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Protein Engineering of Subtilisin BPN′: Enhanced Stabilization through the Introduction of Two Cysteines To Form a Disulfide Bond

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ABSTRACT: Introduction of a disulfide bond by site-directed mutagenesis was found to enhance the stability of subtilisin BPN′ (EC 3.4.21.14) under a variety of conditions. The location of the new disulfide bond was selected with the aid of a computer program, which scored various sites according to the amount of distortion that an introduced disulfide linkage would create in a 1.3-Å X-ray model of native subtilisin BPN′. Of the several amino acid pairs identified by this program as suitable candidates, Thr-22 and Ser-87 were selected by using the additional requirement that the individual cysteine substitutions occur at positions that exhibit some degree of variability in related subtilisin amino acid sequences. A subtilisin variant containing cysteine residues at positions 22 and 87 was created by site-directed mutagenesis and was shown to have an activity essentially equivalent to that of the wild-type enzyme. Differential scanning calorimetry experiments demonstrated the variant protein to have a melting temperature 3.1 °C higher than that of the wild-type protein and 5.8 °C higher than that of the reduced form (–SH HS–) of the variant protein. Kinetic experiments performed under a variety of conditions, including 8 M urea, showed that the Cys-22/Cys-87 disulfide variant undergoes thermal inactivation at half the rate of that of the wild-type enzyme. The increased thermal stability of this disulfide variant is consistent with a decrease in entropy for the unfolded state relative to the unfolded state that contains no cross-link, as would be predicted from the statistical thermodynamics of polymers.

One goal of protein engineering is to learn how to enhance the stability of proteins in order to broaden their utility in medical and commercial applications. Changing the nature of already existing ion bridges, hydrogen bonds, and hydrophobic contacts, or introducing completely new ones through site-directed mutagenesis, could conceivably result in increased stability. The ability to predict where to introduce such changes is, however, poor at present and reflects the complexity of forces that govern the folding of polypeptide molecules (Tanford, 1970).

On the other hand, the theoretical basis for choosing where to introduce disulfide bonds and the anticipated effects of those cross-links on protein stability are more fully understood. Covalent cross-linking in proteins lowers the entropy of the unfolded polypeptide, resulting in a larger free energy difference between the folded and unfolded protein on the assumption that the cross-link does not significantly increase the free energy of the folded form (Schellman, 1955; Flory, 1956; Poland & Scheraga, 1965). Indeed, chemically cross-linked derivatives of ribonuclease A (Lin et al., 1984; Weber et al., 1985) and chicken lysozyme (Ueda et al., 1985) behave as this simple model predicts. One would expect disulfide linkages to result in the same degree of stabilization with the important additional restriction that, unlike most chemical cross-linking reagents, disulfide bonds exhibit well-defined stereochemical properties. Fortunately, these stereochemical requirements are known both from model studies and from the availability of several well-refined disulfide-containing protein structures (Richardson, 1981; Thornton, 1981). Introducing disulfide bonds into a protein by site-directed mutagenesis therefore offers one of the more theoretically sound approaches to increasing thermal stability.

Previous efforts to introduce intrachain disulfide bonds into proteins have utilized proteins that already contain free cysteine residues, making the choice of where to introduce a second cysteine mate relatively straightforward (Perry & Wetzel, 1984; Villafranca et al., 1983). An early attempt to introduce disulfide bonds ab initio into a protein containing no cysteine residues was made by Bryan et al. (1985), using the cloned gene for the bacterial protease subtilisin BPN′. Although none of these initial disulfide-containing subtilisin variants exhibited enhanced stability, these studies provided the first evidence that engineered disulfide bonds will form in vivo in biological systems where the engineered protein can be secreted into the growth medium. The subtilisin BPN′ structure has now been highly refined in our laboratories to 1.3-Å resolution (Finzel et al., unpublished results), and in addition, we have developed a more sophisticated method for selecting where to introduce disulfide linkages into protein molecules. On the basis of these advances, we now report the first example of a designed disulfide bond that enhances the stability of a protein that has no preexisting cysteine residues in the wild-type structure.

Materials and Methods

Molecular Modeling. Sites for the insertion of disulfide linkages were chosen in a three-step process. First, a computer program was used to analyze a highly refined (R = 0.14)[1] 1.3-Å resolution X-ray structure of subtilisin BPN′ for those positions where the main-chain atoms of two juxtaposed residues were in the same geometrical relationship as the main-chain atoms in known disulfide linkages taken from the

[1] $R = \sum|F_o - F_c|/\sum|F_o|$.

