Kinetic Studies on the Peroxidase Activity of Selenosubtilisin†

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ABSTRACT: Selenosubtilisin, a semisynthetic selenoenzyme produced by chemical modification of the serine protease subtilisin, acts as a mimic of glutathione peroxidase, catalyzing the reduction of *tert*-butyl hydroperoxide by 3-carboxy-4-nitrobenzenethiol. To clarify the mechanism of action of this catalyst, detailed kinetic studies have been carried out. Thiol-mediated reduction converts the seleninic acid form of selenosubtilisin ($ESeO_2H$) into a selenenyl sulfide (ESeSAr). Investigations into the reduction of $ESeO_2H$ by the aromatic thiol revealed saturation kinetics and were consistent with a significant lowering of the pK_a of the seleninic acid in the enzyme active site. While the reduction of $ESeO_2H$ was slow compared with a simple model system, the reduced selenoenzyme (ESeSAr) exhibited a much greater peroxidase activity than model compounds. The enzymic selenocysteine residue was shown to be crucial for this activity, and ping-pong kinetics were observed. A catalytic cycle involving interconversion of the ESeSAr, ESeH, and ESeOH forms of the enzyme has been proposed that is consistent with all the available data. The pH-rate profile for the peroxidase activity indicates the involvement of the active site histidine (His64) in the rate-determining step, which these investigations suggest is attack of ArS^- on ESeSAr. The results presented here correlate well with crystallographic and spectroscopic data and provide more detailed information about crucial interactions within the active site of selenosubtilisin.

The generation of enzyme-like catalysts continues to be a fundamental goal for biological chemists. The strategies employed in this area include the chemical synthesis of model systems (Breslow, 1991; Cram, 1988; Lehn, 1985) and the production of antibody molecules which bind carefully designed haptens (Lerner et al., 1991; Lewis & Hilvert, 1991). An alternative strategy involves the mutation of a naturally occurring enzyme, either by genetic or chemical means, in order to alter its pattern of reactivity. The substrate specificity, catalytic behavior, and general stability of enzymes have been manipulated genetically (Knowles, 1987; Wells & Estell, 1988), and the chemical introduction of prosthetic groups into enzyme active sites has allowed new catalytic activities to be incorporated into existing proteins (Hilvert & Kaiser, 1987). We recently reported the use of this "semisynthetic" strategy in the generation of the first artificial selenoenzyme, selenosubtilisin (Wu & Hilvert, 1989).

The bacterial serine protease subtilisin (EC 3.4.21.14) provides a robust, well-characterized template for chemical manipulation, and the incorporation of a selenocysteine at the active site seemed attractive because of the extensive chemical utility of selenium. Furthermore, there is increasing interest in the biochemistry of selenium, and in natural selenoenzymes such as glycine reductase in bacteria and the mammalian enzyme glutathione peroxidase (Stadtman, 1990). This latter enzyme is believed to be crucial in the prevention of oxidative damage in many tissues, since it catalyzes the reduction of hydroperoxides by glutathione (γ-Glu-Cys-Gly) (Ladenstein, 1984). This redox reaction is highly efficient, with second-order rate constants approaching the diffusion limit, and the active site selenocysteine residue is known to be essential for the enzyme's activity (Forstrom et al., 1978).

The structure of bovine glutathione peroxidase has been determined to 2.0-Å resolution (Epp et al., 1983). The protein is a tetramer composed of four identical subunits, each of M_r 21 000, with the essential selenocysteine occupying a shallow depression on the surface of the protein. The key kinetic properties of the enzyme have been elucidated and a number of plausible catalytic mechanisms suggested, although this mechanistic debate remains unresolved.

We developed a methodology for the conversion of the active site serine of subtilisin (Ser221) into selenocysteine. Purified selenosubtilisin is afforded in good yields, and the semisynthetic enzyme has been shown to function as an acyl transferase (Wu & Hilvert, 1989). More recently, we reported that selenosubtilisin can also mimic the catalytic behavior of glutathione peroxidase (Wu & Hilvert, 1990), and the present report describes a more detailed study of this activity.

EXPERIMENTAL PROCEDURES

Materials and Instrumental. Subtilisin Carlsberg (Protease VIII) was purchased from Sigma. Sephadex G-25 was obtained from Aldrich Chemical Company, and thiopropyl Sepharose 6B was from Pharmacia LKB Technology AB. Eglin-c was a generous gift from Dr. H.-P. Schnebli (Ciba-Geigy AG, Basel, Switzerland). 3-Carboxy-4-nitrobenzenethiol was prepared by reducing the corresponding disulfide using the procedure of Silver (1979). The synthesis of *N*-(*tert*-butoxycarbonyl)aminoethaneseleninic acid (**2**) has been described previously (House et al., 1992). [^{35}S]Phenylmethanesulfonyl fluoride (PMSF)[1] was synthesized from ^{35}S-labeled thiourea (Amersham) according to a published procedure (Gold & Fahrney, 1964). All other chemicals were of the

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highest purity commercially available and were used without further purification.

The concentration of subtilisin, or selenosubtilisin, was determined by measuring the UV absorbance at 280 nm and pH 7.0, assuming an extinction coefficient of 23 500 M^{-1} cm^{-1} (Markland & Smith, 1971). IEF and SDS–polyacrylamide gel electrophoresis were performed on a Pharmacia Phastgel system using standard procedures, and liquid phase IEF was achieved using a Rainin Instruments RF3.

The following buffers were routinely used: buffer A (10 mM PIPES, 10 mM $CaCl_2$); buffer B (33 mM citric acid, 33 mM HEPES, 33 mM MES, 10 mM $CaCl_2$, 1 mM EDTA). The buffer pH values were determined with a Radiometer Copenhagen PHM 84 pH meter.

^1H NMR spectra were recorded with a Bruker AM-300 spectrometer. Electrospray mass spectra were obtained with a Sciex API III mass analyzer.

Preparation of Selenosubtilisin. Subtilisin Carlsberg (150 mg, 5.5 μmol) was dissolved in 50 mM PIPES and 10 mM $CaCl_2$, pH 7.0 (4.5 mL), and allowed to react with phenylmethanesulfonyl fluoride (150 μL of a 20 mg/mL solution in acetonitrile, 17 μmol) at 25 °C for 1 h. The resulting sulfonylated enzyme was isolated by gel filtration on Sephadex G-25 using 50 mM PIPES and 10 mM $CaCl_2$, pH 7.0 as eluent. The protein fraction was concentrated to a volume of 10 mL, mixed with an equal volume of 1 M sodium hydrogen selenide (NaHSe) solution prepared according to the procedure of Klayman and Griffin (1973), and incubated at 40 °C for 36 h under an inert atmosphere. The protein was separated from the reaction mixture on a Sephadex G-25 column eluting with buffer A, pH 7.0. Further purification was achieved by affinity chromatography on thiopropyl Sepharose 6B. The crude selenoenzyme preparation was placed in a dropping funnel connected to a round-bottom flask which contained thiopropyl Sepharose 6B (20 μmol of activated thiopropyl groups) and was reduced by treatment with excess sodium borohydride under nitrogen. After unreacted reducing agent was quenched by carefully adjusting the pH to 6.5–7.0 with 1 M HCl and 1 M imidazole, the protein solution was added to the resin and gently stirred for 15 min. The Sepharose was transferred to a small column and washed extensively with buffer A, pH 7.0. Selenosubtilisin was eluted with 20 mM DTT in the same buffer. The enzyme solution was concentrated, dialyzed against 10 mM hydrogen peroxide in buffer A, pH 7.0, for 4 h to oxidize the selenium prosthetic group to the seleninic acid, and finally dialyzed three times against buffer A, pH 7.0.

