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Genetic dissection of pancreatic trypsin inhibitor

(bovine pancreatic trypsin inhibitor/protein stability/protein folding/protease inhibitors/dithiothreitol-sensitive mutants)

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ABSTRACT **In a previous study, a genetic screening procedure was used to identify variants of bovine pancreatic trypsin inhibitor that can fold to an active conformation but that are inactivated much more rapidly than the wild-type protein in the presence of dithiothreitol (DTT). The mechanisms by which 30 of these DTT-sensitive variants are inactivated have now been investigated. Some of the amino acid replacements cause rapid inactivation in the presence of DTT because the three disulfides of the native protein are reduced up to 300-fold faster than for the wild-type protein, leading to complete unfolding. Other substitutions, however, do not greatly increase the rate of complete reduction and unfolding but lead to accumulation of an inactive two-disulfide species. There is a striking correlation between the locations of the DTT-sensitive amino acid replacements in the three-dimensional structure of the protein and the mechanisms by which the variants are inactivated. All of the substitutions that cause rapid unfolding are clustered at one end of the folded protein, in the vicinity of the two disulfides that are reduced most slowly during unfolding of the wild-type protein, while substitutions of the other class are all located at the other end of the protein, near the trypsin binding site. These results indicate that the kinetic stability of native bovine pancreatic trypsin inhibitor and its ability to function as a protease inhibitor are largely influenced by residues in two distinguishable regions of the folded protein.**

Bovine pancreatic trypsin inhibitor (BPTI) is a small protein, composed of 58 amino acid residues, that has served as an important model for experimental studies of protein folding. The folding process has been analyzed in detail by trapping and characterizing disulfide-bonded intermediates that form when the reduced protein refolds under conditions where folding is coupled to re-formation of the three disulfides of the native protein (1–4).§ In addition, site-directed mutagenesis has been used to test the roles of individual residues in the folding pathway (5–8). As a complement to work with site-directed mutants, we have undertaken a genetic analysis of BPTI using variants isolated by screening randomly mutagenized clones (9).

BPTI variants with altered folding energetics were isolated by a screening method that takes advantage of the ability to manipulate the conformational stability of this protein by adjusting the thiol-disulfide redox potential. When the native wild-type protein is incubated with the reducing agent dithiothreitol (DTT), one of the three disulfides is rapidly reduced to produce a two-disulfide intermediate that retains most of the folded conformation of the native protein (10). Although this intermediate (II_N) is thermodynamically less stable than the fully reduced protein under these conditions, further reduction and unfolding is very slow, with a half-time of ≈20 h. The unfolding intermediate, which contains the 30–51 and 5–55 disulfides of the native protein, is also functional as a trypsin inhibitor (11). In the screening assay, randomly mutagenized clones were tested to identify those that produce proteins that can fold to an active conformation but are inactivated by DTT much more rapidly than the wild-type protein. The DTT-sensitive amino acid substitutions might cause rapid inactivation by increasing the rate of further reduction and unfolding of II_N or by rendering this species inactive.¶ Although the screening assay detects kinetic, rather than thermodynamic, differences among mutant and wild-type proteins, variants with altered inactivation kinetics are likely to display differences in folding thermodynamics as well, since the folding equilibrium is determined by both forward and reverse rates.

We have now examined the mechanisms by which these mutants, as well as additional site-directed mutants, are inactivated by DTT. As expected, some of the mutants were found to be inactivated by rapid unfolding. Other mutants, however, are inactivated by DTT because the intermediate in unfolding, II_N, is rapidly cleaved by trypsin and, thus, cannot act as an inhibitor. The sites of the two classes of amino acid replacements are distinctly clustered in the folded protein, indicating that BPTI, which has been generally considered an archetypical single-domain protein, can be genetically divided into two substructures.

