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Flexibility Exists in the Region of [A6–A11, A7–B7] Disulfide Bonds during Insulin Precursor Folding

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To investigate the possible similarity of the proinsulin folding process with insulin-like growth factor I, two swap-like human proinsulin mutant proteins [A7,A11Ser]-HPI and [A11Ser,A12Cys]-HPI were prepared. Their *in vitro* refolding yields, oxidation of free thiol groups, circular dichroism spectra, antibody and receptor binding activities and sensitivity to trypsin digestion were studied and compared with both native HPI and [A6,A11Ser]-HPI. The results indicate that the shift mutation in the disulfide bond caused more conformational change and a greater decrease in biological activity than the deletion mutation on the proinsulin molecule. However, the shift of the intra-A chain disulfide bond had little effect on the refolding rate of the molecule. In vitro refolding yields of HPI analogues with shift or deletion mutations in the region of the [A6–A11,A7–B7] disulfide bonds were almost as high as that of wild type HPI suggesting that the region of the [A6–A11,A7–B7] disulfide bonds possesses some flexibility as is found in the corresponding region of insulin-like growth factor I.

Key words: disulfide bond, flexibility, proinsulin analogue, protein folding, refolding yield.

Abbreviations: DTNB, 5,5′-dithio-bis-(2-nitrobenzonic acid); FPLC, fast protein liquid chromatography; HPI, human proinsulin; IGF-I, insulin-like growth factor I; LB, Luria Bertant medium; MALDI-TOF MS, matrix-assisted laser desorption time-of-flight mass spectrometry; RPC, reverse phase chromatography; TFA, trifluoroacetic acid.

Among members of insulin superfamily, it is known that insulin and IGF-I are 49% homologous in the regions of homology (*1*) and that IGF-I adopts an insulin-like structure (*2*). The folding of IGF-I has been widely studied during the past decade and a folding pathway has been proposed (*3–6*). However, there are still many questions remaining about the folding pathway of insulin, although much progress has been made in recent years and a putative folding pathway has been proposed (*7*). So far, the studies indicate that there are differences between the folding pathways of insulin and IGF-I. It has been demonstrated that the intra-A chain disulfide bond forms first in the folding of the insulin precursor (*8*), a finding that was further confirmed by Qiao *et al.* by trapping an [A6–A11]-one-disulfide-bonded intermediate in the early stages of refolding of fully reduced recombinant porcine insulin precursor (*7*). On the other hand, in studies of the refolding of IGF-I, the disulfide bond 47–52, which corresponds to A6–A11 in insulin, has been shown to be an unfavorable high-energy bond that forms late during the folding process (*4*). In addition, a significant difference between the folding of IGF-I and insulin lies in their major refolding products. The refolded IGF-I and IGF-I swap, and can be distinguished from each other in the pattern of the disulfide linkage corresponding to the region of the [A6–A11,A7–B7] disulfide bonds in insulin, with similar thermodynamic stabilities (*3*, *9*). Meanwhile, the refolding of reduced insulin chains or proinsulin usually gives only one monomeric product with native disulfide bonds (*10–12*). However, it has also been reported that, in the presence of denaturants, small quantities of swap-like insulin isomers could form from native insulin or proinsulin following disulfide rearrangement (*13*, *14*). It is reasonable to think that the kinetic accessibility of the swap-like insulin isomers may indicate something similar between the folding of insulin precursor and that of IGF-I. In order to obtain further information on the folding of insulin precursor, we prepared, mimicking the IGF-I swap, two mutant human proinsulins (HPI) with deleted or position-shifted disulfide bonds in the [A6–A11, A7–B7] region, *i.e.*, [A7, A11Ser]-HPI and [A11Ser, A12Cys]-HPI, respectively. Their physico-chemical properties and biological activities as well as their *in vitro* refolding behaviors, were studied and compared with both native HPI and [A6, A11Ser]-HPI (*15*). The disulfide bond patterns of the HPIs used in this study and the two IGF-I refolding products are shown schematically in Fig. 1A. The three-dimensional structure of insulin with three disulfide bonds is shown in Fig. 1B.

