Stabilizing Mutations and Calcium-Dependent Stability of Subtilisin†

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ABSTRACT: Stability is a property of subtilisin which has proven particularly amenable to enhancement via random mutagenesis and screening, yet the effects of most stabilizing mutations are not understood in structural and energetic detail. This paper seeks to explain the longstanding observation that stabilizing mutations are usually calcium-dependent in their stabilizing effect, irrespective of their proximity to the calcium binding sites. Stabilizing mutations in subtilisin fall into one of three classes. The largest class of mutations stabilize only in the presence of excess calcium. A smaller number of mutations stabilize independently of [calcium], and a few mutations stabilize only in the presence of chelating agents, such as EDTA. This study compares the effects of mutations from each class when introduced into subtilisin BPN′ and two calcium-free versions of subtilisin. The calcium-dependent effects of mutations can be explained by considering subtilisin to be in conformational equilibrium between two structurally similar but energetically distinct states: N and N*. The equilibrium from the N* to the N state can be altered either by calcium binding to site A or by mutation. Mutations which stabilize only in the presence of calcium stabilize the N state relative to N*. Mutations which stabilize only in the presence of chelants stabilize the N* state relative to N. As a byproduct of this analysis, we have developed a hyperstable variant of subtilisin whose inactivation at high temperature in the presence of EDTA is 10^5 times slower than wild-type subtilisin.

The serine protease subtilisin BPN′ from *Bacillus amyloliquefaciens* is an important industrial enzyme and a popular system for protein engineering studies (*1*). Stability is a property of subtilisin which has proven particularly amenable to enhancement, primarily by using random mutagenesis and screening methods to identify stabilizing mutations. Although subtilisins are naturally robust, mutations which measurably increase the half-time of thermal inactivation are fairly common (*2*). Early studies with chemical mutagens found 8 stabilizing mutations in BPN′ by screening through libraries containing 1200 different single amino acid substitutions (*2*−*4*). Misincorporation induced by α-thiodeoxynucleotides identified 3 additional stabilizing mutations in BPN′ (*5*), and studies using error-prone PCR to introduce mutations in subtilisin E identified 11 stabilizing mutations (*6*). Five of the mutations in subtilisin E were previously identified as stabilizing in BPN′. The fact that several of the same mutations have been independently selected indicates that many of the stabilizing mutations which can be produced with single base substitutions have been identified. Since this represents only 30% of the total possible single amino acid substitutions, many other stabilizing single substitutions must exist. Two examples are the directed mutations Y217K,[1] Q206C, which both stabilize significantly but are not accessible by a single point mutation (*7*). Further, Miyazaki and Arnold have shown that targeting random mutagenesis to positions at which stabilizing changes were already found can identify even better amino acids at these positions (*8*).

Contributions from individual stabilizing mutations often accrue cumulatively. Thus, large increases in stability can be achieved with no radical changes in the tertiary protein structure but rather minor, independent alterations. Thus, once stabilizing single amino acid changes have been identified, building a highly stable subtilisin can be accomplished in a step-by-step manner by combining individual mutations into the same molecule. A combination of six stabilizing changes in BPN′ decreased the rate of thermal inactivation by >300-fold (*7*). A similar result was achieved in subtilisin E by performing multiple rounds of random mutagenesis−screening and molecular breeding−screening (*6*). A stable calcium-free subtilisin has also been constructed by a combination of design and random mutagenesis (*9*). A more comprehensive discussion of stabilizing mutations in subtilisin can be found in a recent review (*1*).

Despite the practical successes in increasing subtilisin stability, the effects of most stabilizing mutations are not understood in structural and energetic detail. One reason for this is that subtilisin folding is difficult to study under equilibrium conditions. Biosynthesis of subtilisin requires participation of an N-terminal prodomain (*10*), which is processed from the mature enzyme upon completion of the folding reaction. The folding rate of mature subtilisin without the prodomain occurs so slowly that it generally precludes measurement of the free energy of unfolding.

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[1] A shorthand for denoting amino acid substitutions employs the single-letter amino acid code as follows: N218S denotes the change of asparagine 218 to serine.

