Mechanism-Based Inactivation of the Flavoenzyme Cyclohexanone Oxygenase during Oxygenation of Cyclic Thiol Ester Substrates†

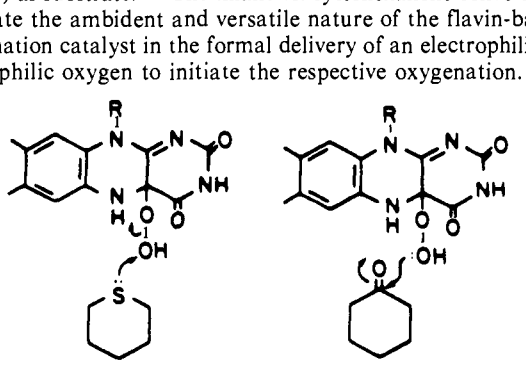
John A. Latham and Christopher Walsh*

Contribution from the Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received May 1, 1986*

Abstract: Cyclohexanone oxygenase from Acinetobacteria can function both as a Baeyer–Villiger catalyst (e.g., cyclohexanone to ε-caprolactone) and a sulfoxidation catalyst (e.g., cyclohexyl sulfide). When confronted with sulfur and a ketone in the same molecule, e.g., 3-thia- and 4-thiacyclohexanones, the enzyme catalyzes the Baeyer–Villiger oxygenative ring expansion exclusively. The corresponding 2-thiacyclohexanone, a thiolactone, is also a substrate but after 14 turnovers irreversibly inactivates the enzyme. Other thiolactones are also suicide substrates with [^{35}S]-γ-thiobutyrolactone leading to stoichiometric labeling of the apoprotein portion of this FAD enzyme. The cyclic thiocarbonate ethylene monothiocarbonate inactivates with a partition ratio of 110 turnovers per inactivation event and in so doing results in the loss of two enzyme SH groups. Cross-linking by the killing species accompanying inactivation is also suggested by the observation that acyclic phenyl thioacetate is turned over but does not inactivate. The killing species could be either an in situ generated acyl sulfoxide or a mixed sulfenic–carboxylic anhydride via enzymic oxygenation at sulfur or the carbonyl, respectively. Either species will be a reactive acylating agent for an enzyme nucleophile (e.g., a cysteine SH) which then unravels to generate an electrophilic sulfenic acid terminus which could readily cross-link to the second cysteine SH group that disappears on inactivation. These cyclic thiol esters constitute a novel class of flavoenzyme mechanism-based enzyme inactivators, activatable by sulfur oxygenation.

In biological oxygen transfer reactions there are two main types of cofactors stoichiometrically associated with enzyme catalysts to facilitate oxygenation chemistry. The redox active transition metals iron and copper are used, but iron is the most versatile, functioning in both heme and non-heme active site environments for mono- and dioxygen transfer. Flavin-linked enzymes are almost exclusively monooxygenases. They are known for phenol to catechol oxygenations[1,2] and N- and S-atom oxygenations[3] but appear to be unable to catalyze olefin epoxidation. In addition, flavin-dependent monooxygenases carry out the oxygenation of the carbonyl moiety, specifically the aldehyde to acid oxygenation in the bacterial luciferase reaction[4] and the conversion of ketones to lactones in the apparent biological Baeyer–Villiger reaction.[5-7] To date no iron-linked Baeyer–Villiger enzyme has yet been purified or characterized.

The best characterized Baeyer–Villiger enzymic catalyst is the FAD-containing cyclohexanone oxygenase, which catalyzes the oxidation of cyclic alkanones to the ring-expanded lactones such as ε-caprolactone.[7] Stereochemical studies showing retention at the migrating carbon center[8] and the migratory aptitudes of substituted cyclohexanones[9] are consistent with a Baeyer–Villiger mechanistic scheme where the 4a-peroxy flavin is the oxygen-transfer agent. We have shown that this enzyme is in fact a potent oxygen-transfer catalyst to other substrates containing a variety of heteroatoms including nitrogen, phosphorus, boron, selenium, and sulfur.[10] With thiane, the cyclic sulfide analogue of cyclohexanone, this oxygenase produces the thiane sulfoxide with almost equivalent k_{cat}/K_m (1×10^6 M^{-1} s^{-1}) to cyclohexanone (5×10^6 M^{-1} s^{-1}) as substrate.[11] The thiane vs. cyclohexanone conversions illustrate the ambident and versatile nature of the flavin-based oxygenation catalyst in the formal delivery of an electrophilic or nucleophilic oxygen to initiate the respective oxygenation.



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In this report, we have explored the regiochemical outcome when sulfur and a ketone group are in the same molecule, e.g., in thiacyclohexanones, and are presented for cyclohexanone oxygenase attack. It will be shown that in the 4-thia- and 3-thiacyclohexanones, only the Baeyer–Villiger product is detected, while 2-thiacyclohexanone (δ-thiovalerolactone) functions as a suicide substrate. For this and other thiolactones, including ethylene thiocarbonate, catalytic flux partitions between turnover to ring-opened, oxidized products and stoichiometric modification involving inactivation of the apoprotein portion of this flavoenzyme.

Experimental Section

^{1}H NMR spectra were obtained on a Bruker 250- or 270-MHz, a Jeol FX 90, or a Hitachi R24B NMR spectrometer; the chemical shifts are expressed in ppm with either Me_4Si or $CDCl_3$ as internal reference. ^{13}C NMR spectra were obtained with a Jeol FX 90 with $CDCl_3$ as internal reference. IR were obtained as a neat oil, a Nujol mull, or in solution on a Perkin-Elmer 283B IR spectrophotometer. Kinetic assays were performed with a Perkin-Elmer 554 or a Lambda 5 spectrophotometer equipped for constant temperature control. Liquid scintillation counting was performed with a Beckman LS1800 liquid scintillation counter using a carbon-14 window for a 10-min acquisition period. Scintillation fluid was from National Diagnostics, Somerville, NJ. Chemicals were obtained from the following sources: bis(Tris)propane dithiothreital (DTT), Tris, NADPH, TPCK–trypsin, soybean trypsin inhibitor, aprotenin, and leupeptin from Sigma; [^{35}S]thiourea from Amersham; γ-thiobutyrolactone, 5-bromovaleric acid, 6-bromohexanoic acid, *S*-phenyl thioacetate, carbonyldiimidazole, 5,5′-dithiobis(2-nitrobenzoic acid) (DTN-

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B), and 3-carboxypropyl disulfide from Aldrich; iodacetamide, 4-thiacyclohexanone, and Matrix Red Agarose from Amicon; No. 2 Spectropor dialysis membrane from American Scientific Products. 3-Thiacyclohexanone was prepared by the method of Young and Heitz.[12] Ethylene monothiocarbonate was prepared by the method of Reynolds, Fields, and Johnson.[13] All other chemicals and solvents used were the highest quality available and were used as obtained without further purification.

