Crevice-forming mutants in the rigid core of bovine pancreatic trypsin inhibitor: Crystal structures of F22A, Y23A, N43G, and F45A

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Abstract

Crystal structures of four mutants of bovine pancreatic trypsin inhibitor (F22A, Y23A, N43G, and F45A), engineered to alter their stability properties, have been determined. The mutated residues, which are highly conserved among Kunitz-type inhibitors, are located in the rigid core of the molecule. Replacement of the partially buried bulky residues of the wild-type protein with smaller residues resulted in crevices open to the exterior of the molecule. The overall three-dimensional structure of these mutants is very similar to that of the wild-type protein and only small rearrangements are observed among the atoms lining the crevices.

Keywords: BPTI; crevices; crystal structure; mutants; rigid core

Bovine pancreatic trypsin inhibitor (BPTI) is a small, globular, highly stable protein (Moses & Hinz, 1983). Its complex with trypsin has an association constant of $>10^{13} \text{ M}^{-1}$, making it one of the tightest binding protein–protein complexes known (Vincent & Lazdunski, 1972). BPTI is a member of the structurally related family of Kunitz serine protease inhibitors, which presumably function in enzyme regulation. Homologs of BPTI have been identified in a wide variety of tissues, both as discrete proteins and as domains contained within larger proteins (Creighton & Charles, 1987). Recently described examples of such domains include the Alzheimer amyloid β -protein precursor (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988) and a lipoprotein-associated coagulation inhibitor (Wun et al., 1988). Crystal structures of two biologically active proteins with extensive sequence similarity to BPTI have been solved and these were shown to be very similar to BPTI (Hynes et al., 1990; Skarżyński, 1992). Because of the very extensive studies on the structure, stability, and function of BPTI, it is considered the prototype of the Kunitz inhibitor family.

Structures of the wild-type BPTI have been determined to high resolution in three different crystal forms (Deisenhofer & Steigemann, 1975; Wlodawer et al., 1984, 1987a,b), and most recently in solution by nuclear magnetic resonance (Berndt et al., 1992). The polypeptide fold is shown schematically in Figure 1. The trypsin binding region, consisting of residues 11–19 and 34–39 is located within two outer loops of the protein (Rühlmann et al., 1973). The central portion of the molecule contains a β -structure comprised of two antiparallel strands formed by residues 18–35. A short pseudostrand (residues 43–45) forms a hydrogen bond network with part of the β -structure and contributes a triple strand character to this region. This network, which involves the main-chain atoms of Phe 45 and the side-chain atoms of Asn 43, is illustrated in Figure 2 and Kinemage 1. Evidence suggests that this region of the protein is particularly critical for stability and folding of BPTI. Mutants with replacements within this region of β -structure, closest to the 5–55 and 30–51 disulfide bonds, have been shown to be more sensitive to unfolding by dithiothreitol than mutants with replacements in other regions (Goldenberg et al., 1992).

Previously we determined the crystal structure of a variant of BPTI with a mutation in the outer loop region. That mutant, Y35G, exhibited large deviations in its three-dimensional structure from the wild-type protein (Hous-

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Fig. 1. Diagram of bovine pancreatic trypsin inhibitor (BPTI) illustrating the secondary structure and the position of the mutated residues. Residues 22, 23, 43, and 45 are associated with the β -structure in the rigid core. Tyr 35 is located in the loops that comprise the trypsin-binding region.

set et al., 1991). In order to probe the relationship of the structural features of BPTI to its stability and internal flexibility, we have also constructed mutants with replacements at the highly conserved positions in the central region of β -structure. Here we report the crystal structures of the mutants F22A, Y23A, N43G, and F45A. The overall three-dimensional structures of these mutants are very similar to that of wild-type protein. The mutations, which

involve removal of bulky side chains, create crevices open to the exterior of the molecule. In the accompanying paper, the differences in stability between these mutants and the wild-type BPTI are reported and analyzed in relation to the expected hydrophobic effects of these crevices.

