Structural Characterization of Selenosubtilisin by ^{77}Se NMR Spectroscopy

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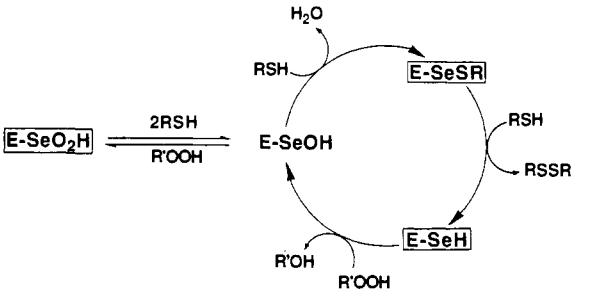
Abstract: The semisynthetic selenoenzyme selenosubtilisin catalyzes the reduction of alkyl hydroperoxides with concomitant oxidation of 3-carboxy-4-nitrobenzenethiol. To gain insight into the mechanism of this process, three oxidation states of the enzyme that are relevant to the redox cycle have been characterized by ^{77}Se NMR spectroscopy. Oxidation of selenosubtilisin with hydrogen peroxide in the absence of thiol yields a species whose spectrum consists of two peaks at 1188 and 1190 ppm, consistent with the selenium prosthetic group being in the seleninic acid oxidation state. The observation of two signals suggests that Met222 in the active site may have been partially oxidized during preparation of the sample and illustrates the sensitivity of the selenium probe to its electronic microenvironment. The pK_a value of the enzyme-bound seleninic acid is at least 1.5 pH units lower than that of simple alkane seleninic acids, as determined by the pH dependence of its NMR signals. This fact indicates significant stabilization of the conjugate base of the seleninic acid by proximal hydrogen bonding groups within the active site and accounts for the relatively sluggish rate at which oxidized selenosubtilisin reacts with thiols. Reduction of the oxidized enzyme with 3 equiv of 3-carboxy-4-nitrobenzenethiol cleanly produces the cognate disulfide and an enzyme-bound selenenyl sulfide derivative (^{77}Se 389 ppm). No other intermediates at the selenenic acid oxidation state were detected spectroscopically, including either the selenenic acid itself or a cyclic selenamide in which the selenium is covalently connected to the protein backbone. Treatment of the enzyme with excess dithiothreitol at neutral pH, on the other hand, reduces the prosthetic group to the selenol, as judged by the single broad ^{77}Se resonance observed at −215 ppm. Like the seleninic acid, the selenol has an unusually low pK_a (<4) and is deprotonated at all accessible pHs. In analogy to the chemistry of glutathione peroxidase, an important naturally occurring selenoenzyme, both the selenenyl sulfide and selenol derivatives of selenosubtilisin are believed to be key intermediates in the peroxidase action of the enzyme, while the seleninic acid is most likely a side product that is formed at high peroxide concentrations. This study thus demonstrates the feasibility of using ^{77}Se NMR spectroscopy to characterize an active selenoenzyme's catalytic center. The enormous chemical shift range and the sensitivity of this technique to subtle variations in chemical environment promise to be invaluable for probing structure–function relationships in this and related selenium-containing proteins.

Introduction

Selenosubtilisin, an artificial enzyme possessing an active-site selenocysteine residue, may be prepared by site-selective chemical modification of the catalytically essential serine residue (Ser221) of the bacterial protease subtilisin Carlsberg. Introduction of the selenium prosthetic group into the binding pocket confers novel hydrolytic and redox properties to the original protease template. For example, although selenosubtilisin is a poor catalyst for amide hydrolysis, it promotes acyl transfer reactions with much higher selectivity than does unmodified subtilisin, greatly favoring aminolysis of the acyl-enzyme intermediate over hydrolysis.[1]

Selenosubtilisin also catalyzes the reduction of alkyl hydroperoxides by thiols.[2] In this respect it mimics the action of the important mammalian enzyme glutathione peroxidase (EC 1.11.1.9), one of several naturally occurring selenoproteins that function in cellular redox reactions. Glutathione peroxidase plays a vital role in the detoxification of organic hydroperoxides in vivo, protecting lipid membranes from oxidative damage.[3] Its biological efficacy is a consequence of high catalytic efficiency: apparent bimolecular rate constants in excess of 10^7 M^{-1} s^{-1} have been measured for the reduction of hydrogen peroxide by glutathione in the presence of the enzyme.[4] The active-site selenocysteine is known to be essential for the peroxidase activity,[5] and a catalytic cycle has been proposed that involves interconversion of the selenol (ESeH), selenenic acid (ESeOH), and selenenyl sulfide (ESeSR) forms of the protein (Scheme I).[6] The seleninic acid (ESe(O)OH) is also formed at high concentrations of hydroperoxide but is not believed to be relevant to normal enzymatic turnover. Despite extensive kinetic and structural studies, however, many questions remain about glutathione peroxidase's detailed mechanism of action. Alternative catalytic cycles have been suggested,[7] and the functional roles of other amino acids in the active site have yet to be elucidated.

Scheme I



The availability of an artificial selenoenzyme with peroxidase activity makes it possible to examine how the protein microenvironment modulates the intrinsic reactivity of the selenium functionality. In contrast to glutathione peroxidase, which is composed of four identical subunits (M_r 21 000), selenosubtilisin is a relatively small (M_r 27 400) monomeric protein. The crystal structure of an oxidized form of selenosubtilisin was recently

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determined at 2.0-Å resolution.[8] The selenocysteine residue, in the seleninic acid oxidation state, is located in a well-defined binding site and is part of what was, in the unmodified protease, a catalytic triad consisting of Asp32, His64, and Ser221 (SeCys221). The prosthetic group is also adjacent to an oxyanion binding pocket that contributes to the stabilization of the tetrahedral intermediates that form during hydrolysis of esters and amides by the native enzyme.[9] The active center of glutathione peroxidase, on the other hand, is located in a flat depression on the surface of each subunit.[10] The crystal structure of the fully reduced enzyme shows that the selenolate anion (ESe^-) forms hydrogen bonds to Nε1 of Trp148 and to Nε2 of Gln70, although the functional role of these residues is uncertain. The seleninic acid derivative, obtained by treating glutathione peroxidase with 5 mM H_2O_2, is also stabilized by multiple hydrogen bonding interactions with nearby active-site residues. The exposed location of the selenium moiety in both forms of the peroxidase provides ready access to substrate and perhaps accounts for the enzyme's high reaction rates.

