Crevice-forming mutants of bovine pancreatic trypsin inhibitor: Stability changes and new hydrophobic surface

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Abstract

Four mutants of bovine pancreatic trypsin inhibitor (BPTI) with replacements in the rigid core result in the creation of deep crevices on the surface of the protein. Other than crevices at the site of the mutation, few other differences are observed in the crystal structures of wild-type BPTI and the mutants F22A, Y23A, N43G, and F45A. These mutants are highly destabilized relative to wild type (WT). The differences between WT and mutants in the free energy change associated with cooperative folding/unfolding, $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$, have been measured by calorimetry, and they are in good agreement with $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$ values from hydrogen exchange rates. For F22A the change in free energy difference is about 1.7 kcal/mol at 25 °C; for the other three mutants it is in the range of 5–7 kcal/mol at 25 °C. The experimental $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$ values of F22A, Y23A, and F45A are reasonably well accounted for as the sum of two terms: the difference in transfer free energy change, and a contribution from exposure to solvent of new surface (Eriksson, A.E., et al., 1992, *Science* 255, 178–183), if the recently corrected transfer free energies and surface hydrophobicities (De Young, L. & Dill, K., 1990, *J. Phys. Chem.* 94, 801–809; Sharp, K.A., et al., 1991a, *Science* 252, 106–109) are used and only nonpolar surface is taken into account. In N43G, three protein–protein hydrogen bonds are replaced by protein–water hydrogen bonds. The value of $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$ computed from model transfer free energies and surface hydrophobicities is 4.0 kcal/mol less than observed, consistent with reports that one hydrogen bond contributes about 1.3 kcal/mol of stabilizing free energy (Shirley, B.A., et al., 1992, *Biochemistry* 31, 725–732).

Keywords: BPTI; crystal structure; hydrogen exchange; protein folding; thermostability

The high-resolution crystal structures of bovine pancreatic trypsin inhibitor (BPTI) with Phe 22, Tyr 23, Asn 43, or Phe 45 replaced by alanine or glycine have been determined (Danishefsky et al., 1993 [companion paper]; kinemage file Dnshfsky.kin). The replaced residues are completely or partially buried in wild type (WT) and are highly conserved in BPTI homologs. Compared to WT, F22A, Y23A, N43G, and F45A have few changes in structure other than removal of atoms of the mutated side chains, which creates deep crevices, composed of large

concave surfaces in contact with solvent water. The molecular surfaces of F22A and F45A in the region of the mutation are shown in Figure 1.

The large differences in the free energy change between WT and mutants, $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$, for the cooperative folding/unfolding transition are reported here. Determination of the contributions of specific interactions to the total stability of a protein, a highly significant question in protein folding and function, remains largely intractable because the interactions involved are numerous, compensating, and/or cooperative. It is difficult, and some argue impossible, to assess the contribution to total stability of a particular interaction if the only data available are folding thermodynamics and structures of mutant proteins with perturbations of the interacting residues in question. Nevertheless, a growing data base of folding

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Fig. 1. Crevices at the mutation sites of F45A (upper) and F22A (lower). Structures for wild-type (WT) and mutant bovine pancreatic trypsin inhibitor (BPTI) are superimposed. The dot surfaces are the molecular surfaces (Connolly, 1983) of the mutant structure. The position of the replaced side chain in the WT structure is evident in the crevices. The locations of crystallographic waters in the crevices of the mutant structures are indicated by *. WT BPTI is 5PTI in the Brookhaven Data Bank. Structures of F45A and F22A are reported in Danishefsky et al. (1993) (see also kinemage file Dnshfsky.kin).

thermodynamics and high-resolution structures of protein variants is becoming available, along with empirical correlations of stability changes and structural changes. In the absence of more fundamental theoretical and experimental approaches, these are important in protein engineering and design.

One correlation of thermodynamic process and molecular structure is widely accepted, namely, hydrophobic transfer and hydrophobic surface area. Solvent transfer free energy of apolar solutes, the standard model for hydrophobic free energy, is approximately proportional to the apolar solute molecular surface area (Hermann, 1972; Chothia, 1974) and carbon number (McAuliffe, 1966; Ben-Naim & Marcus, 1984). Several methods have been developed that are reasonably successful in quantifying thermodynamic parameters of protein folding from

straightforward considerations of protein size and the surface area of atoms buried when the protein folds (Eisenberg & McLachlan, 1986; Ooi et al., 1987; Ooi & Oobatake, 1988; Murphy et al., 1992; Spolar et al., 1992). We have examined the polar/apolar makeup of the new surface area in these BPTI mutant structures and its correlation with stability changes. Specifically, we consider the suggestion of Eriksson et al. (1992b) that destabilization of mutants with a large $\rightarrow$ small apolar side-chain replacement is approximated by two terms, a hydrophobic transfer free energy and a constant number of calories per square Ångström of new surface created by the mutation.

