Probing the Determinants of Disulfide Stability in Native Pancreatic Trypsin Inhibitor†

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ABSTRACT: The effects of single amino acid replacements on the stability of the 14–38 disulfide bond in the native form of bovine pancreatic trypsin inhibitor (BPTI) were measured. A total of 17 mutant proteins, with substitutions at one of 7 residues located 5–15 Å from the disulfide in the native wild-type protein, were examined. The replacements were found to decrease the thermodynamic stability of the disulfide, as measured by exchange with thiol–disulfide reagents, by 0.6–5 kcal/mol, corresponding to a range of nearly 100 mV in redox potentials. The effects of the substitutions on disulfide stability were roughly correlated with the changes in side-chain volume, suggesting that optimal packing is a major factor in determining the stability of the disulfide in the wild-type protein. With only one exception, the substitutions also led to increases, as large as 50-fold, in the rates of disulfide reduction by dithiothreitol. The increased rates of reduction suggest that at least a fraction of the mutational destabilization of the disulfide is due to strain in the native protein that is relieved in the transition state for reduction. The stability of the disulfide in a peptide corresponding to the segments that are linked together by the 14–38 disulfide in native BPTI was found to be about 5 kcal/mol less than that of the disulfide in the intact wild-type protein. Together, the results with the mutant proteins and the peptide indicate that the stability of the disulfide in the native protein depends on both the local environment of the disulfide and on the ability of the rest of the protein to favor a conformation that promotes disulfide formation.

Early discussions of the roles of disulfide bonds in protein folding and stability focused on the effect that a disulfide, or any other cross-link, would have on the unfolded state of the protein. Simple polymer statistics calculations indicated that cross-links can reduce the entropy of an unfolded protein, and thereby increase the relative stability of the native state, by an amount corresponding to 3–5 kcal/mol (Schellman, 1955; Flory, 1956; Poland & Scheraga, 1965; Johnson et al., 1978). More recently, however, it has become apparent that the ability of a disulfide to stabilize the folded conformation also depends on the environment of the disulfide in the native protein. This conclusion has emerged from studies of the roles of naturally-occurring disulfides in protein folding and stability (Creighton, 1983; Creighton & Goldenberg, 1984; Lin & Kim, 1989) and from attempts to stabilize proteins using genetic engineering to introduce new disulfides [e.g., Perry and Wetzel (1984), Pantoliano et al. (1987), Mitchinson and Wells (1989), and Matsumura et al. (1989)]. Both types of study have shown that the contributions of disulfides can vary greatly depending on their location in the native protein.

Because disulfides are reversible covalent bonds, the thermodynamics and kinetics of their formation can be measured directly by following exchange with thiol–disulfide reagents, such as oxidized and reduced glutathione (GSSG[1] and GSH):

$$P_{SH}^{SH} + GSSG \rightleftharpoons P_S^S + 2GSH \qquad (1)$$

where P_{SH}^{SH} and P_S^S represent the forms of the protein in which the two Cys residues are present as free thiols and the disulfide bond, respectively. The thermodynamic stability of the protein disulfide can be expressed as the equilibrium constant for this reaction:

$$K = [P_S^S][GSH]^2/[P_{SH}^{SH}][GSSG] \qquad (2)$$

Since it represents the stability of an intramolecular disulfide (P_S^S) relative to that of a chemically equivalent intermolecular disulfide (GSSG), this equilibrium constant can be thought of as an "effective concentration" (Page & Jencks, 1971; Creighton, 1983; Burns & Whitesides, 1990). Expressing disulfide stabilities as effective concentrations emphasizes the notion that disulfide formation is favored when the conformation of the molecule brings the sulfur atoms into the correct proximity and orientation for reaction. As a consequence of the thermodynamic linkage between disulfide formation and folding, the contribution of a disulfide to the stability of a folded protein depends on the extent to which the equilibrium constant for forming the disulfide, i.e., the effective concentration, is greater in the native protein than in the unfolded state (Creighton 1983, 1988; Goldenberg, 1985; Lin & Kim, 1989, 1991).

Naturally-occurring disulfide bonds in native proteins have been found to have effective concentrations ranging from 10

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor [the disulfides of native BPTI are indicated by the residue numbers of the disulfide-bonded cysteine residues; amino acid replacements are indicated 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]; P_S^S and P_{SH}^{SH}, disulfide and dithiol forms, respectively, of a protein; GSSG and GSH, disulfide and thiol forms, respectively, of glutathione; DTT_S^S and DTT_{SH}^{SH}, disulfide and dithiol forms of dithiothreitol; IAcOH, iodoacetic acid; IAcAm, iodoacetamide; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography.

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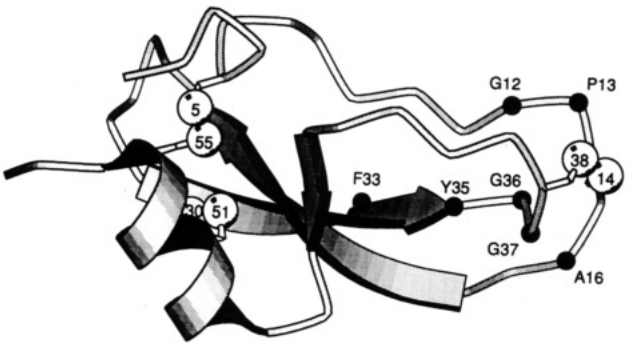


FIGURE 1: Three-dimensional structure of wild-type BPTI, drawn from the coordinates of the Form II crystal (Wlodawer et al., 1987; 5PTI in the Brookhaven Protein Data Bank). Positions of the amino acid replacements used in this study are indicated by filled circles. Drawn with the program "MOLSCRIPT" (Kraulis, 1991).

to 10^5 M (Goto & Hamaguchi, 1981; Creighton & Goldenberg, 1984; Lin & Kim, 1989), and a similarly wide range of stabilities has been observed among engineered disulfides (Matsumura et al., 1989; Mitchinson & Wells, 1989). Studies with small organic molecules have identified steric energy as a major determinant of disulfide stability (Houk & Whitesides, 1987; Burns & Whitesides, 1990), but relatively little is known about what other factors might determine the stability of a disulfide in a folded protein. In addition to steric constraints on the disulfide bond itself, entropic and enthalpic effects on neighboring groups might influence the free energy change for forming a protein disulfide.

To explore the determinants of disulfide stability, we have examined the effects of amino acid replacements on a naturally-occurring disulfide bond in bovine pancreatic trypsin inhibitor (BPTI, Figure 1). Native BPTI contains 3 disulfide bonds, but two of these are buried in the folded protein and are very slow to react with reducing agents. The third disulfide (between Cys 14 and Cys 38) is located on the surface of the protein and links together the two polypeptide segments that form the trypsin-binding surface of the native inhibitor. This disulfide can be readily reduced under conditions where the rest of the protein retains its folded conformation and the other two disulfides remain intact (Kress & Laskowski, 1967). The equilibrium constant for forming the 14–38 disulfide in the otherwise native protein is approximately 200 M (Creighton, 1975; Creighton & Goldenberg, 1984). The studies described here demonstrate that this disulfide is extremely sensitive to mutational perturbations of the native protein.

