Protein Engineering of Disulfide Bonds in Subtilisin BPN′

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ABSTRACT: Five single-disulfide mutants were studied in subtilisin BPN′, a cysteine-free, secreted serine protease from *Bacillus amyloliquefaciens*. The disulfides were engineered between residues 26–232, 29–119, 36–210, 41–80, and 148–243. These bonds connected a variety of secondary structural elements, located in buried or exposed positions at least 10 Å from the catalytic Ser-221, and linked residues that were separated by 39 up to 206 amino acids. All disulfide bonds formed in the enzyme when the expressed protein was secreted from *Bacillus subtilis*, and the disulfides had only minor effects on the enzyme kinetics. Although these disulfide bonds varied by over 50-fold in their equilibrium constants for reduction with dithiothreitol, there was no correlation between the strength of the disulfide bond and the stability it imparted to the enzyme to irreversible inactivation. In some cases, the disulfide-bonded protein was stabilized greatly relative to its reduced counterpart. However, no disulfide mutant was substantially more stable than wild-type subtilisin BPN′. Some of these results can be rationalized by destabilizing effects of the cysteine mutations that disrupt interactions present in the folded enzyme structure. It is also possible that the rate of irreversible inactivation depends upon the kinetics and not the thermodynamics of unfolding and so the entropically stabilizing effect expected from a disulfide bond may not apply.

The techniques of protein engineering permit one to study and modulate protein stability. One attractive strategy to increase the stability of proteins is to introduce intramolecular disulfide bonds [for a review, see Wetzel (1987)] because the formation of natural disulfide bonds (Creighton et al., 1977) or chemical cross-links (Johnson et al., 1978; Lin et al., 1984) is known to be stabilizing. Indeed, nonnatural intramolecular disulfides have been introduced by site-directed mutagenesis in T4 lysozyme (Perry & Wetzel, 1984; Wetzel et al., 1988) and dihydrofolate reductase (Villafranca et al., 1983), and these disulfides have stabilizing effects. Conversely, removal of a disulfide bond in bovine pancreatic trypsin inhibitor (Marks et al., 1987; Goldenberg, 1988) is destabilizing.

The stabilizing effects of disulfide bonds have been rationalized in terms of the cross-link restricting the degrees of freedom for the unfolded polypeptide chain and thus preferentially stabilizing the folded state (Schulz & Shrimer, 1979). Formally, this should apply only when the folded and unfolded states are in dynamic equilibrium, but if refolding competes with an irreversible step, it is possible that a disulfide would have a stabilizing effect. In fact, there are numerous other proteins where reduction of their natural disulfides destabilizes them toward irreversible heat inactivation.

Disulfide bonds have been introduced into subtilisin BPN′ (Wells & Powers, 1986; Pantoliano et al., 1987), a secreted serine protease from *Bacillus amyloliquefaciens*, for which the cloned gene has been expressed (Wells et al., 1983) and high-resolution X-ray structures have been solved (Wright et al., 1969; Drenth et al., 1972; Bott et al., 1988). In each case, two cysteines were introduced into the cysteine-free enzyme, and the disulfide bonds were formed completely in vivo, unlike the disulfide variants of lysozyme and dihydrofolate reductase which were expressed intracellularly and oxidized in vitro. However, neither of the engineered disulfide bonds substantially stabilized subtilisin to irreversible inactivation. Furthermore, X-ray structures showed that the disulfide bond geometries were different from naturally occurring disulfides (Katz & Kossiakoff, 1986).

We have now studied five more disulfide mutants of subtilisin. These were designed to bridge various secondary structures and link proximal as well as distal positions in the polypeptide chain. In one case, a disulfide bond from a natural subtilisin variant (proteinase K) which contains two disulfides was placed into an analogous position in subtilisin BPN′. In another case, a disulfide was introduced across a site normally occupied by a structural calcium ion. In all cases, active mutant proteins were secreted, and the disulfide bonds could be formed completely in vivo. We measured the kinetics of reduction and the redox potential of the disulfide bonds (whenever possible) and the stabilities of the parent single cysteine and disulfide mutant proteins to irreversible thermal inactivation. In no case was the disulfide mutant more stable than wild-type enzyme even though in most cases the disulfide bond strengths were high. The implications of these results for the mechanism of irreversible inactivation in subtilisin are discussed.

Materials and Methods

Materials

Oligonucleotides were synthesized and purified by the Organic Chemistry Department at Genentech. Enzymes for DNA manipulations were from New England Biolabs or Bethesda Research Labs except for *Asu*II/*Fsp*II (Promega Inc.) and *Escherichia coli* DNA polymerase I large fragment (Boehringer Mannheim). DL-Dithiothreitol (DTT),[1] iodo-

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, DL-dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid. Mutant proteins are designated by the wild-type residue (single-letter amino acid code) followed by their position and the mutant residue. Multiple mutants are separated by a slash. For example, A29C/M119C indicates that alanine at position 29 and methionine at position 119 have been replaced by cysteines. C29–C119 refers to the disulfide bond between these residues.

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Table I: Mutations Made in the *B. amyloliquefaciens* Subtilisin Gene

Mutation	Mutagenic Oligomer[a] 5′ → 3′	Method of Identification[d]
A29C[b]	C-ACT-GGA-TCA-AAT-GTT-AAA-GTA-TGC-GTC-ATC-GAC-AGC (C26V, A29C; Bam HI⁻, Hga I⁺)	Restriction analysis
M119C	G-TGG-GCG-ATT-GCA-AAC-AAT-TGT-GAC-GTT-ATT (M119C; Pvu I⁻)	Restriction analysis
D36C	GAC-AGC-GGT-ATC-TGT-TCT-TCT-CAT (D36C; Cla I⁻)	Restriction analysis
P210C	CAA-AGC-ACG-CTG-TGT-GGA-AAC-AAA (P210C; Dra III⁺)	Hybridization
D41C[c]	C-AGC-GGT-ATC-GAT-TCT-TCT-CAT-CCT-TGT-TTA-AAG-GTA (C37S, D41C; Cla I⁺, Pst I⁻)	Restriction analysis
G80C	T-CTT-AAT-AAC-TCC-ATA-TGT-GTA-TTA-GGC-G (G80C; Nde I⁺)	Restriction analysis
V148C	A-TCC-GGC-GTC-TGT-GTC-GCG-GCC-GCA-GCC-GGT-AAC (V148C; Not I⁺)	Restriction analysis and hybridization
N243C	G-AAC-TGG-ACA-TGC-ACT-CAA-GTT-CGA-AGC-AGT-TTA-GAA (N243C; Asu II⁺)	Restriction analysis and hybridization

[a] Asterisks show the locations of mismatches, and underlined sequences show the positions of an introduced ("*Hga*I⁺") or eliminated ("*Bam*HI⁻") restriction site. [b] Made on a V26C template (B. Katz, unpublished results) containing a *Bam*HI site at codon 24. [c] Made on an S37C template (B. Katz, unpublished results) containing a *Pst*I site at codon 37. [d] See Materials and Methods for details.

acetamide, DTNB, β-mercaptoethanol, and the subtilisin substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide were from Sigma. Oxidized DTT was purified by the method of Creighton (1977) and kindly provided by Dr. P. Kosen (Department of Pharmaceutical Chemistry, University of California at San Francisco).

