Nonessential Active Site Residues Modulate Selenosubtilisin's Kinetic Mechanism†

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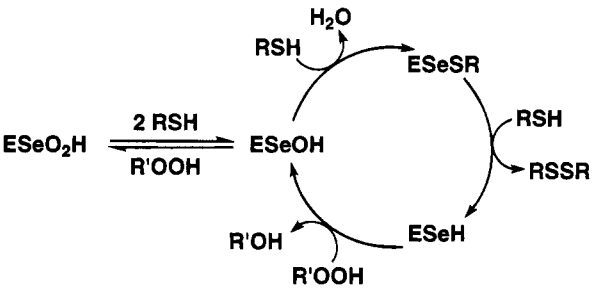
Received January 19, 1995; Revised Manuscript Received March 17, 1995®

ABSTRACT: Selenosubtilisin, a semisynthetic enzyme produced by chemical modification of subtilisin's catalytic serine, mimics the antioxidant enzyme glutathione peroxidase, catalyzing the reduction of hydroperoxides by 3-carboxy-4-nitrobenzenethiol. In analogy with the unmodified protease, selenosubtilisins derived from distantly related subtilisin templates exhibit significantly different kinetic properties. Selenosubtilisin BPN′ not only is less active than the previously studied Carlsberg selenoenzyme but exhibits sequential rather than ping-pong kinetics, indicating the formation of a ternary complex between enzyme, thiol, and peroxide prior to product release. Experiments with subtilisin E and the BPN′ Y217L variant show that the observed differences in kinetic mechanism and chemical efficiency can be attributed largely to amino acid substitutions in the enzyme's S1 and S1′ binding sites, respectively. These contributions appear to be roughly additive, and a BPN′ triple mutant (E156S/G169A/Y217L) has properties that closely approximate those of selenosubtilisin Carlsberg. The kinetic mechanism of selenosubtilisin can thus be controlled by limited mutagenesis of several active site residues not directly involved in the redox chemistry.

Chemical incorporation of prosthetic groups into existing protein binding sites is a useful method for generating novel biocatalysts (Bell & Hilvert, 1994). We recently employed this approach to prepare an artificial selenoenzyme from the serine protease subtilisin. Replacing the active site serine (Ser221) with selenocysteine radically alters the chemical behavior of the enzyme and affords it new hydrolytic and redox activities (Wu & Hilvert, 1989, 1990). For instance, selenosubtilisin mimics the natural selenoprotein glutathione peroxidase (EC 3.4.21.14) by catalyzing the reduction of hydroperoxides by thiols (Bell & Hilvert, 1993; Bell *et al.*, 1993; Wu & Hilvert, 1990). Glutathione peroxidase has been studied in some detail and is thought to be important in the prevention of lipid peroxidation *in vivo* (Flohe, 1989). Detailed characterization of selenosubtilisin's analogous activity may enhance our understanding of the natural system by illuminating how active site microenvironment influences the intrinsic reactivity of the selenium prosthetic group.

Kinetic (Bell & Hilvert, 1993; Bell *et al.*, 1993), spectroscopic (House *et al.*, 1992, 1993), and X-ray crystallographic (Syed *et al.*, 1993) studies of selenosubtilisin suggest a mechanism for the peroxidase reaction that involves interconversion of the selenolate, selenenic acid, and selenenyl sulfide oxidation states of the prosthetic group (Scheme 1). Depending on the reaction conditions, reduction of the enzymic selenenyl sulfide and/or peroxide-mediated oxidation of the resulting selenolate may be rate-limiting. Although His64, a key component of the native protease's catalytic triad, appears to facilitate the peroxidase reaction by serving as a general acid (House *et al.*, 1993), other critical active site residues have yet to be identified. Here, we report the chemical modification of several closely related natural and recombinant subtilisin variants and a comparison of their kinetic properties. These studies shed light on the effect of protein residues in the S1 and S1′ binding pockets on peroxidase activity.

