Peroxide Dependence of the Semisynthetic Enzyme Selenosubtilisin†

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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 natural peroxidase, a variety of hydroperoxides are accepted as substrates for the semisynthetic enzyme, whereas the dialkyl compound *tert*-butyl peroxide is not. Kinetic investigations reveal that k_{max} is dependent upon the nature of the hydroperoxide, indicating that peroxide-mediated oxidation of the enzymic selenolate is at least partially rate-limiting. Experiments with the radical trap 2,6-di-*tert*-butyl-4-methylphenol suggest that, while the nonenzymic reaction between *tert*-butyl hydroperoxide and thiol involves free radicals, the same reaction catalyzed by selenosubtilisin does not. The studies described here support the enzyme's proposed ping-pong mechanism and are consistent with previous mechanistic observations.

Semisynthesis allows the generation of novel biocatalysts by chemical incorporation of a prosthetic group into an existing protein binding site. This technique has yielded new catalysts from both enzymes and antibodies [for a review, see, for example, Hilvert (1991)], and we have employed it in the generation of the first artificial selenoenzyme from the serine protease subtilisin (EC 3.4.21.14) (Wu & Hilvert, 1989). Selenosubtilisin, in addition to having novel acyl transferase properties, mimics the activity of the natural selenoprotein glutathione peroxidase (EC 1.11.1.9) by catalyzing the reduction of hydroperoxides by thiols (Wu & Hilvert, 1990). The natural enzyme is thought to be important in the prevention of lipid peroxidation and the corresponding disruption of membrane function, and it has been studied in some detail (Flohé, 1989). Studies on the corresponding activity of selenosubtilisin may further illuminate the mechanism and evolution of glutathione peroxidase and also increase our understanding of semisynthetic enzymes and biocatalysis in general.

Selenosubtilisin has been studied by both ^{77}Se and ^{1}H NMR spectroscopy (House et al., 1992, 1993), and the crystal structure has been solved to 2.0-Å resolution (Syed et al., 1993). Furthermore, we have recently conducted a more detailed kinetic analysis of the semisynthetic enzyme, and our results correlate well with the structural and spectroscopic data (Bell et al., 1993). Our studies on the enzyme's thiol specificity for the reduction of *tert*-butyl hydroperoxide indicate that, while 3-carboxy-4-nitrobenzenethiol is turned over by the enzyme at least 2000-fold faster than by relevant model systems, most aliphatic thiols are poor substrates for selenosubtilisin. As a logical extension of such kinetic studies, we now present an investigation of the behavior of selenosubtilisin with a number of different peroxide substrates. Specifically, we intended to determine if the peroxides were involved in the rate-determining step of the enzymatic reaction and also to address the contribution of free-radical chemistry.

EXPERIMENTAL PROCEDURES

Materials. Selenosubtilisin was prepared as described previously (Bell et al., 1993). 3-Carboxy-4-nitrobenzenethiol (ArSH) was prepared from the corresponding disulfide by reduction with sodium borohydride in 80% ethanol (Silver, 1979). Following acidification to pH 2, the product thiol was isolated by ethyl acetate extraction and concentration to give a pale yellow solid which had the correct spectroscopic properties. Peroxide substrates were obtained from the Aldrich Chemical Co. All other chemicals were of the highest purity commercially available and were used without further purification.

For concentration determinations, the following extinction coefficients were used: subtilisin (or selenosubtilisin), at 280 nm and pH 7.0, ϵ = 23 500 M^{-1} cm^{-1} (Markland & Smith, 1971); and 3-carboxy-4-nitrobenzenethiol, at 410 nm and pH 5.5, ϵ = 12 600 M^{-1} cm^{-1}. The concentrations of the peroxide stock solutions were determined by iodometric titration (Kolthoff & Medalia, 1949).

All kinetics experiments were performed in buffer A [33 mM citric acid, 33 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 33 mM 4-morpholineethanesulfonic acid (MES), 10 mM $CaCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), and 10% *N*,*N*-dimethylformamide (DMF)], pH 5.50. The buffer pH values were determined with a Radiometer Copenhagen PHM 84 pH meter.

