Insulin analogs with B24 or B25 phenylalanine replaced by biphenylalanine

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B24 and B25 phenylalanines (Phe) play important roles in insulin structure and function. Insulin analogs with B24 Phe or B25 Phe replaced by biphenylalanine (Bip) were prepared by enzymatic semisynthesis. The biological activities were determined by receptor binding assay and in vivo mouse convulsion assay. The results showed that B25 Bip insulin has 139% receptor binding activity and 50% in vivo biological activity, whereas B24 Bip insulin is inactive, when compared with native insulin, suggesting that B24 Phe is crucial for insulin activity. The structures in solution were studied by circular dichroism and fluorometry, and our results suggested that the insulin analogs with low activities tend to be more tightly packed. The association properties were studied by size exclusion chromatography. The Bip-amide replacement of B24 Phe in deshexapeptide insulin or B25 Phe in despentapeptide insulin will cause the monomeric B24 Phe-amide deshexapeptide insulin or B25 Phe-amide despentapeptide insulin to associate and form dimers, whereas the mutations of B24 Phe in insulin will make insulin dimers dissociate into insulin monomers.

Keywords insulin; biphenylalanine; circular dichroism; fluorometry; self-association

Insulin is a protein hormone produced in pancreatic islet cells and stored in the form of hexamers composed of zinc-coordinated dimers. After secreting into the bloodstream, insulin oligomers are dissociated into monomers that bind with insulin receptors to express the biological activities [1]. It has been known that B24 phenylalanine (Phe) and B25 Phe are important not only in maintaining insulin conformation, but also in expressing insulin activity. First, these two residues are highly conserved [2]. Second, the crystal structure of insulin revealed that B24 Phe and B25 Phe play important roles in the formation of dimers and hexamers [3]. Finally, the mutation of B25 Phe to Leu (“insulin Chicago”) and the mutation of B24 Phe to Ser (“insulin Los Angeles”) reduced insulin activity and caused diabetes [4].

It was found that B24 Phe mainly affected the flexibility of its neighboring backbone, important in receptor binding of insulin [5]. Nakagawa and Tager reported that insulin activity was almost totally lost when B25 Phe was replaced by Ser, but when B25 Phe was replaced by a much larger amino acid, naphthyl alanine, 50% of the receptor binding activity and 66% of the in vitro biological activity were retained [6]. Quan et al. [7] reported the receptor binding activities of 19 insulin analogs with B24 or B25 Phe replaced by natural or unnatural amino acids, showing that insulin activity was highly dependent on the aromatic character of these two residues and the position B24 was extremely restrictive to structural modification, whereas B25 was extremely permissive.

Desoctapeptide insulin (DOI, insulin with B23–B30 removed) and desheptapeptide insulin (insulin with B24–B30 removed), both without B24 Phe and B25 Phe, were inactive. Despentapeptide insulin (DPI, insulin with B26–B30 removed), containing both B24 Phe and B25 Phe, were active [8]. Deshexapeptide insulin (DHI, insulin with B25–B30 removed), containing only B24 Phe obtained in our laboratory by enzymatic semisynthesis, was found to be still active, although a little less active than DPI [9]. In addition, B25 Phe-amide DPI with the C-terminal B25 Phe replaced by Phe-amide has higher receptor binding activity than DPI [10].

Biphenylalanine (Bip) is a highly hydrophobic unnatural amino acid with intrinsic fluorescence. Here, we report the enzymatic semisyntheses of analogs of insulin with B24 or B25 Phe replaced by Bip, and analogs of DHI and DPI with B24 Phe and B25 Phe replaced by Bip-amide. The influence of Bip replacement on the structure and...
function of insulin was studied by circular dichroism (CD), fluorometry, in vitro receptor binding assay, and in vivo biological assay.

