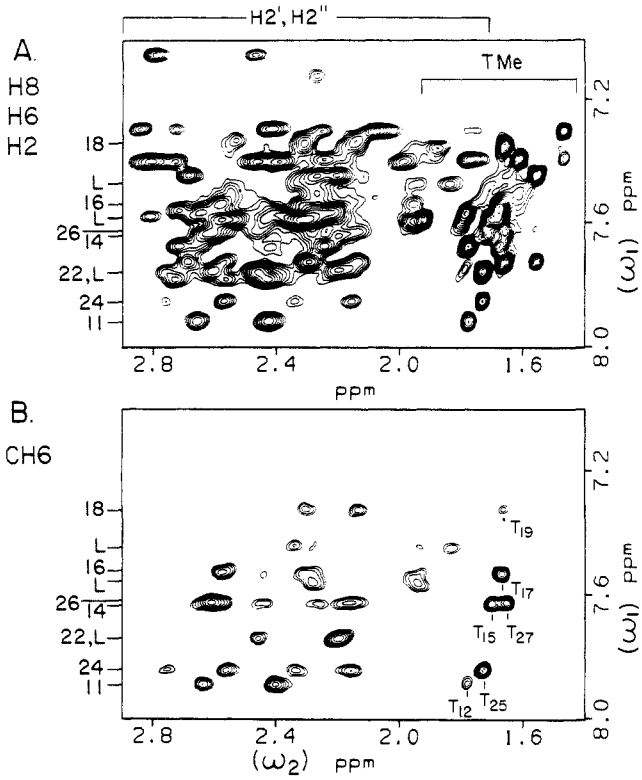


Figure 2. Portion of the 500-MHz (GN500) ^{1}H NMR spectra of d-($G_1A_2G_3A_4G_5A_6A_7C_8C_9C_{10}C_{11}T_{12}T_{13}C_{14}T_{15}C_{16}T_{17}C_{18}T_{19}T_{20}T_{21}C_{22}T_{23}C_{24}T_{25}C_{26}T_{27}T_{28}$) containing the base-H2',H2", TMe cross peaks of (A) the NOESY experiment (5 °C, τ_m = 100 ms) and (B) the HOENOE experiment (10 °C). The sample contained a 2.3-mm DNA strand in 100 mM NaCl, 5 mM $MgCl_2$, pH 5.75, 10 °C. For the HOENOE, both the selective and nonselective excitation were centered at 5.76 ppm with $t_{90(sel)}$ = 0.8 ms, t_r = 0.49 ms, τ_{imix} = 44 ms ($\gamma B_1/2\pi$ = 7.5 kHz), τ_m = 200 ms, and $t_{90(nonsel)}$ = 16 μs. Data result from 2 × 300 × 2048 matrices which were acquired with the acquisition times t_1 = 77 ms and t_2 = 205 ms, respectively. Data were processed with a squared sine-bell phase shifted by 50° in both dimensions and zero-filled to give 2048 × 2048 real points after processing. The software program FTNMR (D. Hare) was used for the data processing. Assignments of the CH6 resonances are indicated on the sides of the spectra. Resonances for C_8-C_{10} in the C-loop region are labeled L. Interbase cross peaks between the TMe and CH6 resonances are labeled in B. Details of the assignment procedures will be presented elsewhere.

experiment does not require any special hardware on commercial NMR spectrometers. Alternative selective excitation schemes such as shaped pulses[10] could replace the selective spin echo. In the next step of the HOENOE experiment, the magnetization of the cytidine H5 protons is selectively transferred to the *J*-coupled H6 ($J_{CH5-CH6} \sim 7$ Hz) protons via isotropic mixing.[11] The in-phase coherence transfer is accomplished with an MLEV[12] (or WALTZ[13]) broadband decoupling cycle. Although complete coherence transfer is obtained for $\tau_{imix} = 1/2J$, for large molecules (and/or low temperatures) where $T_{1\rho}$ is short, a shorter mixing time should be empirically determined and used in order to obtain maximum sensitivity. After the isotropic mixing, the t_1 evolution period of a regular 2D NOE experiment starts. The resulting two-dimensional spectrum obtained is nonsymmetrical, with selective excitation along ω_1 and nonselective excitation along ω_2.