Replacement of a large side chain by a smaller one can lead to relaxation of surrounding atoms into the vacated space (Eriksson et al., 1992a), to the formation of a cavity or crevice (Eriksson et al., 1992b), or to configura-

tional rearrangement of residues in the vicinity of the mutation (Housset et al., 1991). The small number of local rearrangements of neighboring atoms in these BPTI mutants is explained by the location of the replaced side chain in the rigid core of the molecule, defined as the submolecular region containing the slowest exchanging amide protons and the atoms with lowest crystallographic *B*-factors. This may explain, in general, the variable results of replacing a large side chain with a smaller one. In the rigid core there will be little or no rearrangement, whereas in the flexible regions local adjustments are expected.

Results and discussion

Crevice-forming mutants are in the slow exchange core

BPTI has two submolecular domains distinguishable by the ^1H - ^2H isotope exchange rates of their backbone and side-chain NH hydrogens. The core has the slowest exchanging peptide amide hydrogens, which in WT BPTI do not exchange unless the protein first unfolds (Woodward & Hilton, 1980; Hilton et al., 1981). The flexible domain of BPTI contains buried NHs with fast exchange rates in two overlapping loops (residues 9–17 and 36–43), which enclose four buried water molecules (Tüchsen & Woodward, 1987; unpubl.). Hydrogen exchange of buried protons is mediated by internal fluctuations of the protein. Very slow exchange implies inflexibility. Lumry and Gregory (1986) pointed out an expected correlation of the hydrogen exchange core, which they call the knot, with low crystallographic *B*-factors and with high evolutionary conservation of the side chains. This is observed in BPTI. The slowest exchanging cluster of backbone NH groups on residues 21, 22, and 23 exchange on the week-to-month time scale at physiological pH and temperature (Richarz et al., 1979; Wagner, 1983); their side chains are among the most highly conserved residues in BPTI; the vicinity of the backbone in the segment 21–23 coincides with the region of lowest *B*-factors in WT crystal structures. The slow exchange core, taken as heavy atoms within 5 Å of the backbone nitrogens of 21, 22, and 23, consists of atoms in side chains 20, 21, 22, 23, 30, 33, 43, 45, and 51, and of backbone atoms in residues 20, 21, 22, 23, 24, 30, 31, 32, 33, and 45.

The extent of rearrangement of atoms surrounding a replaced residue in BPTI is related to whether the residue is in the flexible loops or the rigid core. The side chains of Phe 22, Tyr 23, and Phe 45 pack against the backbone in the segment 21–23, and the Asn 43 side chain is H bonded to the backbone NH and O of Tyr 23. When atoms are removed in the hydrogen exchange-defined core, as in F22A, Y23A, N43G, and F45A, few rearrangements are observed, even in the immediate vicinity of the mutation (Danishefsky et al., 1993). The most significant

are in N43G, in which three intramolecular H bonds are disrupted (Danishefsky et al., 1993). Little change in structure is observed in BPTI mutants with replacements of cysteines 5, 30, 51, and/or 55 (Eigenbrot et al., 1991; Hurle et al., 1992); their side chains are buried in or near the core. In contrast, when Tyr 35, which is buried within the flexible loops, is changed for glycine, the resulting structure has large adjustments in the region of the mutation, some >4 Å, but essentially no change in the rest of the molecule (Housset et al., 1991). Lattice-packing effects in the Y35G crystals do not explain the rearrangements in the flexible loops. Another example of structural plasticity in the BPTI loops defined as flexible by hydrogen exchange criteria is found in the BPTI-related protein APPI, the protease inhibitor domain of Alzheimer's amyloid β -protein precursor. APPI has many amino acid differences from BPTI, but differences among buried hydrophobic residues are conservative. APPI backbone conformation is almost identical with that of BPTI, except in the loop region 39–41 where significant rearrangements occur, including a flip of the peptide unit of residues 39 and 40 (Hynes et al., 1990). In this region of APPI the sequence Gly–Gly–Asn replaces the WT BPTI sequence Arg 39–Ala 40–Lys 41.