Materials and Methods

Materials
Zn-free insulin was prepared from crystalline porcine insulin purchased from Nova Biomedical (Waltham, USA) as previously described [11]. Tosylphenylalanine chloromethyl ketone (TPCK)-trypsin and trifluoroacetic acid (TFA) were from Sigma-Aldrich (St Louis, USA). 1,4-butanediol was from Tokyo Chemical Industry (Tokyo, Japan). Fmoc-amino acids, H-Thr(tBu)-2-Chlorotryptyl resin and rink amide MBHA resin were from GL Biochem (Shanghai, China). Other reagents were of analytical grade. Sephadex G25 fine, diethylaminoethyl-Sephadex A25, and Superdex G75 HR 10/30 columns were from Amersham Pharmacia Biotech (Uppsala, Sweden). Human placental membrane was a gift from Prof. Youmin Feng at the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

Synthesis of oligo peptides
Peptides were synthesized using the Fmoc strategy on an ABI-433A peptide synthesizer (Applied Biosystems, Foster City, USA). The octapeptides, GlyBipPheTyr(tBu)Thr(tBu) ProLys(Boc)Thr(tBu) and GlyPheBipTyr(tBu)Thr(tBu) ProLys(Boc)Thr(tBu) were cleaved from the H-Thr-(tBu)-2-Chlorotryptyl resin by anhydrous TFA/dichloromethane (F/V=1:99). The products were washed with water, dissolved in 0.1% TFA/90% acetonitrile, and lyophilized. The dipeptide GlyBip-amide and tripeptide GlyPheBip-amide were cleaved from the rink amide MBHA resin with 97% TFA. The cleaved peptides were dissolved in water and lyophilized. Purity of all peptides was checked by HPLC using a Waters Series 600 (Waters, Milford, USA) and mass spectroscopy using an API2000 Q-trap mass spectrometer (Applied Biosystems).

Enzymatic semisynthesis of insulin analogs
The preparation of DOI and the semisyntheses of the insulin analogs were carried out as described previously [10]. The synthetic peptide (60 mM) and DOI (6 mM) were dissolved in a solution containing 30% dimethylformamide and 60% 1,4-butanediol with pH adjusted to 7.0 with Tris. TPCK-trypsin (enzyme/DOI ratio 1:10 by weight) was added and the reaction mixture was incubated at 37 °C overnight. The crude product precipitated by acetone at 4 °C was purified by HPLC and lyophilized. Insulin analogs with protected side chains were treated with anhydrous TFA for 1 h to remove the protecting groups and purified by HPLC.

In vivo biological activity assay
The in vivo biological activity was measured by the mouse convulsion test according to the Chinese Pharmacopoeia [12]. Briefly, ICR mice (18–20 g, purchased from the Sino-British Sippr-BK Experimental Animal Ltd. (Shanghai, China) were fasted overnight. From 1 mg/ml stock solutions, samples of different dosages were prepared by serial dilutions in saline. For each dosage, 10 ICR mice were injected with sample (0.2 ml/mouse) and put into a 35 °C chamber. The convulsion responses of mice were observed and recorded. The activity was calculated by the ratio of the insulin dosage with the insulin analog dosage just producing a convulsion rate over 50%.

In vitro receptor binding assay
Receptor binding assay was carried out using human placental membrane as described previously [13]. The membrane containing approximately 0.2 mg protein was incubated at 4 °C overnight with 125I-labeled insulin (approximately 10^6 cpm) plus a selected amount of insulin or analogs in 0.6 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 1% bovine serum albumin. The unbound 125I-labeled insulin was removed by centrifugation and the precipitate was washed with the same buffer pre-cooled on ice. The radioactivity in the precipitate was counted. The receptor binding activities of the insulin analogs were expressed as the ratio of the insulin IC_50 with the insulin analogs IC_50 each determination was carried out in duplicate.