The selenium content of the product enzyme was determined by titrating the reduced protein with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) using a modification of the method reported by Cavallini et al. (1966). Selenosubtilisin in buffer A was treated with excess sodium borohydride under nitrogen for 20 min, the solution was carefully adjusted to pH 7 with degassed 1 M HCl and 1 M imidazole to quench the reducing agent, and 20 μL of the reduced sample was added to DTNB (980 μL of a 100 μM solution in buffer A, pH 7.0). The concentration of enzyme-bound selenol was determined from the absorption of 3-carboxy-4-nitrobenzenethiolate at 410 nm (ϵ = 13 600 M^{-1} cm^{-1}, pH 7.0).

[1] Abbreviations: ArSH, 3-carboxy-4-nitrobenzenethiol; BCA, bicinchoninic acid; DTNB, dithiobis(2-nitrobenzoic acid); DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IEF, isoelectric focusing; MES, 4-morpholineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; PMS, phenylmethanesulfonyl group; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

Preparation of the Selenenyl Sulfide Form of Selenosubtilisin (ESeSAr). Oxidized selenosubtilisin was treated with 3.1 equiv of 3-carboxy-4-nitrobenzenethiol (**1**) at pH 5.0 for 30 min. The enzyme was separated from the reaction mixture by chromatography on a Sephadex G-25 column, eluting with buffer A, pH 7.0. The yield of the selenenyl sulfide was determined by measuring the release of thiol **1** upon reduction of the enzyme by DTT. Protein concentration was determined by BCA titration, using subtilisin as reference, according to a literature procedure (Smith et al., 1985).

Preparation of (N-Carbobenzyloxy)aminoethaneseleninic Acid (3). (*N*-Carbobenzyloxy)aminoethyl bromide was prepared from the base-catalyzed protection of 2-bromoethylamine hydrobromide (Aldrich) with benzyl chloroformate (Aldrich) (Bergmann & Zervas, 1932) and converted to (*N*-carbobenzyloxy)aminoethyl diselenide according to Syper and Mlochowski (1984). The diselenide was purified by flash column chromatography on silica, eluting with a gradient of dichloromethane and 1–5% diethyl ether. ^1H NMR (300 MHz, $CDCl_3$): δ 3.01 (4 H, t, J = 6 Hz, $CH_2\underline{CH_2}Se$); 3.54 (4 H, q, J = 6 Hz, $NHC\underline{H_2}CH_2Se$); 5.10 (4 H, s, $PhC\underline{H_2}O_2C$); 7.30–7.34 (10 H, m, aromatic H).

The diselenide was oxidized with hydrogen peroxide by the method of McCullough and Gould (1949) to give (*N*-carbobenzyloxy)aminoethaneseleninic acid (**3**). ^1H NMR (300 MHz, CD_3COCD_3): δ 3.11 (2 H, t, J = 6 Hz, $CH_2\underline{CH_2}$-SeO_2H); 3.69–3.75 (2 H, m, $NHC\underline{H_2}CH_2SeO_2H$); 5.08 (2 H, s, $PhC\underline{H_2}O_2C$); 7.29–7.37 (5 H, m, aromatic H).

Preparation of 2-(Aminoethaneselenenyl)-3-carboxy-4-nitrobenzene Sulfide (5). To a solution of selenocystamine dihydrochloride (4.5 mg, 14 μmol, Sigma) in ethanol (500 μL) and water (200 μL) was added sodium borohydride (7 mg, 180 μmol). The reaction mixture was left under nitrogen at room temperature for 3 h, quenched to pH ≈ 6.5 with 1 M HCl and 1 M imidazole (degassed), and made up to 1.0 mL with 100 mM MES and 10 mM $CaCl_2$, pH 5.5 (degassed). A portion of this solution (35.5 μL) was added to 1 mM DTNB, 100 mM MES, and 10 mM $CaCl_2$, pH 5.5, and the yield of selenenyl sulfide **5** was determined from the increase in absorption at 490 nm due to release of thiolate **1** (ϵ = 1390 M^{-1} cm^{-1}, pH 5.5).

Reaction of Oxidized Selenosubtilisin or Model Compound 3 with 3-Carboxy-4-nitrobenzenethiol (1). To a 100 μM solution of **1** in 100 mM MES, pH 5.0, in a cuvette was added 10 μM selenosubtilisin (or 10 μM model compound **3**). The amount of thiol consumed was determined by measuring the UV absorbance change at 410 nm. The reaction was also monitored by reversed-phase HPLC on a C18 column using acetonitrile–0.05% TFA in water as eluent (0–80% gradient of acetonitrile). DTNB formed was identified by comparing its retention time with that of an authentic sample and quantified using *p*-nitrophenol as an internal standard.

Kinetics. The reactions of selenosubtilisin, and model compound **3**, with thiol **1** and the enzyme-catalyzed reduction of *tert*-butyl hydroperoxide by **1** were studied by following the disappearance of the thiolate absorption at 410 nm, at 25 °C. Each initial velocity was measured in triplicate and calculated from the first 5–10% of the reaction. For the peroxidase activity, the rates were corrected for the background reaction between hydroperoxide and **1**. The actual concentration of **1** in the kinetic apparatus was measured from the 410 nm absorbance, and rates were corrected for any variation in the concentration of **1**. Data were fit to equations as described in the text with the program KaleidaGraph (Abelbeck Software).

The concentration of the *tert*-butyl hydroperoxide stock was determined by iodometric titration (Kolthoff & Medalia, 1949). To investigate the dependency of rate on substrate concentration, the reaction rates were determined at several concentrations of one substrate while keeping the concentration of the other constant. In the pH-rate profile investigations, buffer B was used throughout.

Rapid reactions were investigated using a Hi-Tech Scientific SF-51 stopped-flow unit and SU-40 spectrophotometer unit, while slower reaction rates were determined on either Hewlett Packard 8452A or Perkin-Elmer Lambda 4B spectrophotometers. In the stopped-flow investigations on peroxidase activity, equal volumes of *tert*-butyl hydroperoxide and a mixture of enzyme plus thiol **1** were rapidly mixed, and the time-dependent decrease in thiolate absorption was monitored.

Inhibition of Selenosubtilisin. The irreversible inhibition by iodoacetate was effected by treating 0.5 mM selenosubtilisin with 5 mM thiol **1** in 50 mM acetate buffer, pH 5.0, followed by addition of 50 mM iodoacetate. The reaction mixture was treated with 5 mM **1** three times over 45 min. The resulting protein was purified by gel filtration on a Sephadex G-25 column eluting with buffer A, pH 7.0. The redox activity of the enzyme was assayed as described previously.

The effects of eglin-c on the reactions of selenosubtilisin were determined by preincubating the enzyme with various concentrations of the inhibitor and then assaying the enzyme activity as described above.

RESULTS AND DISCUSSION

Preparation of Selenosubtilisin. The Carlsberg variant of subtilisin (M_r 27 300) was chemically converted into selenosubtilisin using an approach which was originally developed for the production of thiolsubtilisin (Polgár & Bender, 1967; Neet et al., 1968). Serine 221 was selectively activated with phenylmethanesulfonyl fluoride (PMSF), and the resulting sulfonate was displaced with hydrogen selenide. The conditions for the incorporation of the selenium prosthetic group were optimized by monitoring the release of ^{35}S-labeled phenylmethanesulfonate from the enzyme. In the presence of a 50-fold molar excess of hydrogen selenide, 99% of the labeled sulfonate groups were displaced from the enzyme after 36 h at 40 °C. The modification reactions were carried out at pH 7 in order to minimize competition from the spontaneous hydrolysis of the sulfonate derivative, which is a significant problem at pH values lower than 5.5 (Polgár & Bender, 1967). The reaction mixtures also contained 10 mM $CaCl_2$ to stabilize the tertiary structure of the enzyme.