MATERIALS AND METHODS

Protein Purification. Mutant forms of BPTI were produced in *Escherichia coli* HB101 harboring plasmids in which the mutant BPTI genes were fused to a sequence encoding the signal peptide of the *E. coli* OmpA protein (6). The BPTI produced in these strains is correctly folded and the signal peptide is removed. For each mutant protein preparation, a 500-ml culture of bacteria was grown at 30°C, harvested by centrifugation, and lysed in a French pressure cell. The resulting extracts were treated with DNase I (10 μg/ml) for 30 min at 4°C and then centrifuged for 30 min at 14,000 × *g*. The BPTI in the supernatants was purified by ion-exchange chromatography on a column of CM-Trisacryl M (IBF) eluted with a step gradient of 0.05–0.6 M NaCl, followed by gel filtration chromatography on a column of Sephadex G-50 equilibrated and eluted with 0.05 M $(NH_4)HCO_3$. The BPTI-containing fractions were pooled, lyophilized, and redissolved in water. The concentration of active BPTI was determined by titration of a known quantity of bovine tryp-

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; DTT, dithiothreitol.

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§The disulfides of native BPTI are indicated by the residue numbers of the disulfide-bonded cysteine residues: 5–55, 14–38, and 30–51.

¶Amino acid replacements are identified by the wild-type residue type (using the one-letter code for the 20 standard amino acids), followed by the residue number and the mutant residue type.

sin, using a spectrophotometric assay to determine the concentration of free trypsin (12). All of the mutants described here appeared to inhibit trypsin stoichiometrically at concentrations in the range of 2 μM. Nondenaturing gel electrophoresis of the BPTI samples indicated that they were 80–95% pure.

Kinetics of Unfolding. The rates of reductive unfolding of the mutant proteins were measured in the presence of 1 mM DTT under the same conditions used to study the folding and unfolding kinetics of wild-type and mutant forms of BPTI (2, 7): 30 μM BPTI/0.1 M Tris·HCl, pH 8.7/0.2 M KCl/1 mM EDTA at 25°C. At time intervals after the addition of DTT, 25-μl samples of the reaction mixture were withdrawn, mixed with 6.2 μl of 0.5 M sodium iodoacetate/0.25 M Tris·HCl, pH 6.8, and allowed to react for 2 min at room temperature. The iodoacetate-trapped molecules were then resolved by nondenaturing gel electrophoresis at pH 4.5 (13). The gels were stained with Coomassie blue R-250, and the half-times for unfolding were estimated by visual inspection of the gels.

Kinetics of Trypsin Cleavage of Selectively Reduced Proteins. Native proteins were selectively reduced by incubation with 1 mM DTT for 5 min under the same conditions as described above, except that the protein concentration was 60 μM. The samples were then mixed, under N_2, with an equal vol of 3 μM trypsin in 20 mM $CaCl_2$/0.5 M Tris·HCl, pH 6.8, to yield a final pH of 7.5. The trypsin used for these experiments (Boehringer Mannheim) was affinity-purified on a column of soybean trypsin inhibitor agarose (Sigma). At time intervals after mixing the selectively reduced protein with trypsin, 25-μl samples were withdrawn, mixed with SDS sample buffer to give a final concentration of 2% SDS, and heated in a boiling water bath for 5 min. The samples were analyzed by SDS/polyacrylamide gel electrophoresis, using either the discontinuous buffer system of Laemmli (14) or the Tricine buffer system of Schägger and von Jaggow (15). The gels were stained with Coomassie blue R-250 and analyzed with a Bio-Med Instruments (Fullerton, CA) laser densitometer.

RESULTS

The mutant proteins used in this study are listed in Table 1. Eighteen of the mutations causing single amino acid replacements were generated by random mutagenesis and identified by the screen for DTT-sensitive mutants (9). Twenty-four additional mutations were constructed by oligonucleotide-directed mutagenesis in order to test further the roles of some of the residues identified in the screen of random mutants. Of the 24 site-directed variants, 12 were found to be DTT-sensitive in the assay used to screen random mutants. Together, the 30 single DTT-sensitive substitutions alter 14 of the 58 residues of wild-type BPTI.