Because there is little or no relaxation of surrounding atoms, the deletion of side-chain atoms in F22A, Y23A, N43G, and F45A results in crevices in the molecular surface. The openings from bulk solvent to the crevice surface, defined by the accessible surface of Lee and Richards (1971), or the molecular surface of Connolly (1983), are large enough to accommodate at least one water molecule. For Y23A, N43G, and F45A the crevices are very deep; the smallest crevice is found in F22A, reflecting the partial exposure of one edge of Phe 22 ring in WT.

The new crevice surface is primarily composed of backbone atoms

Table 1 lists the atoms that comprise the crevice area in each mutant structure. The majority of atoms lining the crevice left by the mutation are backbone atoms. Along with C, N, O, and CA, we include CB atoms in the backbone, as suggested by Richardson et al. (1992). The percentage of the crevice surface created by backbone atoms is 91%, 98%, 81%, and 89%, respectively, for F22A, Y23A, N43G, and F45A. The absence of significant rearrangement of atoms surrounding the site of the mutation is consistent with the packing of the removed side chains against the backbone, which lacks the configurational freedom of side chains. The crevice surface atoms that are not backbone atoms are primarily in rings of tyrosine, phenylalanine, and proline as well as in disulfides, which are expected to be more conformationally restricted than aliphatic side chains.

The crevices in the F22A, Y23A, N43G, and F45A structures contain one or more crystallographic waters

Table 1. Solvent-accessible surface areas of atoms lining the crevices of bovine pancreatic trypsin inhibitor (BPTI) mutants^a

F22A		Å ²	Y23A		Å ²	N43G		Å ²	F45A		Å ²
Pro 9	CG	3.1	Ala 23	CB	2.4	Cys 5	CA	0.6	Phe 4	CE1	10.7
Ala 22	CB	1.3	Asn 24	N	0.1	Glu 7	O	21.6	Arg 42	O	25.4
Asn 24	CB	3.1		C	0.1	Pro 9	CA	9.0	Asn 43	CA	2.0
Gln 31	O	9.7	Gly 28	CA	18.4	Phe 22	CB	1.9	Asn 44	N	1.6
Thr 32	O	20.4		C	2.5		CD1	10.2		C	2.4
				O	19.2	Tyr 23	N	1.6	Asp 50	C	12.2
			Leu 29	C	1.7	Gly 43	N	1.1		O	3.4
				O	0.6		CA	6.7		CB	21.5
			Cys 30	SG	1.3		O	1.7	Cys 51	CA	2.0
			Met 52	SD	0.4				Thr 54	CB	16.3
			Cys 55	C	1.6						
				O	0.1						
				CB	0.1						
			Gly 56	CA	15.0						
Total area		37.6			63.5			54.4			97.5
Nonpolar area		7.5 (20%)			43.5 (69%)			28.4 (52%)			67.1 (69%)
Polar area		30.1 (80%)			20.0 (31%)			26.0 (48%)			30.4 (31%)

^a The static accessible surface (Lee & Richards, 1971) obtained with a probe sphere size of 1.4 Å is given for all atoms lining the crevice in the mutant structures.

(Danishefsky et al., 1993), and atoms lining the crevices are presumed to be in dynamic contact with bulk water. In this sense, the crevices effectively increase the solvent-accessible surface of the protein. There is a difference, however, between the normal external surface of the protein and the surface of crevices and cavities. The outside surface is, on average, convex, whereas the surfaces of crevices and cavities are highly concave. This may be significant in determining the effective hydrophobicity of the solvent-protein interface (Nicholls et al., 1991; Sharp et al., 1992b).

The additional, or new, area of nonpolar surface in each mutant due to crevice formation is shown in Table 2. The new crevice nonpolar area is the area of nonpolar atoms in the crevice at the site of mutation minus the solvent-accessible area of nonpolar atoms in the removed side chain (WT structure). The new crevice polar area is computed similarly (Table 2). The negative sign of the new nonpolar surface area in F22A means that WT has more nonpolar surface than mutant.

Because the crevice surfaces in the mutant structures are largely composed of backbone atoms, we expect polar environments for these side chains in WT. The environment analysis of Bowie et al. (1991) is given in Table 3. Core hydrophobic groups are usually thought of as cluster forming, with extensive contacts between apolar side chains. In BPTI this is not the case. For Phe 22, Tyr 23, and Phe 45, the fraction of the side chain covered by polar atoms, *F_p*, is in the range 41–67%. This is much more polar than the environment of buried aromatic side chains in larger proteins. For example, T4 lysozyme has

a number of hydrophobic side chains with *F_p* in the range 20–35% (Bowie et al., 1991). Like the side chains of 22, 23, and 45, the other aromatic side chains in BPTI (4, 10, 21, 33) are mostly buried and highly conserved, and they also have rather polar environments (Table 3). The only side chains with highly apolar environments, *F_p* ≤ 31%, are the side chains of the buried disulfides formed by cysteines 5, 30, 51, and 55. In other words, BPTI folding does not bring together clusters of aromatic and aliphatic side chains; rather, the apolar side chains packed by other apolar side chains are the buried cysteines 5–55