EXPERIMENTAL PROCEDURES

Production of Mutant Proteins. All of the BPTI variants used in this study were produced in *Escherichia coli* HB101 harboring BPTI-encoding plasmids derived from pTI103 (Goldenberg, 1988). These plasmids direct the synthesis of BPTI as a fusion to the signal peptide of Omp A, a bacterial outer membrane protein. The BPTI accumulates in an active, disulfide-bonded form, from which the OmpA signal peptide has been correctly removed, presumably by the signal peptidase in the *E. coli* periplasmic space. The origins of the various mutants are indicated in Table I.

For each mutant protein preparation, two 500-mL cultures of bacteria were grown at 30 °C and pooled. The mutant proteins were isolated from whole-cell extracts by ion-exchange chromatography [on CM-Trisacryl M (IBF)] and gel filtration chromatography [on Sephadex G-50 (Pharmacia)] as described previously (Goldenberg et al., 1992). The proteins were then further purified by either FPLC ion-exchange chromatography or reversed-phase HPLC. Ion-exchange chromatography was on either a 5-mm diameter × 5-cm long Pharmacia Mono-S column or a similarly sized column of Toyopearl SP-650 resin (Supelco). Both types of ion-exchange columns were eluted with a gradient of 0.1–1 M $NH_4(HCO_3)$. Reversed-phase HPLC utilized a Vydac C_{18} column, which was eluted with a gradient of 12–60% acetonitrile in 0.1% trifluoroacetic acid. Proteins purified by either method were estimated by gel electrophoresis to be greater than 95% pure, and there were no apparent differences in the behavior of samples purified by the two methods.

Measurement of Disulfide Stability by Equilibration with Dithiothreitol. To equilibrate the 14–38 disulfide in the mutant proteins with dithiothreitol, the native proteins were incubated with 40 mM oxidized dithiothreitol (DTT_S^S) and 0.2–20 mM reduced dithiothreitol (DTT_{SH}^{SH}) at 25 °C. In addition to the thiol and disulfide reagents, the solutions contained 30 μM protein, 0.2 M KCl, 0.1 M Tris-HCl (pH 8.7), and 1 mM EDTA. All of the solutions except the protein stock were purged of O_2 by bubbling with N_2 for 10 min, and the reactions were carried out in septum vials under an N_2 atmosphere. Commercial DTT_S^S was purified to remove more potent oxidizing agents (Creighton, 1977).

At 10 and 20 min after the reactions were initiated by addition of the protein, 25-μL samples were withdrawn and mixed with an equal volume of a quench solution containing 0.4 M sodium iodoacetate and 0.063 M Tris-HCl (pH 6.8). The samples were incubated for 2 min at room temperature and then subjected to nondenaturing gel electrophoresis (Goldenberg, 1989) to resolve the disulfide and carboxymethylated dithiol forms of the protein. These gels were stained with Coomassie blue. In all cases, the reactions appeared to have reached equilibrium by 10 min.

Measurement of Disulfide Stability by Equilibration with Glutathione. To measure the equilibrium of the 14–38 disulfide bond in the BPTI variants with glutathione, the native proteins were incubated with 1 mM GSSG and 1–40 mM GSH under the same conditions used for the dithiothreitol equilibrations.

Twenty minutes after the reactions were initiated, two 25-μL samples were withdrawn. One aliquot was mixed with 25 μL of 0.4 M sodium iodoacetate and 0.063 M Tris-HCl (pH 6.8), and the other was mixed with an equal volume of 0.4 M iodoacetamide and 0.063 M Tris-HCl (pH 6.8). After a 2-min incubation at room temperature, the samples were chilled and subjected to nondenaturing gel electrophoresis. Control experiments indicated that the reactions reached equilibrium within 10 min under these conditions.

For both the dithiothreitol and glutathione experiments, the reaction solutions were prepared by diluting a 1 M stock of Tris-HCl (pH 8.7) to a final concentration of 0.1 M, without any further pH adjustment. For all of the dithiothreitol experiments, this procedure resulted in solutions with a final pH of 8.7 ± 0.1. However, the high concentrations of GSH used in some of the glutathione reactions led to pH values as low as 8.0 (with 40 mM GSH). Because the reported values of the pK_a of the glutathione thiol (8.7–9.2) (Jung et al., 1972; Polgar et al., 1973; Snyder, 1987; Chau & Nelson, 1991) are similar to that measured for the thiols of Cys 14 and 38 in selectively-reduced BPTI (pK_a = 8.8) (Creighton, 1975), the errors in the disulfide stability measurements introduced by the variation in pH are expected to be no greater than those arising from other sources. (The equilibrium constants are

Table I: Effects of Amino Acid Replacements on the Thermodynamics and Kinetics of Disulfide Reduction

variant	equilibrium constant for disulfide formation with glutathione (M)	equilibrium constant for disulfide formation with dithiothreitol	effective concentration (M)[d]	rate constant for reduction (s^{-1} M^{-1})	$\Delta\Delta G$ (kcal/mol)[e]	$\Delta\Delta G^{\ddagger}$ (kcal/mol)[f]	$\Delta\Delta G^{\ddagger}/\Delta\Delta G$
wild-type		0.122	150	35			
G12D[a]	0.1		0.1	140	4.3	0.8	0.19
G12V[a]	0.14		0.14	300	4.1	1.3	0.31
P13A[c]		0.0076	9.1	160	1.6	0.9	0.55
P13S[c]		0.011	13	250	1.4	1.2	0.82
A16T[a]		0.0074	8.9	140	1.7	0.8	0.49
A16V[a]		0.014	17	140	1.3	0.8	0.64
F33I[a]	2.9	0.0018	2.5	400	2.4	1.4	0.60
F33L[b]		0.014	17	280	1.3	1.2	0.96
Y35A[b]	0.05		0.05	1400	4.7	2.2	0.46
Y35D[a]	0.23		0.23	400	3.8	1.4	0.38
Y35F[c]		0.043	52	75	0.6	0.4	0.73
Y35G[b]	0.03		0.03	1800	5.0	2.3	0.46
Y35L[b]	0.3		0.3	450	3.7	1.5	0.41
Y35N[a]	0.26		0.26	450	3.7	1.5	0.40
G36D[a]	1.3	0.0018	1.3	13	2.8	−0.6	−0.21
G37A[c]	3.5	0.0025	3.5	120	2.2	0.7	0.33
G37D[b]	7.7	0.0063	7.7	100	1.7	0.6	0.36

[a] These mutants were isolated in a screen of randomly mutagenized clones on the basis of their rapid inactivation by DTT_{SH}^{SH} (Coplen et al., 1990). The rapid inactivation arises because the form of the protein in which the 14–38 disulfide is selectively reduced is inactive as a trypsin inhibitor (Goldenberg et al., 1992). [b] These mutants were constructed by oligonucleotide-directed mutagenesis and display a DTT-sensitive phenotype similar to that described above (Goldenberg, 1992). [c] These mutants were constructed by oligonucleotide-directed mutagenesis and, like the wild-type protein, are active as trypsin inhibitors even after selective reduction (Goldenberg et al., 1992). [d] For those mutants for which the disulfide stability was determined only by equilibration with glutathione, the effective concentration reported is the equilibrium constant for disulfide formation with this reagent. For the disulfide stabilities that were measured only with dithiothreitol, the effective concentration was estimated by dividing the equilibrium constant for protein disulfide formation with dithiothreitol by 1200 M. For the mutants for which data were obtained with both reagents, the two sets of data were combined to estimate a single value for the effective concentration, as described in the text and illustrated for the F33I variant in Figure 3b. [e] The thermodynamic destabilizations of the disulfide were calculated according to $\Delta\Delta G = RT \ln(C_{eff}^{wt}/C_{eff}^{mut})$, where C_{eff}^{wt} and C_{eff}^{mut} are the effective concentrations of the disulfide in the wild-type and mutant proteins, respectively. [f] The destabilizations of the disulfide with respect to the transition state for reduction was calculated according to $\Delta\Delta G^{\ddagger} = RT \ln(k_{mut}/k_{wt})$, where k_{wt} and k_{mut} are the rate constants for reduction of the wild-type and mutant proteins, respectively.

estimated to have uncertainties of about ±50%, as discussed below.)

