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SELENOSUBTILISIN'S PEROXIDASE ACTIVITY DOES NOT REQUIRE AN INTACT OXYANION HOLE

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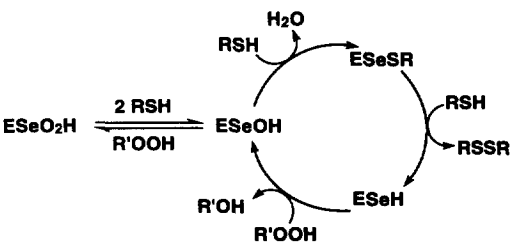
Abstract: Replacement of the active site serine in subtilisin with selenocysteine converts the protease to a peroxidase. The contribution of the oxyanion hole to the efficiency of this novel activity has been investigated by substituting Asn155 with glycine via site-directed mutagenesis. Although the side chain amide of Asn155 provides a hydrogen bond that increases the efficiency of the hydrolytic reactions promoted by native subtilisin, it appears to contribute relatively little to the stability of key intermediates or transition states of the selenosubtilisin-catalyzed peroxidase reaction. Instead, the modest changes in kinetic properties resulting from mutation of the selenoenzyme can be explained by subtle alterations in substrate binding at the S1 pocket which perturb the complex equilibria that determine forward progress along the reaction coordinate. Additional modifications of the subtilisin binding site will apparently be necessary to fully exploit the catalytic potential of the oxyanion hole for selenium-dependent redox chemistry. © 1997 Elsevier Science Ltd.

The ability of enzymes to utilize hydrogen bond formation to stabilize transition states contributes to their remarkable efficiency. In the case of the serine proteases, for example, main chain and side chain amides are believed to play an important role in stabilizing the tetrahedral oxyanion intermediate and flanking transition states formed when the active site nucleophile attacks the scissile carbonyl of the substrate.[1-5] Site-directed mutagenesis experiments on the oxyanion-binding residue Asn155 in the bacterial protease subtilisin suggest that such interactions may be worth up to two or three orders of magnitude in rate.[6-9]

Selenosubtilisin, an artificial selenoenzyme derived from subtilisin through conversion of the catalytically essential serine to selenocysteine,[10] has novel peroxidase activity.[11] In analogy with the natural selenoenzyme glutathione peroxidase (EC 3.4.21.14), it catalyzes the reduction of hydrogen peroxide and alkyl hydroperoxides by aryl thiols via the mechanism shown in Scheme 1.[12,13] X-ray crystallographic[14] and NMR[15,16] characterization of selenosubtilisin suggests that the seleninic acid of the oxidized enzyme has an unusually low pK_a (<4) because of its participation in an extensive hydrogen bonding network with other active site residues, including the side chain of Asn155. Similar interactions with the high energy selenenic acid and selenolate intermediates in the catalytic cycle could conceivably contribute to the efficiency of the peroxidase. In glutathione peroxidase, Gln70 may play such a role, as it is within hydrogen



Scheme 1

bonding distance of both the seleninic acid and selenolate in the oxidized and reduced forms of the enzyme, respectively.[17] To assess the importance of interactions between the selenium redox center in selenosubtilisin and the so-called "oxyanion hole", we have prepared and characterized a variant in which Asn155 has been replaced by glycine. The same change causes a ca. 200-fold drop in the amidase activity of native subtilisin.[9,8]

RESULTS

Stability and Reactivity of Oxidized Selenosubtilisin N155G

Selenosubtilisin is stable at 4 °C for several months in its seleninic acid (ESe(O)OH) oxidation state, losing neither selenium nor catalytic activity upon long-term storage.[15] Hydrogen bonds from the oxygen atoms of the seleninic acid to His64 and to Asn155 in the oxyanion hole confine the prosthetic group to a single well-defined conformation within the active site which prevents the facile *syn* elimination observed with non-enzymic seleninic acids possessing β-hydrogens.[14] Despite the absence of a potentially important interaction, the N155G selenosubtilisin variant is similarly stable for many months.