Table 2. New surface area in BPTI mutant structures

Surface	Area ^a (Å ²)			
	F22A	Y23A	N43G	F45A
Total crevice	37.6	63.6	54.4	97.5
WT ^b NP	21.6	0.6	0.6	13.1
WT ^b P	0	9.5	0	0
New crevice ^c NP	−14	43	28	54
New crevice ^c P	30.1	10.5	26	30.4

^a The static accessible surface (Lee & Richards, 1971) using a probe radius of 1.4 Å.

^b The surface area of nonpolar (NP) and polar (P) atoms in the replaced side chain in the wild-type (WT) structure. The exposed polar surface of Tyr 23 in WT is on the phenolic oxygen.

^c The new nonpolar (NP) surface area is the static accessible surface area of apolar atoms lining the crevice in the mutant structure minus the area of exposed apolar atoms of the replaced side chain in the WT structure. The new crevice polar (P) area is computed in the same way.

Table 3. Environments of WT BPTI side chains^a

Residue	Area buried	Fraction polar	Secondary structure	Residue	Area buried	Fraction polar	Secondary structure
1 Arg	125.8	0.66	C	30 Cys	52.5	0.31	S
2 Pro	66.2	0.60	C	31 Cln	73.4	0.65	S
3 Asp	3.6	0.86	H	32 Thr	58.3	0.65	S
4 Phe	149.7	0.50	H	33 Phe	162.3	0.62	S
5 Cys	56.0	0.23	H	34 Val	85.8	0.69	S
6 Leu	68.4	0.67	H	35 Tyr	181.8	0.61	S
7 Glu	102.4	0.58	C	36 Gly	40.0	0.78	C
8 Pro	19.1	0.75	C	37 Gly	37.9	0.16	C
9 Pro	87.9	0.38	C	38 Cys	23.6	0.62	C
10 Tyr	112.9	0.67	C	39 Arg	33.7	0.79	C
11 Thr	53.1	0.73	C	40 Ala	41.5	0.68	C
12 Gly	40.0	0.54	C	41 Lys	84.1	0.71	C
13 Pro	69.7	0.72	C	42 Arg	39.0	0.80	C
14 Cys	22.6	0.51	C	43 Asn	123.0	0.49	C
15 Lys	24.5	0.97	C	44 Asn	120.2	0.43	S
16 Ala	39.4	0.56	C	45 Phe	166.5	0.59	S
17 Arg	55.0	0.81	C	46 Lys	38.6	0.92	C
18 Ile	96.6	0.70	S	47 Ser	56.6	0.75	C
19 Ile	80.5	0.69	S	48 Ala	45.7	0.56	H
20 Arg	170.9	0.41	S	49 Glu	43.8	0.83	H
21 Tyr	139.6	0.54	S	50 Asp	60.1	0.80	H
22 Phe	163.0	0.55	S	51 Cys	56.0	0.21	H
23 Tyr	192.2	0.42	S	52 Met	97.6	0.67	H
24 Asn	83.0	0.62	S	53 Arg	42.2	0.88	H
25 Ala	57.7	0.37	C	54 Thr	72.1	0.47	H
26 Lys	18.9	1.00	C	55 Cys	56.0	0.18	H
27 Ala	38.7	0.71	C	56 Gly	40.0	0.47	C
28 Gly	32.3	0.80	C	57 Gly	11.2	0.99	C
29 Leu	61.4	0.77	S	58 Ala	0.0	0.95	C

^a The environments of BPTI side chains were determined by the method of Bowie et al. (1991). The area buried is the square Ångstroms of side-chain atoms buried in the crystal structure (5PTI). The fraction polar is the fraction of side-chain atoms covered by polar atoms in the crystal structure. Secondary structure is indicated as S for β -sheet, H for helix, and C for all other. Aromatic residues are in bold type.

and 30–51. The packing of hydrogen-bonded backbone atoms by aromatic side chains may account for the very slow exchange rates of the backbone NH protons in the BPTI core and for the remarkable stability of wild-type BPTI. It is also expected to influence the pathway of disulfide formation during folding from reduced BPTI.