Results and discussion

High-resolution crystal structures were determined for the BPTI mutants F22A, Y23A, N43G, and F45A. Crystal data and refinement statistics are shown in Table 1. Details of the structures were analyzed in order to assess which interactions might contribute to the destabilization of these mutants as compared to the wild-type protein.

Comparison of atomic positions of mutants with wild-type

The mutants analyzed here are very similar structurally to wild-type BPTI and in general, only small (<0.5 Å) rearrangements are observed at the sites of the mutations. Root mean square (RMS) differences between the superposed coordinate sets are shown in Table 2. Comparisons are drawn between each mutant and the wild-type crystal structure of the same space group, in order to minimize any differences that might be related to crystal packing. Therefore N43G is compared to 6PTI⁴ (form III) (Wlodawer et al., 1987b) and the other three mutants are compared to 5PTI⁴ (form II) (Wlodawer et al., 1984).

The local environment of each of the mutation sites and the superposition of mutant on wild-type structures is shown in Figure 3. Asn 43 is located on the short pseudostrand. Its closest contacts are to residues on the N-terminal helix and residues 22 and 23 on the β -strand. In

⁴ 5PTI and 6PTI are the Brookhaven Protein Data Bank codes for the form II and form III BPTI structures, respectively.

Fig. 2. Stereo view of the hydrogen-bonding network in the β -structure of the rigid core of BPTI. The network, comprised of main-chain atoms in the β -strands in addition to main-chain atoms of Phe 45 and side-chain atoms of Asn 43, is represented by thick lines. Hydrogen bonds are shown as dashed lines. The tracing of the carbon- α chain is indicated by the lightly shaded lines. The orientation of this figure is the same as that of Figure 1.

Table 1. Crystal data and refinement statistics for four bovine pancreatic trypsin inhibitor (BPTI) mutants

BPTI mutant	F22A	Y23A	N43G	F45A
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2 ₁
Unit cell dimensions a, b, c (Å)	a = 69.20 b = 22.21 c = 28.71	a = 69.47 b = 23.35 c = 29.19	a = 55.72 b = 37.98 c = 23.79	a = 69.31 b = 23.19 c = 29.24
Number of observations	2,410	2,996	4,421	3,493
Resolution limit (Å)	10.0–2.17	10.0–1.98	10.0–1.85	10.0–1.95
[completeness]	[94%]	[88%]	[98%]	[98%]
Weighted R_{sym} on intensity	6.3%	8.5%	5.8%	6.7%
Crystallographic R -factor	17.2%	16.5%	16.3%	16.9%
Number of water molecules	48	40	64	56
Root mean square deviation (Å) from (restraints applied in parentheses):				
Bond length (0.02)	0.021	0.018	0.013	0.015
Angle distance (0.03)	0.059	0.041	0.036	0.034
Planarity (0.02)	0.033	0.017	0.011	0.015
Chirality (0.15)	0.138	0.207	0.140	0.158

N43G, the main-chain atoms of residues 42 and 44 move away from the crevice so that new water molecules are accommodated. The main-chain atoms of residues 5, 7, and 23 appear to reorganize and adjust to new hydrogen-bonding arrangements (Table 3; Fig. 4). Figure 5A indicates the magnitudes of the positional deviations of superposed N43G coordinates from the wild-type, as a function of residue number for the main-chain atoms. In N43G, the largest displacements from wild-type occur at residues closest to the mutation.

