Mutational Analysis of the Absolutely Conserved B8Gly: Consequence on Foldability and Activity of Insulin

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Abstract B8Gly is absolutely conserved in insulins during evolution. Moreover, its corresponding position is always occupied by a Gly residue in other members of insulin superfamily. Previous work showed that Ala replacement of B8Gly significantly decreased both the activity and the foldability of insulin. However, the effects of substitution are complicated, and different replacements sometimes cause significantly different results. To analyze the effects of B8 replacement by different amino acids, three new insulin/single-chain insulin mutants with B8Gly replaced by Ser, Thr or Leu were prepared by protein engineering, and both their foldability and activity were analyzed. In general, replacement of B8Gly by other amino acids causes significant detriment to the foldability of single-chain insulin: the conformations of the three B8 mutants are essentially different from that of wild-type molecules as revealed by circular dichroism; their disulfide stabilities in redox buffer are significantly decreased; their in vitro refolding efficiencies are decreased approximately two folds; the structural stabilities of the mutants with Ser or Thr substitution are decreased significantly, while Leu substitution has little effect as measured by equilibrium guanidine denaturation. As far as biological activity is concerned, Ser replacement of B8Gly has only a moderate effect: its insulin receptor-binding activity is 23% of native insulin. But Thr or Leu replacement produces significant detriment: the receptor-binding potencies of the two mutants are less than 0.2% of native insulin. The present results suggest that Gly is likely the only applicable natural amino acid for the B8 position of insulin where both foldability and activity are concerned.

Key words insulin; foldability; activity; disulfide; stability

As early as the 1960s, Anfinsen et al. first demonstrated the 3-D structure of a globular protein is uniquely determined by its amino acid sequence [1]. Since then significant advances have been made in the understanding of protein folding through experimental and theoretical approaches. For small proteins with two-state folding, topology is a major determinant of the folding rate and greatly influences the structure of the transition-state ensemble [2–4]. Studies on the disulfide-coupled folding of some small globular proteins, such as bovine pancreatic trypsin inhibitor and ribonuclease A, have revealed a sequence of preferred kinetic intermediates, which define a folding pathway [5–11]. In vitro the protein folding is assisted by the molecular chaperones and folding enzymes, especially for large proteins [12–14], some chaperones can even provide the missing steric information for protein folding [15].

Insulin is a structurally and functionally well-characterized small globular protein containing A- and B-chain linked by three disulfides (one intrachain bond, A6-A11; two interchain bonds, A7-B7 and A20-B19). Its 3-D structure has been well solved by X-ray crystallography [16,17] and nuclear magnetic resonance (NMR) [18]. In vitro the separate A- and B-chain of insulin can be recombined efficiently [19], but in vivo a single-chain polypeptide precursor (preproinsulin) is synthesized. When B29Lys and A1Gly were linked by a peptide bond directly,
the mini-proinsulin still retained the 3-D structure identical to that of insulin [20, 21]. Our laboratory has constructed a single-chain insulin (PIP) in which the C-terminal of porcine insulin B-chain and the N-terminal of its A-chain were linked together by a dipeptide, Ala-Lys. PIP can fold correctly and be secreted efficiently from transformed yeast cells, and can be converted to insulin by enzymatic treatment [22]. It can be reasonably presumed that the 3-D structure of PIP is identical or very similar to that of insulin/mini-proinsulin.

Although significant advances have been made in the understanding of protein folding, an understanding of how the folding information is stored in the amino acid sequence is still elusive. Through protein engineering, various mutants with one specific residue replaced by different amino acids can be prepared and analyzed. This makes it possible to analyze the contribution of individual residues to the foldability and activity of the whole polypeptide chain. The structure and function of insulin have been extensively investigated, and in vitro PIP can spontaneously fold into native structure with a defined folding pathway characterized by kinetically preferred intermediates [23–25]. Therefore, insulin/PIP provides a well-characterized model for the study of protein folding as well as structure-function relationships. In insulins B8Gly is absolutely conserved during evolution, moreover, its corresponding position is always occupied by a Gly residue in other members of the insulin superfamily, such as insulin-like growth factor I and II, relaxin, and bombyxin. B8Gly is located at the conjunction of an invariant α-helix (B9–B19) and a segment of variable conformation (B1–B8). The latter segment undergoes the T (extended)→R (helical) transition in insulin hexamer. In R-state the main-chain dihedral angles are those of an L-amino acid, while in T-state B8Gly lies in the D-region of the Ramachandran plot. Using combinatorial peptide chemistry, Zhao et al. synthesized B8 L-amino acid and D-amino acid peptide libraries [26]. The B8 L-amino acid library yielded only a trace amount of insulin, while the D-amino acid library resulted in native foldability. Replacement of B8Gly by Ala significantly decreased the receptor-binding activity of insulin [27–29] and the foldability of PIP [30]. These suggest that B8Gly contributes to both the activity and foldability of insulin. However, the effects of replacement are complicated, and different substitutions sometimes cause essentially different results.