Kinetics. The enzyme-catalyzed and nonenzymic reductions of peroxides by ArSH were monitored by following the disappearance of the thiolate absorption at 410 nm at 25 °C. To investigate the dependence of rate on substrate concentration, the initial velocities were determined at several concentrations of one substrate while the concentration of the other was kept constant.

Rapid reactions were studied using a Hi-Tech Scientific SF-51 stopped-flow unit equipped with a SU-40 spectrophotometer. Equal volumes of a peroxide solution and a mixture of enzyme plus ArSH were rapidly mixed, and the time-dependent decrease in absorption at 410 nm was recorded. Each initial velocity was measured in triplicate and calculated from the first 5–10% of the reaction. The enzymatic rates were corrected for the background (nonenzymic) reaction between peroxide and thiol. The actual initial concentration

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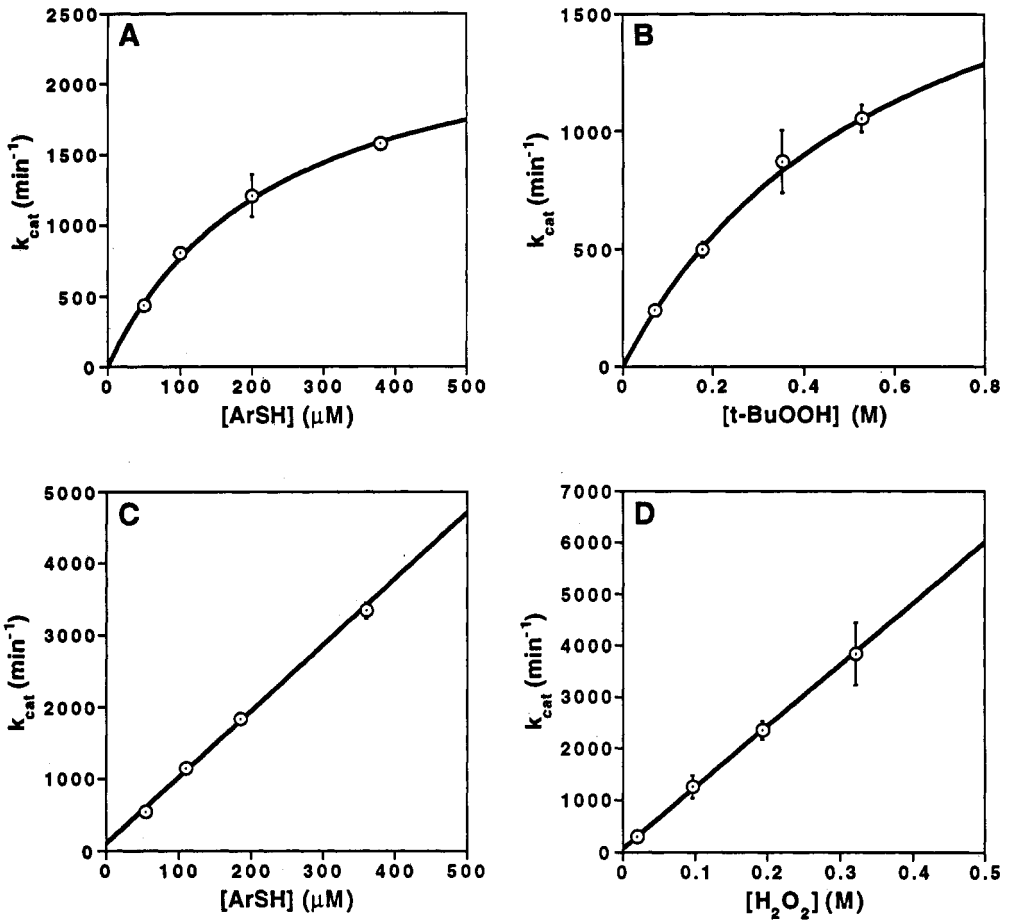


