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Solution Structure of an Active Mini-Proinsulin, M2PI: Inter-chain Flexibility is Crucial for Insulin Activity

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M2PI is an active single chain mini-proinsulin with a 9-residue linker containing the turn-forming sequence 'YPGDV' between the B- and A-chains, but which retains about 50% of native insulin receptor binding activity. The refolding efficiency of M2PI is higher than proinsulin by 20-40% at alkaline pH, and native insulin is generated by the enzymatic conversion of M2PI. The solution structure of M2PI was determined by NMR spectroscopy. The global structure of M2PI is similar to that of native insulin, but the flexible linker between the B- and A-chains perturbed the N-terminal A-chain and C-terminal B-chain. The helix in the N-terminal A-chain is partly perturbed and the β-turn in the B-chain is disrupted in M2PI. However, the linker between the two chains was completely disordered indicating that the designed turn was not formed under the experimental conditions (20% acetic acid). Considering the fact that an insulin analogue, directly cross-linked between the C-terminus of the B-chain and the N-terminus of the A-chain, has negligible binding activity, a flexible linker between the two chains is sufficient to keep binding activity of M2PI, but the perturbed secondary structures are detrimental to receptor binding.

Keywords: mini-proinsulin, solutin structure, inter-chain flexibility

Introduction

Insulin is produced as preproinsulin and processed to proinsulin that has a C-peptide between the B- and A-chains. Mature insulin consists of the B- and A-chains cross-linked by two inter-chain disulfide bonds and is active as a monomer (Knegtel *et al.*, 1991). The released C-peptide is 33 amino acids long while mature insulin has 51 amino acids composed of the B- and A-chains with 30 and 21 amino acids, respectively. Mature insulin plays a critical role in the control of blood glucose levels, but the released C-peptide is also known to have biological activity, such as restoration of the diabetes-induced decrease in cellular Na^+-K^+ dependent ATPase activity (Ido *et al.*, 1997; Steiner and Rubenstein, 1997). Proinsulin, which has a 33 amino acid linker between the two chains, is known to have only 2% of the receptor-binding activity of mature insulin (Chang *et al.*, 1998). On the other hand, an insulin analogue directly cross-linked between the C-terminus of the B-chain and the N-terminus of the A-chain lost bioactivity almost completely, even though the crystal structure of this analogue is virtually the same as native insulin (Derewenda *et al.*, 1991; Hua *et al.*, 1998). This indicates that the length of the cross-link affects the bioactivity of insulin. Tager's group (Brems *et al.*, 1991) systematically investigated the effects of cross-linking between the B- and A-chains and showed that a specific length of the cross-link is required for bioactivity. Site-directed mutagenesis work indicated that secondary structural elements [such as the N-terminal α-helix of the A-chain (A-α1), β-turn between G20-G23 and the α-helix in the B-chain (B-α1)] are crucial for the activity (Pittman *et al.*, 1997). These results suggest that residues involved in receptor binding are distributed over the B- and A-chains and that insulin is active only if the B-, and A-chains are flexible enough to adjust themselves to have proper orientation of interacting residues toward the receptor.

Recently a mini-proinsulin, M2PI, was designed to increase the refolding efficiency of the insulin precursor expressed in *E. coli* (Chang *et al.*, 1998). The refolding yield was increased by 20-40% compared to proinsulin and native insulin was successfully generated by the enzymatic conversion of M2PI. Interestingly, M2PI also exhibited high receptor-binding activity, about 50% as potent as insulin. In order to understand the structure/function relationship of insulin, we determined the solution structure of M2PI.

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The atomic coordinates (code 1 EFE) have been deposited in the protein Data Bank.