BPTI core mutants are highly destabilized

The stabilities of F22A, Y23A, N43G, F45A, and WT are given in Table 4. For comparison, there are also values for the mutant Y35G, which has large structural changes in the flexible loops and essentially no structural changes in the core (Housset et al., 1991). The temperature-induced unfolding transition is measured in two ways, by hydrogen exchange and by calorimetry. The hydrogen exchange experiments are at pH 3.5 where the exchange rates are near their minimum and where the ¹H NMR resonances are assigned. The calorimetry experiments are at pH 2 because at neutral pH BPTI has a $T_m > 90^\circ$, making it too stable to permit accurate characterization of its thermo-

dynamics using ordinary calorimeters. The calculation of T_m , $\Delta H(T_m)$, and $\Delta G^0(\text{cal})$ from the calorimetric data is described in Methods. $\Delta\Delta G^0(\text{cal})$ is the difference in $\Delta G^0(\text{cal})$ for the mutants and $\Delta G^0(\text{cal})$ for WT.

$\Delta\Delta G(\text{WT} \rightarrow \text{mut})$ can be obtained from hydrogen exchange rate constants of the same peptide NH proton in WT and in a mutant, provided the proton exchanges by the unfolding mechanism in both WT and mutant (Tao et al., 1993). For any proton in a given protein, exchange may occur by either of two pathways. One is exchange from the folded state, and the other involves a mechanism in which exchange is preceded by reversible cooperative unfolding (Woodward et al., 1982). Which of these pathways is utilized by an exchanging proton depends on the pH and temperature (Woodward & Hilton, 1980; Woodward et al., 1982; Tüchsen & Woodward, 1987). Under conditions where the unfolding mechanism operates, the ratio of exchange rate constants of the same proton in WT and in a mutant is approximately equal to $K_{\text{eq}}(\text{WT})/K_{\text{eq}}(\text{mut})$, the ratio of folding/unfolding equilibrium constants of WT and mutant. Then $\Delta\Delta G(\text{HX}) =$

Table 4. Stability of WT BPTI and its mutants

	T_m^a (°C, pH 2)	$\Delta H(T_m)^a$ (kcal/mol, pH 2)	$\Delta G(25^\circ\text{C})^a$ (kcal/mol, pH 2)	$\Delta\Delta G(\text{cal})^a$ (kcal/mol, pH 2)	$\Delta\Delta G(\text{HX})^b$ (kcal/mol, pH 3.5)
WT	87	70	9.0		
F22A	78	68	7.8	1.2	2.1
Y23A	54	45	3.1	5.9	7.0
Y35G	69	38	4.0	5.0	5.7
N43G	55	46	3.3	5.7	6.0
F45A	49	36	2.1	6.9	7.2

^a Obtained by differential scanning calorimetry experiments, as described in Methods. The standard deviations for curve fitting and experimental error are 0.5 °C, 1.0 kcal/mol, 100 cal/mol/°K, and <0.7 kcal/mol for T_m , ΔH , ΔC_p , and $\Delta G(25^\circ\text{C})$, respectively.

^b The change in ΔG between WT and BPTI variants, $\Delta\Delta G$, is obtained from hydrogen exchange data at pH 3.5, 30 °C (Tao et al., 1993).

$-RT \ln(K_{eq}(\text{WT})/K_{eq}(\text{mut}))$. Table 4 gives the values of $\Delta\Delta G(\text{HX})$ for the BPTI mutants measured from peptide NH hydrogen exchange rate constants of Tyr 21, Phe 22, and Tyr 23 in wild-type BPTI and in each mutant (Tao et al., 1992; Kim et al., in prep.).

The values of $\Delta\Delta G(\text{WT} \rightarrow \text{mut})$ determined by hydrogen exchange and by calorimetry are in reasonably good agreement, given the difference in pH. BPTI is very stable even at pH 2 where the carboxyl groups have been protonated, including the C-terminal carboxyl, which, when it is deprotonated, forms a salt bridge with the N-terminal amino group. With the exception of F22A, the change in stability in all the other mutants is exceptionally large, around 5–7 kcal/mol.