Scheme 1



EXPERIMENTAL METHODS

Materials. 3-Carboxy-4-nitrobenzenethiol was prepared by reduction of the corresponding disulfide following the procedure of Silver (1979). All other chemicals were of the highest purity commercially available and were used without further purification. Subtilisins Carlsberg and BPN′ were purchased from Sigma. Subtilisin E was overproduced from plasmid pKWZ in *Bacillus subtilis* strain DB501, both the generous gift of Dr. Francis Arnold (California Institute of Technology). Mutant BPN′ enzymes were generously provided by Thomas Graycar of Genencor International. The concentration of the subtilisin isozymes was determined from the absorbance at 280 nm, assuming the following extinction coefficients: Carlsberg, 23 500 M^{-1} cm^{-1}; BPN′, 32 200 M^{-1} cm^{-1}; E, 32 400 M^{-1} cm^{-1} (Markland & Smith, 1971).

Preparation of Selenosubtilisins. Selenosubtilisins were prepared by a slight modification of the procedure of Bell *et al.* (1993). The proteases were treated with PMSF in 10 mM PIPES and 10 mM $CaCl_2$, pH 7.0, isolated by gel filtration (Sephadex G-25, eluted with 100 mM MES and 10 mM $CaCl_2$, pH 5.5), and allowed to react with excess NaHSe under argon for 2 days at pH 5.5, rather than at pH

† This research was supported in part by NSF Grant CHE-9306267.
® Abstract published in *Advance ACS Abstracts,* May 1, 1995.

0006-2960/95/0434-6616$09.00/0 © 1995 American Chemical Society

7.0. The resulting selenoproteins were purified by gel filtration on Sephadex G-25 and affinity chromatography on thiopropyl-Sepharose 6B, and oxidized with H_2O_2 to the seleninic acid oxidation state for long-term storage. The selenium content of each protein was determined by titration with 3-carboxy-4-nitrobenzenethiol ($\Delta\epsilon^{410}$ = 10 800 M^{-1} cm^{-1}, pH 5.0). Three equivalents of thiol is consumed per seleninic acid.

Peroxidase Kinetics. The selenosubtilisin-catalyzed peroxidase reaction was studied by stopped flow. Equal volumes of *tert*-butyl hydroperoxide and a mixture of enzyme plus 3-carboxy-4-nitrobenzenethiol were incubated at 25 °C and rapidly mixed, and the time-dependent absorbance decrease at 410 nm was monitored. Initial velocities were measured in triplicate and calculated from the first 5−20% of the reaction. Rates were corrected for the background reaction between *tert*-butyl hydroperoxide and thiol. The thiol concentration of each sample was calculated directly from the initial absorbance at 410 nm; stock concentrations of *tert*-butyl hydroperoxide were determined by iodometric titration. The program KaleidaGraph (Abelbeck Software) was used to fit the data obtained at fixed thiol concentrations to the equation $v_0/[E] = (k_{cat})_{app}[S]/([S] + K_{m,app})$, where v_0 is the initial velocity, and [E] and [S] are the concentrations of enzyme and peroxide, respectively. Data sets obtained at several thiol concentrations were analyzed simultaneously using an adaptation of Cleland's kinetic program for the Macintosh (J. G. Robertson, Pennsylvania State University, 1988).

RESULTS

Preparation of Selenosubtilisins. Selenosubtilisin is produced in good yield from the bacterial protease subtilisin Carlsberg via selective activation of the catalytic Ser221 with phenylmethanesulfonyl fluoride (PMSF), followed by displacement of the sulfonate with hydrogen selenide at pH 7.0 (Bell *et al.*, 1993). Attempts to modify the related enzymes subtilisin BPN′ and subtilisin E under identical conditions were unsuccessful because the displacement step is impractically slow at pH 7.0 for these proteins. However, as noted many years ago by Polgár and Bender (1967) for the analogous preparation of thiolsubtilisin from subtilisin BPN', the rate of sulfonate displacement increases with decreasing pH, and useful amounts of the desired selenoproteins could be obtained by reacting the PMSF-modified proteases with hydrogen selenide at pH 5.5 rather than 7.0. Although spontaneous hydrolysis of the enzyme-bound sulfonate is a potential complicating factor at the lower pH (Polgár & Bender, 1967), the modified protocol provided selenosubtilisins derived from subtilisin E, BPN' and two BPN' variants in 30−40% yield. All enzymes were >98% pure, as judged by SDS gel electrophoresis, following affinity chromatography on thiopropyl-Sepharose, and were stored as the seleninic acid derivative. In each case, incorporation of 1 selenium atom per protein molecule was confirmed by active site titration. Furthermore, the site-specific nature of the chemical modification was recently established for selenosubtilisin BPN′ by X-ray crystallography (M. McTigue and D. McRee, unpublished results).

