Effects of Negative Charges of a Model for Bovine Pancreatic Trypsin Inhibitor Folding Intermediate on the Peptide Folding

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A peptide model (called PzPβ) of bovine pancreatic trypsin inhibitor (BPTI) for studying the peptide folding structure was designed as a crucial folding intermediate. The PzPβ model folded but it was unstable at low pH. Four acidic groups in PzPβ: Glu-49, Asp-50, and C-termini at Phe-33 and Ala-58, were replaced individually with an Ala (in case of Glu and Asp) and an amide group (for C-terminal carboxyl groups) to study the effects of these negative charged groups on folding structure in terms of thermal stability and pH dependence. Substituting the Glu-49 or Asp-50 with Ala and blocking the C-terminus carboxyl group of Phe-33 in Pβ destabilized the structure, but blocking the negative charge of C-terminus in Ala-58 of Pz stabilized the structure at neutral pH. These results can be interpreted in terms of helix dipole momentum effects and/or salt bridge formation between Asp-50 and Arg-53, and of electrostatic interaction between positive and negative charges, which stabilized the structure. The melting temperature of model peptides (Tm) in low ionic strength buffer were lower than that in high ionic strength buffer except the C-terminus blocking of Pz.

Understanding the relationships between structure and function in protein is one of the goals of protein engineering. To approach the final goal of protein engineering it is necessary to analyze the structure of folding intermediates.1,2) The most well known characterized protein folding pathway is described in terms of disulfide bond formation in the oxidative refolding of bovine pancreatic trypsin inhibitor (BPTI).3,4) Furthermore roles of main subdomains were characterized in the folding of BPTI.5) A crucial early folding intermediate contained a disulfide bond between residues Cys-30 and Cys-51,6) and is denoted [30–51]. A peptide model (called PzPβ) of BPTI, a small analog of [30–51] was designed and synthesized to study the peptide folding structure.7) The synthetic analog (PzPβ) is a disulfide-bond peptide pair in which two short individual peptides containing main α-helix (Pz) and central antiparallel β-sheet (Pβ) are connected by a disulfide bond between Cys-30 of Pz and Cys-51 of Pβ, corresponding to the 30–51 disulfide bond of BPTI (Fig. 1). PzPβ represents approximately one-half of BPTI (30 of 58 residues) and it includes the most important cores, α-helix (residues 47–56), β-sheet (residues 20–33), and short β-strand (residues 43–46), but does not include the N-terminal small γ-helix (residues 2–6) (Fig. 1). This peptide model is very soluble in aqueous solution and does not aggregate, in marked contrast to the authentic intermediate, [30–51], which permits detailed structural studies using 2D-NMR.8) The structure of PzPβ is remarkably native-like, demonstrating the protein subdomain is one of autonomous folding units (AFUs), and providing a structural explanation for why [30–51] is too highly populated in folding of BPTI.8)

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; Pz, α-helix part of BPTI; Pβ, β-sheet part of BPTI; PzPβ, peptide model of BPTI folding intermediate; CD, circular dichroism; and [θ], mean residue ellipticity. One letter abbreviations for amino acids are A, Ala; D, Asp; E, Glu; F, Phe; K, Lys; N, Asn; R, Arg; and Y, Tyr.

Fig. 1. Schematic Drawing of BPTI7) Showing the Regions of BPTI Corresponding to the Peptides Pz and Pβ. It has six positive charges and four negative charges. Some important amino acids are described on the BPTI as an one-letter abbreviation of amino acids. The numbers after one letter abbreviations are the amino acids sequential numbers.
The sequences of the individual peptides (called Pz and Pβ) in PzPβ are:

\[ \text{Pz: } [43-58] \]
\[ +H_2N-Asn-Asn-Phe-^*Lys-Ser-Ala-^*Glu^-Asp-Cys^- \]
\[ \text{Pβ: } [20-33] \]
\[ +H_3N^-Arg-Tyr-Phe-Tyr-Asn-Ala^-*Lys-Ala-Gly-Leu^- \]