Kinetics of Protein Disulfide Reduction. The rates of reduction of the 14–38 disulfide in the mutant proteins were measured in the presence of 50–200 μM DTT_{SH}^{SH} under the same conditions used for the equilibrium measurements. At various times after mixing the protein and thiol reagent, 25-μL samples were withdrawn and mixed with 6 μL of 0.5 M sodium iodoacetate and 0.25 M Tris-HCl (pH 6.8). After reacting with the iodoacetate for 2 min at room temperature, the samples were subjected to gel electrophoresis to resolve the disulfide and blocked dithiol forms of the protein.

Peptide Synthesis and Characterization. A peptide corresponding to residues 34–40 and 11–19 of wild-type BPTI (linked together by four Gly residues) was synthesized on an Applied Biosystems Model 431A solid-phase peptide synthesizer using F-moc chemistry. After removal from the resin and deprotection, the peptide was purified by reversed-phase HPLC and its identity was confirmed by sequencing. After lyophilization, the peptide was dissolved in 0.1 M $NH_4(HCO_3)$ at a final concentration of 0.5 mg/mL and incubated at room temperature while exposed to atmospheric O_2. After 24 h, there was no thiol detectable by reaction with Ellman's reagent (Ellman, 1959). The oxidized peptide was then repurified by reversed-phase HPLC.

To measure the stability of the intramolecular disulfide, the peptide was equilibrated with GSSG and GSH as described above, except that the peptide concentration was 60 μM. After a 30-min incubation at 25 °C, 80-μL samples of the reactions were mixed with 20 μL of 0.5 M sodium iodoacetate and 0.25 M Tris-HCl and incubated for 2 min at room temperature. The samples were then injected into a Vydac C_{18} reversed-phase HPLC column and eluted from the column with a gradient of acetonitrile in 0.1% TFA. The absorbance of the column eluent was monitored at 214 and 280 nm. Four major peptide peaks were resolved by HPLC. The three largest peaks were identified as the disulfide, blocked dithiol, and single mixed-disulfide forms of the peptide by electrospray–ionization mass spectrometry on a Fison Instruments VG Trio 3000 mass spectrometer. The fourth peak was assumed to represent the species with two mixed disulfides, consistent with its behavior as the thiol–disulfide redox potential was varied. The relative concentrations of these four species were determined by integrating the chromatographic profiles generated by monitoring A_{214}.

The kinetics of reduction of the peptide with DTT_{SH}^{SH} were determined as described above for the mutant proteins, except that the disulfide and blocked dithiol forms were separated by reversed-phase HPLC.

Data Analysis. After the gels were stained with Coomassie blue and destained by diffusion, the relative band intensities were determined using either a Biomed Instruments laser densitometer or a video densitometry system composed of a Sony AVC-D7 monochrome video camera, a Data Translation Quick Capture video capture board, and an Apple Macintosh IIci computer. The video images were analyzed using the program "NIH Image". There were no apparent discrepancies between data generated by the two densitometry systems.

Data from the equilibrium experiments utilizing dithiothreitol were analyzed by first calculating the fraction of the protein in the dithiol form (f_{2SH}), according to

$$f_{2SH} = \frac{[P_{SH}^{SH}]}{[P_{SH}^{SH}] + [P_{S}^{S}]} \quad (3)$$

where the concentrations of the two forms of the protein were expressed as relative band intensities. This fraction was then plotted as a function of the ratio of reduced to oxidized

dithiothreitol ($R_{DTT} = [DTT^{SH}_{SH}]/[DTT^{S}_{S}]$), as illustrated in Figure 2b. The equilibrium constant for forming the protein disulfide with DTT^{S}_{S} is given by

$$K_{DTT} = \frac{[P^{S}_{S}][DTT^{SH}_{SH}]}{[P^{SH}_{SH}][DTT^{S}_{S}]} = \frac{[P^{S}_{S}]}{[P^{SH}_{SH}]}R_{DTT} \quad (4)$$

Combining eqs 3 and 4 gives the following expression for the fraction reduced as a function of the ratio of reduced and oxidized dithiothreitol:

$$f_{2SH} = 1/(1 + K_{DTT}/R_{DTT}) \quad (5)$$

A nonlinear least squares fitting routine [in the program "KaleidaGraph" (Synergy software) for the Apple Macintosh] was used to fit the observed data to eq 5, with K_{DTT} as the only adjustable parameter. This analysis procedure tends to minimize the uneven weighting of data corresponding to small values of $[P^{SH}_{SH}]$ that arises when K_{DTT} is calculated directly from eq 4. The values of f_{2SH} estimated by gel electrophoresis are estimated to have errors on the order of ±0.05–0.1, leading to uncertainties of about ±50% in the estimates of K_{DTT}.

Data from the equilibration experiments with glutathione were expressed as the fraction of the protein in the dithiol form, f_{2SH}, and were plotted as a function of $R_{GSH} = [GSH]^2/[GSSG]$. The fraction f_{2SH} is related to the equilibrium constant for disulfide formation and R_{GSH} according to

$$f_{2SH} = \frac{[P^{SH}_{SH}]}{[P^{SH}_{SH}] + [P^{S}_{S}]} = 1/(1 + K_{GSSG}/R_{GSH}) \quad (6)$$

As in the analysis of the dithiothreitol data, the observed data were fit to the hyperbolic function with K_{GSSG} as the only adjustable parameter.

Data from the kinetic experiments were analyzed by comparing the observed time-dependent changes in the concentration of the disulfide and dithiol forms with those predicted by a simple kinetic model:

$$P^{S}_{S} + DTT^{SH}_{SH} \underset{k_f}{\overset{k_r}{\rightleftharpoons}} P^{SH}_{SH} + DTT^{S}_{S} \quad (7)$$

where k_r and k_f are the second-order rate constants for reducing and forming the protein disulfide, respectively. The value of k_r was adjusted to provide a good fit to the kinetics observed with at least two different DTT^{SH}_{SH} concentrations (usually 50 and 100 μM). The value of k_f used in the simulation was constrained so that the ratio k_f/k_r was equal to the measured equilibrium constant for formation of the disulfide with DTT^{S}_{S} (K_{DTT}). The rate constants for reduction are estimated to be accurate within a factor of 2.

RESULTS

The 17 amino acid replacements used in this study alter a total of seven sites located in the two segments of the polypeptide chain, G12 to A16 and F33 to G37, that are linked together by the 14–38 disulfide bond (Figure 1). These replacements include those that (a) replace aromatic residues with smaller nonpolar or polar residues; (b) replace Pro with smaller residues that do not constrain the N–C_α backbone bond; (c) replace Gly or Ala with larger residues.