Selenosubtilisin-Catalyzed Reduction of tert-Butyl Hydroperoxide by 3-Carboxy-4-nitrobenzenethiol. Like selenosubtilisin Carlsberg (Bell *et al.*, 1993), the selenoenzymes derived from subtilisins E and BPN′ catalyze the reduction of hydroperoxides by aromatic thiols. The reaction of *tert*-butyl hydroperoxide (*t*-BuOOH) with 3-carboxy-4-nitrobenzenethiol (ArSH) served as a convenient model system for detailed mechanistic studies. Saturation kinetics were observed for each of the enzymatic peroxidase reactions at all the individual concentrations of ArSH and *t*-BuOOH investigated.

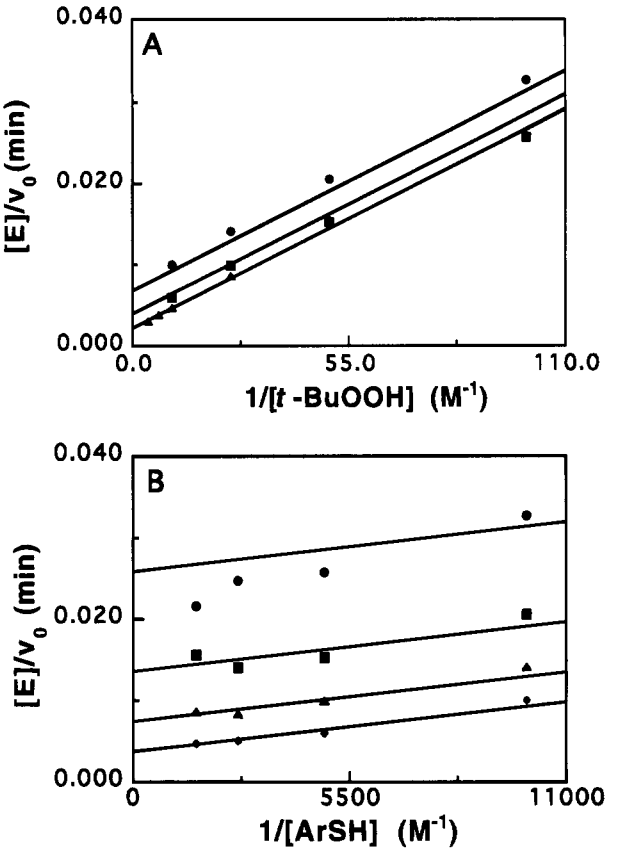


FIGURE 1: (A) Plots of $[E]/v_0$ (min) vs $1/[t\text{-BuOOH}]$ (M^{-1}) for selenosubtilisin E (5 μM) in 100 mM MES and 10 mM $CaCl_2$, pH 5.5 and 25 °C, at [ArSH] = 100 (●), 206 (■),and 625 μM (▲). [ArSH] = 375 μM was omitted for reasons of clarity. (B) Plots of $[E]/v_0$ (min) vs 1/[ArSH] (M^{-1}) for selenosubtilisin E (5 μM) in 100 mM MES and 10 mM $CaCl_2$, pH 5.5 and 25 °C, at [*t*-BuOOH] = 10 (●), 20 (■), 40 (▲), and 100 mM (♦). The data were fit to eq 1.

Table 1: Kinetic Parameters for the Peroxidase Activity of Several Selenosubtilisins[a]

selenosubtilisin	k_{max} (min^{-1})	$K_{t\text{-BuOOH}}$ (mM)	K_{ArSH} (μM)	$K_{i,t\text{-BuOOH}}$ (mM)
E	810 ± 58	199 ± 18	449 ± 61	
BPN′	958 ± 26	128 ± 7	161 ± 14	32 ± 8
Y217L	2140 ± 200	200 ± 20	1100 ± 140	12 ± 3
E156S/G169A/Y217L	2516 ± 104	348 ± 19	461 ± 28	
Carlsberg[b]	2140 ± 120	480 ± 54	130 ± 19	

[a] Reactions were carried out in 100 mM MES and 10 mM $CaCl_2$, pH 5.5, at 25 °C, and followed by stopped-flow spectroscopy as described. The kinetic parameters shown were obtained by fitting the experimental data (Figures 1−4) to either a ping-pong or a sequential mechanism as described in the text. [b] Bell *et al.* (1993).

