Research Article

Engineering and Directed Evolution of a Ca\textsuperscript{2+} Binding Site A-Deficient AprE Mutant Reveal an Essential Contribution of the Loop Leu\textsubscript{75}–Leu\textsubscript{82} to Enzyme Activity

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An aprE mutant from B. subtilis 168 lacking the connecting loop Leu\textsubscript{75}–Leu\textsubscript{82} which is predicted to encode a Ca\textsuperscript{2+} binding site was constructed. Expression of the mutant gene (aprE\textsubscript{Δ}Leu\textsubscript{75–Leu\textsubscript{82}}) produced B. subtilis colonies lacking protease activity. Intrinsic fluorescence analysis revealed spectral differences between wild-type AprE and AprE\textsubscript{Δ}L75–L82. An AprE\textsubscript{Δ}L75–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 AprE\textsubscript{Δ}L75–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 Leu\textsubscript{75}, Asn\textsubscript{77}, Ile\textsubscript{79}, and Val\textsubscript{81} located in the connecting loop Leu\textsubscript{75}–Leu\textsubscript{82} together with Glu\textsubscript{2} and Asp\textsubscript{41} form a calcium binding site (CBS) in subtilisin BPN’ [5]. Furthermore, it is known that residues Gly\textsubscript{83}–Ser\textsubscript{85}, 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 Asp\textsubscript{32}, His\textsubscript{64}, and Ser\textsubscript{221} [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 Ser\textsubscript{85}Ala mutation increased twice the $k_{cat}$ of B. subtilis 168 AprE [6], and a Val\textsubscript{84}Ile 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*AGCGCCAATCGTACC-3′ (asterisk denotes the location of the in-frame deletion).

2.3. Random Mutagenesis of AprEΔL75–L82. Random mutagenesis was carried out as follows. Strain *B. subtilis* PERM570 (Table 1) was grown to an O.D.600 nm 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 μl were inoculated on LB agar plates supplemented with skimmed milk. The plates were incubated 12 hours at 37°C, and colonies exhibiting cascinolytic 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* DH5α. The aprEΔL75–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 aprEΔL75–L82 BamHI/BamHI fragments encoding the preproenzymes were cloned in plasmid pUSH2 [12] to introduce an in-frame six histidine-encoding 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-aprEΔL75–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 μM of either wild-type or mutant AprEΔL75–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

\[ a = \frac{[y(x) - y(x')] - [y(x = 298.16) - y(x')]^{-1}}{y(x = 298.16) - y(x')} \]

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 was analyzed using the thermal following equation:

\[ F_U = \frac{e^{-(\Delta H_m/R+\Delta H_m/R_T)}}{1+e^{-(\Delta H_m/R+\Delta H_m/R_T)}}, \]

where \( T \) is temperature in K, \( T_m \) is the temperature at midpoint, and \( \Delta H_m \) is the enthalpy at the \( T_m \), respectively.

2.7. Enzyme Kinetics. The synthetic peptide Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (sAAPF-pNA, 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 μM–5 mM) and NaCl (0 or 100 mM). The wild-type and variant AprE were either previously treated or not with 100 μM 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 sAAPF-pNA 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:

\[ A_R = A_0 e^{-(k \cdot t)}, \]

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):

\[ N \xrightarrow{k} I. \]
Table 1: Bacterial strains used in this study.

| Bacterial strain | Genotype and description | Reference or source |
|------------------|--------------------------|---------------------|
| Bacillus subtilis |                         |                     |
| 168              | trpC2                    | W. Nicholson        |
| 1A751            | eglSΔ102 bgIT/bgSΔEV aprE nprE his | BGSC*               |
| PERM222          | B. subtilis 1A751 containing pPERM222 (1.2 kbp PCR fragment containing wild-type aprE ORF subcloned in pUSH2) | This study          |
| PERM570          | B. subtilis 1A751 eglSΔ102 bgIT/bgSΔEV aprE nprE his mutT, mutY, sodA-Cm' containing pPERM494 (1.2 kbp BamHI fragment from aprEΔL75–L82 cloned in PUSH2) | This study          |
| PERM505          | B. subtilis 1A751 containing pPERM494 (1.2 kbp BamHI fragment from aprEΔL75–L82 cloned in PUSH2) | This study          |
| PERM658          | B. subtilis 1A751 containing pPERM669 (1.2 kbp BamHI fragment from aprEΔL75–L82 T66M G102D cloned in PUSH2) | This study          |
| PERM200          | B. subtilis 1A751 containing pUSH2 (E. coli-B. subtilis shuttle vector for C-terminal His6-tagging Cm', Kan' [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 AprEΔL75–L82 were recorded with excitation at 280 nm. The AprEΔL75–L82 spectrum showed an emission maximum of ∼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 to the solvent in AprEΔL75–L82. Moreover, as shown in Figure 2(a), the peak emission intensity of AprEΔL75–L82 is ~1.9-fold higher compared to that of wild-type AprE. A comparative amino acid sequence analysis reveals that AprE
Emission spectra of wild-type AprE and AprEΔ 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 (Solid squares) wild-type AprE, (Open circles) AprEΔ 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 AprEΔL75–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 AprEΔL75–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 aprEΔL75–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 aprEΔL75–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 aprEΔL75–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 aprEΔL75–L82 Var1 revealed the existence of two nonsense mutations that resulted in amino acid substitutions, Thr66Met and Gly102Asp. The mutant gene named aprEΔL75–L82 T66MG102D was cloned in pUSH2, and the resulting construction was expressed in the protease deficient strain B. subtilis IA751 (Figure 1). Calculation of kinetic constants kcat and Km from initial rate measurements of hydrolysis of s-AApF-pNa revealed that the relative catalytic efficiency of AprEΔL75–L82 T66MG102D 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 AprEΔL75–L82 T66MG102D enzyme, the medium temperature of denaturation (Tm) was calculated for the three enzymes. Results showed that the Tm value of AprEΔL75–L82 was around four degrees higher than that of the wild-type protein (Figure 3), indicative of a more stable enzyme. Interestingly, the Tm value of the AprEΔL75–L82 T66MG102D mutant was between the Tm values of the AprEΔL75–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 AprEΔL75–L82 T66MG102D were determined.

