Enzyme Modeling

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Direct Synthesis of a Thiolato-S and Sulfinato-S Co^{III} Complex Related to the Active Site of Nitrile Hydratase: A Pathway to the Post-Translational Oxidation of the Protein**

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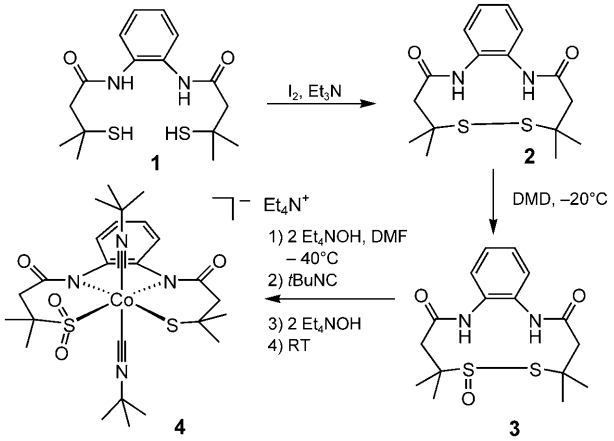
A group of sulfur-oxidized species found in biological systems includes sulfenic, sulfinic, and sulfonic acids derived from cysteine.[1,2] Currently "sulfur-oxidized" proteins are known to carry only one type of modification at a time, except for nitrile hydratase (NHase), which is the only system known to contain thiolate, sulfenate, and sulfinate groups in close proximity through coordination to a metal center.[3] Despite intense studies,[4] the origin of the post-translational modification of NHase remains unclear. The prevalent hypothesis for cysteine sulfur oxidation involves a two-step reaction in which iron or cobalt is bound by the protein ligands within the consensus sequence Cys-X-Y-Cys-Ser-Cys. This results in the formation of an $[Fe(N_2S_3)]^{2-}$ or a $[Co(N_2S_3)]^{2-}$ species which is followed by oxidation of the two bound thiolate groups in the equatorial plane *trans* to two deprotonated amides to give sulfinate and sulfenate. The design of all models synthesized to mimic the NHase active site follows this hypothesis.[5] The result is that only a few iron[6] or cobalt[7,8] complexes show dissymmetrically oxidized thiolate groups, and most of these contain two sulfinate groups irrespective of whether the oxidant used is O_2,[5,9–11b] H_2O_2,[5,11c,d] or dimethyl dioxirane.[11a] This prompted us to find another route to prepare a dissymmetrically oxidized complex. Herein, we describe a new and simple strategy toward mixed thiolate/sulfinate complexes which involves the metalation of a thiosulfinate following cleavage of the S–S bond with HO^-. By using a cyclic pseudopeptidic thiosulfinate, we prepared and structurally characterized a six-coordinate Co^{III} bisamidato/thiolato/sulfinato complex with two axial isonitrile ligands. This enables us to propose an alternate pathway for the post-translational modification of the cysteine residues in NHase, thus extending the implication of disulfide *S*-oxides, a second emerging group of sulfur-oxidized species, in biological systems.[2]

The cyclic disulfide *S*-monoxide **3** shown in Scheme 1 was synthesized in two steps from dithiol **1**, which was previously used to prepare both dithiolato $[CoN_2S_2](Et_4N)$ and disulfinato $[CoN_2(SO_2)_2(t\text{BuNC})_2](Et_4N)$ complexes.[11a] Oxidative cyclization of dithiol **1** with iodine in the presence of triethylamine[12] afforded the cyclic disulfide **2**. Oxidation of **2** with 1 equivalent of 2,2-dimethyl dioxirane (DMD) in acetone at −20 °C afforded the thiosulfinate **3** selectively and in high yield.



Scheme 1. Synthesis and Co metalation of a cyclic thiosulfinate. DMD = 2,2-dimethyl dioxirane, DMF = *N*,*N*-dimethyl formamide.