Fluoremetry
Fluorescence spectra from 290 to 400 nm were recorded on a Hitachi model F-2500 FL spectrophotometer (Hitachi Instruments, San Jose, USA), using a 4 ml cell with 1 cm path length, with the exciting wavelength of 264 nm. Samples were dissolved in 10 mM phosphate-buffered saline (PBS), pH 7.4, to final concentrations of 17 µM. Anisotropies (r) were calculated from the maximal emission intensity I and the instrumental correction factor G according to the following equation (Equation 1):

\[ r = (I_{vv} - GI_v) / (I_{vv} + 2GI_v) \]

where \( G = I_{vv} / I_{hh} \), \( I_{vv} \) is the vertical emission intensity when excited with a horizontally polarized light, \( I_{hh} \) is the horizontal emission intensity when excited with a
horizontally polarized light, $I_{VV}$ is the vertical emission intensity when excited with a vertically polarized light, and $I_{VH}$ is the horizontal emission intensity when excited with a vertically polarized light source. Data were expressed as averages of three scans [14].

**CD analysis**

Samples were dissolved in PBS (pH 7.4) and measured in a Jasco-715 spectropolarimeter (Jasco, Tokyo, Japan) at room temperature [15]. The far-ultraviolet (UV; 250–190 nm) spectra were recorded at a concentration of 0.2 mg/ml and cell path of 0.1 cm. The near-UV (300–245 nm) CD spectra were recorded at a concentration of 0.5 mg/ml and cell path of 1.0 cm.

**Size exclusion chromatography**

Superdex G75 column (HR 10/30) was used for the size exclusion chromatography [16]. Sample (0.04 ml) with different insulin concentrations (38 µM, 75 µM, 150 µM, and 300 µM in PBS, pH 7.4) was loaded onto the column and the column was eluted at room temperature with PBS (pH 7.4) at a flow rate of 0.5 ml/min. The absorbance at 230 nm was monitored.

**Results**

**Characterization of insulin analogs**

Insulin analogs prepared by enzymatic semisynthesis and purified by HPLC were analyzed by mass spectrometry (Table 1) and pH 8.3 native polyacrylamide gel electrophoresis (Fig. 1). The molecular masses of insulin analogs are consistent with their theoretical values. In native polyacrylamide gel electrophoresis, each insulin analog shows a single band.

**Receptor binding activity and in vivo biological activity**

The receptor binding activities and in vivo biological activities are shown in Table 2 and Fig. 2. B25 Bip insulin has 139% receptor binding activity and 50% in vivo biological activity when compared with native insulin, whereas B24 Bip insulin has almost no receptor binding activity.

**Table 1 Molecular masses of insulin analogs determined with an API2000 Q-trap mass spectroscope**

<table>
<thead>
<tr>
<th>Insulin analog</th>
<th>Theoretical value</th>
<th>Experimental value</th>
</tr>
</thead>
<tbody>
<tr>
<td>B24 Bip insulin</td>
<td>5883.68</td>
<td>5883.0</td>
</tr>
<tr>
<td>B25 Bip insulin</td>
<td>5883.68</td>
<td>5883.0</td>
</tr>
<tr>
<td>B24 Bip-amide DHI</td>
<td>5144.30</td>
<td>5143.6</td>
</tr>
<tr>
<td>B25 Bip-amide DPI</td>
<td>5291.50</td>
<td>5291.0</td>
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</tbody>
</table>

Spectroscope supplied by Applied Biosystems, Foster City, USA. Bip, biphenylalanine; DHI, deshexapeptide insulin; DPI, despentapeptide insulin.

**Table 2 Receptor binding and in vivo biological activities of insulin analogs**

<table>
<thead>
<tr>
<th>Insulin analog</th>
<th>Relative activity</th>
<th>Receptor binding activity</th>
<th>In vivo biological activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>100.00±0.00</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>B25 Bip insulin</td>
<td>139.20±1.20</td>
<td>~50</td>
<td></td>
</tr>
<tr>
<td>B25 Bip-amide DPI</td>
<td>58.10±0.60</td>
<td>~50</td>
<td></td>
</tr>
<tr>
<td>B24 Bip insulin</td>
<td>0.87±0.01</td>
<td>≤2</td>
<td></td>
</tr>
<tr>
<td>B24 Bip-amide DHI</td>
<td>0.74±0.01</td>
<td>~20</td>
<td></td>
</tr>
</tbody>
</table>

