The Engineering of Binding Affinity at Metal Ion Binding Sites for the Stabilization of Proteins: Subtilisin as a Test Case†

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ABSTRACT: A weak Ca^{2+} binding site in the bacterial serine protease subtilisin BPN′ (EC 3.4.21.14) was chosen as a model to explore the feasibility of stabilizing a protein by increasing the binding affinity at a metal ion binding site. The existence of this weak Ca^{2+} binding site was first discovered through a study of the rate of thermal inactivation of wild-type subtilisin BPN′ at 65 °C as a function of the free [Ca^{2+}]. Increasing the [Ca^{2+}] in the range 0.10–100 mM caused a 100-fold decrease in the rate of thermal inactivation. The data were found to closely fit a theoretical titration curve for a single Ca^{2+} specific binding site with an apparent log K_a = 1.49. A series of refined X-ray crystal structures ($R \leq 0.15$, 1.7 Å) of subtilisin in the presence of 0.0, 25.0, and 40.0 mM $CaCl_2$ has allowed a detailed structural characterization of this Ca^{2+} binding site. Negatively charged side chains were introduced in the vicinity of the bound Ca^{2+} by changing Pro 172 and Gly 131 to Asp residues through site-directed and random mutagenesis techniques, respectively. These changes were found to increase the affinity of the Ca^{2+} binding site by 3.4- and 2-fold, respectively, when compared with the wild-type protein (ionic strength = 0.10). X-ray studies of these new variants of subtilisin revealed the carboxylate side chains to be 6.8 and 13.2 Å, respectively, from the bound Ca^{2+}. These distances and the degree of enhanced binding are consistent with simple electrostatic theory. Moreover, when both Asp changes were introduced together, the binding affinity for Ca^{2+} was found to be increased about 6-fold over that for the wild-type protein, suggesting an independent and nearly additive effect on the total electrostatic potential at this locus.

Metal ions have been shown to play an important role in stabilizing proteins by binding at specific sites. One well-known example is the neutral protease thermolysin from the thermophilic organism *Bacillus thermoproteolyticus*, which was found to contain four Ca^{2+} binding sites (Matthews et al., 1972, 1974). The collective contribution of Ca^{2+} binding sites to the ΔG of unfolding for thermolysin was reported to be between 8.1 and 9.2 kcal/mol (Voordouw et al., 1976). Similarly, Filimonov et al. (1978) have found that the binding of Ca^{2+} to two noncooperative high-affinity sites (log K_a = 7.5) is accompanied by a 10.6 kcal/mol increase in the overall ΔG of unfolding and a 50 °C increase in the midpoint for the thermally induced unfolding transition of parvalbumin III from carp muscle. In addition, Mitani et al. (1986) have found that the binding of a single Ca^{2+} to the high-affinity site (log K_a = 9.6) in α-lactalbumin causes a 22–34 °C increase in the unfolding transition for this protein, as expected from the interrelationship between ΔG for thermal unfolding and cofactor binding (Schellman, 1975, 1976). Further illustrations of pronounced protein stabilization mediated by metal ions can be found in the literature for Cu–Zn superoxide dismutase (Stellwagen & Wilgus, 1978; Roe et al., 1988) and the zinc metalloenzyme alkaline phosphatase (Chlebowski et al., 1979).

The subtilisin BPN′ structure has now been highly refined (R = 0.14)[1] in our laboratories to 1.3-Å resolution and has revealed structural details for two metal ion binding sites.[2] One of these binds Ca^{2+} with high affinity[3] and is located near the N-terminus (see Ca A in Figure 2A). The second site appears to bind Ca^{2+} and other cations much more weakly and is located 32 Å away (Ca B in Figure 2A). Structural evidence for two calcium binding sites was also recently reported by Bode et al. (1987) for the homologous enzyme subtilisin Carlsberg.

We have chosen these sites as models to test the feasibility of altering the electrostatic potential around metal ion binding sites in proteins to increase the metal ion binding affinity and thereby increase the ΔG for protein unfolding. This paper will focus on the results obtained to date for a redesigned and enhanced version of the weak Ca B site of subtilisin BPN′ with emphasis on the biophysical and functional role of metal ion binding.

Materials and Methods

Protein Purification and Characterization. All variants of subtilisin BPN′ were purified and verified for homogeneity essentially as previously described (Bryan et al., 1986a,b; Pantoliano et al., 1987). Assays were performed by monitoring the hydrolysis of succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (sAAPFna)[4] as described by DelMar et al. (1979).

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[1] $R = \sum|F_o - F_c|/\sum|F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively.

[2] A preliminary disclosure was made by Finzel et al. (1986) and also by Bryan (1987).

[3] log $K_a \sim 8$ (65 °C), unpublished results.

