

Research Article

Engineering and Directed Evolution of a Ca^{2+} Binding Site A-Deficient AprE Mutant Reveal an Essential Contribution of the Loop Leu₇₅–Leu₈₂ to Enzyme Activity

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An *aprE* mutant from *B. subtilis* 168 lacking the connecting loop Leu₇₅–Leu₈₂ which is predicted to encode a Ca^{2+} binding site was constructed. Expression of the mutant gene (*aprE*ΔLeu₇₅–Leu₈₂) produced *B. subtilis* colonies lacking protease activity. Intrinsic fluorescence analysis revealed spectral differences between wild-type AprE and AprEΔL₇₅–L₈₂. An AprEΔL₇₅–L₈₂ variant with reestablished enzyme activity was selected by directed evolution. The novel mutations Thr₆₆Met/Gly₁₀₂Asp located in positions which are predicted to be important for catalytic activity were identified in this variant. Although these mutations restored hydrolysis, they had no effect with respect to thermal inactivation of AprEΔL₇₅–L₈₂ T₆₆M G₁₀₂D. These results support the proposal that in addition to function as a calcium binding site, the loop that connects β-sheet e3 with α-helix c plays a structural role on enzyme activity of AprE from *B. subtilis* 168.

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1. Introduction

Currently, there is a high level of commercial interest for subtilisins that work under extreme biochemical conditions [1, 2]. Therefore, understanding the structure and function of subtilisins is fundamental to employing rational and directed evolution strategies in order to enhance activity and/or change substrate specificity for these proteins [3, 4]. However, there are structural motifs in subtilisin E (AprE) which have proven to affect enzyme activity and still remain uncharacterized. For instance, crystallographic analysis revealed that residues Leu₇₅, Asn₇₇, Ile₇₉, and Val₈₁ located in the connecting loop Leu₇₅–Leu₈₂ together with Gln₂ and Asp₄₁ form a calcium binding site (CBS) in subtilisin BPN' [5]. Furthermore, it is known that residues Gly₈₃–Ser₈₅, conserved among several members of

the subtilisin family [6], form a stretch bend which lies at the C-terminal edge of the loop connecting β-sheet e3 to α-helix c. These residues are located 1.5 nm away from and on the opposite side of the catalytic residues Asp₃₂, His₆₄, and Ser₂₂₁ [7]. Despite their far location from the catalytic residues, mutations in this region induce changes on both substrate specificity and enzyme activity of subtilisins. For instance, a single Ser₈₅Ala mutation increased twice the *k_{cat}* of *B. subtilis* 168 AprE [6], and a Val₈₄Ile mutation not only increased the *K_m* of subtilisin BPN' but also adapted the enzyme to work at a lower than normal temperature [7].

Members of the subtilisin family usually possess two calcium binding sites (CBSs), named CBSA and CBSB [8]. Each CBS displays different affinity for the calcium ion [8]. In this report, evidence is presented supporting the idea that in addition to the role as a calcium binding site, the

loop connecting β -sheet e3 with α -helix c (residues Leu₇₅–Leu₈₂) also plays an important role in the enzyme activity of subtilisin E from *B. subtilis* 168.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids, and Growth Conditions. Bacterial strains used in this work are listed in Table 1. The growth medium used routinely was Luria-Bertani (LB) [9]. Preparation of competent *E. coli* and *B. subtilis* cells and their transformations were performed as previously described [10, 11].

2.2. Site-Directed Mutagenesis of *AprE*. Codons 75 through 82 from wild-type *aprE* [13, 14] were eliminated with the Altered Sites II Site-Directed Mutagenesis System Kit (Promega, Madison, WI) using the oligonucleotide 5'-GCTTGGGCTAACGCC*AGCGGCAATCGTACC-3 (asterisk denotes the location of the in-frame deletion).

2.3. Random Mutagenesis of *AprE* Δ Leu₇₅–Leu₈₂. Random mutagenesis was carried out as follows. Strain *B. subtilis* PERM570 (Table 1) was grown to an O.D._{600nm} of 0.5; the cell culture was supplemented with 2 mM H₂O₂ and incubated for 48 hours at 37°C. Cells were serially diluted, and aliquots of 100 μ L were inoculated on LB agar plates supplemented with skimmed milk. The plates were incubated 12 hours at 37°C, and colonies exhibiting caseinolytic activity were selected and transferred to a fresh plate. The plasmids of selected colonies were isolated and used to retransform *B. subtilis* 1A751 and *E. coli* DH5 α . The *aprE* Δ L₇₅–L₈₂ variant generated through this protocol was fully sequenced on both strands.