FIGURE 1: Plots of the apparent k_{cat} (min^{-1}) vs substrate concentration for the enzymatic peroxidase reactions of selenosubtilisin at pH 5.5 and 25 °C. (A) k_{cat} vs [ArSH] (μM) for *t*-BuOOH reduction. (B) k_{cat} vs [*t*-BuOOH] (M). (C) k_{cat} vs [ArSH] (μM) for H_2O_2 reduction. (D) k_{cat} vs [H_2O_2] (M). For A and B the data were fit to a saturation curve, while for C and D the data were fit to a linear equation. The data were fit to the equations with weighted fits that utilized error estimates for each point (shown as error bars).

of ArSH in the kinetic apparatus was measured from the 410-nm absorbance, and rates were corrected for any variation of this thiol concentration. Slower rates were determined on a Hewlett-Packard 8452A diode array spectrophotometer. The enzyme was preincubated with ArSH in order to ensure that it was present as the selenenyl sulfide form (ESeSAr), and the reaction was initiated by addition of peroxide. The experimental data were processed as detailed above.

Data were fit to equations as described in the text with the program KaleidaGraph (Abelbeck Software). The data for *tert*-butyl hydroperoxide and hydrogen peroxide were also fit to a ping-pong mechanism using an adaption of Cleland's kinetic program for the Macintosh (J. G. Robertson, Pennsylvania State University, 1988).

Table I: Kinetic Parameters for the Peroxidase Activity of Selenosubtilisin[a]

hydroperoxide substrate	$k_{max}/K_{R'OOH}$ (M^{-1} min^{-1})	k_{max}/K_{ArSH} (M^{-1} min^{-1})
t-BuOOH	3900 ± 200	(1.3 ± 0.1) × 10^7
H_2O_2	13 700 ± 800	(1.1 ± 0.1) × 10^7
CuOOH	18 000 ± 1000	

[a] Reactions were carried out in buffer A, pH 5.5, at 25 °C, and followed by stopped-flow spectroscopy as described. For *t*-BuOOH and H_2O_2, the experimental data were fit to a ping-pong mechanism to obtain the parameters shown. For CuOOH, $(k_{cat}/K_{CuOOH})_{app}$ was obtained directly from the linear plot of v_0 vs [CuOOH] at 200 μM ArSH and low [CuOOH], and, assuming a ping-pong mechanism, this value is equivalent to k_{max}/K_{CuOOH}.

RESULTS

Selenosubtilisin catalyzes the reduction of a variety of structurally distinct hydroperoxides, from the hydrophilic H_2O_2 to the bulky aromatic cumenyl hydroperoxide. Saturation kinetics were observed for the enzymatic peroxidase reaction at all the individual concentrations of ArSH and *t*-BuOOH or H_2O_2 investigated, and double-reciprocal plots of initial velocity vs substrate concentration revealed the characteristic parallel lines of a ping-pong mechanism, in analogy with our previous reports (Wu & Hilvert, 1989; Bell et al., 1993). The apparent second-order rate constants for the enzymatic reactions between ArSH and the hydroperoxide substrates *tert*-butyl hydroperoxide and hydrogen peroxide are detailed in Table I. These values were deduced from fitting the experimental data to a ping-pong kinetic scheme. The data did not fit well to other models, such as sequential or equilibrium-ordered mechanisms. In addition to the parameters in Table I, the following values were also yielded for *tert*-butyl hydroperoxide: $k_{max} = 2100 \pm 200$ min^{-1}, $K_{t\text{-BuOOH}} = 0.53 \pm 0.06$ M, and $K_{ArSH} = 160 \pm 20$ μM, in close agreement with our previously reported values (Bell et al., 1993). In contrast, under very similar conditions of substrate concentration, limiting values of k_{cat} and K_m were not obtained when H_2O_2 was employed as the oxidant, as evidenced by plots of apparent k_{cat} values vs substrate concentration (Figure 1). Nonetheless, lower limits on these parameters may be set from an inspection of Figure 1: for ArSH, $K_m > 400$ μM; for H_2O_2, $K_m > 0.3$ M; and $k_{max} > 8000$ min^{-1}. A bulky hydroperoxide, cumenyl hydroperoxide (CuOOH), was also reduced by the enzyme, but its low solubility in aqueous buffer prevented such a detailed analysis of its reduction. However,