To further understand the contribution of B8Gly to both the activity and foldability of insulin, we prepared three new insulin/PIP mutants with B8Gly replaced with Ser, Thr or Leu by means of protein engineering. These three residues were chosen mainly because they are different with Ala in size or in hydrophilic property. The effects of the replacements on the foldability and activity of insulin/PIP were analyzed. The results also suggest that Gly is likely the only applicable natural amino acid for the B8 position of insulin when both activity and foldability are considered.

Materials and Methods

Materials

The Escherichia coli strains DH12S and RZ1032 (dut-, ung-) were used. Saccharomyces cerevisiae XV700-6B (leu2, ura3, pep4) and helper phage R408 were kindly provided by Michael SMITH (University of British Columbia, Vancouver, Canada). Plasmid pVT102-U/αMFL-PIP for secretory expression of PIP was constructed previously [22]. The mutagenesis oligonucleotide primers were chemically synthesized. The other chemical reagents used were of analytical grade.

The Vydc reverse-phase C8 column (5 µm, 4.6 mm×250 mm) (Vydc, Hesperia, USA), Gilson 306 HPLC system (Gilson, Beltline, USA), and Gilson 115 UV (Gilson) detector were used. In the HPLC analysis, a gradient elution was used. Solvent A was 0.15% aqueous trifluoroacetic acid (TFA); solvent B was 60% acetonitrile containing 0.125% TFA. The elution gradient was as follows: 0 min, 0% solvent B; 1 min, 0% solvent B; 2 min, 0% solvent B; 35 min, 80% solvent B; 36 min, 100% solvent B; 38 min, 100% solvent B; 40 min, 0% solvent B; 45 min, 0% solvent B.

DNA manipulation

The expression vectors encoding the B8 mutants were constructed using a gapped duplex DNA approach for site-directed mutagenesis [31]. The plasmid pVT102-U/αMFL-PIP was used as the mutagenesis template, and the expected mutations were confirmed by DNA sequencing.

Expression and purification of B8 mutants

The expression vectors encoding the B8 mutants were transformed into S. cerevisiae XV700-6B (leu2, ura3, pep4), respectively. Then the transformed yeast cells were cultured in a 16-liter fermenter and the secreted PIP mutants were purified from the media supernatant as follows. The target protein in the media supernatant was precipitated by trichloroacetic acid at the final concentration of 5% (W/V). The pellet containing the target protein was
dissolved with 1 M acetic acid several times, and each time the resulting supernatant was applied to a Sephadex G-50 column (Pharmacia, Uppsala, Sweden) pre-balanced with 1 M acetic acid. Then the product separated by gel filtration was lyophilized and applied to an ion-exchange column (DEAE-Sepharose-CL-6B) pre-balanced with 1 M acetic acid. The eluted analog from the ion-exchange column was lyophilized, then dissolved with 2–3 ml water, acidified to pH 2.0 with TFA, and centrifuged. The pellet containing the mutant was further purified by C4 reverse-phase HPLC. The purity of these three analogs was analyzed by native polyacrylamide gel electrophoresis (PAGE), pH 8.3, and analytical C8 reverse-phase HPLC, respectively.