Pz contains 16 residues and corresponds to the residues 43-58, which includes the C-terminal ∝-helix and a short β-strand in BPTI. Cys-55 has been replaced with Ala, so Pz contains only one thiol group (corresponding to Cys-51). Pβ contains 14 residues and corresponds to a part of the antiparallel β-sheet in BPTI (residues 20-33) including Cys-30. There are four acidic (carboxyl) groups in PzPβ; Glu-49, Asp-50, and two C-termini at Phe-33 (Pβ) and Ala-58 (Pz), and there are six amino groups; Arg-20, Lys-26, Lys-46, Arg-53, and two N-termini at Asn-43 and Arg-20 (Fig. 1).

At low pHs the acidic group of amino acids have a neutral charge by protonation, but with the increase of pH these acidic groups are deprotonated to get negative charges. Oas and Kim\(^8\) reported that the structure of PzPβ folded approximately 90% at low temperature and neutral pH but it unfolded at low pH. This means the negative charges stabilize the protein folding structure, therefore, it is interesting to investigate the effects of negative charges on the folding conformation and stability of this peptide model. To study the electrostatic interaction of each of the acidic groups of Glu-49 and Asp-50 was replaced with an Ala residue, which has no charge group, and two carboxyl groups of C-termini were blocked by making amide groups (CO-NH\(_2\)). The subsequent peptide variants replacing Asp-50 and Glu-49 with Ala were called ‘Pz\(_{50}\)Pβ\(_1\)’ and ‘Pz\(_{49}\)Pβ\(_1\)’, respectively. And we used ‘Pz\(_{50}\)Pβ\(_1\)’ and ‘Pz\(_{49}\)Pβ\(_1\)’ for blocked acidic groups of C-termini of Pz (Ala-58) and Pβ (Phe-33), respectively. For comparison, we also used ‘Pz\(_{50}\)Pβ\(_1\)’ for the model BPTI folding intermediate (usually called PzPβ) before.\(^8\)

In this paper, using the peptide model (Pz\(_2\)Pβ\(_1\)) and its variants (Pz\(_{50}\)Pβ\(_1\), Pz\(_{49}\)Pβ\(_1\), Pz\(_{49}\)Pβ\(_1\), and Pz\(_{49}\)Pβ\(_1\)), folding structure and pH and temperature dependences of folding stability were investigated to study the factors that stabilize the structure in these intermediates on protein folding. The peptide folding structure was estimated by measuring the mean residue ellipticity [θ] in circular dichroism (CD).

**Materials and Methods**

*Materials.* All the chemicals and substrates such as amino acid derivatives for synthesizing the peptides were supplied by Applied Biosystems (U.S.A.). Sephadex G-10, G-25, 4-oxyethylphenylacetomidomethyl (PAM) resin, and p-methylbenzhydrolyamine resin were from Bio-Rad Co. (U.S.A.). Trifluoromethane sulfonic acid (TMFSA), guanidine hydrochloride (GuHCl), and dithiothreitol (DTT) were of ultra pure HPLC grade. Acetonitrile, trifluoroacetic acid (TFA), and other chemicals were of HPLC high pure grade.

*Peptide synthesis.* Each peptide, Pz\(_2\), Pβ\(_1\), Pz\(_{50}\), Pz\(_{49}\), Pz\(_{49}\), and Pβ\(_1\), was synthesized individually with solid phase Fmoc methods\(^9\) on an Applied Biosystems Model 430A peptide synthesizer with standard reaction cycles using PAM resin. Especially to give the C-terminal amide in Pz\(_{50}\), p-methylbenzhydrolyamine resin was used.\(^10\) The peptides were cleaved from the resin using TMFSA\(^1\) and desalted on a Sephadex G-10 column in 5% acetic acid.