(10) (a) Loaiza, F.; McCoy, M. A.; Hammer, S. L.; Warren, W. S. *J. Magn. Reson.* **1988**, *77*, 175-181. (b) Emsley, L.; Bodenhausen, G. *J. Magn. Reson.* **1989**, *82*, 211. (c) Kessler, H.; Anders, U.; Gemmecker, G.; Steuernagel, S. *J. Magn. Reson.* **1989**, *85*, 1. (d) Geen, H.; Wimperis, S.; Freeman, R. *J. Magn. Reson.* **1989**, *85*, 620.

(11) (a) Braunschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521-528. (b) Davis, D.; Bax, A. *J. Am. Chem. Soc.* **1985**, *107*, 2821-2923.

(12) (a) Levitt, M. H.; Freeman, R.; Frenkiel, T. *J. Magn. Reson.* **1982**, *47*, 328. (b) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355-360.

(13) (a) Shaka, A. J.; Keeler, J.; Frenkiel, T.; Freeman, R. *J. Magn. Reson.* **1983**, *52*, 335-338. (b) Bax, A. *NMR in Enzymology*; Oppenheimer, N. J., James, T. L., Eds.; Academic Press: San Diego, 1989; Vol. 176, p 151.

Application of the HOENOE experiment to the 28-base DNA oligonucleotide d(GAGAGAACCCCTTCTCTCTTTCTCT-CTT), which folds over to form a triple-stranded structure of the form shown in Scheme I (+ indicates protonated C at N_3), is illustrated in Figure 2. This molecule provides a model for in vivo triplexes,[14] and its detailed study is presented elsewhere.[15] The base-H2',-2", TMe cross-peak region of a standard NOESY experiment is shown in Figure 2A. Figure 2B shows the same region of a HOENOE experiment. Cross peaks in this region of the HOENOE spectrum arise exclusively from NOEs involving the CH6 resonances. These are CH6-TMe cross peaks and intra- and interbase CH6-H2',H2" cross peaks. Although the CH6 resonances can be readily identified in a COSY spectrum from their scalar coupling to CH5, extension of those assignments to the sugar protons is very difficult in this molecule due to spectral overlap with other base-H2',H2" NOEs. The HOENOE experiment provides a way to unambiguously identify H6-sugar cross peaks. This was essential in assigning this molecule, since standard sequential connectivities are not observed for some resonances and many CH6-sugar cross peaks overlap with other cross peaks in the spectrum. This experiment can also be used to observe selective NOEs from the CH5 resonances via selective excitation and coherence transfer of the CH6.

Acknowledgment. This work was supported by National Institutes of Health Grant R01 GM 37254-01, Office of Naval Research Contract No. N00014-88-K-0180, and NSF Presidential Young Investigator Award with matching funds from Sterling Drug, Inc., AmGen, and Monsanto.

Supplementary Material Available: The complete HOENOE and NOESY spectra given in Figure 2 as well as the base-H1',CH5 regions of the NOESY and HOENOE spectra (3 pages). Ordering information is given on any current masthead page.

(14) (a) Htun, H.; Dahlberg, J. E. *Science* **1989**, *243*, 1571-1576. (b) Mirkin, S. M.; Lyamichev, V. I.; Drushlyak, K. N.; Dobrynin, V. N.; Filippov, S. A.; Frank-Kamenetskii, M. D. *Nature* **1987**, *330*, 495-497.

(15) Sklenář, V.; Feigon, J. *Nature*, in press and manuscript in preparation.

(16) States, D. J.; Haberkorn, R. A.; Ruben, D. J. *J. Magn. Reson.* **1982**, *48*, 286-292.

Preparation of a Macrocyclic Polynuclear Complex, $[(en)Pd(4,4'\text{-bpy})]_4(NO_3)_8$,[1] Which Recognizes an Organic Molecule in Aqueous Media

Makoto Fujita,* Jun Yazaki, and Katsuyuki Ogura*

Department of Synthetic Chemistry
Faculty of Engineering, Chiba University
1-33 Yayoicho, Chiba 260, Japan