In the case of selenosubtilisin E, double-reciprocal plots of initial velocity vs substrate concentration exhibited the characteristic parallel lines of a ping-pong mechanism, in analogy with our previous findings for the Carlsberg enzyme (Figure 1). The corresponding kinetic parameters (Table 1) were obtained by fitting the data to eq 1:

$$v/[E]_o = k_{max}[ArSH][t\text{-BuOOH}]/(K_{i\text{-BuOOH}}[ArSH] + K_{ArSH}[t\text{-BuOOH}] + [ArSH][t\text{-BuOOH}]) \quad (1)$$

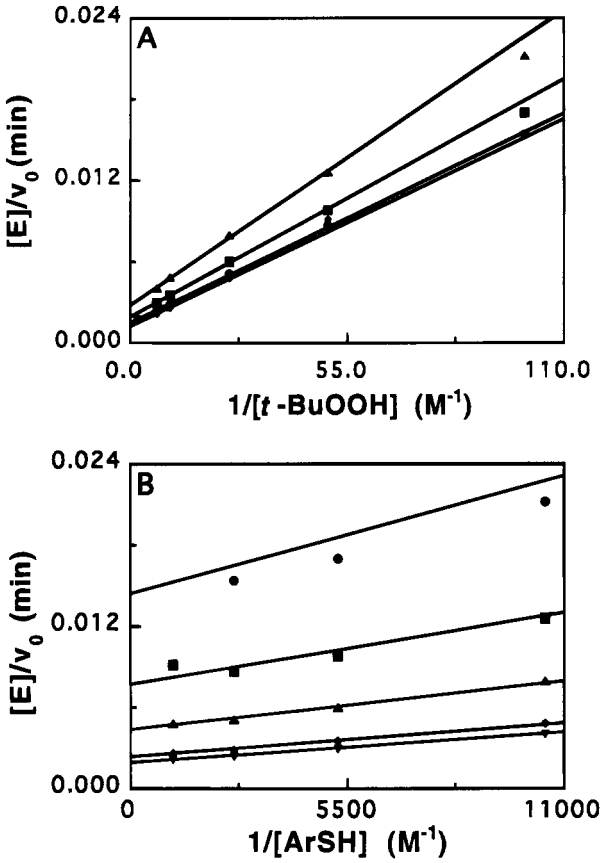


FIGURE 2: (A) Plots of $[E]/v_0$ (min) vs $1/[t\text{-BuOOH}]$ (M^{-1}) for selenosubtilisin BPN′ (5 μM) in 100 mM MES and 10 mM $CaCl_2$, pH 5.5 and 25 °C, at [ArSH] = 95 (▲), 190 (■), 380 (●), and 923 μM (♦). (B) Plots of $[E]/v_0$ (min) vs 1/[ArSH] (M^{-1}) for selenosubtilisin BPN′ (5 μM) in 100 mM MES and 10 mM $CaCl_2$, pH 5.5 and 25 °C, at [t-BuOOH] = 10 (●), 20 (■), 40 (▲), 100 (♦), and 150 mM (▼). The data were fit to eq 2.