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Table I: Occupancy and Crystallographic Temperature Factors for the Wild-Type-like Ca B Site and the Adjacent Na–K Site as a Function of [Ca^{2+}]

[Ca^{2+}] (mM)	resolution[a] (Å)	R-factor	Ca B site[b]		Na–K site[c]	
			Q[d]	B[e]	Q	B
0.0[f]	1.7	0.15	6.5	10.9	18.0	8.1
0.0[g]	1.7	0.14	4.4	16.7	10.8	15.5
25.0[h]	1.7	0.15	13.5	4.4	10.8	19.3
40.0[i]	1.7	0.14	18.0	5.5	10.8	16.5

[a] Diffraction data were collected on each crystal to a maximum resolution of 1.7 Å on a Nicolet area detector mounted on an Elliot GX-21 rotating anode and reduced with a XENGEN software package (Howard et al., 1987). Each structure was refined independently by restrained least-squares methods (Hendrickson, 1985) starting from the 1.3-Å structure of wild-type subtilisin BPN′ (Finzel et al., 1986, and unpublished results). [b] Ligands and geometry are shown in Figure 2B. [c] The monovalent cation site is comprised of seven oxygen ligands which originate from the peptide carbonyl groups of residues Gly 169, Tyr 171, Val 174, and Glu 195, in addition to one O atom of the side-chain carboxylate group of Asp 197 and two water molecules (mean bond distance ~2.9 Å). [d] Q = occupancy in electrons. [e] B = temperature factor (mean square of electrons). [f] The crystal was grown in the presence of 50 mM glycine–KOH and 50 mM KCl, pH 9.0, with no added $CaCl_2$. [g] The crystal was grown in the presence of 50 mM glycine–NaOH, pH 9.0, with no added $CaCl_2$. [h] The crystal was grown in the presence of 20 mM Mes–NaOH, pH 6.0, with 25 mM $CaCl_2$. [i] The crystal was grown in the presence of 100 mM Tris-HCl, pH 8.7, with 40 mM $CaCl_2$.

Metal Analysis. Metal analysis was performed by neutron activation according to the method of Greenberg et al. (1984).

X-ray Crystallography. The purified wild-type and individual mutants of subtilisin BPN′ typically were inactivated with diisopropyl fluorophosphate (DFP) and then dialyzed against a buffer of choice. Four of the five buffers chosen for this study are described in Table I, and the fifth was 50 mM glycine–NaOH, pH 9.0, and 1 mM EDTA. Single crystals were then grown by vapor diffusion against acetone or 2-propanol in hanging drops (Wlodawer & Hodgson, 1975; Bryan et al., 1986b).

Molecular Modeling. The model used to predict the effect of amino acid changes on metal ion binding affinity was kept as simple as possible by considering only electrostatic interactions between Ca^{2+} and negatively charged carboxylates of the amino acid side groups. The interaction between a central metal ion and negatively charged or dipolar ligands has been long understood through the use of the electrostatic crystal field theory (CFT) (Bethe, 1929; Van Vleck, 1932). Because of structural limitations at the Ca B site, however, it was not possible to introduce a carboxylate side group close enough to become a formal ligand (intercharge distance $r \sim 2.4$ Å). Instead, an attempt was made to influence the electrostatic potential at this site through long-range outer-sphere changes where $r > 5$ Å. Electrostatic interactions over this distance can be understood in terms of Coulomb's law, in much the same way that it has been used to model charge–charge interactions that influence the pK_a of the catalytic groups at the active site of enzymes (Matthew, 1985; Russell et al., 1987; Russell & Fersht, 1987). That is, the expected change in the metal ion binding affinity can be calculated by using the form of Coulomb's law that states

$$\Delta pK_a = 244 Z_1 Z_2 / r D_{eff} \quad (1)$$

where in this case K_a is the metal ion binding constant and Z_1 and Z_2 are the two charges measured in units of the charge of an electron (in the current study, $Z_1 = 2+$ for Ca^{2+} and $Z_2 = 1-$ for COO^-). The interatomic distance is r (Å), and D_{eff} is the effective dielectric constant. There are limitations in using such a simple model for proteins since the dielectric medium of folded polypeptide structures is not homogeneous or isotropic (Gilson & Honig, 1987; Sternberg et al., 1987; Hwang & Warshel, 1987). Nonetheless, the utility of such a simple electrostatic model as a first approximation will be evaluated in light of the experimental results.

By use of Coulomb's law as a rudimentary guide, the following three-step process was used to target amino acid residues in subtilisin BPN′ for mutagenesis to Asp or Glu: (1) consider only those amino acid residues that have C_β atoms within 15 Å of the bound metal ion; (2) select only those residues that are variable in the primary sequences of homologous bacterial proteases (Meloun et al., 1985; Pantoliano et al., 1987); (3) give preference to those residues at the protein–solvent interface so that D_{eff} will be close to that of solvent.

