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Contribution of Individual Side-chains to the Stability of BPTI Examined by Alanine-scanning Mutagenesis

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Bovine pancreatic trypsin inhibitor (BPTI) serves as an important model system for the examination of almost all aspects of protein structure. Systematic studies of the effects of mutation on the thermodynamic stability of BPTI, however, have been limited by the extreme stability of the protein. A derivative of BPTI containing only the 5–55 disulfide bond, termed $[5–55]_{Ala}$, has been shown previously to fold into a structure very similar to that of native BPTI and to be a functional trypsin inhibitor. $[5–55]_{Ala}$ undergoes a reversible thermal unfolding transition with a melting temperature of 39°C, and is therefore well suited for stability studies. Using an alanine-scanning mutagenesis approach, we have examined the contribution to stability of each side-chain in the $[5–55]_{Ala}$ derivative of BPTI. These studies demonstrate the importance of the two hydrophobic cores composed largely of clusters of aromatic residues, as well as the internal hydrogen-bonding network, in stabilizing BPTI. Overall, there is a strong relationship between change in buried surface area and stability for both polar and hydrophobic residues, with proportionality constants of 50 and 20 cal/Å^2, respectively. None of the alanine substitutions substantially stabilized $[5–55]_{Ala}$. Nonetheless, approximately 60% (28/46) of the alanine mutants were destabilized by less than 10°C, suggesting that a form of BPTI with up to half of its residues being alanine could fold into a stable structure resembling the native one.

Keywords: protein folding; protein stability; BPTI

Introduction

Bovine pancreatic trypsin inhibitor (BPTI) is a highly stable, 58 residue protein with a native structure (Figure 1) that is stabilized by three disulfide bonds between residues 5 and 55, 30 and 51, and 14 and 38. The native structure of BPTI (Deisenhofer & Steigemann, 1975; Wlodawer *et al.*, 1984, 1987) consists of an amino-terminal 3_{10}-helix, a highly twisted anti-parallel β-sheet, and an α-helix near the carboxy terminus.

BPTI has served as a model system for the examination of almost all aspects of protein structure, including X-ray crystallography and neutron diffraction (Wlodawer *et al.*, 1984), the development of two-dimensional solution nuclear magnetic resonance (NMR) techniques for protein structure determination (Wüthrich *et al.*, 1982), measurement of protein dynamics (Wagner & Wüthrich, 1982; Kim & Woodward, 1993), studies of the position and dynamics of water molecules (Otting *et al.*, 1991) and development of computational approaches to protein folding (Levitt, 1981; Cusack *et al.*, 1988; Dagget & Levitt, 1992). Finally, the oxidative folding pathway of BPTI has been studied in terms of the disulfide-bonded intermediates that accumulate during folding (Creighton, 1978; Creighton & Goldenberg, 1984; Weissman & Kim, 1991, 1992a,b, 1993; Creighton *et al.*, 1993).

Studies of the effects of mutations on the thermodynamic stability of BPTI, however, have been limited by the extremely high stability of the protein. With the three disulfide bonds intact, BPTI is unusually resistant to denaturation by temperature, urea or guanidine hydrochloride (Moses & Hinz, 1983; Makhatadze *et al.*, 1993). This difficulty can be overcome in part by removing the 30–51 (Hurle *et al.*, 1990) or 14–38 (Jullien & Baldwin, 1981; Schwarz *et al.*, 1987) disulfide bonds, either by chemical modification or by mutagenesis. However, even these forms of BPTI with two disulfide bonds are highly stable.

Recently, a derivative of BPTI containing only the 5–55 disulfide bond, termed $[5–55]_{Ala}$, with the remaining cysteine residues changed to alanine, was

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Abbreviations used: BPTI, bovine pancreatic trypsin inhibitor; DSC, differential scanning calorimetry.

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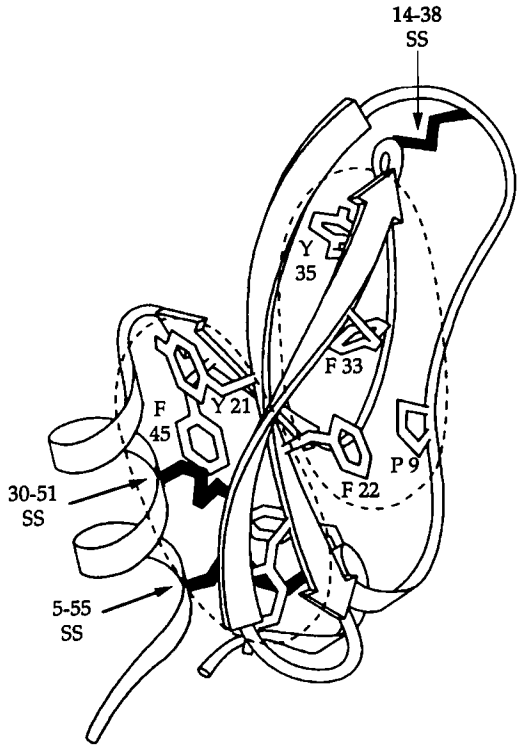

Figure 1. Schematic representation of the crystal structure of BPTI (Richardson, 1985). The 2 hydrophobic cores are indicated by broken lines. Side-chains involved in the cores are included.

shown by two-dimensional NMR to fold into a structure very similar to that of native BPTI, and to be a functional trypsin inhibitor (Staley & Kim, 1992). The crystal structure of $[5–55]_{Ala}$ has not been determined. However, a related derivative of BPTI that contains the 5–55 and the 14–38 disulfide bonds with Cys30 and Cys51 replaced by alanine, termed BPTI(30A/51A), was shown by X-ray crystallography to fold into a structure nearly identical to that of native BPTI (Eigenbrot *et al.*, 1990). The $[5–55]_{Ala}$ molecule undergoes reversible thermal unfolding with a melting temperature (t_m) of 39°C. Thus, $[5–55]_{Ala}$ is an excellent model for assessing the effects of mutations on the stability of BPTI. A different model of the [5–55] species, termed $[5–55]_{Ser}$, in which the remaining cysteine residues are replaced by serine, has also been shown to fold into a structure resembling that of native BPTI (van Mierlo *et al.*, 1991). However, the low stability of $[5–55]_{Ser}$ ($t_m \sim 5$°C) makes it unsuitable for these studies.