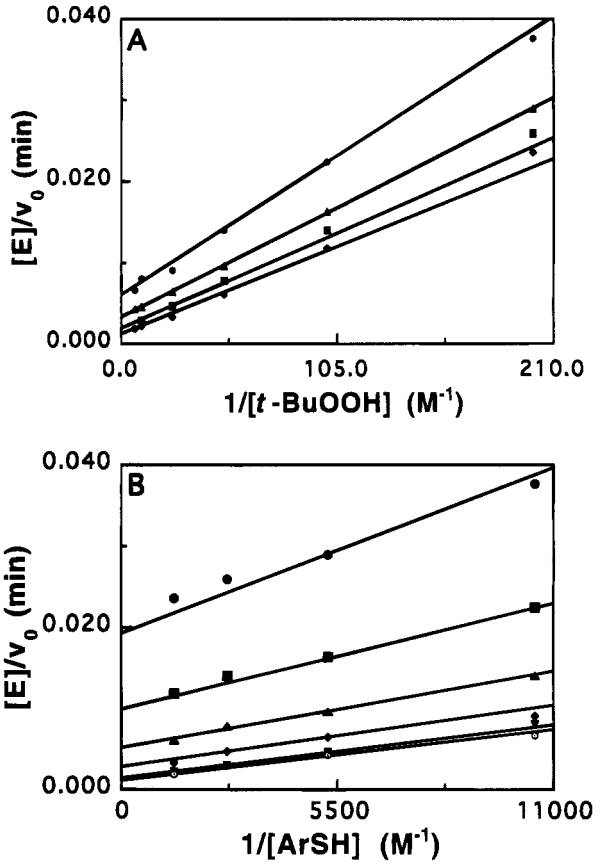


FIGURE 3: (A) Plots of $[E]/v_0$ (min) vs $1/[t\text{-BuOOH}]$ (M^{-1}) for selenosubtilisin BPN′ Y217L (5 μM) in 100 mM MES and 10 mM $CaCl_2$, pH 5.5 and 25 °C, at [ArSH] = 95 (●), 190 (▲), 370 (■), and 740 μM (♦). (B) $[E]/v_0$ (min) vs 1/[ArSH] (M^{-1}) for 5 μM selenosubtilisin BPN′ Y217L in 100 mM MES and 10 mM $CaCl_2$, pH 5.5 and 25 °C, at [t-BuOOH] = 5 (●), 10 (■), 20 (▲), 40 (♦), 100 (▼), and 150 mM (○). The data were fit to eq 2.

where k_{max} is the pseudo-first-order rate constant, and $K_{t\text{-BuOOH}}$ and K_{ArSH} are the apparent Michaelis constants for peroxide and thiol, respectively. Sequential and equilibrium-ordered models yielded poorer fits. The efficiency of selenosubtilisin E, as judged by the value for k_{max}, is about half that of selenosubtilisin Carlsberg. Because of additional variation in the Michaelis constants, the apparent second-order rate constants for reaction of the enzyme with ArSH and hydroperoxide, k_{max}/K_{ArSH} and $k_{max}/K_{t\text{-BuOOH}}$, are respectively 10-fold lower and unchanged relative to their Carlsberg counterparts.

Selenosubtilisin BPN′ also has a substantially smaller k_{max} value than the Carlsberg enzyme (Table 1). More striking is the apparent change in kinetic mechanism. As shown in Figure 2, the BPN′ data are not accommodated by a ping-pong kinetic scheme. Instead, the intersecting pattern of double-reciprocal plots fits well to a sequential model as described by eq 2:

$$v/[E]_o = k_{max}[ArSH][t\text{-BuOOH}]/(K_{i,t\text{-BuOOH}}K_{ArSH} + K_{t\text{-BuOOH}}[ArSH] + K_{ArSH}[t\text{-BuOOH}] + [ArSH][t\text{-BuOOH}]) \quad (2)$$

where $K_{i,t\text{-BuOOH}}$ is the dissociation constant for t-BuOOH, and the other kinetic parameters are defined as for eq 1. The sequential model implies the formation of a kinetically significant ternary complex between enzyme, thiol, and hydroperoxide that is not observed with selenosubtilisin Carlsberg (Cleland, 1977; Rudolph & Fromm, 1979). Furthermore, whereas the value of k_{max}/K_{ArSH} is roughly 3 times smaller than for the Carlsberg enzyme, $k_{max}/K_{t\text{-BuOOH}}$ is 1.7 times larger.

Although subtilisin Carlsberg and BPN′ differ at 83 of 275 amino acids, their respective properties are largely due to the effects of three residues located within 7 Å of the active site (Wells *et al.*, 1987). Site-directed mutagenesis has been used to produce the Carlsberg sequence in the BPN′ protein, and the specificity and catalytic efficiency of the triple mutant E156S/G169A/Y217L approximate those of the Carlsberg enzyme in hydrolytic assays with a panel of amide substrates (Wells *et al.*, 1987). To investigate whether these same residues are responsible for the distinct peroxidase activities of the corresponding selenoenzymes, two BPN′ variants, Y217L and the aforementioned triple mutant, were chemically modified. Double-reciprocal plots for the peroxidase reactions catalyzed by the resulting selenoproteins are shown in Figures 3 and 4. Kinetic parameters are summarized in Table 1.