This selection process targeted residue Pro 172 in WT subtilisin BPN′ as a prime candidate for site-directed mutagenesis. The location of Pro 172 can be seen in Figures 2 and 3. In molecular modeling simulations conducted with the aid of an Evans & Sutherland computer graphic terminal driven by Frodo software (Jones, 1982), the COO^- side group of Asp 172 was expected to be about 7 Å from the bound Ca^{2+}, depending on the rotation around the C_α–C_β bond (χ_1).

Random Mutagenesis. It was also possible to identify amino acid residues that influence the binding affinity at the weak Ca site through the use of random mutagenesis. The application of this technique for the identification of stable variants of subtilisin has been described elsewhere (Bryan et al., 1986b; Rollence et al., 1988). During the course of these earlier studies one of the stabilizing single amino acid changes, G131D, was found to be about 13 Å from the Ca B site. A characterization of this variant of subtilisin was therefore included as part of the current study.

Site-Directed Mutagenesis. The subtilisin 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 elsewhere (Zoller & Smith, 1983; Bryan et al., 1986a). In the current study, five different subtilisin BPN′ genes bearing carboxylate side-group mutations were prepared: P172D, P172E, G131D, D197E, and the double mutant where the changes G131D and P172D exist together.

RESULTS

Metal Analysis. Neutron activation was used for the determination of several different metals in subtilisin BPN′. WT subtilisin BPN′ was first inactivated with DFP and then dialyzed against 20 mM Hepes buffer (4 °C) adjusted to pH 7.0 with NH_4OH.[5] After dialysis against 3 × 1 L changes of this buffer, the sample was lyophilized and analyzed for Ca, Mg, Zn, Al, Mn, Ti, and V. Only Ca was found to be present in

[4] Abbreviations: DFP, diisopropyl fluorophosphate; EDTA, sodium salt of ethylenediaminetetraacetic acid; [E], enzyme concentration; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; sAAPFna, succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide; Tris, tris(hydroxymethyl)aminomethane; $t_{1/2}$, half-life for a kinetic experiment; WT, wild type. A shorthand for denoting amino acid substitutions employs the single letter amino acid code as follows (Knowles, 1987): G131D denotes the change of Gly 131 to Asp, and the variant enzyme containing this single change is denoted subtilisin G131D. When two changes exist together, i.e., P172D and G131D, the variant enzyme is denoted subtilisin P172D/G131D.

[5] NH_4OH was used instead of NaOH or KOH because Na and K interfere with the neutron activation method for determining Ca.

significant quantity: 0.98 mol of Ca/mol of enzyme.

Dialysis against 1.0 mM EDTA (all other conditions being the same) was found to remove more than half of the bound calcium, suggesting a single high-affinity binding site for calcium in this enzyme. These results are consistent with those of Voordouw et al. (1976) except that the apparent binding constant reported by these investigators (log $K_a > 11$) would appear to be too large. Additional experimental evidence for a high-affinity Ca^{2+} binding site but with a log K_a in the range of 8 will be described elsewhere (Pantoliano et al., manuscript in preparation).

Calcium Dependence for the Rate of Thermal Inactivation. Evidence for a second, but much weaker, Ca^{2+} binding site was obtained through the study of the effect of higher concentrations of Ca^{2+} on the kinetic thermal stability of subtilisin BPN′. Increasing the [Ca^{2+}] above 0.10 mM was found to dramatically decrease the rate of irreversible inactivation of WT subtilisin BPN′ at 65 °C as shown in Figure 1A. As the [Ca^{2+}] was increased above 0.10 mM $CaCl_2$, the $t_{1/2}$ for the rate of thermal inactivation at 65 °C was observed to slow from about 2 min to ~200 min at [Ca^{2+}] = 100 mM (Figure 1A). These data were found to closely fit a theoretical titration curve for a single binding site that has an apparent log K_a = 1.49 under these experimental conditions. The upper limit of attainable kinetic stability for WT subtilisin BPN′ at 65 °C corresponds to the form of the enzyme that binds 2 Ca^{2+}/mol of protein. The lower limit corresponds to the kinetic stability of subtilisin which binds 1 Ca, as isolated (see above). The stabilizing effect of Ca^{2+} binding to the high-affinity site cannot be measured at 65 °C since the thermal unfolding transition of the form of subtilisin that binds no Ca^{2+} occurs at 59 °C (Bryan et al., 1986b; Pantoliano et al., 1987). An analysis of the stabilizing effect of metal ion binding to the Ca A site will appear elsewhere (Pantoliano et al., manuscript in preparation).