X-ray diffraction methods provide only one source of structural data on proteins. NMR spectroscopy can yield important complementary information. ^{77}Se NMR spectroscopy, in particular, is a potentially powerful tool for characterizing the oxidation states, ligands, and active-site environment of selenoenzymes. Because selenium-77 has a nuclear spin of $^1/_2$ and a large dynamic chemical shift range (ca. 3400 ppm), it is extraordinarily sensitive to its electronic surroundings.[11] In this regard, the use of ^{77}Se NMR spectroscopy to determine the handedness of chiral centers up to eight atoms from a selenium reporter group is especially noteworthy.[12] The feasibility of applying this technique to the study of large biomolecules was established some years ago with thiol-containing proteins chemically modified with [^{77}Se]-6,6′-diselenobis(3-nitrobenzoic acid).[13,14] ^{77}Se NMR spectroscopy has since been used to monitor the binding of a selenium-containing inhibitor to chymotrypsin and to characterize a selenium-containing acyl–enzyme intermediate on the same enzyme.[15] Experiments with naturally occurring selenoenzymes have been limited, however, by the difficulty of preparing samples highly enriched in ^{77}Se. It was necessary, for example, to maintain sheep for 5 months on a selenium-deficient diet supplemented with $Na_2{}^{77}SeO_3$ (94%) as the only selenium source in order to obtain sufficient quantities of labeled erythrocyte glutathione peroxidase for spectroscopic investigation.[16] Unfortunately, attempts to characterize a catalytically active form of this enzyme by ^{77}Se NMR spectroscopy failed because of the sample's limited solubility; only derivatives of the reduced and denatured protein could be studied.

Unlike glutathione peroxidase, isotopically enriched selenosubtilisin is readily available in large amounts via semisynthesis. Because of its high solubility and stability, the artificial peroxidase is particularly suited for spectroscopic investigation. We report herein the use of ^{77}Se NMR spectroscopy to characterize three derivatives of selenosubtilisin that are pertinent to a detailed understanding of its redox properties: the seleninic acid (ESe(O)OH), the selenenyl sulfide (ESeSAr), and the selenol (ESeH). This work lays the groundwork for future studies aimed at correlating enzyme structure with chemical function.

Experimental Section

Selenosubtilisin. [^{77}Se]Selenosubtilisin was synthesized according to the method of Wu and Hilvert[1] using hydrogen selenide derived from 94.7% isotopically enriched elemental selenium (Oak Ridge National Laboratories). In a typical experiment, ((phenylmethyl)sulfonyl)-subtilisin (PMS-subtilisin) (1.2 mM) and HSe^- (8.5 mM) were allowed to react in 15 mL of 1,4-piperazinebis(ethanesulfonic acid) (PIPES)/$CaCl_2$ buffer (10 mM each, pH 7.0) for 48 h at 40 °C. Yields of purified [^{77}Se]selenosubtilisin were 10–20% based on PMS-subtilisin.

The seleninic acid form of selenosubtilisin (ESe(O)OH) was obtained by sequentially dialyzing the protein against 20 mM dithiothreitol (DTT) and 20 mM H_2O_2 and then exhaustively against buffer alone (10 mM PIPES/10 mM $CaCl_2$, pH 7.0). Reaction of the resulting oxidized enzyme with 3 equiv of 3-carboxy-4-nitrobenzenethiol at pH 5.0 for 15 min at ambient temperature yielded the corresponding selenenyl sulfide derivative (ESeSAr).[2] The fully reduced enzyme (ESeH) was prepared by treating either ESe(O)OH or ESeSAr with 30 mM DTT under an inert atmosphere.

Selenosubtilisin was denatured as described for native subtilisin.[17] At neutral pH, a combination of 8 M urea and 10% sodium dodecyl sulfate (SDS) was required to unfold the protein. At pH ≤4.0, denaturation was achieved in a buffer containing 2 M guanidinium hydrochloride. Conditions were established using a published assay[17] that relies on the differing electrophoretic mobilities of the folded and unfolded enzyme.

Benzeneseleninic Acid. Benzeneseleninic acid was prepared according to the method of McCullough and Gould.[18]

***N*-(*tert*-Butoxycarbonyl)selenocysteamine.** To a stirred solution of selenocysteamine (100 mg, 313 mmol) in 1 mL of H_2O/*tert*-butyl alcohol (1:1 v/v) was added 4 equiv of triethylamine (175 μL, 1.26 mmol), followed by 2 equiv of 2-[((*tert*-butoxycarbonyl)oxy)imino]-2-phenylacetonitrile (154 mg, 0.625 mmol).[19] The resulting mixture was stirred at room temperature until no starting material was detected by TLC (Et_2O/hexane, 2/3). The reaction mixture was extracted with ethyl acetate, and the organic layer was filtered through a silica gel plug to remove excess triethylamine. The desired product was obtained as a colorless oil (110 mg, 78%) after purification by preparative TLC (Et_2O/hexane, 2/3): ^1H NMR (300 MHz, $CDCl_3$) δ 5.11 (s, 1 H), 3.44 (m, 2 H), 2.97 (t, 2 H, J_{H-H} = 6.6 Hz), 1.41 (s, 9 H).

***N*-(*tert*-Butoxycarbonyl)selenohypotaurine.** The preparation of *N*-(*tert*-butoxycarbonyl)selenohypotaurine was analogous to that of benzeneseleninic acid.[18] Hydrogen peroxide (76 μL of a 30 wt % solution in water, 0.672 mmol) was added dropwise to an ice-cooled solution of *N*-(*tert*-butoxycarbonyl)selenocysteamine (100 mg, 0.224 mmol) in 1 mL of 1,4-dioxane. The solution was allowed to warm slowly to room temperature and then to stir for 1 h. Product was precipitated by cooling, collected by filtration, and washed with a small amount of ice water to give a white solid (80.3 mg, 70%): ^1H NMR (300 MHz, D_2O) δ 3.55 (m, br, 2 H), 3.17 (t, br, 2 H), 1.41 (s, 9 H); negative ion, high resolution FAB mass spectrum calcd for $C_7H_{14}O_4N^{76}Se$ 252.0115, found 252.0112.