We have carried out an alanine-scanning mutagenesis study (Cunningham & Wells, 1989) of protein stability in which each amino acid is replaced, one at a time, with an alanine residue. The primary advantage of the alanine-scanning mutagenesis approach is that it allows an unbiased and quantitative evaluation of the contribution of each side-chain to the stability of the protein. In addition, this approach should contribute to the determination of which fraction of the polypeptide sequence of BPTI is required to form a stable folded protein (see Heinz *et al.*, 1992).

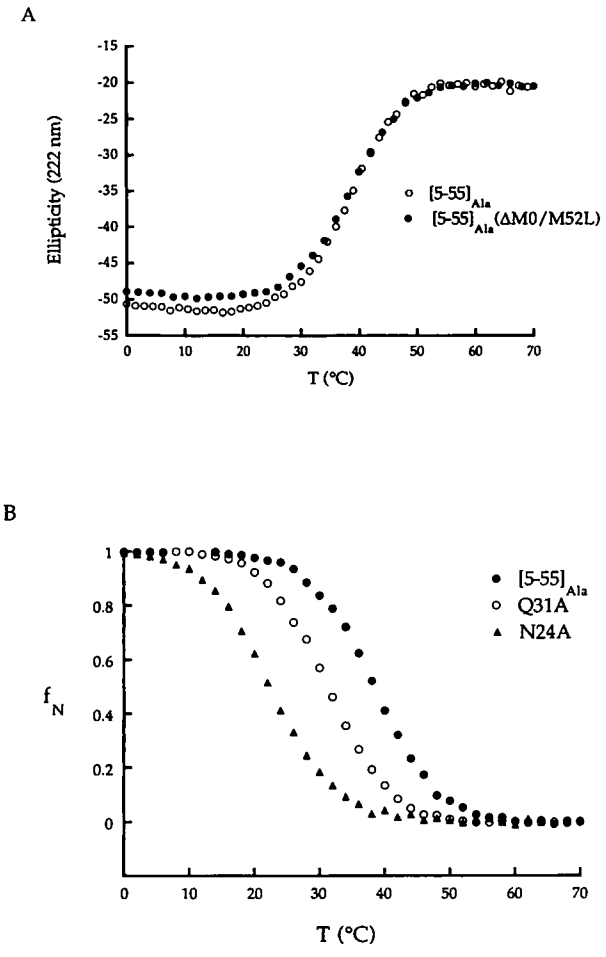

Figure 2. Unfolding of various forms of BPTI monitored by CD. A, The 2 recombinant models of the [5–55] species, $[5–55]_{Ala}$ and $[5–55]_{Ala}$(ΔM0/M52L), have almost identical stabilities. The unfolding transitions were monitored by the change in CD signal (mdeg.) at 222 nm. B, Temperature dependence of the folding transition of $[5–55]_{Ala}$ and the N24A and Q31A mutants, as monitored by the CD signal at 222 nm. Under the conditions used in these studies, the t_m of $[5–55]_{Ala}$ is 39.2°C, the t_m of Q31A is 31.7°C, and the t_m of N24A is 23.4°C, as determined by fitting the CD signal to eqn (1). A linear extrapolation of the folded and unfolded baseline was used to determine the fraction of folded protein. For the CD studies, the protein concentration was 8 μM and the buffer was 10 mM phosphate (pH 7.0), 150 mM NaCl, 1 mM EDTA. T represents temperature.

Results

Thermodynamic parameters for the two state unfolding of $[5–55]_{Ala}$

The circular dichroism (CD) signal of $[5–55]_{Ala}$ at 222 nm exhibits a sigmoidal dependence on temperature, indicating that $[5–55]_{Ala}$ folds (Figure 2). Fitting the melting curve to a two-state unfolding transition indicates that at pH 7.0 the melting temperature (t_m) of $[5–55]_{Ala}$ is 39.2°C, and the change in enthalpy upon unfolding at t_m (ΔH_{vH}) is 43 kcal/mol.