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The measurement of subtilisin stability most amenable to experimental determination is the rate of thermal inactivation. As discussed in the previous paper (*22*), the rate of inactivation of subtilisin at elevated temperature and low concentrations is characterized by a single-exponential decay curve whose time constant is determined predominantly by the rate of subtilisin unfolding. Thus, mutations which decrease the rate of inactivation under these conditions can be considered to have increased the activation energy of unfolding. The previous paper (*22*) has discussed the roles of the two ion binding sites in subtilisin stability. This paper seeks to explain the longstanding observation that stabilizing mutations are usually calcium-dependent in their stabilizing effect, irrespective of their proximity to the calcium binding sites. Stabilizing mutations fall into one of three classes. The largest class of mutations stabilize only in the presence of excess calcium. A smaller number of mutations stabilize independently of [calcium], and a few mutations stabilize only in the presence of calcium chelating agents. This study compares the effects of mutations from each class when introduced into subtilisin BPN′ and two calcium-free versions of subtilisin. By comparing the effects of mutations in each background, the reason for their calcium dependence becomes evident. This study has some practical implications as calcium-independent stability would be useful for many industrial applications of subtilisin. As a byproduct of this analysis, we have developed a hyperstable variant of subtilisin whose stability is independent of calcium and whose inactivation rate at high temperature in EDTA[2] is 10^5 times slower than wt subtilisin.

MATERIALS AND METHODS

Cloning and Expression. The subtilisin gene from *Bacillus amyloliquefaciens* (subtilisin BPN′) has been cloned, sequenced, and expressed at high levels from its natural promoter sequences in *Bacillus subtilis* (*11, 12*). All mutant genes were recloned into a pUB110-based expression plasmid and used to transform *B. subtilis*. The *B. subtilis* strain used as the host contains a chromosomal deletion of its subtilisin gene and therefore produces no background wild-type (wt) activity (*13*). Mutagenesis of the cloned prodomain gene was performed according to the oligonucleotide-directed in vitro mutagenesis system, version 2 (Amersham International plc). Single-strand plasmid DNA was sequenced according to Sequenase (United States Biochemical).

Protein Purification. Subtilisin BPN′ and its variants were expressed in a 1.5 L New Brunswick fermentor at a level of ~100−200 mg/L. Purification was carried out as described in the preceding paper (*22*).

Activity Assays. Assays of peptidase activity were performed by monitoring the hydrolysis of sAAPF-pNA as described by (*14*). The [subtilisin] was determined using 1 mg/mL = 1.17 at 280 nm. For S152 which has one fewer tyrosine, the 1 mg/mL at 280 nm was calculated to be 1.12 (or 0.96 × wt), based on the loss of one Tyr residue (*7*).

Kinetics of Calcium Dissociation. To measure this rate, we used the fluorescent calcium chelator Quin 2 (*15*). Quin 2 binds calcium with a K_a of 1.8×10^8 M^{-1} at pH 7.5 (*16*). The fluorescence of Quin 2 at 495 nm increases by ~6-fold when bound to calcium (*17*). Subtilisin as purified contains one calcium ion per molecule. When mixed with an excess of Quin 2, the kinetics of calcium release from the protein can be followed from the increase in fluorescence at 495 nm using a SPEX FluoroMax spectrofluorometer. The reaction is assumed to follow the pathway:

$$N(Ca) \leftrightarrow N + Ca + Quin2 \leftrightarrow Quin(Ca) + N$$

Determination of Inactivation Rates. The kinetics of inactivation were determined as follows. Subtilisin at 1 μM concentration was dispensed in aliquots of 0.5 mL into 1 mL glass test tubes and covered with Parafilm. The tubes were placed in a circulating water bath at the appropriate temperature. At each time point, a tube was removed and quickly transferred to an ice bath. A 10 μL aliquot was removed, and residual activity was assayed in 990 μL of 1 mM sAAPF-PNA, 0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, and 10 mM $CaCl_2$. The inactivation time course was followed over four half-lives.