Methods

Mutant Construction. Mutations were introduced into the subtilisin gene by site-directed mutagenesis, using previously described methods (Wells et al., 1986; Carter et al., 1986). Briefly, 5′-phosphorylated oligonucleotide primers were used to introduce the desired mutations (Table I) on a pSS5 single-stranded plasmid template (B. Cunningham, D. Powers, and J. Wells, unpublished results) containing the *Bacillus amyloliquefaciens* subtilisin gene (Wells et al., 1983). The heteroduplex was used to transform (Mandel & Higa, 1970) competent *E. coli* Mut L cells (Kramer et al., 1984), and the pool of plasmid DNA was prepared to facilitate segregation of mutant and wild-type plasmids. The pool of DNA either was transformed into *E. coli* JPA101 and the mutation identified by hybridization (Zoller & Smith, 1982) or was transformed into *E. coli* MM 294 cells and clones analyzed by restriction digestion. All mutant sequences were confirmed by dideoxy sequencing (Sanger et al., 1977). Single-strand templates were prepared from JPA101 cells harboring the pSS5 plasmid by coinfection with M13 K07 phage.

Double mutants were constructed either by a second round of mutagenesis using one of the single-mutant templates or by ligation of two restriction fragments from the single-mutant plasmids. The presence of both mutations was confirmed by restriction analyses or hybridization followed by DNA sequencing. Mutant genes in the *E. coli–B. subtilis* shuttle plasmid pSS5 were used to transform *B. subtilis* BG2036 (Anagnostopoulos & Spizizen, 1961), a host strain which contains deletions in the extracellular subtilisin and neutral protease genes (Yang et al., 1984).

Protein Purification and Characterization. Subtilisin genes were expressed in BG2036 by fermentation in shake flasks containing 2×TY medium (Miller, 1972) and 12.5 μg/mL chloramphenicol at 37 °C. The enzymes were purified from the culture supernatant as described (Estell et al., 1985). Samples of pure proteins or samples of culture supernatants were prepared for SDS–PAGE by inhibiting subtilisin with PMSF, blocking any accessible thiols with iodoacetamide, and precipitating the protein with TCA as previously described (Wells & Powers, 1986). Unless otherwise noted, all proteins were greater than 95% pure as analyzed by SDS–PAGE (Laemmli, 1970). Enzyme concentrations were determined spectrophotometrically ($\epsilon_{280_{nm}}^{0.1\%} = 1.17$; Matsubara et al., 1965). Enzymic activities were assayed with the substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (Del Mar et al., 1979) in 0.1 M Tris (pH 8.6) at 24 °C. Kinetic parameters (k_{cat} and K_m) were determined by a method of progress-curve analysis (Estell et al., 1985) by following the time courses of hydrolysis of 68 and 530 μM substrate by subtilisin (5 μg/mL).

Equilibrium constants for reduction of subtilisin disulfides were determined (Wells & Powers, 1986) for purified proteins that were inhibited by PMSF and equilibrated for 2 h in the indicated DTT redox buffers (2 mM $CaCl_2$, 50 mM Tris·HCl (pH 8.0) at 24 °C) followed by a 2-h treatment with iodoacetamide (24 °C, in the dark) to block free cysteines. Amounts of reduced and oxidized proteins were quantified by densitometric scanning of proteins separated on SDS–PAGE and stained with Coomassie blue. In the case of D41C/G80C, the redox equilibrium constant had to be measured on culture supernatants (the mutant enzyme represents about 40% of the total protein) that contained 2 mM $CaCl_2$ and 50 mM Tris·HCl (pH 8.0). Both the incubation and blocking times were reduced to 1 h to avoid autolysis of this unstable protein. In all cases, the incubation time with the redox buffers was sufficient to reach equilibrium (data not shown).