Received March 23, 1990

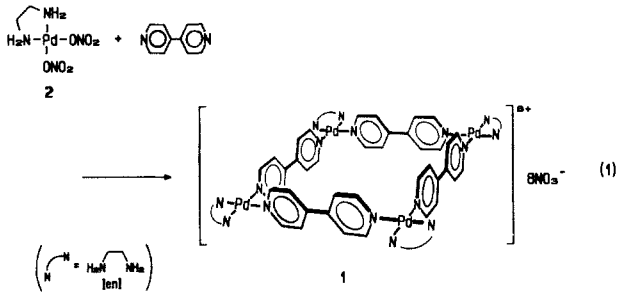
Recognition of molecular shapes and functions is becoming an urgent problem in both organic and inorganic chemistry.[2] In contrast to such flexible organic hosts as crown ethers or cyclophanes, some inorganic metal complexes are known to bind organic substances in their latticed or layered infinite framework.[3]

(1) en = ethylenediamine; bpy = bipyridine.

(2) For recent reviews, see: *Molecular Inclusion and Molecular Recognition—Clathrates I (Topics in Current Chemistry, Vol. 140)*; Weber, E., Ed; Springer-Verlag: New York, 1987. *Molecular Inclusion and Molecular Recognition—Clathrates II (Topics in Current Chemistry, Vol. 149)*; Weber, E., Ed; Springer-Verlag: New York, 1987. Iwamoto, T. *Inclusion Compounds*; Atwood, J. L., Davies, J. E. D., MacNicol, D. D., Eds; Academic Press: New York, 1984; Vol. 1, p 29.

0002-7863/90/1512-5645$02.50/0

Although the inclusion phenomena by these complexes are highly shape specific, the structural design or modification of the framework is difficult. If the inorganic host is constructed *as a molecule*, its structural design is easy and it might recognize organic guests even in a solution. This idea prompted us to prepare macrocyclic metal complexes in which metals are bridged by linear bidentate ligands. We report here a novel preparation of a polynuclear Pd(II) complex, $[(en)Pd(4,4'\text{-bpy})]_4(NO_3)_8$ (**1**), wherein the square-planar Pd atoms are bridged by 4,4'-bpy (eq 1).[4]



An ethanol (4 mL) solution of 4,4'-bpy (0.5 mmol) was added at room temperature to a methanol–water (1:1) solution (4 mL) of $(en)Pd(NO_3)_2$[5] (**2**), prepared from $(en)PdCl_2$[5] (0.5 mmol) and $AgNO_3$ (1.0 mmol), and the solution was stirred for 10 min at that temperature. Upon addition of ethanol (4 mL), a pale yellow powder immediately precipitated. The elemental analysis of the solid agreed with the empirical formula $[(en)Pd(4,4'\text{-bpy})](NO_3)_2 \cdot 0.9H_2O$,[6] from which the isolated yield based on $(en)PdCl_2$ was calculated to be 91%. Physical data are as follows: mp >220 °C dec; ^1H NMR (400 MHz, D_2O)[7] δ 2.70 (s, CH_2), 7.64 (d-like,[8] J = 7.0 Hz, Ar H_β), 8.64 (d-like,[8] J = 7.0 Hz, Ar H_α); ^{13}C NMR (100 MHz, D_2O)[9] δ 49.57 (CH_2), 127.43 (C_β), 149.59 (C_γ), 154.70 (C_α); IR (KBr) 1607 (ν_{C-N}), 820 (ν_{Ar-H}) cm^{-1}.

The structure of this complex is estimated to be a macrocyclic tetramer **1** by the following facts: (i) all pyridine nuclei of the complex are completely equivalent in NMR spectrometry (vide ante); (ii) the empirical formula predicted by CHN analysis[6b] was reproduced even if the complex was prepared with excess 4,4'-bpy (2 equiv); (iii) right bond angles (N–Pd–N(cis) = 90°) rule out the formation of other cyclic oligomers which must have significant ring strain.[10]