MATERIALS AND METHODS

Materials—Escherichia coli DH5 α was used as host for the expression of recombinant genes. Plasmid pBV220, with λ-phage CI heat-shock promoter P_RP_L, was used as a cloning vector. Plasmids pJG401 and pJG103 were previously constructed in our laboratory with the insertion of

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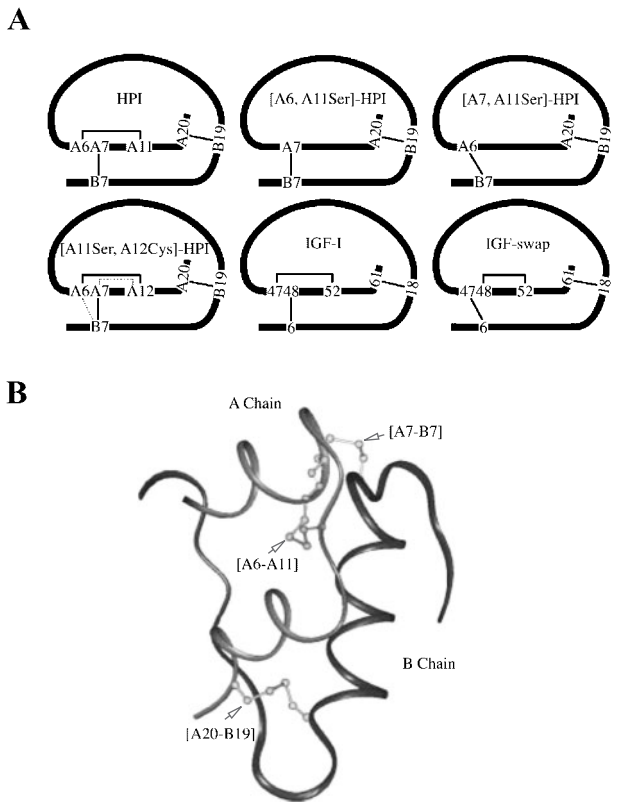

Fig. 1. **Demonstration of some insulin disulfide bond related structures.** (A) shows a brief illustration of the disulfide bonds in HPI and its three analogues, and also the IGF-I and IGF-swap. The known disulfide linkages in HPI, [A6,A11Ser]-HPI, IGF-I and IGF-swap are indicated. In other analogues, the probable disulfide linkages are also shown (The second probable disulfide linkages in [A11Ser,A12Cys]-HPI are indicated by the dotted line). (B) three-dimensional structure of insulin with three disulfide bonds indicated. The structure was derived from 2HIU in the protein data bank (Brookheaven, CA) by program Insight II.

the [A6,A11Ser]-*HPI* or *HPI* gene into pBV220 (*15*, *16*). PCR primers for the construction of HPI mutant genes were synthesized by Sagon, Shanghai. Taq DNA polymerase, T_4 DNA ligase and restrictive enzymes were from Promega. DTT was purchased from Sagon, Shanghai. Endoproteinase Glu-C (V8) of sequencing grade and trypsin with an activity of 10,400 units/mg were from Sigma. Chromatography columns and media were products of Amersham Pharmacia, Sweden. DTNB and other chemicals of analytical or chromatographic grade obtained locally. The [^{125}I]-insulin radioimmunoassay kit was kindly provided by the Navy Radioimmunoassay Technique Center (Beijing, China).

Construction of Mutant Genes and Preparation of Proteins—The mutant gene of [A7,A11Ser]-HPI was obtained by PCR with plasmid pJG401 containing the [A6,A11Ser]-*HPI* gene as a template. The mutant primer (5′-GTCGACGGATCCTCAGTTGCAGTAGTTCTCCAGTTGGTAGAGGGAAGATGCTGGT**AGAGCA**CTG-3′) introduced a Ser to Cys mutation at A6 and Cys to Ser mutation at A7 of [A6, A11Ser]-*HPI*. The mutant gene of [A11Ser,A12Cys]-HPI was obtained by PCR with plasmid pJG103 containing the *HPI* gene as template. The mutant primer (5′-CGACGGATCCTCAGTTGCAGTAGTTCTCCAGTTGGTAGAG**GCAGCT**GATGCTGG-3′) introduced a Cys to Ser mutation at A11 and Ser to Cys mutation at A12 of *HPI*. The mutant gene was then inserted into pBV220 and the constructed expression plasmid was used to transform *E. coli* strain DH5α. The target mutations were confirmed by DNA sequencing. The two HPI mutants as well as *HPI* and [A6,A11Ser]-*HPI* were expressed and their proteins purified following procedures described previously (*16*), and then subjected to a final purification on a RPC Resource™ (3 ml) column. The RPC column was equilibrated with distilled water containing 0.1%TFA, and eluted with a linear gradient of 0–100% acetonitrile at 2 ml/min for 10 column volumes. The FPLC-purified proteins with purity above 95% were lyophilized and stored at –20°C for later testing.