Growth of Acinetobacter NCIB 9871. Cells were grown from a culture, the gift of Dr. P. W. Trudgill of the University College of Wales, Aberystwyth, U.K. A single colony was generated in rich media for subculture into minimal media where cyclohexanol was used as the sole carbon source. The media used for growth was the following in g/L: KH_2PO_4 9; Na_2HPO_4, 4; $(NH_4)_2SO_4$, 13.5; yeast extract, 1; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2$, 0.11. A single colony was used to inoculate a 50-mL starter culture that was grown up overnight by using 1 g/L of cyclohexanol in the medium. The starter culture was used to inoculate a 1-L culture which was then transferred to a carboy containing 19 L of the media described above. At the initial time of inoculation, 0.5 g/L of cyclohexanol was added to the growth. After the bacteria completed the first doubling cycle, this was increased to 1 g/L. The cells were harvested when they were judged to be in late log phase of growth. A normal preparation generates 250–350 g of wet cell paste.

Cell Disruption and Ammonium Sulfate Fractionation. Cells (119 g) were thawed to a cell paste and mixed with 120 mL of 21 mM KPi and 10 mM β-mercaptoethanol, pH 7.2. The phosphate buffer described above is used throughout the preparation and in subsequent descriptions will be referred to as the prep buffer. This was then passed 2 times through a French pressure cell with a disruption pressure of 14–16 000 psi at the orifice while collecting the effluent of the pressure cell on ice. The resulting material was spun for 60 min at 14 000 rpm (23.6 K × G, 4 °C). All subsequent operations were performed at 4 °C.

The supernatant solution was recovered and the pellets discarded. The pooled supernatent was brought to 50% saturation in $(NH_4)_2SO_4$ by the addition of 29.1 g/100 mL of solid enzyme grade $(NH_4)_2SO_4$ to the stirring solutions. Upon complete addition and solubilization of the $(NH_4)_2SO_4$, the solution was stirred for an additional 30 min. At this time it was subjected to centrifugation at 14 000 rpm (23.6 K × G, 4 °C) for 45 min. The resultant supernatant was pooled and the pellets discarded. The solution was brought to 90% saturation in $(NH_4)_2SO_4$ by adding an additional 26.8 g/100 mL of enzyme grade $(NH_4)_2SO_4$ to the solution. Upon complete addition of the $(NH_4)_2SO_4$, the solution was stirred for an additional 60 min. The precipitated protein was then pelleted by centrifugation at 14 000 rpm (23.6 K × G, 4 °C) for 60 min.

The supernatant was poured off and the resulting yellow pellets were resuspended in the breakage buffer. This was transferred to a No. 2 Spectropor dialysis membrane and left to dialyze overnight against 2 L of the same buffer.

DE-52 Anion-Exchange Chromatography. DE-52 (preswollen, Whatman) was washed and prepared in 0.5 M KPi. A slurry was slowly stirred in 0.5 M KPi and brought to pH 3 with concentrated HCl. This was stirred for 30 min and collected via vacuum filtration. The resin was resuspended in 0.5 M KPi and brought to pH 10 with 50% (w/w) NaOH. The slurry was stirred for 30 min and again collected via vacuum filtration. Finally, the resin was suspended in pH 7 distilled water. This was stirred for 30 min and collected. The resin was suspended in distilled water and allowed to settle. After 30 min the fines were removed via suction. The resin was resuspended a second time and allowed to settle, and the fines were removed. A 13- × 2.5-cm column was poured (70-mL bed volume) and equilibrated with prep buffer overnight at 4 °C.

The resuspended $(NH_4)_2SO_4$ fraction was loaded on the column over 50 min. Elution was done by using a linear gradient established by mixing 250 mL of 0.5 M KCl in prep buffer into 250 mL of prep buffer. Collected were 8.6-mL fractions. These were analyzed by absorbance at 280 nm, 440 nm, and specific activity toward 4-methylcyclohexanone. Fractions containing activity were pooled and either dialyzed or subjected to $(NH_4)_2SO_4$ precipitation (90%) to concentrate the sample, followed by dialysis.

Amicon Red Dye Matrix Affinity Purification. A 21.5- × 2.5-cm column (100-mL bed volume) of Amicon Matrix Red agarose was poured. This was washed with 300 mL of 8 M urea/0.5 N NaOH and 300 mL of distilled water and transferred to 4 °C. The column was equilibrated overnight at 4 °C with 300 mL of prep buffer, at a flow rate of 25 mL/h.

The pooled eluent from the DE-52 column was then applied to this column in 25-mL volume over 50 min. The sample was washed into the column with 5 mL of prep buffer, and the column was allowed to stand for 40 min undisturbed.

At this time, the column was washed with 250 mL of prep buffer. At one column volume, yellow tinted fractions were collected for 80 mL. The eluent then became clear and colorless and continued in this manner for 50 mL. A 5 mM NADP solution was then applied to the column at a 25 mL/h flow rate. After 120 mL, yellow fractions eluted for 33 mL. These were assayed for enzymatic activity toward 4-methylcyclohexanone and found to contain all the activity eluted from the column.

These fractions were pooled and concentrated by using an Amicon ultrafiltration device, equipped with a PM-10 membrane. This was repeated until all the NADP had been removed (as judged by the 260-nm absorbance of the pooled protein solution, <1.0 OD).

Spectrophotometric Kinetic Assays. Assays were performed in 1-mL total volume of solution containing 80 mM glycine–NaOH, pH 9, 200 μM NADPH, and various enzyme concentrations. Substrate concentrations normally were saturating unless specified (concentration for standard 4-methylcyclohexanone assays was 82 μM). All assays were performed at 15 °C, where the enzyme has been shown to be stable to autoinactivation.[10] The background uncoupled oxidase rate of the enzyme used in these experiments was a maximum of 1.4% the normal specific activity exhibited toward 4-methylcyclohexanone (8–10 U/mg where U = μmol/min). Enzyme activity was monitored continuously by following NADPH consumption at 340 nm.

Synthesis of Thiolactones. Thiolactones were made via pyrolysis of the corresponding mercapto alkylcarboxylic acids. These mercapto alkylcarboxylic acids were generated via isothiouronium exchange of 5-bromovaleric acid and 6-bromohexanoic acid followed by base hydrolysis, respectively. Product characteristics were identical with literature physical characteristics for each of the thiolactones synthesized:[15,16] ^1H NMR δ (δ-thiovalerolactone) 3.17 (t, 2 H), 2.65 (t, 2 H), 1.96 (m, 4 H), (ε-thiocaprolactone) 3.16 (t, 2 H), 2.61 (t, 2 H), 1.99 (m, 4 H), 1.71 (t, 2 H).

K_m and V_{max} Determination for γ-Thiobutyrolactone. Assays were performed as described above with 0.5 and 0.77 μM cyclohexanone oxygenase and substrate concentrations ranging from 24.5 to 122.3 μM and 12.3 to 97.8 μM, respectively. Kinetic constants were obtained from Lineweaver–Burk double reciprocal plots.