2.4. Expression and Purification of Wild-Type and *AprE* Mutants. Wild-type *aprE* and *aprE* Δ L₇₅–L₈₂ BamHI/BamHI fragments encoding the preproenzymes were cloned in plasmid pUSH2 [12] to introduce an in-frame six histidine-coding sequence at the 3' end of both *aprE* sequences. This strategy generated the strains, *E. coli* PERM223 harboring pPERM222 (pUSH2-*aprE*) and *E. coli* PERM494 harboring pPERM494 (pUSH2-*aprE* Δ L₇₅–L₈₂), respectively. Wild-type and *AprE* variants were expressed and purified from the culture media of *B. subtilis* 1A751 by metal affinity chromatography on a Ni-NTA-agarose column (Quiagen; Valencia, CA) as previously described [6]. Protein concentrations were determined by using the Coomassie (Bradford) Protein Assay Kit (Pierce; Rockford, IL).

2.5. Subtilisin Intrinsic Fluorescence (IF) Assays. Fluorescence spectra data were obtained after equilibration of a mixture containing 4 μ M of either wild-type or mutant *AprE* Δ L₇₅–L₈₂ in 10 mM Pipes pH 7.5 at 25°C in the presence or absence of 0.5 mM EGTA in a spectrofluorophotometer RF-5301PC (Shimadzu, Japan) equipped with both a thermostated cell and constant stirring. Fluorescence spectra were recorded between 280–450 nm upon exciting the protein at 280 nm.

2.6. Thermal Unfolding Followed by Intrinsic Fluorescence. Subtilisin E samples were placed into a 2 mL quartz cuvette; changes in intrinsic fluorescence were measured at 340 nm using an excitation wavelength of 280 nm (4 nm bandwidth) and emission wavelength from 300 to 400 nm (4 nm bandwidth). Temperature was ramped from 25 to 90°C with a 1°C min⁻¹. Thermal unfolding data were normalized to

$$\alpha = \frac{[y(x) - y(x')]}{[y(x = 298.16) - y(x')]} \quad (1)$$

where x' is the temperature in Kelvin where the enzyme was completely unfolded. Thermodynamic parameters were calculated by nonlinear least-squares fitting to following scheme.

Two-state model between native (N) and unfolded (U) states $N \rightarrow U$. Data were analyzed using the thermal following equation:

$$F_U = \frac{e^{-(\Delta H_m/RT + \Delta H_m/RT_m)}}{1 + e^{-(\Delta H_m/RT + \Delta H_m/RT_m)}} \quad (2)$$

where T is temperature in K, T_m is the temperature at midpoint, and ΔH_m is the enthalpy at the T_m , respectively.

2.7. Enzyme Kinetics. The synthetic peptide Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (s-AAPF-*p*-Na, Sigma Chemical Co. St. Louis, MO) was used as substrate; assays were performed in 100 mM Tris-HCl (pH 8.0) and 5 mM CaCl₂ at 37°C. The amount of *p*-nitroanilide released was measured by recording the absorbance increase at 410 nm. Enzyme activity was expressed as units/mg protein. Velocity data were fitted to the Michaelis-Menten equation by nonlinear regression.

2.8. Thermal Stability of the Enzymes. Purified wild-type or variant *AprE* enzymes (0.7 mg/mL) were incubated in 100 mM Tris-HCl (pH 8.0) and varying concentrations of both CaCl₂ (100 μ M–5 mM) and NaCl (0 or 100 mM). The wild-type and variant *AprE* were either previously treated or not with 100 μ M EDTA and then incubated on ice for 15 minutes before testing for thermal stability. The activity remaining after a given time of incubation was determined at 37°C using s-AAPF-*p*Na as the substrate. The temperatures tested for enzyme stability were between 50–65°C.

2.9. Data Analysis. Thermal inactivation kinetics for both WT and *AprE* variant were studied fitting the inactivation data to (3) by nonlinear regression and using the iterative program Microcal Origin, as described in studies of thermal enzyme inactivation [15]. The equation used was the following:

$$A_R = A_0 e^{-(k \cdot t)} \quad (3)$$

where A_R represents the (%) of residual activity at a given time (t), A_0 is the initial relative activity, considered as 100%, and k is the rate constant for enzyme inactivation in min⁻¹. Equation (3) describes a one-step process (4) for enzyme inactivation; from the native (N) to the inactive state (I):



TABLE 1: Bacterial strains used in this study.