Bip, biphenylalanine; DHI, deshexapeptide insulin; DPI, despentapeptide insulin.
activity or in vivo biological activity. That is, B25 Phe but not B24 Phe could be replaced by the unnatural amino acid Bip. In our earlier studies, the coupling of B23 Gly and B24 Phe to inactive DOI could convert it into active DHI [9]. Furthermore, in insulin from different species, B24 Phe is highly conserved. These results indicate that B24 Phe is indispensable for insulin activity, whereas B25 Phe only helps to reinforce the insulin activity. In DPI and DHI analogs with Bip-amide replacement, B25 Bip-amide DPI showed 50% in vivo biological activity, similar to B25 Bip insulin, whereas B24 Bip-amide DHI still retained 20% in vivo biological activity.

Fluorescence analysis

Bip has strong fluorescence, so these insulin analogs can be studied by fluoremetry. The results are shown in Table 3 and Fig. 3. Compared to B24 Bip insulin and B24 Bip-amide DHI, the fluorescence intensity of B25 Bip insulin and B25 Bip-amide DPI is larger, indicating that B25 Bip is more exposed than B24 Bip. This is consistent with the crystal structure of native insulin [3]. Compared to B24 Bip insulin, the r value of B24 Bip-amide DHI is smaller, indicating that B24 Bip is a little less tightly packed with the removal of B25–B30. Compared to B25 Bip insulin, the r value of B25 Bip-amide DPI is larger, indicating that B25 Bip is more tightly packed with the removal of B26 to B30 [14].

CD analysis

B25 Bip insulin and insulin have similar CD spectra with troughs at 222 nm and 208 nm, respectively (Fig. 4), indicating that they have similar conformations in solution. The CD spectrum of B24 Bip insulin also has double troughs but with increased absolute value of the mean residue molar ellipticity |θ| at 222 nm and 208 nm, indicating that B24 Bip insulin has a more compact structure [Fig. 4(A)] [17, 18]. The CD spectra of B24 Bip-amide DHI and B25 Phe-amide DHI are similar [Fig. 4(B)], whereas |θ|222 nm and |θ|208 nm of B25 Bip-amide DPI are larger than those of B25 Phe-amide DPI, indicating that B25 Bip-amide DPI has a more compact structure [Fig. 4(C)]. Fig. 4(D) is the near-UV CD spectra of insulin and insulin analogs with B24 Bip or B25 Bip. Their trough values show the following differences:

|θ|224 Bip insulin<|θ|insulin<|θ|B25 Bip insulin
indicating that the association tendency of B24 Bip insulin is lower, but that of B25 Bip insulin is higher, than that of insulin [19–22].

Association behavior analyzed by size exclusion chromatography

The association behaviors of insulin analogs studied by size exclusion chromatography are shown in Fig. 5. From the profiles of size exclusion chromatography, it can be seen that the Bip-amide replacement of B24 Phe in DHI or B25 Phe in DPI will cause the monomeric B24 Phe-amide DHI or B25 Phe-amide DPI to associate and form dimers, whereas the replacement of B24 Phe in insulin will make insulin dimers dissociate into insulin monomers. The dissociation of insulin by Bip replacement at B24 is consistent with an earlier report that B24 Ala insulin is monomeric [23], and also consistent with the CD spectrum of B24 Bip insulin that has the smallest |θ| in the near-UV region [Fig. 4(D)]. B25 Bip-amide DPI tends to form dimers, whereas B25 Phe-amide DPI is monomeric at the same concentration, indicating that the increased hydrophobicity of B25 Bip facilitates the dimer formation.