Selenosubtilisin was isolated from the crude reaction mixture by gel filtration and purified by affinity chromatography on thiopropyl Sepharose. We found the thiopropyl Sepharose affinity resin to be superior to mercury–agarose because of the apparent irreversible binding of the selenoenzyme to mercurial columns. The yield of purified selenosubtilisin was typically found to be around 40%, based on PMS-subtilisin. The selenium content of the isolated protein was determined, following anaerobic reduction of the sample with sodium borohydride, by titration with DTNB, and 0.95 ± 0.03 equiv of selenol were detected per protein molecule by this method. Aerial oxidation of the selenol form of the enzyme was found to be rapid, and therefore ESeH was always handled under an inert atmosphere.

In order to convert the purified enzyme into a distinct oxidation state, it was treated with 10 mM hydrogen peroxide and then dialyzed exhaustively against buffer A, pH 7.0, in which it was stored at 4 °C. This procedure produced a seleninic acid derivative as judged by X-ray crystallography (Syed et al., 1993), ^{77}Se NMR spectroscopy (House et al., 1992), and electrospray mass spectrometry. The latter technique yielded an M_r value of 27 380 ± 3, (calculated M_r = 27 384 for $ESeO_2H$). For comparison, native subtilisin gave M_r = 27 289 ± 2 (calculated M_r = 27 289) and PMS-subtilisin was found to have M_r = 27 441 ± 1 (calculated M_r = 27 443). One ambiguity in the characterization of selenosubtilisin was the presence of an additional seleninic acid resonance in the ^{77}Se NMR spectrum, which may be due to partial oxidation of the proximal methionine residue (Met222) by H_2O_2 during preparation of the enzyme (House et al., 1992). However, the X-ray crystallographic data did not allow us to confirm the presence of any methionine sulfoxide at this position (Syed et al., 1993) nor was such an oxidized form of the enzyme detected in the electrospray mass spectrum. It should be noted that the absence of positive evidence of a methionine sulfoxide residue from the latter technique does not rule out the possibility of such oxidation: although the *precision* of the electrospray mass spectrum allows the M_r of a species to be determined to within a few daltons, the *resolution* is approximately 1 part in 2000. Thus, the detection of a minor contaminant with a mass increment of only 16 Da (oxidized Met vs Met) is at the limit of the technique and was not possible in this case.

The oxidized selenoenzyme had the same mobility as PMS-subtilisin on SDS–polyacrylamide gels, and both were contaminated by small amounts of lower molecular weight material. An examination of the electrospray mass spectra of selenosubtilisin and PMS-subtilisin revealed similar contaminants which appeared to be due to a single autolytic cleavage of subtilisin to give two major fragments, with approximate M_r values of 15 600 and 11 600. The fragments accounted for approximately 15% of the total protein, and the fact that they were only observed by denaturing techniques and not, for example, by NMR spectroscopy suggests that under normal conditions they may associate to give a complex that is essentially identical to the uncleaved enzyme. Recent analysis of pI values, on both the Pharmacia Phastgel and the RF3 liquid phase isoelectric focusing systems, has revealed that the isoelectric points of selenosubtilisin and the native enzyme are essentially identical, in contrast to our earlier report (Wu & Hilvert, 1990). The incorrect value previously quoted appears to be a consequence of the difficulty of focusing subtilisin with the Phastgel system.

It is known that simple organic seleninic acids with β-hydrogens tend to be unstable because of facile syn-elimination (Reich & Jasperse, 1987). The seleninic acid form of selenosubtilisin is quite stable, however, and can be stored in solution at 4 °C for several months without loss of selenium or activity. Interactions of the enzyme-bound seleninic acid with the proximal oxyanion hole and His64 apparently prevent this undesired reaction (Syed et al., 1993).

Reduction of Oxidized Selenosubtilisin by Thiols. Kice and Lee (1978) have shown that benzeneseleninic acid reacts rapidly with 3 equiv of thiol to give the corresponding selenenyl sulfide (PhSeSR) and disulfide (RSSR) under a variety of conditions. The seleninic acid form of selenosubtilisin behaves analogously (eq 1):

$$ESeO_2H + 3\ ArSH \xrightarrow{-2H_2O} ESeSAr + ArSSAr \quad (1)$$

When 3-carboxy-4-nitrobenzenethiol (**1**) is employed as the reducing agent, this reaction provides a convenient method

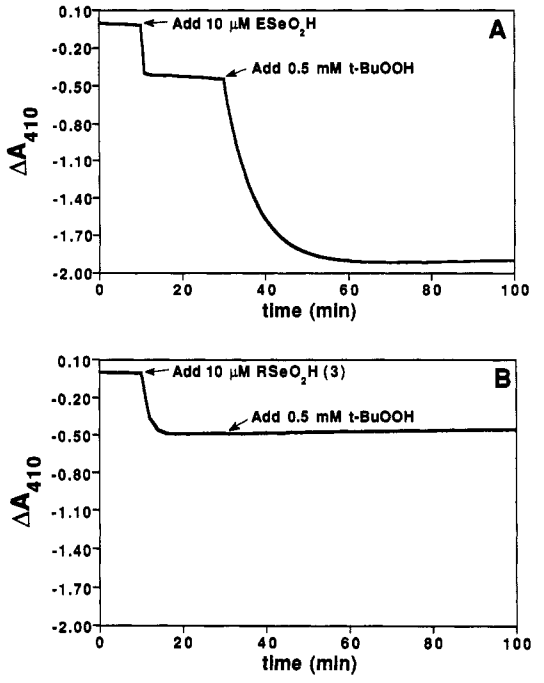


FIGURE 1: (A) 10 μM selenosubtilisin was added to 185 μM thiol **1** in 100 mM MES, pH 5.0, at 25 °C, and the disappearance of the thiolate absorption at 410 nm was monitored. After the initial consumption of 3 equiv of **1**, 0.5 mM *t*-BuOOH was added. (B) As for panel A but with 10 μM model **3** substituted for the enzyme. The absorbance change was corrected for the background reaction between peroxide and **1**.

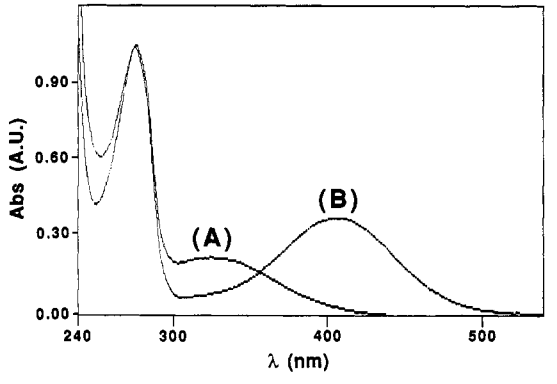
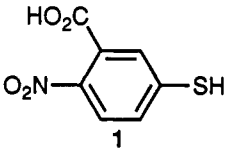


FIGURE 2: (A) UV spectrum of isolated ESeSAr in buffer A, pH 7.0 (ϵ_{326} = 7600). (B) After addition of 16 mM DTT (for ArSH, pH 7.0, ϵ_{410} = 13 600).

for titrating the enzyme active site, since the disappearance of thiolate is readily monitored spectroscopically at 410 nm (ϵ = 10 800 M^{-1} cm^{-1}, pH 5) (Ellman, 1959). Thus, the enzymic seleninic acid was found to react with 3 equiv of 3-carboxy-4-nitrobenzenethiol at pH 5.0 (Figure 1). One equivalent of 5,5′-dithiobis(2-nitrobenzoic acid) was produced in the course of the reaction as determined by reversed-phase HPLC and comparison with an authentic sample.