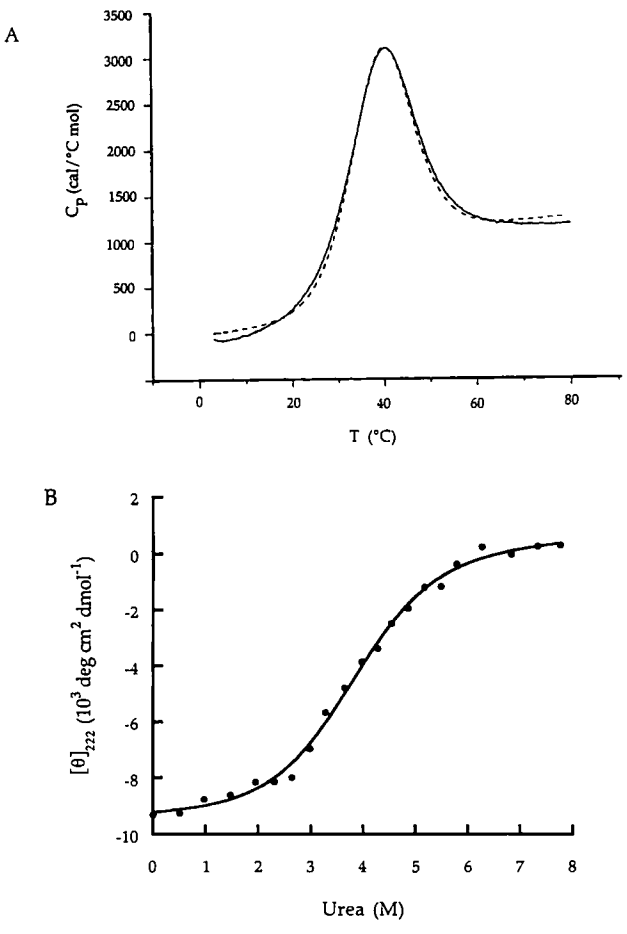

Figure 3. Unfolding of $[5–55]_{Ala}$. A, DSC scan for the unfolding of $[5–55]_{Ala}$ in 10 mM phosphate (pH 7.0), 150 mM NaCl and 1 mM EDTA. The continuous line is the measured excess enthalpy, and the broken line is the best fit to a 2-state unfolding transition with constant ΔC_p. B, Urea-induced unfolding transition of $[5–55]_{Ala}$ as measured by CD spectroscopy at 222 nm. The continuous line is the best fit to eqn (2). The protein concentration was 15 μM and the solvent conditions were 10 mM phosphate (pH 7.0), 150 mM NaCl and 1 mM EDTA at 5°C.

To check the validity of the two-state unfolding model, $[5–55]_{Ala}$ was subjected to differential scanning calorimetry (DSC) (Figure 3A). The specific enthalpy of unfolding (ΔH_{cal}) and the van't Hoff enthalpy (ΔH_{vH}) as measured by DSC were 43 kcal/mol and 42 kcal/mol, respectively. The change in heat capacity (ΔC_p) was approximately 900 cal/(mol deg.). The similarity in the value of ΔH_{vH}, as measured by CD and DSC and the value of ΔH_{cal}, indicates that no thermodynamically relevant intermediates are significantly populated during the thermal denaturation of $[5–55]_{Ala}$.

The CD signal at 222 nm was also used to monitor the urea-induced unfolding transition of $[5–55]_{Ala}$ (Figure 3B). The free energy of unfolding in the absence of urea (ΔG^{H_2O}) was estimated by extrapolating the free energy of unfolding at each urea concentration to zero urea, assuming that they are linearly related (Pace, 1986). This procedure indicated that at pH 7.0, ΔG^{H_2O} for $[5–55]_{Ala}$ is 2.7 (±0.1) kcal/mol at 5°C. This value is in good agreement with the value of 2.9 kcal/mol estimated from the DSC measurements.

Stability of alanine mutants

Altered forms of $[5–55]_{Ala}$ containing a single alanine substitution at each of the 46 non-alanine residues, with the exception of Cys5 and Cys55, were produced in *E. coli*. Four alanine mutants, Phe4 (F4A), Pro8 (P8A), Thr11 (T11A) and Tyr35 (Y35A), were not expressed well by *E. coli*. These mutants were studied in the context of a slightly different model of $[5–55]_{Ala}$, termed $[5–55]_{Ala}$(ΔM0/M52L) (see Materials and Methods). The thermal unfolding transition of $[5–55]_{Ala}$(ΔM0/M52L) is almost identical to that of $[5–55]_{Ala}$ (Figure 2A).

Unfolding of the alanine mutants was followed by a change in CD signal at 222 nm as a function of temperature. The melting curves for $[5–55]_{Ala}$ and two mutants, Q31A and N24A, are depicted in Figure 2B. The data for all the mutants are summarized in Table 1. The melting temperature (t_m) and ΔH_{vH} for 39 of the 46 alanine mutants were determined by fitting the thermal unfolding transition to a two-state model. Four of the 46 alanine mutants (Y23A, F33A, N43A and F45A) showed no evidence of folded structure, even at 0°C. Another, Y21A, was predominantly unfolded at 0°C. Two other mutants (F4A and N44A) did not have sufficient folded baseline to fit the thermal unfolding transition accurately, but were predominantly folded at 0°C. For these two mutants, the t_m was estimated by the minimum of the first derivative, with respect to T^{-1} (Kelvin), of the CD signal at 222 nm (Cantor & Schimmel, 1980).

For the 39 mutants in which it was possible to measure both t_m and ΔH, estimates of the change in the free energy of unfolding compared to that of the wild type (ΔΔG) at the melting temperature of $[5–55]_{Ala}$ (39°C) were made using two different approximations (Table 1). The first method was to assume that ΔΔG and the change in t_m (Δt_m) are related linearly by the change in entropy of unfolding (ΔS) for $[5–55]_{Ala}$ (Becktel & Schellman, 1987):

$$\Delta\Delta G = \Delta t_m * \Delta S$$

This estimate should be valid for mutants with small Δt_m values. The second method was to calculate the ΔΔG of unfolding at 39°C by assuming a constant value for ΔC_p of 900 cal/(mol deg.) for all the mutants, and to use the values of ΔH, ΔS and T_m (t_m in Kelvin) determined from the CD melts for each mutant, with the Gibbs–Helmholz equation (Becktel & Schellman, 1987):

$$\Delta G(T) = \Delta H - T\Delta S + \Delta C_p(T - T_m - T\ln(T/T_m))$$

In this equation, $\Delta G(T)$ is the free energy of folding at the temperature T, ΔH and ΔS are the enthalpy and entropy of unfolding, respectively, at the T_m of the given mutant. The two estimates of ΔΔG are reported in Table 1, and generally agree within 10%.