Buffers and pH Measurements. All NMR experiments were conducted in buffer A (a modified Morrison buffer[20] containing 50 mM acetate, 50 mM 4-morpholineethanesulfonic acid (MES), 100 mM tris(hydroxymethyl)aminomethane (Tris), and 10 mM $CaCl_2$) or buffer B (50 mM PIPES, 10 mM $CaCl_2$). Buffer composition and ionic strength had no effect on the ^{77}Se chemical shift or the half-height line width ($\nu_{1/2}$) of the protein samples or the model compounds. The pH of all samples was measured at the probe temperature of the relevant experiment. Measurements were made using a Microelectrode MI-410 probe coupled to an Orion SA 720 pH meter.

Instrumental. ^{77}Se NMR spectra were recorded on either a Bruker WP200 (38.17 MHz) or a Bruker AM300 (57.24 MHz) FT-NMR spectrometer equipped with a 10-mm probe. A tip angle of 45° was routinely used in all experiments, and the probe temperature in each experiment was maintained within ±1 °C of the set temperature. A recycle time of 3 s was used with the WP200 to minimize sample heating due to the high power levels required by the broad band decoupler. The AM300 spectrometer is equipped with a composite pulse decoupler which operates at a much lower power level and does not initiate sample heating. Therefore, the recycle time for the AM300 was typically 0.3 s. Between 20000 and 120000 FID's were acquired for each sample, and all chemical shifts were referenced externally to 60% $(CH_3)_2Se$ in

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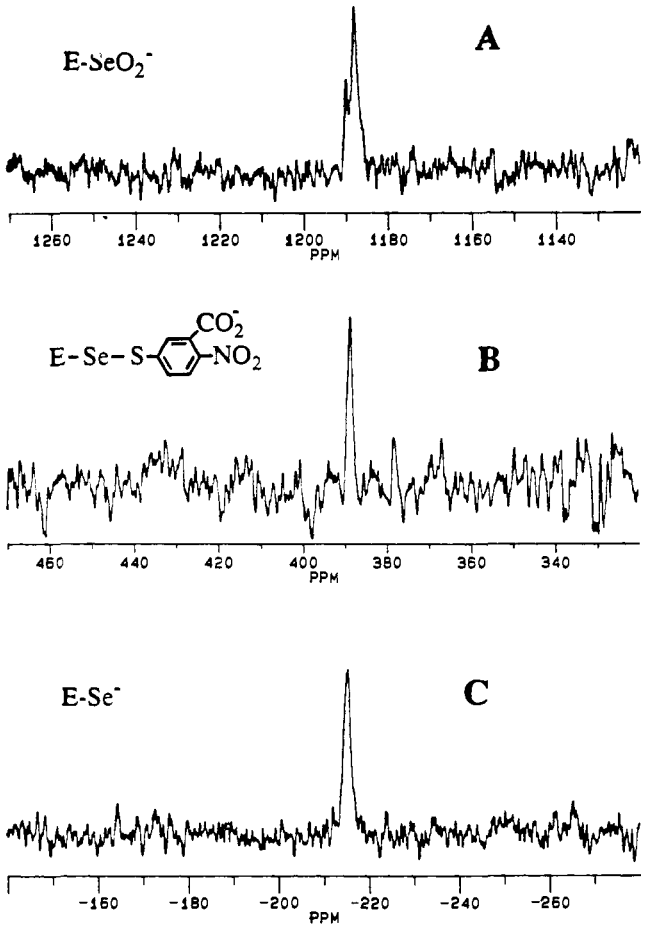


Figure 1. ^{77}Se NMR spectra of (A) 1.38 mM oxidized selenosubtilisin ($ESe(O)O^-$) at pH 7.0 in buffer A (57.24 MHz), (B) 0.61 mM selenenyl 3-carboxy-4-nitrophenyl sulfide form of selenosubtilisin (ESeSAr) at pH 5.0 in buffer B (38.16 MHz), and (C) 0.93 mM selenol form of selenosubtilisin (ESe^-) at pH 7 in buffer A (57.24 MHz).

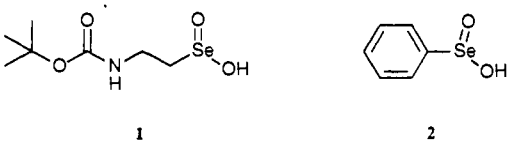
$CDCl_3$.[21] Sample volume was typically 2 mL with protein concentrations between 0.4 and 2.0 mM. The FID's were acquired with a 32K data set and zero-filled to 64K before Fourier transformation. A line broadening of 20 Hz was also applied to each FID before Fourier transformation. ^{77}Se NMR spectra of the seleninic acid model compounds were recorded in a manner analogous to that for the protein samples. The concentrations of benzeneseleninic acid and *N*-(*tert*-butoxycarbonyl)selenohypotaurine were 423 and 41 mM, respectively. A line broadening of 7 Hz was applied to each FID before Fourier transformation.

Results and Discussion

Oxidized Selenosubtilisin. Many oxidation states are available to organic selenium compounds. To convert the prosthetic group in selenosubtilisin into a single, chemically well-defined state, the initially isolated protein was first reduced with excess DTT and then oxidized with 5 mM H_2O_2. This procedure yields a form of the enzyme that is stable in air and can be stored for many months at 4 °C without change. Oxidized selenosubtilisin is also soluble at relatively high concentrations (up to ca. 5 mM) and thus is an ideal starting point for the NMR structural investigations described herein. Because of the low natural abundance of ^{77}Se (7.5%), however, the enzyme was prepared in an isotopically enriched form (94.7% ^{77}Se).

Chemical evidence had previously suggested that the selenocysteine in selenosubtilisin was present as the seleninic acid ($ESeO_2H$) after treatment with H_2O_2.[2] Consistent with this tentative assignment, the ^{77}Se NMR spectrum of the enzyme exhibits two resonances at 1188 ppm ($\nu_{1/2}$ 53 Hz) and 1190 ppm ($\nu_{1/2}$ 29 Hz) in a ratio of 3:2 (Figure 1A). For comparison, the chemical shifts reported for organoseleninic acids ($RSeO_2H$) in DMSO-d_6 are typically between 1175 and 1240 ppm.[11b] Selenenic acids (RSeOH) and selenonic acids ($RSeO_3H$) resonate outside this range at roughly 1090[22] and 1300[11a] ppm, respectively.