Circular dichroism analysis and guanidine titration

The samples were dissolved in 35 mM phosphate buffer (pH 7.0). The protein concentration was determined by UV absorbance at 276 nm with the extinction coefficient of 1.0 ml-mg⁻¹-cm⁻¹, and the final concentration was adjusted to 0.2 mg/ml. Circular dichroism (CD) measurements were performed on a Jasco-715 CD spectropolarimeter (Jasco Corp., Tokyo, Japan) at 25 ºC. The near-UV spectra were scanned from 300 nm to 245 nm with a cell of 1.0 cm path length; the far-UV spectra were scanned from 250 nm to 190 nm with a cell of 0.1 cm path length. The data were expressed as molar ellipticity. The software J-700 for Windows Secondary Structure Estimation (Version 1.10.00; Jasco Corp.) was used for secondary structural content evaluation from CD spectra.

For guanidine titration the samples were dissolved to a final concentration of 0.2 mg/ml in 35 mM phosphate buffer (pH 7.0) containing different concentrations of guanidine chloride. The spectra of samples in different concentrations of denaturant were recorded from 230 nm to 220 nm at 25 ºC. The CD signal at 222 nm was used to monitor the denaturation, and the measured data were fitted with the two-state model.

Disulfide stability in redox buffer

The wild-type PIP and the B8 mutants were dissolved in the buffer (0.1 M Tris-HCl, 1 mM EDTA, pH 8.7) containing different concentrations of reduced glutathione (GSH; Amresco, Solon, USA) and oxidized glutathione (GSSG; Amresco), respectively. The final protein concentration was 0.15 mg/ml. The reaction was carried out at 0 ºC overnight. After incubation, one-fifth volume of freshly prepared 0.5 M sodium iodoacetate solution was added to modify the free thiol groups. Then the mixture was analyzed by native PAGE, pH 8.3.

In vitro refolding analysis

The sample was reduced in buffer containing 0.5 M Gly-HCl, 1 mM EDTA, pH 9.5, 10 mM dithiothreitol (DTT; Sigma, St. Louis, USA) at 15 ºC for 30 min. The final concentration of sample is 0.5 mg/ml. After reduction 6 µl of solution was removed and immediately mixed with 3 µl sodium iodoacetate solution (0.5 M). The carboxymethylation was carried out at room temperature for 5 min, then the sample was analyzed by native PAGE, pH 8.3, to examine whether the samples were fully reduced. The fully reduced sample was 10-fold diluted with the refolding buffer. The refolding was carried out under the conditions indicated in Table 1 for 2 h. After incubation, 100 µl of refolding mixture was acidified to pH 2.0 with TFA, then analyzed by C8 reverse-phase HPLC and detected at 230 nm. The refolding yield was calculated from the peak area of the folded sample.

**Table 1** Refolding yields of B8 mutants under different refolding conditions

<table>
<thead>
<tr>
<th>Sample</th>
<th>T (ºC)</th>
<th>pH</th>
<th>GSSG (mM)</th>
</tr>
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<tbody>
<tr>
<td>PIP</td>
<td>65</td>
<td>44</td>
<td>65</td>
</tr>
<tr>
<td>[B8Ser]PIP</td>
<td>35</td>
<td>26</td>
<td>35</td>
</tr>
<tr>
<td>[B8Thr]PIP</td>
<td>39</td>
<td>22</td>
<td>39</td>
</tr>
<tr>
<td>[B8Leu]PIP</td>
<td>33</td>
<td>19</td>
<td>33</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th>Sample</th>
<th>T (ºC)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>PIP</td>
<td>40</td>
<td>65</td>
<td>60</td>
</tr>
<tr>
<td>[B8Ser]PIP</td>
<td>26</td>
<td>35</td>
<td>22</td>
</tr>
<tr>
<td>[B8Thr]PIP</td>
<td>25</td>
<td>39</td>
<td>32</td>
</tr>
<tr>
<td>[B8Leu]PIP</td>
<td>14</td>
<td>33</td>
<td>30</td>
</tr>
</tbody>
</table>

* in pH 9.5 buffer and 2 mM GSSG; † at 16 ºC and 2 mM GSSG; ‡ in pH 9.5 buffer and at 16 ºC. The refolding was carried out under the conditions indicated for 2 h. GSSG, oxidized glutathione; PIP, single-chain insulin.

Conversion of single-chain mutants to double-chain mutants

The purified B8 PIP analogs were dissolved in the reaction buffer (0.1 M NH₄HCO₃, pH 8.5) at a final concentration of 3 mg/ml. Then Lys-C endoproteinase was added to the solution at a mass ratio of 1:500. The enzymatic cleavage was carried out at 25 ºC overnight, then [desB30]insulin analogs were purified by C8 reverse-phase HPLC.