Table 1. Stability of various $[5–55]_{Ala}$ mutants

Mutant	t_m (°C)	Δt_m (°C)	ΔH (kcal/mol)	$\Delta\Delta G^a$ (kcal/mol)	$\Delta\Delta G^b$ (kcal/mol)
$[5–55]_{Ala}$	39.2	0.0	43	0.0	0.0
R1A	35.3	3.9	41	0.5	0.5
P2A	29.4	9.8	37	1.3	1.3
D3A	40.6	−1.4	44	−0.2	−0.2
F4A[c,d]	18	21		3	
L6A	34.6	4.6	40	0.6	0.6
E7A	28.0	11.2	42	1.5	1.8
P8A[c]	36.7	2.5	40	0.3	0.3
P9A	33.7	5.5	42	0.8	0.8
Y10A	30.2	9.0	32	1.2	1.1
T11A[c]	39.0	0.2	42	0.0	0.0
G12A	26.1	13.1	34	1.8	1.8
P13A	30.2	9.0	36	1.2	1.2
K15A	36.6	2.6	37	0.4	0.3
R17A	37.3	1.9	41	0.3	0.3
I18A	28.3	10.9	33	1.5	1.4
I19A	24.1	15.1	35	2.1	2.1
R20A	26.0	13.2	38	1.8	1.9
Y21A	<0				
F22A	24.5	14.7	37	2.0	2.2
Y23A	≪0				
N24A	23.4	15.8	40	2.2	2.5
K26A	39.1	0.1	44	0.0	0.0
G28A	31.9	7.3	43	1.0	1.1
L29A	39.5	−0.3	42	0.0	0.0
Q31A	31.7	7.5	39	1.0	1.0
T32A	38.8	0.4	40	0.1	0.1
F33A	≪0				
V34A	30.2	9.0	41	1.2	1.3
Y35A[c]	30.9	8.3	29	1.1	0.9
G36A	23.8	15.4	31	2.1	2.0
G37A	22.0	17.2	34	2.3	2.4
R39A	39.2	0.0	41	0.0	0.0
K41A	36.2	3.0	34	0.4	0.4
R42A	35.6	3.6	40	0.5	0.5
N43A	≪0				
N44A[d]	15	29		3.3	
F45A	≪0				
K46A	39.8	−0.6	42	−0.1	−0.1
S47A	27.3	11.9	42	1.6	1.9
E49A	37.9	1.3	43	0.2	0.2
D50A	36.6	2.6	44	0.4	0.4
M52A	27.1	12.1	38	1.7	1.8
R53A	38.3	0.9	45	0.1	0.1
T54A	38.5	0.7	43	0.1	0.1
G56A	37.8	1.4	43	0.2	0.2
G57A	37.7	1.5	44	0.2	0.2

[a] $\Delta\Delta G$ estimated by $\Delta t_m{}^*\Delta S$ (see the text).
[b] $\Delta\Delta G$ estimated from the Gibbs–Helmholz equation (see the text).
[c] These mutants were studied in the $[5–55]_{Ala}$(ΔM0/M52L) background.
[d] t_m estimated by taking the derivative of the CD signal with respect to T^{-1}.

Discussion

In the alanine-scanning mutagenesis study described here, each non-alanine residue (with the exception of Cys5 and Cys55) was replaced, one at a time, with an alanine, and the effect of the mutation on stability was measured. Alanine was chosen as the replacing residue because it eliminates the side-chain beyond the β-carbon without introducing new flexibility in the main-chain, as glycine might (see discussion by Lau & Fersht, 1987; Cunningham & Wells, 1989). In addition, alanine is the most abundant residue, and is found frequently in both buried and exposed positions, and in all types of secondary structure.

This alanine-scanning mutagenesis study was carried out on a recombinant model of BPTI, termed $[5–55]_{Ala}$, containing only the 5–55 disulfide bond, with the remaining cysteine residues changed to alanine. $[5–55]_{Ala}$ was shown to fold into a structure very similar to that of native BPTI, and to be a functional trypsin inhibitor (Staley & Kim, 1992). In

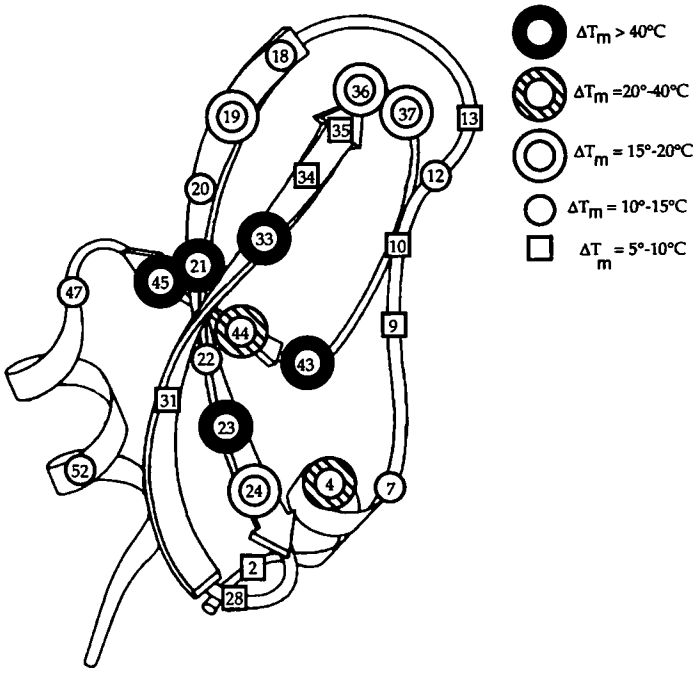

Figure 4. Schematic representation of the effects of the alanine substitutions on the stability of $[5–55]_{Ala}$. The mutations are grouped according to the degree of loss of stability (see key in Figure). Mutations at positions not marked decrease the t_m by less than 5 deg. C.