Like other seleninic acids,[18,13] oxidized selenosubtilisin N155G reacts with three equivalents of 3-carboxy-4-nitrobenzenethiol (ArSH) to give a selenenyl sulfide plus a molecule of free disulfide. Reduction of the selenium prosthetic group probably occurs via thiolseleninate ester (ESe(O)SAr) and selenenic acid (ESeOH) intermediates, and the kinetics are multiphasic. As seen previously for selenosubtilisin Carlsberg,[13] the overall process is acid catalyzed, with initial rates increasing with decreasing pH (Fig. 1). The slope of the log(v_{obs}) vs pH plot is greater than unity (= 1.26), however, reflecting the kinetic complexity of reduction. The initial rates of reaction are comparable to those obtained with the parent BPN' selenoenzyme over the entire pH range examined. The greatest differences are observed at low pH, where the rates for the N155G variant start to level off. Although a true inflection is not observed in the accessible pH range, the apparent curvature would be consistent with a higher pK_a for the N155G seleninic acid.

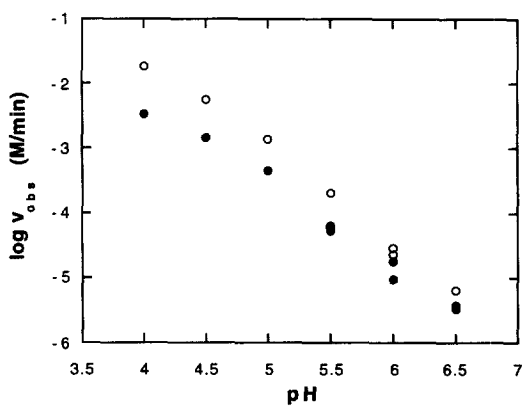


Figure 1. Plots of log (v_{obs}) vs pH for reduction of 10 μM $ESeO_2H$ by 500 μM 3-carboxy-4-nitrobenzenethiol at 25 °C. Selenosubtilisin BPN' (○); selenosubtilisin N155G (●).

Peroxidase Activity of Selenosubtilisin N155G

For detailed comparisions of the peroxidase activities of the N155G and parent BPN' enzymes, the reduction of *tert*-butyl hydroperoxide (*t*-BuOOH) by ArSH was investigated by stopped flow at pH 5.5 and 25 °C. Initial rates were measured by varying peroxide concentration while maintaining thiol concentration constant. Saturation kinetics were observed at each individual thiol concentration investigated (Table 1).

Table 1. Kinetic Parameters for the Peroxidase Activity of Selenosubtilisin BPN' N155G as a Function of Thiol Concentration.*

[ArSH] μM	$k_{cat,app}$ min^{-1}	$K_{m,app}$ mM	$(k_{cat}/K_m)_{app}$ M^{-1} min^{-1}
97	18.2	12.5	1450
278	53.2	23.6	2260
734	164	43.7	3840

*Assays were performed in triplicate at 25 °C in 100 mM MES and 10 mM $CaCl_2$ (pH 5.5). Standard errors for the individual parameters was less than 10% in each case.

Table 2. Apparent Second Order Rate Constants for Selenosubtilisin BPN' and the Corresponding N155G Variant.*

Catalyst	k_{max}/K_{ArSH} M^{-1} min^{-1}	$k_{max}/K_{t\text{-}BuOOH}$ M^{-1} min^{-1}
BPN' [a]	5.95×10^6	7480
BPN' N155G	0.23×10^6	5530

*Assays were performed as described in the legend for Table 1.

[a] From reference 19

Unlike the initial rate patterns obtained with the BPN' and Carlsberg selenoenzymes,[11,13,19] the available data for the N155G variant cannot be fit to a sequential mechanism or to other simple kinetic models, such as ping-pong or equilibrium-ordered mechanisms. Replots of the apparent k_{cat} values versus [ArSH] are linear up to 800 μM (Fig. 2a), indicating that $K_{ArSH} > 1$ mM. Furthermore, the apparent second-order rate constant for reaction of the enzyme with thiol, $k_{max}/K_{ArSH} = 2.3 \times 10^5$ M^{-1} min^{-1}, is more than an order of magnitude smaller than the value of 5.95×10^6 M^{-1} min^{-1} obtained with selenosubtilisin BPN' (Table 2).