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FIGURE 1: Designed disulfide linkage. An α-carbon backbone model of the refined 1.3-Å resolution subtilisin BPN′ structure. The sites of mutations Thr-22 and Ser-87 are highlighted, as well as the catalytic triad (Ser-221, His-64, Asp-32).

Brookhaven Protein Data Bank (Ladner, 1987). Second, the hypothetical sulfur atoms were inserted into the structure via computational modeling, and those candidates with unfavorable steric interactions were discarded. Third, the several amino acid pairs identified by this program were subjected to the final requirement that the individual cysteine mutations occur at positions that exhibit some variability in evolutionally related subtilisin primary sequences (Meloun et al., 1985). This three-step analysis selected residues Thr-22 and Ser-87 as prime candidates for cysteine substitutions that would lead to formation of a stabilizing disulfide bond (see Figure 1).

Site-Directed Mutagenesis. The subtilisin BPN′ gene from *Bacillus amyloliquefaciens* has been cloned and expressed at high levels in *Bacillus subtilis* by using its natural promoter sequence (Vasantha et al., 1984; Wells et al., 1983). Procedures for producing site-directed variants of this subtilisin have also been described (Bryan et al., 1986; Zoller & Smith, 1983). In the current study, three different subtilisin BPN′ genes bearing cysteine mutations were produced: Ser-87 → Cys, denoted 7126; Thr-22 → Cys, denoted 7158; and the double mutant Thr-22 → Cys and Ser-87 → Cys, designated 7159. The *B. subtilis* host strain used contains a chromosomal deletion of its subtilisin gene and therefore produces no background subtilisin activity (Fahnestock & Fisher, 1987). All three mutant enzymes were efficiently expressed in *B. subtilis* and were secreted into the culture medium at a concentration of ~1 g/L.

Protein Purification and Characterization. All variants of subtilisin BPN′ were purified from the fermentation broth essentially as described previously (Bryan et al., 1986) except that acetone or 2-propanol was used instead of ammonium sulfate in precipitation steps. A slight modification was employed for those variants that contained single cysteine residues, i.e., 7126 and 7158. In these cases all purification steps were carried out in the presence of 10 mM dithiothreitol (DTT)[2] to prevent oxidation of sensitive free thiol groups.

RESULTS

Protein Characterization. Disulfide bond formation can be detected by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) run under nonreducing conditions because a protein that contains a disulfide linkage has a smaller radius of gyration and therefore migrates farther down the gel (Perry & Wetzel, 1984; Pollitt & Zalkin, 1983). Figure 2 shows that under nonreducing conditions subtilisin 7159 does indeed migrate faster than the wild-type protein, while under reducing conditions the two bands are virtually indistinguishable. This evidence of disulfide bond formation is supported by our recent analysis of a 1.8-Å electron density map of subtilisin containing cysteines at positions 22 and 87 (unpublished results). It thus appears that the intended disulfide bond was formed spontaneously either as the protein was synthesized or as the protein was secreted into the fermentation broth. These observations are consistent with results previously reported for five other disulfide-containing variants of subtilisin BPN′ (Bryan et al., 1985; Pantoliano et al., 1985).

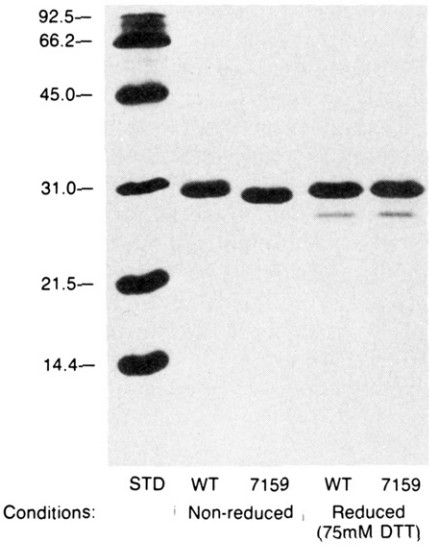

FIGURE 2: SDS–PAGE analysis of subtilisin 7159. Purified samples of subtilisin 7159 and the wild-type (WT) enzyme were inactivated with phenylmethanesulfonyl fluoride (PMSF) before boiling in 1% SDS in order to limit the autolysis that accompanies denaturation. For the samples in the two right-hand lanes, the SDS solution also contained 75 mM DTT as a reducing agent. The DTT was omitted for the nonreduced samples on the left. Otherwise the conditions are essentially those of Laemmli (1970) with about 15 μg of protein loaded in each lane. Molecular weight standards were from Bio-Rad (low M_r) and have the kilodalton values shown at the left.