addition, unlike mature BPTI which is highly resistant to denaturation (Moses & Hinz, 1983), $[5–55]_{Ala}$ undergoes a two-state thermal unfolding transition with a t_m of 39°C.

A schematic representation of the effects of the alanine substitutions on the BPTI structure is shown in Figure 4, and the data are summarized in Table 1. None of the alanine mutants results in a significant increase in stability. However, approximately 60% (28/46) of the mutations caused less than a 10 deg. C change in t_m (~ 1.3 kcal/mol). Four of the 46 alanine substitutions (Y23A, F33A, N43A and F45A) resulted in total loss of structure even at 0°C. Three of these four residues (Tyr23, Asn43, and Phe45) have been observed previously to be highly sensitive to replacement with alanine or glycine, in the context of native BPTI with the three disulfide bonds intact (Goldenberg *et al.*, 1989; Kim *et al.*, 1993).

Correlation with structural parameters

Despite its small size, BPTI contains two hydrophobic cores (Richardson, 1985). The major core is located at the bottom of the molecule as depicted in Figure 1, and comprises the 30–51 disulfide bond (replaced by alanines in $[5–55]_{Ala}$), the 5–55 disulfide bond, and the aromatic residues Phe4, Tyr21, Tyr23 and Phe45. The minor core is located near the top of the molecule and comprises the side-chains of residues Pro9, Phe22, Phe33 and Tyr35.

We found that the two hydrophobic cores respond differently to mutations. The major hydrophobic core is extremely intolerant to alanine substitutions. Mutations in all but one of the aromatic residues (Phe4) in this hydrophobic cluster cause $[5–55]_{Ala}$ to be predominantly or completely unfolded at 0°C (Table 1). Replacement of Phe4 with alanine destabilizes $[5–55]_{Ala}$ by approximately 3 kcal/mol. The minor hydrophobic core is somewhat more tolerant to alanine substitutions. F33A is completely unfolded at 0°C, and F22A is destabilized by 2 kcal/mol. However, alanine substitutions at Pro9 and Tyr35 lower the stability by only 0.8 and 1.0 kcal/mol, respectively.

The relatively small change in the stability of the Y35A mutant, as well as the small change in stability of native BPTI when Y35 is replaced with alanine (Goldenberg *et al.*, 1992), is surprising, since Tyr35 is over 80% buried in BPTI (30A/51A) (Eigenbrot *et al.*, 1990) and is highly conserved among BPTI homologs (Creighton & Charles, 1987). Structural determination of a mutant of native BPTI in which Tyr35 was replaced with Gly (Housset *et al.*, 1991) has shown that BPTI compensates for the loss of the Tyr35 side-chain by undergoing a large conformational change. We find that the thermal unfolding transition of Y35A is unusually broad (for example, compare ΔH_{vH} for Y35A with that of V34A, which has a comparable t_m; Table 1). Thus, it seems likely that the replacement of Tyr35 by Ala also leads to significant structural adjustment in the $[5–55]_{Ala}$ background. By contrast, a recent X-ray crystallographic analysis of four highly destabilizing mutants (F22A, Y23A, N43G and F45A) (Danishefsky *et al.*, 1993) shows that in the context of wild-type BPTI with the three disulfide bonds intact, these mutations result in only modest changes in the protein structure.

This alanine-mutagenesis study also demonstrates the importance of buried side-chain hydrogen bonds in the stability of BPTI. For example, the side-chain of Asn43 forms hydrogen bonds with the backbone atoms of Tyr23, thus mimicking a β-sheet hydrogen bonding pattern, as well as with the backbone carbonyl group of Glu7. Replacement of Asn43 with

Ala causes $[5–55]_{Ala}$ to be completely unfolded at 0°C. Similarly, the side-chain oxygen of Asn44 accepts a hydrogen bond from the side-chain NH of Arg20, donates a hydrogen bond to the ring of Tyr35, and is involved in a hydrogen bond with a water molecule. The N44A and R20A mutants are destabilized by approximately 3 and 1.9 kcal/mol, respectively. The δ-nitrogen of Asn24 donates a hydrogen bond to the ϵ-oxygen of Gln31, and the δ-oxygen of Asn24 accepts hydrogen bonds from the main-chain nitrogens of Lys26 and Ala27. The N24A and Q31A mutants are destabilized by 2.3 and 1.0 kcal/mol, respectively. Finally, the side-chain hydroxyl group of Ser47 accepts a hydrogen bond from the backbone amide of residue 50 at the beginning of the α-helix. Removal of this hydrogen bond in S47A decreases the stability of $[5–55]_{Ala}$ by 1.8 kcal/mol.