Unfolding Kinetics of the DTT-Sensitive Mutants. To determine whether the DTT sensitivity of the mutant proteins was due to rapid unfolding in the presence of DTT, each of the proteins was partially purified and the rates of unfolding in the presence of 1 mM DTT at 25°C (pH 8.7) were measured. At time intervals after the addition of DTT, samples were treated with iodoacetic acid, to block any free thiols, and analyzed by nondenaturing gel electrophoresis (Fig. 1). The native proteins are readily separated from partially and fully reduced forms because each free thiol is blocked with a carboxymethyl group, which decreases the net positive charge of the protein. In addition, unfolding increases the hydrodynamic volume of the protein, further decreasing the electrophoretic mobility.

Some of the DTT-sensitive mutants, such as A16V in Fig. 1*a*, displayed unfolding kinetics similar to those of the wild-type protein. One of the three disulfides of the native protein was reduced by 2 min, but no further reduction was detectable after 40 min, indicating that the half-time for complete unfolding and reduction was >250 min. In contrast, other mutants, such as F45L in Fig. 1*c*, unfolded with half-times of 3 min or less. Intermediate rates of unfolding

Table 1. Properties of BPTI variants

Amino acid replacement	Sensitivity to DTT*	$t_{1/2}$ for unfolding,† min	$t_{1/2}$ for cleavage of II_N,‡ min
P2A	Resistant	70	ND
P2S	Resistant	40	ND
P9A	Resistant	>250	ND
P9I	Resistant	20	ND
P9L	Resistant	10	ND
P9T	Resistant	7	ND
G12D§	Sensitive	>250	9
G12R§	Sensitive	>250	2
G12V§	Sensitive	>250	3
P13A	Resistant	250	ND
P13S	Resistant	250	ND
A16T§	Sensitive	>250	20
A16V§	Sensitive	>250	20
Y21N§	Sensitive	3	ND
F22I§	Sensitive	3	ND
F22L§	Sensitive	2	ND
Y23L¶	Sensitive	<2	ND
Y23S§	Sensitive	<2	ND
N24P§	Sensitive	<2	ND
G28A	Resistant	>250	ND
G28K	Resistant	>250	ND
G28V	Resistant	30	ND
L29P§	Sensitive	<2	ND
C30S§	Sensitive	<2	ND
F33A	Sensitive	7	3
F33G	Sensitive	7	7
F33I§	Sensitive	20	20
F33L	Sensitive	20	41
F33V	Sensitive	15	7
Y35A	Sensitive	>250	2
Y35D§	Sensitive	100	2
Y35G¶	Sensitive	>250	4
Y35L	Sensitive	40	2
Y35N§	Sensitive	40	3
G36D§	Sensitive	>250	2
G37A	Resistant	250	ND
G37D	Sensitive	100	20
N43A¶	Sensitive	<2	ND
N43G¶	Sensitive	2	ND
N43L	Sensitive	<2	ND
F45L§	Sensitive	<2	ND
F45S§	Sensitive	<2	ND

*Sensitivity to DTT was determined by assaying bacterial extracts for trypsin inhibitor activity after a 20-min incubation with 2 mM DTT at 25°C and pH 8 (9).

†The half-times for unfolding in the presence of 1 mM DTT at 25°C and pH 8.7 were determined by gel electrophoresis of iodoacetate-trapped samples, as illustrated in Fig. 1.

‡For those variants that unfolded sufficiently slowly, the rate of cleavage of the native-like two-disulfide intermediate, II_N, by trypsin was determined at pH 7.5 as illustrated in Fig. 3. For some of the mutants, such as those with replacements of F33, the half-time for cleavage at pH 7.5 is comparable to the half-time of unfolding at pH 8.7 indicated in the previous column. At pH 7.5, however, the rate of unfolding is ≈10-fold slower than at pH 8.7, making it possible to estimate the rate of cleavage of II_N. ND, not determined.

§These mutants were isolated in a screen of randomly mutagenized clones (9). The other mutants described here were generated by oligonucleotide-directed mutagenesis.

¶The construction and folding kinetics of these mutants have been described (7).