Methods and Material

Sample preparation All NMR experiments were performed using the purified over-expressed protein, as described previously (Chang *et al.*, 1998). Cultured *E. coli* cells were collected and the cell pellet was disrupted with sonication, then the cell lysate was centrifuged at 6,000×g for 10 min. The pellet was solubilized in 50 mM Tris-HCl, pH 9.0, 6 M guanidine-HCl at the concentration of 10 mg/ml. Oxidative sulfitolysis was performed by adding sodium sulfite and sodium tetrathionate to final concentrations of 300 mM and 60 mM, respectively, then incubating for 12 h at room temperature. The proteins were precipitated by adding 2 volumes of 0.1 M $ZnCl_2$ solution and then white precipitates were collected. The precipitates were dissolved in 8 M urea, 0.3 M HCl at the concentration of 10 mg/ml and cleaved by CNBr at room temperature for 16 h under dark conditions. Proteins were precipitated again by adding 2 volumes of 0.5 M $ZnCl_2$ solution. The precipitates were solubilized in 7 M Urea, 20 mM formic acid buffer (pH 4.0) and loaded onto a S-Sepharose cation exchange column (Pharmacia Biotech., Upsala, Sweden) equilibrated with the same buffer. The column was eluted using a linear gradient of 0- 0.5 M NaCl in equilibration buffer. Eluted fractions were pooled. Two volumes of distilled water were added and after incubation for 30 min at room temperature, precipitates were collected. After washing with the same volume of water again, the protein was lyophilized and stored at -20°C. For the purpose of obtaining heteronuclear a NMR spectra, the M2PI was uniformly labeled in a M9 minimal medium containing 1 g/L $^{15}NH_4Cl$. NMR Samples were prepared by dissolving the lyophilized protein powder in 70% H_2O/20% deuterated acidic acid/10% D_2O (350 ul H_2O, 50 ul D_2O and 100 ul acetic acid) or 79.96% D_2O/20% deuterated acidic acid. The concentration of the samples was about 2 mM.

NMR spectroscopy The NMR spectra were recorded on a Varian UNITY*plus* 600 (in Advanced Analysis Center in KIST) spectrometer at 30°C. Deuterium exchange of amide protons in M2PI was initiated by dissolving the lyophilized sample in 76.96% ^2H_2O/20% acetic acid and 2D ^1H-^{15}N HSQC spectra were recorded at 30°C. The cross-peaks observed after 40 mins of acquisition are classified as slowly exchanging protons. The mixing time of 45 ms was used for TOCSY and ^{15}N-HSQC-TOCSY and 150 ms was used for NOESY and ^{15}N-HSQC-NOESY. Heteronuclear ^{15}N-{^1H}-NOEs were obtained by acquiring spectra with and without ^1H saturation of 3 sec using the pulse sequences from Farrow *et al.* (1994) with 2 sec of relaxation delay as recommended by Gagné *et al.* (1998). 3 D spectra were processed by a program nmrPipe (Delaglio *et al.*, 1995) and assigned using a peak-picking program PIPP (provided by Dr. Garrett). The spectral widths of ^1H- and ^{15}N-dimensions were 7500 and 1600 Hz, respectively.

Assignments and distance restraints The basic assignments were achieved by use of ^1H homonuclear NMR spectra and confirmed by ^{15}N-edited experiments. This procedure offered a useful check of the previously obtained ^1H chemical shifts that had ambiguities in some assignments. The initial chemical shift assignments on all 60 amino acids were accomplished by homonuclear NMR experiments. Previously determined ^1H assignments for native insulin were used as a starting point for the ^1H assignment of the M2PI and confirmed by use of ^{15}N-HSQC-TOCSY and ^{15}N-HSQC-NOESY experiments. The stereospecific assignments of Hβ protons, and methyl groups of Val and Leu, were based on the intensity of HN-Hβ or HN-Hγ cross-peaks in ^1H-^{15}N TOCSY-HSQC and ^1H-^{15}N NOESY-HSQC spectra (Clore *et al.*, 1991) and NOE intensity of stereospecifically assigned Hβ protons to δ-methyl protons of Leu. The NOE distance restraints were derived from ^1H NOESY and 3D ^1H-^{15}N NOESY-HSQC all with a mixing time of 150 ms. The NOE intensity was converted into three groups of classes (1.8-2.7, 1.8-3.5, and 1.8-5.0) and pseudo atom corrections were made appropriately (Wüthrich *et al.*, 1983). Hydrogen bond restraints (r_{NH-O}, 1.7-2.3; r_{N-O}, 2.5-3.3) were given where secondary structures were indicated based on NOE connectivity (residues B9-B19 and A13-A19). The scalar coupling constants of the α-proton to the amide proton ($^3J_{HNH\alpha}$) were obtained from 3D-HNHA experiments. The backbone torsion angle φ was restrained to 25 to 85 for $^3J_{HNH\alpha}$<6.0 Hz, -60 to 180 for $^3J_{HNH\alpha}$ 7-8 Hz, -90 to 150 for $^3J_{HNH\alpha}$>8 Hz, and 10 to 100 for where the helix is defined by NOE connectivity.