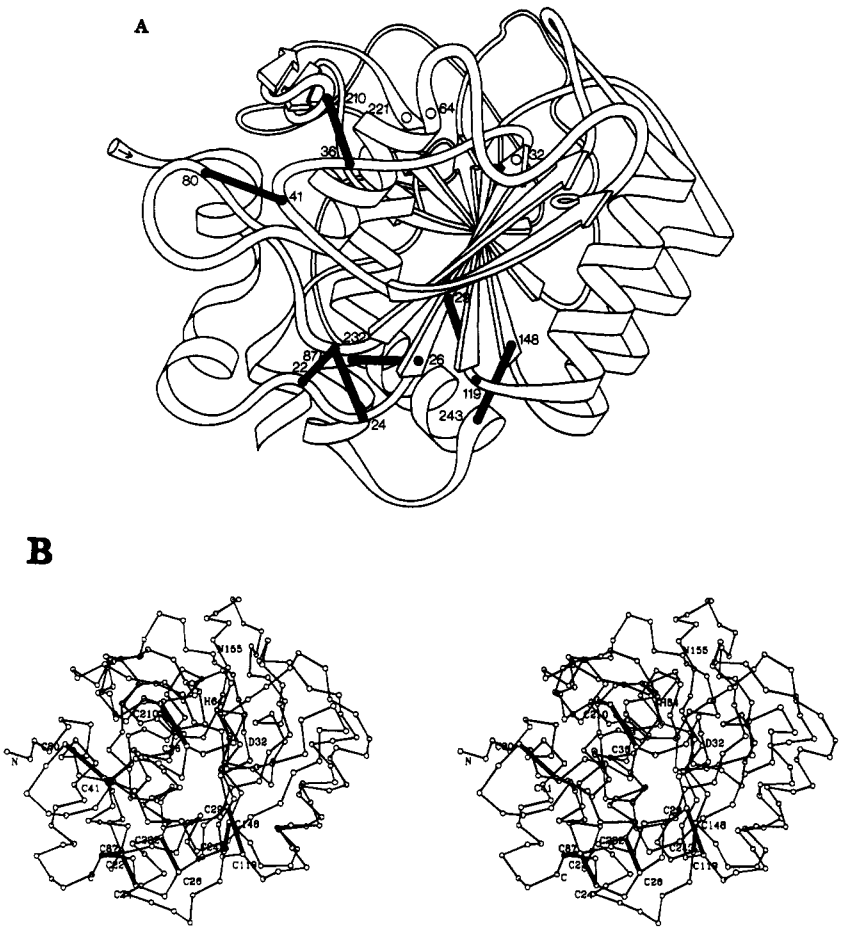


FIGURE 1: Positions of disulfides engineered into subtilisin. (Panel A) Ribbon diagram of subtilisin (Richardson, 1981) with cysteine positions and active-site residues (Ser-221, His-64, and Asp-32) labeled. Disulfide bonds are represented by dark lines. (Panel B) Stereoview from the same perspective as above showing α-carbons, disulfide cross-links, and active-site residues including Asn-155. The C24–C87, C22–C87, and C26–C232 disulfides were made previous to this study [see Wells and Powers (1986), Pantoliano et al. (1987), and B. Katz (unpublished results), respectively].

Thermal Inactivation. The stabilities of wild-type and variant subtilisins were measured by their rates of inactivation at elevated temperatures (50–61 °C). At temperatures where subtilisin BPN′ becomes unstable (Wells & Powers, 1986), the rates of inactivation were very temperature dependent. For this reason, a wild-type reference sample was incubated alongside every set of samples, and temperatures were monitored constantly with a microprobe in a blank sample. At indicated times, aliquots of enzymes (10–20 μL) were assayed for activity toward succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide. Sometimes, aliquots were quenched on ice, and this had no effect on their activities. Inactivation rates of purified enzymes (80 μg/mL) were assayed in 2 mM $CaCl_2$, 0.01% Tween 20, and 50 mM MOPS, pH 7.0. The Tween 20 was added to reduce adsorption of subtilisin to the tube surface. Typically, inactivation was followed until greater than 80% of the enzyme activity has been lost and plots of the logarithm of residual activity versus time were linear. Under these conditions, the rate of inactivation of wild-type subtilisin was virtually independent of its concentration (Table II). Values of half-times for duplicate samples within the same experiment (at one fixed temperature) had standard deviations less than or equal to ±8%. Large variations in the inactivation rates between experiments (often ±20%) were caused by the strong dependence of the inactivation rate on temperature (Wells & Powers, 1986) and variation in the recorded temperature due to the exact placement of the microprobe in the sample. For mutants that were much less stable than wild-type subtilisin, it was necessary to determine their inactivation rates (along with wild-type subtilisin) at lower temperatures to obtain accurate comparisons.

Table II: Concentration Independence for the Half-Time of Irreversible Thermal Inactivation of Subtilisin BPN′

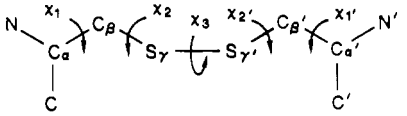
concn of subtilisin BPN′ (μg/mL)	half-time (min) for thermal inactivation[a]
15	68
47	69
127	63
376	59

[a] Inactivations were performed as described under Materials and Methods in 2 mM $CaCl_2$, 0.01% Tween 20, and 50 mM MOPS (pH 7.0) at 61.3 ± 0.3 °C.

RESULTS

Choice of Sites for Engineered Disulfides. Model building was performed on an Evans and Sutherland PS 300 using the program FRODO (Jones, 1978) and coordinates from a 1.8-Å resolution highly refined structure of subtilisin BPN′ (Bott et al., 1988). A list of potential sites for disulfides was generated by using the program PROTEUS (Pabo & Suchanek, 1986; B. Barnett, personal communication). Candidate sites were limited to those at least 10 Å from the catalytic Ser-221 to reduce possible effects on enzyme activity (Figure 1). As a further constraint, only those disulfides were considered that could be modeled with the minimal nonbonded contacts and energetically favorable dihedral angles (especially at χ_3; Katz & Kossiakoff, 1986). In previous studies (Katz & Kossiakoff, 1986; B. Katz and A. Kossiakoff, unpublished results), the

Table III: Observed and Predicted Geometries of Engineered Disulfides in Subtilisin[a]



disulfide	χ_1	χ_2	χ_3	χ_2'	χ_1'	C_α–C_α distance (Å)	bond dihedral[b] energy (kcal/mol)	structural context
natural LH[c]			−85 ± 9			5.88 ± 0.49	1.7 ± 1.5	
natural RH[c]			99 ± 11			5.07 ± 0.73	3.2 ± 1.4	
C22–C87	53 (64)	121 (112)	−98 (−85)	143 (130)	−49 (−57)	5.37 (5.37)	4.8 (4.3)	loop to loop
C24–C87	−65 (−70)	−50 (−35)	96 (92)	−171 (−152)	−157 (−174)	4.59 (4.76)	2.5 (2.7)	loop to loop
C26–C232	177 (79)	−126 (114)	−71 (115)	−47 (86)	−90 (−117)	5.57 (5.91)	5.0 (10.1)	β-sheet to α-helix; buried
C29–C119[d]	−86 (−65)	62 (61)	83 (113)	160 (144)	−99 (−136)	5.88 (6.27)	5.4 (6.9)	β-sheet to turn; buried
C36–C210	(−81) (−89)	(−151) (134)	(−90) (92)	(96) (−87)	(−160) (−73)	(5.54) (5.54)	(5.0) (5.6)	loop to turn
C41–C80[e]	(−175) (−45)	(178) (169)	(−98) (77)	(126) (111)	(−149) (−76)	(6.30) (6.30)	(5.1) (3.9)	loop to loop; high-affinity Ca^+ binding site
C148–C243	(−40)	(−10)	(−87)	(−63)	(−73)	(5.12)	(3.9)	β-sheet to α-helix

[a] Values are from X-ray crystallography (Katz & Kossiakoff, 1986; B. Katz and T. Kossiakoff, unpublished results), or from molecular modeling when shown in parentheses. For modeled structures, the C_α–C_α separation is assumed to be fixed from the C_α atoms in the wild-type structure. The dihedral angles in a disulfide are defined in the diagram at the top of this table. [b] Calculated from a subroutine of the program AMBER (Weinger et al., 1984). [c] LH and RH are left-handed and right-handed disulfides, respectively, and these values are averages from at least 10 natural disulfides [taken from Katz and Kossiakoff (1986)]. [d] C29–C119 modeled by B. Katz and C. Mitchinson. [e] The probability of flexibility in this region of subtilisin in the absence of the structural calcium makes it particularly difficult to judge how close to the actual structure the modeled structure will be.

disulfide bond geometries predicted from such model building agreed reasonably well (in three out of four instances) with the geometries determined from X-ray crystallography (Table III).