The selenenyl sulfide form of the enzyme (ESeSAr) could be isolated on a preparatively useful scale in greater than 90% yield by gel filtration on Sephadex G-25. It was found that treatment with only 3.1 equiv of thiol gave optimal yields, as judged by release of thiol **1** upon treatment of the sample with excess DTT (Figure 2). Use of a large excess of **1** significantly decreased the yield of recovered ESeSAr, presumably because excess thiol further reduces the selenenyl sulfide to the selenol, which may be reoxidized aerobically once the enzyme has been separated from thiol by gel filtration. The enzymic selenenyl sulfide moiety was characterized by ^{77}Se NMR



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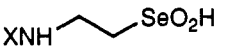
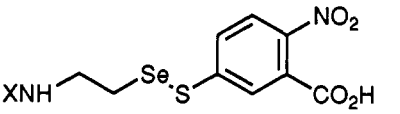


2: X = (CO)O*t*-Bu
3: X = (CO)OCH$_2$Ph



4: X = (CO)OCH$_2$Ph
5: X = H

spectroscopy (House et al., 1992). It exhibited a single resonance at 388 ppm, which is characteristic of such derivatives. The selenenyl sulfide form of the enzyme, like the seleninic acid, was found to be stable in solution at 4 °C for extended periods.

The kinetics of the reduction of oxidized selenosubtilisin ($ESeO_2H$) by 3-carboxy-4-nitrobenzenethiol were examined using the stopped-flow technique. Complete reduction of the enzyme is kinetically complex and presumably proceeds via thioselininate (ESe(O)SAr) and selenenic acid (ESeOH) intermediates in analogy with the chemistry of benzeneseleninic acid (Kice & Lee, 1978). Under conditions where the enzyme was in large excess, however, pseudo-first-order kinetics were observed for the disappearance of the thiolate absorption at 410 nm. Plots of the observed first-order rate constants against enzyme concentration exhibited saturation behavior at high concentrations of selenosubtilisin. The data were fitted to eq 2

$$k_{obs} = \frac{k_2[ESeO_2H]}{K_s + [ESeO_2H]} \quad (2)$$

to give k_2 = 2.29 ± 0.08 min^{-1} and K_s = 125 ± 9 μM at pH 5.0 and 25 °C (Figure 3A).

In contrast to the behavior of selenosubtilisin, saturation kinetics were not observed for the nonenzymic model reaction between excess (*N*-carbobenzyloxy)aminoethaneseleninic acid (**3**) and thiol **1** (Figure 3B). Moreover, comparison of the second-order rate constant for the model system (3.3 × 10^6 M^{-1} min^{-1}) with the apparent bimolecular rate constant k_2/K_s for the enzymic reaction (1.8 × 10^4 M^{-1} min^{-1}) indicates that the initial reduction of selenosubtilisin is approximately 200 times slower than reduction of a simple alkaneseleninic acid at pH 5.

Reduction of both the selenoenzyme and model **3** by thiol **1** is acid catalyzed. In the case of the enzyme, the observed rate increases monotonically with a slope of 1 over the entire pH range 7.5–4.0. The observed rate for the model system, however, increases to a maximum value as pH is lowered (Figure 4). The data for the model reaction rate were fitted to eq 3:

$$v_{obs} = \frac{V_{max}}{1 + K_1/[H^+]} \quad (3)$$

where K_1 represents the dissociation constant of an ionizing

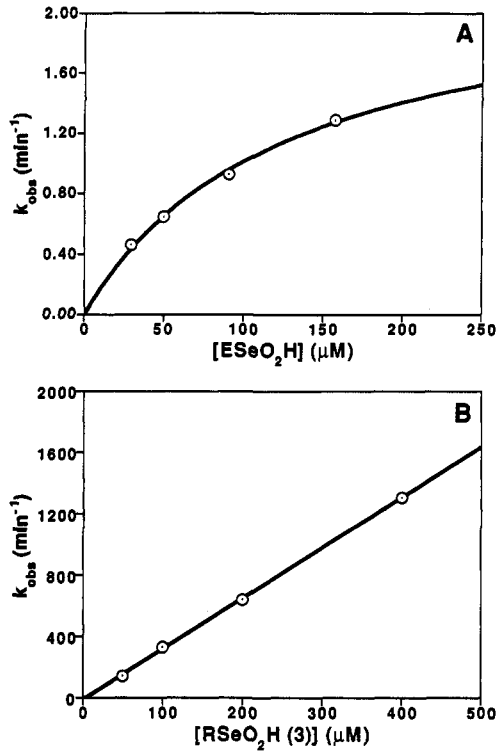


FIGURE 3: (A) k_{obs} (min^{-1}) vs [$ESeO_2H$] (μM) at 3 μM thiol **1** in buffer B, pH 5.0 and 25 °C. (B) k_{obs} (min^{-1}) vs [model **3**] (μM) at 3 μM thiol **1** in buffer B, pH 5.0 and 25 °C. The data for both enzymic and model systems were fitted to first-order exponential decays to obtain k_{obs}.

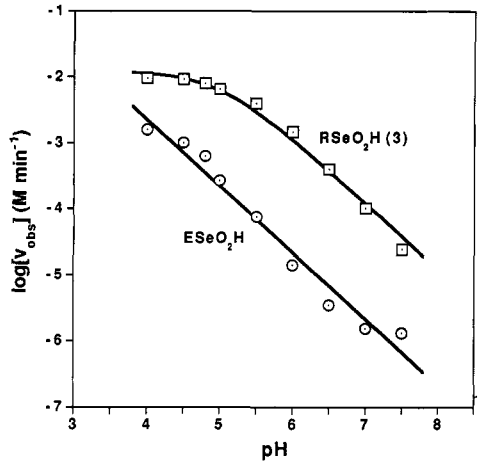


FIGURE 4: Plots of log (v_{obs}) (M min^{-1}) vs pH for reduction of 5 μM $ESeO_2H$, and 5 μM model **3**, by 200 μM thiol **1** in buffer B at 25 °C. The data for the model reaction were fit to the equation $\log(v_{obs}) = \log(v_{max}) - \log(1 + K_1/[H^+])$; the data for the enzymic reaction were fit to a linear equation to give a slope of −1.01.

group. At 25 °C, $pK_1 = 5.3 \pm 0.04$, in excellent accord with the expected pK_a of an alkaneseleninic acid. In fact, the pK_a of the closely related *N*-(*tert*-butoxycarbonyl)aminoethaneseleninic acid (**2**) has been found to be 5.4 by titration of its ^{77}Se NMR signal (House et al., 1992).

^{77}Se NMR spectroscopic data (House et al., 1992) have shown that the pK_a values of both the seleninic acid and selenol forms of selenosubtilisin are considerably perturbed, with the prosthetic group being far more acidic than analogous alkyl compounds. X-ray crystallographic data (Syed et al., 1993) are consistent with these observations, indicating that the seleninic acid group is hydrogen-bonded to several residues, especially those of subtilisin's oxyanion hole and the histidine of subtilisin's catalytic triad, which would be expected to stabilize the seleninate form of the side chain, thus lowering the pK_a of the acid. Therefore, the kinetic data for the reduction of the seleninic acids by **1** may be explained by a mechanism which requires the seleninate group to be protonated to achieve maximal rates. The attenuated reactivity of selenosubtilisin relative to the alkyl model system is consistent with a lowered pK_a value for the enzymic seleninic acid.