Bacterial strain	Genotype and description	Reference or source
<i>Bacillus subtilis</i>		
168	<i>trpC2</i>	W. Nicholson
1A751	<i>eglSΔ102 bgIT/bglSΔEV aprE nprE his</i>	BGSC*
PERM222	<i>B. subtilis</i> 1A751 containing pPERM222 (1.2 kbp PCR fragment containing wild-type <i>aprE</i> ORF subcloned in pUSH2)	This study
PERM570	<i>B. subtilis</i> 1A751 <i>eglSΔ102 bgIT/bglSΔEV aprE nprE his mutM:Tet^r, mutY:Sp^r, sodA:Cm^r</i> containing pPERM494 (1.2 kbp <i>Bam</i> HI fragment from <i>aprEΔL75-L82</i> cloned in PUSH2)	This study
PERM505	<i>B. subtilis</i> 1A751 containing pPERM494 (1.2 kbp <i>Bam</i> HI fragment from <i>aprEΔL75-L82</i> cloned in PUSH2)	This study
PERM658	<i>B. subtilis</i> 1A751 containing pPERM669 (1.2 kbp <i>Bam</i> HI fragment from <i>aprEΔL75-L82 T66M G102D</i> cloned in PUSH2)	This study
PERM200	<i>B. subtilis</i> 1A751 containing pUSH2 (<i>E. coli-B. subtilis</i> shuttle vector for C-terminal His ₍₆₎ -tagging Cm ^r , Kan ^r [12])	This study

*BGSC: *Bacillus* Genetic Stock Center.

3. Results and Discussion

Mutations in the stretch bend Gly₈₃-Ser₈₅ lying at the C-terminal edge of the loop connecting the β -sheet e3 with the α -helix c of AprE led to changes on both substrate specificity and enzyme activity of subtilisins [6, 7]. These findings strongly suggest that this region has an important structural role for enzyme activity in AprE. Therefore, this loop was eliminated by site-directed mutagenesis, and the resulting *aprEΔL75-L82* mutant gene (Figure 1) was expressed in *B. subtilis* 1A751, a strain lacking protease activity as determined on casein plates (Figure 1). In fact, the cell free culture medium of this strain possessed no activity against hide powder azure and only 3% of the activity showed by the strain expressing the wild-type *aprE* gene against azocasein (Results not shown). A version of subtilisin BPN' lacking the CBSA and containing stabilizing mutations has been previously produced [16, 17]. Refolding of this protein was greatly facilitated by the absence of the Ca-loop while retaining high levels of activity [17]. However, as described here in the absence of stabilizing mutations deletion of the CBSA on AprE resulted in a dramatic loss of enzyme activity. Therefore, the loop L₇₅-L₈₂ may be important for structural integrity of not only the binding site but also the active site.

Changes in intrinsic fluorescence are excellent for monitoring the polarity of Trp environment and hence are sensitive to protein conformation [18, 19]. Therefore, the emission fluorescence spectra of AprE and AprE Δ L₇₅-L₈₂ were recorded with excitation at 280 nm. The AprE Δ L₇₅-L₈₂ spectrum showed an emission maximum of \sim 358 nm which was red-shifted by 14 nm relative to the peak of the wild-type AprE spectrum (Figure 2(a)). These data suggest that the side chains of the aromatic residues are more exposed