The disulfide C29–C119 was chosen on the basis of structural homology to the disulfide C34–C123 of proteinase K, a fungal protease that contains two disulfide bonds (Pahler et al., 1984) and shares about 37% sequence identity with subtilisin BPN'. The C148–C243 disulfide was picked partly because it was similar to the other proteinase K bond, C178–C249. The C36–C210 disulfide was introduced to replace the hydrogen bond between the carboxylate of Asp-36 and the main-chain nitrogen of Gly-211. Several stabilizing point mutations have been isolated by random mutagenesis and screening [N218S and N218D (Bryan et al., 1986); K213R (Cunningham & Wells, 1987); S204C (R. Caldwell and J. Wells, unpublished results)], and these are located near Gly-211 in the twisted β-hairpin structure that is composed of residues 204 to 218. The C41–C80 disulfide was designed to try to replace the stabilizing effect of a structural calcium (Drenth et al., 1972; Voordouw et al., 1976). Gly-80 is in the calcium binding loop, and the carboxylate of Asp-41 is one of the calcium binding ligands. However, the model of the C41–C80 disulfide showed structural adjustments would be necessary to relieve potential steric hindrance with the side chain of Tyr-214 and main-chain atoms of residues 79 to 81. These engineered disulfides (Figure 1), including those constructed previously (Wells & Powers, 1986; B. Katz and A. Kossiakoff, unpublished results), connect loops, turns, helices, or regions of β-sheet in buried or exposed positions (Table III) and span residues separated from 39 up to 206 residues (mature subtilisin contains 275 residues).

Disulfide Formation. All of the disulfides introduced by site-directed mutagenesis formed spontaneously in vivo, as shown by the gel mobility characteristics (Pollitt & Zalkin, 1983) of the mutant subtilisins taken directly from culture supernatants (Figure 2). In the presence of β-mercaptoethanol, the double-cysteine mutants migrated with the mobility of the relevant single mutants (data not shown). For C41–C80, which cross-linked the shortest disulfide loop, it was necessary to use an 18% acrylamide gel or a 10 to 15% "PHAST" gel (Pharmacia) to resolve the reduced and oxidized forms. Disulfide formation was confirmed for at least two independent clones of each double-cysteine mutant and for the purified disulfide proteins (data not shown). In almost all instances, disulfide formation was quantitative (Figure 2). However, some cultures of V26C/A232C confirmed previous observations of 50% disulfide bond formation (B. Katz and A. Kossiakoff, unpublished results), and cultures of A29C/M119C showed up to 10% migrating as the reduced protein. Other cultures of these same mutants showed quantitative disulfide formation. The source of variation in formation of these two disulfides is unknown, and no dependence on the time of culturing or the presence of reducing agent was observed. One possibility is that there was variation in the heavy-metal content of the culture media that could catalyze thiol oxidation. For example, the formation of the C26–C232 bond can be forced to completion in the presence of cupric ion (B. Katz and A. Kossiakoff, unpublished results).

Expression Levels. All of the variant proteins were expressed at 40% or more of the wild-type level, except for V148C (Table IV). This mutant enzyme was expressed at less than 10% of the wild-type level even though the purified V148C enzyme had the same specific activity as wild type and was completely stable for at least 7 h at 37 °C. No correlation was observed between the half-time for thermal inactivation and expression level of the mutant enzymes. In addition, fermentation of wild type or D41C/G80C in the presence of EGTA, or 1–50 mM calcium chloride, did not markedly influence the expression levels (data not shown), despite the known stabilizing effect of calcium on wild type (Voordouw et al., 1976). Steady-state levels of intracellular expression of mutant proteins are sometimes used as a basis for a stability screen (Pakula et al., 1986; Shortle & Meeker, 1986) because susceptibility to proteolytic degradation in vivo can be correlated with thermodynamic stability. The lack of a correlation for subtilisin may be due to the fact that subtilisin is translated as prepro-subtilisin and secreted and/or that it is observed to resist autolysis at the growth temperature.

All of the purified mutant enzymes had virtually the same specific activity as wild-type enzyme, except for V148C/N243C. This enzyme had a 25% lower activity that was

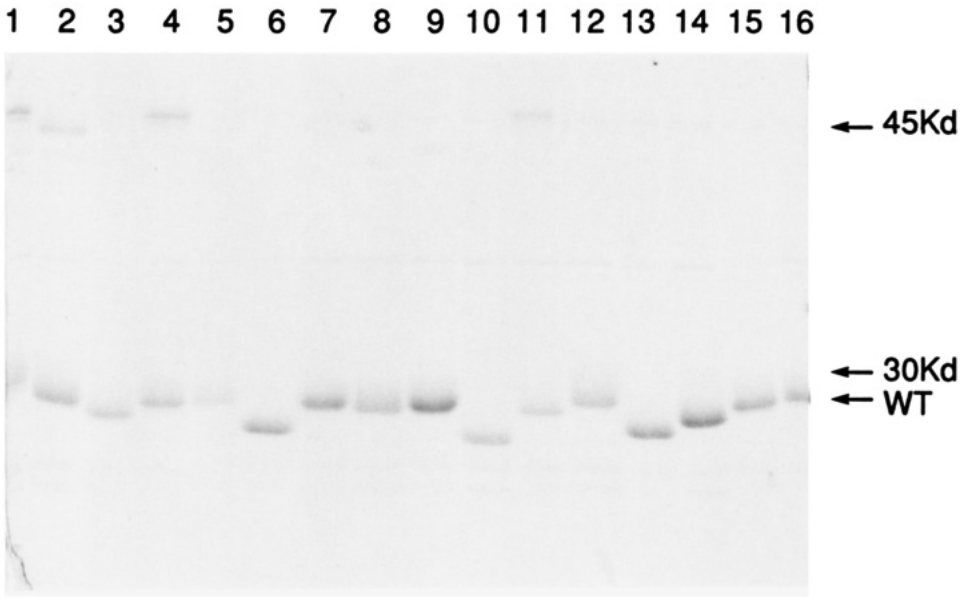


FIGURE 2: Spontaneous formation of disulfide bonds in mutant subtilisins in vivo. Lane 1, V26C; lane 2, A232C; lane 3, V26C/A232C; lane 4, A29C; lane 5, M119C; lane 6, A29C/M119C; lanes 7 and 16, wild type; lane 8, D36C; lane 9, P210C; lane 10, D36C/P210C; lane 11, D41C; lane 12, G80C; lane 13, D41C/G80C; lane 14, V148C/N243C; lane 15, N243C. Lanes 10, 12, and 14 are from 40 μL of culture supernatant (~1.8 μg of subtilisin) and the rest from 20 μL of supernatant (~1.6 μg of subtilisin).