Structure calculation A model structure was built by inserting 9 amino acids between B30 and A1 of the human insulin structure. Either this model structure, or an extended chain, was used as an initial structure in the structure calculations. Hydrogen bond, dihedral angle, and $^3J_{HNH\alpha}$ coupling constants were included in the simulated annealing procedure in addition to the NOE distance restraints. Distance restraints with a large violation were excluded in the first round of calculation. If NOE restraints are

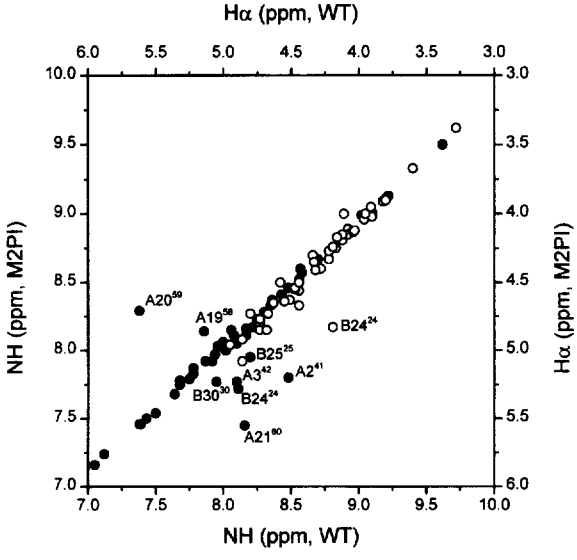

Fig. 1. Comparison of chemical shifts of M2PI and native insulin. Chemical shifts of M2PI and native insulin (Hua and Weiss, 1991) are compared. The closed and open circles are for NH and Hα protons, respectively. The residues with a chemical shift difference over 0.2 ppm from the correlation line are numbered. The residues 1-30 and 40-60 correspond to the B-chain and A-chain of the native insulin, respectively. Superscript at each number indicates the sequence number in M2PI.