Table 3 Fluorescence anisotropy values (r) of insulin analogs

<table>
<thead>
<tr>
<th>Insulin analog</th>
<th>r</th>
</tr>
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<tbody>
<tr>
<td>B25 Bip insulin</td>
<td>0.091±0.001</td>
</tr>
<tr>
<td>B25 Bip-amide DPI</td>
<td>0.209±0.008</td>
</tr>
<tr>
<td>B24 Bip insulin</td>
<td>0.150±0.010</td>
</tr>
<tr>
<td>B24 Bip-amide DHI</td>
<td>0.110±0.010</td>
</tr>
</tbody>
</table>

Bip, biphenylalanine; DHI, deshexapeptide insulin; DPI, despentapeptide insulin.

Fig. 3 Fluoresmetric spectra of insulin analogs with dipeptide (Gly-Bip) and insulin as positive and negative controls, respectively. The exciting wavelength was 264 nm and the emission spectra from 290 to 400 nm were recorded. DHI, deshexapeptide insulin; DPI, despentapeptide insulin.
Discussion

B25 Bip insulin is highly fluorescent and its structure in solution is similar to insulin as shown by their CD spectra. The replacement of B25 Phe of insulin by Bip increases its receptor binding activity and retains 50% in vivo biological activity. Bip at B25 is a good probe in fluorescent analysis of insulin [24], and we expect Bip might be a useful probe for other proteins as well. The fluorescence intensities of insulin analogs with B25 Phe replaced by Bip are higher in comparison with insulin analogs with B24 Phe replaced by Bip, indicating that B25 Bip is more exposed than B24 Bip (Fig. 3). The CD spectra of B25 Bip insulin and insulin are similar, indicating that they have similar structures in solution. The solution structures of B24 Bip-amide DHI and B24 Phe-amide DHI are also similar, as shown by their CD spectra (Fig. 4). The negative values of peaks at 208 nm and 222 nm of B24 Bip insulin and B25 Bip-amide DPI, respectively, are higher than those of insulin and B25 Phe-amide DPI, indicating that they have more compact structures [17,18]. The structures of Bip-replaced insulin analogs in solution are consistent with their receptor binding activities (Table 2). The fluorescence of B24 Bip insulin is the lowest, as shown in Fig. 3. Its structure is more compact than that of insulin, as shown in its CD spectrum, so its buried Bip can not bind with insulin receptor, resulting in its very low binding activity. The solution structure of B24 Bip-amide DHI is similar to that of B24 Phe-amide DHI. Its fluorescence intensity is higher than that of B24 Bip insulin but not as high as B25 Bip-replaced analogs, indicating that its Bip is more exposed but not enough to show significant receptor binding. From our results, we think in insulin and Bip-replaced analogs, the exposure of the aromatic side chain is very important for receptor binding. B24 Bip-amide DHI has 0.74% binding activity.

Fig. 4  Circular dichroism (CD) spectra of insulin analogs in 10 mM phosphate-buffered saline, pH 7.4  (A) Far-ultraviolet (UV) CD spectra of B24 biphenylalanine (Bip) insulin and B25 Bip insulin with insulin control. (B) Far-UV CD spectra of B24 Bip-amide deshexapeptide insulin (DHI) and B24 phenylalanine (Phe)-amide DHI. (C) Far-UV CD spectra of B25 Bip-amide despentapeptide insulin (DPI) and B25 Phe-amide DPI. (D) Near-UV CD spectra of B24 Bip insulin and B25 Bip insulin with insulin control.
Insulin analogs with B24 or B25 phenylalanine replaced by biphenylalanine

Fig. 5 Size exclusion chromatography of insulin analogs in 10 mM phosphate-buffered saline (pH 7.4) on a Superdex G75 column (HR 10/30)  The number near each peak is the retention volume (ml). Superdex G75 column supplied by Amersham Pharmacia Biotech, Uppsala, Sweden. Bip, biphenylalanine; DHI, deshexapeptide insulin; DPI, despentapeptide insulin.

and 20% in vivo biological activity. This difference was also observed in DHI, indicating that higher biological activity can be expressed with lower receptor binding because of the presence of spare receptors [25].

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Insulin analogs with B24 or B25 phenylalanine replaced by biphenylalanine

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