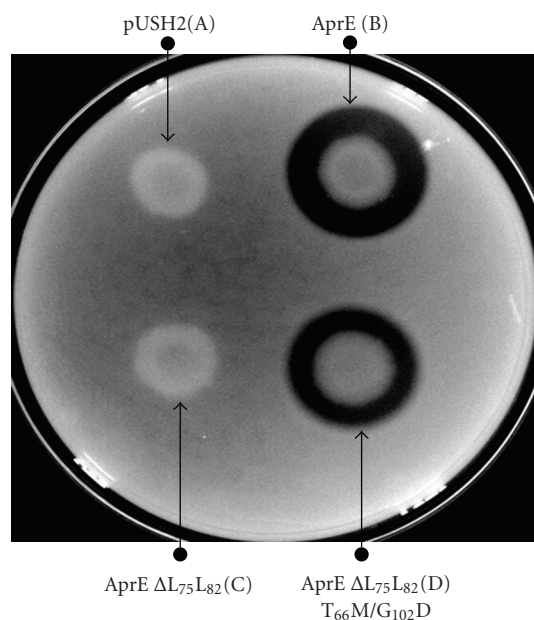


FIGURE 1: Extracellular proteolytic activity of strains *B. subtilis* PERM200 (A), PERM222 (B), PERM505 (C), and PERM658 (D). 10 μ L aliquots were taken from cell cultures (O.D._{600nm} of \sim 0.5) and deposited on LB agar plates containing 2% (w/v) skimmed milk. The plates were incubated overnight at 37°C to allow the developing of halos of hydrolysis.

to the solvent in AprE Δ L₇₅-L₈₂. Moreover, as shown in Figure 2(a), the peak emission intensity of AprE Δ L₇₅-L₈₂ is \sim 1.9-fold higher compared to that of wild-type AprE. A comparative amino acid sequence analysis reveals that AprE

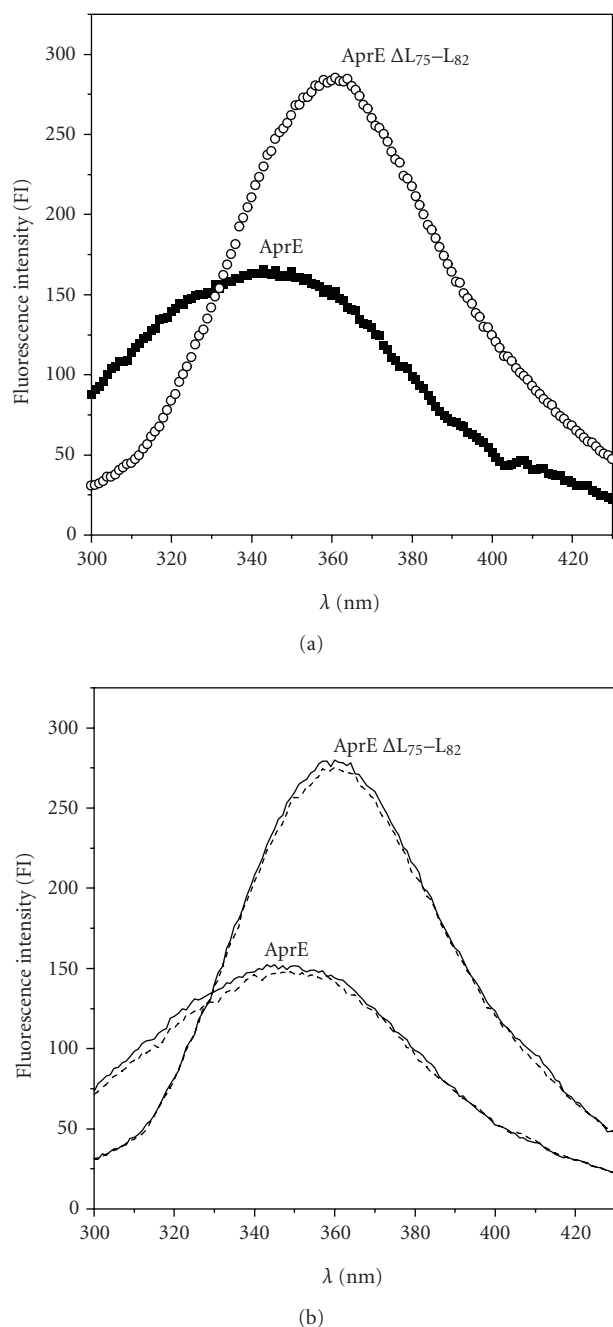


FIGURE 2: (a) Emission spectra of wild-type AprE and AprE Δ L₇₅-L₈₂. (Solid squares) wild-type AprE, (Open circles) AprE Δ L₇₅-L₈₂. (b) Emission spectra of wild-type AprE and AprE Δ L₇₅-L₈₂ in the presence of 0.5 mM EGTA. (Continuous line) native state; (Dashed line) 0.5 mM EGTA. Assays were performed on 10 mM Pipes, pH 7.5 buffer at room temperature (25°C) using a 1 cm cuvette of 1.5 mL with continuous shaking.