The formation of a disulfide bond in substilisin 7159 does not appear to greatly affect the catalytic parameters of the enzyme toward hydrolysis of either azocasein or the synthetic peptide substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitro-

[2] Abbreviations: Dns-BBA, *N*-dansyl-3-aminobenzeneboronic acid; DSC, differential scanning calorimetry; DTT, dithiothreitol; EDTA, sodium salt of ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; sAAPFna, succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide; SDS, sodium dodecyl sulfate; T_m, temperature at the midpoint of the thermal transition; Tris, tris(hydroxymethyl)aminomethane; $t_{1/2}$, half-life for a kinetic experiment; WT, wild type.

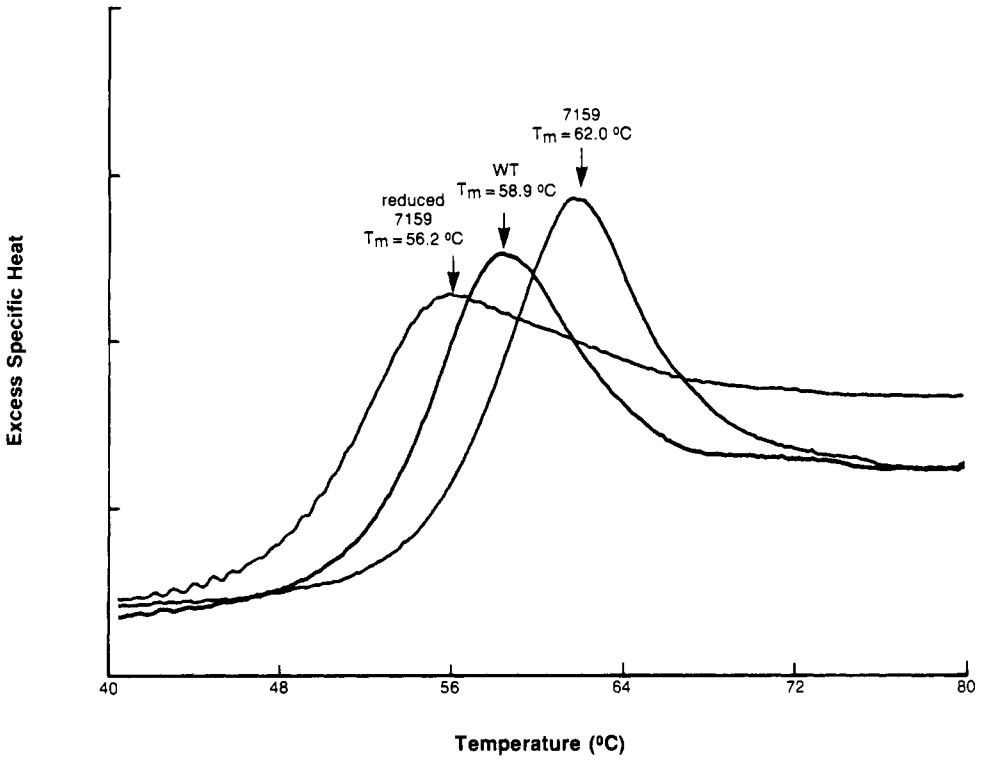

FIGURE 3: Differential scanning calorimetry data for wild-type and subtilisin 7159. These results were obtained in 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM KCl, 10 mM EDTA, and 2 mM Dns-BBA, a competitive inhibitor of subtilisin (see text). Data for the reduced forms of these proteins were obtained in the same buffer plus 10 mM DTT. The excess specific heat as a function of temperature was measured with a Hart Scientific microcalorimeter interfaced with an IBM personal computer (XT). The temperature was increased at a rate of 1 °C/min from 20 to 90 °C. The protein concentration for all samples was 3.0 ± 0.1 mg/mL, and the sample size was 0.7 mL.

anilide (sAAPFna), when assayed according to DelMar et al. (1979). The specific activity of subtilisin 7159 for the latter substrate was virtually unchanged from that of the wild-type enzyme—78 ± 6 units/mg vs. 82 ± 3 units/mg for the wild type.