Identification of Disulfide Match by V8 Mapping—Ten micrograms of HPI or its analogues was digested with 0.5 μg of endoproteinase V8. Enzymatic digestion was performed in 0.1 M Tris-HCl (pH 7.8) at 37°C for 18 h. Then, 0.5 μl of the digest solution was mixed with 0.5 μl of matrix (saturated α-cyanocinnamic acid in 30% aqueous acetonitrile) and applied to do MALDI-TOF MS using a Bruker Biflex™ III instrument (Bremen, German) in linear mode at 19 kV.

In Vitro Refolding Assay of Fully Reduced Proteins—One milligram of HPI or its analogues was dissolved in 1 ml of 0.05 M Tris-HCl (pH 8.0), containing 8 M urea, 1 mM EDTA and 20-fold (in terms of thiol groups) excess DTT. After 2 h incubation at 37°C, the reaction mixture was dialyzed against 2,000 volumes of refolding buffer (0.05 M glycine-NaOH, pH 10.8) at 4°C for 24 h to remove the reducing reagent and denaturant. Ten microliters of each refolding product was then analyzed by both 15% native PAGE and 15% non-reductive SDS-PAGE analyses. After staining the gel with Coomassie Brilliant Blue, the proinsulin bands were quantified by densitometry using software Glyko Bandscan (Glyko, USA).

Measurement of Thiol Group Oxidation—Two milligrams of HPI or its analogues was dissolved in 1 ml of 0.05 mol/L Tris-HCl (pH 8.0), containing 8 M urea, 1 mM EDTA, and 20-fold (in terms of thiol group) excess of DTT. After 2 h incubation at 37°C, the pH of the reaction mixture was adjusted to 2–3 and then the reaction mixture was loaded onto a desalting FPLC HiTrap™ column (5 ml). The column was eluted with 0.1% TFA to remove excess DTT. The collected protein peak was mixed with 1/10 volume 0.5 M glycine-NaOH (pH 10.8) and incubated at 4°C for refolding. The numbers of thiol groups at various time periods during refolding were determined with DTNB according to the method of Ellman's (*17*).

CD Studies—HPI or its analogues was dissolved in double-distilled water (pH 7.0). Protein concentration was determined by UV absorbency and adjusted to 0.2 mg/ml. CD measurement was performed on a Jasco-715 circular dichroism spectropolarimeter using a cell with a pathlength of 2 mm. The spectrum was recorded at 25°C and was the average of four scans from 200 to 250 nm. The relative secondary structure contents were calculated according to the method of Chen & Yang (*18*).

Receptor and Antibody Binding Activities Assay—The receptor binding activity of the mutant HPI was assayed as described previously (*19*). The immune assay with

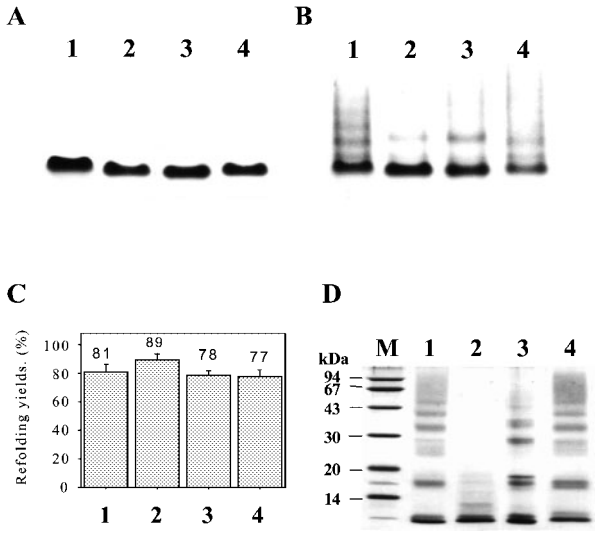

Fig. 2. **Refolding analysis of HPI and its analogues.** (A) 15% native PAGE analysis of FPLC-purified HPI and its analogues. (B) 15% native PAGE analysis of HPI and its analogues after *in vitro* refolding. (C) refolding yields of HPI and its analogues under the given conditions. The values represent mean ± SD of three independent experiments as shown in (B). (D) 15% non-reductive SDS-PAGE analysis of HPI and its analogues after *in vitro* refolding. In each panel, 1, 2, 3, 4 represent HPI, [A6,A11Ser]-HPI, [A7,A11Ser]-HPI, [A11Ser,A12Cys]-HPI, respectively.