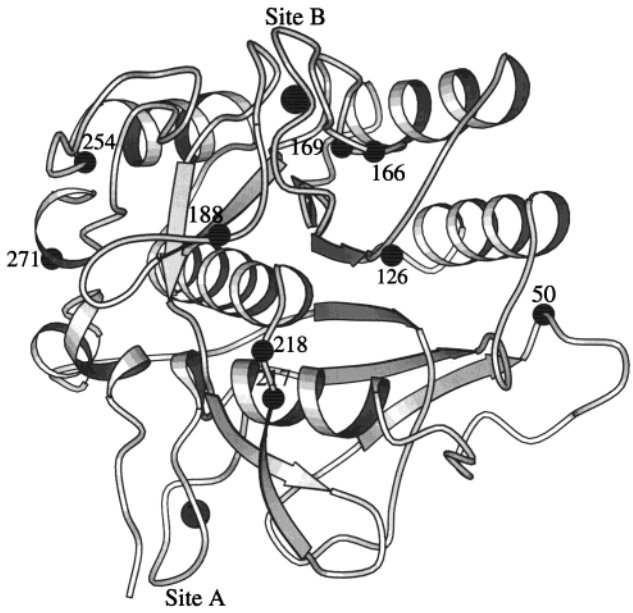

FIGURE 1: Ribbon drawing depicting the α-carbon backbone of subtilisin. Cations at sites A and B are depicted as gray spheres. The α-carbon positions of mutations sites are depicted as black spheres.

RESULTS

Calcium-Dependent Stability of Mutations in Wild-Type Subtilisin. We have chosen to analyze nine stabilizing mutations which have been identified by earlier studies (*2, 3, 7*). The positions of these mutations in the subtilisin structure are shown in Figure 1. Each of the nine were introduced as single mutations into subtilisin BPN′, and rates of inactivation of each mutant were determined in 100 μM $CaCl_2$ at 60 °C and in 100 μM EDTA at 50 °C. The invariant buffer components were 100 mM Tris-HCl, pH 8.0, 100 mM NaCl. As discussed in the previous paper (*22*), in 100 μM $CaCl_2$ and 100 mM NaCl, the calcium site A is >95% bound with calcium and site B is ~95% bound with sodium. In the presence of 100 μM EDTA, the calcium site A is <0.02% bound with calcium and site B is >98% bound with sodium.

[2] Abbreviations: EDTA, disodium salt of ethylenediaminetetraacetic acid; sAAPF-pNA, 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.

Table 1: Mutations in Subtilisin BPN′ [a]

mutation	100 μM $CaCl_2$	100 μM EDTA	ref
L126I	1.4	1.1	(*2*)
G166S	2.0	1.0	(*2*)
G169A	5.0	1.1	(*7*)
S188P	1.8	1.0	(*2*)
T254A	2.0	1.0	(*2*)
N218S	3.5	2.6	(*3*)
Y217K	3.3	2.7	(*7*)
Q271E	1.3	1.3	(*20*)
M50F	0.75	1.5	(*7*)

[a] The $t_{1/2}$ of thermal inactivation relative to wild-type subtilisin BPN′ (1.0) is shown for each mutation in $CaCl_2$ at 60 °C and in EDTA at 50 °C. The invariant buffer components were 100 mM Tris-HCl, pH 8.0, 100 mM NaCl.

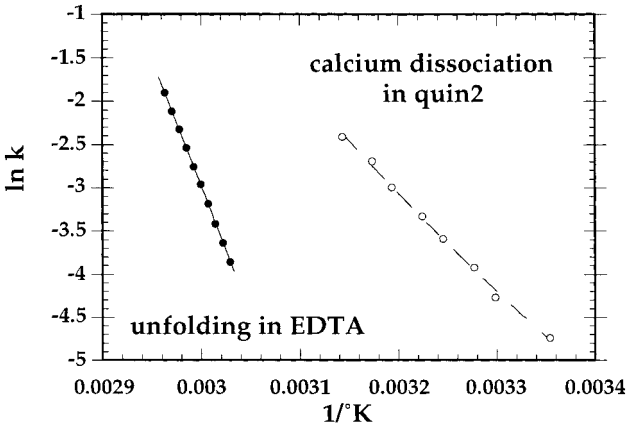

FIGURE 2: Comparison of the rates of calcium dissociation in excess fluorescent chelator (quin2) with the rate of thermal unfolding for the inactive subtilisin mutant S11 (*14*). The activation energies are 23 kcal/mol for calcium dissociation in quin2 and 60 kcal/mol for unfolding in 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM EDTA, at 45 °C. Data are plotted as the natural logarithm of the rate constants vs 1/K. Solid circles show the rate of unfolding, and open circles show the rate of calcium dissociation.