Differential Scanning Calorimetry (DSC). In a DSC experiment T_m is defined as the midpoint in the thermally induced transition from the folded to the unfolded state. A comparison of the T_m for wild-type and mutant proteins thus provides an estimate of relative thermodynamic stability. Figure 3 shows the DSC transitions for wild-type subtilisin BPN′ and the oxidized and reduced forms of subtilisin 7159. These experiments were performed in the presence of 10 mM ethylenediaminetetraacetic acid (EDTA) in order to measure the intrinsic stability of the protein in the absence of the well-known calcium ion stabilization effect (Voordouw et al., 1976). A competitive inhibitor of subtilisin, *N*-dansyl-3-aminobenzeneboronic acid (Dns-BBA), with a K_i of 2 μM (Philipp & Maripuri, 1981) was also included in these experiments in order to reduce the amount of autolysis that accompanied the unfolding process. This inhibitor was found to have no effect on the T_m values observed.

The subtilisin 7159 variant exhibits a T_m of 62.0 ± 0.1 °C, which is 3.1 °C higher than that observed for the wild-type enzyme. Under reducing conditions (10 mM DTT), however, the 7159 variant was observed to have a T_m of 56.2 ± 0.1 °C, while the T_m for the wild-type enzyme was unaffected. The unfolding profile for reduced 7159 appears broader than for the others, which may be due to the presence of a small amount of unreduced subtilisin 7159.

The single-site variants, where the changes Thr-22 → Cys (7158) and Ser-87 → Cys (7126) exist alone, were also characterized by DSC. These two proteins gave T_m values of 56.4 ± 0.1 and 57.2 ± 0.2 °C, respectively (see Table I).

Table I: T_m Values for Subtilisin BPN′ Variants

variant	change	T_m (°C) (10 mM EDTA)[a]
wild type		58.9 ± 0.2
7159	Thr-22 → Cys plus Ser-87 → Cys	62.0 ± 0.1
7159 (10 mM DTT)	Thr-22 → Cys plus Ser-87 → Cys	56.2 ± 0.1
7126	Ser-87 → Cys alone	57.2 ± 0.2
7158	Thr-22 → Cys alone	56.4 ± 0.1

[a] The conditions for these experiments are described in the legend of Figure 3.

Thus, each of these single-site changes results in a less stable protein. No change was observed for these variants when they were melted in the presence of 10 mM DTT.

A DSC analysis in the presence of 10 mM $CaCl_2$ showed the same trend as discussed above, but the magnitude of the effect was not as great. Subtilisin 7159 was observed to have a T_m of 78.7 ± 0.1 °C, which is about 0.5 °C higher than that of the wild-type protein under these conditions.

Kinetics of Thermal Inactivation. The rate of thermal inactivation of subtilisin 7159 was found to be 1.5–2.0 times slower than that for the wild-type enzyme under a variety of conditions. For example, it was observed that the 7159 enzyme had a half-life ($t_{1/2}$) of 22.5 ± 2.3 min in 8 M urea at 40 °C while the wild-type enzyme had a $t_{1/2}$ of 11.2 ± 0.7 min under identical conditions (see Figure 4A). The inactivation rates for both enzymes appeared to be first order with respect to the enzyme concentration. In addition, it was observed that the rate of inactivation of subtilisin 7159 was significantly faster ($t_{1/2}$ = 9.5 ± 0.5 min) under reducing conditions (10 to 40 mM DTT) while the inactivation rate for the wild-type enzyme remained essentially unchanged (data not shown).

Similar results were obtained under less vigorously denaturing conditions. Figure 4B illustrates the rates of thermal