Comparison of the three mutants F22A, Y23A, and F45A with the wild-type structure indicate that concerted movements of residues surrounding the crevices formed by the mutations are uncommon (Fig. 3). The main-chain atoms of Phe 22 and Tyr 23 are located in one of the core β -strands. The side chain of Phe 22 is in contact with residues on both strands of the β -structure and with Pro-9. A displacement of about 0.8 Å of Pro 9 toward Ala 22

is observed in F22A (Kinemage 2). No concerted movements of other residues are observed in this region of F22A, and displacements of atoms from wild type are generally less than 0.5 Å. The side chain of Tyr 23 is in the vicinity of the β -turn and the C- and N-termini. Displacements from wild type observed in this region of Y23A (Kinemage 3) are less than 0.5 Å except for the

Table 3. Comparison of hydrogen bonds at the mutation site in N43G and wild type (6PTI)

Atom	Atom	Wild type		Water atom	N43G	
		Distance (Å)	Angle ^a (degrees)		Distance (Å)	Angle ^a (degrees)
O-7	ND2-43	2.8	157	WAT-301	2.8	140 ^b
	WAT-152	2.8	119 ^b	WAT-152	2.8	122 ^b
O-5	WAT-143	3.5	134	WAT-300	3.1	120
				WAT-152	3.4	133
O-23	ND2-43	3.3	129	WAT-143	3.5	137
	WAT-143	3.1	129			
N-23	OD1-43	2.9	3	WAT-300	2.8	6
O-43	WAT-112	2.8	116 ^b	WAT-112	3.0	116 ^b
				WAT-301	3.0	117

^a Where main-chain carbonyl oxygens are in the first column, the angle refers to the C–O–X angle, in which C–O is the carbonyl group and X is hydrogen bond donor. Where N-23 is in the first column the angle represents the deviation of the acceptor atom from an axis drawn along the main-chain N–H bond.

^b Though the two atoms are within hydrogen-bonding distance, the position of the water molecules deviates substantially from the plane, formed by the α carbon and the carbonyl carbon and oxygen, in which the oxygen lone pair electrons lie. Therefore the nature of these interactions is uncertain (Thanki et al., 1991).

Table 2. Root mean square (RMS) deviations of mutant coordinates from wild type^a

BPTI mutant	RMS deviation of main-chain atoms	RMS deviation of all atom types
F22A	0.34 (218)	0.40 (381)
Y23A	0.24 (223)	0.28 (366)
N43G	0.20 (215)	0.24 (415)
F45A	0.21 (222)	0.24 (370)

^a RMS deviations were calculated using ALIGN (Satow et al., 1986). The numbers of target pairs are shown in parentheses. Each mutant is compared to the wild-type structure of the same crystal form. Therefore F22A, Y23A, and F45A are compared to 5PTI, and N43G is compared to 6PTI.

Fig. 4. Stereo view of the geometry of atoms at the site of the N43G mutation for wild type (**A**) and N43G (**B**). Wat 300 and Wat 301 are the two new water molecules in the crevice of N43G. (Only those water molecules not present in the wild-type structure are represented.) Dashed lines are drawn to indicate where distances between two atoms are appropriate for hydrogen bond formation.

terminal residues, which appear disordered in many of the crystal structures. Phe 45 is located on the pseudo-strand and its side-chain atoms contact other residues on this strand as well as residues on the C-terminal helix (Kinemage 4). Displacements of atoms of F45A from wild type in the region of this mutation are generally less than 0.4 Å and in no case greater than 0.5 Å.

The largest positional deviations of main-chain atoms from wild type occur at residues 25–27, particularly in F22A (Fig. 5B). Residues 25–29 comprise a β -turn, which is not in direct contact with the Phe 22 side chain in the wild-type crystal structure. Relative flexibility of this

β -turn region is reflected by the fact that displacement of residues 25–29 are among the largest when comparing the two wild-type structures, 5PTI and 6PTI (Fig. 5E) and by the finding that temperature factors of this region in Y23A and F45A are the highest in these structures (Fig. 6C,D). In addition, Kossiakoff et al. (1992) have noted that this region has the most intermolecular crystallographic contacts and the most variability among the crystal structures they have compared. Main-chain coordinates at the region of residues 46–50 also have above average deviations from wild type in the F45A and F22A mutants. Though this region does make contacts with

Fig. 5. Root mean square deviations of mutant main-chain coordinates from wild type of same crystallographic space group. **A:** N43G. **B:** F22A. **C:** Y23A. **D:** F45A. **E:** Comparison of two wild-type crystal structures: 6PTI and 5PTI.