γ-Thiobutyrolactone Partition Ratio Determination. In reference and sample cells of the UV–vis spectrophotometer, 200 μM NADPH and 0.446–1.338 μM cyclohexanone oxygenase were placed in 1-mL total volume of 80 mM glycine–NaOH pH 9 buffer. γ-Thiobutyrolactone, (1.07 mM) in methanol (10 μL, 1% final volume) was added to the sample cell and an equal volume of methanol added to the reference cell. The total NADPH consumption for inactivation was measured from the difference.

Partition Ratio Determination for ε-Thiocaprolactone and δ-Thiovalerolactone. Partition ratios were performed in the manner described above for the five-member thiolactone δ-thiobutyrolactone under the following thiolactone conditions: δ-thiovalerolactone, 2.11 mM; ε-thiocaprolactone, 1.85 mM.

Substrate Protection Studies. Substrate protection studies were undertaken by using the following concentrations of 4-methylcyclohexanone, 41 μM, and γ-thiobutyrolactone, 87 μM, under standard kinetic conditions with 200 μM NADPH in 1-mL total volume of 80 mM glycine-NaOH pH 9 buffer at 15 °C.

Oxygen Electrode Assays. All assays were performed by using a YSI 5331 oxygen probe in 3-mL total volume under standard assay conditions. Oxygen consumption was measured by addition of substrate to a base line stabilized solution at 15 °C.

Synthesis of [^{35}S]-γ-Thiobutyrolactone. To a one-neck 25-mL round-bottom flask equipped with magnetic stirring bar, motor, condenser, Ar inlet, and outlet was added 4-bromobutyronitrile (163 mg, 1.1 mmol). Next the flask was charged with thiourea (84 mg, 1.1 mmol; 73 mg cold and 11.5 mg [^{35}S]-labeled at 29.33 mCi/mmol) in water (2.4 mL). The system was flushed with argon and allowed to reflux for 6 h. The solution was cooled to room temperature and 3 mL of 5 N NaOH added. Argon was flushed through the system, and the reaction was brought to reflux for 20 h. At this time the solution was allowed to cool to room temperature and was diluted to 25-mL total volume with water. The aqueous solution was acidified to pH 1 with concentrated HCl. This solution was washed 4 times with diethyl ether (4–25 mL). The organic layers were combined, dried with $MgSO_4$, and filtered, and the solvent was removed by rotary evaporation. The resulting [^{35}S]-4-mercaptobutyric acid was then heated neat to 160 °C under argon to afford [^{35}S]-γ-thiobutyrolactone (57 mg, 0.56 mmol) at 3.96 mCi/mmol specific radioactivity

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(51% yield overall). The radiolabeled intermediate 4-mercaptobutyric acid and the final product, γ-thiobutyrolactone, were identical with authentic standards prepared with this synthetic scheme using nonradiolabeled thiourea and with commercially available δ-thiobutyrolactone (Aldrich). The intermediates in the procedure were characterized and showed data identical with literature data:[13] ^{1}H NMR δ (4-mercaptobutyric acid) 8.87 (s, 1 H), 2.74 (s, 2 H), 2.44 (t, 2 H), 1.93 (m, 2 H), 1.37 (t, 1 H), (γ-thiobutyrolactone) 3.41 (t, 2 H), 2.48 (t, 2 H), 2.31 (m, 2 H).

Inactivation Stoichiometry Studies with [^{35}S]-γ-Thiobutyrolactone. Cyclohexanone oxygenase (1.80 μM, 5.75 mg of enzyme, M_r 66 000) was pretreated with 1 mM γ-thiobutyrolactone in 50 mL of 80 mM glycine–NaOH pH 9 buffer for 15 min at 15 °C to eliminate background labeling. This incubation was performed in the absence of NADPH. The incubation was then concentrated by using ultrafiltration with an Amicon ultrafiltration device equipped with an Amicon PM-10 membrane. This pressurized dialysis was repeated to result in a net 1000-fold dilution, eliminating any small molecule contamination from the enzyme solution. The resulting protein was then added to a solution of 168 μM NADPH in 50-mL total volume of the above buffer at 15 °C. Next, 0.5 mL of a methanolic solution of [^{35}S]-γ-thiobutyrolactone (3.96 mCi/mmol) was added to arrive at a final concentration of 1 mM. Specific activity assays were performed at 5 and 15 min by removal of 10 μL of the 0.115 mg/mL protein solution and assayed under 20-fold molar excess of 4-methylcyclohexanone. At 15 min, the incubation was immersed in ice, transferred to 4 °C, subjected to pressurized dialysis under the conditions described above, and concentrated to 1.5 mL. This sample was applied to a 15- × 1.5-cm Biorad P6DG gel filtration column in 21 mM KP-*i*, pH 7.2. Material was eluted with the same buffer at a flow rate of 20 mL/h in 0.65-mL fractions. Fractions were assayed by absorbance at 280 nm, 440 nm, and ^{35}S by counting 25-μL aliquots in 15 mL of liquid scintillation fluid. The stoichiometry was determined by counting four samples containing 25 and 100 μL of the peak protein fraction in 15 mL of scintillation fluid. A control experiment was performed under identical conditions but in the absence of NADPH. Activity and stoichiometry measurements are compared to this control.

Distribution of ^{35}S Label in Enzyme. Labeled protein solution (2750 dpm) was brought to 0.5-mL total volume and precipitated by TCA addition to a final concentration of 10%. This was done for varying amounts of time, ranging from 1 to 20 min, in 5-min intervals. The solution was then centrifuged and the supernatant removed. The entire supernatant was counted in 18 mL of liquid scintillation fluid. The pellet was resuspended in 0.3 mL of 6 M guanidine–HCl in 0.3 M Tris, pH 8, and counted in 18 mL of liquid scintillation fluid.

Trypsin Digestion of Radiolabeled Cyclohexanone Oxygenase and Attempted Peptide Isolation. Cyclohexanone oxygenase (3.4 mmol, 3660 dpm) was dissolved in 100 mM $(NH_4)HCO_3$ pH 7.9 buffer (1 mL). Iodoacetamide (20 mmol) was added under argon and the reaction left to incubate at 37 °C for 60 min. At this time 5 μL of 1 mg/mL TPCK-treated trypsin was added, and the digestion was run for 1.75 h. An additional 5 μL of 1 mg/mL TPCK–trypsin was added at this time, and digestion was allowed to continue for an additional 1.75 h. The reaction was quenched by addition of trypsin inhibitors at the following concentration: 10 μL of 0.01 mg/mL soybean trypsin inhibitor, 0.01 mg/mL aprotenin, and 0.005 mg/mL leupeptin. The solvent was removed by lyophilization. The resulting solid was resuspended in 100 μL of water and applied to waters phenyl reverse-phase HPLC column and elution performed with a water/acetonitrile gradient with 0.08% TFA. Initial conditions were 10% acetonitrile; final conditions were 40% acetonitrile with a linear gradient over 60 min at a flow rate of 2 mL/min. Fractions were collected every 60 s and counted in 18 mL of liquid scintillation fluid.