Thiosulfinates are very sensitive to nucleophiles, which cleave the S(O)–S bond, and nucleophilic attack can occur at the sulfenyl or sulfinyl sulfur atom.[13] However, alkaline

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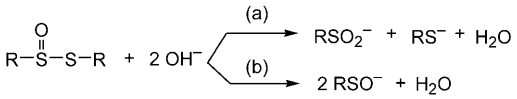
hydrolysis of thiosulfinate has been described as quite selective for sulfinyl sulfur to afford a thiolate and a sulfinate as the predominant products [Eq. (1), route (a)].[13a] We used



(1)

a combination of alkaline hydrolysis of compound **3** and metalation with a Co^{III} salt, $Na_3[Co(NO_2)_6]$, to trap the open species. Incorporation of a Co^{II} salt followed by a single-electron oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone is also possible, but the final purification of the complex is very tedious. This is why the use of the new hexanitritocobalt(III) sodium salt is much more convenient. Typically, the addition of 2 equivalents of Et_4NOH to a solution of **3** in DMF at −40 °C is followed by 1 equivalent of the Co^{III} salt. At this stage, the amide nitrogen atoms are likely coordinated in their imine tautomer form. After the addition of *tert*-butylisocyanide in excess, two other base equivalents are required to deprotonate the amides. The final complex is stable as a six-coordinate species with two isonitrile groups as axial ligands as previously observed for the disulfinate complex.[11a]

Complex **4** (Scheme 1) was thoroughly characterized. All the spectroscopic data are in agreement with a dissymmetrical thiolate/sulfinate structure, $[CoN_2S(SO_2)(t\text{BuNC})_2](Et_4N)$, with an S-bonded sulfinate, as in Co-NHase.[3b] There is no evidence for a disulfenate species resulting from the cleavage of the S(O)–S bond by HO^- group attack at the sulfenyl sulfur atom [Eq. (1), route (b)]. The IR spectrum of **4** (Supporting Information) exhibits the two SO_2 stretching frequencies expected for an S-bound sulfinate at 1185 ($\tilde{\nu}_{as}(SO_2)$) and 1047 cm^{-1} ($\tilde{\nu}_s(SO_2)$). There is no strong absorption around 950 cm^{-1} that could be attributed to the stretching frequency of a disulfenate species.[11c] ESI MS (negative ion) analysis of **4** shows a molecular peak at $m/z =$ 426.9, which corresponds to the mass of the anion of **4** with loss of the two isonitrile axial ligands. Further MS–MS analysis of this peak gives a daughter peak at $m/z = 363.1$ resulting from the loss of SO_2. As with all six-coordinate Co^{III} complexes in this series, **4** is diamagnetic. In contrast to the previously characterized $[CoN_2(SO_2)_2(t\text{BuNC})_2](Et_4N)$ species, which is completely symmetrical and exhibits only one resonance in its ^1H NMR spectrum for the methyl groups and another for the CH_2 protons,[11a] both the methyl and the CH_2 protons of **4** are split and each appears at two different chemical shifts. The cyclic voltammogram of **4** in CH_3CN with NBu_4BF_4 as supporting electrolyte exhibits an oxidation step at +510 mV versus standard calomel electrode (SCE). This oxidation wave is located between that observed for $[CoN_2(SO_2)_2(t\text{BuNC})_2](Et_4N)$ ($E_{pa} = +640$ mV (vs. SCE)) and $[CoN_2S_2(t\text{BuNC})_2](Et_4N)$ ($E_{pa} = +390$ mV (vs. SCE)).[11a] The anodic shift is about 125 mV for each addition of two oxygen atoms. The same trend has been observed upon sequential thiolate oxygenation of Ni complexes.[14]

The dissymmetry of the coordination sphere is further supported by the crystal structure of the anion of **4** (Figure 1)

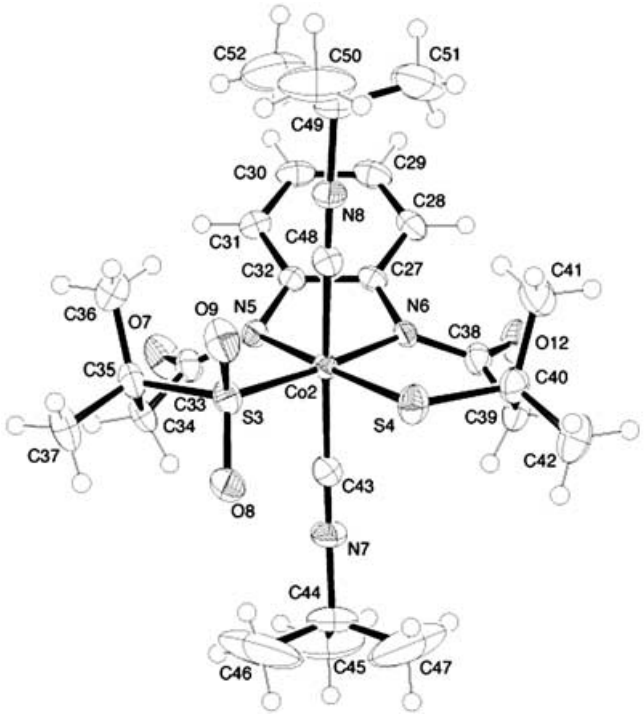
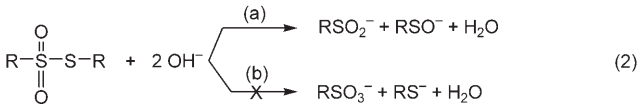


