

## The Role of $\beta$ -Turn in the Folding of Mini-Proinsulins

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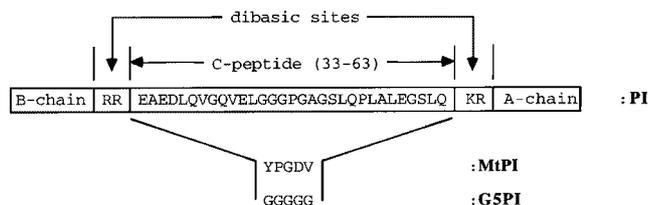
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The problem of whether turns actively participate in protein folding by directing subsequent folding events or play a passive role with chain reversal merely formed by the context surrounding the turn, has attracted considerable attention.<sup>1-3</sup> Previous studies indicate that certain amino acid sequences have a high preference of turn formation in proteins or peptides<sup>1,3,4</sup> and turns can also be formed in the early stages of protein folding.<sup>5</sup> However, whether turns affect the protein folding is still unclear.

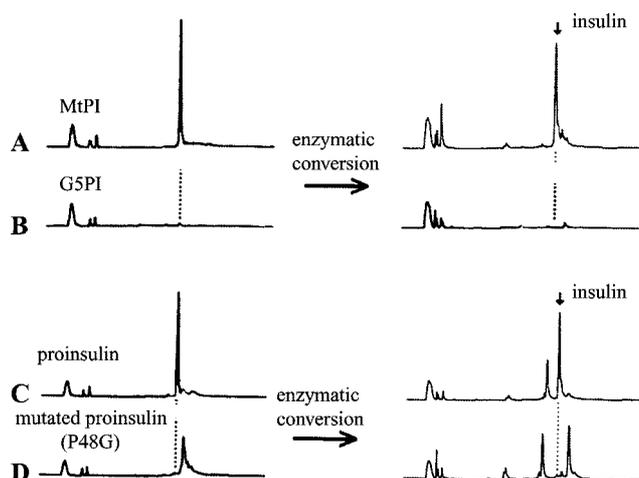
To examine the role of turn in protein folding, we have designed mini-type proinsulin analogs (mini-proinsulins) where the connecting C-peptide of 31 amino acids between the two dibasic (enzyme processing) sites in proinsulin was replaced with pentapeptide sequences, forming either turn (YPGDV) or random coil (GGGGG) (Fig. 1). YPGDV sequence was selected for this study since this peptide has shown to display highly populated turn conformations in isolation.<sup>3</sup>

The mini-proinsulins were produced as fusion proteins which form inclusion bodies in the *E. coli* cell.<sup>6</sup> The gene for the fusion protein was placed under the control of the T7 promoter in the expression plasmid pET-3a<sup>7</sup> and expressed in *E. coli* BL21(DE3) by IPTG induction. The fusion proteins were sulfonated in 6 M GdnHCl, purified using Ni-chelation column and cleaved by CNBr reaction. The mini-proinsulins as a sulfonated form were purified by cation-exchange chromatography and the purities were confirmed with SDS-PAGE analyses.

Refolding reactions of the sulfonated mini-proinsulins were performed in 50 mM glycine buffer at various pH and



**Figure 1.** Amino acid sequences of proinsulin and mini-proinsulins. Human proinsulin (PI) comprises the insulin B-chain, an Arg-Arg sequence, the connecting C-peptide of 31 amino acids, a Lys-Arg sequence, and the insulin A-chain. The connecting C-peptide with 31 amino acids was replaced with pentapeptide sequences, YPGDV (MtPI) and GGGGG (G5PI), respectively.