*PzPβ complex preparation.* Each Pz and Pβ (reduced by DTT after desalting) was purified by reverse phase HPLC (Waters, U.S.A.) on a preparative Vydac C\(_{18}\) column at 50°C, using a water-acetonitrile gradient in the presence of 0.1% TFA. Each PzPβ peptide complex was cross-linked from each Pz and Pβ by air oxidation in 5 mM GuHCl, 0.2 M Tris–buffer (pH 8.4, at 25°C for 48 h (48 h oxidation was not enough for Pz\(_{50}\)Pβ\(_1\), so the oxidation time was elongated to 72 h)). GuHCl was used to prevent the precipitation of the Pβ homodimer.\(^9\) After oxidation, which was confirmed by analytical HPLC for checking the disappearance of each monomer, PzPβ was purified from Pz homodimer and Pβ homodimer using HPLC with the same solvent. The peptide complex was lyophilized from 5% acetic acid and stored in a desiccator.

*Identification of peptides.* The PzPβ peptides were identified by measuring the molecular weight at the MIT Mass Spectrometry NIH Facility at MIT B.\(^{1,10}\)

*Measurement of peptide concentration.* All peptide concentrations were measured by tyrosine and cystine absorbance at 275.5 nm in 6 M GuHCl, 0.2 M phosphate buffer, pH 6.5. We used AVIV Model 115DS spectrophotometer.\(^14\) We used the molar extinction coefficients of tyrosine and cystine at 275.5 nm of 1500 and 145, respectively.

*CD experiments.* All CD experiments were done on an AVIV Model...
60DS CD spectropolarimeter using a 1 nm path-length cell. Temperatures were controlled by a HP Model 89100A Pelletier temperature control unit. The CD samples (optimum concentrations; about 0.15 mg of peptide/1 ml of CD buffer, which was kindly provided by T. G. Oas) were prepared by diluting the stock solutions (about 5 mg of peptide/1 ml of 1 mm HCl) into the standard buffer (0.2 M Na₂SO₄, 10 mM Na₂HPO₄, pH 6.0). The pH of the solutions was adjusted with H₂SO₄ and HCl at room temperature. The pH was measured before and after CD work, and the changes in pH were less than 0.1 unit. For measuring the melting curve, the temperature of CD spectrometer was programmed for every temperature from 0°C to 80°C as follows: rising time, 1 min; equilibrium time, 2 min; and data collecting time, 2 min. All samples were degassed under a vacuum before CD experiments.

Measurement of melting temperature. The melting temperatures (midpoint of transition, Tₘ) for the peptides were calculated by the first derivatives of the temperature dependence of the CD signal at 220 nm using the methods in previous papers. Figure 2b shows the first derivatives of temperature dependence from the melting curve of P₅₈₆ Pβ₁ (Fig. 2a) at pH 6.0 in 0.2 M Na₂SO₄ and 10 mM Na₂HPO₄ solution. Tₘ is the temperature at which the first derivative of the melting curve is maximum.

Results

Identity of peptides

The identity of peptides was confirmed by analysis of molecular weight with a mass-spectrometer. The molecular weights of P₂₅ Pβ₁, P₂₅₀ Pβ₁, P₂₅₀ Pβ₁, P₂₅₅ Pβ₁, and P₂₅₂ Pβ₃₃, were 3352.3, 3308.5, 3294.1, 3351.9, and 3352.1, respectively. Each datum corresponds with the expected molecular weight, which was calculated as 3351.8, 3307.7, 3293.7, 3350.8, and 3350.8, for P₂₅ Pβ₁, P₂₅₀ Pβ₁, P₂₅₅ Pβ₁, P₂₅₈ Pβ₁, and P₂₅₂ Pβ₃₃, respectively.

Concentrations of peptides for CD

The peptide concentrations used for calculating the mean residue ellipticity by CD experiments were 24.8, 24.2, 22.6, 27.2, and 28.7 μM for P₂₅ Pβ₁, P₂₅₀ Pβ₁, P₂₅₀ Pβ₁, P₂₅₅ Pβ₁, and P₂₅₂ Pβ₃₃, respectively. At these concentrations, no aggregation took place even at high temperature and low pH. For every additional CD experiment, each peptide concentration was measured (data not shown).