About half of the mutants examined were isolated in a screen of randomly mutagenized clones (Coplen et al., 1990). This screen identified two classes of mutants that are inactivated by DTT^{SH}_{SH} more rapidly than the wild-type protein: one class in which the rate of complete reduction and unfolding is enhanced and a second class for which selective reduction of the 14–38 disulfide leads to loss of protease inhibitor activity (in contrast to the wild-type protein, which retains activity after selective reduction) (Goldenberg et al., 1992). All of the "DTT-sensitive" mutants examined in the present study are members of this second, slowly unfolding, class. Although there may be some correlation between disulfide stability and the activity of the selectively reduced protein, there was no direct selection for substitutions that alter the kinetics or equilibrium of reducing the 14–38 disulfide.

Measurements of Disulfide Stability by Equilibration with Dithiothreitol. Although the stability of a protein disulfide could, in principle, be measured by exchange with any thiol–disulfide pair, many protein disulfides are so stable that it is not practical to measure the equilibrium by exchange with a reagent that forms an intermolecular disulfide, such as glutathione. With the methods and conditions used for these studies, the most stable protein disulfides for which a measurable equilibrium could be established with glutathione have effective concentrations of about 5 M. To determine the stabilities of more stable disulfides, including that of the wild-type protein, the equilibrium of the protein disulfide with the oxidized and reduced forms of dithiothreitol (DTT^{S}_{S} and DTT^{SH}_{SH}) was measured:

$$P^{SH}_{SH} + DTT^{S}_{S} \rightleftharpoons P^{S}_{S} + DTT^{SH}_{SH} \quad (8)$$

Because dithiothreitol forms a stable intramolecular disulfide, it is a much more potent reducing agent than reagents such as GSH (Cleland, 1964). In addition, mixed disulfides between dithiothreitol and protein thiols are very unstable and do not contribute significantly to the distribution of molecules present at equilibrium, thus simplifying the analysis of the equilibrium mixture.

The mutant proteins were allowed to equilibrate with mixtures of DTT^{S}_{S} and DTT^{SH}_{SH} at 25 °C, pH 8.7, and then reacted with iodoacetate to quench any further thiol–disulfide exchange and to introduce two negative charges into the dithiol form of the protein. The disulfide and carboxymethylated dithiol forms were then separated by nondenaturing gel electrophoresis on the basis of this charge difference, as illustrated in Figure 2a. The relative concentrations of the two forms of the protein were determined by densitometry of the gels and used to estimate the equilibrium constant for protein disulfide formation (Figure 2b). For the wild-type protein, the value of K_{DTT} determined in this way was 0.12, versus values of 0.15–0.25 determined previously (Creighton, 1975, 1977; Creighton & Goldenberg, 1984).

For 10 of the BPTI variants, it was possible to measure the stability of the 14–38 disulfide by exchange with dithiothreitol. The mutant proteins with the least stable disulfides for which this measurement was practical were F33I and G36D, for which K_{DTT} was 0.0018, corresponding to a disulfide stability 2.5 kcal/mol lower than that of the wild-type protein.

Measurements of Disulfide Stability by Equilibration with Glutathione. For those mutants with disulfides too unstable to establish an equilibrium with dithiothreitol, the disulfide stabilities were measured by equilibration with glutathione. Although the reaction of interest for comparing protein disulfide stabilities (eq 1) involves only the dithiol and disulfide forms of the protein, significant levels of molecules with one or two mixed disulfide ($P^{S\text{-}SG}_{SH}$ and $P^{S\text{-}SG}_{S\text{-}SG}$) can also accumulate when a protein disulfide is equilibrated with glutathione:

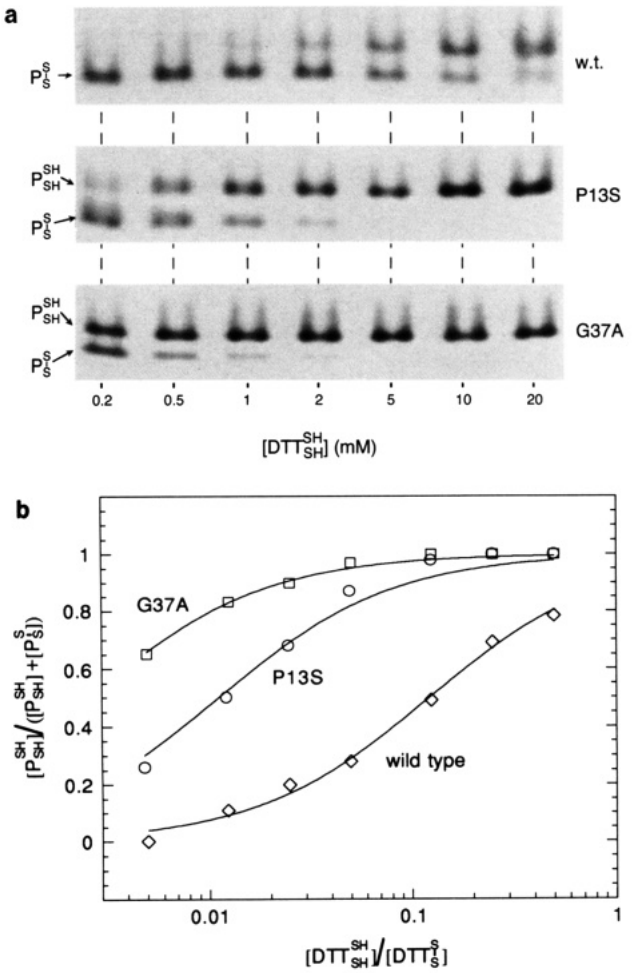
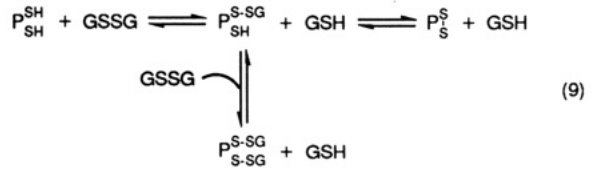


FIGURE 2: Equilibration of BPTI variants with oxidized and reduced dithiothreitol. (a) Gel electrophoresis of samples equilibrated for 20 min with 40 mM DTT^{S}_{S} and the indicated concentration of DTT^{SH}_{SH}. Samples were trapped with iodoacetate to facilitate separation of the selectively reduced protein (P^{SH}_{SH}) from the protein with all three disulfides (P^{S}_{S}). This image and that shown in Figure 3a were generated from digitized video images. (b) The fraction of protein selectively reduced as a function of the ratio of reduced to oxidized dithiothreitol. The curves were calculated from the equilibrium expression (eq 5) using the estimates for K_{DTT} (Table I) determined by nonlinear least-squares fitting.