Product Characterization from Enzymatic Incubation with 4-Thiacyclohexanone. To a 125-mL Erlenmeyer flash equipped with stirring bar and motor, NADPH (25 mg, 30 μmol) was added to 50 mL of 80 mM glycine–NaOH pH 9 buffer. Purified cyclohexanone oxygenase (200 μL, 0.59 mg) was added to a final concentration of 0.18 μM. The solution was cooled to 15 °C with a ice–water bath and maintained at this temperature throughout the incubation. After a 10-min equilibration period, 0.5 mL of a methanolic solution of 4-thiacyclohexanone (70 mM) was added to the stirring solution (final concentration of 0.7 mM). The reaction mixture was stirred for 2.5 h. A control experiment was conducted in parallel where no enzyme was present to test for substrate stability under incubation conditions.

The reaction was quenched at 2.5 h by the addition of 10 drops of concentrated HCl. Brine (25 mL) was added to the aqueous phase, and the reaction mixture was extracted with methylene chloride (3–80 mL). This was dried with $MgSO_4$ and filtered, and the solvent was removed. High-field ^{1}H NMR was obtained in $CDCl_3$. The enzymatic product was characterized as the nonenzymatic methanolysis product methyl (2-hydroxyethyl)-3-thiopropionate: ^{1}H NMR δ 3.68 (t, 2 H), 3.64 (s, 3 H), 2.65 (m, 6 H), 1.95 (br, 1 H), identical with an authentic standard prepared from methyl acrylate and β-mercaptoethanol via ^{1}H NMR and TLC.[17]

Product Characterization of Enzymatic Incubation with 3-Thiacyclohexanone. Conditions were the same as described above with 0.9 mM 3-thiacyclohexanone and 0.67 mM NADPH. Cyclohexanone oxygenase was present at 1–2 μM. A control was performed in parallel in the standard manner.

After 2.5 h at 15 °C, the aqueous layer was extracted with ether (2–100 mL). These were combined, dried with $MgSO_4$, and filtered. The solvent was then removed. The aqueous layer was acidified with 20 drops of concentrated HCl and extracted with ether (3–100 mL). The ether layers were combined and washed with brine (150 mL). The organic layers were dried with $MgSO_4$ and filtered, and the solvent was removed. The ^{1}H NMR spectrum was obtained on $CDCl_3$ soluble material of both fractions. No organic product was detected in the basic organic layer. The acidic organic layer contained the nonenzymic hydrolysis product of oxidation, 4-mercaptobutyric acid, identical with the synthetic standard described above in the [^{35}S]-γ-thiobutyrolactone preparation via ^{1}H NMR and HPLC. ^{1}H NMR δ (4-mercaptobutyric acid) 8.87 (s, 1 H), 2.74 (t, 2 H), 2.44 (t, 2 H), 1.93 (m, 2 H), 1.37 (t, 1 H).

Product Characterization of Enzymatic Incubation with *S*-Phenyl Thioacetate. Conditions were similar to those described above for 4-thiacyclohexanone. Concentrations used were 0.7 mM *S*-phenyl thioacetate, 0.96 mM NADPH, and 0.18 μM cyclohexanone oxygenase. The control was identical, except cyclohexanone oxygenase was absent.

After 2.5 h at 15 °C, the aqueous layer was acidified with 10 drops of concentrated HCl and extracted with chloroform (3–100 mL). These organics were combined and washed with brine solution (100 mL). The organic layer was dried with MgSO and filtered, and the solvent was removed. The organic soluble material was dissolved in $CDCl_3$ and characterized via 250-MHz ^{1}H NMR. TLC and ^{1}H NMR comparison to authentic standard (Aldrich) showed the organic soluble material to be phenyl disulfide.

Spectroscopic Investigation of γ-Thiobutyrolactone Inactivation and Reactivation. A large-scale inactivation of cyclohexanone oxygenase (6 mg, 91 nmol with δ-thiobutyrolactone) was performed in the normal manner with 50 mL of 80 mM glycine–NaOH pH 9 buffer. It was then concentrated with an Amicon ultrafiltration system equipped with PM-10 membrane. Controls were run in parallel in the absence of NADPH. A UV–vis spectrum was then taken.

Protein solution (0.7 mL) from inactive and control protein samples were dialyzed for 12 h in No. 2 spectrapor dialysis tubing against 100 mL of the following: (i) 10 mM β-mercaptopethanol, (ii) 10 mM hydroxylamine, and (iii) 10 mM aliquots of both. All were in 21 mM KPi, pH 7.2, at 4 °C. Catalytic assays were performed on each sample after dialysis under standard conditions. A UV–vis spectrum was taken of these samples after dialysis.

Spectroscopic Investigation of ε-Thiocaprolactone Inactivation. Cyclohexanone oxygenase was added to a solution of 80 mM glycine–NaOH pH 9 buffer containing 220 μM NADPH. Inactivation was initiated by the addition of a methanolic solution of ε-thiocaprolactone to a final concentration of 1.8 mM, to bring the assay to 1-mL total volume. After 60 min at 15 °C under ambient oxygen concentration, the sample was transferred to 4 °C and subjected to ultrafiltration by using identical conditions described above. A UV–vis spectrum was obtained after a 10 000-fold net dilution.

***K*$_i$ and *k*$_{inact}$ Determination for Mechanism-Based Inactivators.** Incubation was performed at 15 °C in a solution of 20 mM bis(Tris)-propane, pH 7.2, containing 500 μM NADPH, 0.06 or 0.03 μM cyclohexanone oxygenase, and various concentrations of inactivator in methanol (final organic level is less than 2.5%). At fixed time points the incubation was diluted 50–100-fold into 80 mM glycine–NaOH, pH 9, containing 210 μM NADPH to bring it to a final volume of 990 μL. The residual activity was assayed by addition of 10 μL of a methanolic solution of 4-methylcyclohexanone (final concentration 1.2 mM). Apparent k_{inact} was determined from a semilog plot of remaining residual activity vs. time at various fixed concentrations. k_{inact} and K_i at infinite substrate concentration were determined from a double reciprocal plot.

DTNB Thiol Titrations during Mechanism-Based Inactivation with Ethylene Monothiocarbonate and γ-Thiobutyrolactone. Purified cyclohexanone oxygenase (2.25 μM) was mixed with 1.1 mM ethylene monothiocarbonate or 1.1 mM γ-thiobutyrolactone and 196 μM DTNB in

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Table I. Substrate Profile of Cyclohexanone Oxygenase

substrate	K_M, μM	V_{max}, %
cyclohexanone[b]	6	100
4-methylcyclohexanone	16	114[a]
thiane[c]	24	87
4-thiacyclohexanone	21	108
3-thiacyclohexanone	10	152

[a] See Experimental Section for assay details; 100 % = ca. 689 min^{-1}. [b] From ref 7. [c] From ref 11.

80 mM glycine–NaOH, pH 9. This was incubated for 5 min at 15 °C, to establish the background rate of change in 412-nm absorbance. A solution of NADPH in the same buffer was added to bring the total volume to 1 mL and to initiate the turnover/suicide inactivation reaction. The absorbance increase at 412 nm was recorded (ϵ_{412} 13.6 mM^{-1} cm^{-1}). A second series of experiments were performed in an identical manner at 1.125 μM cyclohexanone oxygenase.