CD spectra of peptides

At pH 6.0 and pH 2.0, the CD spectra of the peptides, P₂₅ Pβ₁, P₂₅₀ Pβ₁, P₂₅₀ Pβ₁, P₂₅₅ Pβ₁, and P₂₅₂ Pβ₃₃ at 0°C are shown in Fig. 3. At neutral pH, the spectra were very similar to the typical α-helix structure and showed clear minima at 218 nm and 204 nm and show the maximum around 198 nm, but CD spectra of P₂₅₂ Pβ₃₃ were slightly different from those of any other peptides. As the temperature increased to 50°C (data not shown) and the acidity of solution (pH 2.0) increased, the minimum at 218 nm was severely reduced. At high temperature and low pH the minima at 218 nm disappeared but minima at 204 nm still appeared.

![CD Spectra of P₅₈₆ Pβ₁(a), P₂₅₀ Pβ₁(b), P₂₅₅ Pβ₁(c), and P₂₅₂ Pβ₃₃(d) in 200 mm Na₂SO₄ and 10 mm Na₂HPO₄ at 0°C, pH 6.0 (○) and pH 2.0 (●).](image-url)
pH dependences of $[\theta]_{220}$ for the peptides

The pH dependences of $[\theta]_{220}$ for the peptides between pH 2.0–pH 8.0 at 0°C are shown in Fig. 4. $[\theta]_{220}$ instead of $[\theta]_{218}$ (218 nm is minimum) was used because the maximum difference of CD spectra between 0°C and 40°C ($-[\theta]_{220}$ was decreased up to 40°C in most of the PzPβ)13,16) and between pH 2.0 and pH 6.0 appeared not at 218 nm but at 220 nm. The $-[\theta]_{220}$ was increased with the increase of pH up to pH 8.0. At higher pH (over pH 9.0), spectra for all peptides did not show a typical α-helical spectrum due to tyrosine. Therefore, we included the spectrum data from pH 2.0 to pH 8.0 only. For all peptides, $-[\theta]_{220}$ were increased sharp from pH 3.0 to pH 5.0. At pH 6.0 PzPβ, showed the highest mean residue ellipticity among the peptides followed by PzPβ, PzPβ, and PzPβ, and finally PzP2β, had the smallest value. At lower pH, however, PzPβ had the stabler α-helix folding structure among the peptides, although the spectrum was slightly different.

Melting curves of peptides

The temperature dependences of $[\theta]_{220}$ for the peptides were determined as shown in Fig. 5 at pH 6.0 and pH 2.0. The thermal unfolding transition for PzPβ was broad as in case of PzPβ (Fig. 4a), and a fully folded base line could not be reached even at 0°C. At 0°C over 90% of the peptides were folded.13) The transition was completely reversible at temperatures up to 80°C provided that the sample was degassed before use. At low pH, however, the thermal transition changed to a lower temperature, and nonlinearity was observed below 20°C for PzPβ, similar to C-peptide analog RN-54, which was called a “weak helix former”.18) Nonlinearity is shown at the temperature point of the unfolding transition which corresponds to zero of the first derivative of the melting curve of the peptide. Adding GuHCl (6 M, pH 6.0) eliminated the transition (Fig. 2a), and showed a linear temperature dependence of the CD signal as in case of PzPβ model by GuHCl.18) By the same method the melting curves of PzPβ, PzPβ, and PzPβ, at pH 6.0 and pH 2.0 were measured as shown in Figs. 5a, 5b, and 5c. The melting curve for PzPβ was also shown in Fig. 5d to compare the results. At the lower pH the CD signals for all peptides looked like that of a weak helix former like PzPβ.