Indeed, the enzyme with an intact oxyanion hole exhibits substantially larger apparent k_{cat} values than the N155G variant at all accessible thiol concentrations (Fig. 2a). Fitting the BPN' data to the equation $k_{cat,app} = k_{max}[E][ArSH]/(K_{ArSH} + [ArSH])$ gives $k_{max} = 983 \pm 18$ min^{-1} and $K_{ArSH} = 170 \pm 9$ μM, as reported previously.[19] The apparent second-order rate constant $k_{max}/K_{t\text{-}BuOOH}$ for the two selenoenzymes can be similarly obtained by fitting plots of apparent k_{cat}/K_m values versus [ArSH] (Fig. 2b) to the equation $(k_{cat}/K_m)_{app} = (k_{max}/K_{t\text{-}BuOOH})[ArSH]/(K_{i,t\text{-}BuOOH}K_{ArSH}K_{t\text{-}BuOOH}^{-1} + [ArSH])$. As shown in Table 2, the limiting values of $k_{max}/K_{t\text{-}BuOOH}$ are comparable for both catalysts. While it is not possible to deconvolute the values of K_{ArSH} and $K_{t\text{-}BuOOH}$ for the N155G variant, $K_{i,t\text{-}BuOOH}$ can be estimated from the other parameters to be 14 mM. For BPN', $K_{t\text{-}BuOOH}$ and $K_{i,t\text{-}BuOOH}$ are 130 mM and 32 mM, respectively.[19]

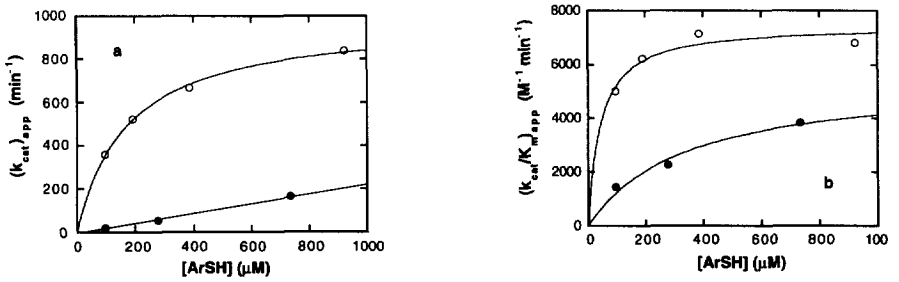


Figure 2. Plots of (a) $(k_{cat})_{app}$ vs [ArSH] and (b) $(k_{cat}/K_m)_{app}$ vs [ArSH] for the peroxidase reaction of selenosubtilisin BPN' (○)[19] and its N155G derivative (●) at pH 5.5 and 25 °C. With the exception of the N155G data in part (a), which were fit to a linear equation, all data were fit to a hyperbolic curve, as described in the text.

DISCUSSION

Crystallographic investigations of the serine protease subtilisin have suggested that the side chain of Asn155 contributes to catalysis by providing a hydrogen bond to the oxyanionic intermediates and transition states that occur during cleavage of amide and ester substrates.[2] Consistent with this notion, replacement of this residue with other amino acids results in 10^2 to 10^3-fold losses in specific activity,[6-9] although the precise energetic contribution of hydrogen bonding is still a matter of debate.[5,4,20] Asn155 is similarly poised to influence the reactivity of the selenium moiety in selenosubtilisin,[14] but our mutagenesis studies indicate that it provides surprisingly little stabilization to Sec221 derivatives.