Fig. 6. Average temperature factors of main-chain atoms for each residue of N43G (**A**), F22A (**B**), Y23A (**C**), F45A (**D**), and 6PTI (**E**).

Phe 45 in wild type, it is not in direct contact with Phe 22. Therefore the largest deviations of atomic coordinates from the wild type may be less directly related to the mutations themselves than to an inherent flexibility of certain regions of the protein. The deviations in these regions may be an indirect result of the destabilizing effects of the mutations, a response to subtle changes in crystal packing, or a reflection of slightly larger positional errors of this region.

Two features of the structures at the sites of mutation probably contribute to the seeming inelasticity. First, the regions of residues 22, 23, 43, and 45 are among the most rigid in the BPTI structure. This point is evidenced by the findings that these regions have temperature factors that are among the lowest (Fig. 6E), and their backbone amide exchange rates are among the slowest (Wagner & Wüthrich, 1982). Second, the formed crevices are lined largely with structurally restrained backbone atoms (Kim et al., 1993 [companion paper]). The implication of recent studies on T4 lysozyme mutants is that when atoms repack around a mutation site, there is less destabilization relative to wild type than when atoms surrounding a mutation site are rigid (Eriksson et al., 1992). If this is the case, the rigidity of the structure of BPTI at these mutation sites may be contributing to their large decrease in stability.

Hydrogen bonds

Of the residues mutated in this study, only Asn 43 and Tyr 23 have side chains capable of hydrogen bonding. In all crystal structures of wild-type BPTI, Asn 43 is in position to form intramolecular hydrogen bonds with the main-chain atoms O-23 and N-23 in the β -strand and with O-7 in the N-terminal helix. This scheme is illustrated in Figure 4 and Kinemage 5. In the N43G crystal structure these three atoms can form hydrogen bonds with solvent molecules (Table 3). Two ordered water molecules, Wat 300 and Wat 301, are observed in the space left by removal of Asn 43 in the wild-type structure (Fig. 4B; Kinemage 5). The two water molecules are offset from the exact positions occupied by OD1-43 and ND2-43 in the wild-type structure. Specifically, Wat 300 is located between the positions of OD1-43 and ND2-43 and Wat 301 is located on the other side of ND2-43 position. The temperature factors for Wat 300 and 301 are $10 \text{ \AA}^2$ and $20 \text{ \AA}^2$, respectively. Only four internal water molecules, which have been observed in all three wild-type BPTI crystal structures (Wlodawer et al., 1987b), have temperature factors lower than $10 \text{ \AA}^2$, indicating that the position of Wat 300 is a relatively stable one. The difference in temperature factors between Wat 300 and Wat 301 is consistent with the geometry of their hydrogen bonds. Ligands to Wat 300 are approximately tetrahedral, whereas the geometry of ligands bound to Wat 301 suggests that this

water could not interact simultaneously with all its observable potential hydrogen-bonding partners.

Studies of T4 lysozyme mutants have suggested that mutations that remove hydrogen-bonding residues are less destabilizing if the replacements are small residues that allow room for a hydrogen-bonding water molecule to be positioned at the site of the mutation (Alber et al., 1987). Therefore, it is interesting to compare the stability of N43G to those of mutations in which Asn 43 is replaced by a bulkier residue. The finding reported by Goldenberg et al. (1989) that N43G is somewhat more stable than N43A is consistent with the notion that water molecules in the crevice created by the N43G mutation may contribute to the stability of this mutant. Nevertheless, the thermal stability of N43G compared to wild type is greatly reduced (Kim et al., 1993 [companion paper]). The protein-water hydrogen bonds are likely to be less stable than the protein-protein hydrogen bonds. The loss of water entropy associated with the intermolecular hydrogen bonds as well as the less than optimal geometry of hydrogen-bonding partners around Wat 301 probably reduce the stability of these hydrogen bonds, as compared to those in the wild-type protein.