X-ray Crystallography. Structural evidence for two Ca^{2+} binding sites has been obtained from a highly refined (R = 0.14) X-ray diffraction (1.7-Å resolution) model of subtilisin BPN′ shown in Figure 2A. Only a brief description of the structural characterization of these binding sites is presented here. The Ca A site, located near the N-terminus, is believed to be the high-affinity site characterized by metal analysis. Support for this assignment comes from X-ray structures (1.7 Å) obtained from crystals of subtilisin BPN′ that were grown in the absence and presence of 1 mM EDTA (50 mM glycine–NaOH, pH 9.0). An F_{native}–F_{EDTA} difference Fourier analysis indicated that no change had taken place at site A, implying that the Ca^{2+} bound here is difficult to remove under the conditions of crystallization. This result is consistent with the metal analysis after dialysis against a 1 mM EDTA solution (see above). It seems likely, therefore, that the Ca A site corresponds to the high-affinity site first reported by Voordouw et al. (1976). Structural details for Ca^{2+} coordination at this site are very similar to that recently reported by Bode et al. (1987) for subtilisin Carlsberg.

The Ca B site is located some 32 Å away from the Ca A site in a shallow crevice between two segments of polypeptide chain near the surface of the molecule (shown in Figure 2B). This structure was obtained from an X-ray model for subtilisin BPN′ crystals grown in the presence of 40 mM $CaCl_2$. The coordination geometry of this site closely resembles a distorted pentagonal bipyramid. Three of the formal ligands are derived from the protein and include the carbonyl oxygen atom of Glu 195 and the two side-chain carboxylate oxygens of Asp 197. Four water molecules comprise the remainder of the first

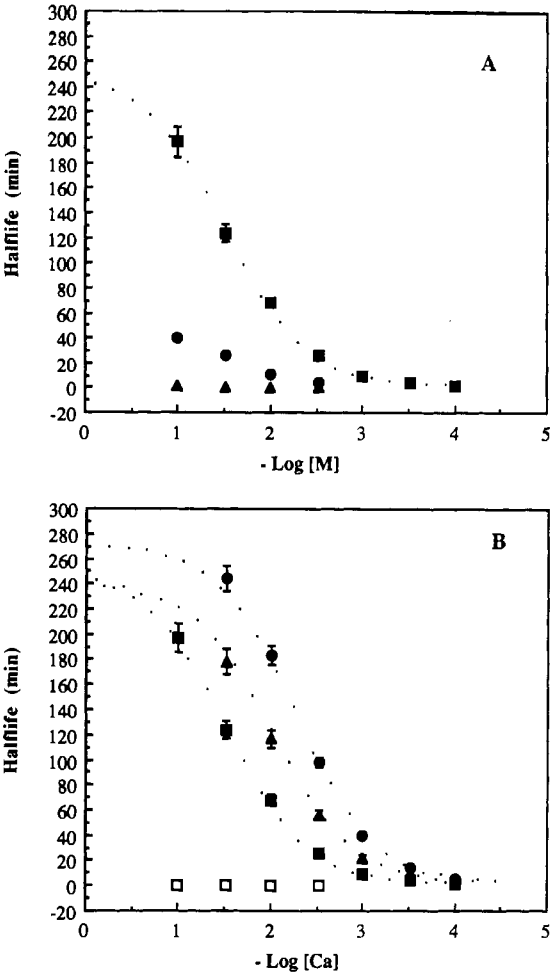

FIGURE 1: Metal ion binding titration curves from the rates of thermal inactivation. (A) $t_{1/2}$ for thermal inactivation of wild-type subtilisin BPN′ as a function of the −log [M] (pM). The rates for thermal inactivation were measured by immersing enzyme solutions, [E] = 50 μg/mL (1.8 μM), in a thermostated circulating water bath set at 65 °C. The sample was dissolved in 50 mM Tris-HCl buffer (pH 8.0) containing varying amounts of $CaCl_2$ (■), $SrCl_2$ (●), $MgCl_2$ (▲), or $BaCl_2$ (superimposable with the data for Mg^{2+}). The ionic strength, I, was adjusted to 0.10 for all data points (except [M^{2+}] = 100 mM) by addition of KCl. At various time intervals 10-μL aliquots were removed and diluted into 0.99 mL of a 1.0 mM solution of sAAPFna for the measurement of residual activity at 25 °C. Log of % activity vs time plots were found to be linear (least-squares fit with a regression coefficient of 0.998 or better) for 3–4 half-lives ($t_{1/2}$), indicative of a first-order decay process under the conditions employed. The reported $t_{1/2}$'s are the averages for three independent experiments, and the error bars represent the range of values (no error bars indicate the range to be smaller than the size of the symbol). In addition, NaCl and KCl in the range of 0.10–100 mM (I not adjusted to 0.10) were also found to have an insignificant effect on the stability of the WT enzyme when compared with the results for Ca^{2+}. For the purpose of clarity, the data for NaCl and KCl are not shown. The dotted curve drawn through the data for Ca^{2+} is a theoretical titration for a single binding site {pCa = pK_a + log ([E]/[E–Ca])} with an apparent log K_a = 1.49 (upper and lower limits are 252 and 2 min). (B) $t_{1/2}$ of thermal inactivation of subtilisin P172D (▲), subtilisin P172D/G131D (●), and subtilisin D197E (□) as a function of −log [Ca^{2+}]. Wild-type subtilisin BPN′ (■) is shown for comparison. The conditions are the same as in (A). The theoretical titration curve for subtilisin P172D has an apparent log K_a = 2.02 with upper and lower limits of 242 and 2 min, respectively. The theoretical titration curve for subtilisin P172D/G131D has an apparent log K_a = 2.27 with upper and lower limits of 272 and 2 min, respectively.