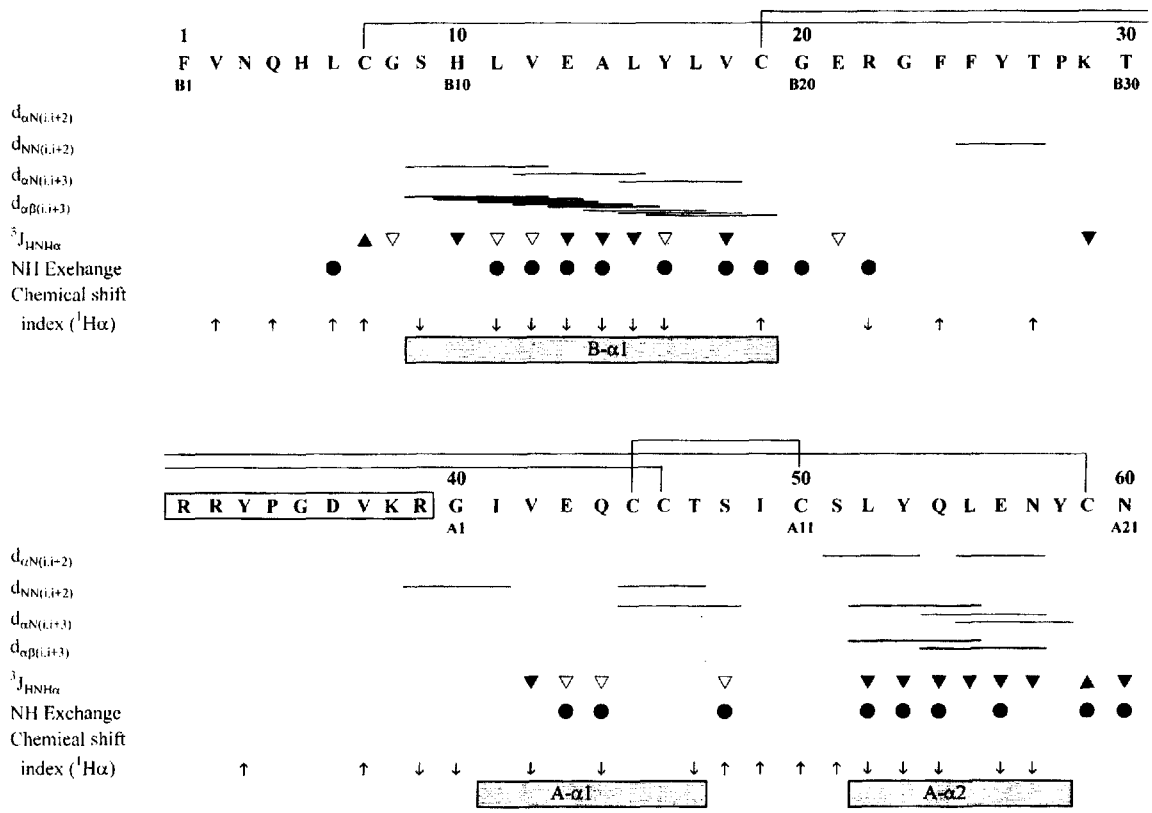

Fig. 2. Summary of short- and medium-range NOE, $^3J_{HNH\alpha}$ coupling constants and deuterium exchange. The cross peaks observed after 40 min at 30°C in 20% acetic acid are shown in the circle. The thickness of line correlates with the intensity of NOE. The $^3J_{HNH\alpha}$ coupling constants are measured from 3D-HNHA experiments and indicated by triangles with downward and up-ward for <6.0 and >8.0 Hz, respectively. Open triangles indicate $^3J_{HNH\alpha}$ coupling constants, which are too small to observe off-diagonal peaks in 3D-HNHA spectrum. The linker between the two chains is boxed and the sequence number of the native insulin is shown below the amino acid sequence. Chemical shift indeces of Ha proton are shown by arrows, up-ward for '1' and down-ward for '-1', respectively.

correctly assigned, the same NOE restraints are used again. However, if NOE assignments are ambiguous, new assignments were made. The ten calculated initial structures were averaged and minimized. The averaged structure was used as an initial structure for refinement. In refinements, database potential, implemented by the Clore group (Kuszewski *et al.*, 1997) are included. All calculations were made by the program XPLOR (Brünger, 1993).

Results

Chemical shifts of M2PI Chemical shifts of nuclei represent the surrounding chemical environments. Chemical environments represented by chemical shifts are also related to the local protein structure. When chemical shifts of M2PI and native insulin (Hua and Weiss, 1991) are compared (Fig. 1), most of them are similar to each other, but chemical shifts of a few residues (Ile^{A2}, Val^{A3}, Tyr^{A19}, Cys^{A20}, Asn^{A21}, Phe^{B24}, Phe^{B25}, and Thr^{B30}) in M2PI have changed significantly, compared to the wild type insulin. The residues of chemical shift perturbation are in the helices A-α1 (Ile^{A2}, Val^{A3}), A-α2 (Tyr^{A19}, Cys^{A20}, Asn^{A21}) and the functionally critical Phe^{B24} and Phe^{B25}. The chemical shift perturbation of A-α1, Phe^{B24}, Phe^{B25}, and Thr^{B30} is the direct consequence of the flexible linker and that of A-α2 is likely to be affected by the disrupted β-turn that is close to helix A-α2. These chemical shift perturbations imply that regions corresponding to helix A-α1 and β-turn in native insulin are disrupted in M2PI.