(9)

In order to measure and correct for the concentrations of mixed disulfide species in the equilibrium reactions, we took advantage of the net negative charge of the glutathione, as illustrated in Figure 3a. When the equilibrium mixtures were trapped with iodoacetate, all of the forms in which the protein disulfide is broken (including the blocked dithiol form and molecules with one or two mixed disulfides) contained two negative charges not present in the disulfide form of the protein. These three species all migrated together with a mobility less than that of the disulfide form of the protein (upper panel of Figure 3a). To determine the concentrations of the mixed disulfides, parallel samples of the reactions were quenched with iodoacetamide, which results in neutral carboxyamidomethyl groups. When these samples were electrophoresed, the disulfide and blocked dithiol forms migrated together, but the mixed disulfide forms migrated more slowly (lower panel

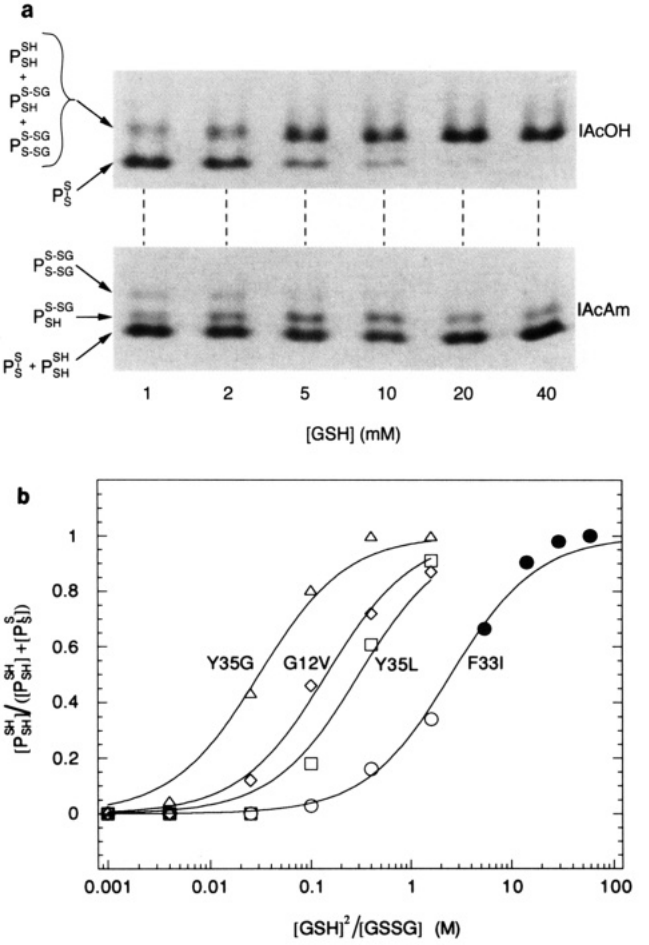


FIGURE 3: Equilibration of BPTI variants with oxidized and reduced glutathione. (a) Gel electrophoresis of Y35G BPTI equilibrated for 20 min with 1 mM GSSG and the indicated concentrations of GSH. The samples in the upper panel were trapped with iodoacetate (IAcOH) to separate the disulfide form of the protein (P^{S}_{S}) from the blocked dithiol form (P^{SH}_{SH}) and the forms with one or two mixed disulfides ($P^{S\text{-}SG}_{SH}$ and $P^{S\text{-}SG}_{S\text{-}SG}$). The samples in the lower panel were trapped with iodoacetamide (IAcAm), leading to separation of the disulfide and blocked dithiol forms (in the lower band) from molecules with one or two mixed disulfides. The data from the two trapping procedures were combined to determine the relative concentrations of P^{SH}_{SH} and P^{S}_{S}, as plotted in (b). The open symbols represent data obtained directly from equilibration with glutathione. The filled symbols represent data for the F33I variant obtained by equilibration with dithiothreitol and converted to the "glutathione scale" as described in the text. The curves were calculated from the equilibrium expression (eq 6) using the estimates for K_{GSH} (Table I) determined by nonlinear least-squares fitting. The curve shown for the F33I mutant was obtained by fitting the glutathione and dithiothreitol data simultaneously.

of Figure 3a). By combining the results obtained with the two blocking reagents, it was thus possible to estimate the relative concentrations of the dithiol and disulfide forms of the protein.

The relative concentrations of the dithiol and disulfide forms were then used to calculate the fraction of protein in the dithiol form, as plotted in Figure 3b as a function of the ratio $[GSH]^2/[GSSG]$. The curves shown in Figure 3b represent the least-squares fits of the expected equilibrium expression to the experimental data.

The equilibrium of the protein disulfide with glutathione was measured for 11 BPTI mutants, including 4 for which the equilibrium with dithiothreitol was also measured. The values of K_{GSSG}, which represent effective concentrations, for the 11 mutants ranged from 0.03 M (for Y35G) to 9 M (for G37D).

For a given protein disulfide, the equilibrium constants for forming the disulfide by exchange with glutathione and dithiothreitol (K_{GSSG} and K_{DTT}) are expected to be related according to

$$\frac{K_{GSSG}}{K_{DTT}} = \frac{[P_S^S][GSH]^2}{[P_{SH}^{SH}][GSSG]} \frac{[P_{SH}^{SH}][DTT_S^S]}{[P_S^S][DTT_{SH}^{SH}]}$$

$$= \frac{[DTT_S^S][GSH]^2}{[DTT_{SH}^{SH}][GSSG]} = K_{DTT,GSSG} \quad (10)$$

where $K_{DTT,GSSG}$ is the equilibrium constant for forming the disulfide of dithiothreitol by exchange with glutathione, i.e, the effective concentration of dithiothreitol. Thus, the value of $K_{DTT,GSSG}$ can be used to calculate the effective concentration of the protein disulfide (K_{GSSG}) from K_{DTT} for those disulfides that are too stable to be measured by direct equilibration with glutathione.

Two laboratories have recently determined the value of $K_{DTT,GSSG}$ by directly measuring the equilibrium between glutathione and dithiothreitol (Chau & Nelson, 1991; Rothwarf & Scheraga, 1992). Both laboratories report values of 380 M at pH 8.7, 25 °C. For the four BPTI variants for which the 14–38 disulfide was equilibrated with both reagents, the ratio K_{GSSG}/K_{DTT} ranged from 600 M to 2000 M, with an average of 1400 M. This value is significantly greater than the value of $K_{DTT,GSSG}$ determined directly, but is in reasonable agreement with the value (1200 M) estimated previously from the apparent equilibrium constants for the individual steps in the BPTI folding pathway (Creighton & Goldenberg, 1984). The reason for the discrepancy between the direct measurements and the BPTI studies is not clear, but it may arise from some interaction between one of the reagents and BPTI. Because it seems to provide the most consistent correlation between K_{DTT} and K_{GSSG} for the BPTI disulfides under the conditions of these measurements, the value 1200 M was used to compare the estimates of disulfide stability obtained with glutathione and dithiothreitol.

For those mutants that were equilibrated with both dithiothreitol and glutathione (F33I, G36D, G37A, and G37D), the data obtained with the two reagents were combined as illustrated for F33I in Figure 3b. For this purpose, the dithiothreitol concentrations were converted to a "glutathione scale" by calculating the ratio $[GSH]^2/[GSSG]$ corresponding to the concentrations of GSH and GSSG that would be in equilibrium with the actual concentrations of DTT_{SH}^{SH} and DTT_S^S used for the measurements (according to eq 10, assuming a value of 1200 M for $K_{DTT,GSSG}$). These hypothetical values of $[GSH]^2/[GSSG]$ were then used to plot the dithiothreitol data (represented by filled symbols) along with the glutathione data (open symbols), and a single value of K_{GSSG} was estimated by fitting all of the data to the glutathione equilibrium expression. As shown in Figure 3b, the dithiothreitol and glutathione data for F33I appear to be very consistent when treated this way. A similar degree of consistency was observed for the other three mutants for which data with both reagents were available.

When combined, the measurements using the two sets of thiol–disulfide reagents indicate that single amino acid replacements can cause a remarkably wide range of changes in the stability of the 14–38 disulfide in native BPTI. The values of K_{GSSG} (determined directly or from K_{DTT}) range from 0.03 M, for Y35G, to 150 M, for the wild-type protein. This range of equilibrium constants corresponds to a range of 5 kcal/mol in the free energy change for disulfide formation.