The change in $t_{1/2}$ relative to wild type was determined for each mutation in $CaCl_2$ and EDTA. The results are summarized in Table 1. The nine stabilizing mutations can be classified into one of three groups: (1) stabilizing only in calcium, (2) stabilizing in both conditions, (3) stabilizing only in EDTA. Five mutations stabilized only in the presence of excess calcium (L126I, G166S, G169A, S188P, T254A), three mutations stabilized significantly either with or without calcium (Y217K, N218S, Q271E), and M50F stabilized in EDTA but destabilized slightly in $CaCl_2$.

This partitioning of mutations suggests that the mechanism of thermal inactivation differs depending on whether site A is occupied with calcium. To understand why this is so, one must understand how the kinetics of inactivation are related to the kinetics of calcium loss in EDTA.

Inactivation in Excess EDTA. Thermal inactivation in EDTA is a two-step process as shown below:

$$N(Ca) + EDTA \leftrightarrows N + Ca{\cdot}EDTA \rightleftharpoons U \rightleftharpoons I$$

In this mechanism, only occupancy of site A is considered, since site B's occupancy with sodium in the native state is almost invariant. Figure 2 compares the rates of calcium dissociation from site A with the rate unfolding of an inactive variant (S221C) of subtilisin BPN′ (*15*). Repartitioning of calcium from site A into the EDTA-like, fluorescent chelator (Quin2) occurs at a rate of 5 h^{-1} at 45 °C. The kinetic barrier to calcium removal is 23 kcal/mol. Calcium is an integral part of the subtilisin structure, and its association or dissociation from site A requires significant but transient disruption in surrounding protein−protein interactions. This disruption in structure would explain the high activation energy and slow kinetics of calcium dissociation. For example, breaking main-chain hydrogen bonds between the N-terminal region and the 75−83 loop region would allow the relatively buried calcium a passageway out of the protein. Global unfolding of the S221C mutant in 10 mM EDTA at 45 °C is much slower than calcium dissociation, however, occurring at a rate of 0.04 h^{-1}, with an activation energy of ∼60 kcal/mol.

Because calcium binding reaches equilibrium quickly relative to the rate of unfolding, mutations which stabilize in EDTA must stabilize apo-subtilisin. Increasing the binding constant of site A would not help unless the increase in binding affinity were enormous. Consider a typical experiment in which 100 μM EDTA is added to 10 μg/mL subtilisin (0.36 μM) bound to a stoichiometric amount of calcium. The calcium will partition between subtilisin and EDTA according to the equation:

$$[SCa]/[S_{total}] = K_{S-Ca}[S]/(1 + K_{S-Ca}[S] + K_{E-Ca}[E])$$

where $[SCa]/[S_{total}]$ is the fraction of subtilisin bound to calcium, [S] ∼ total subtilisin, and [E] ∼ total EDTA. Since the binding constant of subtilisin for calcium at site A (K_{S-Ca}) $= 7 \times 10^6$ M^{-1} (*15*) and the binding constant of EDTA for calcium (K_{E-Ca}) $= 2 \times 10^8$ M^{-1}, then less than 0.02% subtilisin would be bound to calcium at equilibrium.

The fact that most mutations do not stabilize the apo-form of subtilisin suggests that there are some global structural differences between the conformations of apo-subtilisin (which we will refer to as N*) and the calcium-bound conformation (which we will refer to as N). The preferential stabilization of N* at the expense of N by the mutation F50M also implies the existence of differences in the two conformations. The two conformations must be similar in many respects, however, as evidenced by the observations that some mutations stabilize both conformations and that both apo- and calcium-bound subtilisin have very similar catalytic properties.