Structure of M2PI The structure of M2PI has been defined by 602 experimentally derived NOEs, 41 dihedral angle restraints, and 36 $^3J_{HNH\alpha}$ coupling constants. The backbone atomic root mean square deviation around mean structure for the structured region (residues 9-19, 40-60) is 0.7 Å and for the B-, and A-chain region (residues 1-30, 40-60) is 1.2 Å. More than 85% of the residues of the B-, and A-chains are in the most favorable region in the Ramachandran plot (Table 1). M2PI consists of three helices (Figure 2-3). Helices B-α1 (B9-B19), A-α1 (A2-A8), and A-α2 (A13-A19) are the same secondary structural elements found in native insulin. But the helix A-α1 (A2-A8) is not as well defined as that in native insulin. Only one backbone helical NOE was found in the A-α1 helix, but disulfide bonds (Cys^{A6}-Cys^{A11}, Cys^{A7}-Cys^{B7}) and side chain NOEs induced the helical conformation in this

Table 1. Summary of structural restraints derived from experimental measurements and structural statistics for the 20 final structures

NOE distance restraints		
Intra-residual	346	
Sequential	149	
Medium (1<li-jl=4)	65	
Long (li-jl>5)	42	
Hydrogen bonds	2*10[a]	
Angular restraints		
Dihedral angle ϕ	32	
Dihedral angle χ^1	9	
Coupling constants	36	
Ramachandran plot[b]		
(%, residues 1-30, 40-60 for all 20 structures)		
Most favorable region	87.8	
Additional allowed region	11.1	
Generously allowed region	0.9	
Disallowed region	0.3	
Atomic root mean square deviation (Å)		
	Backbone	All heavy atoms
Residues 1-30, 40-60	1.2	1.7
Residues 9-19, 40-60	0.7	1.1

[a]For each hydrogen bond, two distance restraints are used, r_{HN-O}, 1.7-2.3 and r_{N-O}, 2.5-3.3. Coupling constants are obtained from HNHA experiments.

[b]The program PROCHECK_nmr (Laskowski *et al.*, 1996) was used to analyze the quality of structure.

region. The $^3J_{HNH\alpha}$ coupling constants and hydrogen exchange data also indicate that the A-α1 helix is not well defined. The residues B24-B28 do not form a preferential secondary structure, whereas this region forms a β-sheet at neutral pH in native insulin (Olsen *et al.*, 1996). And a turn for B20-B23 was also lost in M2PI, but some of side-chain NOEs that are similar to native insulin were observed in this region. The linker, including a designed turn sequence YPGDV, did not show any preferred structures.

Flexibility of Structure The flexibility of the protein was assessed by hydrogen exchange rates and backbone dynamics using ^{15}N-{^{1}H}-heteronuclear NOE. As shown in Figure 2, the secondary structure regions showed slow proton exchange indicating relative rigidity. This is also consistent with the backbone dynamics measured by heteronuclear NOE. ^{15}N-{^{1}H}-NOEs indicates that two α-helix (B9-B19 and A13-A20) regions are relatively rigid, but other regions are highly flexible (Fig. 4). The residues Gly^{B23} and Phe^{B24} show high ^{15}N-{^{1}H}-NOE and high angular order parameters, but atomic displacements indicate that this region is not well defined. This indicates that Gly^{B23} and Phe^{B24} are flexible in the slow-time scale due to the segmental motion of the C-terminus of the B-chain, but are relatively rigid in the faster time scale.