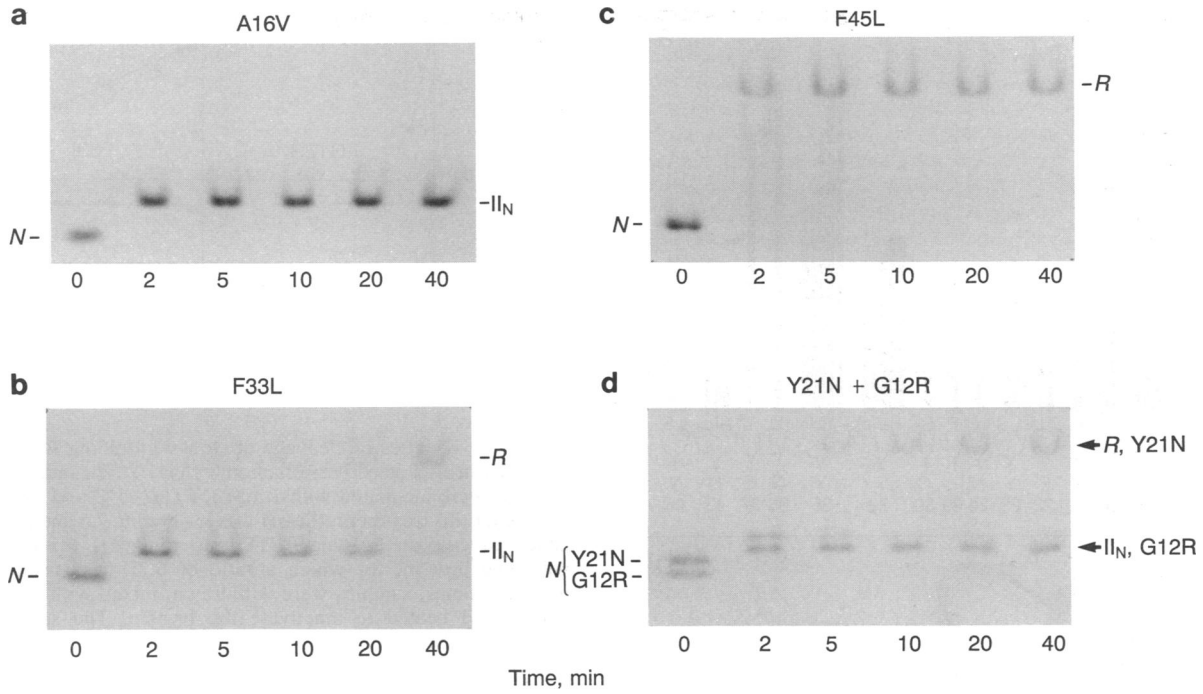

FIG. 1. Unfolding kinetics of DTT-sensitive BPTI mutants. The BPTI variants were incubated at 25°C and pH 8.7 in the presence of 1 mM DTT to promote disulfide reduction and unfolding. At the indicated times, samples were withdrawn, underwent reaction with sodium iodoacetate, and were subjected to gel electrophoresis. The mobilities of the native (*N*), reduced (*R*), and native-like two-disulfide (II_N) forms are indicated. (*d*) Two proteins, Y21N and G12R, were incubated together. Because these two proteins have different net charges, their behaviors can be distinguished electrophoretically. Under these conditions, the wild-type protein displays unfolding kinetics similar to those shown for the A16V mutant.

were observed for proteins with substitutions at F33 and Y35, as shown for the F33L mutant in Fig. 1*b*. The half-times for unfolding of the DTT-sensitive mutants, as well as the 14 DTT-resistant site-directed mutants, are reported in Table 1.

Because the protein samples used in these experiments were not completely purified, it was possible that some of the observed variation in unfolding rates might be due to the presence of differing amounts of other proteins in the samples. Thioredoxin, for example, is known to reduce protein disulfides much more rapidly than does DTT (16), and it is conceivable that the increased rate of unfolding of mutants such as F45L was due to the presence of a higher concentration of this or other *E. coli* proteins in the samples. To address this concern, mixing experiments with mutants displaying different unfolding rates were carried out, as illustrated in Fig. 1*d*. In this experiment, two mutant proteins, Y21N and G12R, which unfold at very different rates, were mixed at equal concentrations and incubated with DTT. Because the G12R substitution increases the net charge of the protein, the behavior of the two proteins in the mixture could be easily distinguished electrophoretically. The two proteins both displayed the same unfolding kinetics in the mixture as they did in isolation. Similar results were obtained with three other pairs of slowly and rapidly unfolding mutants. Thus, the rates of unfolding appear to reflect the intrinsic properties of the individual proteins, rather than differences in the sample preparations.