DTNB Thiol Titrations of Native Enzyme, Catalytically Active Treated Enzyme, Ethylene Monothiocarbonate Inactivated Enzyme, and γ-Thiobutyrolactone Inactivated Enzyme. Cyclohexanone oxygenase (1.125 or 2.25 μM) was mixed in 300 mM Tris pH 8 buffer (990 mL) and brought to 15 °C. This was placed in a spectrophotometer and monitored at 412 nm. After a background absorbance level was established for the sample, 196 μM DTNB was added (10 ML), the solution was mixed, and the absorbance at 412 nm was measured. No significant absorbance increase could be observed under nondenaturing conditions.

Cyclohexanone oxygenase (1.125 or 2.25 μM) was then mixed in 6 M guanidine–HCl/300 mM Tris pH 8 buffer and brought to 15 °C. A background absorbance was established, and 196 μM DTNB was added. The solution was mixed and the absorbance at 412 nm measured.

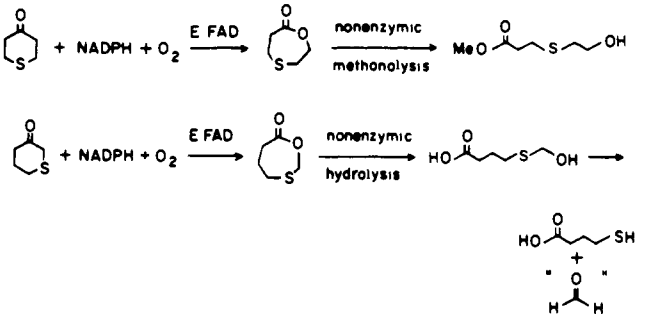
Treated enzyme was prepared by mixing 1.875 or 3.75 μM cyclohexanone oxygenase with 1.83 mM ethylene monothiocarbonate or γ-thiobutyrolactone (in the *absence* of NADPH) in 0.58-mL total volume of 300 mM Tris, pH 8, and incubating for 5 min at 15 °C. At this time the protein was denatured by the addition of 0.4 mL of 6 M guanidine–HCl in the same buffer. A background 412-m absorbance was established, and 196 μM DTNB was added. The solution was mixed and the absorbance increase recorded.

Ethylene monothiocarbonate inactivated or γ-thiobutyrolactone inactivated enzyme was prepared identically with that described for the [^{35}S] stoichiometry determination experiment (see above). Thiol determination was done by using the conditions described for native protein under denaturing conditions.

Results and Discussion

Cyclohexanone oxygenase is a FAD-linked monoxygenase isolated from bacterial strains able to carry out oxygen insertion–ring expansion reactions on cyclic ketones (C_4–C_8).[7] The enzymic process appears chemically equivalent to peracid-mediated Baeyer–Villiger reactions in terms of stereochemistry at the migrating center (retention)[8] and in migratory group aptitude (oxygen insertion at the secondary carbon of 2-methylcyclohexanone).[9] The enzyme-bound FAD 4a-hydroperoxide is the likely oxygen-transfer reagent, and Bruice's studies have shown such flavin hydroperoxides to be 10^7-fold more reactive than *tert*-butyl hydroperoxide in S and N oxygenations.[19] Indeed, in previous work we have reported that cyclohexanone oxygenase processes the cyclohexyl sulfide, thiane, to the thiane-*S*-oxide, with k_{cat}/k_m equivalent to that of cyclohexanone.[11] Thus the enzyme can use bound FAD–4a-OOH as an ambident oxygen-transfer reagent in analogy with peroxide reagents.

Baeyer–Villiger vs. Sulfoxidation Regiochemistry. We have now analyzed the selectivity of cyclohexanone oxygenase when confronted with a substrate containing a ketone and sulfide functional groups in the same molecule. In particular, the enzyme processes 3-thiacyclohexanone and 4-thiacyclohexanone with the catalytic parameters noted in Table I, as detected by O_2 consumption and NADPH oxidation assays. The V_{max} values are actually higher than for cyclohexanone conversion, and the k_{cat}/k_m values are comparable. To analyze whether sulfur (chemically more facile) or the carbonyl group was the site of enzymic oxygenation, large-scale incubations (0.6 mg of enzyme) were performed to isolate sufficient product for spectroscopic characterization and chromatographic comparison to standard thialactone analogues and derivatives. In both the 4-thia- and 3-thiacyclohexanones, only the Baeyer–Villiger type products derived from the thialactones were detected. No sulfur oxidation competed.



This is in contrast to the greater ease of sulfur oxidation in nonenzymic oxygen-transfer chemistry.[10] The enzymic regiochemistry may be rationalized if a ketone binding determinant predominates in orientation of the substrate toward the FAD–4a-OOH in the active site.

Thiolactone Substrates. The corresponding 2-thiacyclohexanone is in fact a thiolactone. It is still a substrate at least for ca. 14 turnover events, but then it causes irreversible inactivation of cyclohexanone oxygenase. The inactivation as well as turnover requires both O_2 and NADPH and so appears mechanism based. As shown in Table II, the five-member γ-thiobutyrolactone and the analogous seven-member ε-thiocaprolactone are also suicide substrates with the indicated partition ratios of 33/1 and 17/1, respectively. The kinetic parameters described in Table II indicate that these thiolactones are in fact poor substrates for cyclohexanone oxygenase (15- to 50-fold slower in turnover than the corresponding cyclic alkanone). However, these substrates generate potent inactivating reagents once oxidized by the enzyme, as

Table II. Kinetic Parameters of Mechanism-Based Inactivators of Cyclohexanone Oxygenase

inactivator	k_{inact}, min^{-1}	K_i, mM	partition ratio	k_{cat}, min^{-1} [a]
γ-thiobutyrolactone	1.25	2.0	33	41
δ-thiovalerolactone	1.03	2.9	14	14.4
ε-thiocaprolactone	0.8	2.0	17	13.6
ethylene monothiocarbonate	1.3	2.0	110	143

[a] Estimated from the product of the observed partition ratio and k_{inact}.

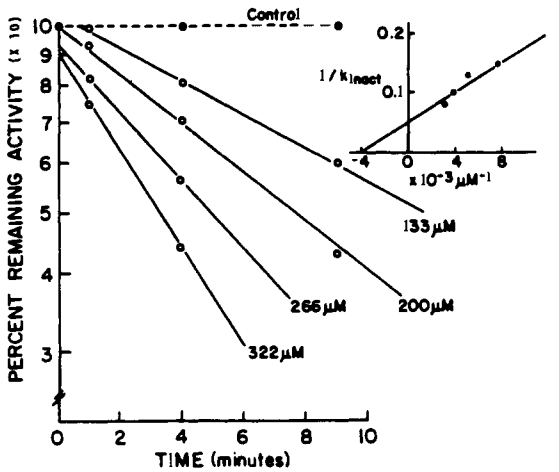


Figure 1. Determination of K_i and k_{inact} values for inactivation of cyclohexanone monooxygenase by γ-thiovalerolactone. Enzyme was preincubated with the indicated concentrations of inactivator under turnover conditions and then diluted into assay buffer with saturating substrate to assay residual activity as described in materials and methods. The K_i and k_{inact} values were then determined from the double reciprocal plot of $1/k_{inact}$ vs. $1/I$ as shown in the inset and are listed in Table II.