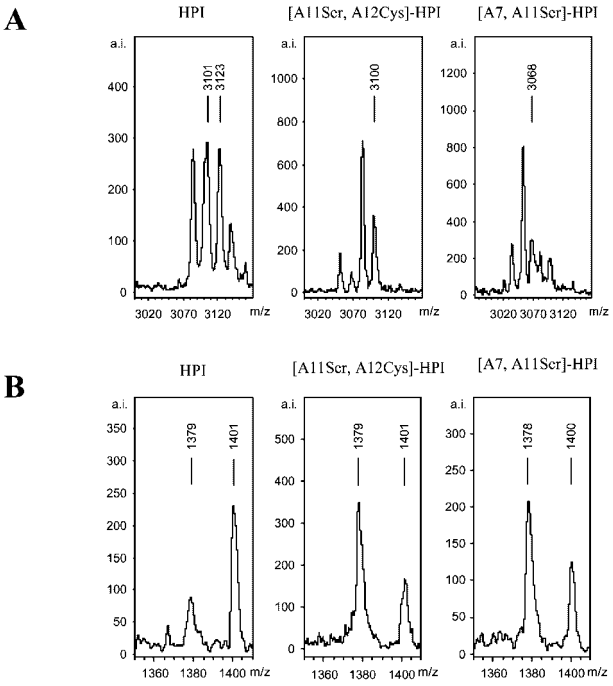

Fig. 3. **MALDI-TOF MS analysis of products of V8 digested HPI and its analogues.** (A) peaks corresponding to A (5–17)/Met + B (1–13). Measured MH^+ indicated. The theoretical MH^+ values for native HPI, [A11Ser,A12Cys]-HPI and [A7,A11Ser]-HPI are 3101.5, 3101.5, and 3067.6, respectively. The theoretical MNa^+ for native HPI is 3123.5. (B) peaks corresponding to A (18–21)/B (14–21). Measured MH^+ and MNa^+ are indicated. For HPI, [A11Ser,A12Cys]-HPI and [A7,A11Ser]-HPI, the theoretical MH^+ is 1,378.5, and the theoretical MNa^+ is 1,400.5.

insulin polyclonal antibodies was performed according to the manual of [^{125}I]-insulin radioimmunoassay kits.

Sensitivity to Tryptic Digestion—Two milligrams of HPI or its analogues was dissolved in 1 ml of 0.05 M Tris-HCl, pH 7.2. Trypsin was dissolved in 1 mM HCl at different concentrations, and 2 μl was then added to 20 μl of the HPI or analogue solutions at defined enzyme/substrate (w/w) ratios. After incubation at 37°C for 0.5 h, the reaction was stopped by adding an equal volume of 2× native PAGE loading buffer. Ten microliters of reaction mixture was applied immediately to 15% native PAGE. The corresponding des-B30Thr-insulin bands were quantified by software Glyko Bandscan.

RESULTS

Identification of Disulfide Linkages in Mutant HPIs—HPI and HPI analogues with deletion and shift mutations in the intra-A chain disulfide bond purified by reverse phase FPLC are shown to yield single major bands with similar rates of mobility on native PAGE (Fig. 2A). Unlike IGF-swap found in the *in vitro* refolding of IGF-I, no isomer was observed for HPI or its analogues. Reverse phase FPLC analysis of these proteins also showed single peaks (data not shown). HPI or the disulfide mutant HPI was digested with V8 and analyzed by MALDI-TOF MS. For HPI, two proteolytic segments with [A6–A11,A7–B7] and [A20–B19] linkages were found. The corresponding segments from the digestion products of [A7,A11Ser]-HPI and [A11Ser,A12Cys]-HPI were also found as shown in Fig. 3. The results indicate that for [A7,A11Ser]-HPI, the swap disulfide bond A6-B7, like that in IGF-swap, exists exactly as expected. All HPI analogues contain the correct A20-B19 disulfide bond.