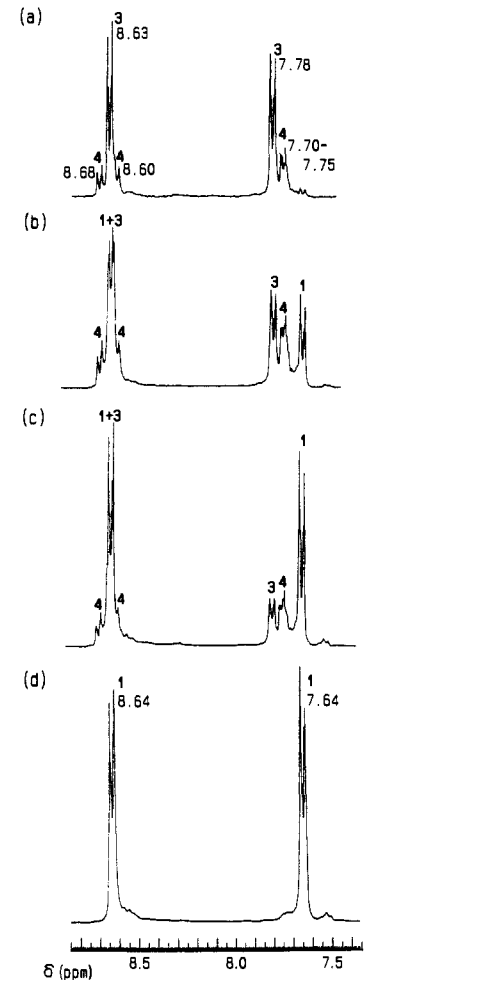
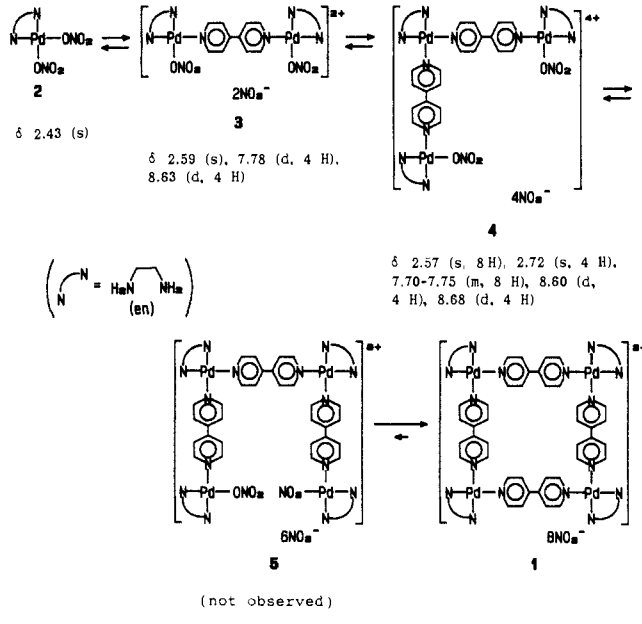


Figure 1. ^1H NMR spectra (270 MHz, D_2O)[8] obtained from mixtures of **2** and 4,4'-bpy: (a) **2**:bpy = 1:0.2; (b) **2**:bpy = 1:0.4; (c) **2**:bpy = 1:0.6; (d) **2**:bpy = 1:0.9.

Scheme I



It is particularly interesting that complex **1** is formed under thermodynamic control. This was proved by ^1H NMR experiments. In Figure 1 is shown the spectral change observed when 4,4'-bpy was portionwise added to **2** in D_2O (25 °C). Two main products at 0.2 equiv of bpy can be assigned as complexes **3** and **4**.[11] The formation of **1** became outstanding above 0.4 equiv of

(3) For examples of metal complexes as inorganic hosts, see the following. (a) [Ni(diamine)Ni(CN)$_4$]: Rayner, J. H.; Powell, H. M. *J. Chem. Soc.* **1952**, 319. Walker, G. F.; Hawthorne, D. G. *Trans. Faraday Soc.* **1966**, 166. (b) [Cd(diamine)Ni(CN)$_4$]: Nishikiori, S.; Iwamoto, T.; Yoshino, Y. *Bull. Soc. Chem. Jpn.* **1980**, *53*, 2236. Hasegawa, T.; Nishikiori, T.; Iwamoto, T. *J. Inclusion Phenom.* **1984**, *1*, 365; *Chem. Lett.* **1985**, 1659. Nishikiori, S.; Iwamoto, T. *Ibid.* **1982**, 1035; **1983**, 1129; **1984**, 319. Also see: Idem. *Ibid.* **1987**, 1127. (c) Cu[C(C$_6$H$_4$CN)$_4$]BF$_4$: Hoskins, B. F.; Robson, R. *J. Am. Chem. Soc.* **1989**, *111*, 5962. (d) Cd(CN)$_2$: Kitazawa, T.; Nishikiori, S.; Kuroda, R.; Iwamoto, T. *Chem. Lett.* **1988**, 1729. Abrahams, B. F.; Hoskins, B. F.; Robson, R. *J. Chem. Soc., Chem. Commun.* **1990**, 60. Hoskins, B. F.; Robson, R. *J. Am. Chem. Soc.* **1990**, *112*, 1546. (e) [(Me$_3$Sn)$_3$M(CN)$_6$] (M = Co, Fe): Eller, S.; Brandt, P.; Brimah, A. K.; Schwarz, P.; Fischer, R. D. *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 1263. Brandt, P.; Fischer, R. D.; Martinez, E. S.; Calleja, R. D. *Ibid.* **1989**, *28*, 1265. Yünlü, K.; Hock, N.; Fischer, R. D. *Ibid.* **1985**, *24*, 879.