and subtilisin BPN' share 86% similarity; in fact the three tryptophan residues existing in mature subtilisin BPN' (i.e., Trp₁₀₆, Trp₁₁₃, and Trp₂₄₁) are present in equivalent positions in AprE (i.e., Trp residues 105, 112, and 240, resp.) [5, 20, 21]. On the other hand, a previous study suggested that in subtilisin BPN', Trp₁₁₃, is virtually nonfluorescent; the largely

exposed Trp₂₄₁ contributes 20% of the fluorescence, whereas the partially exposed Trp₁₀₆ accounts for the majority of the emission [22]. Therefore, the increased fluorescence intensity observed in AprE Δ L₇₅-L₈₂ could be attributed to perturbations in the local environment of residues Trp₁₀₅ and/or Trp₁₁₂ which are located near to the deleted loop L₇₅-L₈₂.

As noted above, deletion of the loop 75–82 abolished the calcium binding potential at site A while leaving intact the calcium binding site B. To further investigate this notion, wild-type and mutant AprE proteins were incubated in the presence of 0.5 mM EGTA, a concentration enough to chelate Ca²⁺ only from the CBSB [23]. As shown in Figure 2(b), elimination of Ca²⁺ from CBSB induced in both enzymes a small decrease in their fluorescence intensity with respect to the nontreated native enzymes (Figure 2(b)). These results are in agreement with the presence of an intact CBSB in both the wild-type and the AprE Δ L₇₅-L₈₂ enzymes.

The structural consequences of loop L₇₅-L₈₂ removal from AprE resulted in the lost not only of the CBSA but also of enzyme activity. Therefore, a directed evolution strategy was used to search for amino acid substitutions in the mutant enzyme that could restore enzyme activity. A plasmid containing *aprE* Δ L₇₅-L₈₂ was expressed in a hypermutagenic strain of *B. subtilis* deficient on the *mutM* *mutY* and *sodA* genes that also lacked protease activity as described above. After several rounds of mutagenesis for *aprE* Δ L₇₅-L₈₂, three colonies exhibiting extracellular protease activity against casein were recovered. The colony with the highest protease activity was selected to further characterize its phenotype; the clone was called *aprE* Δ L₇₅-L₈₂ Var1. Interestingly, the cell free culture medium of this strain recovered 27% and 65% of the activity exhibited by the strain expressing the wild-type *aprE* gene against hide powder azure and azocasein, respectively (Results not shown).

Analysis of the nucleotide sequence of *aprE* Δ L₇₅-L₈₂ Var1 revealed the existence of two nonsense mutations that resulted in amino acid substitutions, Thr₆₆Met and Gly₁₀₂Asp. The mutant gene named *aprE* Δ L₇₅-L₈₂T₆₆M G₁₀₂D was cloned in pUSH2, and the resulting construction was expressed in the protease deficient strain *B. subtilis* IA751 (Figure 1). Calculation of kinetic constants *k*_{cat} and *K*_m from initial rate measurements of hydrolysis of s-AAPF-*p*Na revealed that the relative catalytic efficiency of AprE Δ L₇₅-L₈₂-T₆₆M G₁₀₂D was of around 7.4% as compared with the wild-type AprE enzyme (Table 2).

In order to understand the effect of these mutations in the structure of the AprE Δ L₇₅-L₈₂ T₆₆M G₁₀₂D enzyme, the medium temperature of denaturation (*T*_m) was calculated for the three enzymes. Results showed that the *T*_m value of AprE Δ L₇₅-L₈₂ was around four degrees higher than that of the wild-type protein (Figure 3), indicative of a more stable enzyme. Interestingly, the *T*_m value of the AprE Δ L₇₅-L₈₂ T₆₆M G₁₀₂D mutant was between the *T*_m values of the AprE Δ L₇₅-L₈₂ and wild-type enzymes (Figure 3). These results suggest that the stabilities of the two variants are essentially the same.

The CBSA absence and compensatory mutations on the activity of AprE Δ L₇₅-L₈₂ T₆₆M G₁₀₂D were determined.

TABLE 2: Kinetic parameters of AprE and AprE Δ L_{75–L82} T₆₆M G₁₀₂D during hydrolysis of *s*-AAPF-*p*Na. Reactions were carried out in 100 mM Tris-HCl, pH 8.0, 5 mM CaCl₂, at 37°C, using as substrate *s*-AAPF-*p*Na. Values are triplicate determinations in two separate experiments \pm SD.