Comparison of the In Vitro Refolding of Native and Mutant HPIs—FPLC-purified HPI and mutant HPIs were fully reduced by DTT and then dialyzed against refolding buffer (pH 10.8) for renaturation. The *in vitro* refolding products analyzed by native PAGE are shown in Fig. 2B. The relative percentage of the band representing the correctly refolded product was quantified by densitometry. The refolding yields are summarized in Fig. 2C. The reduced HPI, [A6,A11Ser]-HPI, [A7,A11Ser]-HPI, and [A11Ser,A12Cys]-HPI all refolded into a proinsulin-like conformation at a level of 80–90% under the present conditions. The refolding yield of [A6,A11Ser]-HPI was slightly higher than those of HPI and the other two HPI analogues. The same results can be seen by the non-reducing PAGE assay of the refolded products (Fig. 2D). Apart from the monomeric product, the refolding of HPI and its three mutants all gave dimers and other polymers to different degrees. Both [A6,A11Ser]-HPI and [A7,A11Ser]-HPI, especially [A6,A11Ser]-HPI, showed fewer dimers and other polymers, probably because both possess only four thiol groups as compared with HPI and [A11Ser,A12Cys]-HPI, which have six.

In Vitro Oxidation of Reduced Proteins—The time courses of oxidation of reduced HPI and mutant HPIs are shown in Fig. 4. The oxidation of thiol groups at pH 10.8 is much faster than at pH 8.3 (*8*). During the first half-hour of refolding, the thiol groups of reduced [A11Ser,A12Cys]-HPI were oxidized at a high speed similar to that for HPI. However, the oxidation of thiol

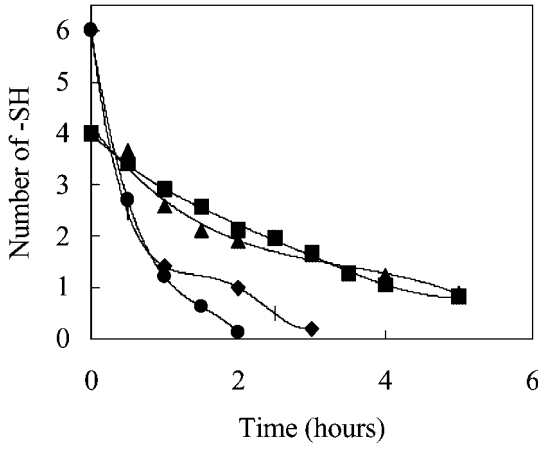

Fig. 4. **Time course of oxidation of reduced HPI and its analogues.** The values represent means of triplicate data. Circles, squares, triangles, diamonds represent HPI, [A6,A11Ser]-HPI, [A7,A11Ser]-HPI, [A11Ser,A12Cys]-HPI, respectively.

groups in the intra-A chain disulfide bond deleted HPI analogues [A6,A11Ser]-HPI and [A7,A11Ser]-HPI was much slower. One hour after the beginning of refolding, the oxidation of [A11Ser,A12Cys]-HPI became slower than that of HPI. This agrees well with previous findings that the intra-A chain disulfide bond forms first at high speed (*7*, *8*). Even shifted, the intra-A chain disulfide bond can still form quickly, and the slower oxidation of thiol groups of reduced [A6,A11Ser]-HPI and [A7,A11Ser]-HPI is due to the loss of the intra-A chain disulfide bond.

CD Spectra of Native and Mutant HPIs—The CD spectra of FPLC-purified HPI and mutant HPIs are shown in Fig. 5. The relative secondary structure contents were calculated and are listed in Table 1. Compared with native HPI, the analogues [A6,A11Ser]-HPI and [A7,A11Ser]-HPI with one disulfide bond deletion exhibited similar spectra with a large decrease in α-helical structure, while [A11Ser,A12Cys]-HPI, containing three disulfide linkages, showed less decrease in α-helix content.