Structural analysis of the oxidized BPN' (M. McTigue and D. McRee, unpublished results) and related Carlsberg[14] selenoenzymes shows the amide side chain of Asn155 hydrogen bonded to one of the oxygen atoms of the proximal seleninic acid, in analogy to the contacts made by Gln70 at the active site of glutathione peroxidase.[17] Although this interaction was postulated to contribute to the unusually low pK_a of the seleninic acid (<4) and to its resistance to facile β-elimination,[14] the results of Fig. 1 and the extended stability of the oxidized N155G mutant demonstrate that Asn155 has little effect on selenosubtilisin's redox center between pH 4 and 8. The reaction of thiols with the oxidized enzyme is >10^2-times slower than with simple alkyl seleninic acids, as seen previously for selenosubtilisin Carlsberg,[13] and replacement of Asn155 with glycine causes only a 2 to 5-fold further reduction in rate. The slight leveling off observed for the mutant at low pH may reflect some perturbation of the seleninic acid pK_a, but the enzyme-bound functionality must still be substantially more acidic than a typical alkyl seleninic acid (p$K_a \approx 5.4$).[15] Clearly, other factors, including a hydrogen bond from the backbone amide of Sec221, the proximity of positively charged His64 and the complementary macrodipole of the α-helix bearing Sec221, are sufficient to stabilize the seleninate ion in the absence of a hydrogen bonding interaction with the side chain of Asn155. Sluggish reduction of both the parent BPN' and mutant N155G selenoenzymes can then be attributed to the stability of the conjugate base of the seleninic acid (the reaction is acid catalyzed) and/or suboptimal reaction trajectories for the attacking thiol imposed by the steric constraints of the active site. In glutathione peroxidase,[17] the essential selenocysteine residue is sterically more accessible than in selenosubtilisin, and positively charged residues capable of stabilizing its seleninate (or selenolate) oxidation states electrostatically are conspicuously absent; the nearest positive charge (Arg167) is 5.91 Å away.

The peroxidase reaction of selenosubtilisin is seemingly more sensitive to the N155G substitution, as judged by substantially slower rates of 3-carboxy-4-nitrobenzenethiol oxidation by *t*-BuOOH achieved by the mutant enzyme at all substrate concentrations tested. Greater insights into the consequences of mutation are provided by comparison of the initial rate patterns of the parent BPN' selenoenzyme and the N155G variant. The peroxidase reaction promoted by selenosubtilisin is kinetically complex, involving multiple substrates and covalent modification of the enzyme (Scheme 1). The kinetic behavior of selenosubtilisin BPN' is described by a sequential mechanism,[19] which requires the formation of a kinetically significant ternary complex between enzyme, thiol, and peroxide prior to product release. In contrast, neither the sequential model nor other simple kinetic schemes accommodate the N155G data, because the mutant enzyme is not saturated by thiol at concentrations below 1 mM (Fig. 2a), precluding accurate determination of a k_{max} value. Nevertheless, the apparent second-order rate constants for reaction of the enzyme with thiol (k_{max}/K_{ArSH}) and peroxide ($k_{max}/K_{t\text{-BuOOH}}$) can be extracted and compared with the analogous parameters obtained with selenosubtilisin

BPN' (Table 2). Removal of the Asn155 side chain apparently has little effect on the $k_{max}/K_{t\text{-}BuOOH}$ term, but causes a 26-fold drop in k_{max}/K_{ArSH}.

The conclusion that Asn155 is important for transition state stabilization in subtilisin-catalyzed proteolysis derives from the large reductions in k_{cat} and modest changes in K_m that are observed when the putative hydrogen bond it provides to the tetrahedral oxyanion is eliminated by site-directed mutagenesis. For the hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, for example, the 180-fold drop in k_{cat}/K_m caused by the N155G mutation results from a 150-fold decrease in k_{cat} and 1.2-fold increase in K_m.[9] In the case of selenosubtilisin, on the other hand, the N155G mutation leads to a significant increase in the Michaelis constant for the thiol substrate, K_{ArSH}, which accounts for much of the reduction in k_{max}/K_{ArSH}. The deleterious effect of the N155G substitution on the k_{max} parameter can be no greater than 4-fold, and it seems likely that a hydrogen bond from Asn155 adds little to the stability of the selenenic acid or selenolate intermediates (or the various transition states) in the catalytic cycle.

