Suicide Inactivation of Hydroxylamine Oxidoreductase of *Nitrosomonas europaea* by Organohydrazines†

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ABSTRACT: In the presence of a suitable electron acceptor such as mammalian cytochrome *c*, hydroxylamine oxidoreductase (HAO) from the chemolithotrophic bacterium *Nitrosomonas europaea* catalyzes the oxidation of hydroxylamine or hydrazine to nitrite or dinitrogen, respectively. Each subunit of HAO contains 7 *c*-hemes and a chromophore of the active site called heme P460, a *c*-heme bridged from a methylene carbon to a ring carbon of a tyrosine of the peptide chain. Reaction with either substrate results in reduction of several *c*-hemes of HAO. The reaction of organohydrazines with HAO was investigated in this work. HAO was inactivated by (phenyl-, (methyl-, or (hydroxyethyl)hydrazine. The process followed first order kinetics and was inhibited by the substrates, hydroxylamine or hydrazine. Complete loss of enzyme activity and absorbancy characteristic of native heme P460 of HAO occurred at a 1:1 ratio of phenylhydrazine and HAO. HAO was covalently derivatized by two molecules of [^{14}C]-phenylhydrazine per subunit. Heme P460 was derivatized with high affinity, and an amino acid residue was derivatized with lower affinity. *c*-Hemes were not derivatized except for the partial reaction of (hydroxyethyl)hydrazine with one heme. As with hydroxylamine and hydrazine, incubation with organohydrazines resulted in reduction of *c*-heme of HAO. Derivatized *minus* native optical difference spectra of ferric or ferrous HAO revealed changes in the optical properties of heme P460 which were generally similar to shifts seen in the reaction of the heme of other hemoproteins with organohydrazines. The data indicate that organohydrazines are suicide substrates of HAO and constitute direct evidence that P460 is at the active site. The data also establish conditions for the use of organohydrazines as probes for structural and mechanistic analysis of HAO.

The chemolithotrophic bacterium, *Nitrosomonas europaea*, obtains energy from the oxidation of ammonia to nitrite. The intermediate, hydroxylamine (Hollocher et al., 1981), is oxidized by hydroxylamine oxidoreductase (HAO)[1] $NH_2OH + H_2O \rightarrow HNO_2 + 4e^- + 4H^+$ (Andersson & Hooper, 1982). HAO also catalyzes the oxidation of hydrazine to a gas presumed to be dinitrogen (Anderson, 1964), a reaction of unknown significance in nature. HAO is a dimer or trimer of a 63 kDa (Terry & Hooper, 1981; Masson et al., 1990) subunit which contains 7 *c*-hemes and one heme P460 (Arciero & Hooper, 1993). Several *c*-hemes of HAO (but not heme P460) are reduced in the presence of hydroxylamine or hydrazine, and HAO catalyzes the multiple turnover steady-state oxidation of hydroxylamine or hydrazine and concomittant reduction of exogenous oxidants such as mammalian cytochrome *c* or redox dyes (Hooper & Nason, 1965).

Heme P460 of HAO, which has a Soret band at 463 nm in the ferrous form but no clearly identified optical feature in the ferric state (Collins et al.,1993), consists of a *c*-heme covalently bridged from a meso carbon to a ring carbon of a tyrosine of the polypeptide chain (Arciero et al., 1993). The cross-link is from Cys 229 and 232 to Tyr 467 (Sayavedra-Soto et al., 1994). Mossbauer and EPR spectra indicate that the irons of heme P460 and a *c*-heme are electronically coupled (Andersson et al., 1984) and bridged by a single moiety, possibly histidine (Hendrich et al., 1994). Model compounds which mimic the Mossbauer and optical properties of ferrous heme P460 are five-coordinate porphyrins with an oxyanionic ligand (Nasri et al., 1987). Considerable evidence implicates heme P460 as a component of the active site. In the ferrous form it reacts with CO, O_2, or H_2O_2; i.e., it is accessible to small molecules (Hooper & Balny, 1982; Hooper et al., 1983). More directly, reaction of ferric HAO with H_2O_2 appears to simultaneously eliminate heme P460 (the absorbancy of ferrous heme P460 is lost), the substrate reducibility of hemes of HAO, and enzyme catalysis (Hooper & Terry, 1977).

Alkyl- and arylhydrazine derivatives are thought to inactivate hemoproteins by the reaction: $RN_2H_3 \rightarrow [RNNH] \rightarrow N_2 + R^{\bullet+} + 3H^+ + 4e^-$ (Lavallee, 1987). The cation radical is initially bound to the iron and, depending upon the redox state of the iron and the environment of the heme, migrates to a meso carbon or an amino acid. For most monoheme proteins, an exogenous oxidant such as oxygen, peroxide, or ferricyanide must be present for maximum inactivation. Reaction of organohydrazines with a ferrous heme often produces an organic cation radical which bonds with the iron. If the heme iron is ferric, the R group typically migrates to a pyrrolic N as seen with myoglobin, hemoglobin, cytochrome $P450_{cam}$, or catalase (Augusto et al., 1982; Ortiz de Montellano & Kerr, 1983). The crystallographic struc-

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[1] Abbreviations: HAO, hydroxylamine oxidoreductase, SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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tures of the *N*-phenyl porphyrin forms of myoglobin and $P450_{cam}$ are known (Ringe et al., 1984; Raag et al., 1990).