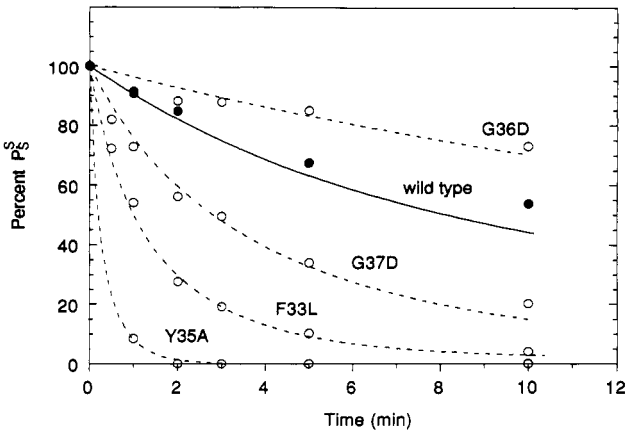
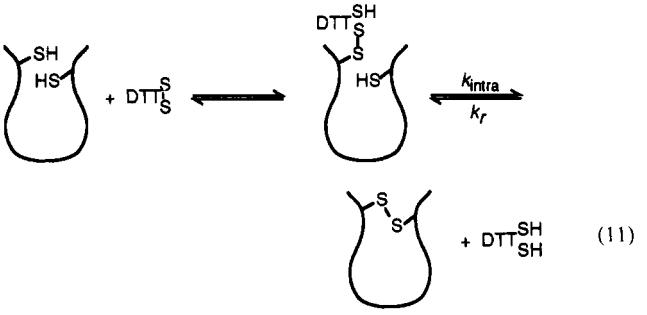


FIGURE 4: Kinetics of reduction of wild-type BPTI (filled symbols and solid line) and 4 mutants (open symbols and dashed lines) in the presence of 0.1 mM DTT_{SH}^{SH}. At the indicated times, samples were reacted with iodoacetate and the disulfide and selectively reduced forms were separated by nondenaturing gel electrophoresis. The curves shown are those generated by computer simulations based on the rate and equilibrium constants in Table I.

Effects of Amino Acid Replacements on the Kinetics of Protein Disulfide Reduction. The observed thermodynamic destabilization of the protein disulfide might be expressed kinetically as a decreased rate of disulfide formation, an increased rate of reduction, or a combination of the two. Formation or reduction of the protein disulfide takes place via two thiol–disulfide exchange steps, as illustrated below for the reaction involving dithiothreitol:



(11)

Because the mixed-disulfide intermediate between the protein thiol and dithiothreitol is rapidly broken down to regenerate DTT_S^S, the mixed disulfide is in rapid equilibrium with the dithiol form of the protein, and the apparent rate of disulfide formation is proportional to the rate (k_{intra}) of the intramolecular process in which the second protein thiol displaces the reagent (Creighton & Goldenberg, 1984; Creighton, 1986). The rate of disulfide formation with DTT_S^S is expected to reflect the probability that the two sulfur atoms in the dithiol form are favorably oriented for disulfide formation. The apparent rate of reduction of the protein disulfide by DTT_{SH}^{SH} is usually the rate of forming of the mixed-disulfide intermediate from the protein disulfide (k_r), since the intermediate is rapidly converted to the dithiol form (Creighton & Goldenberg, 1984; Creighton, 1986). The rate of reduction is likely to depend on the accessibility of the disulfide to the reagent and the presence of any strain in the protein disulfide that can be relieved in the transition state for reduction (Creighton, 1975; Singh & Whitesides, 1990).

The kinetics of reduction of the wild-type protein and each of the variants were measured under the same conditions used for the equilibrium measurements (Figure 4). For each protein, the rate of reduction was measured with DTT_{SH}^{SH} at

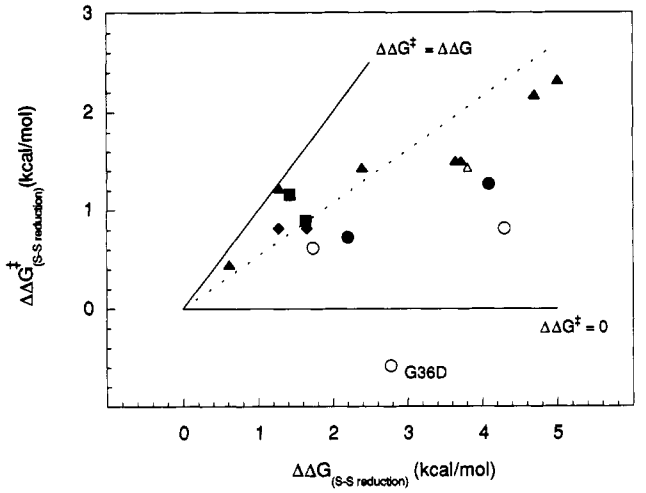


FIGURE 5: Correlation between the thermodynamic destabilization ($\Delta\Delta G$) of the 14–38 disulfide by amino acid replacements and the effects of the replacements on the rate of disulfide reduction ($\Delta\Delta G^{\ddagger}$). Different types of amino acid replacements are indicated by the symbols: ■, Pro → other; ▲, aromatic → other; ♦, Ala → other; ●, Gly → other. Open symbols indicate replacements that introduce a net negative charge. The two solid lines represent positions on the plot corresponding to the hypothetical cases where all of the thermodynamic destabilization is expressed as an enhanced rate of reduction ($\Delta\Delta G^{\ddagger} = \Delta\Delta G$) or the substitutions do not affect the reduction rate and all of the thermodynamic destabilization is expressed as a decreased rate of disulfide formation ($\Delta\Delta G^{\ddagger} = 0$). The dashed line corresponds to the average value of $\Delta\Delta G^{\ddagger}/\Delta\Delta G$ for the substitutions that do not alter the charge of the protein.

two concentrations, and in each case the observed rate was proportional to the thiol reagent concentration, as expected for the second-order reaction.

For all but one of the mutants (G36D), the rate of disulfide reduction was significantly greater than that observed with the wild-type protein. The apparent second-order rate constants varied from 13 s^{-1} M^{-1} for (G36D) to 1800 s^{-1} M^{-1} (for Y35G), versus 35 s^{-1} M^{-1} for the wild-type protein. Under these conditions, the rate constant for reducing the disulfide of GSSG, which should be fully accessible and unstrained, is about 20 s^{-1} M^{-1} (Creighton, 1975; Rothwarf & Scheraga, 1992).

For nearly all of the mutants, a large fraction (30–80%) of the thermodynamic destabilization is reflected in an increased reduction rate. This correlation is illustrated in Figure 5, where the change in free energy of activation ($\Delta\Delta G^{\ddagger}$) is plotted versus the change in disulfide stability ($\Delta\Delta G$). The major exception to this trend is the G36D mutant, which is reduced at a lower rate than the wild-type protein, even though its disulfide is thermodynamically 2.8 kcal/mol less stable. This unusually low rate might be due to electrostatic effects, since the negative charge of the Asp side chain in this mutant, if properly positioned, would tend to destabilize the negatively-charged transition state (Szajewski & Whitesides, 1980). Some of the other variants in which negative charges have been introduced also display relatively low values of the ratio $\Delta\Delta G^{\ddagger}/\Delta\Delta G$ (open symbols in Figure 5). Other than this possible electrostatic effect, there is no apparent correlation between the relative increases in reduction rate and the natures of the amino acid replacements.