Comparison of experimental $\Delta\Delta G^0$ with the sum of $\Delta\Delta G_{tr}^0$ and $\Delta\Delta G^0$ from new hydrophobic surface

Any attempt to identify specific interactions contributing to the total stability of a protein by comparing the difference in structure between WT and mutant to the difference in ΔG^0 between WT and mutant is hampered by uncertainty. This is clearly illustrated by Sturtevant and associates (Connelly et al., 1991) with T4 lysozyme mutants having multiple side-chain replacements at the same site. The calorimetrically measured $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$ is small and destabilizing for all of the mutants. However, the changes in the enthalpy difference, $\Delta\Delta H_d$, are highly variable; in some mutants $\Delta\Delta H_d$ is small or zero, but in others it is quite large and of varying sign. Mutants in the latter category will therefore also have large, compensating changes in $\Delta\Delta S_d$, likewise of varying sign. Further, the value of $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$ for the T4 lysozyme mutants depends on the pH. The cautionary point is that underlying large changes in enthalpic and entropic interactions are not necessarily reflected in $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$ nor are they necessarily indicated by the crystal structure, since they may consist of numerous small contributions spread throughout the molecule (Connelly et al., 1991).

Nevertheless, solvent transfer free energy of apolar solutes is approximately proportional to the apolar solute surface area (Hermann, 1972; Chothia, 1974) and carbon number (McAuliffe, 1966; Ben-Naim & Marcus, 1984). A number of groups have developed computer-based techniques that relate protein folding thermodynamics and protein size and/or buried surface (Eisenberg & McLachlan, 1986; Ooi et al., 1987; Ooi & Oobatake, 1988; Murphy et al., 1992; Spolar et al., 1992). Given this, it seems appropriate to examine whether the changes in stability are explained by approaches suggested for hydrophobic free energy associated with transfer of apolar side chains and with exposure of apolar surface. In the BPTI mutants discussed here, the changes in ΔG^0 are very large, and the changes in ΔH_d are of the same sign and of similar magnitude except for F22A (Table 4).

Eriksson et al. (1992b) pointed out that mutants with large cavity surfaces are more highly destabilized than accounted for by the difference in transfer free energy of the side-chain replacements. They reported a series of cavity-forming mutants of T4 lysozyme in which destabilization is accounted for by the sum of two terms, the transfer free energy, ΔG_{tr}^0 , for the side-chain replacement Leu $\rightarrow$ Ala, and 20 cal/Å² for the new cavity surface (Connolly, or molecular surface) in the mutants. Similar to the cavity-forming mutants of T4 lysozyme, the crevice mutants of BPTI have $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$ values that are not explained by model compound transfer free energies. The observed $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$ for the three aromatic $\rightarrow$ alanine BPTI mutants are approximated by two terms as suggested by Eriksson et al. (1992b), but only if we use the transfer free energy values and about 47 cal/mol/Å² of new apolar surface (De Young & Dill, 1990; Sharp et al. 1991a,b). The results of this calculation are shown in Table 5.

In Table 5, a new static accessible surface arising from apolar atoms only (Lee & Richards, 1971) is multiplied by 47 cal/mol/Å² (De Young & Dill, 1990; Sharp et al., 1991a) to get the contribution from the new surface; this

Table 5. Comparison of the observed values for BPTI mutants of $\Delta\Delta G^0$ (WT→mut) with the computed change in free energy, taken as the sum of the change in transfer free energy for the amino acid replacement plus a constant factor for new apolar surface in the mutants^a

Mutant	$\Delta\Delta G_{\text{surf}}^0$ ^b	$\Delta\Delta G_{\text{tr}}^0$ ^c	$\Delta\Delta G_{\text{total}}^0$ ^d	$\Delta\Delta G^0$ observed ^e
F22A	-0.66	3.1	2.4	1.2
Y23A	2.0	2.9	4.9	5.9
F45A	2.5	3.5	6.0	6.9
N43G	1.3	0.4	1.7	5.7

^a $\Delta\Delta G^0$ is in kilocalories/mole.

^b The change in free energy due to the exposure of new apolar surface, taken as 47 cal/mol/Å² of new apolar surface area.

^c Values of transfer free energy are corrected water-octanol transfer free energies in Table III of Sharp et al. (1991b). In WT, Phe 22 and Phe 45 side chains are 18% and 11% exposed to the static accessible surface (Lee & Richards, 1971); adjustment for this is made by subtracting 18% and 11%, respectively, of the ΔG_{tr} values for Phe. The listed $\Delta\Delta G_{\text{tr}}$ values are ΔG_{tr} for the replaced side chain, minus ΔG_{tr} for alanine or glycine.

^d The total computed change in free energy, $\Delta\Delta G_{\text{surf}}^0 + \Delta\Delta G_{\text{tr}}^0$.