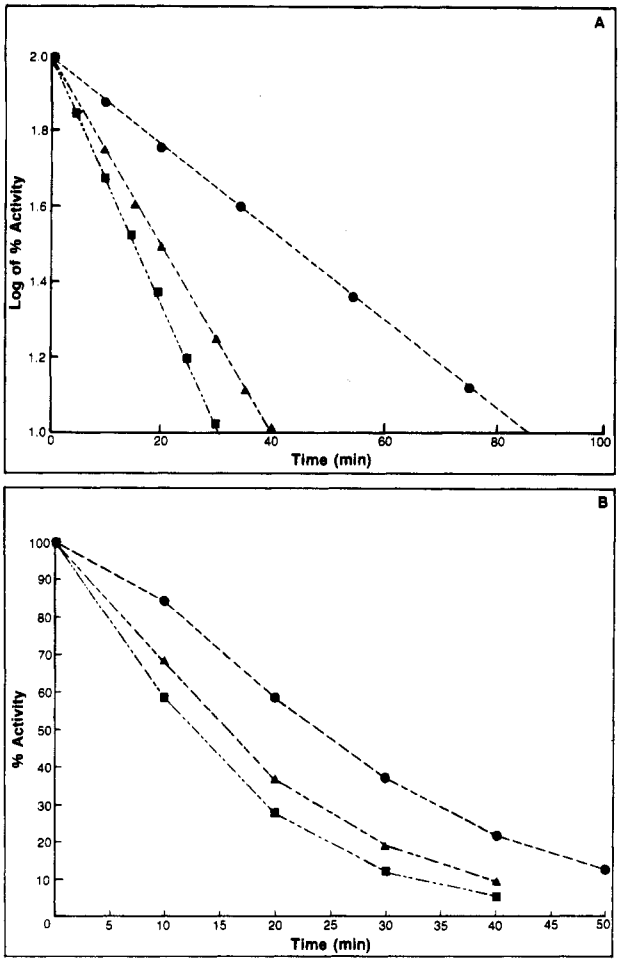

FIGURE 4: Kinetics of thermal inactivation of wild-type subtilisin (▲), subtilisin 7159 (●), and the reduced form of subtilisin 7159 (■) under the following conditions: (A) in 8 M urea at 40 °C [the urea was dissolved in 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM KCl]; (B) in 50 mM Tris-HCl (pH 8.0) buffer containing 50 mM KCl and 1.0 mM EDTA at 45 °C. The enzyme concentration in each case was 50 μg/mL, and the samples were immersed in a thermostatic circulating water bath equilibrated at the indicated temperature. At the designated time intervals, 10-μL aliquots were removed and diluted into 1.0 mL of the peptidase assay solution (sAAPFna) equilibrated at 25 °C. The data for the reduced form of subtilisin 7159 were obtained in the presence of 10 mM DTT.

inactivation at 45 °C in 50 mM Tris-HCl (pH 8.0)/50 mM KCl buffer containing 1.0 mM EDTA. The only difference between these results and those obtained in 8 M urea is that the inactivation process does not follow simple first-order kinetics. Thermal inactivation of the subtilisin 7159 protein as isolated (i.e., no added EDTA) gave results almost indistinguishable from those in Figure 4B except that it was necessary to conduct the experiment at 60 °C in order to obtain comparable rates (data not shown).

The rates of thermal inactivation for the variants of subtilisin that contain only the single cysteine changes (i.e., 7126 and 7158) were also examined. They were found to have rates that were intermediate between the wild-type enzyme and the reduced form of subtilisin 7159 when incubated at 45 °C in 50 mM Tris-HCl (pH 8.0), 50 mM KCl, and 1.0 mM EDTA. Again, DTT was not found to greatly affect the rates of inactivation for these variants.

When the thermal inactivation experiment was conducted in the presence of 10 mM $CaCl_2$, the 7159 variant gave a half-life that was only about 10% greater than that for the wild-type enzyme. This result is in agreement with the DSC data in 10 mM $CaCl_2$.

Discussion

The statistical thermodynamics of polymers predicts that covalent cross-links should reduce the entropy of the unfolded state of polypeptide chains by restricting the conformational degrees of freedom relative to the unfolded form that contains no cross-links (Schellman, 1955; Flory, 1956; Poland & Scheraga, 1965). The reduction in entropy that results from such a cross-link has been expressed as

$$\Delta S = -\frac{3}{4}R[(\ln n') + b] \quad (1)$$

where n' = total number of residues between the cross-link, b = constant ($^9/_4$ is usually used), and R = gas constant. Thus the formation of the disulfide linkage in subtilisin 7159 is expected to diminish the entropy of the unfolded polypeptide by ~9.5 eu relative to that of the unfolded reduced form of 7159. In terms of free energy, the unfolded state of the disulfide-containing form of subtilisin 7159 is expected to be ~3.2 kcal/mol less stable than that for the reduced form near the T_m for the wild-type protein (59 °C). This should result in a ~3.2 kcal/mol increase in the free energy change for the unfolding reaction.