Disulfide Formation in a Peptide Corresponding to the 14–38 Region of Native BPTI. The results with the BPTI variants indicate that the stability of the 14–38 disulfide in the native protein is very much dependent on the sequence of the two loop segments that are linked together by the disulfide. To examine the extent to which the disulfide is also dependent on the rest of the native protein, a peptide corresponding to the isolated loop segments was synthesized. In order to create a model in which disulfide formation would be an intramolecular process, as in the intact protein, the two BPTI segments were linked together with four Gly residues between the residues corresponding to Ala 40 and Thr 11, which are approximately 8 Å apart in the native protein. The sequence of the entire peptide was

34 35 36 37 38 39 40 11 12 13 14 15 16 17 18 19
Val-Tyr-Gly-Gly-Cys-Arg-Ala-$(Gly)_4$-Thr-Gly-Pro-Cys-Lys-Ala-Arg-Ile-Ile

where the numbers indicate the corresponding residues in the intact protein. The four Gly residues were chosen as a linker on the basis of computer modeling using the Biosym programs "Insight II" and "Discover". The modeling studies indicated that this linker could be incorporated into the structure of the two BPTI segments in the structure of the native protein [in the Form II crystals, Wlodawer et al. (1987)] without introducing any strain.

The equilibrium constant for forming the disulfide in the peptide by exchange with glutathione was measured in the same way as described above for the mutant proteins, except that the various forms of the peptide were separated by reversed-phase HPLC. The effective concentration of the peptide disulfide was estimated to be 0.045 M, comparable to some of the least stable mutant disulfides examined, and was not significantly altered by the addition of 8 M urea. The stability of the disulfide in this peptide is comparable to that measured for peptides of similar size in the presence of strong denaturants (Snyder, 1987; Lin & Kim, 1989; Huyghues-Despointes & Nelson, 1992). These results suggest that, in the absence of the rest of the folded protein, the segments linked together by the 14–38 disulfide do not contain any specific structure that stabilizes the disulfide. The peptide also did not appear to have any trypsin inhibitor activity.

The second-order rate contant for the reduction of the peptide disulfide by DTT^{SH}_{SH} was 130 s^{-1} M^{-1}, about 4-fold greater than that observed for the wild-type protein (35 s^{-1} M^{-1}). From the equilibrium constant for disulfide formation and the rate of reduction, the rate constant for forming the peptide disulfide by exchange with DTT^{S}_{S} can be estimated to be 4.9 × 10^{-3} s^{-1} M^{-1}, versus 4.3 s^{-1} M^{-1} for the wild-type protein. Thus, the much lower stability of the disulfide in the peptide than in the native protein is expressed kinetically as a very large difference in the rate of disulfide formation and a smaller difference in the rate of reduction. The low rate of forming the disulfide in the peptide very likely reflects the substantial loss of entropy that occurs when the two peptide sulfur atoms must come together in the intramolecular step of the reaction (eq 11). The greater rate of reduction in the peptide may be due, in part, to greater exposure to the thiol reagent, since the disulfide is about 50% buried in the native wild-type protein.

DISCUSSION

Effects of Amino Acid Replacements on Disulfide Stability. Previous studies have indicated that the stability of a disulfide and, therefore, the ability of the disulfide to contribute to conformational stability depend greatly on the context of the disulfide in the native protein (Goto & Hamaguchi, 1982; Creighton & Goldenberg, 1984; Lin & Kim, 1989; Matsumura et al., 1989; Mitchinson & Wells, 1989). The results presented here demonstrate that single amino acid replacements in the vicinity of a disulfide can alter its stability by as much as 5

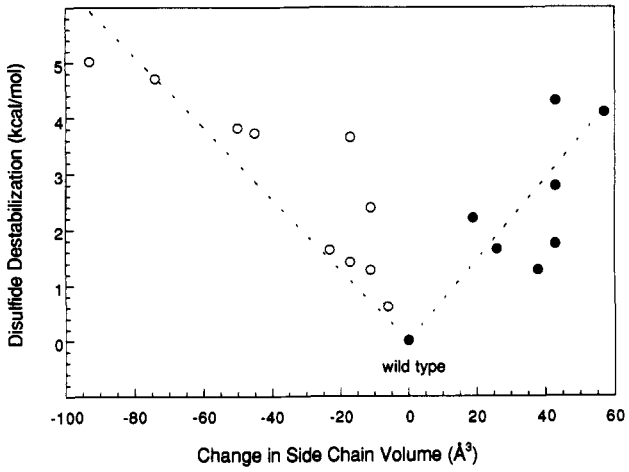


FIGURE 6: Correlation between the destabilization of the disulfide ($\Delta\Delta G$, calculated as in Table I) and the change in side-chain volume [calculated from the side-chain van der Waals volumes calculated by Creighton (1992)]. Solid symbols represent replacements that increase side-chain volume, while open symbols represent replacements that decrease the volume. The dashed lines represent linear least-squares fits to the two sets of data, with the lines constrained to pass through the point corresponding to the wild-type protein.

kcal/mol. These results emphasize the importance of the coupling between individual interactions in a protein and indicate that a disulfide can be sensitive to perturbations as far as 15 Å away in the native protein.

The large changes in disulfide stability observed here are particularly striking in comparison to the relatively small effects of amino acid replacements on the fully reduced and unfolded form of BPTI (Goldenberg & Zhang, 1992). The effects of mutations on unfolded BPTI have been examined by nondenaturing gel electrophoresis and by measuring the rate of disulfide formation in the fully reduced protein. These experiments indicate that amino acid replacements alter the hydrodynamic volume of the reduced polypeptide by less than ~3% and alter the average rate of initial disulfide formation by no more than 2-fold. Thus, the folded protein appears to be much more sensitive to amino acid replacements than is the unfolded state, probably because the unfolded polypeptide can more readily accommodate a sequence change than can the closely packed native protein.

The measurements described here are formally similar to double-mutant studies in which the effects of multiple amino acid replacements on conformational stability are measured in isolation and together. In these studies, interactions between sites in a protein are detected by nonadditivity of the effects of two or more substitutions [e.g., Shortle and Meeker (1986), Sandberg and Terwilliger (1989), Serrano et al. (1990), and Sun et al. (1991)]. In many of the cases reported so far, the effects of multiple replacements on protein stability appear to be nearly additive, suggesting that the individual replacements do not influence one another. In contrast, every amino acid replacement examined here causes a measurable change in the equilibrium for reducing the 14–38 disulfide, which can be thought of as a reversible mutational change. The greater apparent interdependence between sites in the native protein detected here may reflect the more severe steric constraints imposed by disulfide bonds than by noncovalent interactions.

Among the substitutions examined, there appears to be a rough correlation between the change in side-chain volume associated with the substitution and the degree of disulfide destabilization (Figure 6). Either increasing or decreasing the side-chain size from that of the wild-type protein by the volume of a methyl group (27 Å^3) leads to an average disulfide destabilization of 1.8 kcal/mol. This correlation suggests that optimal packing of the region surrounding of the disulfide is a major determinant of disulfide stability in the wild-type protein.