coordination sphere. The formal ligands have a mean r of ~2.4 Å from the centrally bound metal, as might be expected for a bona fide Ca^{2+} binding site (Hardman et al., 1982). The deficiency in protein-derived ligands suggests a low affinity

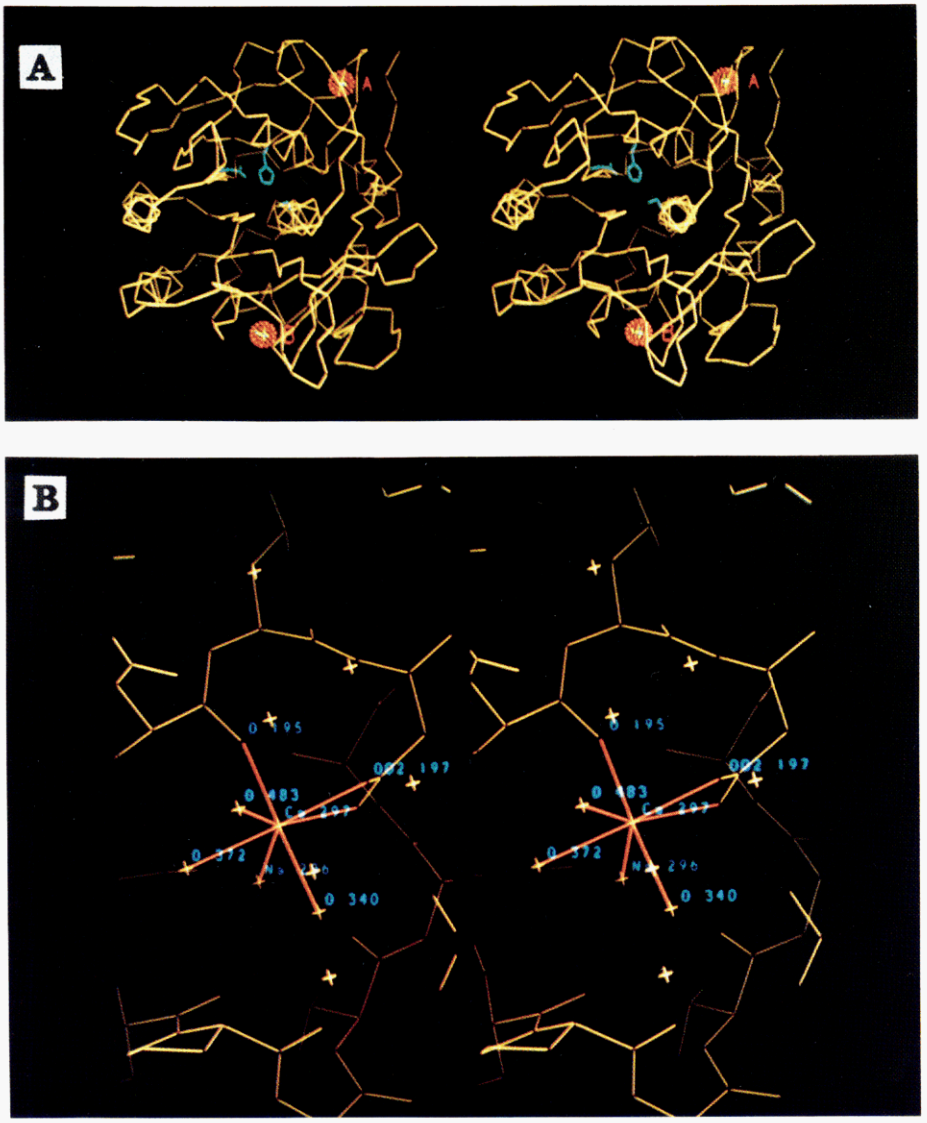

FIGURE 2: X-ray structural data for the Ca^{2+} binding sites of subtilisin BPN′. (A) C_α backbone model of wild-type subtilisin BPN′ showing the location of the two Ca^{2+} binding sites, red globes labeled Ca A and Ca B. The catalytic triad His 64, Ser 221, and Asp 32 is shown in green. (B) Structural details for the Ca B binding site. This model was obtained from an X-ray structure at 1.7-Å resolution (R = 0.14) for a crystal of subtilisin grown in the presence of 40 mM $CaCl_2$ (100 mM Tris-HCl, pH 8.7). Solvent molecules of high occupancy are shown as stars. Na 296 shows the location of a Na^+ or K^+ that is bound in the absence of added Ca^{2+}, depending on whether the crystallization buffer contains 50 mM glycine–NaOH, pH 9.0, or 50 mM glycine–KOH and 50 mM KCl, pH 9.0 (see Table I).

of this site for divalent cations.