under conditions where [CuOOH] < 10 mM, a plot of the enzymatic rate for the peroxidase reaction against [CuOOH] was linear, and the gradient gave the parameter $(k_{cat}/K_{CuOOH})_{app}$ = 18 000 ± 1000 M^{-1} min^{-1} at 200 μM ArSH. A similar method could not be used to obtain $(k_{cat}/K_{ArSH})_{app}$ in this case because the value of $(K_{ArSH})_{app}$ is too low to allow an accurate investigation of the linear region of the saturation curve at the accessible peroxide concentrations. Investigations on *tert*-butyl peroxide (*t*-BuOO*t*-Bu) failed to reveal any enzymatic rate enhancement above background.

Initial rates for the spontaneous reduction of hydroperoxides by ArSH were found to vary linearly with the concentrations of hydrogen peroxide and cumenyl hydroperoxide, although a small deviation from this behavior was observed for *tert*-butyl hydroperoxide at high peroxide concentrations (>0.5 M), with the rate being slightly lower than expected as the limit of solubility of this peroxide in aqueous buffer was approached. At 200 μM ArSH, the observed pseudo-first-order rate constants for the background reactions were as follow: *tert*-butyl hydroperoxide, k_1 = (1.3 ± 0.1) × 10^{-3} min^{-1}; cumenyl hydroperoxide, k_1 = (4.3 ± 0.3) × 10^{-3} min^{-1}; and hydrogen peroxide, k_1 = (5.7 ± 0.4) × 10^{-3} min^{-1}.

The spontaneous reaction between 3-carboxy-4-nitrobenzenethiol and *tert*-butyl hydroperoxide was investigated in the presence and absence of the radical trap 2,6-di-*tert*-butyl-4-methylphenol (BHT). The peroxide concentration was varied at constant [ArSH] (120 μM), and the inclusion of 100 μM BHT revealed a marked effect on the initial velocities as compared with identical experiments run in the absence of BHT, as illustrated in Figure 2A. In fact, the pseudo-first-order rate constant calculated from these plots of velocity vs [*t*-BuOOH] was more than halved by the addition of 100 μM BHT. In contrast, analogous experiments carried out in the presence of 5 μM selenosubtilisin (ESeSAr) revealed no inhibition of the enzymatic reaction by 100 μM BHT within experimental error (Figure 2B).