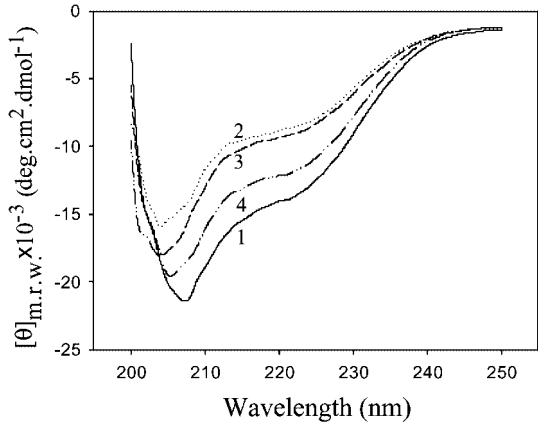

Fig. 5. **CD spectral analysis of HPI and its analogues.** The spectra are the averages of four scans from 200 to 250 nm. Curves 1, 2, 3, 4 represent HPI, [A6,A11Ser]-HPI, [A7,A11Ser]-HPI, [A11Ser,A12Cys]-HPI, respectively.

Table 1. **Secondary structure contents of native and mutant HPIs.**

Structure content (%)	α-helix	β-sheet	random coil
HPI	39.1	7.7	53.3
[A6,A11Ser]-HPI	16.1	31.6	52.4
[A7,A11Ser]-HPI	19.0	27.3	53.6
[A11Ser,A12Cys]-HPI	26.0	11.6	62.3

In Vitro Receptor and Antibody Binding Activities—The results of receptor and antibody binding assays are shown in Fig. 6 and summarized in Table 2. [A7,A11Ser]-HPI and [A11Ser,A12Cys]-HPI did not reach an IC_{50} point in receptor binding assay under our working conditions. The receptor binding activities of these two HPI analogues were much less than 1% as compared with HPI. The antibody binding activity of the two HPI analogues was also much lower as compared with HPI.

Sensitivity of Mutant HPIs to Tryptic Digestion—FPLC-purified HPI and mutant HPIs were digested with trypsin, and analyzed by native PAGE (Fig. 7). The tryptic mapping patterns of mutant HPIs were similar to that of HPI as all proteins share the same tryptic cleaving sites. Des-B30Thr-insulin and des-octapeptide-insulin as well as some intermediates could be observed during tryptic digestion. Des-B30Thr-insulin shows a similar mobility rate on native PAGE to that of porcine insulin

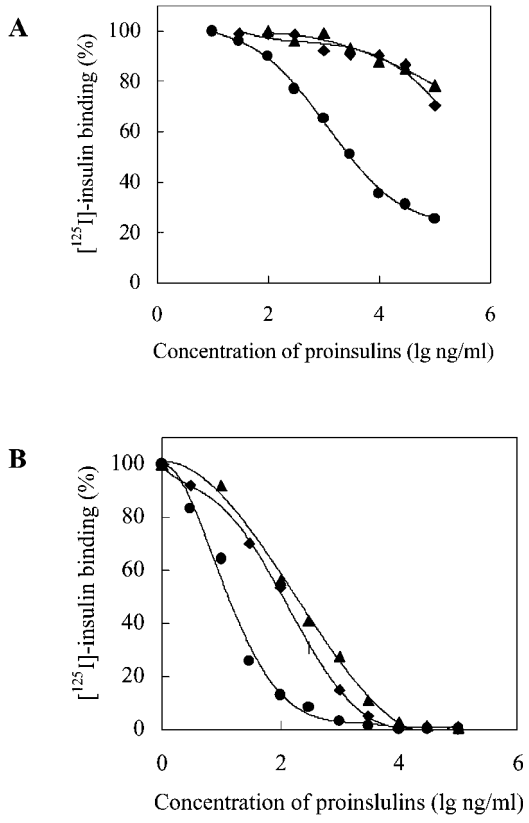

Fig. 6. **Receptor and antibody binding assays of HPI and its analogues.** The values represent means of triplicate data. (A) receptor binding assay, (B) antibody binding assay. In each panel, circles, triangles, diamonds represent HPI, [A7,A11Ser]-HPI, [A11Ser,A12Cys]-HPI, respectively.

Table 2. **Receptor and antibody binding activities of mutant HPIs as compared with HPI.**

	Receptor binding activity (%)	Antibody binding activity (%)
[A6-A11Ser]-HPI*	5.4	110
[A7-A11Ser]-HPI	≪1	2
[A11Ser, A12Cys]-HPI	≪1	6

*Data previously reported in Ref. *15*.