In this regard, evidence that the bound metal ion is Ca^{2+} comes from the observation that the occupancies of the metal ion in a series of crystal structures are correlated with the [Ca^{2+}] of the solution from which the crystals were grown (Table I). When the [Ca^{2+}] is increased from 0 to 40 mM $CaCl_2$,[6] the occupancy of the Ca B site was found to increase from 4.0 electrons (interpreted as 0.5 occupied water molecules) to 18 electrons (see Table I), which was interpreted as 1.0 occupied Ca^{2+}. At 25 mM $CaCl_2$ the occupancy of this site was found to be 13.5 electrons, which was interpreted as being about 0.5 occupied Ca^{2+} and 0.5 occupied water molecules.

In the absence of added Ca^{2+} this general locus was found to bind a monovalent cation, Na^+ or K^+, when present as a counterion in the buffer solutions during crystallization (see Table I). The exact location of this bound Na^+ or K^+ ion, however, was observed to be about 2.7 Å away from the position of the bound Ca^{2+} as shown in Figure 2B. The occupancy in electrons increases from 10.8 to 18.0 when the crystallization buffer is changed from 50 mM glycine–NaOH, pH 9.0, to 50 mM glycine–KOH and 50 mM KCl, pH 9.0, with no added Ca^{2+} in each case (Table I). This is the expected result if Na^+ (10 electrons) or H_2O is replaced by the more electron dense K^+ (18 electrons) at this site.

The existence of a monovalent ion binding site near this locus was first reported by Drenth et al. (1972) in a 2.8-Å resolution X-ray structure for crystals of subtilisin Novo grown in the presence of excess Na^+, K^+, or Tl^+. The binding of Ca^{2+} and monovalent cations appears to be mutually exclusive so that when Ca^{2+} binds, the monovalent cation ion is displaced, and a water molecular appears in its place directly coordinated to the bound Ca^{2+}.

The X-ray structures for the two variant enzymes subtilisin P172D and subtilisin G131D were solved to 1.7-Å resolution (R = 0.14) after suitable crystals were obtained in 50 mM glycine–NaOH, pH 9.0, with no added $CaCl_2$. Figure 3 reveals a superposition of the X-ray structures of subtilisin

[6] Due to the nature of growing crystals using the vapor diffusion method, it is conceivable that the free [Ca^{2+}] is higher in the resulting mother liquor than in the starting buffer due to evaporation.

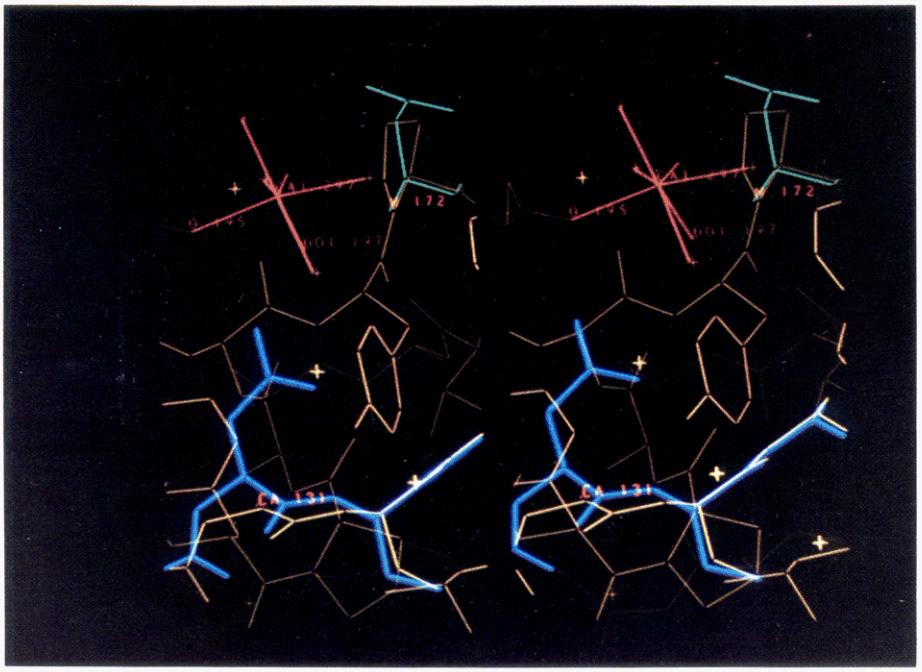

FIGURE 3: Structural details for the variant subtilisins. Superposition of the X-ray structures of subtilisin P172D (green) and subtilisin G131D (blue) on top of the wild-type-like Ca B structure (yellow) shown in Figure 2B. Only atoms with significant rms difference are shown for clarity. These two variant structures were also solved to 1.7-Å resolution and refined to $R = 0.14$. The crystallization conditions for both mutant forms were 50 mM glycine–NaOH, pH 9.0, with no added $CaCl_2$.