We have examined simple alkane- and areneseleninic acids under our experimental conditions as models of the prosthetic group in selenosubtilisin. The ^{77}Se NMR spectra of *N*-(*tert*-butoxycarbonyl)selenohypotaurine (**1**) and benzeneseleninic acid (**2**) were found to have single, sharp resonances at 1187 and 1152



1 2

ppm, respectively, in aqueous buffer (pH 8.0). The chemical shift of the alkane seleninic acid is thus very close to that observed for selenosubtilisin, while the areneseleninic acid appears at a more shielded position. The spectra of the model systems differ strikingly from that of the enzyme, however, in terms of signal multiplicity.

Preliminary data from the 2.0-Å resolution crystal structure of selenosubtilisin show that the seleninic acid occupies a well-defined conformation within the active site.[8] It is embedded in an extensive hydrogen bonding network with one of the seleninic acid oxygens forming a direct hydrogen bond with Nε2 of His64, an essential general base in the unmodified protein, and the other oxygen interacting with the oxyanion hole which is comprised of the side chain amide of Asn155 and the backbone amide of residue 221. The observation of two signals in selenosubtilisin's ^{77}Se NMR spectrum was therefore surprising but may simply reflect partial overoxidation of the enzyme. The loss of activity observed when native subtilisin is treated with hydrogen peroxide is known to result from oxidation of the active-site residue Met222 to the corresponding methionine sulfoxide.[23] If Met222 were partially oxidized during the preparation of oxidized selenosubtilisin, the electronic microenvironment of the selenium prosthetic group could be significantly perturbed. The quality of the X-ray diffraction data does not allow the oxidation state of Met222 in selenosubtilisin to be determined with confidence, but structural studies of oxidized native subtilisin[24] show that the resulting sulfoxide would have the *S* configuration and that the sulfoxide oxygen would consequently point directly toward the seleninic acid. Given the estimated distance of roughly 4–4.5 Å between the seleninic acid and the sulfoxide oxygen, the observed 2-ppm chemical shift difference for selenosubtilisin containing or lacking the methionine sulfoxide would underscore the sensitivity of the selenium probe to subtle through-space interactions and its potential for addressing important questions about structure and function.

Specific hydrogen bonding interactions between the seleninic acid and active-site residues, as seen in the crystal structure, would be expected to stabilize the seleninate form of the prosthetic group ($ESe(O)O^-$) significantly. This stabilizing influence is indeed reflected in the pH dependence of the enzyme's ^{77}Se NMR spectrum. The chemical shifts of simple alkane- and areneseleninic acids are sensitive to pH as expected for weak acids. For example, analysis of standard titration curves yielded pK_a values of 5.4 for the selenohypotaurine derivative **1** and 4.8 for benzeneseleninic acid (**2**) (Figure 2). The latter value is in good agreement with the pK_a of 4.79 reported by McCullough and Gould for **2**.[18] In contrast, the ^{77}Se chemical shifts of *both* selenosubtilisin signals are invariant over the pH range of 4.5–8.0 (Figure 2). Although precipitation of the protein precluded examination at lower pHs, it is clear from the data shown in Figure

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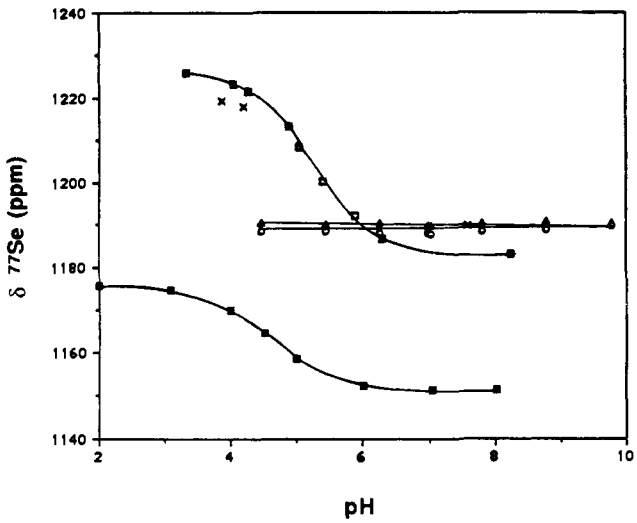

Figure 2. Titration data for *N*-(*tert*-butoxycarbonyl)selenohypotaurine (□), benzeneseleninic acid (■), native selenosubtilisin (ESe(O)O⁻ form) (▲, O), and denatured selenosubtilisin (ESe(O)O⁻ form) (×).

2 that the pK_a of the enzyme-bound seleninic acid is depressed by at least 1.5 pH units compared to **1**. Given their similar pH dependences, we can conclude that both forms of the enzyme are stabilized by a similar complement of hydrogen bonds.

The fact that the seleninic acid in selenosubtilisin is deprotonated at all accessible pH values has obvious functional significance. Reduction of seleninic acids by thiols is an acid-catalyzed process with protonation of the seleninate occurring prior to addition of the attacking thiolate.[25] Reduction of the seleninic acid form of selenosubtilisin also increases with decreasing pH, but the rate of the enzymic reaction is roughly 180 times slower than reduction of *N*-(*tert*-butoxycarbonyl)selenohypotaurine (**1**) under identical conditions.[26] The lower reactivity of the enzyme toward thiols corresponds roughly to the diminished stability of the conjugate acid of the prosthetic group within the active site. It is interesting to note that despite its sluggish stoichiometric reaction with thiols, selenosubtilisin is a much better catalyst for the reduction of alkyl hydroperoxides by 3-carboxy-4-nitrobenzenethiol under turnover conditions than is the model alkaneseleninic acid.[2,26] This fact suggests that the seleninic acid is not an essential intermediate during normal catalytic turnover, although its formation may become important at high peroxide concentrations, as proposed for glutathione peroxidase (Scheme I).