(Fig. 7A). The mobility rates of the mutant des-B30Thr-insulins are all somewhat slower than that of porcine insulin. Compared with HPI, analogues [A6,A11Ser]-HPI, [A7,A11Ser]-HPI and [A11Ser,A12Cys]-HPI were much more easily digested by trypsin. The production of des-B30Thr-insulin analogues at different enzyme/substrate ratios was quantified from the gels and the results are shown in Fig. 7B. [A7,A11Ser]-HPI and [A11Ser,A12Cys]-HPI acquired a 50% yield of des-B30Thr-insulin analogues at lower enzyme/substrate ratios than [A6, A11Ser]-HPI. This indicates that [A7,A11Ser]-HPI and [A11Ser,A12Cys]-HPI are more sensitive to tryptic digestion than [A6,A11Ser]-HPI.

DISSCUSION

The single band for each mutant HPI protein as shown on the native PAGE gel, suggests a unique disulfide linkage pattern for each HPI analogue (Fig. 2A). The disulfide linkages in [A6,A11Ser]-HPI are [A7–B7,A20–B19] by three-dimensional structure determination, as previously reported (*20*). The disulfide pattern in [A7,A11Ser]-HPI was confirmed to be [A6–7,A20–B19] by V8 peptide mapping. The V8 peptide mapping also indicated that a disulfide linkage between A20 and B19 exists in [A11Ser,A12Cys]-HPI. However, the pattern of intra-A chain disulfide bonding in [A11Ser,A12Cys]-HPI could not be determined because its tertiary structure is not available. Its possible disulfide linkages are [A6–A12, A7–B7,A20–B19] or [A6–B7,A7–A12,A20–B19]. The three-dimensional structure simulation of the two possible analogues indicated that the structure with the [A6–A12,A7–B7,A20–B19] linkages exhibits lower free energy and less conformational distortion than the other (data not shown). Since only one refolded product was observed for [A11Ser,A12Cys]-HPI, it is likely that the disulfide bond pattern of [A11Ser,A12Cys]-HPI is [A6–A12,A7–B7,A20–B19]. However, whatever disulfide bond pattern forms in [A11Ser,A12Cys]-HPI, position-shifted disulfide bonds do form in the region of [A6–A11,A7–B7].

The data on the physico-chemical properties and biological activities of the HPI analogues with shifted disulfide bonds provide some information on the flexible conformational changes. The CD spectra indicate that the mutant [A11Ser,A12Cys]-HPI, which still has three disulfide bonds, exhibits a smaller decrease in α-helix content than either [A6,A11Ser]-HPI or [A7,A11Ser]-HPI. This suggests that the structure of [A11Ser,A12Cys]-HPI is more compact than those of [A6,A11Ser]-HPI and [A7,A11Ser]-HPI. However, the very low receptor binding activities of [A7,A11Ser]-HPI as well as [A11Ser,A12Cys]-HPI indicate that large conformational changes occur in the receptor binding regions of these disulfide-shifted