Table IV: Thermal Inactivation and Expression Levels of Subtilisins

	half-time (min) for thermal inactivation[a] at						secreted subtilisin act. (% of wild type)[b]
	55.6 ± 0.2 °C		60.6 ± 0.2 °C		60.8 ± 0.2 °C		
variant	-DTT	+DTT	-DTT	+DTT	-DTT	+DTT	
wild type	1500[c]		117	117 (85)	83	87 (62)	100
V26C/A232C			121	(93)			87
V26C							100
A232C							76
A29C/M119C			75	(51)			72
A29C					77	(52)	95
M119C					149	(126)	86
D36C/P210C			120	4			37
D36C					42	52	72
P210C					98	93	120
V148C/N243C	9	102		16			43
V148C	191	135		26			8
N243C	191	209		34			120
D41C/G80C			4[c]	2[c]			80
D41C			1[c]				89
G80C			4[c]				47

[a] Thermal inactivations of purified subtilisins (80 μg/mL) were measured in 2 mM $CaCl_2$, 0.01% Tween 20, and 50 mM MOPS (pH 7.0) with or without 25 mM DTT or, when indicated by parentheses, with 100 mM DTT. The variation of the wild-type half-times over the three temperatures shows the extreme temperature dependence of the rates of inactivation (see Materials and Methods). For this reason, the values listed in each column were obtained from samples inactivated in parallel and always together with a wild-type standard. [b] Two-milliliter cultures (in triplicate) were grown 22 h at 37 °C to similar cell densities (A_{570} = 4.0 ± 0.3). Cells were removed by centrifugation and culture supernatants assayed for subtilisin activity as described under Materials and Methods. Wild-type expression level was 95 μg/mL. [c] This value is an approximate because reactions were too slow or too fast to measure half-times accurately.

increased to almost the wild type by treatment with DTT (25 mM, pH 8.0 for 2 h at 25 °C, data not shown). More detailed kinetic analysis showed the V148C/N243C mutant to have k_{cat} and K_m values of 23 ± 1 s^{-1} and 190 ± 40 μM, respectively. These compared with higher k_{cat} values (44 ± 1 and 43 ± 2 s^{-1}) and lower K_m values (165 ± 5 and 140 ± 25 μM) for the single mutants V148C and N243C, respectively. The k_{cat} and K_m values previously determined for wild-type subtilisin are 50 s^{-1} and 140 μM, respectively (Estell et al., 1985).

Subtilisin Stability. The half-time for irreversible inactivation at high temperature (determined as the time to reach 50% of the initial activity) was used as the measure of stability (Table II). The D41C/G80C family of mutants was so unstable (Tables IV and V) that these could not be purified from culture supernatant before they autolyzed completely. Successive purification steps gave progressively less stable samples of all three proteins, suggesting that there was (were) some stabilizing factor(s) in the culture supernatants. Therefore, inactivation studies on these enzymes had to be conducted on samples of culture supernatants and compared to a wild-type control. Nevertheless, under conditions used to measure the inactivation rates, the wild-type enzyme in culture supernatant inactivated at about the same rate as its purified form.

Several points are apparent from these data (Table IV). Firstly, none of the disulfide mutants are significantly more stable than wild-type subtilisin. Secondly, in most cases, disulfide bond formation made a positive contribution to stability compared to their reduced counterparts. For example, the C36–C210 disulfide provides more than a 30-fold increase in the half-time for inactivation relative to its reduced form. The only case where the oxidized form was seen to be less stable

Table V: Effect of Calcium on Thermal Inactivation of Subtilisins

subtilisin	half-time (min) for inactivation at 51 ± 1 °C[a]	
	-EGTA	+10 mM EGTA
wild type	≫240[b]	8.0
D41C	18	17
G80C	9.0	3.5
D41C/G80C	39	46
D41C/G80C + 25 mM DTT	8.7	8.1

[a] Thermal inactivations were carried out on wild-type and mutant subtilisins (40 μg/mL final concentration) directly from culture supernatants containing 2×TY media and 0.005% Tween 20. Under these conditions, plots of logarithm of activity versus time were linear to less than 10% residual activity. [b] No inactivation of wild-type subtilisin was observed over 4 h.

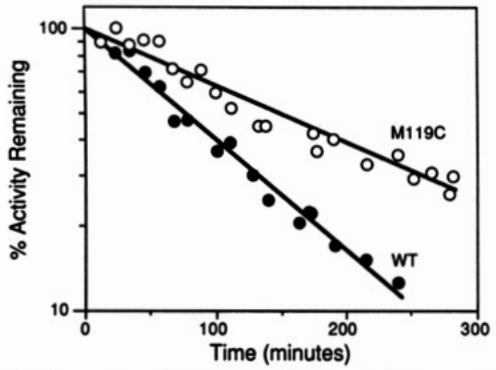


FIGURE 3: Thermal inactivation of M119C and wild-type subtilisins. Purified enzymes [(▲) WT; (○) M119C] were incubated in 2 mM $CaCl_2$ at 60.8 ± 0.2 °C as described in Table IV.

than the reduced form was for C148–C243. Third, the introduction of single cysteines either had no effect or was destabilizing (e.g., D36C, V148C, N243C, D41C, and G80C). The one exception was M119C, which was found to have a stabilizing effect (Figure 3). Finally, mutations in the high-affinity calcium binding site were very unstable compared to wild-type enzyme in the presence of calcium. However, the D41C and D41C/G80C mutants showed no calcium dependence on stability and were more stable than wild type in the absence of calcium (Tables IV and V).