Enzyme	K_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	% of relative activity
WT AprE	21.8 \pm 1.4	1.7 \pm 0.2	12.9 \pm 0.7	100
AprE Δ L _{75–L82} T ₆₆ M G ₁₀₂ D	2.6 \pm 0.4	2.7 \pm 0.3	0.96 \pm 0.6	7.4

TABLE 3: Thermal inactivation parameters (k_i and $t_{1/2}^*$) of wild-type AprE and AprE Δ L_{75–L82} T₆₆M G₁₀₂D (Var1).

Assay condition	WT k_i (10 ⁻⁴ min ⁻¹)	WT $t_{1/2}$ (min)	Var1 k_i (10 ⁻⁴ min ⁻¹)	Var1 $t_{1/2}$ (min)
5 mM Ca ²⁺ , 50°C	8 \pm 2	856	51 \pm 4	135
0.1 mM Ca ²⁺ , 100 mM NaCl, 50°C	130 \pm 16	52	2220 \pm 141	3.1
0.1 mM EDTA, 100 mM NaCl, 50°C	2700 \pm 170	2.6	3255 \pm 32	2.1
5 mM Ca ²⁺ , 65°C	490 \pm 100	14	4550 \pm 9	1.5

* $t_{1/2}$ were obtained considering an $A_R = 50\%$ and using (3) (Materials and Methods). Enzymes were dissolved in 100 mM Tris-HCl, pH 8.0. Values are triplicate determinations in two separate experiments \pm SD.

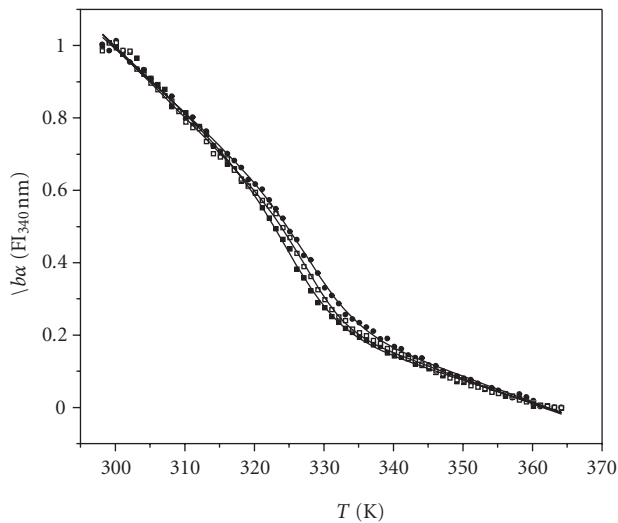


FIGURE 3: Thermal unfolding of subtilisin E. Fluorescence intensity unfolding data for wild-type AprE (■), AprE Δ L_{75–L82} (●), and AprE Δ L_{75–L82} T₆₆M G₁₀₂D (□). The data were obtained with a heating rate of 1 K min⁻¹. Solid lines represent the best fit of unfolding data with $\Delta H_m = 233.3 \pm 18.2$ kJ mol⁻¹ and $T_m = 326.9 \pm 0.4$ K, $\Delta H_m = 237.3 \pm 21.1$ kJ mol⁻¹ and $T_m = 329.8 \pm 0.5$ K, and $\Delta H_m = 304.5 \pm 29.2$ kJ mol⁻¹ and $T_m = 329.3 \pm 0.4$, respectively.

To this end, AprE and AprE Δ L_{75–L82} T₆₆M G₁₀₂D were incubated with 0.1 mM Ca²⁺ and 100 mM Na⁺, respectively. Under these incubation conditions, binding sites A and B of subtilisin BPN' were saturated 95% with Ca²⁺ and Na⁺, respectively [24]. The kinetic parameters for thermal inactivation were calculated using (3) to better correlate the effect of amino acid residues substitutions (Thre₆₆Met and Gly₁₀₂Asp) on the calcium dependent stability of AprE Δ L_{75–L82}. Table 3 shows that at 50°C and Ca²⁺ saturation, the wild-type AprE enzyme had a $t_{1/2}$ (half life) of 856 minutes. This value is six times higher than that of the AprE Δ L_{75–L82}