P172D and subtilisin G131D on the wild-type-like Ca B site. Only atoms with significant rms difference are shown for clarity.

Functional Characterization of Variant Subtilisins. The Ca^{2+} dependence of the $t_{1/2}$ for thermal inactivation of subtilisin P172D is clearly shifted toward lower Ca^{2+} ion concentrations (Figure 1B). The theoretical titration curve that best approximates the data is one for a single site with an apparent log $K_a = 2.02$.

A similar result was found for the G131D change, but the apparent increase in Ca^{2+} affinity was not as great. The data in this case were best approximated by an apparent log K_a of 1.82 (Table II). Importantly, when both changes occur together in one subtilisin molecule, the Ca^{2+} dependence on the kinetic thermal stability is shifted even further so that the data can be simulated by a theoretical titration curve of apparent log $K_a = 2.27$ (Figure 1B).

Besides the shift in pK_a there is also an increase in the upper limit of kinetic stability attainable when the site is fully saturated as shown for subtilisin P172D/G131D (Figure 1B). This upward shift to $t_{1/2} = 272$ min from 252 min is smaller, however, than one might expect, considering the enhanced electrostatic interactions that exist for the saturated form of this variant. The reason for this is due to a small decrease in the base-line stability for the P172D change at low free [Ca^{2+}] which is not apparent in Figure 1B because of the scale of this plot, but is reflected in a diminished upper limit of attainable kinetic stability.[7]

It was also possible to decrease the affinity of this site for Ca^{2+} to the point that it was effectively abolished. This was accomplished by changing the Asp residue at 197 to a much larger Glu. The stabilizing effect of $CaCl_2$ in the millimolar range does not exist for this version of subtilisin (Figure 1B).

While the introduction of carboxylate groups in the vicinity of the Ca B site was found to influence the pK_a for Ca^{2+} binding, they were found to have only minor effects on the hydrolysis of sAAPFna.[8]

Table II: Comparison of Experimental Shifts in pK_a for Ca^{2+} Binding to Subtilisin with That Expected from Simple Electrostatic Theory

variant	intercharge radius[a] r (Å)	ΔpK_a[b] (exptl at $I = 0.10$ and $T = 65$ °C)	ΔpK_a[c] (theory)
P172D	6.8	0.53 ± 0.05	0.56
G131D	13.2	0.33 ± 0.05	0.15
P172D/G131D		0.78 ± 0.05	0.71[d]

[a] The r values were measured directly from the X-ray data for these variant enzymes (Figure 3) and are the average for OD1 and OD2 to Ca^{2+} distances. [b] Observed from the data in Figure 1B. $\Delta pK_a = pK_a(\text{variant}) - pK_a(\text{WT})$ so that (+) values denote increased binding affinity for these variants. [c] The ΔpK_a was calculated from the expression $\Delta pK_a = 244 Z_1 Z_2 / r D_{eff}$, where D_{eff} was calculated by using Debye–Hückel theory. In pure water at 65 °C, $D_{eff} = D_w = 65$ (Handbook of Chemistry & Physics, 55th Edition), but in dilute electrolyte solutions the D_{eff} is understood in terms of $D_{eff} = D_w \exp(\kappa r)$, where κ is the Debye–Hückel screening parameter (Castellan, 1971). In aqueous buffer (25 °C), $\kappa = 0.33 I^{1/2}$ Å^{-1} (Cantor & Schimmel, 1980), where I = ionic strength. Thus, at $I = 0.10$, the Debye length ($1/\kappa$) is 10 Å, and the D_{eff} for an r of 6.8 Å can be calculated to be 128 at 65 °C. [d] X-ray data for subtilisin P172D/G131D was not available, but the theoretical ΔpK_a assumes additivity for the two individual changes.

Discussion

The Ca^{2+} dependence of the rate of thermal inactivation provides convincing evidence for the existence of a weak calcium binding site in subtilisin BPN′ with an apparent log

[7] If residue 172 is changed to Glu, $\Delta pK_a = 0.5$, but the upper limit of stability is increased by 16% to $t_{1/2} = 292$ min. The G131D change alone gives a 12% increase in the upper limit ($t_{1/2} = 282$; not shown). Thus we would predict that subtilisin P172E/G131D should exhibit an upper limit of stability of $t_{1/2} = 330$ min.

[8] The relative specific activities of subtilisins P172D, G131D, D197E, P172D/G131D, and wild type at pH 8.0 and 25 °C are 1.00, 1.23, 1.09, 1.29, and 1.00, respectively.