The reduction of selenosubtilisin by **1** was competitively inhibited by eglin-c, a 70 amino acid polypeptide isolated from the leech *Hirudo medicinalis* that is known to bind unmodified subtilisin with high affinity (Seemüller et al., 1986). A Dixon analysis indicated that the dissociation constant for the selenosubtilisin–eglin-c complex was 10 μM. This figure is approximately 6 orders of magnitude higher than typical subtilisin–eglin-c K_i values (Heinz et al., 1991), suggesting that the seleninic acid moiety significantly hinders binding of the peptide. A high-resolution X-ray crystal structure of the subtilisin Carlsberg–eglin-c complex reveals that a hydrophobic leucine side chain of the inhibitor projects into the enzyme's active site (Bode et al., 1987), and it is probable that the negatively charged seleninate group, and the altered hydrogen-bonding network in which it resides, greatly reduce the affinity of eglin-c for the binding site. Furthermore, the increased steric bulk of the prosthetic group ($ESeO_2^-$ vs EOH) would also be expected to hinder inhibitor binding, relative to native subtilisin.

Selenosubtilisin-Catalyzed Reduction of tert-Butyl Hydroperoxide by 3-Carboxy-4-nitrobenzenethiol. The naturally occurring selenoenzyme glutathione peroxidase (EC 1.11.1.9) couples the reduction of hydroperoxides with the oxidation of glutathione in vivo. Clearly, the ability to bind this thiol substrate is a necessity for the enzyme's activity, and it has been proposed that two arginine residues near the active site form salt bridges with the terminal carboxylate groups of glutathione (Epp et al., 1983). An examination of subtilisin's X-ray crystal structure reveals that the protein template is unlikely to offer a suitable binding site for glutathione. However, subtilisin favors aromatic groups at its S_1 subsite (Philipp & Bender, 1983), so we were not surprised to discover that the aromatic thiol **1** is a reasonable substrate for the enzymatic reduction of hydroperoxides by selenosubtilisin. As shown in Figure 1A, addition of *tert*-butyl hydroperoxide to thiol **1** in the presence of selenosubtilisin resulted in the rapid and complete consumption of **1**, as judged by the disappearance of the absorption at 410 nm. In parallel experiments with (*N*-carbobenzyloxy)aminoethaneseleninic acid (**3**) (Figure 1B) or benzeneseleninic acid, the rate of oxidation of thiol was not increased appreciably over the background rate upon addition of *tert*-butyl hydroperoxide, even in the presence of high concentrations of the selenium derivative. Control experiments also showed that the peroxidase activity of selenosubtilisin was linearly proportional to enzyme concentration and that this activity was destroyed by heat denaturation of the protein.

The reduction of $ESeO_2H$ by thiol **1** produces the selenenyl sulfide, ESeSAr (eq 1), which was found to be a competent catalyst of the peroxidase reaction and which was employed for kinetic investigations of this activity. The relative efficiencies of selenosubtilisin and the nonenzymic model system **4** [2-((*N*-carbobenzyloxy)aminoethaneselenenyl)-3-carboxy-4-nitrobenzene sulfide, generated in situ by reduction of **3** with thiol **1**] were investigated at pH 5.5 and 25 °C. At 200 μM **1** and 10 mM *t*-BuOOH, the initial rate of reaction (corrected for the spontaneous oxidation in the absence of

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

[ArSH] (μM)	k_{cat}(app) (min^{-1})	$K_{t\text{-BuOOH}}$(app) (M)	k_{cat}(app)/$K_{t\text{-BuOOH}}$(app) (M^{-1} min^{-1})
51	610 ± 44	0.15 ± 0.02	4000 ± 230
95	960 ± 78	0.23 ± 0.02	4100 ± 50
195	1320 ± 98	0.32 ± 0.03	4100 ± 110

[t-BuOOH] (M)	k_{cat}(app) (min^{-1})	K_{ArSH}(app) (μM)	k_{cat}(app)/K_{ArSH}(app) (M^{-1} min^{-1})
0.068	246 ± 7	15 ± 2	(1.6 ± 0.26) × 10^7
0.203	631 ± 37	41 ± 3	(1.5 ± 0.19) × 10^7
0.508	1070 ± 59	72 ± 9	(1.5 ± 0.10) × 10^7

[a] Reactions were carried out in buffer B, pH 5.5 at 25 °C, and followed by stopped-flow spectroscopy as described. The data in the table were obtained from the plots in Figure 5.

catalyst) for the enzyme (5 μM) was 2.0 × 10^{-4} M min^{-1}. For the model compound, the reaction rate was not measurably increased above the background rate of 1.9 × 10^{-5} M min^{-1} even at concentrations of **4** in excess of 50 μM, under conditions where catalytic rates of 15% of the background rate would have been detected. Because of the negligible reactivity of **4**, coupled with its low solubility in aqueous buffer, we investigated the activity of the water-soluble model 2-(aminoethaneselenenyl)-3-carboxy-4-nitrobenzene sulfide (**5**). This model was synthesized from the corresponding diselenide, which was reduced to the selenol with sodium borohydride. The selenol (1 mM) was added to 1 mM DTNB to give **5** in 97% yield based on the diselenide, as determined from the increase in thiolate absorption at 490 nm. At pH 5.5 and 25 °C, the initial rate of the reaction between 0.93 mM **1** and 10 mM *t*-BuOOH catalyzed by 0.97 mM **5** was found to be 9.5 × 10^{-5} M min^{-1}. Assuming that the rate has a first-order dependence on thiol concentration, these data indicate that the enzyme is at least 2000-fold more efficient than **5**. Thus, although the protein template makes the seleninic acid form of the selenoenzyme less easy to reduce with thiol compared to the simple model **3**, selenosubtilisin is a substantially better turnover *catalyst* than the simple models **4** and **5** for the reduction of alkyl hydroperoxides by thiol **1**.

In order to probe the mechanism by which selenosubtilisin (ESeSAr) promotes the peroxidase reaction, detailed kinetic studies were undertaken. The initial rates for the reduction of *t*-BuOOH by 3-carboxy-4-nitrobenzenethiol were determined as a function of substrate concentration at 25 °C and pH 5.5 (buffer B) by stopped-flow spectroscopy, varying one substrate concentration while the other was fixed. Michaelis–Menten kinetics were observed for both substrates under all the conditions investigated. The apparent kinetic parameters obtained at several thiol and *t*-BuOOH concentrations are summarized in Table I. Double-reciprocal plots of initial velocity vs substrate concentration yielded families of parallel lines for both substrates (Figure 5), consistent with a ping-pong mechanism involving at least one covalent enzyme intermediate. The relevant steady-state equation for this system is

$$\frac{v_o}{[E]_o} = \frac{k_{max}[ArSH][t\text{-BuOOH}]}{K_{t\text{-BuOOH}}[ArSH] + K_{ArSH}[t\text{-BuOOH}]} \quad (4)$$

where k_{max} is a pseudo-first-order rate constant and $K_{t\text{-BuOOH}}$ and K_{ArSH} are the Michaelis constants for the peroxide and **1**, respectively. From the data in Table I, k_{max} = 2140 ± 120 min^{-1}, $K_{t\text{-BuOOH}}$ = 0.48 ± 0.054 M, and K_{ArSH} = 130 ± 19 μM. Thus, $k_{max}/K_{t\text{-BuOOH}}$ = 4500 M^{-1} min^{-1} and k_{max}/K_{ArSH} = 1.6 × 10^7 M^{-1} min^{-1}.