Table Tm of PzPβ, PzPβ, PzPβ, and PzPβ, Peptide Variants in Standard Buffer (200 mM Na2SO4 and 10 mM Na2HPO4, pH 6.0) and in Lower Salt Buffer (10 mM NaCl and 10 mM Na2HPO4, pH 6.0) Which were Measured from the Melting Curves

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200 mM Na2SO4</td>
</tr>
<tr>
<td>PzPβ</td>
<td>24.0 ± 1.10 a</td>
</tr>
<tr>
<td>PzPβ (D50A)</td>
<td>15.8 ± 1.17</td>
</tr>
<tr>
<td>PzPβ (E49A)</td>
<td>21.4 ± 0.41</td>
</tr>
<tr>
<td>PzPβ (A58CONH2)</td>
<td>29.4 ± 1.30</td>
</tr>
<tr>
<td>PzPβ (F33CONH2)</td>
<td>17.1 ± 1.75</td>
</tr>
</tbody>
</table>

* SD for at least three measurements.

$T_m$ of peptides

From the melting curves at pH 6.0 in 0.2 M Na2SO4 and 10 mM Na2HPO4 solution, midpoints of transition ($T_m$), were found as shown in Fig. 2b, 2d, and 15) Table shows the $T_m$ of each peptide. PzPβ had a higher $T_m$ (29.4°C) than PzPβ (24.0°C). $T_m$ of PzPβ (15.8°C), PzPβ (21.4°C), and PzPβ (17.1°C) were lower than that of PzPβ. $T_m$s of peptides were directly proportion to $[\theta]_{220}$ at pH 6.0 as shown in Fig. 3. The $T_m$ of model PzPβ (PzPβ) was about 24°C, which was the almost same temperature reported by Oas and Kim.8) The $T_m$s of peptides at a low ionic strength (10 mM NaCl and 10 mM Na2HPO4, pH 6.0) are also shown in Table. $T_m$ of PzPβ was increased to 33°C from 29°C by reducing the salt concentration, while all other peptides were less thermally stable in low salt concentration than in high salt.

Discussion

The CD spectra of the peptides at 0°C and pH 6.0 and pH 2.0 (Fig. 3) suggested that all of the peptides have α-helical structures at low temperature and neutral pH.19) At the present time, there is no evidence that the PzPβ could be aggregated or that $[\theta]_{218}$ could be dependent on peptide concentration. In fact, the mean residue ellipticity of unfolded peptides at 0°C, $[\theta]$, was independent of the peptide concentration over the range of 15 μM to 0.3 mM.8,19) These data showed apparently that PzPβ is more stable than model peptide (PzPβ), however, PzPβ was less stable. Also PzPβ and PzPβ were much less stable than PzPβ at neutral pH.

From these results, we suggest that the negative charges of Glu-49, Asp-50, and Phe-33 were important in the α-helix forming of model BPTI folding intermediate, but the negative charge of Ala-58 was adverse. Data of pH dependence of $[\theta]_{220}$ at neutral pH PzPβ had the most stable structure and PzPβ had the most unstable structure and at lower pH PzPβ had the most stable
x-helix folding structure (Fig. 4), supported this suggestion. The $-[\theta]_{220}$ was increased sharply from pH 3.0 to pH 5.0, due to the carboxylation of the C-terminal $\alpha$-helix (Ala-58) and C-terminal $\beta$-sheet (Phe-33). NMR data (unpublished data) indicated that these four groups were titrated between pH 3.0 and pH 5.0. The pK$_a$ of Glu-49 of P$_{2\alpha0}$$\beta_1$ and Asp-50 of P$_{4\alpha9}$$\beta_1$ were expected to be larger than those of P$_{2\alpha}$$\beta_1$, because of less polarity, when the vicinal charged residues were replaced to Ala.