As shown in Fig. 2*a*, the half-times for unfolding of the 30 DTT-sensitive mutants vary by >100-fold. For eight residues of the protein, two or more DTT-sensitive amino acid replacements were studied. In most cases, different mutants with replacements of the same residues behaved similarly, indicating that the sites of the substitutions are primary determinants of the unfolding rates, although the natures of the replacements undoubtedly also influence the behavior of the mutants.

The sites of the DTT-sensitive replacements in the native structure of wild-type BPTI are indicated in Fig. 2*b*, with the most darkly shaded circles indicating the sites at which substitutions cause the largest increases in unfolding rate. There is a clear pattern in the distribution of the substitutions in the native protein. All of the variants that unfold most rapidly are altered at sites surrounding the 5–55 and 30–51 disulfides. Those substitutions that cause inactivation of BPTI without rapid unfolding are located at the opposite end of the molecule, closer to the 14–38 disulfide and the trypsin binding site.

Cleavage of Selectively Reduced Mutant Proteins by Trypsin. The mutants that are rapidly inactivated by DTT but do not unfold rapidly might be inactivated because the native-like two-disulfide intermediate II_N does not interact with trypsin at all, or because this species is rapidly cleaved by trypsin. To distinguish between these possibilities, these variants were selectively reduced by a brief treatment with DTT and were then incubated with trypsin. At designated time intervals after addition of trypsin, samples were withdrawn and the concentration of intact BPTI was determined by SDS gel electrophoresis. The kinetics of hydrolysis for three mutants and wild-type BPTI are shown in Fig. 3.

The 17 DTT-sensitive mutants examined in this way were all rapidly cleaved by trypsin after selective reduction. In the absence of the DTT treatment, however, there was no measurable decrease in the concentration of intact protein after 90 min. As expected from previous studies (11, 18), there was no detectable cleavage of wild-type BPTI after 90 min, with or without DTT treatment, even in the presence of a 4-fold greater trypsin concentration.

DISCUSSION

The assay used to identify the DTT-sensitive mutants used in this study relies on two properties of the native-like two-disulfide intermediate II_N: This species is very slow to be