**Figure 2:** (a) Emission spectra of wild-type AprE and AprEΔL75–L82. (Solid squares) wild-type AprE, (Open circles) AprEΔL75–L82. (b) Emission spectra of wild-type AprE and AprEΔL75–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.
Table 2: Kinetic parameters of AprE and AprEL75–L82 T66MG102D during hydrolysis of s-AAPF-pNa. Reactions were carried out in 100 mM Tris-HCl, pH 8.0, 5 mM CaCl2, at 37°C, using as substrate s-AAPF-pNa. Values are triplicate determinations in two separate experiments ± SD.

| Enzyme                  | $K_{cat}$ (s$^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$) | % of relative activity |
|-------------------------|----------------------|------------|-----------------------------------|------------------------|
| WT AprE                 | 21.8 ± 1.4           | 1.7 ± 0.2  | 12.9 ± 0.7                        | 100                    |
| AprEΔL75–L82 T66MG102D  | 2.6 ± 0.4            | 2.7 ± 0.3  | 0.96 ± 0.6                        | 7.4                    |

Table 3: Thermal inactivation parameters ($k_i$ and $t_{1/2}$) of wild-type AprE and AprEΔL75–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 ± 2                           | 856               | 51 ± 4                            | 135                 |
| 0.1 mM Ca$^{2+}$, 100 mM NaCl, 50°C | 130 ± 16                       | 52                | 2220 ± 141                        | 135                 |
| 0.1 mM EDTA, 100 mM NaCl, 50°C | 2700 ± 170                     | 2.6               | 3255 ± 32                         | 135                 |
| 5 mM Ca$^{2+}$, 65°C | 490 ± 100                      | 14                | 4550 ± 9                          | 135                 |

$t_{1/2}$ 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 ± SD.

To this end, AprE and AprEΔL75–L82 T66M G102D were incubated with 0.1 mM Ca$^{2+}$ and 100 mM Na$^+$, respectively. Under these incubation conditions, binding sites A and B of subtilisin BPN’ were saturated 95% with Ca$^{2+}$ and Na$^+$, respectively [24]. The kinetic parameters for thermal inactivation were calculated using (3) to better correlate the effect of amino acid residues substitutions (Thr66Met and Gly102Asp) on the calcium dependent stability of AprEΔL75–L82. Table 3 shows that at 50°C and Ca$^{2+}$ 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 AprEΔL75–L82 T66M G102D mutant. At 65°C and Ca$^{2+}$ saturation the half life of AprE was 9 times higher than that of AprEΔL75–L82 T66M G102D (Table 3). On the other hand, in the presence of 0.1 mM Ca$^{2+}$, 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 (Thr66Met and Gly102Asp) led to recover of enzyme activity but had no effect with respect to thermal inactivation of AprEΔL75–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 Thr96Met was found to be in close contact with the active site of the enzyme, in particular interacting with His96 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 AprEΔL75–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 Thr96Met possibly had a positive effect in reestablishing the core environment (polarity) in the active site of AprEΔL75–L82. Mutations directed to this region might be useful in identifying amino acid substitutions that reestablish the full activity to AprEΔL75–L82. On the other hand, it has been reported that substitutions of the residues Gly102Phe and Ser128Phe in savinase, a subtilisin ortholog, partially compensated by the substitutions T66MG102D which are located in close vicinity with the catalytic triad of AprE. Therefore, the introduction of a polar and bulky residue like Asp in position 102 of AprEΔL75–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, AprEΔL75–L82 and AprEΔL75–L82 T66MG102D proteins, strongly suggest that the local perturbation induced by deletion of the loop L75–L82 were partially compensated by the substitutions T66MG102D 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 loop L75–L82 has an important structural role in the enzyme activity of AprE.

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