Insulin receptor-binding assay

The receptor-binding assay of [desB30]insulin mutants with insulin receptor was performed using human placental
membrane as previously described [32]. A total of 250 µg membrane insulin receptor was incubated with 125I-insulin (approximately 10^5 cpm) and 0.4 ml native insulin or sample solution (50 mM Tris-HCl, 1% BSA, pH 7.5) at 4 °C overnight. After incubation the unbound 125I-insulin was washed with ice-cold buffer (50 mM Tris-HCl, 0.1% BSA, pH 7.5) three times and the radioactivity of the precipitate was measured. The receptor binding activity of the sample was calculated from the concentration that caused 50% inhibition of 125I-insulin binding to the receptor.

Results

Expression and purification of the B8 mutants

Three B8 mutants, [B8Ser]PIP, [B8Thr]PIP and [B8Leu] PIP, were purified from the fermentation supernatant as described in “Materials and Methods”. After purification, the purities were analyzed by analytical C8 reverse-phase HPLC and native pH 8.3 PAGE. As shown in Fig. 1, the three PIP analogs showed a single peak on HPLC and a single band on native PAGE, therefore all of them were homogeneous. Their molecular weights were analyzed by electrospray mass spectrometry and are listed in Table 2. All of the measured values were well consistent with the theoretical values, suggesting the expected mutations had occurred in the three analogs. As far as the secretion yield was concerned, [B8Leu]PIP was approximately 3–4 mg obtained from 8 liters of fermentation supernatant; [B8Ser]PIP and [B8Thr]PIP were approximately 1–2 mg; while the wild-type PIP was approximately 50 mg from the same volume of fermentation supernatant. Therefore, mutation of B8Gly had significant detriment on the secretion efficiency of PIP from transformed yeast cells. Additionally, the secretion efficiency of [B8Leu]PIP was higher than that of [B8Ser]PIP and [B8Thr]PIP. This difference was probably caused by their different structural stability: [B8Leu]PIP was much more stable than the other two B8 mutants as shown by following stability analysis. This result was confirmed by direct analysis of the secretion product in the medium supernatant by native PAGE (pH 8.3, silver staining) and densitometry (data not shown).

Circular dichroism analysis on secondary structure

The conformational changes of the three B8 mutants were analyzed by CD. As shown in Fig. 2, both near-UV and far-UV spectra of the three mutants were different from that of the wild-type PIP. The estimated α-helix content of [B8Ser]PIP, [B8Thr]PIP, [B8Leu]PIP and wild-type PIP was 33%, 29%, 36% and 46%, respectively. Therefore, replacement of B8Gly by Ser, Thr, or Leu had significant effect on the conformation of PIP. Although the present CD analysis suggests that the secondary/tertiary structures of the three mutants had been disturbed, it is difficult to deduce where the disturbance occurred. High-resolution analyses (crystal or NMR) will be used to analyze the structural disturbance of these mutants in future.

Structural stability analysis

Our previous results showed that Ala substitution at the B8 position significantly decreased the structural stability of PIP [30]. Here, the effects of different mutations on the structural stability of PIP were analyzed using guanidine titration. The denaturation was monitored by CD, and the signals at 222 nm, a helix-sensitive wavelength, were used to plot against the concentrations of denaturant, guanidine.

Table 2 Molecular masses of B8 mutants measured by electrospray mass spectrometry

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Theoretical MW (Da)</th>
<th>Measured MW (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[B8Ser]PIP</td>
<td>5988.9</td>
<td>5989.0</td>
</tr>
<tr>
<td>[B8Thr]PIP</td>
<td>6003.0</td>
<td>6003.0</td>
</tr>
<tr>
<td>[B8Leu]PIP</td>
<td>6015.0</td>
<td>6016.0</td>
</tr>
</tbody>
</table>

PIP, single-chain insulin. MW, molecular weight.
Fig. 2  Circular dichroism analysis of three B8 single-chain insulin (PIP) mutants

(A) In far-UV region. (B) In near-UV region.

