR-GGGGG-KR		----- (G5)

To minimize X-Pro isomerization and to populate in the β-turn forming trans-isomer, we have selected Ala, His, Tyr in the position-1, by reference to the previously published data (Dyson et al. 1988; Shin et al. 1993). Gly-Pro-Gly sequence was selected to see the role of this sequence in the folding of proinsulin since this sequence locates in the center of C-peptide region of proinsulin . A peptide having only 5 Gly residues (G5) was also made as a control. According to the molecular modelling study on mini-proinsulin M1 (data not shown), the distance between α-carbons of C-terminal Thr-30 of B-chain and N-terminal Gly-1 of A-chain was similarly maintained as in insulin. This indicates that the insertion of nona-peptide sequence between the two chains has no significant structural constraints on the remaining parts of the mini-proinsulin. The modelling shows that the turn structure could be stabilized by the charge interaction between Asp and Arg, and hydrophobic interaction between Ala and Val.

Proinsulin and mini-proinsulins were produced as fusion proteins in *E. coli* in the form of inclusion bodies. After isolation process of the sulfonated mini-proinsulins, the subsequent refolding experiments were carried out. As predicted, the refolding yields of mini-proinsulins were much greater than that of proinsulin (Fig. 1). Refolded mini-proinsulins were enzymatically processed into human insulins using trypsin and carboxypeptidase B. The insulin production well correlated with the refolding yields. The successful generation of insulin from the mini-proinsulins indicates that the efficiency of enzyme processing was not altered by the modification of C-peptide region. The correct disulfide formation was verified by subsequent finger print analysis using Glu-C endoproteinase (Fig. 6). The mini-proinsulin containing 5 Gly residues in the turn region shows significantly reduced refolding yield

compared with the mini-proinsulins having β-turn forming sequences, suggesting the importance of the role of β-turn in the folding of mini-proinsulins. The effects of pH, temperature and concentration on the folding efficiency were also investigated and the results were shown in Figures 2, 3 and 4. The pH has dramatic effect on the folding of M2 mini-proinsulin, providing only narrow range of pH region for folding, between pH 10 and 12. Under pH 10, no proper folding species was detected. This is probably due to the strong repulsive force between the two enzyme processing sites, Arg-Arg and Lys-Arg. In the case of proinsulin, this repulsive force could be neutralized by the presence of highly acidic residues adjacent to the Arg-Arg doublet at the B chain/C-peptide junction.

The conformations of human insulin (HI), M2 mini-proinsulin (M2PI) and proinsulin (HPI) were studied using circular dichroism in the far-UV region. These proteins exhibit somewhat similar circular dichroic patterns as shown in Fig. 7, in oxidized (A) and reduced (B) forms.

In summary, we have designed mini-proinsulins with β-turn motif which show substantially increased folding yields compared with the proinsulin. Since the low refolding yield of proinsulin has been the main obstacle for the study of folding mechanism, these novel mini-proinsulins could be used for the study of folding mechanism of proinsulin.

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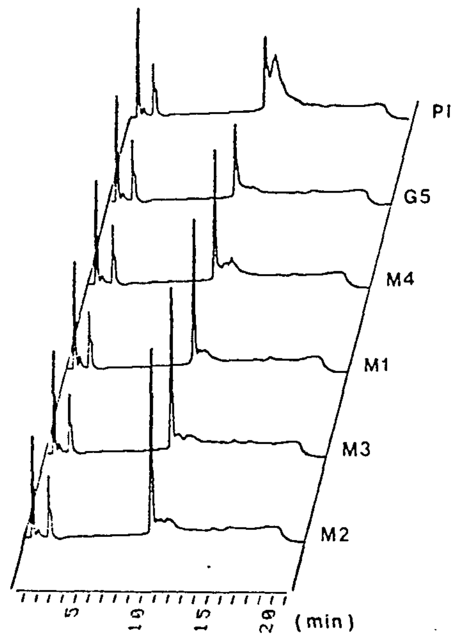