Figure 1. Thermal ellipsoid plot (50% probability level) of the anion of **4**. The hydrogen atoms, countercation, and solvent molecules have been omitted for clarity. Only one anionic enantiomer is shown. Selected bond lengths [Å]: Co2–C43 1.872(4), Co2–C48 1.870(4), Co2–N5 1.980(3), Co2–N6 1.997(3), Co2–S3 2.2205(11), Co2–S4 2.2505(12), S3–O8 1.463(4), S3–O9 1.467(4), N7–C43 1.143(5), N8–C48 1.142(5). Selected bond angles [°]: C48-Co2-C43 177.31(18), C48-Co-N5 89.84(15), C48-Co2-N6 87.55(15), C43-Co2-N6 92.60(15), C43-Co2-N5 92.84(15), N5-Co2-N6 81.90(13), N5-Co2-S3 95.53(10), N5-Co2-S4 178.63(10), N6-Co2-S4 96.87(10), O8-S3-O9 113.9(2).

and by comparison with the disulfinate complex.[11a] The Co^{III} center exhibits an octahedral geometry as does the disulfinate complex, but with one thiolate group and one S-bonded sulfinate group *trans* to the two carboxamido nitrogen atoms in the equatorial plane. The Co–S distances show significant variation (2.221–2.259 Å), which underscores the inequivalence of the two sulfur sites; these distances are almost equal in the disulfinate complex owing to the equivalence of the sulfur atoms. Whereas ^1H NMR analysis reveals a plane of symmetry in solution, the aromatic ring and the lateral chains relating N5 to S3 and N6 to S4 are on either side of the N_2S_2 plane, and the molecule is asymmetrical in the solid state. This can be related to the fact that the complex crystallizes with two enantiomers in the asymmetric unit (Experimental Section). The crystal structure of **4** also reveals the presence of hydrogen bonds between the co-crystallized water molecules and the oxygen atoms of the amides and one sulfinate, as previously observed in the crystal structure of the disulfinate complex.

Our results show that cyclic pseudopeptidic thiosulfinates can be efficiently trapped by a metallic cation under basic conditions. The alkaline cleavage occurs upon reaction of HO^- at the sulfinyl sulfur atom to give the selective formation of the thiolate/sulfinate complex. As in other six-coordinate Co^{III} complexes,[9,11] the sulfinate has a strong preference for a

sulfur-to-cobalt binding mode. Clearly, this is the only route to selectively prepare such a complex, as H_2O_2 oxidation of the dithiolate $[CoN_2S_2(t\text{BuNC})_2](Et_4N)$ affords a mixture of sulfur-oxygenated species, whereas DMD oxidation gives the S-bonded disulfinate complex.[11a] Such a reaction could be biologically relevant and could probably be extended to alkaline hydrolysis and metalation of cyclic pseudopeptidic thiosulfonates. Disulfide *S*-dioxides are much more sensitive to nucleophiles such as oxy anions than are disulfide *S*-monoxides; their alkaline hydrolysis has been described as selective for the sulfenyl sulfur atom [Eq. (2), route (a)].[13]

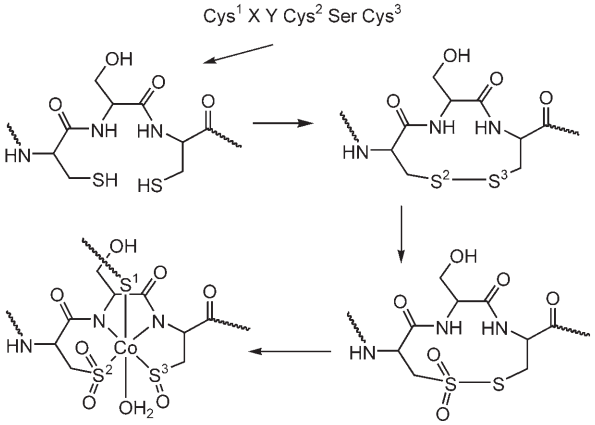


(2)