Although partial overoxidation can account for the fact that selenosubtilisin exhibits two seleninic acid resonances, we have explicitly considered two other explanations for this observation. First, multiple signals would also be seen if the seleninic acid side chain adopted two slowly interconverting conformations within the binding pocket. Although only one orientation is observed in the crystal structure of selenosubtilisin, additional energetically favorable conformers possessing distinctive NMR signatures may be accessible in solution. For example, seleninic acids have a pyramidal geometry, and simple rotation about the C–Se single bond would place the diastereotopic oxygen atoms in different electronic microenvironments. To investigate the potential conformational lability of the prosthetic group, we examined the effect of temperature on selenosubtilisin's ^{77}Se NMR spectrum. At low temperatures the enzyme exhibits only a single, broad resonance with a low signal-to-noise ratio that is centered at 1189 ppm (data not shown). As the sample temperature is raised from 4 to 22 °C, the resolution of the spectrum improves and signals for two seleninic acid species become apparent, as shown in Figure 1A. The improvement in the signal-to-noise ratio is a consequence of lower sample viscosity, and the temperature dependence of the

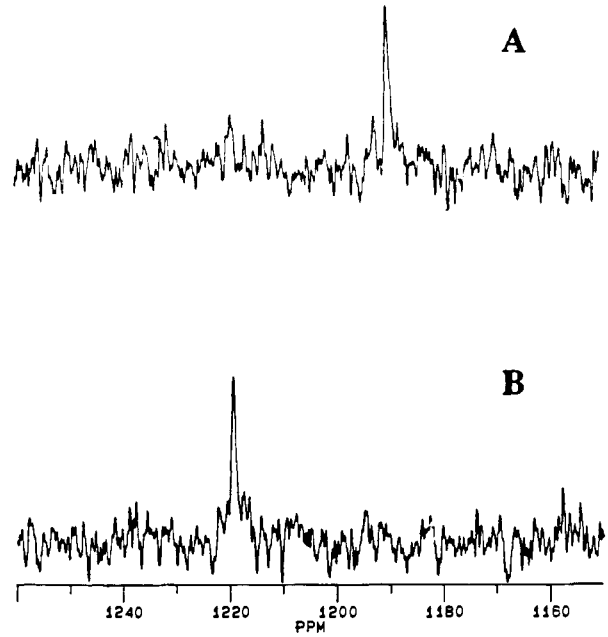

Figure 3. ^{77}Se NMR spectra (57.24 MHz) of ~0.5 mM denatured selenosubtilisin (ESe(O)O⁻ form) in buffer A at pH 7.58 (A) and at pH 3.87 (B).

^{77}Se chemical shifts is consistent with the results of earlier model studies.[21] Further increases in temperature to 35 °C did not result in coalescence of the seleninic acid NMR signals; higher temperatures could not be studied because of problems associated with protein precipitation. While these data do not allow us to rule out the possibility of multiple orientations of the prosthetic group, the fact that coalescence was not observed indicates that the putative alternate conformation must be locked into place by multiple hydrogen bonding interactions. Examination of the active site shows, however, that any rotation of the seleninic acid side chain from the conformation seen in the crystal structure necessitates loss of at least one set of stabilizing interactions, either with His64 or with the oxyanion binding site.[8]

Second, two seleninic acid resonances would also be observed in the ^{77}Se NMR spectrum of selenosubtilisin if our enzyme samples were contaminated with denatured protein. We examined this possibility by preparing and characterizing the unfolded enzyme directly. Selenosubtilisin, like the unmodified protease from which it is derived, is very resistant to denaturation.[27] A combination of urea (8 M) and sodium dodecyl sulfate (10%) was required to unfold the enzyme completely at ambient temperature and neutral pH. The denatured protein exhibited a single ^{77}Se resonance at 1189 ppm at pH 7.58 (Figure 3A), which shifted to 1219 ppm when the pH was lowered to 3.87 (Figure 3B). It was not possible to determine a complete titration curve with this sample, however, due to the steady decrease in signal intensity with time. A second batch of enzyme was therefore denatured, and a peak at 1217 ppm was observed at pH 4.1. Time-dependent loss of the ^{77}Se signal again precluded acquisition of additional titration data. As shown in Figure 2, the 30-ppm chemical shift difference observed for the denatured enzyme at low and high pH values mirrors the titration behavior of the model alkaneseleninic acid **2** (Figure 2), contrasting with the pH invariance of *both* signals of native selenosubtilisin over the same range. Consequently, neither peak in the spectrum of native selenosubtilisin can be attributed to denatured protein.

The apparent instability of denatured selenosubtilisin contrasts with the long-term stability of the correctly folded enzyme. To determine whether the selenium moiety remains attached to the unfolded protein, a denatured sample was subjected to minidialysis

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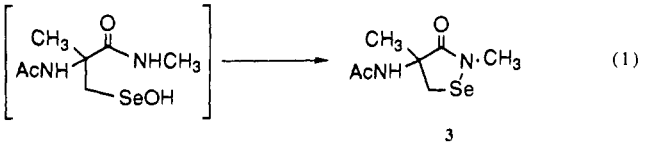
using a centrifugal microconcentrator (Amicon) and the buffer pass fraction was reduced with an acidic solution of $SnCl_2$.[28] This treatment resulted in the precipitation of elemental selenium as a red solid. The precipitate was collected as a pellet by centrifugation and shown to be readily resolubilized upon oxidation with aqueous sodium hypochlorite. Elemental selenium was not detected during analogous treatment of an 8-month-old sample of native selenosubtilisin that had not been exposed to denaturants. These results demonstrate that selenium loss, probably via a facile β-elimination process, accompanies denaturation of the protein. The concomitant appearance of multiple inorganic selenium species in fast chemical exchange in solution would account for the disappearance of the ^{77}Se NMR signal during an overnight run. In the X-ray structure, the seleninic acid is held in a geometry that is not conducive to β-elimination through its interactions with His64 and the oxyanion hole. It is expected that such constraints would be eliminated when the protein is unfolded.

Reaction of Selenosubtilisin with 3-Carboxy-4-nitrobenzenethiol. In analogy to the chemistry of benzeneseleninic acid,[25] oxidized selenosubtilisin reacts with 3 equiv of 3-carboxy-4-nitrobenzenethiol at pH 5.0.[2] In this process, 2 equiv of thiol are oxidized to the disulfide, 5,5′-dithiobis(2-nitrobenzoic acid). The remaining thiol is incorporated covalently into the enzyme and can be released upon subsequent treatment with DTT. The modified enzyme is also competent as a catalyst for the reduction of alkyl hydroperoxides at the expense of additional thiol.