These predictions are made with the assumption that no other components of the total free energy equation are affected by the formation of a disulfide cross-link. This assumption could therefore be invalid if the two cysteines are not ideally suited to the geometric and spatial limitations of disulfide bridges. Under these circumstances the increase in ΔG for unfolding gained by the reduction in entropy for the unfolded state could be negated by conformational strain or other forces that disrupt stabilizing elements present in the folded state of the wild-type protein. For this reason the 3.2 kcal/mol gain in free energy predicted from eq 1 for the unfolding transition for subtilisin 7159 should be considered an approximate upper limit because it assumes an idealized linkage that has no effect on the folded state.

In theory, we should be able to use the calorimetry results obtained for subtilisin 7159 to evaluate the utility of eq 1 in predicting the effect of disulfide linkages on the stability of proteins. However, the accurate determination of thermodynamic state functions necessary for such an analysis (Privalov & Khechinashvili, 1974) is difficult with subtilisin because the protein undergoes partial autolytic degradation (Takahashi & Sturtevant, 1981). Nevertheless, the T_m can be accurately determined and compared with the thermal unfolding data available for other protein systems with similar physical and thermal properties, but which lack the complications of autolysis. For example, Hecht et al. (1984) have reported a 5.9 °C increase in T_m (DSC) over that of the wild-type protein for the unfolding of the NH_2-terminal domain of the phage λ repressor (M_r 24 000) when Gln-33 is changed to Tyr. This change was found to correspond to an increase of 1.3 kcal/mol for the ΔG of unfolding at 52 °C. Similarly, Ueda et al. (1985) report a chemically cross-linked derivative of lysozyme to give a T_m that is 7.3 °C higher than that of the unmodified form and have calculated the ΔG for unfolding to be increased by 1.6 kcal/mol near the T_m (55 °C). It is reasonable, therefore, to estimate that the disulfide bond of subtilisin 7159 contributes about 1.3 kcal/mol to the ΔG of unfolding for this protein relative to the reduced form. This value corresponds to less than half of the estimated upper limit expected from theoretical considerations and thus suggests some deviation from an idealized fit. This point will be more fully analyzed when the refinement of the 1.8-Å resolution electron density map for this disulfide bond containing subtilisin is completed.

A second parameter that is important for the design of stabilizing disulfide linkages, but independent of the proper three-dimensional alignment discussed above, is related to the individual contributions of single amino acids toward the ΔG of unfolding. For example, in subtilisin 7158 the single Thr-22 → Cys change causes a 2.5 °C decrease in T_m, and the variant is also kinetically less stable than the wild-type protein in the thermal inactivation experiments. This change alone could thus account for the difference in stability between the wild-type protein and the reduced form of subtilisin 7159 (see Table I). Among the over 50 single-site variants of subtilisin BPN′ that we have so far made, it has been rare to find isolated changes that do not alter the stability of the protein, and the majority of these changes are, in fact, destabilizing. These observations are similar to those reported in the rapidly expanding literature for single-site changes in many different proteins (Hecht et al., 1984; Matthews et al., 1980; Schultz & Richards, 1986).

The mutagenesis of the wild-type protein to introduce individual cysteine residues therefore establishes a new level of free energy difference between the folded and unfolded states that in this case is smaller (less stable) than that of the wild type. Only after establishment of this new base line of ΔG of unfolding for the reduced form of the mutated protein can one attempt to predict the effect of covalent cross-linking. In this particular case, the stabilizing effect of the disulfide cross-link more than compensates for the destabilizing effects of the single-site changes so that the 7159 protein melts 3.1 °C higher than the wild-type protein. Without the destabilizing effects of the single-site changes subtilisin 7159 would be expected to melt 5.8 °C higher. On the other hand, one can easily imagine a situation where the single-site changes are so deleterious that even with the stabilizing effect of the covalent cross-link the resulting protein is still less stable than the wild type. This could be one reason for the lack of success, until this report,[3] in engineering disulfide bonds to yield more stable proteins where more than one cysteine needs to be introduced. The necessity of making two amino acid changes doubles the chances for destabilizing the base line free energy of the protein beyond recovery by cross-links. Our attempt to minimize the effect of single amino acid changes has been to avoid residues that are highly conserved in related subtilisin primary sequences.