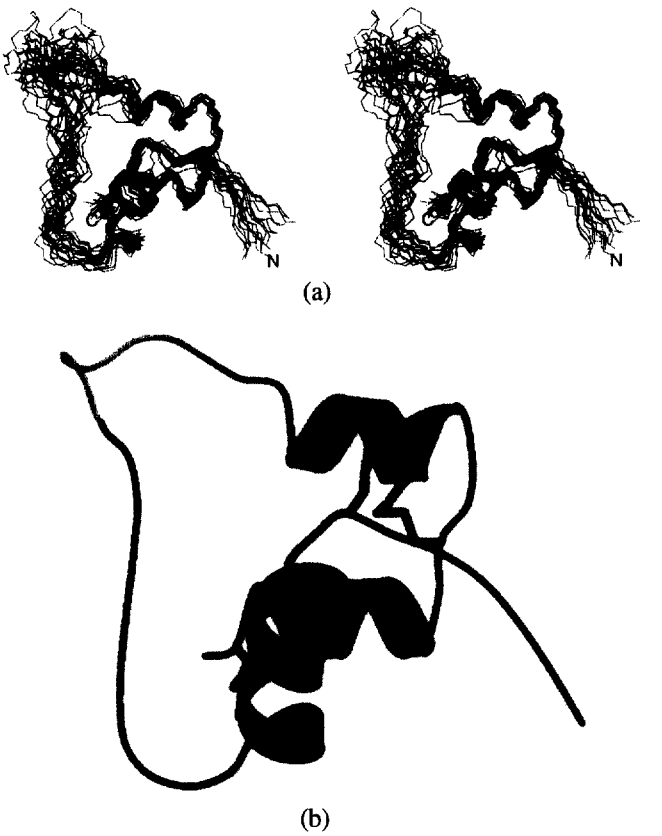

Fig. 3. Structure of M2PI. A: Stereoview of the backbone atoms (N, C_α, C') of 20 structures of M2PI. The root mean square deviation about the mean coordinate position for residues 9-19 and 40-60 is 0.7 Å for backbone atoms and 1.1 Å for all heavy atoms. No distance restraints are violated more than 0.5 Å in any structure, and no torsion angle restraints are violated more than 5°. B: Ribbon drawing of the NMR structure of M2PI. The residues corresponding to the A-chain are in magenta, the B-chain is in dodger blue, and the linker is in gold. The disulfide bonds are shown in dark orange. Ribbon drawing was generated by the program MOLSCRIPT (Kraulis, 1991).

^{15}N-{^{1}H}-NOEs indicate that the A-α1 helix is not well defined in M2PI and the designed turn in the mini-C-peptide also showed no restrained movement compared to the other flexible regions.

Discussion

In M2PI, the central α-helix (B-α1) in the B-chain, and the N-(A-α1) and C-terminal (A-α2) α-helices of the A-chain, are defined as in native insulin, but a turn and the B-chain C-terminal β-sheet are not observed in M2PI. Also, the linker between the two chains is completely disordered. However, the A-chain α1 helix is not well defined, as indicated by the chemical shift differences, backbone helical NOEs, and ^{15}N-{^{1}H}-NOE.

The residues implied in receptor binding are Ile^{A2}, Val^{A3},

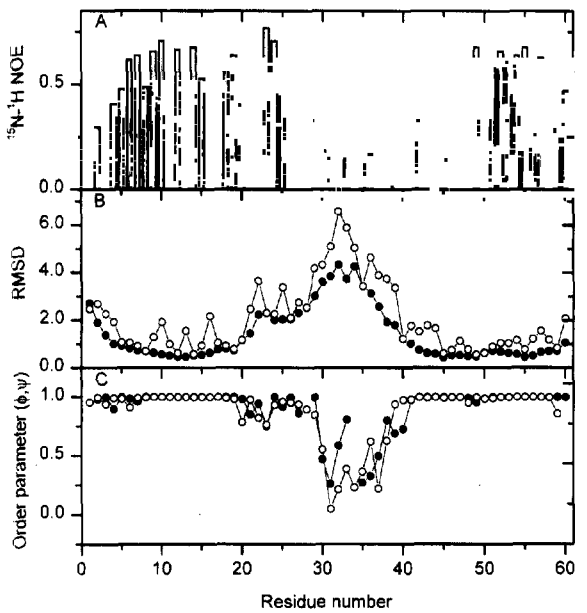

Fig. 4. Atomic displacements of heavy atoms and ^{15}N-{^{1}H}-NOE. A: ^{15}N-{^{1}H}-NOE of backbone M2PI. The values are calculated from the ratio of ^{15}N-^{1}H cross-peaks intensity between with and without ^{1}H saturation. B: The atomic root mean square deviation about the mean coordinate is shown in closed and open circles for backbone and side-chain atoms, respectively. C: Angular order parameters of ϕ and ψ angles are shown in closed and open circles, respectively. Angular order parameters are calculated by the program MOLMOL (Koradi, 1996).