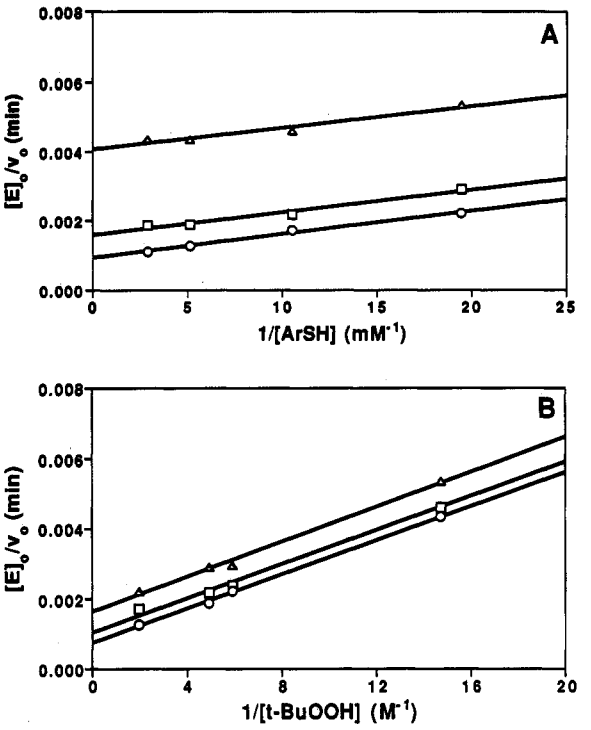
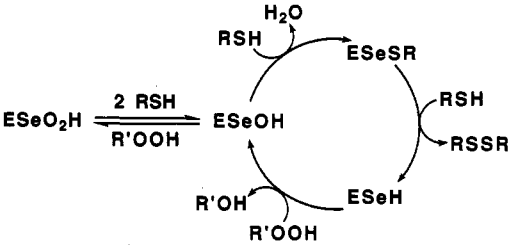


FIGURE 5: (A) $[E]_o/v_o$ (min) vs $1/[ArSH]$ (mM^{-1}) for 5 μM selenosubtilisin in buffer B, pH 5.5 and 25 °C, at [*t*-BuOOH] = 0.0678 M (△), 0.203 M (□), and 0.508 M (○). (B) $[E]_o/v_o$ (min) vs $1/[t\text{-BuOOH}]$ (M^{-1}) for 5 μM selenosubtilisin in buffer B, pH 5.5 and 25 °C, at [ArSH] = 51 μM (△), 95 μM (□), and 195 μM (○).

Scheme I



The peroxidase activity could be initiated with either the selenenyl sulfide (ESeSAr) or the seleninic acid ($ESeO_2H$). For the former derivative, the observed absorbance decays were more rapid than those observed for the seleninic acid under similar conditions. Moreover, with $ESeO_2H$ a lag phase was apparent for the peroxidase reaction: an initially slow rate of thiol consumption, which gave way to a faster "turnover" rate, was observed. We interpret this lag as initial reduction of the seleninic acid group by thiol, followed by turnover as the selenenic acid oxidation state is reached. Indeed, the lag time was reduced as thiol concentration was increased, and the initial rate for the peroxidase activity of $ESeO_2H$ approaches that of ESeSAr at high thiol concentrations. These observations are consistent with the mechanism shown in Scheme I, originally proposed for glutathione peroxidase (Ladenstein, 1984; Ganther & Kraus, 1984). In this mechanism, the seleninic acid lies off the main catalytic cycle but may become important at very high concentrations of hydroperoxide. The proposed catalytic cycle itself processes the substrates via the selenenic acid, selenenyl sulfide, and selenol forms of selenosubtilisin as shown, and it is clearly consistent with our observation of ping-pong kinetics.

No inhibition of selenosubtilisin's peroxidase activity by eglin-c was observed, even at concentrations of eglin-c as high as 160 μM. This is consistent with the increased disruption

of the binding pocket which would be expected in ESeSAr. The catalytic cycle (Scheme I) also contains the intermediates ESeH and ESeOH, for which some inhibition by eglin-c might be expected, since the binding site in these cases is presumably no more disrupted than in the case of the seleninic acid. The absence of any observed inhibition of the peroxidase activity suggests that the concentrations of ESeH and ESeOH may not be significant under steady-state conditions and hence that ESeSAr is the predominant species in the turnover reaction. This idea is reinforced by the fact that the selenenyl sulfide form is stable and may be isolated, whereas selenolates are prone to rapid oxidation and selenenic acids are known to be quite unstable.

The peroxidase activity of selenosubtilisin was examined from pH 5 to 8 at a constant thiol concentration of 200 μM. Values of k_{cat}(app) and $K_{t\text{-BuOOH}}$(app) were obtained by fitting the experimental data to the Michaelis–Menten kinetic scheme. This treatment yielded the profiles for k_{cat}(app) and k_{cat}(app)/$K_{t\text{-BuOOH}}$(app) shown in Figure 6. The data for k_{cat}(app) vs pH were fit to

$$k_{cat}(\text{app}) = \frac{k_{max}(\text{app})}{1 + K_1/[H^+] + [H^+]/K_2} \quad (5)$$

while the data for k_{cat}(app)/$K_{t\text{-BuOOH}}$(app) vs pH were fit to

$$\frac{k_{cat}(\text{app})}{K_{t\text{-BuOOH}}(\text{app})} = \frac{k_{max}(\text{app})/K_{t\text{-BuOOH}}(\text{app})}{1 + K_1/[H^+]} \quad (6)$$

Although a good deal of caution should be exercised in the interpretation of pH-rate profiles (Knowles, 1976), particularly when the reaction mechanism is as complicated as that proposed for this peroxidation, it can be seen that the curves in Figure 6 are in accord with the anticipated mechanism. Both profiles suggest that maximal rate requires the protonation of a group with a p$K_a \approx 7$. This is consistent with a requirement that His64, which has a reported pK_a value of 7.22 in native subtilisin (Jordan et al., 1985), must be protonated for optimal enzymic efficiency. ^{77}Se NMR spectroscopy has shown that the prosthetic group in selenosubtilisin is deprotonated above pH 4 in the seleninic acid and selenol oxidation states (House et al., 1992), presumably because of hydrogen-bonding interactions which stabilize the anionic forms of the selenium side chain. Furthermore, recent ^{1}H NMR spectroscopic studies indicate that the pK_a of His64 is raised significantly, perhaps by 2–3 pH units, in the seleninate ($ESeO_2^-$) and selenolate (ESe^-) forms of selenosubtilisin (House et al., 1993). It therefore seems unlikely that the titrating group seen in the pH-rate profiles (Figure 6) could be His64 in the seleninate or selenolate forms of the enzyme, since the pK_a of the apparent ionizing group is far too low. Furthermore, no such ionizing group is seen in the plot of initial rate vs pH for the conversion of $ESeO_2H$ to ESeSAr (Figure 4). If the proposed mechanism (Scheme I) is correct, therefore, it seems most likely that the critical protonated group would be His64 in the selenenyl sulfide form of the enzyme, and ^{1}H NMR studies on ESeSAr indicate that its active site histidine has a p$K_a \approx 7$ (House et al., 1993). This interpretation means that the rate-determining transformation would be conversion of (ESeSAr + ArS^-) to (ESe^- + ArSSAr), with the protonated His64 perhaps facilitating displacement of the selenolate group. Interaction of the selenol and His64 apparently lowers the pK_a of the prosthetic group, hence stabilizing the selenolate form, and this may account for much of the enhanced reactivity relative to the model system.