(19) Bruice, T. C.; Noar, J. B.; Ball, S. S.; Uenkataram, U. V. *J. Am. Chem. Soc.* **1963**, *105*, 2452–2463.

indicated by the low partition ratios observed for this class of mechanism-based inactivators. As shown in Figure 1, the δ-thiovalerolactone shows first-order inactivation kinetics, allowing a k_{inact} to be extrapolated at infinite suicide substrate concentration. Mechanism-based inactivation is rapid and efficient as judged by the observed partition ratio. In the case of δ-thiovalerolactone (2-thiacyclohexanone) on average every 15th catalytic turnover is suicidal. The substrate 4-methylcyclohexanone shows significant kinetic protection against inactivation as expected for mechanism-based inactivation (retention of greater than 90% of control activity when preincubated with the cyclic alkanone). The similar high killing efficiency in oxygenative processing of the five- and seven-ring thiolactones suggests this will be a general mode for the thiolactone functional group with this enzyme.

To understand the route of the mechanism-based inactivation we have tried to analyze the nature of the few product molecules forming in kinetic competition with inactivation and by analysis of site and stoichiometry of the flavoenzyme modification. Efforts toward each point are delineated below.

Stoichiometry of Inactivation. To determine the stoichiometry of enzyme labeling during inactivation, [^{35}S]-γ-thiobutyrolactone was prepared from [^{35}S]thiourea and 4-bromobutyronitrile by an isothiouronium exchange reaction described in the Methods section followed by pyrolysis to yield the radiolabeled thiolactone.

Because thiolactones are themselves potential acylating agents, even in the absence of oxidant, we tested the reaction of [^{35}S]-γ-thiobutyrolactone with enzyme in the absence of NADPH and O_2 and isolated the enzyme after gel filtration. Typically, enzyme treated in this manner retained ≥90% of its specific activity, but has in fact incorporated 1 mol of ^{35}S label per mole of enzyme. Thus, there is one reactive enzyme nucleophile stoichiometrically acylated by the thiolactone. This is however a catalytically indifferent event because the acylated enzyme is essentially fully active. It is only when NADPH and O_2 are also present with thiolactone that enzyme becomes inactivated. We do not yet know the nature of this catalytically irrelevant acylation event.

For determining the stoichiometry of inactivation caused by [^{35}S]-γ-thiobutyrolactone in the presence of NADPH and O_2, the enzyme was pretreated as above with unlabeled thiolactone in the absence of NADPH and O_2, recovered by ultrafiltration (and shown to be ≥90% active), and then exposed to [^{35}S]-γ-thiobutyrolactone, NADPH, and O_2 until ≥95% activity loss. Subsequent gel filtration to quantitate enzyme-associated radioactivity showed 0.9 ^{35}S equiv/enzyme molecule inactivated.

For other flavoenzymes inactivated by suicide substrates, some such as 2-hydroxybutynoate modify the flavin coenzyme[20] while others such as butynyl CoA modify the apoprotein.[21] On inactivation with γ-thiobutyrolactone, cyclohexanone oxygenase shows a UV change with broad absorbance in the 320–370-nm region but otherwise an essentially unchanged oxidized flavoprotein spectrum. The other thiolactone mechanism-based inactivators generate similar spectra after inactivation has occurred. When inactive enzyme from gel filtration chromatography noted above was treated with 5% cold trichloroacetic acid, 80% of the radiolabel precipitated with apoenzyme while FAD was quantitatively released in the supernatant. On further analysis the released FAD was unmodified and unlabeled, suggesting oxygenated thiolactone-mediated inactivation modifies an amino acid residue(s) stoichiometrically.

Attempts to characterize the ^{35}S-labeled apoprotein further have not yet been conclusive. On treatment of ^{35}S-inactive enzyme with 21 mM β-mercaptoethanol (12 h) or with 21 mN NH_2OH, only small amounts of activity regain were detected (less than 10% of the control). On the other hand, dialysis against both of these reagents did diminish the broad absorbance in the 320–370-nm region associated with the initial inactive enzyme and returned the spectrum to that of a control sample. This occurred without regain of activity. Efforts to isolate a radioactive tryptic peptide

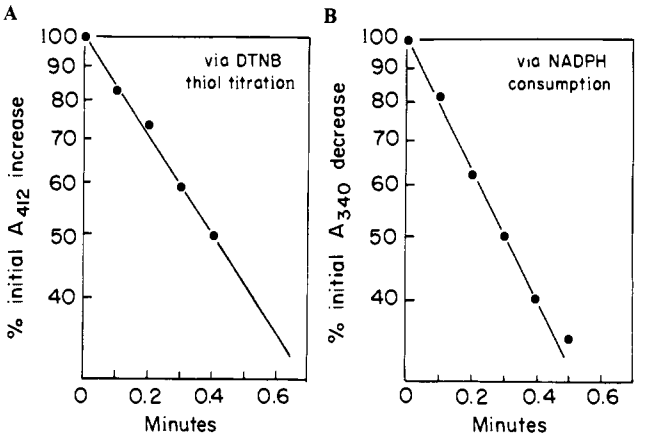
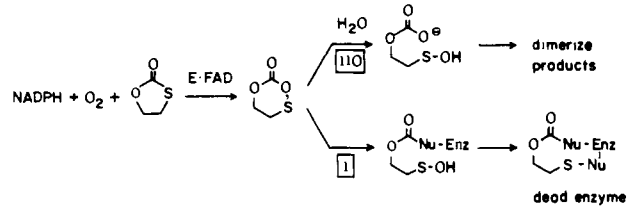


Figure 2. Correlation of rate of appearance of a single titratable enzyme thiol during turnover-dependent inactivation by ethylene monothiocarbonate with rate of activity loss: (A) rate of appearance of a stoichiometric thiol group of cyclohexanone monooxygenase during inactivation as measured by DTNB reduction, as noted in methods; (B) rate of loss of enzyme activity by NADPH consumption assay, as described in methods.

failed on multiple occasions. Although trypsin-generated peptides were well separated on HPLC, there was no discreate peak of radioactivity associated with any fragment. These results were obtained even if the reduction and alkylation were omitted prior to tryptic digest. Thus, the ^{35}S-labeled apoprotein adduct appears labile under these conditions of protein digestion.

Inactivation with the Cyclic Thiol Ester Ethylene Monothiocarbonate. In addition to the five-, six-, and seven-membered thiolactones of Table II, we have also determined that a heteroatom-containing analogue of γ-thiobutyrolactone is a suicide substrate of the enzyme. Ethylene monothiocarbonate has a similar k_{inact} (1.3 min^{-1}) but a considerably higher k_{cat} (143 min^{-1}) for a partition ratio of 110 turnovers per inactivation event. This



cyclic thiocarbonate is hydrolytically more stable than γ-thiobutyrolactone, a less reactive acylating agent and a 3-fold less effective suicide substrate than thiobutyrolactone (Table II). These properties made ethylene monothiocarbonate the suicide substrate of choice in studies with DTNB to monitor any changes in the level of enzyme thiol groups during inactivation. Unlike γ-thiobutyrolactone, the cyclic thiocarbonate did not exhibit non-enzyme-dependent thiol interchange with DTNB.