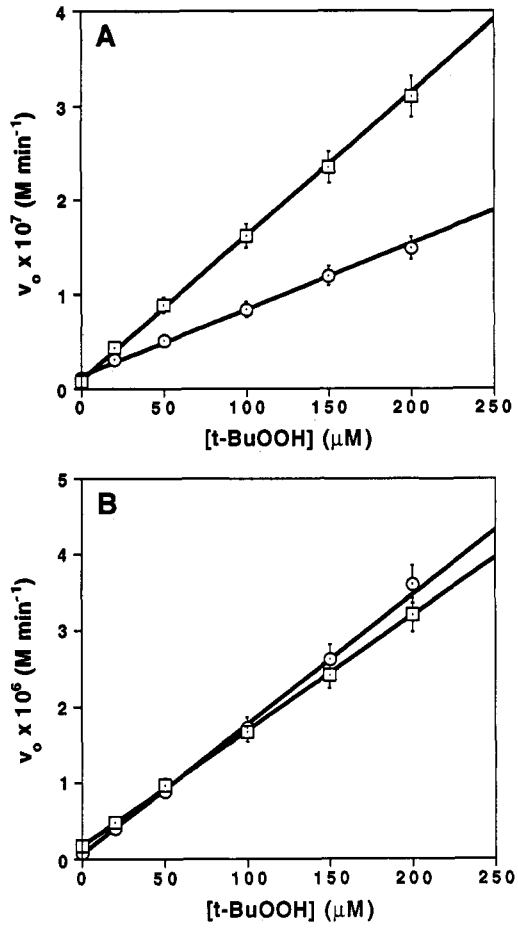


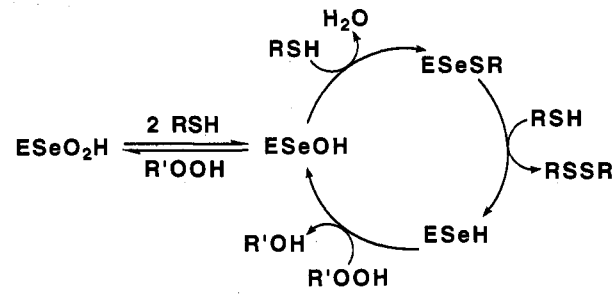
FIGURE 2: (A) Plots of v_0 (M min^{-1}) vs [*t*-BuOOH] (μM) for 120 μM ArSH in buffer A containing 5% acetonitrile, pH 5.5 and 25 °C, at [BHT] = 0 μM (□) and 100 μM (○). Data were fit to a first-order equation with a weighted fit that utilized error estimates for each point (shown as error bars). (B) As for panel A but with the addition of 5 μM selenosubtilisin. The enzymatic rates (v_0) were corrected for the background reaction.

DISCUSSION

Selenosubtilisin is produced in good yield from the bacterial protease subtilisin via selective activation of the catalytic serine with phenylmethanesulfonyl fluoride (PMSF) followed by displacement of the sulfonate with hydrogen selenide (Wu & Hilvert, 1989; Bell et al., 1993). The resulting single-atom mutation (from oxygen to selenium) radically alters the chemical behavior of the enzyme and affords it a novel peroxidase activity. In analogy with the natural selenoenzyme glutathione peroxidase (Flohé, 1989), selenosubtilisin exhibits ping-pong kinetics; exists in a sluggish, oxidized form ($ESeO_2H$) which can be reduced to a more reactive species by thiol; and may be described mechanistically by the catalytic cycle illustrated in Scheme I, which is consistent with all the available data. In addition to kinetic studies, the seleninic acid ($ESeO_2H$), selenol (ESeH), and selenenyl sulfide (ESeSAr) forms of selenosubtilisin may be isolated and have been characterized chemically (Bell et al., 1993) and also by NMR spectroscopy (House et al., 1992; 1993).

The peroxidase activity of the semisynthetic enzyme exhibits a pH–rate profile that suggests a requirement for a protonated group with a pK_a of 7 (Bell et al., 1993). This is consistent with the pK_a of the catalytic residue His64 in native subtilisin (Jordan et al., 1985). For selenosubtilisin, NMR (House et al., 1992, 1993), crystallographic (Syed et al., 1993), and kinetic (Bell et al., 1993) data all indicate the presence of strong interactions between the deprotonated selenium prosthetic group and the protonated His64 residue in the selenol (ESeH) and seleninic acid ($ESeO_2H$) species. Indeed the

Scheme I



pK_a values of these prosthetic groups are considerably lower than expected, while that for the histidine is raised well above 7. A consideration of Scheme I, coupled with the fact that no such ionizing group is seen in a pH–rate profile for the reduction of $ESeO_2H$ to ESeSAr, suggests that if the apparent pK_a is representative of His64, it is most likely the histidine in ESeSAr (Bell et al., 1993). This interpretation implies that the conversion of (ESeSAr + ArS^-) to (ESe^- + ArSSAr) is rate-determining to some degree, although it should be emphasized that the interpretation of pH–rate profiles requires several assumptions (Knowles, 1976). One such assumption is that only one state of ionization of the active site is capable of catalysis, and in this complex system it is difficult to make such statements with certainty.