The higher selectivity of thiosulfonates towards hydrolysis relative to thiosulfinates results from the fact that the sulfenyl sulfur is much more readily accessed than is the sulfonyl sulfur, and from the fact that a sulfinate is a better leaving group than a thiolate.[13]

X-ray analysis of both Fe- and Co-NHases,[3] as well as enzymatic inhibition studies[4c] support the presence of a sulfenate group in the metal environment of NHase. Moreover, a Co complex with two S-bonded sulfenates has been shown recently to promote nitrile hydration.[11d] With the aim of isolating a mixed sulfinate/sulfenate complex, the reactivity of **4** toward oxidants was studied. The products were identified by ^{1}H NMR spectroscopy. Oxidation of **4**, even at low temperature and with less than 1 equivalent of H_2O_2 or DMD, provides the previously isolated S-bonded disulfinato species, either alone or as a mixture with the starting product.[11a]

On the basis of our results with a thiosulfinate, we can propose an alternate pathway for the specific thiolate oxidation of NHases into sulfinate and sulfenate species (Scheme 2). This sequence involves a post-translational modification of the protein prior to metal insertion as follows: the two cysteine residues of the consensus sequence that are separated by a serine residue are initially oxidized to a disulfide, then to a disulfide *S*-dioxide. This is followed by alkaline hydrolysis and then by iron or cobalt insertion. A selective HO^- attack at the sulfenyl sulfur should directly afford the mixed sulfenate/sulfinate in the mean plane of the active site. Indeed, there is now clear evidence that disulfide *S*-oxides have important biological implications.[2] Their production is either mediated by reactive oxygen, nitrogen species generated under oxidative stress conditions,[2] or catalyzed by monooxygenases[15a] or dioxygenases.[15b] These disulfide oxides, mainly studied as their glutathione derivatives, lead to (gluta)thionylation of proteins or metallothionein by reaction of the free or zinc-bound cysteinate group at the sulfenyl sulfur atom of the disulfide *S*-oxide.[16] We suggest that these disulfide oxides might also result from a post-translational oxidation of proteins, with NHase possibly being the first example. Finally, we have shown that the reactivity of disulfide *S*-oxides is not limited to reaction with thiolates in proteins, but that they can also react with metallic cations after hydrolytic cleavage of the S–S bond. A more complete study of such reactions of thiosulfinates and thiosulfonates is in progress.



Scheme 2. Alternate pathway to the post-translational oxidation of nitrile hydratase.

Experimental Section

All procedures were carried out under argon with standard Schlenk techniques. Solvents were dried following standard procedures and stored under argon.

3: Elemental analysis (%) calcd for $C_{16}H_{22}N_2O_3S_2\cdot 0.33\,H_2O$ (360.49): C 53.31, H 6.34, N 7.77; found: C 53.50, H 6.21, N 7.48. ^{1}H NMR (250 MHz, $CDCl_3$): $\delta = 1.52$ (s, 3H), 1.63 (s, 3H), 1.67 (s, 3H), 1.79 (s, 3H), 2.66–2.74 (m, 2H), 2.92–2.99 (m, 2H), 7.23 (m, 2H), 7.41 (m, 2H), 8.02 (s, 1H), 8.33 ppm (s, 1H). IR (neat): $\tilde{\nu} = 3253$ (N–H), 1665 (C=O), 1069 cm^{-1} (S=O).