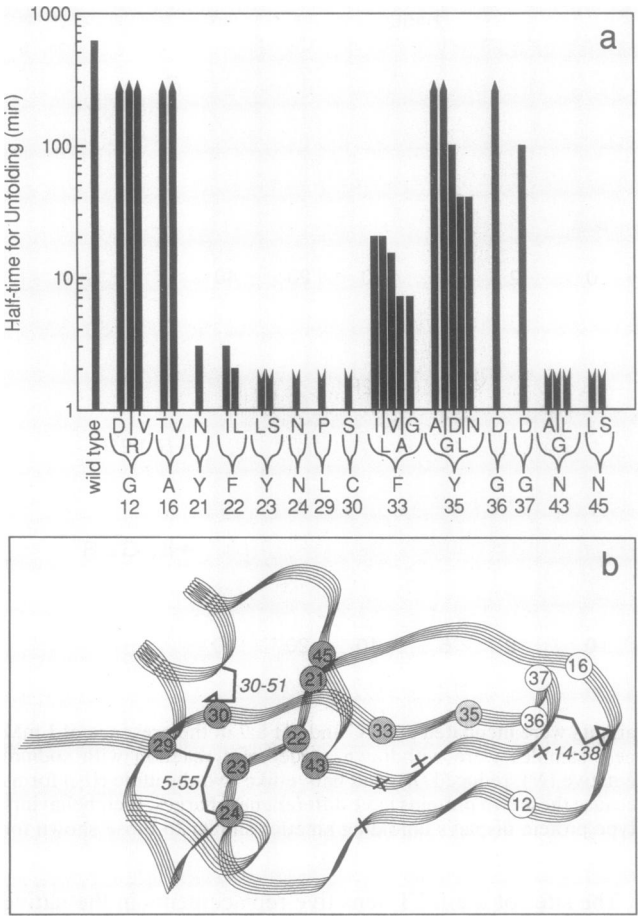

FIG. 2. Unfolding rates of DTT-sensitive mutants. Half-times for unfolding were estimated from gel electrophoresis of iodoacetate-trapped reactions, as illustrated in Fig. 1. (*a*) Half-times are plotted for each DTT-sensitive replacement at the indicated sites. Bars topped with upward-pointing triangles indicate that the half-time for unfolding was estimated to be >250 min, while bars with downward-pointing "v"s indicate unfolding half-times of <2 min. (*b*) Half-times for mutants with replacements at different sites in the native protein are indicated by shading circles at the positions of α-carbons in the ribbon diagram [drawn from coordinates of the form II crystal structure (17)]. Darkest shading indicates sites at which replacements cause unfolding half-times of 3 min or less. Unshaded circles identify sites of replacements in DTT-sensitive mutants with unfolding half-times of 100 min or more. Lighter shading is used to indicate that variants with substitutions at F33 or Y35 display a range of intermediate unfolding rates. The positions of the three disulfide bonds of the native protein are indicated by thick lines; ×, positions of the four buried water molecules. The trypsin binding site is at the right of the structure as drawn here, near the 14–38 disulfide.

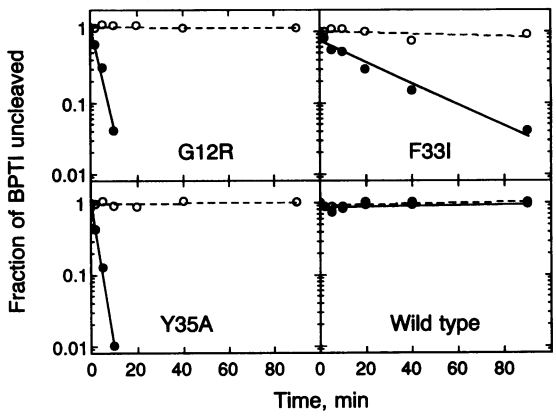

FIG. 3. Kinetics of cleavage of slowly unfolding BPTI variants with trypsin after selective reduction with DTT. The indicated native proteins were incubated with 1 mM DTT at 25°C and pH 8.7 for 5 min. After this treatment, the pH was lowered to 7.5 and trypsin was added to give an enzyme/BPTI ratio of 0.05:1 (except for the wild-type protein, for which a ratio of 0.2:1 was used). At the indicated times, samples were withdrawn, mixed with SDS sample buffer, and heated to inactivate the trypsin. The samples were subjected to SDS/polyacrylamide gel electrophoresis, and the relative concentrations of uncleaved BPTI were determined by densitometry of the Coomassie blue-stained gels. ●, Time course of cleavage after treatment with DTT; ○, results of control experiments in which the native proteins were incubated with trypsin without prior treatment with DTT.

reduced and unfolded, and it is a moderately good trypsin inhibitor. The results presented here indicate that these two properties of II_N are largely determined by amino acid residues in different regions of the native protein.

Seventeen of the amino acid replacements examined give rise to the DTT-sensitive phenotype by causing the two-disulfide intermediate to be cleaved by trypsin. Although the selectively reduced disulfide in these variants has not yet been identified directly, it is almost certainly the same as the first disulfide to be reduced in the wild-type protein 14–38. It seems unlikely that a substitution near the 14–38 disulfide would simultaneously make this disulfide very unreactive and greatly increase the reduction rate of a disulfide buried at the other end of the protein. Some of the substitutions that allow cleavage of II_N alter residues that are located on the surface of the protein and form direct contacts with trypsin in the enzyme–inhibitor complex. Substitutions at these sites, which include A16, G36, and G37, may affect BPTI only functionally. There are, however, other sites that are altered in mutants of this class that are buried in the native protein and are likely to contribute to conformational stability. Y35, for instance, is >90% buried in the native wild-type protein, and the Y35G and Y35L substitutions both destabilize the native protein by >5 kcal/mol (1 cal = 4.184 J) with respect to the reduced and unfolded state (ref. 7; J.-X.Z. and D.P.G., unpublished results). Substitutions that increase the rate of hydrolysis of II_N may act by increasing the flexibility of the trypsin binding site or by altering the interaction of the inhibitor with trypsin in a way that makes formation of the tetrahedral transition state more facile.