A simple way to access information concerning the peroxide dependence of the rate-limiting step(s) is to conduct parallel experiments with hydroperoxide substrates of different intrinsic

reactivity. The relevant steady-state equation for the enzymatic peroxidase reaction is

$$\frac{v_0}{[E]_0} = \frac{k_{max}[ArSH][R'OOH]}{[ArSH][R'OOH] + K_{R'OOH}[ArSH] + K_{ArSH}[R'OOH]} \quad (1)$$

where k_{max} is a pseudo-first-order rate constant and $K_{R'OOH}$ and K_{ArSH} are the Michaelis constants for the peroxide and thiol, respectively. If the maximal k_{cat} value for the enzymic reaction (k_{max}) is altered, then the peroxide substrate must be involved in a step which is at least partially rate-determining. The results of parallel experiments with *t*-BuOOH and H_2O_2 are illustrated by the plots of apparent k_{cat} values vs thiol and peroxide concentrations (Figures 1A and 1B, *t*-BuOOH, and Figures 1C and 1D, H_2O_2). Clearly, the rate constants observed for H_2O_2 are significantly higher than the corresponding values for *t*-BuOOH. Furthermore, the linearity of the plots for hydrogen peroxide indicates that the enzyme has not been fully saturated with respect to both substrates in this case and hence that accurate k_{max} and K_m values may not be obtained. Nonetheless, lower limits for the parameters may be set, and they indicate that k_{max} for H_2O_2 is at least 4-fold greater than that for *t*-BuOOH, allowing qualitative conclusions to be drawn. The low aqueous solubility of cumenyl hydroperoxide prevented such an analysis in its case, but the linear plot of rate against [CuOOH] provides a value of $(k_{cat}/K_{CuOOH})_{app}$ = 18 000 ± 1000 M^{-1} min^{-1} at 200 μM ArSH. Like glutathione peroxidase, selenosubtilisin seems to accept only hydroperoxides as substrates; the dialkyl *tert*-butyl peroxide was not turned over to any measureable degree.

As discussed above, k_{max} for the reaction with hydrogen peroxide is significantly greater than that for *tert*-butyl hydroperoxide, and it must be concluded that the more-reactive H_2O_2 has either increased the velocity of a rate-limiting step or that it has altered the mechanism to give a different rate-determining process. The ping-pong kinetic patterns observed with both hydroperoxides argue against a dramatic difference in mechanism between the two. The only apparent dissimilarity was observed at very high concentrations of H_2O_2 (≈1 M) which, coupled with its greater reactivity, resulted in unexpectedly low rates for the enzymatic reaction. Indeed, the double-reciprocal plots of initial velocity vs substrate concentration showed apparent substrate inhibition at these very high peroxide concentrations. This behavior is consistent with overoxidation of the ESeOH form of the enzyme in Scheme I, which would shift the equilibrium toward $ESeO_2H$ and hence inhibit the reaction. This has presumably not been observed with *t*-BuOOH because it is not as strong an oxidizing agent as H_2O_2 and also because it is not soluble at such high concentrations in aqueous buffer. Such anomalous data for the H_2O_2 reaction were not used to derive the kinetic parameters, and the simplest conclusion with regard to the values of k_{max} is that the hydroperoxide substrate is involved in a step which is at least partially rate-determining. From Scheme I, this step may be expected to be conversion of (ESe^- + R'OOH) to (ESeOH + R'OH). The pH–rate data discussed above implicated the conversion of ESeSAr to ESe^- as a rate-limiting process, and the present results may simply mean that *both* the selenolate oxidation and the reduction of the enzymic selenenyl sulfide are partially rate-determining under all the conditions investigated. Alternatively, the reduction of ESeSAr may become rate-limiting only when His64 is *not* protonated. When the histidine side chain *is* protonated, conversion of ESeSAr to ESe^- could be far more rapid, and other processes, such as oxidation of the selenolate by hydroperoxide, may be completely rate-determining. In either case, the rate-determining nature of hydroperoxide turnover is consistent with the relatively low values of $k_{max}/K_{R'OOH}$, as compared with k_{max}/K_{ArSH}. This suggests that attempts to enhance the catalytic activity of selenosubtilisin should focus on the transformation of ESe^- to ESeOH, perhaps by reengineering the subtilisin active site either to relieve any steric hinderance of hydroperoxide attack upon the selenolate or to slightly destabilize the selenolate residue. Mutation of Asn155, which may donate a hydrogen bond to the selenolate anion and slightly blocks the active-site cleft, would be a suitable starting point for such engineering experiments.