Other examples in the literature of mutations which preferentially stabilize apo-subtilisin are C22−C87 and C206−C216 (*18*). Ironically, a mutation which preferentially stabilizes N* relative to N will weaken calcium binding and catalyze inactivation under conditions of excess calcium and high temperature. This phenomenon is displayed in the M50F mutant, which is more stable than wild type in 100 μM EDTA but less stable in 100 μM $CaCl_2$ (Table 1).

Stabilizing Effects of Mutations in Calcium-Free Subtilisin. None of the five mutations which are calcium-dependent in their stabilizing effects directly involve binding site A. This suggests that calcium binding can be influenced by increasing the global stability of the N conformation, and not just by affecting direct interactions with calcium. To test this idea, we introduced stabilizing mutations into a version of subtilisin from which the calcium site A was deleted.

The deletion mutant was constructed as follows. The calcium binding loop of site A is formed from a nine amino

Table 2: Mutations in Δ75−83 Subtilisin[a]

mutation	100 μM $CaCl_2$	100 μM EDTA
L126I	1.0	1.1
G166S	1.1	1.0
G169A	0.9	1.1
S188P	0.9	0.9
T254A	0.8	0.8
N218S	3.5	3.5
Y217K	3.3	3.0
Q271E	1.3	1.3
M50F	1.5	1.5

[a] The $t_{1/2}$ of thermal inactivation relative to Δ75−83 subtilisin (1.0) is shown for each mutation in $CaCl_2$ and in EDTA at 50 °C. The invariant buffer components were 100 mM Tris-HCl, pH 8.0, 100 mM NaCl.

acid bubble in the last turn of the α-helix comprising amino acids 63−85, termed helix C (*19*). Deleting amino acids 75−83 created an uninterrupted helix and abolishes the calcium binding potential at site A (*15, 20*). The inactivation kinetics of Δ75−83 subtilisin in 0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl are very similar to those of wild-type subtilisin in the same conditions plus EDTA.

Each of the nine mutations was introduced into Δ75−83 subtilisin. The change in $t_{1/2}$ relative to Δ75−83 subtilisin was determined for each mutation in 100 μM $CaCl_2$ and 100 μM EDTA. The results are summarized in Table 2. The mutations M50F, Y217K, N218D, and Q271E all stabilized the Δ75−83 subtilisin in either $CaCl_2$ or EDTA. The mutations L126I, G166S, G169A, S188P, and T254A did not stabilize Δ75−83 subtilisin in either condition. We would suggest that Δ75−83 subtilisin has a conformation analogous to the N* conformation of apo-subtilisin. This suggestion is based on the observation that mutations which stabilize wild-type subtilisin in the presence of EDTA also stabilize Δ75−83 subtilisin, while those mutations that only stabilize wt subtilisin in the presence of calcium do not stabilize Δ73−83 subtilisin.

The X-ray structure of the Δ75−83 mutant has shown that except for the region of the deleted calcium binding loop, the structures of the mutant and wild-type protein are remarkably similar considering the size of the deletion. The structures of subtilisin with and without the deletion superimpose with an rms difference between 261 Cα positions of 0.17 Å. Following deletion of amino acids 75−83, the α−helix C is uninterrupted and shows normal helical geometry over its entire length (*21*). Nevertheless, regions of subtilisin which were adjacent to the 75−83 loop have lost many favorable interactions. The N-terminus of the wild-type protein lies beside the site A loop, furnishing one calcium coordination ligand, the side chain oxygen of Q2. The deletion leaves residues 1−4 disordered. In addition to the N-terminal amino acids, several regions of the mutant exhibit significant increases in *B*-factors as a result of the deletion: the 36−44 ω-loop, the 63−85 α-helix, and the 202−219 β-strand. We suggest that such changes are characteristic of the N* state.