Almost all of the alanine substitutions that destabilize $[5–55]_{Ala}$ by more than 10 deg. C occur at residues involved in regular secondary structure. The exceptions occur at the conserved (Creighton & Charles, 1987) glycine residues (Gly12, Gly36, Gly37) found in the loops at the top of the molecule (Figure 1). Gly12 and Gly37 adopt dihedral angles in the BPTI (30A/51A) crystal structure that are in regions of Ramachandran space which are not available to the other amino acids (Schulz & Schirmer, 1979). Gly28 also adopts a backbone conformation which is not possible for other amino acids, but substitution with alanine results in a relatively modest (1.1 kcal/mol) decrease in stability. In contrast to the glycine residues mentioned above, however, Gly28 is not highly conserved evolutionarily.

Comparison of thermal unfolding with reductive unfolding

Goldenberg and co-workers (Coplen *et al.*, 1990; Goldenberg *et al.*, 1992) have carried out extensive genetic screening to identify mutants of BPTI that can fold to an active form, but which are inactivated readily by the reducing agent dithiothreitol (DTT). In the presence of DTT, one of the disulfide bonds of BPTI, 14–38, is readily reduced to yield a two-disulfide intermediate termed N^{SH}_{SH}. The N^{SH}_{SH} intermediate is an active trypsin inhibitor, and is very resistant to further reduction (Creighton, 1977). Two classes of DTT-sensitive mutants have been identified (Goldenberg *et al.*, 1992). For the first class, inactivation results from complete reduction and unfolding of N^{SH}_{SH}. For the second class, the rate of complete reduction is not increased greatly, but the resulting N^{SH}_{SH} intermediate is not an active trypsin inhibitor.

There is good correlation between mutations that greatly increase the rate of reduction of the N^{SH}_{SH} intermediate, and the residues identified in this paper as being most sensitive to replacement with alanine. Mutations that result in a dramatic increase in the rate of reduction of N^{SH}_{SH} are found (Goldenberg *et al.*, 1992) at each of the five residues (Tyr21, Tyr23, Phe33, Asn43 and Phe45) that are most intolerant to alanine substitution in $[5–55]_{Ala}$.

The agreement between other mutants that increase the rate of reduction of N^{SH}_{SH} and our results is not as good. For example, no DTT-sensitive mutants were found at residue Phe4 or Asn44, even though alanine substitutions at these residues decreased the stability of $[5–55]_{Ala}$ by approximately 3 kcal/mol. In addition, two alanine substitutions (G12A, G37A) were found to have little effect on the rate of reduction of N^{SH}_{SH} by DTT (Goldenberg *et al.*, 1992), whereas these changes destabilized the $[5–55]_{Ala}$ protein by 1.8 and 2.4 kcal/mol, respectively. It is possible that the rate of reduction by DTT is not sensitive to slight changes in the thermodynamic stability of BPTI. Alternatively, these Ala mutations might cause a greater destabilization in $[5–55]_{Ala}$ than in N^{SH}_{SH}.

Correlation between buried surface area and stability

It is generally believed that the hydrophobic effect is the major factor that stabilizes the folded structures of globular proteins (review, Dill, 1990). While some studies have found a good correlation between the change in the buried hydrophobic surface area and the change in the free energy of unfolding due to mutations (e.g. see Matsumura *et al.*, 1988; Kellis *et al.*, 1988; Eriksson *et al.*, 1992) other studies have found a poor correlation (e.g. see Shortle *et al.*, 1990; Sandberg & Terwilliger, 1991; Serrano *et al.*, 1992; Milla *et al.*, 1994).

Measurements of the change in buried surface area for our alanine mutations are complicated by the fact that an X-ray structure for $[5–55]_{Ala}$ is not yet available. However, the structure of BPTI (30A/51A) has been determined at a resolution of 1.6 Å (Eigenbrot *et al.*, 1990). Using the BPTI (30A/51A) structure, the static solvent-accessible surface area of each side-chain at non-glycine positions was calculated, and the buried surface area was determined using the value for Ala-X-Ala as a reference for the unfolded state (Lee & Richards, 1971). Effects of alanine substitutions at each position were approximated by the removal of the atoms beyond C^{β}, assuming no significant structural change resulted from the mutations.

For the Ala mutants in $[5–55]_{Ala}$, the change in free energy is linearly related to the loss of buried hydrophobic surface area among the hydrophobic residues (Val, Ile, Leu, Met, Phe and Pro) of $[5–55]_{Ala}$ (Figure 5). The slope of the correlation corresponds to a loss of about 20 cal/mol per $Å^2$ buried area ($r = 0.92$). This is similar to the value for the transfer of hydrophobic surfaces from non-polar solvents to water (Nozaki & Tanford, 1971; Damodaran & Song, 1986), although it is less than that observed in some other studies (Pace, 1992; Jackson *et al.*, 1993). Two features of the hydrophobic residues studied here distinguish them from most of the hydrophobic substitutions studied previously. First, none of the

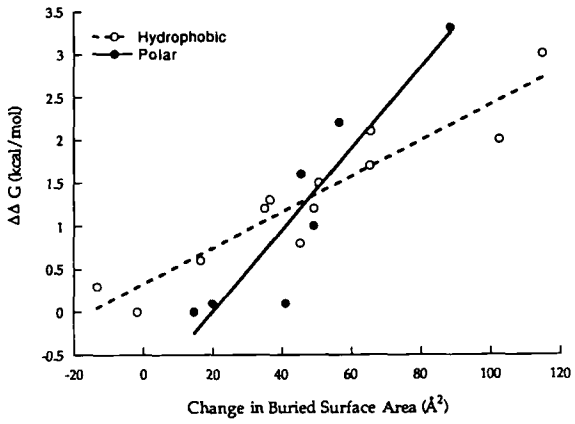

Figure 5. Relationship of $\Delta\Delta G$ of unfolding as a function of buried surface area. Substitutions of the hydrophobic residues (○, Leu, Val, Phe, Met, Ile and Pro) are plotted separately from substitutions of the polar residues (●, Ser, Thr, Asn and Gln). The straight lines are the least squares fit to the data. The slope of the lines are 20 and 50 cal/mol Å^2 (r = 0.92 and 0.93) for the hydrophobic and polar residues, respectively. Solvent-accessible static surface area of each atom was calculated from the structure of BPTI (30A/51A) (Eigenbrot *et al.*, 1990).