The values obtained for $k_{max}/K_{R'OOH}$ provide a measure of the rate of reaction between free enzyme and hydroperoxide. For glutathione peroxidase, the equivalent bimolecular rate constants approach the diffusion limit. This is believed to reflect the fact that in the natural enzyme the selenolate sits in a shallow depression on the protein's surface and may essentially react with any approaching hydroperoxide: the enzyme has no real substrate specificity for hydroperoxides, provided that steric hinderance does not prevent their reaction (Flohé, 1989). For comparison with the kinetic parameters for selenosubtilisin and subject to the caveat that they were obtained under different conditions (pH 6.7 and 37 °C), the bimolecular rate constants for the reactions between glutathione peroxidase and the same hydroperoxides are as follow: k_{+1}(*t*-BuOOH) = 4.5 × 10^8 M^{-1} min^{-1}; k_{+1}(CuOOH) = 7.7 × 10^8 M^{-1} min^{-1}; and $k_{+1}(H_2O_2)$ = 3.5 × 10^9 M^{-1} min^{-1} as determined previously (Flohé et al., 1972; Günzler et al., 1972). The bimolecular reactions between selenosubtilisin and these peroxides are approximately 4–5 orders of magnitude slower than those for the natural enzyme. This may be due in part to the fact that the selenium side chain in the semisynthetic enzyme is buried in a much deeper pocket and is presumably less accessible than its natural counterpart to hydroperoxides. Furthermore, the strong interactions between the prosthetic group and the residues in subtilisin's oxyanion hole and His64 may stabilize the selenolate anion to such a degree that it loses catalytic efficiency. In highly-evolved enzymes, the fine balance between stabilizing a high-energy species and lowering its energy to the point of unreactivity has been perfected. It is not surprising that the subtilisin protein template, which was not intended for peroxidase activity, lacks such fine-tuning.

It is interesting to note that for both the background reaction between ArSH and hydroperoxide and the bimolecular reaction between glutathione peroxidase and hydroperoxide, the rate constants vary in magnitude in the order $k(H_2O_2)$ > k(CuOOH) > k(*t*-BuOOH). In contrast, the analogous bimolecular rate constants for selenosubtilisin ($k_{cat}/K_{R'OOH}$) vary as k(CuOOH) > $k(H_2O_2)$ > k(*t*-BuOOH). It is possible that the first series reflects the intrinsic rate of reaction between the hydroperoxides and a selenolate or thiolate in the absence of any significant binding effects, while the latter series indicates that cumenyl hydroperoxide is able to take some binding advantage of the subtilisin protein template and hence raise its $k_{cat}/K_{R'OOH}$ above that of hydrogen peroxide by lowering K_{CuOOH}. Subtilisin is known to favor aromatic groups at its S_1 subsite (Philipp & Bender, 1983), but further work will be necessary to clarify this situation.

Importantly, Table I reveals that k_{max}/K_{ArSH} is identical, allowing for experimental error, for the enzymatic reactions with *t*-BuOOH and H_2O_2. This is expected from the mechanism illustrated in Scheme I, in which the thiol reacts