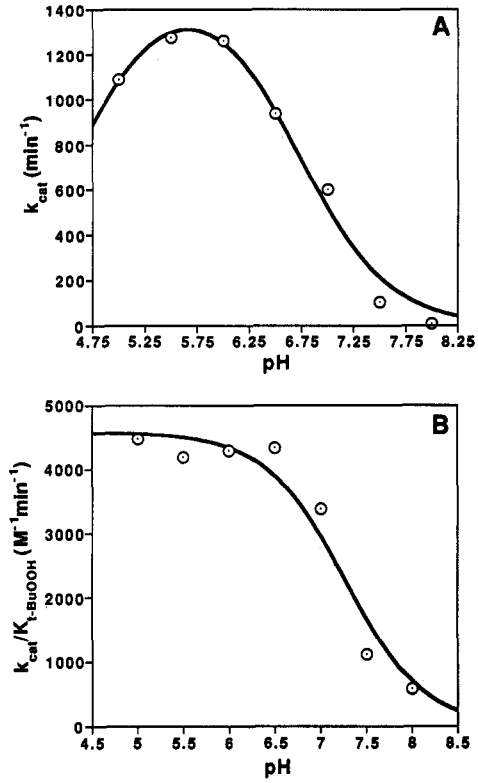


FIGURE 6: (A) k_{cat}(app) (min^{-1}) vs pH for 5 μM selenosubtilisin and 200 μM thiol **1** in buffer B at 25 °C. The data were fit to eq 5, to give pK_1 = 4.6 ± 0.1, pK_2 = 6.7 ± 0.1, and k_{max} = 1500 ± 100 min^{-1}. (B) k_{cat}(app)/$K_{t\text{-BuOOH}}$(app) (M^{-1} min^{-1}) vs pH for 5 μM selenosubtilisin and 200 μM thiol **1** in buffer B at 25 °C. The data were fit to eq 6, to give pK_1 = 7.3 ± 0.1 and k_{max}(app)/$K_{t\text{-BuOOH}}$(app) = 4600 ± 200 M^{-1} min^{-1}.

The bell-shaped curve seen for k_{cat}(app) vs pH reflects the ionization of the enzyme–substrate complex whose decomposition is rate-limiting. This profile suggests that efficient catalysis also depends upon the deprotonation of a group with a p$K_a \approx 4.6$ in the enzyme–substrate complex. Although a number of factors may be responsible for this decrease in k_{cat} at low pH, it is interesting to note that the estimated pK_a is remarkably close to that of thiol **1**, whose pK_a = 4.43 ± 0.02 by spectrophotometric titration. This would be consistent with a requirement for thiolate, ArS^-, as a nucleophile in the rate-determining step, again suggestive of the conversion of ESeSAr to ESe^-.

Inactivation of Selenosubtilisin with Iodoacetamide. The dependence of the yield of isolated selenenyl sulfide on thiol concentration suggested to us that this form of the selenoenzyme was in equilibrium with the corresponding selenolate:

$$\text{ESeSAr} + \text{ArS}^- \rightleftharpoons \text{ESe}^- + \text{ArSSAr} \quad (7)$$

The position of equilibrium lies far to the left, but the selenolate, if removed from the reducing environment (for example, by gel filtration), would be readily oxidized aerobically. We anticipated that the selenol might also be irreversibly trapped by a suitable alkylating agent (Forstrom et al., 1978). To test this hypothesis, ESeSAr was incubated with a large excess of iodoacetamide at pH 5 in the presence of thiol **1**. Protein recovered by gel filtration after three successive additions of thiol had completely lost its peroxidase activity. As a control, selenosubtilisin was similarly treated with iodoacetamide, but in the absence of thiol. This batch of enzyme was recovered with full activity. These results show that the selenol group, produced by reduction of selenosubtilisin, can be irreversibly modified by iodoacetamide

and that this modification of the prosthetic group abolishes peroxidase activity.

Thiol Specificity. In addition to 3-carboxy-4-nitrobenzenethiol, a number of thiols have been examined as potential substrates for the peroxidase reaction catalyzed by selenosubtilisin. Preliminary investigations on glutathione, DL-dithiothreitol, DL-dihydrolipoamide, and DL-dihydrolipoic acid indicate that these thiols are not particularly good substrates for the enzyme. Although the enzymic reaction exhibited a large rate enhancement compared to the spontaneous oxidation of each thiol by hydroperoxide, at a variety of different pH values and different concentrations of thiol and hydroperoxide selenosubtilisin exhibited only a moderate advantage over the model diselenide compound, *N*-(*tert*-butoxycarbonyl)aminoethyl diselenide. With DL-dihydrolipoamide, at pH 5.5 and 25 °C, the rate advantage of the enzyme over the model system was approximately 20-fold. For glutathione, the enzymic advantage was even lower. These results should not be surprising, since enzymes have evolved to bind specific substrates, and this specificity is one of their most desirable properties. A semisynthetic enzyme retains some of the substrate specificity of its native template, and this should be exploited if optimal rates are to be achieved. The aromatic thiol **1** may be able to take some advantage of the binding site of the subtilisin template, since the enzymic system exhibits a significant rate advantage over the model system for this peroxidase activity, but there is no reason to believe that this activity is optimal. We are currently employing molecular modeling techniques to elucidate the conformational preferences and stability of the (ESeSR·RS$^-$) complex in order to identify thiol substrates which may utilize the enzyme's binding energy more efficiently.

Selenosubtilisin and Glutathione Peroxidase. The kinetic data for both selenosubtilisin and glutathione peroxidase can be fit to a triple-transfer mechanism and may be described by the following general rate law (Dalziel, 1969):

$$\frac{[E]_o}{v_o} = \phi_o + \frac{\phi_{RSH}}{[RSH]} + \frac{\phi_{t\text{-BuOOH}}}{[t\text{-BuOOH}]} \quad (8)$$

For glutathione peroxidase, acting on *tert*-butyl hydroperoxide and glutathione, at pH 6.7 and 37 °C, $\phi_{t\text{-BuOOH}} = 2.25 \times 10^{-9}$ M min, and $\phi_{RSH} = 3.73 \times 10^{-8}$ M min (Ladenstein, 1984). The equivalent parameters for selenosubtilisin, acting on thiol **1** and *tert*-butyl hydroperoxide, at pH 5.5 and 25 °C, are $\phi_{t\text{-BuOOH}} = 2.5 \times 10^{-4}$ M min and $\phi_{RSH} = 6.5 \times 10^{-8}$ M min, by inspection of eq 4. These parameters cannot be directly equated, since they were measured at different pH and temperature for different thiol substrates, but they do allow an approximate comparison of the two enzymes.

Glutathione peroxidase is believed to have evolved to near optimal efficiency for the decomposition of hydroperoxides, having an apparent second-order rate constant for the reaction between hydroperoxide and enzyme of the order of 10^8 M^{-1} min^{-1}. Selenosubtilisin falls far short of this ideal, giving an equivalent rate constant for hydroperoxide consumption of approximately 4000 M^{-1} min^{-1}. The rates of reaction between the two enzymes and their respective thiol substrates are, however, very similar, as evidenced by the values of ϕ_{RSH} above. This shows that a semisynthetic enzyme, exhibiting a different activity from its native template counterpart and acting upon a substrate which the protein has not evolved to bind, can exhibit a rate of reaction which is similar to that of a highly-evolved natural enzyme reacting with its natural substrate.