^e Calorimetric values from Table 4.

is added to the transfer free energies taken from corrected octanol–water transfers in Table III of Sharp et al. (1991b) and recently supported by Pace (1992). Comparison of columns four and five in Table 5 shows reasonable, though not compelling, agreement of observed and computed values for the mutants with replacements of non-polar residues, F22A, Y23A, and F45A. Mutant N43G is not expected to follow this type of behavior, since it has protein–water H bonds replacing three protein–protein H bonds, as discussed below. When the approach used by Eriksson et al. (1992b) is used, computed values for $\Delta\Delta G_{\text{total}}^0$ are 2.4, 2.2, 1.5, and 3.5 kcal/mol for F22A, Y23A, N43G, and F45A. The difference between calculation of Eriksson et al. and the one used in Table 5 is as follows. Eriksson et al. compute the total new cavity molecular surface area using the method of Connolly (1983) and multiply by 20 cal/Å² to get the contribution from the total new surface. To this they add the transfer free energies taken from uncorrected octanol–water or ethanol–water transfers. Our use of new apolar surface area, rather than total surface exposed, is an important difference because F22A is stabilized, rather than destabilized, by the surface term, since WT has 14 Å² more apolar surface area exposed to solvent than does the mutant (Table 2).

Several uncertainties, besides the ones discussed above, might explain the differences in observed and computed changes for F22A, Y23A, and F45A. First, the area of the Y23A crevice surface is underestimated due to the uncertainty in the position of the Arg 1 side chain (Danishefsky et al., 1993). Second, it has been suggested that the hydrophobicity of the solvent–protein interface at the protein

surface is shape dependent (Nicholls et al., 1991). The crevices created in these mutants increase the surface area of the molecule, but the external surface in WT is, on average, convex, while the cavity surface is deeply concave, especially in Y23A and F45A. In the calculations of Nicholls et al. (1991), based on surface tension considerations, a convex surface is more highly hydrophobic than a concave surface.

Spolar et al. (1992) have developed a method for calculating thermodynamic functions using the apparent reduction in water-accessible surface when a protein goes from an extended peptide to the folded state as represented by the crystal structure. Their method gives values in good agreement with experimental thermodynamic values for the set of proteins for which both crystal structures and thermodynamic data are known. However, it is not clear that their method should work as well for predicting enthalpy and/or free energy changes for mutant proteins since it is based on a WT protein data set, and in addition, BPTI differs from the average in their protein data set (cf. Table III in Spolar et al., 1992).

H-bond replacements in N43G

Unlike most polar side chains, the Asn 43 primary amide is fully buried. Its removal disrupts three intermolecular H bonds involving the buried NH of Tyr 23, and the buried carbonyl oxygens of Glu 7 and Tyr 23. These are replaced in N43G by protein–water H bonds (Danishefsky et al., 1993). The large $\Delta\Delta G^0$ for N43G is not approximated by the sum of the transfer free energy and new surface area (Table 5). The difference between the observed and computed $\Delta\Delta G^0$ is 4.0 kcal/mol. Dividing by three gives 1.3 kcal/mol as the apparent contribution of each intramolecular H bond changed for an intermolecular H bond. Although this cannot be taken as a demonstration of the difference in stability between a buried protein–protein H bond and an exposed protein–water H bond, it is notable that this value is entirely consistent with the values found by Shirley et al. (1992). It has been suggested that one reason for a difference in stability between protein–protein H bonds and protein–water H bonds may be the loss of water entropy associated with intermolecular H bonds (Vriend et al., 1991).

Summary

The structures of four variants of BPTI with replacements of buried conserved side chains have been solved (Danishefsky et al., 1993). The crevices formed at the mutation site add significant new concave surface to the protein. Three of the mutants, F22A, Y23A, and F45A, have replacement of a conserved, buried aromatic ring by a methyl group. The other mutant, N43G, has replacement of a conserved, buried primary amide by hydrogen. Dif

ferences in the free energy change for the cooperative denaturation reaction, $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$, measured by calorimetry, are 1.2, 5.9, 5.7, and 6.9 kcal/mol for F22A, Y23A, N43G, and F45A. These agree fairly well with values of $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$ from hydrogen exchange (Tao et al., 1993).

The experimental $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$ values of F22A, Y23A, and F45A are, to a first approximation, accounted for as the sum of two terms, the difference in transfer free energy change, and a contribution from exposure to solvent of new surface, as suggested by Eriksson et al. (1992). However, we get reasonable agreement between observed and calculated $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$ only if the recently corrected transfer free energies and surface hydrophobicities (De Young & Dill, 1990; Sharp et al., 1991a,b) are used, and new nonpolar surface, rather than total surface, is taken into account.