Replacement of Tyr217 with leucine alone is insufficient to alter BPN's kinetic mechanism. As with the parent protein, the data are best fit by a sequential model (Figure 3). However, the Y217L substitution alters the enzyme's activity substantially, increasing k_{max} and both Michaelis constants (Table 1). These results contrast with those obtained with the triple mutant. As shown in Figure 4, the double-reciprocal plots acquired at several thiol concentra-

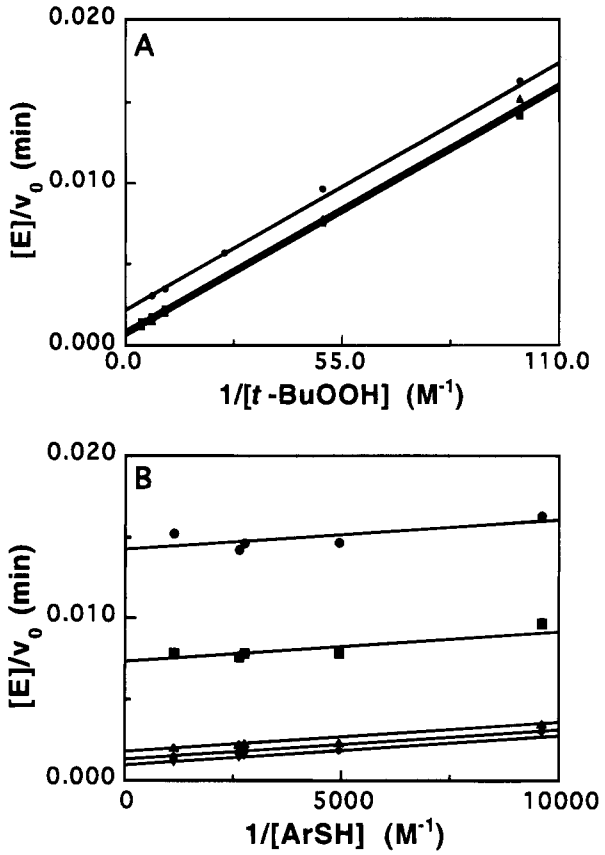


FIGURE 4: (A) Plots of $[E]/v_0$ (min) vs $1/[t\text{-BuOOH}]$ (M^{-1}) for selenosubtilisin BPN′ E156S/G169A/Y217L (5 μM) in 100 mM MES and 10 mM $CaCl_2$, pH 5.5 and 25 °C, at [ArSH] = 104 (●), 379 (■), and 882 μM (▲). (B) Plots of $[E]/v_0$ (min) vs 1/[ArSH] (M^{-1}) for selenosubtilisin BPN′ E156S, G169A, Y217L (5 μM) in 100 mM MES and 10 mM $CaCl_2$, pH 5.5 and 25 °C, at [t-BuOOH] = 10 (●), 20 (■), 100 (▲), 150 (♦), and 250 mM (▼). The data were fit to eq 1.

tions are parallel, as seen previously for the Carlsberg enzyme. The experimental data were fit to both ping-pong and sequential mechanisms, but inclusion of the additional $K_{i,t\text{-BuOOH}}K_{ArSH}$ term required by the sequential model does not improve the quality of the fit or influence the calculated values of k_{max}, K_{ArSH}, or $K_{t\text{-BuOOH}}$, which approach those obtained with selenosubtilisin Carlsberg (Table 1). Moreover, the standard error on the value of $K_{i,t\text{-BuOOH}}$ derived from the sequential model is high (3.7 ± 6.8 mM). Replacement of Glu156, Gly169, and Tyr217 in selenosubtilisin BPN′ with the corresponding Carlsberg sequence is apparently sufficient to confer the peroxidase activity and kinetic mechanism of the Carlsberg enzyme on the BPN′ template.