with enzymic intermediates that do not depend upon the nature of hydroperoxide. As k_{max} increases upon changing from *t*-BuOOH to H_2O_2, a concomitant increase in K_{ArSH} preserves the ratio of the two parameters. Glutathione peroxidase exhibits a strong specificity for its donor substrate glutathione (γ-Glu-Cys-Gly), with small structural changes in the thiol leading to large reductions in catalytic efficiency (Flohé et al., 1971). Similarly, selenosubtilisin seems to have a preference for 3-carboxy-4-nitrobenzenethiol, although possibly for different reasons. A clue is provided in another group of proteins which can mimic the glutathione peroxidase activity: the glutathione *S*-transferases, a group of enzymes which catalyze the nucleophilic addition of glutathione (GSH) to a variety of acceptor substrates, such as hydroperoxides (Mannervik & Danielson, 1988). In this latter case, the GSH donor is oxidized to the sulfenic acid GSOH, which then reacts nonenzymatically with glutathione to give GSSG and H_2O (Prohaska, 1980). The glutathione *S*-transferases are thought to enhance the nucleophilicity of GSH via several interactions which lower its pK_a and hence increase the local concentration of the more-reactive thiolate anion (Ji et al., 1992). This may explain why normal aliphatic thiols are poor substrates for selenosubtilisin, which has no known mechanism for increasing their acidity, whereas ArSH, which is inherently quite acidic (pK_a = 4.4), is accepted as a good donor substrate by the semisynthetic enzyme. We are currently investigating this line of reasoning and its implications for selenosubtilisin and the natural glutathione peroxidase.

When examining the reactions of peroxides, it is natural to consider the possibility of free-radical chemistry. Harman and co-workers used spin-trapping ESR methodology to demonstrate that no thiyl radical was formed during normal glutathione peroxidase catalysis, providing strong evidence against radical involvement for the natural enzyme (Harman et al., 1986). In contrast, there is evidence that the spontaneous reduction of hydroperoxides by thiols does involve free radicals (Capozzi & Modena, 1974). To address this issue with respect to selenosubtilisin, the enzymatic and background rates were measured in the presence and absence of the radical trap 2,6-di-*tert*-butyl-4-methylphenol (BHT). The results are illustrated in Figure 2. The pronounced effect of the inclusion of BHT on the background reaction confirms the ability of this phenol to inhibit the reaction between ArSH and *t*-BuOOH in free solution and also indicates that this nonenzymic reaction does indeed involve a radical pathway (Figure 2A). Repetition of this experiment under identical conditions, except for the addition of 5 μM selenosubtilisin, resulted in plots of initial velocity vs [*t*-BuOOH] which are essentially identical to each other within experimental error (Figure 2B). The lack of inhibition by BHT for the enzymatic reaction suggests that selenosubtilisin, in analogy with glutathione peroxidase, catalyzes the reduction of hydroperoxides by thiol ArSH via a nonradical mechanism. If this is the case, the intriguing conclusion is that the chemical intervention of the selenocysteine residue, in both the natural and semisynthetic enzymes, provides a more favorable pathway for the peroxidation reaction which obviates the necessity for free-radical intermediates. However, it is possible that the selenosubtilisin-catalyzed reaction is not inhibited by BHT simply because the radical trap cannot access enzyme-bound radical intermediates. This assumes, however, that the putative radical intermediates do not exist extensively in free solution, where they could react with BHT, and this assumption seems somewhat unlikely since neither thiol nor hydroperoxide are high-affinity binders of selenosubtilisin.

In conclusion, these studies extend our previous investigations on selenosubtilisin by examining the dependence of its peroxidase activity upon the peroxide substrate. The semisynthetic enzyme accepts a variety of structurally distinct hydroperoxides as substrates, and the turnover number (k_{max}) is dependent upon the nature of the hydroperoxide. This latter observation offers mechanistic insights which may facilitate the rational improvement of the enzyme's efficiency. Kinetic experiments with the radical trap BHT suggest that, while the spontaneous reduction of *t*-BuOOH by ArSH involves free radicals, the same reaction catalyzed by selenosubtilisin does not. These investigations help to define the mechanism of selenosubtilisin's peroxidase activity, many aspects of which closely mirror those of the natural enzyme glutathione peroxidase. This is apparently due to the intrinsic chemical properties of the amino acid selenocysteine, which can seemingly engender a novel activity in a totally unrelated protein template. In addition to demonstrating the potential of semisynthesis as a route to novel biocatalysts, the mutant enzyme selenosubtilisin continues to give a mechanistic perspective on primitive biocatalysts and the evolution of glutathione peroxidase itself.

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