Effects of Amino Acid Replacements on the Kinetics of Disulfide Reduction and Formation. The thermodynamic destabilization of the disulfide is also correlated with increased rates of disulfide reduction in the mutant proteins (Figure 5). This suggests that factors that destabilize the disulfide with respect to the dithiol form of the protein also tend to destabilize it with respect to the transition state for reduction. The mechanism of thiol–disulfide exchange reactions has been studied extensively and is known to be an S_N2 nucleophilic displacement (Szajewski & Whitesides, 1980). In the transition state for this reaction, the three participating sulfur atoms come together in a linear arrangement in which the two sulfur–sulfur bonds are longer and weaker than the ground-state disulfide (Pappas, 1977; Rosenfield et al., 1977). An amino acid replacement might increase the rate of reduction by increasing the accessibility of the disulfide to the thiol reagent or by introducing strain that is relieved in the more loosely constrained transition state.

In the native structure of wild-type BPTI, the sulfur atom of Cys 38 is nearly completely buried, but the Cys 14 sulfur is fully exposed to an attack along the axis of the disulfide bond. Thus, by increasing the accessibility of the disulfide, an amino acid replacement might increase the rate of reduction by at most a factor of 2, much less than the 50-fold increase observed for some of the substitutions. High reduction rates have been observed for disulfide bonds within structures that are believed to be strained, such as five-membered rings (Creighton, 1975; Singh & Whitesides, 1990) and small peptides (Zhang & Snyder, 1988, 1989), and the large increases in reduction rate observed here may indicate that many of the substitutions strain the disulfide in native BPTI. Singh and Whitesides (1990) have presented evidence that all of the strain present in a disulfide-bonded five-membered ring is relaxed in the transition state for reduction. If this is the case for the protein disulfides examined here, the ratio $\Delta\Delta G^{\ddagger}/\Delta\Delta G$ would represent the fraction of destabilization arising from strain, disregarding any possible enhancement of the reduction rate from increased accessibility.

When the changes in reduction rate are expressed relative to the thermodynamic destabilization of the disulfide ($\Delta\Delta G^{\ddagger}/\Delta\Delta G$), similar effects are observed for several different types of amino acid replacements (Figure 5). Some of the substitutions examined here were replacements of Gly with larger residues, a type of substitution that might be expected to be particularly likely to introduce strain in the native protein. Other substitutions, however, represent deletions of only a few atoms (e.g., Y35F, P13A, F33L, Y35L). Surprisingly, both types of replacement lead to comparable values of $\Delta\Delta G^{\ddagger}/\Delta\Delta G$, suggesting that even small deletions may cause energetically significant perturbations in nearby interactions.

The dihedral angle of the sulfur–sulfur bond is frequently cited as a major determinant of disulfide stability, and several authors have attempted to calculate relative stabilities from this angle (Katz & Kossiakoff, 1986, 1990; Kuwajima et al., 1990; Pjura et al., 1990). The crystal structure of the BPTI variant with the least stable disulfide examined in this study (Y35G) has recently been determined, and the structure surrounding the 14–38 disulfide in this mutant is very different from that of the wild-type protein (Housset et al., 1991). The dihedral angle of the 14–38 disulfide bond is 95° in the wild-

type protein and −70° in the Y35G mutant. Because both of these values are relatively close to minima in the dihedral energy function (at ±85°), the difference in disulfide dihedral energies is not expected to be more than about 0.5 kcal/mol (Katz & Kossiakoff, 1986; Burns & Whitesides, 1990). The low effective concentration and high rate of reduction of the disulfide in this mutant suggest that other steric factors may be at least as important as the dihedral angle in determining disulfide stability. It is also possible, however, that the crystal structure of the mutant protein does not accurately reflect the dominant conformation in solution, since there are extensive crystal contacts in the region of the 14–38 disulfide (Housset et al., 1991).

In addition to direct effects on the geometry of the disulfide, amino acid replacements might also introduce strain in other bonds near the disulfide. A substitution might cause many small perturbations that together lead to a substantial strain energy that can be partially relieved when one bond, the disulfide, is weakened in the transition state for reduction. Connelly et al. (1991) have suggested that this sort of aggregate effect on multiple interactions in the native protein may be the underlying mechanism of the enthalpy–entropy compensation commonly observed in mutational studies of protein stability.

Although the kinetics of disulfide formation were not measured directly, the rate constants for forming the disulfides in the mutant proteins by exchange with DTT_S^S can be calculated from the equilibrium and rate constants for reduction. The second-order rate constants calculated in this way vary from 0.012 M^{-1} s^{-1} for the G12D variant to 4.3 M^{-1} s^{-1} for the wild-type protein, a range comparable to that observed for the rates of reduction. The decreases in disulfide formation rate caused by the mutations might arise from either entropic or steric effects on the selectively reduced protein. If, for instance, a substitution increases the conformational entropy of the selectively reduced protein and some of this excess entropy is lost in the transition state for the intramolecular step in disulfide formation, the rate of this process would be decreased. In addition, substitutions that alter the charge distribution might destabilize the transition state with respect to the selectively reduced protein through unfavorable electrostatic interactions.

Implications for the Functional Morphology of BPTI. The 14–38 disulfide bond in BPTI is known to play an important role in the ability of the native protein to function as a protease inhibitor. When this disulfide is selectively reduced, the protein retains activity as a trypsin inhibitor, but is cleaved with a half-time of about 24 h after it binds to trypsin (Kress et al., 1968; Jering & Tschesche; 1976). Many of the substitutions examined in this study greatly increase the rate at which the selectively reduced protein is cleaved (Goldenberg et al., 1992). Indeed, about half of the substitutions studied were identified by this property in a genetic screen of randomly mutagenized clones (Coplen et al., 1990).

Since trypsin inhibitor activity and disulfide stability both depend on the native structure of the protein, it might be expected that both would be sensitive to amino acid replacements. There is, however, only a limited correlation between the effects of the replacements on the two properties. All nine of the substitutions that destabilize the disulfide by 2.4 kcal/mol or more also cause the selectively reduced protein to be rapidly cleaved by trypsin. Among the eight substitutions that are destabilized by less than 2.4 kcal/mol, however, four (P13A, P13S, Y35F, and G37A) retain inhibitor activity after the 14–38 disulfide is reduced (Goldenberg et al., 1992). This suggests that the structural requirements for disulfide stability and function are not identical.

The results presented here demonstrate that the sequences of the loop segments are crucial to the stability of the 14–38 disulfide in the native protein. But the stability of this disulfide also depends on the ability of the rest of the protein to stabilize a conformation in which forming the disulfide is favored. In the absence of the rest of the protein, a peptide model of the loop region has an effective concentration of only 45 mM, versus 150 M for the native wild-type protein. It also appears that the rest of the protein is necessary for an effective trypsin inhibitor, although other peptides corresponding to the trypsin-binding segments have been shown to have temporary inhibitor activity before being cleaved by trypsin with half-times of 5–15 min (Tan & Kaiser, 1977; Kitchell & Dyckes, 1982).

Summary. The experiments described here demonstrate that the stability of a disulfide bond in a native protein can be highly sensitive to a variety of nearby amino acid replacements. The mutational effects on the 14–38 disulfide appear to be correlated with changes in side-chain volume, and in most cases a substantial fraction of the thermodynamic destabilization is expressed kinetically as an increase in the rate of reduction. These correlations suggest that steric factors play an important role in determining disulfide stability, but the structural bases of these effects are not known. Structural and theoretical analyses of the mutant proteins are now needed to determine the details of how the disulfide is influenced by its surroundings. Further studies of the effects of amino acid replacements on nearby interactions may aid in developing a more fundamental and quantitative understanding of the cooperativity among the many covalent and noncovalent interactions that determine protein stability.

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