Table VI: Properties of Engineered Disulfides in Subtilisin

disulfide bond (S^1–S^2)	% reduction obsd in 100 mM DTT after 5 min at 25 °C	accessibility[a] of S_γ atoms (Å²)		equilibrium constant for reduction by DTT[b]
		S^1	S^2	
C22–C87	>90	4.1	12	82 ± 22
C24–C87	100	22	25	20 ± 5
C26–C232	0	0	1.1	NR[d]
C29–C119	0	0	0–2.7	NR
C36–C210	100	5.3	22	26 ± 10
C41–C80	35	0[c]	0[c]	0.55 ± 0.29
C148–C243	50	0.1	5.4	1.6 ± 0.60

[a] Calculated by using the method of Lee and Richards (1971) as the surface area of contact of a 1-Å radius probe with the effective surface. This was carried out by using the program "ACCESS" modified by M. Handschumacher at Yale. S^1 and S^2 refer to the first and second cysteines in the mutant sequence, respectively. The first four values are for the observed X-ray structures (Katz & Kossiakoff, 1986; unpublished results). The range for C29–C119 represents the two observed structural conformers of A29C/M119C. Accessibility values for the bottom three disulfides were calculated from modeled structures. [b] Calculated from $K_{eq} = [Enz]_{red}[DTT]_{ox}/[Enz]_{ox}[DTT]_{red}$ for 8–10 determinations. Values for the top two disulfides are from Wells and Powers (1986), and the next two were resistant to reduction so they could not be measured. [c] These accessibility values are very uncertain because of large approximations in the modeled disulfide structure (Table III). [d] NR, not reducible.

Disulfide Bond Strength. The difference in mobility on SDS–PAGE of reduced and oxidized forms of the disulfide mutants (Figure 4) was used to measure the equilibrium constants for reduction by DTT (Table VI). Collectively, the disulfides varied over 100-fold in equilibrium constant from the strongest, C41–C80, to the weakest, C22–C87 (Wells & Powers, 1986). No correlation was found between the strength of the disulfide and its contribution to the stability of subtilisin

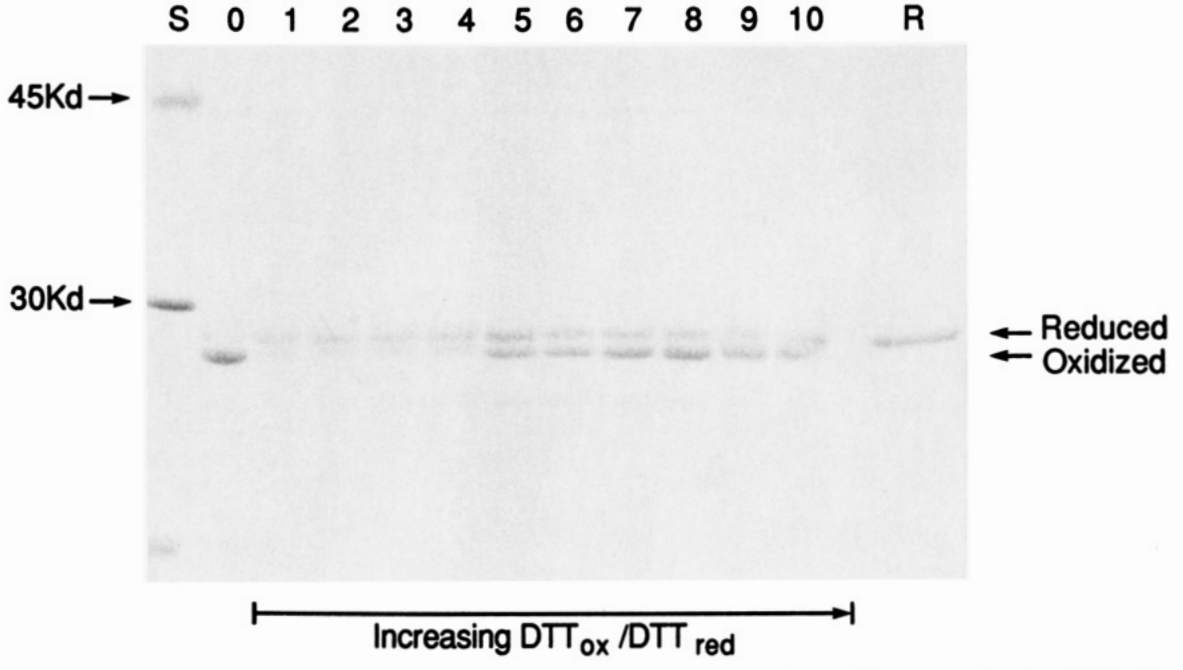


FIGURE 4: Representative SDS–PAGE (18% gel) of V148C/N243C (1.6 μg per lane) after equilibration with various DTT redox buffers. Incubations were carried out as described under Materials and Methods. S, molecular weight standards; R, V148C/N243C + β-mercaptoethanol. Remaining lanes are V148C/N243C subtilisin incubated in varying proportions of oxidized and reduced DTT, expressed as the ratio $[DTT]_{ox}/[DTT]_{red}$: lane 0, 0/0; lane 1, 1.08/16.8; lane 2, 5.3/18.1; lane 3, 10.4/16.8; lane 4, 19.8/16.9; lane 5, 17.9/12.5; lane 6, 18.4/9.86; lane 7, 18.9/7.5; lane 8, 19.5/4.85; lane 9, 20.4/3.99; lane 10, 20.5/1.47. The subtilisin at the outer edges of lanes 0 and 10 is reduced by reducing agent diffusing from lanes S and R, respectively.

Table VII: Summary of Properties of Subtilisin Disulfides

subtilisin disulfide mutant,[b] $-S^1/S^2-$	half-time for thermal inactivation[a] (as % of wild-type value)				ΔG for reduction by DTT[c] (kcal/mol)
	single-Cys mutants		double-Cys mutant		
	$-S^1H$	$-S^2H$	reduced $-S^1H/-S^2H$	oxidized $-S^1-S^2-$	
V26C/A232C	100[d]	100[d]	NA	100	NA
A29C/M119C	85	173	NA	69	NA
D36C/P210C	50	110	3	110	−1.9
D41C/G80C	0.80	3.0	1.5	3.0	+0.35
V148C/N243C	16	19	15	<1.5	−0.28
Y21A/T22C/S87C[e]	18	87	29	41	−2.6
S24C/S87C[e]	100	87	73	96	−1.8

[a] Half-time values shown have standard deviations of less than or equal to ±8% on the basis of three to five separate determinations. [b] S^1 and S^2 are defined in Table III. The residues underlined are conserved in all known *Bacillus* subtilisins. NA indicates the disulfide was not accessible to reduction by DTT. [c] Calculated from equilibrium constants in Table VI; $\Delta G_{reduction} = -RT \ln K_{eq}$. [d] Values from B. Katz and A. Kossiakoff (unpublished results). [e] Half-time and redox data from Wells and Powers (1986). It is likely that the Y21A mutation contributes substantially to the instabilities of the Y21A/T22C and Y21A/T22C/S87C mutants.