The solubility of selenosubtilisin decreases after treatment with thiols, and considerable precipitation was observed at protein concentrations above ca. 1 mM. Nevertheless, it was possible to obtain a clean ^{77}Se NMR spectrum of the covalent intermediate, as shown in Figure 1B, consisting of a single resonance at 389 ppm with a half-height line width of 30 Hz. The observed chemical shift suggests that the prosthetic group in the modified enzyme is present as a selenenyl sulfide (ESeSAr). By way of comparison bis(alkyl selenenyl) sulfides have been reported to have chemical shifts in aqueous buffer in the range of 250–333 ppm.[29] It is probable that the electron-withdrawing nature of the aromatic thiol induces the modest downfield shift relative to the model compounds that we observe. The observation of a single, sharp peak provides strong evidence that the selenenyl sulfide moiety adopts one predominant conformation within the binding pocket. It further suggests that the prosthetic group is held distant from the electronic influence of any Met222 sulfoxide that may be present, since reduction of the methionine sulfoxide is unlikely under the conditions used to prepare the selenenyl sulfide (3 equiv of thiolate per active site).

Formation of the selenenyl sulfide from the seleninic acid probably occurs via a multistep process involving an enzyme-bound thiolseleninate ester (ESe(O)SAr) and selenenic acid (ESeOH) as intermediates.[25] The kinetics of reduction are multiphasic,[26] as would be expected for such a complex process. However, we have not been able to prepare either of the postulated intermediates for spectroscopic investigation. Organic thioseleninate esters are known to be highly reactive species that have only been detected at temperatures well below 0 °C.[22] Selenenic acids are also very unstable, and only areneselenenic acids with electron-withdrawing substituents in the ortho position have been isolated and characterized.[22] Although selenenic acids derived from selenocysteine have been trapped as cyclic selenenamides in model studies (eq 1),[7b] we found no evidence for an analogous reaction at the active



(1)

3

site of selenosubtilisin. The cyclic selenenamide **3** was reported to have a ^{77}Se NMR shift of 861 ppm, 472 ppm deshielded with respect to the resonance detected for the selenenyl sulfide of selenosubtilisin.[7b]

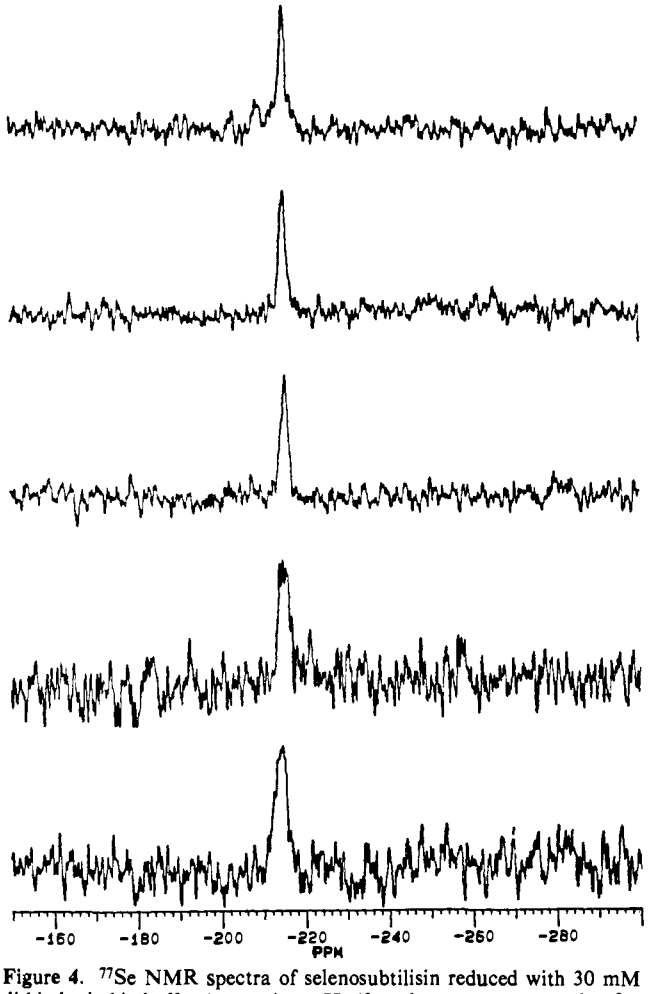


Figure 4. ^{77}Se NMR spectra of selenosubtilisin reduced with 30 mM dithiothreitol in buffer A at various pHs (from bottom to top): 4.39, 4.97, 5.67, 7.12, and 8.36.

As shown in Scheme I, a selenenyl sulfide and selenenic acid are key intermediates in the postulated mechanism of action of glutathione peroxidase. That the selenenyl sulfide derivative of selenosubtilisin promotes the reduction of alkyl hydroperoxides in the presence of excess thiol is consistent with this scheme. An alternative mechanism involving a central cyclic selenenamide intermediate seems less likely given our inability to detect such a species spectroscopically under conditions in which it would be expected to accumulate.

Reduction of Selenosubtilisin with Dithiothreitol. Treatment of selenosubtilisin ($ESeO_2H$ or ESeSAr) with an excess of DTT under a nitrogen atmosphere produced a third derivative whose ^{77}Se NMR spectrum consists of a single resonance at −215 ppm with a half-height line width of 85 Hz at pH 7 (Figure 1C). The prosthetic group in the reduced form of the enzyme is apparently the selenolate (ESe^-), as its chemical shift agrees well with that reported for deprotonated selenocysteamine (−212 ppm, pH 7.1)[11a] and for reduced and denatured glutathione peroxidase (−212, pH 8).[16] Like the selenenyl sulfide derivative, the fully reduced selenocysteine appears to adopt one predominant conformation within the active site. Reoxidation of the reduced enzyme with H_2O_2 yielded spectra that were identical to Figure 1A.