During catalytic turnover and inactivation with the thiocarbonate a stoichiometric burst of one DTNB-titratable enzyme thiol was observed. There was no such titratable thiol, stoichiometric with the amount of enzyme present, produced during normal turnover of cyclic ketone substrates. Since 110 product molecules are generated per enzyme inactivation, it cannot be the enzymic oxidation product which is contributing an SH group (this would give a burst of 110 mol of SH). It suggests the SH burst is protein associated. Further, the titratable thiol burst occurs with a first-order rate constant equivalent to the rate constant for enzyme inactivation, as measured by NADPH consumption, depicted in Figure 2. Thus, the enzymic suicidal event proceeds with liberation of one DTNB-titratable SH per enzyme molecule inactivated at a rate constant controlled by the stoichiometric inactivation process. The SH burst ceases as enzyme is inactivated.

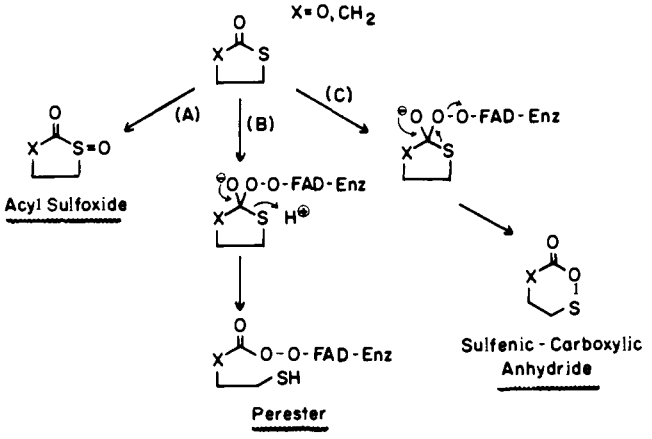
In principle this titratable thiol appearing concomitant with enzyme inactivation could be contributed by enzyme via a conformation change on killing or from a newly generated SH group in the covalent adduct formed during stoichiometric modification

(20) Walsh, C. T.; Schonbrunn, A.; Lockridge, A.; Massey, V.; Abeles, R. H. *J. Biol. Chem.* **1972**, *247*, 6004–6006.
(21) Fendrich, G.; Abeles, R. H. *Biochemistry* **1982**, *21*, 6685–6695.

by the ethylene monothiocarbonate skeleton upon oxygenative processing. We evaluated the total number of DTNB-titratable thiols in control enzyme and, after full inactivation by ethylene thiocarbonate, enzyme was used in 4-mg quantities followed by gel filtration to remove small molecules and denaturation in 6 M guanidine before DTNB titration. The control, native enzyme, shows no DTNB-accessible thiols at 15 °C, the assay and inactivation temperature utilized. At 30 °C one can titrate thiols in native enzyme, but the enzyme is unstable in catalytic assays at this temperature. For the past 5 years we have assayed at 15 °C where cyclohexanone oxygenase is stable for hours in catalytic assay, thereby permitting accurate and quantitative determination of mechanism-based inactivator kinetics.

At 15 °C denatured control enzyme reproducibly shows five cysteine SH groups while ethylene monothiocarbonate inactivated enzyme shows only three SH groups in 6 M guanidine–HCl. Thus the enzyme inactivation process involves net loss of two enzyme cysteine SH groups, both with ethylene monothiocarbonate and with γ-thiobutyrolactone, upon stoichiometric inactivation. A mechanism involving cross-linking of two enzyme SH groups to one doubly activated killing species is discussed below. Coupled with the loss of two SH groups is the appearance of one SH, *under native conditions*, either an enzymic cysteine as a DTNB-accessible group or, less likely, an SH derived from that doubly activated species.

Studies on the Nature of Oxygenation of Thiol Ester Substrates. Given the precedents that cyclohexanone oxygenase can attack ketone carbonyls in a Baeyer–Villiger oxygenation or can attack sulfur in sulfoxidation, three pathways for enzymic processing of cyclic thiol esters seem possible, for γ-thiobutyrolactone or ethylene monothiocarbonate as noted below.

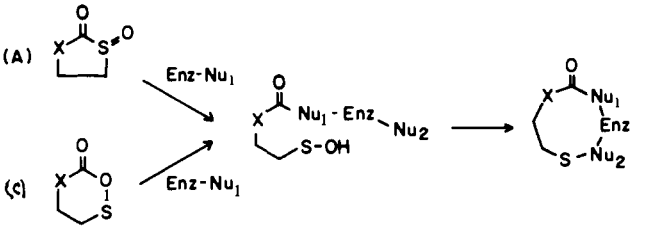


Route A involves oxygenation at sulfur to yield an acyl sulfoxide. Routes B and C both propose attack by FAD-4a-OOH on the carbonyl carbon to yield the tetrahedral adduct. In path B, the sulfur is proposed as the leaving group, with acid catalysis to yield a perester intermediate. In path C, the Baeyer–Villiger type ring-expansion process is proposed with the sulfur atom as the migrating group to yield not a ring-expanded ester product but a ring-expanded sulfenic–carboxylic anhydride. All three intermediates, the acyl sulfoxide, the acyl perester, and the mixed anhydride, would be reactive acylating agents. Precedents in the organic and natural product literature for acyl sulfoxides and mixed carboxylic–sulfenic anhydrides exist,[18,22,23] unlike route B where little has been established about the perester acyl transfer product. For example, the anhydride structure is represented in the recently isolated thiotropocin,[22] which may be stabilized by the adjacent tropolone-like ring in this antibiotic.

Chemical precedent exists for conversion of thio esters to acyl sulfoxides by oxygenation at sulfur. In studies by Minato[18] and colleagues, and by the Barton group,[23] a very reactive acyl transfer reagent could be generated by oxidizing thio esters with peracids. Minato proposed that oxidation of *S*-phenyl thioacetate generated the corresponding acyl sulfoxide. This species contains a highly electrophilic carbonyl activated for acyl transfer to a nucleophile. The putative acyl sulfoxide was not isolable but was inferred by characterization of the resulting products of acyl transfer, namely, the acylated alcohols used as nucleophiles to "trap" the acyl sulfoxide. In addition, the sulfur-containing fragment from the reactive intermediate (a phenyl sulfenic acid) was detected as the phenyl disulfide monosulfoxide, a characteristic self-condensation product of sulfenic acids.[24-26]

In Barton's work, oxidations[23] were performed with peracids on a similar thio ester moiety, specifically aryl monothiocarbonates. In the case of dibenzyl monothiocarbonate, the intermediate acyl sulfoxide is an isolable species, which was crystallized and spectroscopically characterized. This acyl sulfoxide, when treated with an amine, rapidly undergoes acyl transfer to generate a carbamate quantitatively under mild conditions (methylene chloride or chloroform at room temperature). We have also found that with dibenzyl monothiocarbonate in a comparison of the reactivity between the parent monthiocarbonate and the acyl sulfoxide generated on oxidation, acyl transfer to primary amines occurs at a minimum 400 times faster from the acyl sulfoxide than from the starting thiocarbonate.