Recently, Wells and Powers (1986) reported an attempt to stabilize subtilisin BPN′ by introducing a disulfide linkage between positions 22 and 87 and, in a separate protein, between positions 24 and 87. They concluded from their investigation that neither one of these disulfide linkages serves to stabilize subtilisin beyond that of the wild-type protein. In fact, they reported the Cys-22/Cys-87 variant to be less stable in thermal inactivation experiments when compared with the wild-type protein. The reason for such a large discrepancy between their experimental results and those reported here is not entirely clear. One potential source of the difference, however, is that their version of subtilisin with the 22/87 disulfide linkage actually contains a third change, Tyr-21 → Ala, that was introduced as part of an unrelated study. This third change may destabilize the reduced form of the triple mutated subtilisin to a level that is beyond recovery by the effect of a covalent cross-link on the entropy of the unfolded protein.

Although the 22/87 disulfide linkage has a significant effect on the intrinsic stability of subtilisin, that effect is less pronounced in 10 mM $CaCl_2$. Calcium is known to stabilize subtilisin (Voordouw et al., 1976), and Finzel et al. (unpublished results) have recently observed two metal binding sites in the X-ray structure for wild-type subtilisin BPN′. One of these sites binds calcium with sufficient strength to remain fully occupied even after dialysis against 50 mM Tris-HCl (pH 8.0), consistent with the results of Voordouw et al. (1976). However, we have also observed that by increasing the concentration of Ca^{2+} above 0.1 mM one begins to titrate the second weaker metal binding site (unpublished results). A possible simple explanation for the smaller stabilizing effect of the disulfide bond in the presence of 10 mM $CaCl_2$ is that the binding of calcium at the second weaker site results in conformational and/or dynamic changes that introduce strain at the disulfide linkage, thereby diminishing its effectiveness in stabilization under these conditions.

[3] Another computer-aided design of disulfide bonds has been recently reported by Pabo and Suchanek (1986). It has been successfully applied to the design of an interchain disulfide linkage between the dimer interface of the N-terminal domain of λ repressor (Sauer et al., 1986), but has not yet been reported for the design of an intrachain disulfide linkage as described here for subtilisin BPN′.

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Registry No. L-Cys, 52-90-4; L-Thr, 72-19-5; L-Ser, 56-45-1; subtilisin, 9014-01-1.

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Epidermal Growth Factor Binding Protein: Identification of a Different Protein†

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ABSTRACT: Partial amino acid sequence analysis of epidermal growth factor binding protein (EGF-BP), an arginine esteropeptidase that specifically associates with EGF to form a high molecular weight complex in male mouse submandibular glands, has revealed a single, distinct protein that is different from three previously reported forms of EGF-BP. This protein shows substantial sequence homology with these other putative forms of EGF-BP as well as with a large family of kallikreins expressed in the mouse submandibular gland. Purified EGF-BP contains three polypeptide chains as a result of two internal cleavages at residues 87–88 and 140–141. These modifications may represent processing events that are critical for determining the binding specificity of EGF-BP, since they occur within regions surrounding the substrate binding site.

Epidermal growth factor (EGF)[1] has a number of different biological effects, including potent mitogenic activity for a variety of tissue culture cells, and appears to play important roles in development and growth control throughout life (Cohen, 1962; Carpenter & Cohen, 1979). Sequence analysis of the cDNA clone for mouse EGF (Gray et al., 1983; Scott et al., 1983b) has revealed a much larger precursor form containing 1217 amino acids compared to the 53 amino acid mature form. ProEGF is predicted to be a membrane-bound protein due to the presence of a transmembrane segment near the C-terminus (Doolittle et al., 1984). In addition to EGF, the precursor contains seven or eight regions that show sequence homology to EGF (Gray et al., 1983; Scott et al., 1983b; Doolittle et al., 1984). It is unclear if EGF generally acts as a membrane-bound protein in cell–cell interactions (or even as a receptor) or if it is normally processed and acts as

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[1] Abbreviations: EGF, epidermal growth factor; EGF-BP, epidermal growth factor binding protein; β-NGF, β-subunit of mouse nerve growth factor; γ-NGF, γ-subunit of mouse nerve growth factor; α-NGF, α-subunit of mouse nerve growth factor; HMW-EGF, high molecular weight EGF; TAME, N^{α}-*p*-tosylarginine methyl ester; BAPNA, N^{α}-benzoylarginine-*p*-nitroanilide; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

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