Given the perturbed pK_a of the enzyme-bound seleninic acid, it was of interest to titrate the selenol form of selenosubtilisin. ^{77}Se NMR titration curves have been reported previously for simple alkaneselenols with pK_a values between 5 and 5.5.[11a,29] The chemical shift of a protonated selenol is typically shielded by approximately 150 ppm relative to that of its conjugate base. Although sharp signals are readily detected at both the high and

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low pH extremes, ^{77}Se spectra are difficult or impossible to obtain for these compounds at pH values near their pK_a. Under these conditions, direct proton exchange causes extensive line broadening. As illustrated in Figure 4, we obtained ^{77}Se NMR spectra for selenosubtilisin that had been reduced with DTT at several pH values spanning the range of 4.4–8.4. As the sample pH was lowered, the resonance arising from the selenolate broadened by 121 Hz but shifted only a total of 0.81 ppm to a more shielded position. Clearly the selenol is still deprotonated at pH 4.4; its pK_a must be substantially lower than that of a normal alkaneselenol.

As in the case of the seleninic acid form of selenosubtilisin, we presume that the increased stabilization of the enzyme-bound selenolate relative to the model compounds is a result of interactions with the surrounding amino acid residues in the binding pocket. Previous work with thiolsubtilisin,[30] in which Ser221 has been replaced by cysteine rather than selenocysteine, provided evidence for the formation of a stable thiolate–imidazolium ion pair between the cysteine residue and the catalytically essential histidine, even at low pH. The similarity between sulfur and selenium chemistry suggests that SeCys221 and His64 would form an ion pair at the active site of selenosubtilisin, as well. Additional stabilization of the selenolate might derive from hydrogen bonding to the oxyanion hole. The three-dimensional structure of thioltrypsin,[31] another reengineered serine protease, has been solved to 2.5 Å and shows that the thiol forms a hydrogen bond with the backbone amide of Gly193 which is part of trypsin's oxyanion hole. Although diffraction data are not available for fully reduced selenosubtilisin, examination of the active site suggests that analogous interactions are feasible between SeCys221 and Asn155.

The selenolate is the third major intermediate in the catalytic cycle proposed for glutathione peroxidase (Scheme I). We believe that it is also required for the peroxidase activity of selenosubtilisin. Treatment of the selenenyl sulfide form of the artificial selenoenzyme with iodoacetamide at pH 5.0 in the presence of 3-carboxy-4-nitrobenzenethiol leads to efficient and irreversible inactivation of the enzyme via alkylation of the free selenolate.[2] Because the enzyme-bound selenol is deprotonated at all accessible pHs, it is expected to be highly reactive and susceptible to both alkylation and oxidation. The pK_a value of the selenol in glutathione peroxidase is not known, but the corresponding selenolate is also likely to be considerably more stable than is expected from simple model systems. The 2-Å crystal structure of the fully reduced enzyme shows that the selenolate forms specific hydrogen bonds with Nϵ1 of Trp148 and Nϵ2 of Gln70 and that it is further stabilized by the helical dipole of the α_1 helix.[10] Together these interactions will enhance the nucleophilicity of this critical residue in the natural peroxidase.

Concluding Remarks. We have exploited ^{77}Se NMR spectroscopy for the first time to investigate the catalytic center of an active selenoenzyme. Three distinct oxidation states of selenosubtilisin that are relevant to its peroxidase activity have been characterized, including a seleninic acid, a selenenyl sulfide, and a selenolate. In each case, well-resolved resonances are observed at chemical shifts appropriate for the assigned structures. The insights gained in the course of these experiments about the conformational flexibility, protonation state, and pK_a's of the individual selenosubtilisin derivatives thus complement and extend results from ongoing *x*-ray crystallographic and kinetic studies. As depicted in Scheme I, the selenolate and selenenyl sulfide—together with an uncharacterized selenenic acid derivative—are believed to constitute the catalytic cycle by which alkyl hydroperoxides are reduced by thiols. The relative concentrations of these species under catalytic conditions will vary depending on the concentrations of thiol and hydroperoxide, but our ability to isolate and study the selenenyl sulfide and the selenolate suggests that they will dominate the equilibrium population. Consistent with this conclusion, alkaneselenenic acids are known to be very unstable and, even at the active site, may have a fleeting existence. The seleninic acid, which is stable in the absence of reductant, probably lies off the main catalytic pathway and is likely to become important only at high peroxide concentrations. Our results are thus in accord with the previously proposed mechanism of action of the naturally occurring selenoenzyme glutathione peroxidase.[6]

Previous ^{77}Se NMR studies have shown that chemical shift anisotropy is the primary relaxation mechanism for selenium-containing macromolecules and that the T_1 and T_2 values for these systems should allow ready detection of the appropriate ^{77}Se signal at low field strengths (<9.4 T).[11a,13-16] The half-height line widths ($\nu_{1/2}$) for the three selenosubtilisin derivatives reported here are fully consistent with these observations.[32] Extension of these studies to naturally occurring selenoenzymes like glutathione peroxidase is therefore feasible. Comparison of the active-site microenvironments of selenosubtilisin and the natural enzyme will be particularly valuable for correlating the effects of structure on the reactivity of the various oxidation states of the selenium prosthetic group. The principal challenge in this regard will be to prepare sufficient quantities of the respective isotopically enriched proteins. To achieve reasonable signal-to-noise ratios, our spectra were typically acquired in overnight runs with high concentrations (>0.5 mM) of ^{77}Se-labeled selenosubtilisin; longer acquisition times were required for samples at lower concentrations. While specifically labeled selenoenzymes and genetically engineered variants thereof may become available through overproduction in microorganisms,[33] selenoproteins are often available only in small quantities from natural sources. Even when relatively large amounts of labeled enzyme can be isolated, as was the case of glutathione peroxidase itself,[16] poor solubility can limit or preclude detailed spectroscopic investigation of the catalytically active molecules. Approaches to overcoming the solubility problem will have to be developed, perhaps through optimization of buffer conditions or the use of mild detergents.