Despite this relatively rapid rate of reaction between thiol and selenosubtilisin, the enzyme is clearly far less efficient than the natural peroxidase. In this context, it is interesting to note that ϕ_o has a finite value for selenosubtilisin but is equal to zero for glutathione peroxidase. This indicates that there is no accumulation of catalytic intermediates for the latter enzyme but that such intermediates do build up in the case of selenosubtilisin (Ladenstein, 1984). Our kinetic data suggest that the attack of ArS$^-$ on ESeSAr may be rate-determining to some degree and hence that ESeSAr would accumulate, while the concentration of ESe$^-$ would be low, under steady-state conditions. Such a low concentration of the selenolate may be responsible for the slow rate of consumption of peroxide, which reacts with this enzymic intermediate. As discussed above, it may be possible to design a thiol substrate for selenosubtilisin which could utilize the enzyme's binding energy to overcome the rate-determining step and hence circumvent this problem.

CONCLUSION

The semisynthetic approach to enzyme mutation allows the introduction of a new side chain to the active site of an existing enzyme. We have demonstrated how a single atom change, from oxygen to selenium, in the active site serine of subtilisin can radically alter the enzyme's pattern of reactivity. Not only is its behavior with ester substrates dramatically shifted, favoring aminolysis over hydrolysis as compared with the native subtilisin (Wu & Hilvert, 1989), but the selenoenzyme also acts as a glutathione peroxidase mimic.

In this latter capacity, selenosubtilisin catalyzes the oxidation of 3-carboxy-4-nitrobenzenethiol with concomitant reduction of *tert*-butyl hydroperoxide. Our investigations support the mechanism illustrated in Scheme I and show that the enzyme has a substantial rate advantage over model compounds. These studies also indicate that the selenenyl sulfide oxidation state of selenosubtilisin is the catalytically important one, rendering a variety of catalytic schemes which process via the seleninic acid unlikely. The rate-determining step appears to be conversion of (ESeSAr + ArS$^-$) to (ESe$^-$ + ArSSAr), and His64, which is the catalytic general base in native subtilisin, is implicated as a general acid or electrostatic catalyst, facilitating the departure of the selenolate group.

In order to optimize the peroxidase activity of selenosubtilisin, the thiol and peroxide must be able to fully utilize the active site geometry and the binding specificity of the enzyme. While it is very likely that there are potential substrates which would achieve much higher enzymic efficiency, determining the exact nature of these substrates is not straightforward. We expect that a molecular modeling approach will help in the search for such optimal combinations of thiol and peroxide. Modeling studies may also enable us to rationally mutate the subtilisin template by genetic methods in order to enhance reactivity and specificity. Such site-directed mutagenesis techniques should also allow us to further probe the mechanism of selenosubtilisin, for instance by investigating the importance of the oxyanion hole for stabilizing ESe$^-$, or the role of Asp32 in raising the pK_a of His64 in the ESeO$_2^-$ and ESe$^-$ forms of the enzyme.

The studies presented here, in conjunction with X-ray crystallography and NMR spectroscopy, are enhancing our understanding of the peroxidase activity of selenosubtilisin and may help resolve some of the mechanistic questions about glutathione peroxidase itself. Moreover, this investigation reinforces the idea that a protein template can be transformed

by a simple mutation, to give an enzymic activity for which it did not evolve: a different prosthetic group placed in the environment of a protein binding site can equal a new enzyme. This approach may yield useful catalysts for industrial and medical applications and also a greater understanding of the mechanisms, and the evolution, of biological catalysts.

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REFERENCES

Bergmann, M., & Zervas, L. (1932) *Ber. Dtsch. Chem. Ges. 65B*, 1192–1201.
Bode, W., Papamokos, E., & Musil, D. (1987) *Eur. J. Biochem. 166*, 673–692.
Breslow, R. (1991) *Ciba Found. Symp. 158*, 115–127.
Cavallini, D., Graziani, M. T., & Dupré, S. (1966) *Nature (London) 212*, 294–295.
Cram, D. J. (1988) *Science 240*, 760–767.
Dalziel, K. (1969) *Biochem. J. 114*, 547–556.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.
Epp, O., Ladenstein, R., & Wendel, A. (1983) *Eur. J. Biochem. 133*, 51–69.
Forstrom, J. W., Zakowski, J. J., & Tappel, A. L. (1978) *Biochemistry 17*, 2639–2644.
Ganther, H. E., & Kraus, R. J. (1984) *Methods Enzymol. 107*, 593–602.
Gold, A. M., & Fahrney, D. (1964) *Biochemistry 3*, 783–791.
Heinz, D. W., Preistle, J. P., Rahnel, J., Wilson, K. S., & Grütter, M. G. (1991) *J. Mol. Biol. 217*, 353–371.
Hilvert, D., & Kaiser, E. T. (1987) *Biotechnol. Genet. Eng. Rev. 5*, 297–318.
House, K. L., Dunlap, R. B., Odom, J. D., Wu, Z.-P., & Hilvert, D. (1992) *J. Am. Chem. Soc. 114*, 8573–8579.
House, K. L., Dunlap, R. B., Odom, J. D., & Hilvert, D. (1993) *Biochemistry* (in press).
Jordan, F., Polgár, L., & Tous, G. (1985) *Biochemistry 24*, 7711–7717.
Kice, J. L., & Lee, T. W. S. (1978) *J. Am. Chem. Soc. 100*, 5094–5102.
Klayman, D. L., & Griffin, T. S. (1973) *J. Am. Chem. Soc. 95*, 197–199.
Knowles, J. R. (1976) *CRC Crit. Rev. Biochem. 4*, 165–173.
Knowles, J. R. (1987) *Science 236*, 1252–1258.
Kolthoff, I. M., & Medalia, A. I. (1949) *J. Am. Chem. Soc. 71*, 3789–3792.
Ladenstein, R. (1984) *Pept. Protein Rev. 4*, 173–214.
Lehn, J. M. (1985) *Science 227*, 849–856.
Lerner, R. A., Benkovic, S. J., & Schultz, P. G. (1991) *Science 252*, 659–667.
Lewis, C. T., & Hilvert, D. (1991) *Curr. Opin. Struct. Biol. 1*, 624–629.
McCullough, J. D., & Gould, E. S. (1949) *J. Am. Chem. Soc. 71*, 674–677.
Markland, F. S., Jr., & Smith, E. L. (1971) in *The Enzymes* (Boyer, P. D., Ed.) 3rd ed., Vol. 3, pp 561–608, Academic Press, New York.
Neet, K. E., Nanci, A., & Koshland, D. E., Jr. (1968) *J. Biol. Chem. 243*, 6392–6401.
Philipp, M., & Bender, M. L. (1983) *Mol. Cell. Biochem. 51*, 5–32.
Polgár, L., & Bender, M. L. (1967) *Biochemistry 6*, 610–621.
Reich, H. J., & Jasperse, C. P. (1987) *J. Am. Chem. Soc. 109*, 5549–5551.
Seemüller, U., Dodt, J., Fink, E., & Fritz, H. (1986) in *Proteinase Inhibitors* (Barret, A. J., & Salvesen, G., Eds.) pp 347–355, Elsevier, Amsterdam.
Silver, M. (1979) *Methods Enzymol. 62D*, 135–136.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76–85.
Stadtman, T. C. (1990) *Annu. Rev. Biochem. 59*, 111–127.
Syed, R., Hogle, J., Wu, Z.-P., & Hilvert, D. (1993) *Biochemistry* (submitted).
Syper, L., & Mlochowski, J. (1984) *Synthesis*, 439–442.
Wells, J. A., & Estell, D. A. (1988) *Trends Biochem. Sci. 13*, 291–297.
Wu, Z.-P., & Hilvert, D. (1989) *J. Am. Chem. Soc. 111*, 4513–4514.
Wu, Z.-P., & Hilvert, D. (1990) *J. Am. Chem. Soc. 112*, 5647–5648.