(Table VII). The equilibrium constants for the two buried disulfides, C26–C232 and C29–C119, could not be measured because they were completely resistant to reduction. In the native enzyme, these disulfides were not reduced to a significant extent by treatment overnight at 25 °C with either 0.25 M β-mercaptoethanol or 0.1 M DTT, or when treated for 2 h at 60 °C with 0.1 M DTT.

Discussion

Disulfide Bond Formation. Our results show that engineered disulfide bonds which connect various secondary structural elements both in surface-accessible and in buried environments, and which vary widely in redox equilibrium constant (Table VI), will form spontaneously in vivo (Figure 2). The fact that the buried disulfides, C26–C232 and C29–C119, can form incompletely in vivo may reflect burial of thiols during folding, making their subsequent oxidation slow. In the folded protein, these disulfides are resitant to reduction by small alkyl thiols. This is probably due to the inaccessibility of these two disulfides, as there is a good correspondence between the bond accessibilities and rates of disulfide reduction (Table VI). The only possible exception is C41–C80, but the validity of the modeled structure here is particularly uncertain (Table III). Resistance to disulfide bond formation in vitro has been previously reported for a partially buried disulfide bond engineered into dihydrofolate reductase (Villafranca et al., 1987). Our results support that surface accessibility of the thiols is an important factor in determining the kinetics of disulfide bond formation in vivo and in vitro. In fact, the low accessibilities for the V26C, A232C, A29C, and D41C mutants may account for the disulfide dimer band that is associated with only these proteins in SDS–PAGE (Figure 2). These inaccessible thiols in the native enzyme may not have reacted completely with iodoacetamide. However, when these were exposed during the nonreducing SDS–PAGE, the thiols could oxidize to give the observed dimer forms.

Disulfide Bond Strength. The measured strengths of the disulfide bonds introduced into subtilisin are comparable to those measured in natural or genetically altered proteins (H. Gilbert, personal communication). For example, the reduction equilibria constants with glutathione for the C14–C38 and C5–C55 disulfides in bovine pancreatic trypsin inhibitor (Creighton, 1983), for the C6–C103 and C2–C10 disulfides in ribonuclease T1 (Pace & Creighton, 1986), and for the C88–C88 intermolecular disulfide engineered into the λ repressor (Sauer et al., 1986) are 1500, 1.1×10^7, 34, 1000, and 100 M, respectively. On this glutathione scale and under similar conditions, the reduction equilibrium constants measured for the subtilisin disulfides C22–C87, C36–C210, C24–C87, C148–C243, and C41–C80 are 120, 390, 500, 6300, and 18 000 M, respectively. The range of subtilisin disulfide bond strengths spans a free energy difference of nearly 3 kcal/mol (Table VII). The basis for this large difference in redox potential is not easily explained by dihedral bond strain in itself. For example, the optimal model disulfide for C148–C243 is predicted to be 1.4 kcal/mol weaker than the observed C24–C87 disulfide, yet C148–C243 is actually 1.5 kcal/mol stronger (Tables III and VII). This poor correlation may stem from inaccuracies in the models or theory that predicts dihedral bond strain. It is also likely that other factors may contribute to the free energy of disulfide bond formation including microenvironmental effects on the pK_a of the thiols, constraints on packing for the disulfide compared to the dithiol form, and the flexibility of the main chain to accommodate a more stable disulfide geometry.

Stability of Disulfide Mutants to Irreversible Inactivation. The parameter often used to measure subtilisin stability is the rate of irreversible inactivation and autolysis at elevated temperature. Although others have attempted to interpret the effects of mutations in subtilisin in thermodynamic terms (Pantoliano et al., 1987), the irreversible nature of the inactivation process (even in the presence of inhibitors) precludes rigorous thermodynamic analysis. Nonetheless, this inactivation exhibits properties typical of protein unfolding. For example, agents known to disrupt the conformation of subtilisin (denaturants, high pH, high temperature, chelants) increase the rate of irreversible inactivation (Voordouw et al., 1986). Furthermore, the inactivation rate exhibits a cooperative "melting curve" (Wells & Powers, 1986) and shows no dependence on subtilisin concentration under the conditions used here (Table II). In addition, a subtilisin variant (H64A) in which the catalytic efficiency is reduced 10^6-fold (Carter & Wells, 1987) inactivates irreversibly and at the same rate as wild type (C. Mitchinson, unpublished results). Thus, autolytic digestion of wild-type enzyme under these conditions appear to be a consequence, and not the cause, of the irreversible inactivation. The data suggest that the thermally induced inactivation is mediated by an inactivating irreversible denaturation.

Theory and practice suggests that disulfide bonds should impart some thermodynamic stability to proteins [for a review, see Wetzel (1987)] and could still be stabilizing toward irreversible inactivation. Yet at least seven disulfides have been introduced into subtilisin (this study; Wells & Powers, 1986; Pantoliano et al., 1987), and none of the disulfides impart greater stability to irreversible inactivation compared to the wild-type enzyme in the presence of calcium. It is possible that none of these engineered disulfides provides thermodynamic stability to subtilisin. Alternatively, if the folded and unfolded states are not in equilibrium, disulfides cannot be expected to stabilize the inactivation process by preferential destabilization of the unfolded state. In this case, inactivation would be driven by the kinetics and not the thermodynamics of unfolding. The rate of unfolding is dependent upon the free energy difference between the folded state and the transition state, a structure which may closely resemble the folded state (Creighton, 1988). Thus, even thermodynamically stabilizing disulfides may not be expected to slow the rate of unfolding

because the entropies of the two states may be quite similar. In fact, the naturally occurring disulfide in the immunoglobulin C_L fragment (Goto & Hamaguchi, 1982a,b) has little effect on the rate of unfolding. In contrast to results on T4 lysozyme (Wetzel et al., 1988), the engineered disulfide bond in DHFR stabilizes the enzyme to reversible but not to irreversible inactivation (Villafranca et al., 1987).