As pointed out by Mock and Chua[20] for subtilisin itself, a variety of factors in addition to hydrogen bonding, especially changes in hydration and conformational distortion of the active site induced by mutation, undoubtedly contribute to the magnitude of the observed effects. With selenosubtilisin such factors may dominate. Indeed, removal of the Asn155 side chain is likely to perturb the complex equilibria that determine forward progress along the peroxidase reaction coordinate by subtly influencing substrate binding. Asn155 is located at the edge of the S1 binding site, and mutations elsewhere in this pocket have been shown to alter the kinetic mechanism and raise the value of K_{ArSH}.[19] In this context it is worth noting that molecular dynamics simulations indicate that the side chain of Asn155 partially blocks the preferred trajectory for thiol attack on the selenenyl sulfide intermediate (ESeSAr).[14] Because replacement of Asn155 with an amino acid like glycine that lacks a bulky side chain diminishes, rather than augments, the efficiency of the peroxidase reaction, the detrimental consequences of mutation on other steps in the catalytic cycle must outweigh any beneficial effects.

Although the interdependence of the catalytic triad and oxyanion binding site is a critical feature of the normal workings of the serine protease subtilisin,[8,9] functional interplay between the selenium redox center and Asn155 in selenosubtilisin appears modest at best. The relative unimportance of Asn155 contrasts with the significant role played by His64 both in stabilizing anionic oxidation states of Sec221[15,16] and in proton transfer,[13] and may reflect the distinct structural and energetic demands of the peroxidase reaction (as opposed to proteolysis) at the subtilisin active site. To fully exploit the catalytic potential of the oxyanion hole for selenosubtilisin-promoted redox chemistry additional modifications of the binding pocket will apparently be necessary.

MATERIALS AND METHODS

Materials. 3-Carboxy-4-nitrobenzenethiol was prepared by reduction of the corresponding disulfide following the procedure of Silver[21] for the preparation of dihydrolipoamide. All other chemicals were of the highest purity commercially available and were used without further purification. Subtilisin BPN' was purchased from Sigma. The N155G BPN' variant was generously provided by Thomas Graycar of Genencor International. The concentration of subtilisin was determined from the absorbance at 280 nm, assuming an extinction coefficient of 32,200 M^{-1} cm^{-1}.[22]

Preparation of Selenosubtilisins. Selenosubtilisin BPN' was prepared as previously described.[19] Selenosubtilisin N155G was obtained by a slight modification of this procedure. Ser221 of subtilisin N155G was activated in 10 mM PIPES, 10 mM $CaCl_2$, pH 7.0 through periodic additions of phenylmethanesulfonyl fluoride (50 μL aliquots from a 20 mg/ml acetonitrile stock to 10 mL of a 10 mg/mL enzyme solution) over a period of 1 to 2 days. The progress of the reaction was monitored with the reporter substrate succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. When the specific activity for hydrolysis dropped below 5% of the initial activity, the sulfonylated protein was isolated by gel filtration (Sephadex G-25, eluted with 100 mM MES, 10 mM $CaCl_2$, pH 5.5) and allowed to react with excess NaHSe under argon for two days at pH 5.5. The resulting selenoprotein was purified by gel filtration on Sephadex G-25 and affinity chromatography on thiopropyl Sepharose 6B, and oxidized with H_2O_2 for long-term storage.[13,19] Typical yields were approximately 40% based on phenylmethanesulfonyl-subtilisin N155G. The selenium content of the selenoproteins was determined by titration with 3-carboxy-4-nitrobenzenethiol at pH 5.0 (ε_{410} = 10.8 mM^{-1} cm^{-1}).[13]

Kinetics. Reduction of the selenoenzymes by 3-carboxy-4-nitrobenzenethiol was monitored spectroscopically after rapidly mixing enzyme and substrate solutions at the proper pH at 25 °C. For the selenosubtilisin-catalyzed peroxidase reactions, equal volumes of *tert*-butyl hydroperoxide and a mixture of enzyme plus 3-carboxy-4-nitrobenzenethiol were incubated at 25 °C and rapidly mixed by stopped flow, and the time-dependent absorbance decrease at 410 nm was monitored. In all cases, 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. Thiol concentration 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 peroxidase data obtained at fixed thiol concentrations to the equation $v_o/[E] = (k_{cat})_{app}[S]/(K_{m,app} + [S])$, where v_o is the initial velocity, and [E] and [S] are the concentrations of enzyme and peroxide, respectively.

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