Fig.1 HPLC profiles of refolded proinsulin and miniproinsulins

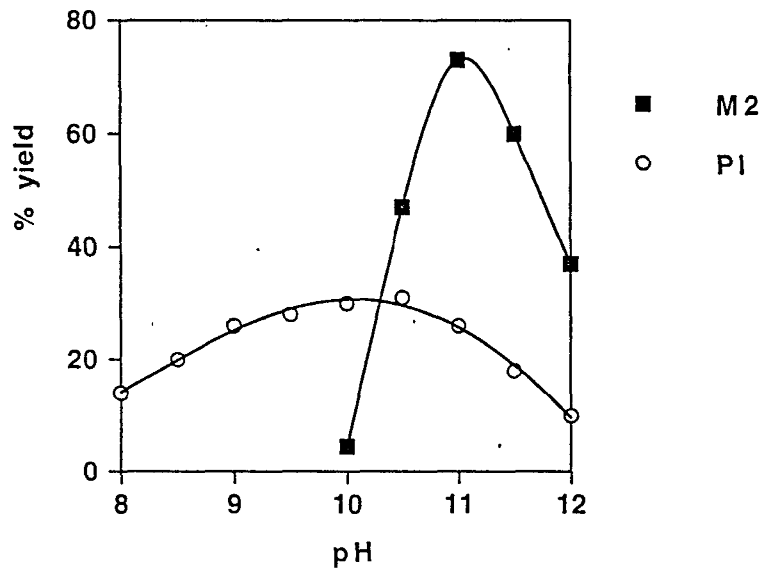


Fig.2 Effect of pH on the refolding of miniproinsulin-M2 and proinsulin

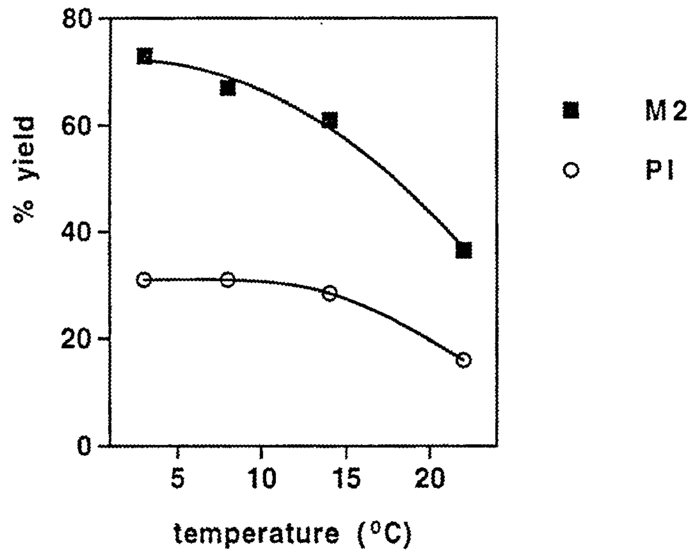


Fig3. Effect of temperature on the refolding of Miniproinsulin-M2 and proinsulin

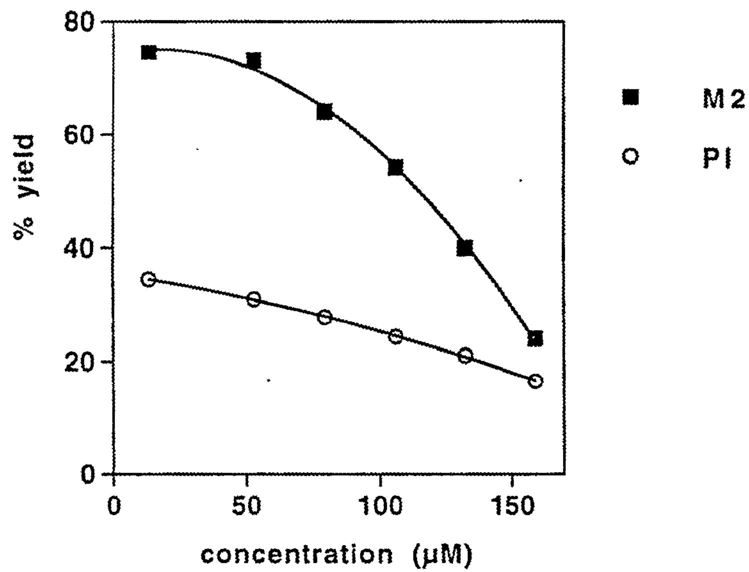
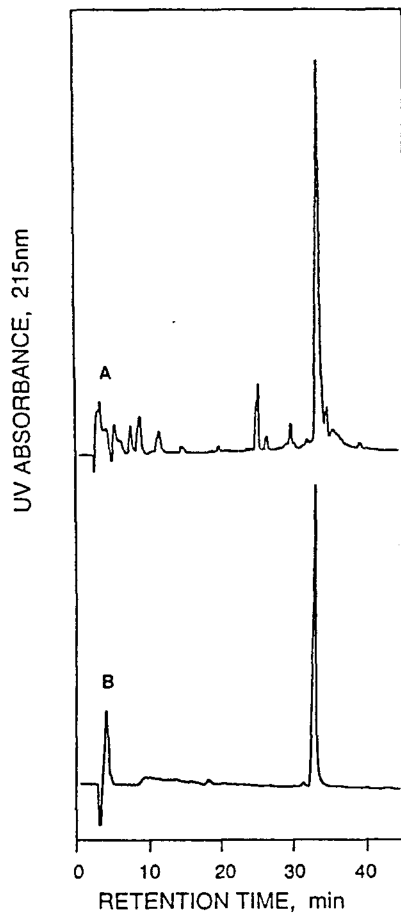


Fig4. Effect of protein concentration on the refolding of miniproinsulin-M2 and proinsulin