The extraordinary dispersion in chemical shift that is observed for structurally dissimilar selenium compounds is certainly the most striking feature of ^{77}Se NMR spectroscopy. In the current study, the resonance for the seleninic acid of selenosubtilisin is deshielded by ~800 and ~1400 ppm relative to that of the corresponding selenenyl sulfide and selenol derivatives, respectively. These enormous chemical shift differences leave no doubt about the oxidation state of the prosthetic group or about its attached ligands. The smaller shifts dictated by the chemical and electronic environment around the reporter group are potentially even more informative about the selenium functionality's orientation and reactivity and how both are influenced by the surrounding structure. These factors can now be investigated directly by introducing second-site mutations into the binding pocket. Selenosubtilisin is a particularly convenient model system for examining how the electronic microenvironment of a protein affects the intrinsic chemical shift of the selenium probe, as its parent template has been extensively mutagenized to investigate mechanism, structure, and stability.[34] For example, Met222 can be replaced by nonoxidizable amino acids[23] to confirm that partial oxidation of this residue gives rise to the second ^{77}Se resonance seen in the spectrum of the oxidized enzyme. The amino acids responsible for lowering the pK_a of the enzyme-bound seleninic acid and the selenol moieties can be studied in a similar fashion.

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(32) In response to a reviewer's suggestion we have compared the experimentally determined half-height line widths ($\nu_{1/2}$) with calculated values. Using a CSA value of ~400 ppm (determined experimentally for diphenyl diselenide[14]) and a rotational correlation time of ~11 ns (estimated for chymotrypsin, M_r 25 000; Kuznetsov, A. N.; Ebert, B.; Gyul'khandanyan, G. V. *Mol. Biol. (Moscow)* **1975**, *9*, 697), excellent agreement was observed between theory and experiment for the three selenosubtilisin derivatives we examined. For example, the calculated $\nu_{1/2}$ for the selenenyl sulfide derivative is 31 Hz vs a measured value of 30 Hz. These results demonstrate that the protein is not highly associated at the high concentrations used for the NMR experiments.

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More generally, the sensitivity of ^{77}Se NMR spectroscopy to the microenvironment is likely to provide a useful means of monitoring the binding of various ligands, including potential substrates, products, or inhibitors, to the active site.

In summary, ^{77}Se NMR spectroscopy represents a powerful and sensitive tool for examining the subtle interplay between structure and function in the active site of selenoenzymes. Its continued application to the characterization of the artificial peroxidase selenosubtilisin will undoubtedly enhance our understanding of this molecule's detailed catalytic mechanism and may ultimately yield insights that result in improved glutathione peroxidase mimics.

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Mössbauer and Integer-Spin EPR of the Oxidized P-Clusters of Nitrogenase: P^{OX} is a Non-Kramers System with a Nearly Degenerate Ground Doublet

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Abstract: The molybdenum–iron protein of nitrogenase contains 2 Mo atoms and ca. 28–30 Fe atoms. Approximately 16 Fe atoms belong to the P-clusters, a novel type of iron–sulfur cluster of unknown structure. Mössbauer studies have established that P-clusters are diamagnetic in the semireduced state, P^N. Upon oxidation of the protein with redox dyes such as thionin the state P^{OX} is attained. Previous studies have revealed that the low-temperature (≤4.2 K) Mössbauer spectra of P^{OX} exhibit magnetic hyperfine patterns even in the absence of external magnetic fields. Such behavior is generally characteristic of a Kramers system, i.e., of a system with an odd number of electrons. The spectra had features typical of those observed for a Kramers doublet with extremely anisotropic g-values ($g_1 \gg g_2, g_3$). Recent Mössbauer and EPR studies of integer spin systems in our laboratory have suggested the possibility that P^{OX} may be a very unusual non-Kramers system. Here we report Mössbauer and EPR studies of the proteins from *Azotobacter vinelandii* (Avl), *Clostridium pasteurianum* (Cpl), *Klebsiella pneumoniae* (Kpl), and *Xanthobacter autotrophicus* (Xal) which prove that the electronic ground doublet of P^{OX} is not a Kramers doublet but rather a nearly degenerate doublet (splitting Δ) of a system with an even number of electrons. Cpl, Avl, and Kpl have $\Delta \leq 10^{-3}$ cm^{-1}, and the magnetic patterns observed in the zero-field Mössbauer spectra result from mixing of the two electronic levels by ^{57}Fe hyperfine interactions ($|A_z| \approx 10^{-3}$ cm^{-1}). For Avl and Kpl we have observed integer-spin EPR transitions, at $g_{eff} = 11.9$, between two excited-state spin levels at 10–15 cm^{-1}. The ground doublet of Xal exhibits an integer-spin resonance at $g_{eff} = 15.6$. Analysis of the Xal Mössbauer spectra yields $\Delta \approx 0.010$ cm^{-1}. Using this value of Δ, quantitation of the EPR spectra yielded ca. two spins per MoFe protein for P^{OX}. The observed g-values suggested that P^{OX} of Xal has $S = 3$ or $S = 4$. However, the ground- and excited-state Δ-values of Kpl and Avl indicate that the electronic ground manifold may not consist of an isolated multiplet with definite spin S. Recognition of P^{OX} as a non-Kramers state implies that two electrons are removed from each P-cluster in the transformation $P^N \rightarrow P^{OX}$. Since ca. four electrons per MoFe protein are removed and since ca. 16 Fe atoms are involved, it follows that the $\alpha\beta$ dimer of nitrogenase contains two identical P-clusters and that each cluster has ca. 8 Fe sites. The Mössbauer data are reviewed for a model that considers the P-clusters to consist of two bridged cubanes.

Introduction

In 1978 Zimmermann and co-workers[1] published a Mössbauer study of the MoFe protein of nitrogenase from *Azotobacter vinelandii* (Avl).[2] This study concluded that Avl contains (30 ± 2) Fe atoms per $\alpha_2\beta_2$ unit. Approximately 12 Fe atoms were found to be associated with the two identical cofactor centers (labelled M-centers), and ca. 16 Fe atoms were assigned to a novel cluster type, called the P-clusters. In the semireduced (i.e., when kept in excess dithionite) protein the M-centers have cluster spin $S = {}^3/_2$, state M^N, whereas the P-clusters are in the diamagnetic state P^N. When the protein is oxidized with the redox dye thionin, the oxidation proceeds in two phases. In the first oxidation step, approximately four electrons are removed from the protein without

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(2) Abbreviations used: MoFe protein of *Azotobacter vinelandii*, Avl; *Klebsiella pneumoniae*, Kpl; *Clostridium pasteurianum*, Cpl; *Xanthobacter autotrophicus*, Xal; EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism.

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