(4) Cyclophanes containing 4,4'-bpy units: Odel B.; Reddington, M. V.; Slawin, A. M. Z.; Spencer, N.; Stoddart, J. F. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 1547. Ashton, P. R.; Odell, B.; Reddington, M. V.; Slawin, A. M. Z.; Stoddart, J. F.; Williams, D. J. *Ibid.* **1988**, *27*, 1550.

(5) Drew, H. D. K.; Pinkard, F. W.; Preston, G. H.; Wardlaw, W. *J. Chem. Soc.* **1932**, 1895.

(6) (a) Calcd for $C_{12}H_{16}N_6O_6Pd \cdot 0.9H_2O$: C, 31.13; H, 3.88; N, 18.15. Found: C, 31.13; H, 3.67; N, 17.93. (b) The hydrate number varied in the range 0.9–1.3 owing to high hygroscopicity of this complex.

(7) TMS (a CCl_4 solution) was used as an outer standard.

(8) Signals at δ 7.64 and 8.64 showed an AA'BB' pattern.

(9) TMS (a $CDCl_3$ solution) was used as an outer standard.

(10) Although formation of other cyclic oligomers like a hexamer are permitted, each of them has more than two isomers, which should be differentiated in NMR spectrometry.

bpy, and then, the spectrum converged to that of **1** at ~1.0 equiv of bpy. Furthermore, the spectrum of Figure 1c (0.6 equiv of bpy) was completely identical with the spectrum obtained when pure **1** and **2** were mixed so that the ratio Pd:bpy became the same. These observations support rapid equilibrium which mainly lies on the stable cyclic tetramer **1** as shown in Scheme I. It is noteworthy that *the thermodynamic cyclization realized quantitative formation of* **1** without employing any special conditions such as high dilution.

A significant feature of this complex is its ability for molecular recognition in aqueous media.[12,13] When **1** was added to a D_2O solution of 1,3,5-trimethoxybenzene (**6**), high field shifts in ^1H NMR were observed for the signals of **6** ($\Delta\delta$ = 1.56 ppm for Ar *H*, 0.59 ppm for CH_3; [**1**] = [**2**] = 0.005 M, D_2O, 25 °C). The CPK model showed that **1** has an inner cavity (7.8 Å × 7.8 Å × 6.5 Å)[14] surrounded by π electrons of eight pyridine nuclei. The high field shift is most likely attributed to complexation in the cavity. Analysis of the chemical shift change ($\Delta\delta$ vs [**1**]/[**6**]) by the Benesi-Hildebrand[15] and nonlinear least-squares methods[12a] predicted that the complexation of **1** and **6** is 1:1 and the association constant (K_a) at 25 °C is 7.5 × 10^2 L mol^{-1}.[16]

(11) Fully characterized by ^1H NMR (270 MHz, D_2O).[7] Complex **3**: δ 2.59 (s, 8 H), 7.78 (d-like, J = 7.0 Hz, 4 H), 8.63 (d-like, J = 7.0 Hz, 4 H). Complex **4**: δ 2.57 (s, 8 H), 2.72 (s, 4 H), 7.70-7.75 (m, 8 H), 8.60 (d-like, J = 7.0 Hz, 4 H), 8.68 (d-like, J = 7.0 Hz, 4 H).