To convert the Δ75−83 subtilisin from the N* to the N conformation, a number of compensating mutations were identified and introduced to produce a mutant denoted S88 (Q2K, S3C, P5S, K43N, A73L, Δ75−83, Q206C, N212G, K217L, N218S, and Q271E). This work is described in detail in Strausberg et al. (*9*). S88 subtilisin has about the same stability as wt subtilisin does in 0.1 M $CaCl_2$ ($t_{1/2}$ = 90 min for S88 and 60 min for wt at 65 °C). We therefore suspected that the rehabilitating mutations in S88 had shifted the equilibrium from the N* conformation to the more stable N conformation. To test this idea, we introduced the "N-specific" mutations L126I, G166S, G169A, S188P, and T254A into S88 subtilisin. Each mutant was tested in 100 μM $CaCl_2$ and 100 μM EDTA. The results are summarized in Table 3. As predicted, these mutation stabilize S88 subtilisin ($CaCl_2$ or EDTA) to about the extent they stabilized wild-type subtilisin in 100 μM $CaCl_2$. The rehabilitated S88 subtilisin behaves as if it is constitutively in the N state independent of ligand binding. When the mutations L126I, G166S, G169A, S188P, and T254A are combined in S88, a hyperstable and calcium-independent variant of subtilisin results. The combination variant inactivates ∼10^5 times slower than wt subtilisin in 100 μM EDTA, 70 °C.

Table 3: Mutations in S88 Subtilisin[a]

mutation	100 μM $CaCl_2$	100 μM EDTA
L126I	1.2	1.2
G166S	2.4	2.4
G169A	5.0	5.0
S188P	1.4	1.3
T254A	3.5	3.5

[a] The $t_{1/2}$ of thermal inactivation relative to S88 subtilisin (1.0) is shown for each mutation in $CaCl_2$ and in EDTA at 65 °C. The invariant buffer components were 100 mM Tris-HCl, pH 8.0, 100 mM NaCl.

DISCUSSION

To explain the calcium-dependent effects of mutations on stability, we suggest the subtilisin conformation can be thought of as a equilibrium between two structurally similar but energetically distinct states: N and N*. This equilibrium from the N* to the N state can be altered either by calcium binding to site A or by mutation. According to the model, mutations which stabilize only in the presence of calcium stabilize the N state relative to N*. The model raises the following question, however. If the N conformation is more stable than N* and "calcium-specific" mutations shift the equilibrium toward the N state, why do they not stabilize in EDTA? This observation can be explained by considering the following mechanism for inactivation:

$$\begin{array}{ccc} & \mathbf{K_{eq}} & \\ \mathbf{N} & \Leftrightarrow & \mathbf{N^*} \\ \Downarrow \mathbf{k_1} & & \Downarrow \mathbf{k_2} \\ \mathbf{U} & & \mathbf{U} \\ & \Downarrow > \mathbf{k_2} & \\ & \mathbf{I} & \end{array}$$

In the model, the rate of inactivation depends on the relative stabilities of N and N* and the equilibrium constant for the two states ($K_{eq} = N^*/N$). Because the inactivation rate of N* is much faster than N, the observed inactivation rate will be dominated by the fraction of subtilisin in the N* state times the inactivation rate for N*: $k_2(K_{eq}/(1 + K_{eq})$.

If the fraction of subtilisin in the N* state is large, then the inactivation rate will be insensitive to small changes in K_{eq}. For example, suppose K_{eq} = 1000 in 100 μM EDTA

and that a mutation decreases the equilibrium constant to K_{eq}(mut) = 500. The mutation would decrease the fraction of subtilisin in the N* state by <0.1% and hence barely affect the inactivation rate. In contrast, suppose that K_{eq} (100 μM calcium) = 0.001. If the same mutation were analyzed in 100 μM calcium, then K_{eq} (calcium + mutation) would be 0.0005. Thus, the fraction of subtilisin in the N* state would decrease by 2-fold, and the inactivation rate would decrease by 2-fold.

In summary, as the equilibrium is shifted more and more toward the N state, the mutations which stabilize the N state will approach their maximum effect. Shifting of the equilibrium can be done either by titrating site A with calcium or, in the case of Δ75−83 subtilisin, by introducing rehabilitating mutations. In Δ75−83 subtilisin, it was possible to find enough mutations which stabilize the N state to shift the equilibrium to the point where the N state predominates. In principle, this might be possible even for the apo-form of wt subtilisin if enough mutations were accumulated to compensate for the unoccupied oxygens in the binding loop. In practice, it is easier to remove the baggage of unoccupied ligands by deleting the loop.

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