residues examined in our study was completely buried, while most mutation sites analyzed in the other studies were (Serrano *et al.*, 1992; Pace, 1992; Jackson *et al.*, 1993). Second, unlike most proteins, many of the hydrophobic residues of BPTI are buried in rather polar environments (Danishefsky *et al.*, 1993).

The change in buried surface area and the change in free energy is also correlated (Figure 5) for the alanine substitutions of the polar residues (Asn, Gln, Ser and Thr). Surprisingly, the proportionality constant ($\sim$50 cal/mol per Å^2; r = 0.93) is 2.5-fold greater than the value for the hydrophobic residues. By contrast, an analysis of polar to alanine mutations in staphylococcal nuclease found only a weak correlation between change in stability and degree of burial of the amino acid side-chain (Green *et al.*, 1992). It is likely that in BPTI the large destabilization upon substitution of the buried polar residues results from the loss of buried hydrogen bonds (see above).

Conclusions

The stability changes observed in the alanine mutants of $[5\text{–}55]_{Ala}$ correlate well with structural parameters. In particular, these studies demonstrate the importance of the two hydrophobic cores composed largely of clusters of aromatic residues, as well as the internal hydrogen bonding network, in stabilizing BPTI. Our studies also suggest that the sequence of BPTI is substantially optimized for stability, as none of the alanine substitutions stabilized $[5\text{–}55]_{Ala}$ significantly, and virtually all the side-chains make a measurable, albeit often modest, contribution to the stability of the protein. Overall, our results are qualitatively in good agreement with a recent alanine-scanning mutagenesis study of the Arc repressor (Milla *et al.*, 1994).

Numerous studies on the effect of mutations on protein stability have established that proteins are generally highly tolerant to single residue substitutions (for reviews see Alber, 1989; Matouschek & Fersht, 1991; Matthews, 1993). Where this has been examined in other proteins, it has been found that the introduction of multiple mutations generally destabilizes a protein by an amount that is less than or equal to the sum of the individual mutations (Wells, 1990; Gregoret & Sauer, 1993; Green & Shortle, 1993). Assuming that the effects of the alanine substitutions on the stability of BPTI will also be largely additive, this scanning alanine mutagenesis study suggests that, in addition to the ten alanine residues already present in $[5\text{–}55]_{Ala}$, up to 21 additional residues could be replaced by alanine while still destabilizing the protein by less than 5 kcal/mol. Although the native structure of $[5\text{–}55]_{Ala}$ is stabilized by only 2.7 kcal/mol, BPTI containing two or three disulfide bonds has a significantly greater stability than 5 kcal/mol (Moses & Hinz, 1983; Schwarz *et al.*, 1987; Hurle *et al.*, 1990). These studies, therefore, raise the interesting possibility that a form of BPTI with up to half of the residues being alanine could fold into a stable structure resembling the native one.

Materials and Methods

Molecular biology reagents were obtained from New England Biolabs and Boehringer Mannheim. Ultra-pure urea was purchased from Nacalai Tesque (Japan), and ultra-pure guanidine hydrochloride from Schwarz/Mann Biotech. All other chemicals were reagent grade.

Plasmid construction

All plasmids were constructed using standard cloning procedures (Sambrook *et al.*, 1989) and the sequences were confirmed by DNA sequencing. The gene coding for $[5\text{–}55]_{Ala}$ (Staley & Kim, 1992) was ligated into the *Nde*I/*Bam*HI site of a phagemid/T7 expression vector, pAED4 (Doering, 1992) to construct an expression plasmid called p5–55. The plasmid backbone of pAED4 is pUC-f1 (Pharmacia Fine Chemicals), and the bacteriophage T7 expression sequences of pAED4 are from the pET3a plasmid (Studier *et al.*, 1990). Alanine substitutions were introduced individually into the gene coding for $[5\text{–}55]_{Ala}$ by oligonucleotide-directed mutagenesis (Kunkel, 1985). Mutagenic oligonucleotides were synthesized on an Applied Biosystems DNA/RNA synthesizer (model 392).

Protein expression and purification

All the variants of BPTI used in these studies formed inclusion bodies upon expression in *Escherichia coli*. Proteins were purified from inclusion bodies and refolded as described previously (Staley & Kim, 1992), except that the reverse-phase high performance liquid chromatography (HPLC) purification step prior to the oxidation of the disulfide bond was omitted. Amino-terminal sequencing of several mutant proteins and laser desorption mass spectrometry (Finnigan LASERMAT) indicated that expression in *E. coli* resulted in the addition of a methionine

residue to the amino terminus. Laser desorption mass spectrometry also confirmed the amino acid substitutions in each mutant protein (measured molecular masses were within 5 AMU (atomic mass units) of the expected molecular masses).