The side chain of Tyr 23 in the various wild-type BPTI crystal structures is not consistently in a good position for intramolecular hydrogen bonding. In some of the structures it may form weak hydrogen bonds with the guanidinium group of the Arg 1. However, this interaction is difficult to assess because the Arg 1 side chain adopts multiple conformations and appears disordered in some of the crystal structures. This disorder most likely reflects a high degree of flexibility at pH >9 where these crystals are obtained. However, in the pH range 4–8 a salt bridge between the N- and C-terminus is indicated by NMR experiments (Brown et al., 1978; Tüchsen & Woodward, 1985). In the Y23A crystal structure, the guanidinium group of Arg 1 is repositioned into the crevice formed by the mutation and hydrogen bonded to Wat 400 of this mutant. Considering the uncertainty and the pH dependence of the position of the Arg 1 side chain, we suspect that the movement of this side chain into the crevice may not occur in solution when the N- to C-salt bridge is intact.

All intramolecular hydrogen bonds not involving the replaced side chains are present in the mutant structures reported here. The intramolecular hydrogen bonds in the wild-type structures are listed in Table 3 of Wlodawer et al. (1987b). Hydrogen bonds involving Arg 1, Glu 7, and Arg 53 side chains were not considered in this comparison because these have alternate conformations or appear disordered in wild-type and some mutant structures.

Ordered water molecules at the crevices

The crevices created by the mutations must be in contact with the surrounding solvent. Electron density corre-

sponding to positions of ordered water molecules is visible in difference Fourier maps for all four mutant crystal structures (Fig. 7), indicating that, to some degree, definable positions for solvent molecules exist in the crevices.

The two new waters that occupy the former position of Asn 43 side chain in N43G were described above. These

water molecules are in contact with the channel of interior water molecules found in all native BPTI crystal structures (waters 111, 112, and 113) and might be viewed as an extension to this channel. The interior water molecules in native BPTI are a dynamic component, and movement of protein atoms in their vicinity allows rapid

Fig. 7. Stereo diagrams of atomic coordinates and electron density maps ($2F_o - F_c$) at the site of each mutation. Solid lines correspond to mutant coordinates. Dashed lines correspond to position of wild-type superposed on the mutant. The crosses indicate waters, in the crevices formed by the mutations. **A:** N43G. **B:** F22A. **C:** Y23A. **D:** F45A.

exchange with bulk solvent. The exchange rates of these buried waters were measured by an isotope exchange method, which showed that they exchange with bulk solvent on the subsecond time scale (Tüchsen et al., 1987), and more recently by an NMR method, which showed these waters have residence times in the range 10^{-2} to 10^{-8} s (Otting et al., 1991).

The solvent molecules visible in the crevices formed by the mutations of F45A, F22A, and Y23A are generally close to the surface of the protein. Their temperature factors, which are higher than those of Wat 300 in N43G, reflect their comparatively less stable positions. Upper limits for exchange rates with bulk solvent for surface water molecules, such as these, have been calculated to be less than 500 ps (Otting et al., 1991).

Conclusion

High-resolution structures are reported for four BPTI mutants having single amino acid replacements of highly conserved residues in the rigid core of the molecule. The structural consequences of these mutations may affect stability of BPTI through a variety of interactions including hydrogen bonding, van der Waals forces, and the hydrophobic effect in both the native and the unfolded state. Of the four mutants, only N43G exhibits significant changes in hydrogen bonding. The mutants do not undergo much reorganization at the site of the mutation. In place of the removed atoms there are crevices at the surface of the protein. In the accompanying paper we report the stabilities of these mutants and an analysis of the expected hydrophobic effects of the amino acid replacements and these newly exposed surface areas (Kim et al., 1992).