K_a of 1.49 under the conditions employed. These experiments clearly illustrate the extent to which Ca^{2+} binding at this site protects subtilisin BPN′ from thermal inactivation at elevated temperatures. This function of metal ion binding is specific for Ca^{2+} since both Mg^{2+} and Ba^{2+} are ineffective in affording kinetic stability to subtilisin BPN′ in the same concentration range. Such an observation is common for Ca^{2+} binding sites in proteins where it is not unusual to find a 10 000-fold difference in binding affinity for Ca^{2+} and Mg^{2+}. This degree of discrimination has been explained by Stuart et al. (1986) in terms of rigidity in the area of Ca^{2+} binding to α-lactalbumin where the protein is apparently unable to collapse around the smaller Mg^{2+} (Ladd ionic radius = 0.78 Å vs 1.06 Å for Ca^{2+}; Cotton & Wilkinson, 1972). Indeed, comparison of the four different X-ray structures obtained for various conditions of cation occupancy (Table I) reveals very little change in main-chain or side-chain coordinates in the vicinity of the Ca B site of subtilisin BPN′.

Structural characterization of this weak calcium binding site was only possible after crystals of subtilisin BPN′ were grown in the presence of 0.0, 25, and 40 mM $CaCl_2$. The occupancy of this site was found to be a function of the $[Ca^{2+}]$ for a series of X-ray structures obtained for crystals grown with increasing amounts of Ca^{2+} in the range 0.0–40 mM. The highest Ca^{2+} occupancy observed was 1.0 for $[Ca^{2+}] = 40$ mM.[6] These structural observations are consistent with the kinetic thermal stability data shown in Figure 1A. Together, these results provide a more complete physical–chemical explanation for the long-standing observation that added Ca^{2+} in the millimolar range causes an increase in kinetic thermal stability for the subtilisin-like proteases (Matsubara et al., 1958).

The correlation of the X-ray structural results with the Ca^{2+}-dependent kinetic thermal stability data provides an opportunity to explore the feasibility of increasing the stability of proteins through the engineering of the attractive binding forces at a metal ion binding site. Two outer-sphere changes that successfully increased the binding affinity for this calcium binding site were P172D and G131D. Individually, these two changes increased the binding affinity by $\Delta pK_a = 0.53 \pm 0.05$ and 0.33 ± 0.05, respectively (Table II). Moreover, when both changes existed together in one protein structure, they increased the ΔpK_a by 0.78 ± 0.05, nearly the amount expected for the case where both Asp's work independently and additively in influencing the electrostatic potential at this site.

Crystallographic data at 1.7-Å resolution ($R = 0.14$) for subtilisin P172D and subtilisin G131D reveal that the Asp changes are introduced into the protein structure in such a way that the main-chain protein fold is not very different from that for the WT protein. In each case the carboxylate side groups are projected out into the solvent with no protein atom neighbors within 4 Å. Further inspection of the subtilisin P172D structure reveals that there are no protein atoms between the carboxylate group of 172 and the position where the Ca^{2+} is bound. These structural results suggest that the dielectric shielding problem should be greatly minimized for analysis of the electrostatic interactions for this particular case (Gilson & Honig, 1987).

In this regard, the experimentally derived shifts in pK_a were compared with that expected from simple electrostatic theory (see Table II). The comparison is quite good for the pK_a shift due to the P172D change (Table II). This kind of agreement is consistent with the X-ray data for this variant (Figure 3), which reveals the two charged ions to be highly exposed to solvent with no protein structure intervening between them; not unlike two simple ions in solution. On the other hand, the agreement between experiment and theory for the pK_a shift due to the G131D change is not as satisfactory. This disagreement, however, is also consistent with the X-ray data for the G131D variant since there is evidence of interposed protein structure between the two charged ions (see Figure 3). Indeed, this is one of the shortcomings of using the Debye–Hückel theory for electrostatic calculations in proteins since it ignores the low dielectric constant of protein structure (vs water) and also the fact that proteins exclude electrolyte (Gilson & Honig, 1988).

This study demonstrates the ability to increase the K_a for metal ion binding to a protein through the engineering of electrostatic interactions in the vicinity of the binding site.[9] Importantly, the dramatic increase in kinetic stability that accompanies Ca^{2+} binding to this site is also shifted to lower free $[Ca^{2+}]$. The extrapolated outcome of incremental shifts in pK_a is the evolution from a weak binding site to a relatively high-affinity site ($\log K_a > 5$) where the upper limit of kinetic stability attained upon saturation is available even at very low free $[Ca^{2+}]$. Additional support for this concept, even for sites buried within the protein structure, will be described in another paper concerning the engineering of electrostatic interactions at the Ca A site of subtilisin (Pantoliano et al., manuscript in preparation).

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[9] Serpersu et al. (1987) have reported a 10-fold decrease in the K_a for Ca^{2+} binding to a weak site ($\log K_a = 3$) in staphylococcal nuclease by changing the formal ligand Asp 40 to Gly.

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