Fig. 3  Normalized equilibrium denaturation curves of three B8 single-chain insulin (PIP) mutants

Table 3  Thermodynamic parameters of B8 mutants analyzed by equilibrium guanidine denaturation

<table>
<thead>
<tr>
<th>Mutant</th>
<th>C_{mid} (M)</th>
<th>ΔGº (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type PIP</td>
<td>5.5±0.1</td>
<td>20.0±0.5</td>
</tr>
<tr>
<td>[B8Ser]PIP</td>
<td>4.7±0.1</td>
<td>14.6±0.5</td>
</tr>
<tr>
<td>[B8Thr]PIP</td>
<td>4.7±0.1</td>
<td>14.6±0.5</td>
</tr>
<tr>
<td>[B8Leu]PIP</td>
<td>5.6±0.1</td>
<td>20.2±0.5</td>
</tr>
</tbody>
</table>

PIP, single-chain insulin.

chloride. The normalized denaturation curves of wild-type PIP and the three B8 mutants are shown in Fig. 3, and the calculated thermodynamic parameters, $C_{\text{mid}}$ and $\Delta G^\circ$, are listed in Table 3. Both Ser and Thr replacement of B8Gly significantly decreased the structural stability of PIP; this is similar to the effect of Ala substitution. Unexpectedly, Leu replacement of B8Gly has little effect on the structural stability of PIP.

Disulfide stability analysis

The structure of PIP/insulin must be stabilized by its three disulfides. In turn, the structure affects the disulfide stability. Here, the effect of B8 mutation on the disulfide stability of PIP was analyzed. As shown in Fig. 4, on each gel the upper band is the native species with intact disulfides (N), and the lowest band is the species whose disulfides are fully reduced (R). For each sample, the disulfides of some species were reduced after incubation in the redox buffer, and these reduced species ran faster on the native PAGE after modification of their free thiol groups with sodium iodoacetate. On each gel, the different lanes represent different redox potentials (GSH:GSSG) where the sample is incubated. From lane 2 to lane 8, the ratio of GSH:GSSG was increased gradually, therefore more and more native species were converted into reduced species. The more stable the disulfides, the higher the ratio of GSH:GSSG needed in order to convert the same percentage of native species into reduced species. As shown in Fig. 4, for wild-type PIP, when 50% of native species were converted into reduced species, the ratio of GSH:GSSG is
is concerned, the B8 position is tolerant to Ser substitution, but not tolerant to Thr or Leu replacement.

Discussion

In the present study, three new PIP/insulin mutants in which the absolutely conserved B8Gly was replaced by Ser, Thr or Leu, were prepared and their foldability and activity were analyzed. Together with previous results [30], it is known that B8Gly is critical for the foldability of the insulin. Once B8Gly was mutated, the foldability of insulin decreased significantly; the native structure was disturbed; the in vitro refolding efficiency was decreased; the disulfides were more easily reduced in redox buffer; and the structural stability was decreased (except in the instance of Leu replacement). In terms of biological activity, B8Gly replacement with Ala, Thr or Leu has serious detriment. According to the insulin-receptor interaction model [28, 33], B8Gly does not directly interact with the insulin receptor. Therefore, the direct contribution of the B8 position is likely to be on foldability, and the decrease in biological activity is the result of local and/or global structural disturbance caused by B8 replacement. The B8 position is located at the conjunction of an invariant α-helix (B9–B19) and a segment of variable conformation (B1–B8). Although the R-state conformation has been observed in crystal, in solution the conformation of insulin/mini-proinsulin is similar to the T-state of the crystal structure [17,18,20]. In the T-state, the main-chain dihedral angles of the B8 position are those of a D-amino acid. In
the natural amino acids, only Gly is a non-chiral residue and can adopt the main-chain dihedral angles of D-amino acid. Therefore, Gly is likely the only applicable natural amino acid for the B8 position as far as foldability and activity are concerned.

Some interesting phenomena were observed in the present study. B8 substitutions usually significantly decrease the structural stability as measured by guanidine titration. However, it is tolerant to Leu replacement. Because Leu has a much larger side-chain than Ala, Ser, or Thr, it is difficult to deduce how the large side-chain of Leu is accommodated in the mutant. In terms of biological activity, the B8 position is tolerant to Ser substitution but not to Ala, Ser or Leu replacement. Ser replacement has serious detriment on foldability, why is it tolerant for activity? To answer these questions, the high-resolution structures of these mutants need to be solved.

References

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281