Materials and methods

Crystallizations

BPTI mutants were constructed and the protein was expressed and purified as described in Housset et al. (1991). The wild-type gene was kindly provided by Dr. David Goldenberg. Crystallizations of the mutants were carried out by vapor diffusion. For N43G and F45A, approximately 50 μ L of an 8-mg/mL protein solution in 0.6 M potassium phosphate buffer, pH 9, was equilibrated with a reservoir containing 1.2–1.3 M potassium phosphate. Microcrystals grew first and were used to seed fresh protein solutions, which were allowed to equilibrate for 1–2 weeks at 4 °C. Crystals of F22A and Y23A were obtained by the hanging drop method (Wlodawer & Hodgson, 1975). Protein solutions at a concentration of 8 mg/mL in 0.3–0.4 M potassium phosphate (pH 9 for Y23A and pH 9.7 for F22A) were equilibrated with a reservoir containing 1.5 M potassium phosphate, at ambient temperature.

Data collection

Diffraction data were collected on a Siemens electronic area detector mounted on a Rigaku RU-200 rotating anode generator, operating at 50 kV and 100 mA. The measurements for each mutant were performed using only one crystal. The area detector chamber was mounted 10 cm from the crystal with a carriage angle set to 15° for F22A, 20° for Y23A and F45A and 25° for N43G. Diffraction data were collected as a series of 0.25° oscillations and were processed using the XGEN program system (Howard et al., 1987). Details of data processing are listed in Table 1.

Crystal structure refinement and model building

The crystal forms of F22A, Y23A, and F45A are isomorphous and are also closely related to the native form II (Wlodawer et al., 1984). However, there is a 7% reduction in the length of the unit cell *a*-axis in the crystals of the mutant proteins as compared to the native form II. The crystal form of N43G is isomorphous with the native form III (Wlodawer et al., 1987b). To obtain initial phase sets for F22A and Y23A, the coordinates of residues 2–56 of form II were first translated so that the center of the non-hydrogen atoms had the same fractional coordinates in the mutant unit cell as in the form II unit cell. Rigid body refinement was then carried out with three successive resolution windows of 10–6 Å, 10–5 Å, and 10–3 Å. The *R*-factors subsequent to this rigid-body refinement were 38.8% and 40.5% for F22A and Y23A, respectively, using reflections between 10.0 Å and 3.0 Å. The initial phase sets used for F45A were based on the final coordinates of F22A, while N43G refinement was started from the final coordinate sets of the native form III.

Simulated annealing refinement using the slow cool procedure of the XPLOR package was used for initial refinement of all the structures (Brünger et al., 1987; Brünger, 1990). Reflections between 10 Å and the highest resolution range available for each data set were used in this procedure, and an overall temperature factor of 15 Å² was applied at first. After this initial refinement, the mutated residues (and in some cases, the terminal residues), which had been omitted until this point, were built into the model by examination of difference Fourier maps in FRODO (Jones, 1985). Individual temperature factors were refined in XPLOR. Several sets of cycles of restrained least-squares refinement of structure and individual temperature factors were performed using PROLSQ (Hendrickson, 1985; Finzel, 1987; Sheriff, 1987), except for the F22A case in which refinement was carried out using only XPLOR. Water molecules were added to the models in stages, by use of the PEKPIK program in the PROTEIN package (Steigemann, 1974) and by examination of model coordinates and difference Fourier maps in FRODO. Refinement statistics are shown in Table 1.

The final electron density maps are of high quality (Fig. 7). However, the last two residues on the carboxyl terminus appear to be disordered in all the crystal structures. In difference Fourier maps calculated for N43G, no density was observed for these residues, and they were not built into the model. In maps calculated for the other structures, some discontinuous density was observed, and the two residues were fit to this density and refined, though temperature factors are high. The coordinates of N43G and Y23A contain a phosphate group whose position was clear in the electron density maps. This group could not be unambiguously identified in the maps calculated for the F22A and F45A structures. All coordinates have been deposited at the Brookhaven Protein Data Bank.

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