Critically $T_m$ data of all peptides, which was representative of the thermal stability of peptides in folding, consolidated this conclusion. Substituting the Glu-49 (P$_{4\alpha9}$$\beta_1$) and Asp-50 (P$_{2\alpha0}$$\beta_1$) to Ala, which has no charge group, destabilized the structure, moreover, the $T_m$ of P$_{2\alpha0}$$\beta_1$ (15.8°C) was much lower than that of P$_{4\alpha9}$$\beta_1$ (21.5°C). That means these two negative charges stabilize the structure of the model BPTI folding intermediate, furthermore Asp-50 is more important than Glu-49 in the peptide folding. The $T_m$ of P$_{2\alpha58}$$\beta_1$ was about 29°C, which means that P$_{2\alpha58}$$\beta_1$ is more stable than model P$_{2\alpha}$$\beta_1$. This indicates the negative charge of C-terminal $\alpha$-helix destabilizes the folding structure. On the other hand, $T_m$ of P$_{2\alpha}$$\beta_{13}$ (17.1°C) was lower than that of the P$_{2\alpha}$$\beta$ model but higher than that of P$_{2\alpha0}$$\beta_1$ (15.8°C). The crystal structure showed that N-terminus (Arg-20) of $\beta$-sheet had two positive charges and the C-terminus (Phe-33) of $\beta$-sheet had one negative
The result indicated the electrostatic interaction between positive charge and negative charge stabilizes the PzPβ model.

However, three questions remained: 1) How these two amino acids, Glu-49 and Asp-50, stabilize the BPTI folding intermediate structure; 2) Why Asp-50 is so important in peptide folding; and 3) Why the carboxyl group in C-terminus of Pz destabilizes PzPβ1. These can be interpreted in terms of a salt bridge, electrostatic interaction, and the helix dipole moment. There are two possibilities of salt bridge between Arg-53 and Asp-50 or Glu-49. The result, Pz50Pβ1 was less stable than Pz49Pβ1 at neutral pH, shows that Asp-50 is more important than Glu-49 in folding, which indicates Asp-50 is more likely to make the salt bridge with Arg-53 than Glu-49. The data of Pz49Pβ1 shows that negative charge of Asp-49 also has a weak but substantial interaction with the positive charge of Arg-53. This result is consistent with the data of X-ray crystallography in crystal form I of BPTI, which showed a salt bridge within Asp-50 and Arg-53, thus α-helix of BPTI was stabilized by the salt bridge in the α-helix turn as in case of ribonuclease.

In addition the result confirmed the suggested helix dipole moment theory. In the α-helix, all COO− groups are directed to the C-terminal of helix and make peptide bonds with the next turn of NH3+ groups which were directed to the N-terminal of helix. Thus negative polarity (δ−) was accumulated toward C-terminal while positive polarity (δ+) was found in the N-terminal of the α-helix. Therefore, in the N-terminal of the α-helix the structure can be stabilized by the electrostatic interaction between the negative charge and positive end of the helix dipole or can be destabilized by repulsion of the same charges. The α-helices in Pz of BPTI and C-peptide (1–13 residues) of ribonuclease must be stabilized by the electrostatic interaction within the negative charge and positive end of helix dipole around N-terminus.

This helix dipole moment model in α-helix was confirmed again by blocking the C-terminal negative charges of Ala-58 in Pz and of Phe-33 of Pβ. Electrostatic repulsion between negative charges of helix dipole and C-terminus carboxyl group of Pz destabilized the structure, although Ala-58 was not a part of α-helix because Gly-56 was identified as the last helical residue in native BPTI. This is the main reason why Pz58Pβ1 (blocking the C-terminus of Pz in Pz) was so stable. However, in antiparallel β-sheet of Pβ1, the positive charge of the N-terminus (Arg-20) and the negative charge of the C-terminus (Phe-33) are so close they can make electrostatic binding between the ends, which can stabilize the PzPβ structure. Thus substituting the C-terminal carboxylic acid with amide destabilizes the structure (Pz2Pβ3).

Shoemaker et al. concluded that the helix dipole structure was dependent on salt concentration due to the interaction between a bond charged group and the helix dipole. Therefore at low salt concentrations the divergence of mean residue ellipticity at 220 was greater than at high salt concentrations. Less stable peptides will be stable in high salt by this reaction. This is the reason why Tm data of Pz58Pβ1 at lower salt concentration (Tm is 31°C) was greater than at higher salt concentration (Tm is 29°C), but the Tm (22°C) of Pz2Pβ1 at lower salt concentration was lower than that (24°C) at higher concentration.

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