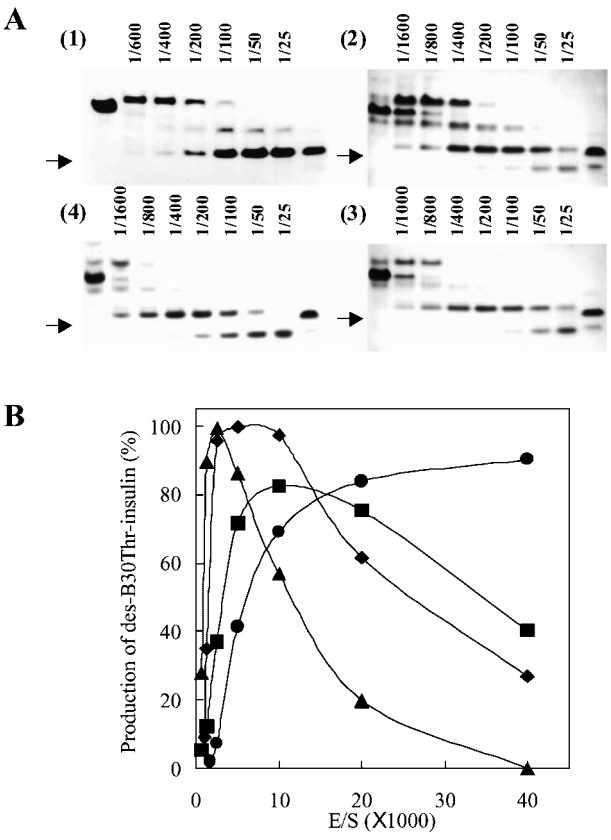

Fig. 7. **Electrophoretic analysis of the tryptic digestion products of HPI and its analogues.** (A) 15% native PAGE analysis of tryptic products of FPLC-purified HPI and its analogues at different E/S ratios. 1, 2, 3, 4 represent HPI, [A6,A11Ser]-HPI, [A7,A11Ser]-HPI, [A11Ser,A12Cys]-HPI, respectively. In each panel, the far-left lane is HPI or the corresponding mutant analogues, the far-right lane is porcine insulin as a control. Enzyme/substrate (w/w) ratios are indicated at the top of each lane. The lanes for des-B30Thr-insulin and its analogues are indicated by arrows. (B) with a plot indicating the formation of des-B30Thr-insulin analogues for each mutant HPI analogue at different E/S ratios. Curves circles, squares, triangles, diamonds represent HPI, [A6,A11Ser]-HPI, [A7,A11Ser]-HPI, [A11Ser,A12Cys]-HPI, respectively.

mutants. It has been demonstrated that the A-chain N-terminal helix is very important for the interaction of insulin with its receptor (*21*). The shift mutation of the [A6–A11,A7–B7] disulfide bonds may have a greater effect on the A-chain N-terminal helix than the deletion mutation [A6,A11Ser]-HPI. The significant decrease in the immune activities of [A7,A11Ser]-HPI and [A11Ser, A12Cys]-HPI shows larger conformational changes in the molecule as a whole. This is further supported by their higher sensitivity to tryptic digestion than [A6,A11Ser]-HPI, which is very likely due to the greater exposure of their tryptic cleaving sites. The above data indicate that flexibility in the region of the [A6–A11,A7–B7] disulfide bonds facilitates the formation of these disulfide bond shifted mutants. Interestingly, all HPI analogues with either deletion or shift mutations on the [A6–A11,A7–B7] disulfide bonds obtained high levels of *in vitro* refolding. We also reported previously a similar high *in vitro* refolding yield for an HPI analogue with an A7–B7 deletion (*22*). However, the *in vitro* refolding yields of HPI ana-

logues with shift or deletion mutations on the A20-B19 disulfide bond were found to be decreased significantly (unpublished observation). This further indicates that flexibility in the region of the [A6–A11,A7–B7] disulfide bonds is important for folding of the insulin precursor. The region in IGF-I corresponding to [A6–A11,A7–B7] in insulin is quite flexible, and disulfide exchange reaction in this region results in two refolding products with similar thermodynamic stabilities (*3*). In the case of insulin, similar flexibility is very likely to be present in the region of the [A6–A11,A7–B7] disulfide bonds. Unlike IGF-I, the refolding of insulin or its precursor gives only one monomeric product with a correct disulfide pattern. This indicates that the native disulfide pattern of insulin is the most stable among other possible patterns in term of thermodynamics. However, in the presence of denaturant, small quantities of swap-like insulin isomers could be kinetically trapped (*13*, *14*). In our case, the substitution of Cys to Ser at the region of the [A6–A11,A7–B7] disulfide bonds prevents the mutant HPIs from refolding into the native structure. This can be regarded as a kinetic trap in the folding pathway. Instead of aggregating, the flexibility in the region allows the mutant molecules to refold into a relatively stable structure. It has recently been reported that the B chain/domain mainly controls the folding behavior of insulin and IGF-I (*23*). In that study, single chain hybrids of the insulin A chain and IGF-I B domain were found to fold into two thermodynamically stable disulfide isomers just like IGF-I. This also suggests that the region of the A6–A11 disulfide bond in the insulin A chain has similar flexibility to that of IGF-I. In a recent study by Tang *et al.* on the oxidation of several Cys to Ser A-chain analogues of relaxin, another member of the insulin superfamily, a possible folding intermediate with a shifted intra-A chain disulfide bond was proposed (*24*). This indicates that some flexibility also exists in this region for the formation of the intra-A chain disulfide bond in relaxin. We thus conclude that flexibility exists not only in the region of the [A6–A11,A7–B7] disulfide bonds to allow insulin precursor folding, but also the same is also the case for other family members including IGF-I and relaxin.

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