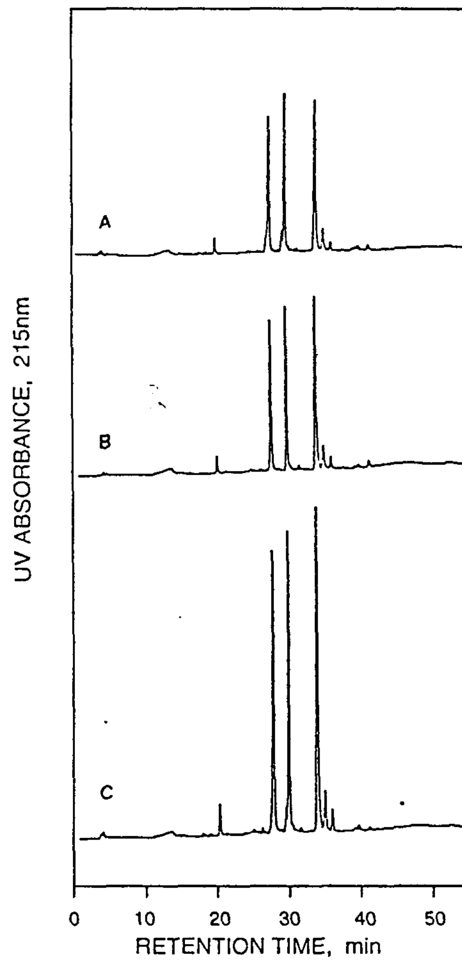


Fig.5 (A) HPLC analysis of the enzymatic conversion of the miniproinsulin to insulin. (B) Human insulin.

Fig.6 Glu-C endoprotease peptide mapping of (A) insulin derived from miniproinsulin, (B) human insulin and (C) A+B.

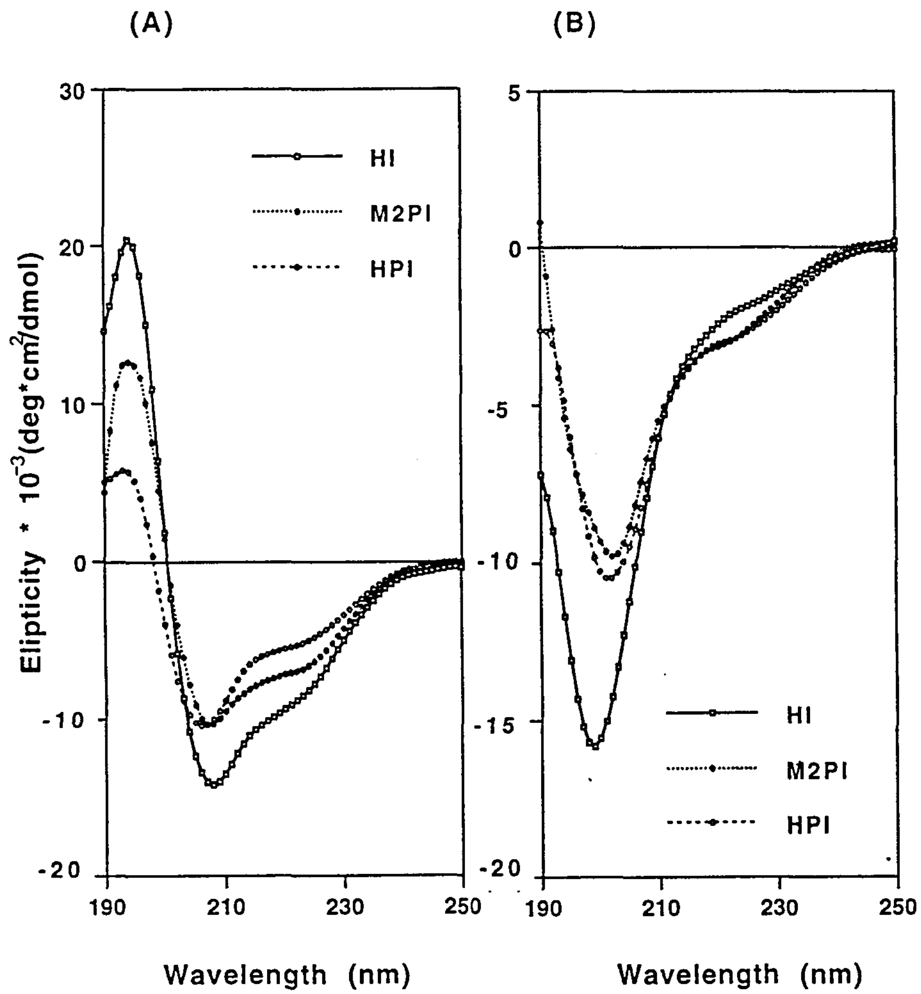


Fig.7 CD spectra of various insulins (A) Oxidized forms (B) Reduced forms.