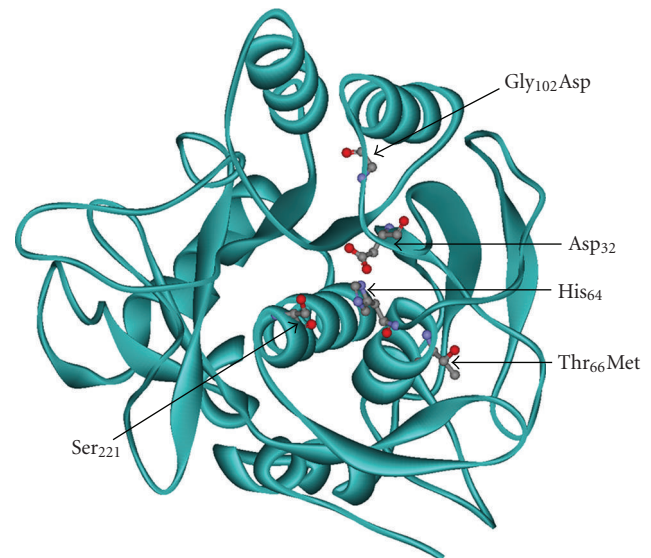


FIGURE 4: Ribbon diagram of the crystal structure of native subtilisin BPN (from coordinates obtained from [21]), drawn using the program Discovery Studio (<http://www.accelerys.com/>). Relative locations of the catalytic residues and mutations are indicated.

T₆₆M G₁₀₂D mutant. At 65°C and Ca²⁺ saturation the half life of AprE was 9 times higher than that of AprE Δ L_{75–L82} T₆₆M G₁₀₂D (Table 3). On the other hand, in the presence of 0.1 mM Ca²⁺, the $t_{1/2}$ for the wild-type enzyme was around 17 times higher than that of the mutant enzyme. However, in the presence of 0.1 mM EDTA, that is, in the absence of calcium, both enzymes showed a similar inactivation rate (Table 3). Therefore amino acid residues substitutions (Thre₆₆Met and Gly₁₀₂Asp) led to recover of enzyme activity but had no effect with respect to thermal inactivation of AprE Δ L_{75–L82}.

The three-dimensional structure of AprE has not been determined but it has been reported for subtilisin BPN' [21]. In fact, as noted above both proteins share 86% identity; therefore their three-dimensional structures are likely to be similar. Thus, the structural analysis using subtilisin BPN' as a model [21] revealed that the mutation Thr₆₆Met was found to be in close contact with the active site of the enzyme, in particular interacting with His₆₄ which acts as a general-base catalyst to activate the γ -OH group of the nucleophile Ser₂₂₁. On the other hand, the mutation Gly₁₀₂Asp was found to occur in the substrate binding subsite S₄ of AprE (Figure 4).

The bulky and nonpolar functional side group of Met suggests that the microenvironment in the active site of the AprE Δ L₇₅-L₈₂ mutant was disturbed as a consequence of a polarity change. This alteration may impair the nonpolar residues present in the substrate s-AAPF-pNa (i.e., Phe) that enter in contact with the catalytic residues. Thus, substitution of Thr₆₆Met possibly had a positive effect in reestablishing the core environment (polarity) in the active site of AprE Δ L₇₅-L₈₂. Mutations directed to this region might be useful in identifying amino acid substitutions that reestablish the full activity to AprE Δ L₇₅-L₈₂. On the other hand, it has been reported that substitutions of the residues Gly₁₀₂Phe and Ser₁₂₈Phe in savinase, a subtilisin ortholog, blocked the entrance of aromatic residues into the active site pocket, eliminating thus the preference for these residues [25]. Therefore, the introduction of a polar and bulky residue like Asp in position 102 of AprE Δ L₇₅-L₈₂ may anticipate an important structural change in the affinity for the substrate.

Overall, the results of the structural and biochemical analysis of the wild-type, AprE Δ L₇₅-L₈₂ and AprE Δ L₇₅-L₈₂ T₆₆M G₁₀₂D proteins, strongly suggest that the local perturbation induced by deletion of the loop L₇₅-L₈₂ were partially compensated by the substitutions T₆₆M G₁₀₂D which are located in close vicinity with the catalytic triad of AprE. Therefore, the results described in this work strongly support the idea that in addition to function as a Ca²⁺ binding domain, the loopL₇₅-L₈₂ has an important structural role in the enzyme activity of AprE.

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