In light of the above discussion, it is less surprising that no correlation was found between the stability of subtilisin disulfide mutants to irreversible thermal inactivation and the strength of the disulfide bond that it accommodates. For example, the C41–C80 disulfide is the strongest bond, yet it produces the least stable mutant (Table VII). Furthermore, disulfide bond strength is a poor predictor of the stabilizing effects the disulfide imparts relative to its reduced counterpart. For example, the most stable disulfide bond (C41–C80) imparts only a 2-fold greater stability to the mutant enzyme compared to its reduced form, while a much weaker disulfide (C36–C210) occurs in a mutant that is 30-fold more stable than its reduced counterpart. In fact, the protein containing the C148–C243 disulfide is more than 10-fold *less* stable than its reduced counterpart, yet the disulfide bond is much stronger than C36–C210. We conclude that disulfide bond strength alone is a poor indicator of the stabilizing effect the disulfide bond provides to subtilisin toward irreversible thermal inactivation. In addition, there is not a clear correlation between the size of the cross-linked loop and its redox potential or the effect of the disulfide on the rate of thermal inactivation.

A plausible approach to circumvent the above uncertainties associated with the design of stabilizing disulfides in subtilisin was the introduction of disulfide bonds in naturally occurring positions. The reduced form of protease K is much less stable than the oxidized form to irreversible inactivation (B. Barnett and J. Sullivan, personal communication). Yet the introduction of the disulfides C29–C119 or C148–C243 into subtilisin BPN′ that are in structurally analogous positions to those in protease K was not stabilizing. Indeed, C148–C243 was destabilizing. There are at least two possible explanations for these results. First, the structural homology is quite divergent between these two proteases in the regions around these disulfides (Bott et al., 1988; Pahler et al., 1984). Thus, to get the true benefit of the protease K disulfides may require more of the protease K structure. Second, the stabilizing effects of these disulfides may require them both to be present in the same molecule.

Effects of Single-Cysteine Mutations on Stability to Irreversible Inactivation. Some of the effects of the mutations on irreversible inactivation rates can be accounted for by the single cysteine mutations and not the disulfide that is derived from them. In some cases, the cysteine mutations were destabilizing, and these effects could be rationalized by disruption of an interaction present in the folded protein structure. For example, the D41C and G80C mutations substitute residues in the tight binding calcium site and increase the rate of inactivation dramatically (Tables IV and V). In the presence of EGTA, which has little or no effect on these mutants, the wild type is destabilized over 50-fold (Table V). Under these conditions, the D41C mutant is twice as stable as wild type, perhaps because in the absence of calcium the wild type would contain disruptive electrostatic interactions that the D41C mutation can alleviate. The disulfide mutants D41C/G80C and T22C/S87C (Pantoliano et al., 1987) are 6-fold and 2-fold more stable than wild type, respectively, but only in the absence of calcium. It should be stressed that these apoproteins are extremely unstable, in comparison to the native enzyme, and it is difficult to consider these mutants as having truly enhanced stability.

Introduction of cysteines at position 148 or 243 is also destabilizing. Model building suggests that the V148C mutation will disrupt the hydrophobic packing about the 147–152 β-sheet. The N243C mutation would break side-chain hydrogen bonds in the same region (to main-chain atoms of 143, 147, and 244). The reduced double-cysteine mutant is no less stable than the single mutants, but all three enzymes inactivate 5–10-fold more rapidly than wild type. The protein with this strong disulfide formed is 10-fold less stable than its reduced counterpart, and modeling suggests that bond formation will cause these residues, and the sheet and helix they are in, to move closer together to accommodate a less strained disulfide.

D36C/P210C illustrates the potential problem in having to introduce two cysteine substitutions in too close proximity (Table VII). The D36C mutant is destabilized as would be expected from the elimination of the hydrogen bond between the carbonyl of Asp-36 and the main-chain amide of Gly-211 in the wild-type protein. However, the reduced D36C/P210C mutant is much less stable than either of its single-cysteine parents. Modeling suggests that the preferred side-chain rotamers (Ponder & Richards, 1987) would tend to bring the two Sγ atoms in the reduced enzyme within 2.5 Å of each other, introducing steric hindrance in an area known to be important for subtilisin stability (Bryan et al., 1986; Cunningham & Wells, 1987). The formation of the C36–C210 disulfide bond gives a large increase in stability, probably by relieving the steric hindrance (the S–S distance in a disulfide is 2 Å) but also by successfully substituting for the lost hydrogen bond; the disulfide protein is more stable than the single D36C mutant, but not more so than wild type. This illustrates that a comparison of the stability of the reduced and oxidized protein (as is normally done for natural disulfide-containing proteins) can reflect both destabilization by steric hindrance of the two cysteines and the loss of the cross-link.

Sequence conservation is not a good predictor of destabilizing effects that may result from cysteine mutations. Val-26 and Ala-232 occupy buried positions and are highly conserved in all *Bacillus* subtilisins; Pro-210 is also highly conserved. Yet these residues can be freely replaced with cysteines without producing deleterious effects on the rate of irreversible inactivation (Table VII).

In contrast to destabilizing the enzyme by disrupting the folded structure, a mutant (N218S) has been isolated by random mutagenesis methods that is more stable to irreversible inactivation and found to have enhanced hydrogen-bonding interactions in the native enzyme (Bryan et al., 1986). Other mutants show that cysteine substitutions are not inherently destabilizing. The M119C mutation provides a significant stabilizing effect relative to wild type (Figure 3, Table IV) as does another cysteine mutant (S204C) isolated by random mutagenesis and screening (R. Caldwell and J. Wells, unpublished results).

Summary

To date, seven engineered disulfides of greatly varying redox strength have been introduced into subtilisin BPN′ without greatly affecting the enzyme activity or expression levels. However, in no case does the disulfide variant have greater than wild-type stability to irreversible thermal inactivation, and sometimes this can be rationalized by the deleterious effects of the parent cysteine mutations. It may be that none of these disulfides are thermodynamically stabilizing. It is also possible that disulfides do not stabilize the enzyme to irreversible thermal inactivation under these conditions because

the folded and unfolded forms are not in thermodynamic equilibrium. This is consistent with how disulfides and single cysteines can be destabilizing, because disruption of stabilizing interactions in the native enzyme could increase the rate of unfolding and therefore the rate of irreversible inactivation.

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