4: The sodium hexanitrocobaltate(III) salt is not soluble in DMF, but a solution was prepared as follows: DMF (3 mL) and trimethylorthoformate (10 mL) as a dehydrating agent were added to an aqueous solution (1.5 mL) of $Na_3[Co(NO_2)_6]$ (114 mg, 0.282 mmol). After stirring for 30 min, excess orthoformate as well as CH_3OH and $HCOOCH_3$ (products derived from the reaction of $HC(OMe)_3$ with H_2O) were removed under controlled vacuum to prevent the complete evaporation of DMF. Then, Et_4NOH (1.4 M in MeOH, 405 μL, 2 equiv) and the Co^{III} solution were added to a solution (2 mL) of **3** (100 mg, 0.282 mmol) in DMF at −40 °C. After stirring for a few minutes, a large excess of *t*BuNC (1 mL in 1 mL DMF) and 2 further equivalents of Et_4NOH (405 μL) were added to the mixture. The solution was then allowed to warm to room temperature. After evaporating to dryness in vacuo, the residue was dissolved in CH_3CN (1 mL) and a powder containing **4**, $NaNO_2$, and Et_4NNO_2 was isolated upon precipitation with Et_2O. This powder was dissolved in acetone (2 mL) and nitrite salts were removed through careful precipitation by dropwise addition of Et_2O. After centrifugation, the supernatant was slowly poured into Et_2O while stirring to afford **4** as a brown powder. Yield: 130 mg (60%). Crystals suitable for X-ray crystallographic analysis were grown by diffusion of Et_2O into a CH_3CN solution of **4**. Elemental analysis (%) calcd for $C_{34}H_{58}CoN_5O_4S_2\cdot 3\,H_2O$ (777.96): C 52.49, H 8.29, N 9.00; found: C 52.77, H 8.17, N 9.29. ^{1}H NMR (250 MHz, CD_3CN): $\delta = 1.01$ (m, 12H, CH_3), 1.15 (s, 6H, CH_3), 1.3 (s, 18H, *t*BuNC), 1.39 (s, 6H, CH_3), 2.55 (s, 2H, CH_2), 2.69 (s, 2H, CH_2), 2.95 (m, 8H, CH_2, Et_4N), 6.54 (m, 2H$_{ar}$), 7.94 ppm (m, 2H$_{ar}$). IR (neat): $\tilde{\nu} = 2200$ (C≡N), 1538 (C=O), 1185 and 1047 ($\tilde{\nu}_s$ and $\tilde{\nu}_{as}$ SO_2), 1173, 1002 cm^{-1} (Et_4N^+). Cyclic voltammetry (vs. SCE, nBu$_4$NBF$_4$ (0.1 M), 20 mV s^{-1}, CH_3CN): $E_{pc} = -1860$ mV, $E_{pa} = +510$ mV. FAB MS (positive ion): m/z (%) = 853.39 (100) {[CoN_2S-

$(SO_2)(t\mathrm{BuNC})_2](Et_4N)_2]^+$; ESI MS (negative ion): m/z (%) = 426.9 (40) $[CoN_2S(SO_2)]^-$, 363.1 (100) $[M-SO_2]^-$.

Crystal data for complex **4**: $(C_{26}H_{38}CoS_2O_4N_4)_2(C_8H_{20}N)_2$-$(C_2H_3N)_2(H_2O)_3$, $M_w = 1583.96$, pale-yellow crystal ($0.6 \times 0.4 \times 0.15\ mm^3$), triclinic, space group $P\bar{1}$, $a = 11.727(4)$, $b = 20.031(9)$, $c = 20.614(9)$ Å, $\alpha = 116.804(2)°$, $\beta = 90.384(3)°$, $\gamma = 95.913(3)°$, $V = 4291.3(3)$ Å^3, $Z = 2$, $\rho = 1.226\ g\,cm^{-3}$, $\mu(Mo\,K_\alpha) = 5.43\ cm^{-1}$, $T = 223$ K, $\theta = 1.18$–$28.62°$. 47639 reflections measured at on a Bruker–Nonius Kappa CCD diffractometer, 20515 unique reflections, 946 parameters refined on F^2 (20515 reflections) using SHELXL-97 to final indices $R[F^2 > 4\sigma(F^2)] = 0.099$, $wR = 0.149$ $[w = 1/[\sigma^2(F_o^2) + (0.0001P)^2 + 12.4629P]$ in which $P = (F_o^2 + 2F_c^2)/3]$. Compound **4** crystallized as two independent anionic monomers in the asymmetric unit, with two Et_4N cations, two molecules of acetonitrile, and three water molecules. The anionic moiety was found to be a disordered mixture of two isomers corresponding to the two possible sulfur oxidation sites that afford sulfinates. The refinement was therefore carried out by considering two positions for the sulfinate group on each monomer: the occupancy factors for both the oxygen atoms of the sulfinate group were fixed to 0.8 and 0.2 on the two sites for the first monomer and to 0.2 and 0.8 on the two sites for the second monomer. Most of the H atoms, including one hydrogen of one water molecule, were found experimentally. The remaining H atoms (excluding those on the water molecules) were introduced in theoretical positions. They were all included in the calculations but not refined. The final residual Fourier positive and negative peaks were equal to 0.91 and $-0.804\ e\,Å^{-3}$, respectively. CCDC-262627 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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