For undetermined reasons, four mutants, Phe4 (F4A), Pro8 (P8A), Thr11 (T11A) and Tyr35 (Y35A) were not expressed well in *E. coli*. These alanine mutants could be expressed as a fusion protein consisting of the TrpLE leader sequence (Kleid *et al.*, 1981), followed by a single methionine residue and the BPTI gene. The BPTI mutants were produced from the fusion proteins as described previously (Staley, 1993; Staley & Kim, 1994). Briefly, (1) the fusion proteins were purified from inclusion bodies and subjected to cyanogen bromide (CNBr) cleavage (1 liter of cells was resuspended in 5 ml of 70% (v/v) formic acid containing approximately 200 mg of CNBr, and cleavage was allowed to proceed for 2 to 3 hours). The single methionine residue in BPTI at residue 52 was replaced with leucine to prevent cleavage of the protein during the CNBr treatment. (2) The cleaved BPTI protein was subjected to air oxidation in 6 M guanidine hydrochloride. (3) The oxidized protein was purified by reverse-phase HPLC. Production as a fusion protein yields a slightly different model of BPTI, termed $[5\text{–}55]_{Ala}$(ΔM0/M52L), which is identical to $[5\text{–}55]_{Ala}$, except that it does not contain an amino-terminal methionine, and Met52 has been replaced with Leu. The four mutants (F4A, P8A, T11A and Y35A) were studied in the context of the $[5\text{–}55]_{Ala}$(ΔM0/M52L) molecule.

Thermal unfolding transition

Temperature-dependent unfolding was monitored by circular dichroism (CD) spectroscopy at 222 nm. Samples consisted of 8 μM protein in a buffer containing 10 mM phosphate (pH 7.0), 150 mM NaCl and 1 mM EDTA. Protein concentrations were determined by tyrosine and cystine absorbance of stock solutions (Edelhoch, 1967). Samples were degassed under negative pressure shortly before melting. Measurements were made in a 10 mm pathlength cell in an Aviv model 62DS CD spectrophotometer. Two-degree steps between 0 and 70°C were used, with 90 seconds equilibration between steps, and 60 seconds of signal averaging per step. The melting temperatures (t_m) and the van't Hoff enthalpies of the unfolding transitions (ΔH_{vH}) were determined by fitting the experimental data to equation (1) using a non-linear least squares fitting program (Nonlin, Robelco software):

$$\theta(T) = \frac{\theta_f + m_f T + (\theta_u + m_u T)\exp[-\Delta H_{vH}((1/T)-(1/T_m))/R]}{1 + \exp[-\Delta H_{vH}((1/T)-(1/T_m))/R]} \quad (1)$$

T is the absolute temperature in K; R is the gas constant (1.98 cal/mol K); $\theta(T)$ is the measured CD signal at 222 nm; θ_f is the value of the folded signal extrapolated to 0 K; m_f is the slope of the temperature dependence of the CD signal for the folded protein; θ_u is the value of the folded signal extrapolated to 0 K; m_u is the slope of the temperature dependence of the CD signal for the unfolded protein. For two mutants, F4A and N44A, there was not sufficient folded baseline to accurately fit the unfolding transition. For these proteins, the T_m (Kelvin) was determined by taking the first derivative of the CD signal (θ) with respect to T^{-1} (Kelvin) and finding the minimum of this function (Cantor & Schimmel, 1980).

Urea-induced unfolding transition

Native protein (15 μM) was incubated in 10 mM sodium phosphate (pH 7.0), 150 mM NaCl and 1 mM EDTA, and various concentrations of urea at 5°C. The CD signal at 222 nm was measured for each sample. The free energy of unfolding (ΔG^{H_2O}) was determined by fitting the experimental data to a two-state unfolding model, equation (2), assuming a linear relationship between free energy of unfolding and the concentration of urea (Pace, 1986);

$$\theta = \frac{\theta_f + m_f[U] + (\theta_u + m_u[U])\exp(-(\Delta G^{H_2O} - m[U])/(RT))}{1 + \exp(-(\Delta G^{H_2O} - m[U])/(RT))} \quad (2)$$

θ is the CD signal at 222 nm; T is temperature in K; R is the gas constant; θ_f is the value of the folded signal in the absence of urea; m_f is the slope of the urea dependence of the CD signal for the folded protein; θ_u is the value of the unfolded signal in the absence of urea; m_u is the slope of the urea dependence of the signal for the unfolded protein; $[U]$ is the urea concentration; and m is the dependence of the free energy of folding on the denaturant concentration.

Calorimetry

Differential scanning calorimetry (DSC) was performed on a MC-2 calorimeter (Microcal, Inc.) at a protein concentration of 1.5 to 3 mg/ml. Samples were dialyzed exhaustively against folding buffer (10 mM sodium phosphate (pH 7.0), 150 mM NaCl and 1 mM EDTA), centrifuged to remove any particulate matter, and degassed under negative pressure. The melts were performed at 45 to 60°C/hour. The values of ΔH_{cal} and estimates of the change in heat capacity upon unfolding (ΔC_p) were determined by fitting the data to a two-state model with non-zero ΔC_p and a progress baseline (Origin, Microcal, Inc.). The van't Hoff enthalpy was determined from the calorimetric data according to equation (3):

$$\Delta H_{vH} = 4RT_m^2 \Delta C_{p,m} \Delta H_{cal}^{-1} \quad (3)$$

T_m is the midpoint of the melting transition (Kelvin); $\Delta C_{p,m}$ is the excess specific heat capacity at T_m; and ΔH_{cal} is the corresponding specific enthalpy change. The melting transition was highly reversible under these conditions, showing an approximately 5% loss in ΔH after each scan. A similar degree of reversibility has been reported previously for calorimetric studies of a derivative of BPTI in which Cys14 and Cys38 were chemically modified (Schwarz *et al.*, 1987).

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