(12) Aqueous phase complexation of organics by cyclophanes: (a) Shinkai, S.; Araki, K.; Manabe, O. *J. Am. Chem. Soc.* **1988**, *110*, 7214. (b) Arimura, T.; Nagasaki, T.; Shinkai, S.; Matsuda, T. *J. Org. Chem.* **1989**, *54*, 3766. (c) Coleman, A. W.; Bott, S. G.; Morley, S. D.; Means, C. M.; Robinson, K. D.; Zhang, H.; Atwood, J. L. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 1361. (d) Diederich, F.; Lutter, H.-D. *J. Am. Chem. Soc.* **1989**, *111*, 8438.

(13) In spite of many attempts, a solid clathrate compound between **1** and aromatic compounds was not obtained.

(14) For the Pd-N bond length, 2.1 Å is adopted: Maitlis, P. M.; Espinet, P.; Russell, M. J. H. In *Comprehensive Organometallic Chemistry*; Wilkinson, G., Ed.; Pergamon Press: New York, 1982; Vol. 6, Chapter 38-1.

(15) (a) Benesi, H. A.; Hildebrand, J. H. *J. Am. Chem. Soc.* **1949**, *71*, 2703. (b) The NMR method: Hanna, M. W.; Ashbaugh, A. L. *J. Phys. Chem.* **1964**, *68*, 811. (c) Scope and limitation: Deranleau, D. A. *J. Am. Chem. Soc.* **1969**, *91*, 4044, 4050.

(16) The K_a value roughly estimated by the Benesi-Hildebrand method[15b] was defined by the nonlinear least-squares method.[12a] Calculation was done according to the following equation: $\delta_{calcd} = ([\mathbf{1}]_0 + [\mathbf{6}]_0 + K_a^{-1} - (([\mathbf{1}]_0 + [\mathbf{6}]_0 + K_a^{-1})^2 - 4[\mathbf{1}]_0[\mathbf{6}]_0)^{1/2})(2[\mathbf{6}]_0)^{-1}(\delta_{complex} - \delta_{free}) + \delta_{free}$, where $[\mathbf{1}]_0$ and $[\mathbf{6}]_0$ are initial concentrations of **1** and **6**, respectively.

Selenosubtilisin as a Glutathione Peroxidase Mimic†

Zhen-Ping Wu and Donald Hilvert*

Departments of Chemistry and Molecular Biology
Research Institute of Scripps Clinic
10666 North Torrey Pines Road
La Jolla, California 92037

Received February 26, 1990

There is considerable interest in the biosynthesis and mechanism of action of naturally occurring selenoenzymes, like glycine reductase and glutathione peroxidase.[1] The latter enzyme catalyzes the reduction of hydroperoxides by glutathione, thereby protecting mammalian cells against oxidative damage.[2] An active-site selenocysteine residue is essential for catalytic activity, but its mechanistic role is still debated.[2] We recently reported the preparation of an artificial selenoenzyme, selenolsubtilisin, which also contains a selenocysteine residue in its active site.[3] Here we report that this semisynthetic enzyme mimics key aspects of the chemistry of glutathione peroxidase.

Selenosubtilisin was prepared by chemically converting the active-site serine (Ser 221) of the protease subtilisin into a selenocysteine.[3] Treatment of the freshly made selenoenzyme with hydrogen peroxide[4] leads to an oxidized form with the prosthetic group most likely in the seleninic acid oxidation state (ESe(O)OH). The p*I* value of the oxidized protein is 5.7, considerably lower than that of the wild-type enzyme (7.8).[5] Moreover, the oxidized enzyme reacts with 3 equiv of 3-carboxy-4-nitrobenzenethiol (**1**) according to eq 1, in analogy to the reduction of benzeneseleninic acid by thiols.[6] In contrast to other alkaneseleninic acids with β-hydrogens which can syn eliminate,[2d] oxidized selenolsubtilisin is stable for months at 4 °C. Ongoing structural studies[7] are likely to show whether the protein provides specific stabilizing interactions for the seleninic acid moiety or sterically blocks the elimination pathway.

$$\mathrm{ESe(O)OH} + 3\mathrm{ArS^-} \xrightarrow{-H_2O} \mathrm{ESeSAr} + \mathrm{ArSSAr} \qquad (1)$$

Like glutathione peroxidase, selenosubtilisin catalyzes the reduction of alkyl hydroperoxides by thiols. The reduction of *tert*-butyl hydroperoxide (*t*-BuOOH) by 3-carboxy-4-nitrobenzenethiol (**1**) was studied in detail since it can be conveniently followed spectroscopically. Both the seleninic acid (ESe(O)OH)[4] and the selenenyl sulfide (ESeSAr)[6] forms of the enzyme substantially accelerate the rate of this reaction with multiple (>100) turnovers in processes that are first order in protein concentration. In both cases, enzymatic activity was observed to increase with decreasing pH, with the maximal rate occurring below pH 5.5.