The results cited above provide chemical precedents that cyclohexanone oxygenase could function as a peracid equivalent for the same type of sulfur oxidation. We have not yet isolated products from 33 γ-thiobutyrolactone turnovers per killing event in the enzymic reaction. Just as the cyclic sulfoxide species has a reactive acyl group which will undergo nucleophilic capture and generate a cleaved product with an unmasked sulfenic acid group, a sulfenic–carboxylic anhydride should also acylate nucleophiles and likewise liberate an electrophilic sulfenic acid moiety. It does not seem readily possible to distinguish path A from path C at present. Each has the propensity to yield a reactive acylating agent which could capture an enzyme nucleophile. In so doing, this uncovers at the cleaved oxidized sulfur atom an electrophilic sulfenic acid group capable of capturing a second, nearby enzyme nucleophile and thus act as a bifunctional, cross-linking suicide substrate.



In an initial effort to see whether an initial acylation of the enzyme by the proposed reactive acylating equivalent was the killing reaction, whether subsequent sulfenylation was the inactivating event, or lastly whether both were needed in a cross-linking sequence, we turned to *S*-phenyl thioacetate as a possible inactivator. The two halves of the potential inactivating species generated upon oxidation (the acylic acyl sulfoxide or acyclic sulfenic–carboxylic anhydride) would be disconnected after the initial acylation.

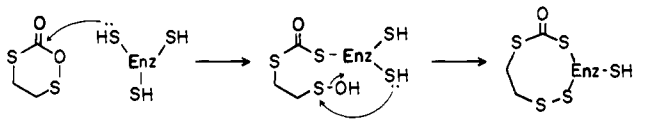
We found the enzyme did process *S*-phenyl thioacetate catalytically. During the processing event, however, *no inactivation occurred*. Isolation and characterization revealed diphenyl disulfide as one of the enzyme products. This product conceivably arises from nucleophilic addition of the distal 4a-FAD hydroperoxide oxygen to bound substrate carbonyl to generate the tetrahedral intermediate. Collapse would generate the FAD-4a-acetyl perester and the phenylthiolate anion, which subsequently oxidizes to the phenyl disulfide isolated. The flavin adduct would

(22) Kintaka, K.; Ono, H.; Tsubotani, S.; Harada, S.; Okazaki, H. *J. Antibiot.* **1984**, 1294–1300.
(23) Barton, D. H. R.; Manly, D. P.; Widdowson, D. A. *J. Chem. Soc., Perkin Trans. 1* **1975**, 1568–1574.
(24) Davis, F. A.; Freidman, A. J.; Nadir, U. K. *J. Am. Chem. Soc.* **1978**, *100*, 2844–2852.
(25) Hogg, D. R. *Compr. Org. Chem.* **1979**, *4*, 261–310.
(26) Kice, J. L. *Adv. Phys. Org. Chem.* **1980**, *17*, 65–181.

hydrolyze to acetate, H_2O, and oxidized FAD.

It would seem that cross-linking may be essential in cyclohexanone oxygenase inactivation by the *cyclic* thiol ester class of mechanism-based inactivators.

Possible Scheme for Cyclohexanone Oxygenase Inactivation. Cyclic thiolactones and monothiocarbonates are enzymically oxygenated to species which are reactive acylating agents capable of further derivatization via an unmasked sulfenylating agent. Since the enzyme appears to lose two thiol groups on inactivation by these suicide substrates, it is tempting to suggest the following hypothesis: one active site cysteine SH initially undergoes acylation and then a second, nearby cysteine SH reacts with the tethered sulfenate terminus to generate a cross-linked enzyme acyl thiol ester disulfide species. The process is illustrated for a sulfenic–carboxylic anhydride (a similar sequence for an acyl sulfoxide is equally plausible), formed from ethylene monothiocarborate. This proposal suggests the single titratable SH accessible on inactivation is one of the original five cysteine SH groups. This cross-linking adduct would likely be labile during trypsin digestion and peptide separation, consistent with failure to isolate a ^{35}S-labeled peptide. It would predict release of label and regain of activity on combined exposure to β-mercaptoethanol and hydroxylamine. Some activity was returned, from 1% residual back to 10% of control, by 21 mM NH_2OH and β-mercaptoethanol with regain of the normal UV–vis spectra. It is unclear why more recovered activity was not observed if this proposal is correct. However the enzyme conformation may no longer be native (to it the titratable enzyme SH group is only accessible on inactivation).



These cyclic thiol esters constitute a novel class of mechanism-based enzyme inactivators for flavoproteins, activatable by oxygenation. These mechanisms may be relevant to the mode of autodestruction of adrenal and testicular steroid P 450 hydroxylase activity by the antihypertensive 7-thioacetyl steroid spironolactaone.[27] Similarly, thiocarbamate herbicides such as butylate and benzothiocarb are converted during microsomal oxidations[28-33] to the corresponding acyl sulfoxides which act as reactive carbamylating agents of important thiol groups involved in susceptible plant fatty acid metabolism.

Abbrevations: NADPH, dihydronicotinamide adenine dinucleotide phosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; FAD, flavin adenine dinucleotide.

Acknowledgment. We thank Dr. Bruce Branchaud for initial experiments on thiolactone inactivators and for drawing attention to these molecules as mechanism-based inactivators of cyclohexanone monooxygenase.

Registry No. Cyclohexanone oxygenase, 52037-90-8; 4-methylcyclohexanone thiane, 589-92-4; 4-thiacyclohexanone, 1072-72-6; 3-thiacyclohexanone, 19090-03-0; γ-thiobutyrolactone, 1003-10-7; δ-thiovalerolactone, 1003-42-5; ϵ-thiocaprolactone, 17689-16-6; ethylene monothiocarbonate, 3326-89-4.

(27) Menard, R. H.; Guenthuer, T. M.; Kon, H.; Gillette, J. R. *J. Biol. Chem.* **1979**, *254*, 1726–1733.
(28) Casida, J. E.; Kimmel, E. C.; Ohkawa, H.; Ohkawa, R. *Pestic. Biochem. Physiol.* **1975**, *5*, 1–11.
(29) Chen, Y. S.; Casida, J. E. *J. Agric. Food Chem.* **1978**, *26*, 263–267.
(30) Chen, Y. S.; Schuphan, I.; Casida, J. E. *J. Agric. Food Chem.* **1979**, *27*, 709–712.
(31) Hubbell, J. P.; Casida, J. E. *J. Agric. Food Chem.* **1977**, *25*, 404–413.
(32) Lay, M. M.; Hubbell, J. P.; Casida, J. E. *Science (Washington, DC)* **1975**, *189*, 287–289.
(33) Lay, M. M.; Casida, J. E. *Pestic. Biochem. Physiol.* **1976**, *6*, 442–456.