DISCUSSION

Subtilisins comprise a large family of structurally homologous serine proteases (Markland & Smith, 1971). Although distantly related and functionally divergent subtilisin variants can exhibit dramatically different substrate specificity and catalytic efficiency, their differences can be attributed to a relatively small number of amino acid substitutions. For example, subtilisins Carlsberg and BPN′ share only ~70% sequence identity, but the proteolytic properties of the former have been successfully grafted onto the latter by site-directed mutagenesis of only three active site residues (Wells *et al.*, 1987). The novel peroxidase activity of the analogous selenoenzymes appears equally sensitive to active site sequence.

Residues 156 and 169, located in subtilisin's S1 subsite, are two of the amino acids that influence substrate specificity. The third, residue 217, is part of the S1′ pocket. The relatively apolar nature of the S1 and S1′ sites has been assumed to account for selenosubtilisin's preference for aromatic thiols and hydrophobic alkyl hydroperoxides as substrates (Bell & Hilvert, 1993; Bell *et al.*, 1993). This inference is consistent with the crystallographically determined structure of selenosubtilisin Carlsberg (Syed *et al.*, 1993). The proximity of the side chains of Ser156, Ala169, and Leu217 to the selenium prosthetic group is illustrated in Figure 5, and although the seleninic acid depicted is believed to lie off the main catalytic cycle and must be reduced by thiol to achieve efficient turnover (Bell *et al.*, 1993; Scheme 1), thiol and/or peroxide bound at the S1 and S1′ sites should have easy access to the selenium moiety throughout the catalytic cycle.

Selenosubtilisin BPN′, which has the active site sequence Glu156, Gly169, and Tyr217 (Table 2), exhibits kinetic properties substantially different from those of the Carlsberg enzyme. In addition to a drop in activity as measured by the pseudo-first-order rate constant k_{max}, these substitutions result in an apparent switch in kinetic mechanism from ping-pong to sequential. Although initial rate patterns provide only qualitative information about chemical mechanism, the sequential model requires formation of a ternary complex between enzyme, thiol, and peroxide prior to product release (Cleland, 1977; Rudolph & Fromm, 1979). Such a model is not necessarily incompatible with Scheme 1; it is conceivable, for example, that both thiol and peroxide must bind to the selenenyl sulfide intermediate if the selenolate, which exists in an unfavorable equilibrium with ESeSAr, is to be effectively trapped by t-BuOOH rather than ArSSAr. Ternary complexes are not detected with selenosubtilisin Carlsberg, possibly because $K_{i,t\text{-BuOOH}} \ll K_{t\text{-BuOOH}}$ (Cleland, 1977). The observation that the Carlsberg enzyme has the largest $K_{t\text{-BuOOH}}$ of all variants investigated (Table 1) is consistent with this interpretation.

Given the approximate additivity of contributions made by residues 156, 169, and 217 to the specificity of unmodified subtilisin [Wells *et al.*, 1987; but see Bonneau *et al.* (1991)], it was of interest to determine how changes at the S1 and S1′ sites separately influence the initial rate patterns and magnitudes of the rate constants for the peroxidase reaction. The availability of natural and recombinant subtilisin variants makes this possible. For example, subtilisins BPN′ and E are closely related (86% sequence identity). Except for the replacement of Gly169 by alanine, the amino acid found in the active site of the Carlsberg enzyme (Table 2), their active sites are identical. Although residue 169 is not believed to make direct contact with bound substrate, it appears to modulate the shape of the S1 pocket and thereby influence substrate specificity (Wells *et al.*, 1987). Consistent with this modest change, the Michaelis constants and k_{max} values for selenosubtilisins E and BPN′ are very similar (Table 1). However, the glycine to alanine mutation does have a dramatic effect on the apparent kinetic mechanism. Like its Carlsberg counterpart, but unlike the more closely related BPN′ enzyme, selenosubtilisin E displays apparent ping-pong kinetics (Figure 1). This finding suggests that binding interactions at the S1 site are essential for the formation of a productive ternary complex.