The initial rates for the reduction of *t*-BuOOH by thiol **1** were determined as a function of substrate concentration at 25.0 °C and pH 5.5 by stopped-flow spectroscopy. These experiments were carried out by varying one substrate's concentration while keeping the other constant. Although the enzyme can be saturated by thiol, the kinetic behavior is complicated. On the other hand, typical Michaelis-Menten kinetics were observed with respect to the hydroperoxide. The apparent k_{cat} and $(K_m)_{t\text{-BuOOH}}$ values at 60 μM of thiol, for example, were determined to be 430 ± 10 min^{-1} and 160 ± 10 mM, respectively. As shown in Figure 1, the kinetic data at several thiol concentrations give characteristic parallel Lineweaver-Burk plots, indicating a ping-pong mechanism with at least one covalent intermediate.[8] Glutathione peroxidase behaves analogously,[9] and our observation that the oxidized enzyme reacts stoichiometrically with thiol to give an isolable selenenyl sulfide derivative is consistent with this kinetic pattern. Although further experiments are needed to characterize each of the intermediates in the catalytic cycle, the turnover reaction may proceed via the mechanism shown in Scheme I, which was

†Contribution No. 6291-MB from the Research Institute of Scripps Clinic.

(1) (a) Stadtman, T. C. *FASEB J.* **1987**, *1*, 375. (b) Stadtman, T. C. *Annu. Rev. Biochem.* **1980**, *49*, 93.

(2) (a) Wendel, A. In *The Enzymatic Basis of Detoxification*; Jakoby, W. B., Ed.; Academic Press: New York, 1980; Vol. I, p 333. (b) Ladenstein, R. *Pept. Protein Rev.* **1984**, *4*, 173. (c) Ganther, H. E.; Kraus, R. J. *Methods Enzymol.* **1984**, *107*, 593. (d) Reich, H. J.; Jasperse, C. P. *J. Am. Chem. Soc.* **1987**, *109*, 5549.

(3) Wu, Z.-P.; Hilvert, D. *J. Am. Chem. Soc.* **1989**, *111*, 4513.

(4) Freshly prepared selenosubtilisin was dialyzed exhaustively against 10 mM DTT, then against 20 mM H_2O_2, and finally against PIPES buffer (10 mM, pH 7.0). The resulting protein was homogeneous as judged by polyacrylamide gel electrophoresis.

(5) Markland, F. S., Jr.; Smith, E. L. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1971; Vol. III, p 561.

(6) See: Kice, J. L.; Lee, T. W. S. *J. Am. Chem. Soc.* **1978**, *100*, 5094. The stoichiometry of the enzymatic reaction was determined by monitoring the disappearance of thiol **1** spectroscopically at 412 nm in aqueous buffer (100 mM Mes, pH 5.0, 1.0 mM EDTA). Formation of 1 equiv of disulfide was verified by analytical reversed-phase HPLC and comparison with authentic samples. Moreover, as judged by the release of free **1** upon addition of DTT, ESeSAr was isolable in greater than 90% yield following treatment of the oxidized enzyme for 30 min at pH 5.0 with 3.1 equiv of thiol and gel filtration on G-25 Sephadex.

(7) High-quality crystals of oxidized selenosubtilisin that diffract at high resolution have been obtained, and structural elucidation is in progress. Syed, R.; Hogle, J. M.; Wu, Z.-P.; Hilvert, D., unpublished data.

(8) Dalziel, K. *Biochem. J.* **1969**, *114*, 547.

(9) Flohé, L.; Loschen, G.; Günzler, W. A.; Eichele, E. *Hoppe-Seyler's Z. Physiol. Chem.* **1972**, *353*, 987.

0002-7863/90/1512-5647$02.50/0