Thirteen amino acid replacements that increase the rate of unfolding of II_N by 200-fold or more were identified. These substitutions were all located in the vicinity of the 30–51 and 5–55 disulfides in the native protein. During the unfolding of the wild-type protein, these disulfides can be broken by two different mechanisms (10). One of these processes is an intramolecular rearrangement of the buried disulfides by exchange with the thiol of either C14 or C38. Since C14 and C38 are located at the opposite end of the protein from the 30–51 and 5–55 disulfides, these rearrangements must involve extensive distortion or unfolding of the native conformation. The two-disulfide intermediate II_N can also be directly reduced by DTT, but under the conditions used here direct reduction of the wild-type protein is ≈100-fold slower than the rearrangements (6). Since both the 30–51 and 5–55 disulfides are completely buried in the native conformation, the direct reduction reaction must also involve at least local unfolding of II_N. The DTT-sensitive amino acid replacements in the region of the buried disulfides may lead to rapid reduction and unfolding by destabilizing the protein so as to facilitate the unfolding required for either direct disulfide reduction or rearrangement. The increased rates of reduction may also be due, in part, to increased exposure of the disulfides to the thiol reagent, since many of the substitutions decrease the sizes of residues close to the disulfides.

The most striking feature of the distribution of mutational effects illustrated in Fig. 2*b* is the clustering of substitutions

that increase the rate of reductive unfolding. Since the collection of mutants characterized here was assembled by screening a large number of clones carrying mutations throughout the BPTI gene, the failure to identify rapidly unfolding proteins with substitutions far from the 30–51 and 5–55 disulfides indicates that such variants are rare. Thus, the kinetic stability of II_N appears to be determined primarily by residues and interactions in the region of the protein where the α-helix and β-sheet are closely packed around these disulfides.

The amino acid replacements that allow II_N to be rapidly cleaved by trypsin, but do not greatly increase the rate of its unfolding, are all located in the polypeptide segments that form two closely packed loops making up the trypsin-binding site of the native protein. The crystal structure of one of the DTT-sensitive mutants with a replacement in this region, Y35G, has recently been determined and found to differ markedly from that of the wild-type protein, with some backbone atoms shifted by as much as 6 Å (19). These structural changes are largely limited, however, to two regions of the primary structure, from residue 9 to 18 and from residue 35 to 41—the same segments in which all of the DTT-sensitive substitutions that do not cause rapid unfolding are located. The ability of the loops to accommodate amino acid replacements locally may be due in part to the presence of four buried water molecules that contribute to a network of hydrogen bonds in this region of the folded protein (17, 20). Like the wild-type structure, the Y35G variant contains buried water molecules in the loop region, but their positions are quite different from those found in the wild-type protein. The ability of the hydrogen-bonding pattern to rearrange by the shifting of solvent molecules may allow amino acid replacements to have minimal effects on the rest of the protein.

In contrast to the loop region, in which many interactions are mediated by solvent molecules, the other end of the folded protein is characterized by a high density of intramolecular hydrogen bonds (refs. 17 and 20; see figure 8 of ref. 9). The amide hydrogen atoms in this region include the most slowly exchanging in the folded protein (21, 22), suggesting that this part of the protein is particularly rigid. The rigidity of this structure may contribute to both the stability of the 30–51 and 5–55 disulfides in the wild-type protein and the resistance of this part of the protein to structural changes induced by amino acid substitutions in the loops.

BPTI is among the smallest known proteins with a well-defined native conformation. The finding that amino acid replacements in different regions of the folded protein can cause clearly distinguishable effects indicates that even a protein as small as BPTI may be dissectable into substructures by genetic means. Further characterization of these mutants should enable us to better define the mechanisms by which the substitutions act and may reveal underlying properties of the native protein that give rise to the distinct pattern of mutational effects described here.

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