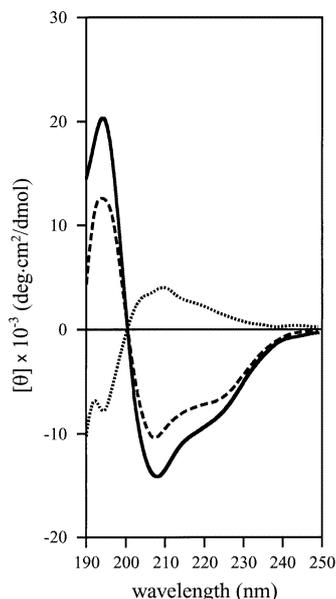


**Figure 2.** HPLC analysis of refolded proteins and subsequent enzymatic conversion products. (A) mini-proinsulin (YPGDV sequence inserted) (B) mini-proinsulin (GGGGG sequence inserted) (C) wild-type proinsulin (D) P48G proinsulin.

temperature conditions. MtPI mini-proinsulin with YPGDV sequence shows high refolding capacity towards native conformer (Fig. 2A). Refolded MtPI was enzymatically processed into human insulin and it was found that the insulin production was proportional to the refolding yields. The successful generation of insulin from the MtPI 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.<sup>8</sup> Under the same conditions, white aggregates were formed during the refolding of G5PI and no proper folding species was detected in the HPLC chromatograms (Fig. 2B). These results suggest the important role of  $\beta$ -turn in the folding of mini-proinsulins. The positive role of  $\beta$ -turn in the folding reaction was also found with such turn-forming sequences as HPGDV, APGDV and GPG, which were inserted between the two dibasic sites of mini-proinsulin (data not shown).

To see whether the turn conformation is preserved in the final folded structures, the conformations of human insulin (HI) and MtPI mini-proinsulin were investigated by circular dichroism (CD) spectroscopy in the far-UV region. They exhibit somewhat similar circular dichroic patterns as shown in Figure 3. A difference spectrum obtained by subtracting the CD spectrum of insulin from that of mini-proinsulin

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**Figure 3.** Far-UV circular dichroism spectra of human insulin (solid line) and MtPI mini-proinsulin (dashed line). The difference spectrum (dotted line in a) was obtained by subtracting the CD spectrum of human insulin from that of MtPI. All spectra were recorded at the protein concentration of 0.2 mg/mL at room temperature.

contains a broad positive band centered around 210 nm, similar to that of a type-II  $\beta$ -turn.<sup>9</sup> This result suggests that the turn connecting B-chain and A-chain of insulin is well maintained in the final folded structure.

Proline is commonly found in the residue i+1 of  $\beta$ -turns.<sup>10</sup> Two to four prolines are found in proinsulin C-peptide sequences from various species, suggesting some putative turn-forming sites. One of these regions (residues 47-51, GPGAG), which is located in the center of the C-peptide was selected for mutation study. Single amino acid substitution at the position 48 (Pro  $\rightarrow$  Gly) in the C-peptide of proinsulin was carried out (Fig. 1) and the folding property of the P48G proinsulin was compared with that of proinsulin (Fig. 2C and 2D). The P48G proinsulin did not show a proper folding towards the native conformer, in contrast to the proinsulin which folds into the native form with high yield. This result potentiates the active role of turn found in the folding of mini-proinsulin.

Brunet *et al.* examined the role of turns in the structure of an  $\alpha$ -helical protein using cytochrome b-562 which forms four-helix bundle.<sup>11</sup> They randomly substituted an inter-helical tripeptide in the protein with many different amino acid sequences and found that all of the substituted proteins fold into native structures. Based on the results, they suggested that the inter-helical turn does not play a dominant role in determining the folded structure of four-helix bundle. However, since four-helix bundle can also be formed by helical peptides without joining sequences,<sup>12,13</sup> four-helix bundle is not a good model to understand the role of turn in protein folding.

In this study, we found that the existence of turn between B- and A-chains of mini-proinsulin strongly increases the

folding yield, indicating that turn can act as one of the key elements in dictating the folded structures of native proteins. So far the production of mini-type proinsulin analogs has been only successful in our laboratory, with some reports in other laboratories of single-chain cross-linked insulins without enzyme processing dibasic sites.<sup>14</sup> The successful design of mini-proinsulin utilizing strong turn-forming sequence has implications not only for our understanding of the role of turn in protein folding but also for the application of the mini-proinsulin analog to gene therapy for the treatment of diabetes.<sup>15</sup>

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