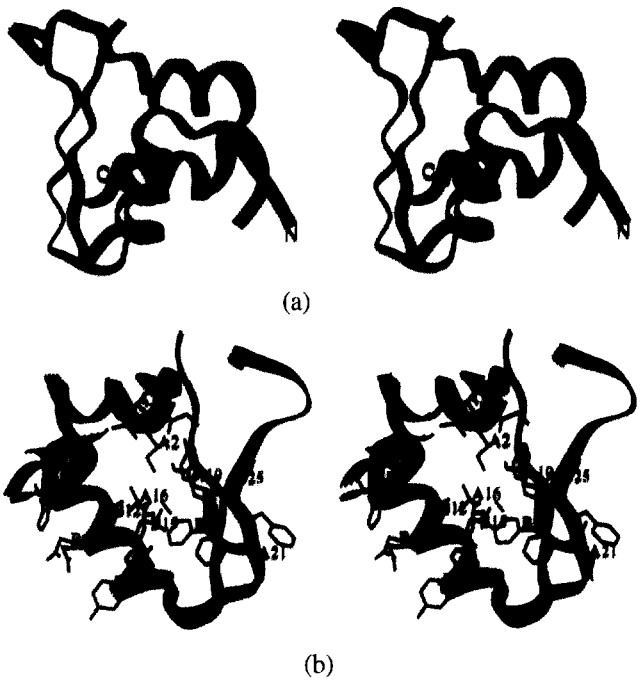

Fig. 5. Superposition of M2PI and native insulin (Hua *et al.*, 1995; 2HIU in PDB code). A: The backbone trace of M2PI and native insulin are compared in the same view as in Fig. 3. B: The side-chains of the putative receptor binding site are shown with the residue number of native insulin. Orientation is rotated about 180 in x-direction from Fig. 5a. M2PI is in dodger blue and native insulin is in magenta.

Val^{B12}, Phe^{B24}, Phe^{B25}, Tyr^{A19}, Asn^{A21}, Leu^{A16} and Leu^{B15} as the major determinants and the residues of Ser^{B9}, His^{B10}, Glu^{B13}, and Tyr^{B16} are also implied to constitute a minor binding site (Mynarcik *et al.*, 1997). Thr^{A8} appears to have an effect on the binding activity by influencing the helix formation of the N-terminal α-helix (Olsen *et al.*, 1998). In receptor binding, these two sites are suggested to bind to different receptors forming a 1 : 2 complex in a manner similar to the human growth hormone (Schaffer, 1994). The major binding site can be formed only if the A- and B-chains have enough flexibility to rearrange residues involved in receptor binding. In mutants that restrained the A- and B-chain movement by direct cross-linking, or by a short peptide linker, negligible activity was observed (Brems *et al.*, 1991; Derewenda *et al.*, 1991). These results indicate that the major binding site is formed by the rearrangement of residues involved in the receptor-binding, rather than as they are in free ligand. The rearrangement of residues involved in receptor binding requires inter-chain flexibility. However, some secondary structures are also required in order to maintain the proper location on the binding surface. M2PI has about 50% receptor binding compared to native insulin, indicating that some detrimental factors in receptor binding still exist in the M2PI conformation. The global structure of M2PI is similar to native insulin, but the B-chain C-terminus is very different, as shown in Fig. 5. The locations of the side-chains of the putative biding sites are relatively well conserved in M2PI, except the N-terminus of the A-chain and the β-turn region. The reduced binding activity could be due to the disrupted A-α1 helix and β-turn. Several mutants that affect the structure of A-α1 showed that helical conformation is required for receptor binding (Hua *et al.*, 1996). The β-turn is also reported to be crucial for activity (Bao *et al.*, 1997). Other factors influencing the activity could be steric hindrance of the linker that might affect receptor binding. In fact, the long linker between the B- and A-chains of proinsulin may well affect binding affinity by conformational hindrance. Upon binding to the receptor, insulin would have a conformational change to expose the secondary binding site to the binding site in the receptor (Kurapkat *et al.*, 1999). This process would require not only inter-chain flexibility, but also appropriate local conformation. Thus far, the C-terminal β-sheet in the B-chain is known to be dispensable (Nakagawa *et al.*, 1986; Casaretto *et al.*, 1987), but other secondary structures are required for full activity. Some of them are required for insulin folding, and others are required for direct interaction with the receptor.

The reason for the improved folding efficiency of M2PI could not be deduced from the M2PI structure in 20% acetic acid. However, at alkaline pH, where M2PI shows higher refolding efficiency than proinsulin, the designed turn in the

linker could play a critical role in refolding. In fact, the refolding efficiency of M2PI in acidic pH was much lower than that of proinsulin (Chang *et al.*, 1998).

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