In contrast to the effects of the G169A change, substitutions in the S1′ subsite appear to affect catalytic efficiency

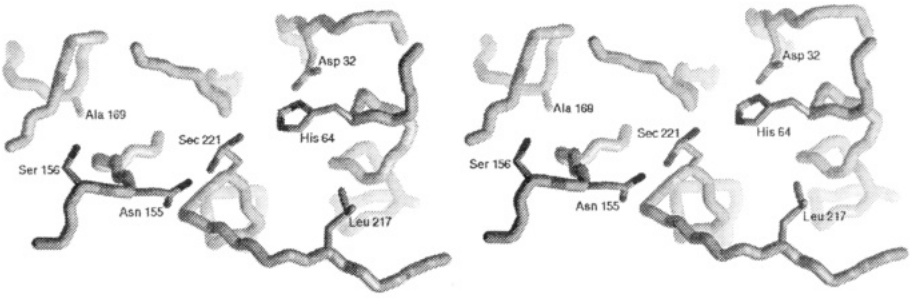


FIGURE 5: Stereoview of the active site of selenosubtilisin Carlsberg. The crystal structure was solved with the selenium prosthetic group, Sec221, in the seleninic acid oxidation state (Syed *et al.*, 1993). The seleninic acid participates in an extended hydrogen bonding network involving Asn155 and the catalytic triad residues His64 and Asp32. Ala169 lies at the bottom, and Ser156 lies on the rim, of the S1 binding site, while Leu217 defines part of the S1′ binding site.

Table 2: Amino Acid Variation within the Active Site of Several Subtilisin Variants

	subtilisin		
amino acid position	Carlsberg	E	BPN′
156	Ser	Glu	Glu
169	Ala	Ala	Gly
217	Leu	Tyr	Tyr

more than kinetic mechanism. The selenosubtilisin BPN′ variant in which Tyr217 is replaced by leucine has a turnover number that is within experimental error of the k_{max} for selenosubtilisin Carlsberg (Table 1), but exploits the same sequential mechanism as the parent BPN′ template (albeit with a somewhat smaller $K_{i,t\text{-BuOOH}}$). The Y217L mutation also causes a ~10-fold increase in K_{ArSH}, with comparatively little change in $K_{t\text{-BuOOH}}$, suggesting that thiol may bind in the vicinity of this amino acid [see also Wells *et al.* (1987)]. This conclusion warrants some caution, however, given that the Michaelis parameters are not equivalent to microscopic binding constants in this system. Specific interactions between the selenenyl sulfide intermediate and residue 217 (Syed *et al.*, 1993) might also explain the high K_{ArSH} value if they influenced binding of the second substrate thiol elsewhere on the enzyme.

Although the selenosubtilisin-promoted peroxidase reaction is mechanistically complex, our experiments establish the importance of both the S1 and S1′ subsites in substrate binding and catalysis. Mutations at both sites have separable and distinct consequences for reactivity. Moreover, in analogy with the experiments in which the proteolytic specificity of subtilisin Carlsberg was recruited for BPN′ (Wells *et al.*, 1987), the properties of one selenoenzyme can be conferred on the other by combining a limited number of substitutions. Thus, the BPN′ triple mutant E156S/G169A/Y217L and selenosubtilisin Carlsberg have comparable activities and exhibit ping-pong kinetics (Table 1; Figure 4). The largest difference between them is the 3.5-fold larger K_{ArSH} value for the triple mutant, which must be due to indirect effects of residues outside the binding site. In fact, many of the additional amino acid differences between BPN′ and Carlsberg are clustered near the S_1′ pocket and may further perturb substrate interactions with residue 217. Although the effects of E156S have not been separately investigated in this study, it is notable in this context that two S1 modifications are able to partially compensate for the large perturbation on K_{ArSH} resulting from the Y217L substitution.

In conclusion, these studies extend our previous investigations on selenosubtilisin by showing how subtle changes in active site microenvironment can be used to alter peroxidase activity. Most notably, site-directed mutagenesis has revealed the existence of a kinetically significant ternary complex in the peroxidase catalytic cycle. Chemical modification in conjunction with site-directed mutagenesis appears to have considerable potential as a means of creating new catalysts with tailored properties.

ACKNOWLEDGMENT

We are grateful to S. Zhou for preliminary experiments on the preparation of selenosubtilisin BPN′ and T. Macke for assistance in preparing Figure 5.

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BI950127E