In N43G, three buried intramolecular H bonds involving Asn 43 in WT are lost and the remaining H-bonding partners are in protein-water H bonds. Therefore, the difference between the computed and observed $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$ should be due to the difference in stability between three intramolecular and intermolecular H bonds. We find that the difference between the computed and observed $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$ is 4.0 kcal/mol. This is consistent with the approximate value of 1.3 kcal/mol per H bond reported by Shirley et al. (1992) to be the difference between one intramolecular H bond and one intermolecular H bond.

Methods

The surface area of WT and mutant BPTI was calculated both as static accessible surface area (Lee & Richards, 1971) using the program ACCESS and as the molecular surface (Connolly, 1983) using the program MS. Atoms lining the crevice surface were identified by inspection of the overlapped surfaces of WT and mutant displayed on Insight (Biosym). The cavity surface area is the summed surface areas of atoms lining the crevices.

Environmental profiles of side chains in WT BPTI were calculated by the program ENVIRONMENTS included in the 3D Profile program package (Bowie et al., 1991). This program calculates the area of the residue buried in the protein and the fraction of side-chain area that is covered by polar atoms. The solvent-accessible area of the side-chain is the sum of the solvent-accessible areas of the side-chain atoms, including the α -carbon atom. The total area of a side chain buried in the protein is the difference between the solvent-accessible side-chain area in the protein and in a Gly-X-Gly tripeptide.

Thermodynamic stabilities of WT BPTI and mutants were measured by differential scanning calorimetry (DSC) using an MC-2 instrument (Microcal, Inc.) at a scan speed of 1°/min. Protein (2 mg/mL) was buffered at pH 2 with

10 mM glycine/HCl, 0.1 N NaCl. DSC measurements were carried out at pH 2.0 for several reasons. BPTI is too stable to permit the gathering of reliable data at higher pH. Further, we find that, at pH >3.5, the high temperatures used in calorimetry hydrolyze the peptide bonds of Pro 2-Asp 3 (75%) and Asp 3-Phe 4 (25%). We attribute the hydrolysis to catalysis by the charged side-chain carboxyl group of Asp 3 at high temperature. Other reasons favoring calorimetry experiments at pH 2 are that the enthalpy of unfolding of BPTI is almost independent of pH around pH 2.0 (Moses & Hinz, 1983), and that the buffer effect on the enthalpy of unfolding is negligible because the titration enthalpy of $-\text{COO}^-$ is negligible (Privalov et al., 1986). Variation of concentration over the range 1.0–3.0 mg/mL did not affect the thermodynamic behavior of proteins.

The midpoint temperature, T_m , and $\Delta H^0(T_m)$ values in Table 4 for WT and for mutants are determined from fits to the differential scanning calorimetry curves obtained from the MC-2 instrument, assuming two-state denaturation (Jackson & Brandts, 1970). The ratio of the calorimetric ΔH to the van't Hoff ΔH is within experimental error of unity. The free energy of each protein at 25 °C was calculated from a form of the Gibbs-Helmholtz equation

$$\Delta G = \Delta H_m * (1 - T/T_m) + \int_{T_m}^T \Delta C_p * dT - T * \int_{T_m}^T \Delta C_p * d(\ln T)$$

where $\Delta G(T)$ is the ΔG at temperature T , T_m is the midpoint of the thermal unfolding, ΔH_m is the enthalpy change for unfolding measured at T_m , and ΔC_p is the difference in heat capacity between the unfolded and folded states (Privalov, 1979). Recent experiments carried out in collaboration with Dr. Peter Privalov on the DASM-4M scanning microcalorimeter show that ΔC_p of WT BPTI is temperature dependent, ~ 700 cal/mol/°K at 25 °C and ~ 70 cal/mol/°K at 125 °C (Makhatadze et al., unpubl.). In Table 4, with the exception of Y35G, the calorimetric values of $\Delta G^0(\text{mut})$ and $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$ are calculated using the temperature-dependent ΔC_p derived from the data of Makhatadze et al. (unpubl.), which gives a value of ~ 400 cal/mol/°K at T_m of 87 °C. We also carried out numerous calorimetric experiments using the MC-2 instrument with WT BPTI over the pH range 2.0–3.5, in the presence and absence of 2.0 M GuHCl, and with mutant BPTIs at pH 2.0. We observe an apparent ΔC_p at T_m of about 400 cal/mol/°K for WT and 400 cal/mol/°K or less for mutants. For Y35G the apparent ΔC_p at T_m is about half of WT. Therefore, for Y35G, a ΔC_p value of half that used for the WT is employed for calculation of $\Delta G^0(\text{mut})$ and $\Delta\Delta G^0(\text{WT} \rightarrow \text{mut})$ in Table 4.

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