The present work shows that organohydrazines are suicide substrates of HAO and that heme P460 is covalently modified. The general characteristics of the inactivation resemble the reaction of other hemoproteins with hydrazine derivatives. This work provides more compelling evidence than was previously available that heme P460 is at the active site of HAO and provides base-line data necessary for the use of organohydrazines as probes of the structure and catalytic mechanism of this complex enzyme.

MATERIALS AND METHODS

All chemicals were reagent grade or better. Water was double distilled or Millipore Super Q. Uniformly labeled [^{14}C]phenylhydrazine (7 mCi/mmol; 259 MBq/mmol) was from ICN Radiochemicals, Irvine, CA. Type III horse heart cytochrome *c*, bovine liver catalase, Sephadex & resins, and DEAE-Sephacel were from Sigma Chemical Co., St. Louis, MO. Phenylhydrazine hydrochloride was from the J. T. Baker Chemical Co., Phillipsburg, NJ. (2-Hydroxyethyl)-hydrazine and 1,1-dimethylhydrazine were from Aldrich Chemical Co., Milwaukee, WI. Methylhydrazine was from Kodak, Rochester, NY. Sephadex and ampholines for isoelectric focusing were from either Sigma or Pharmacia, Inc., Piscataway, NJ. Because most alkyl- and arylhydrazines were somewhat unstable in the light at room temperature, a concentrated stock solution, dispensed in aliquots, was frozen in dry ice, stored in the dark, and thawed immediately before use.

Growth of Bacteria. N. europaea (Schmidt strain) was grown either in 15 L carboys as previously described (Hooper et al., 1972) or in continuous culture. For the latter, a 110 L cylindrical polypropylene tank (Nalgene) was the media reservoir. Medium was transferred by peristaltic pump at a constant flow rate into the reactor (55 L volume in a cylindrical Nalgene polypropylene tank). Media were made from the following separate stock solutions: (a) 38 g/L KH_2PO_4; (b) 50 g/L $NaHCO_3$; (c) 375 g/L $(NH_4)_2SO_4$; (d) 68 g/L $MgCl_2{\cdot}6H_2O$ and 3.1 g/L $CaCl_2{\cdot}2H_2O$; (e) 6.7 g/L $FeSO_4$, 1 g/L $CuSO_4$, and 8 g/L tetrasodium EDTA. To make 15 L of media, 20 g/L Na_2HPO_4, 45 mL of solution a, 150 mL of solution b, and 160 mL of solution c were made up to 15 L with distilled water and autoclaved for 45 min. Solutions d and e were autoclaved separately, and 45 and 15 mL volumes, respectively, were added to the cooled 15 L solution in the carboy.

During growth, the pH was maintained at a value of 7.8 by automated titration with 50% w/v K_2CO_3. Three spargers provided aeration and mixing. The reactor sat in a larger, covered tank containing water maintained at 28 °C. A 110 L cylindrical polypropylene tank (Nalgene) in a refrigerator served as the collection vessel for the reactor output. Harvesting was within 5 days using a Millipore Pellicon system. Typically, the dilution rate was 0.32 day^{-1}, and growth yields were 200 mg/L of cells (wet weight), approximately twice that obtained from carboy growth of cells. Once established, the continuous culture could be operated for many months without interruption. Heterotrophic bacteria were present at about 10^6/mL as determined by plating dilutions of a sample from the reactor on nutrient agar. Direct microscopic counts of the reactor solution revealed the presence of $\sim 5 \times 10^8$ cells/mL; thus the contaminants were apparently present at ~0.2% relative to *Nitrosomonas*. By Gram stain there were typically several hundred small, Gram negative ovoids (*Nitrosomonas*) per contaminant.

Preparation of Enzyme. Protein purification was generally conducted at 4 °C. The major concern during purification was loss of heme P460 of HAO by possible peroxide inactivation. Because ferrous HAO can reduce O_2 to H_2O_2 (Hooper & Balny, 1982), the enzyme was not exposed aerobically to chemical reductants or light (which was found to photoreduce a small amount of the *c*-heme of HAO). Cells were broken by freezing and thawing three times and centrifuged at 20000*g* for 30 min to remove large debris (Hooper et al., 1972). Purification was based on the method of Arciero et al. (1991), the 60−80% ammonium sulfate fraction was applied to a Sephadex G-100 column, and the HAO-containing fractions were pooled and dialyzed against 30% ammonium sulfate with 100 mM KCl and 25 mM potassium phosphate buffer, pH 7.5, and then loaded onto a 30 × 2.5 cm octyl-Sepharose column. The bulk of the HAO eluted between a concentration of 25% and 20% ammonium sulfate in a 40−10% gradient (in 100 mM KCl and 50 mM potassium phosphate, pH 7.5). HAO eluted from the 20−10% ammonium sulfate were rechromatographed and eluted at 25−20% ammonium sulfate as above. The final 408/280 nm ratio for this preparation was 3.8. Unless otherwise stated, the concentration of HAO is expressed as the concentration of the 63 kDa subunit (which is equal to the concentration of heme P460 of the catalytic site) based on an extinction coefficient of 700 mM^{-1} cm^{-1} at 408 nm in the ferric state, or of 140 mM^{-1} cm^{-1} at 552 nm in the ferrous state.

SDS−polyacrylamide electrophoresis was performed with a Bio-Rad minigel electrophoretic apparatus and protocols. Fluorography for the detection of [^{14}C]phenylhydrazine-labeled HAO in acrylamide gels was by the technique of Bonner (1983). Scintillation counting was performed by a Beckman LS3801 counter in 10 mL volumes of Econofluor (ICN Biomedicals, Irvine, CA) to which 0.5 mL or less of solution was added.

Optical Measurements. Unless otherwise stated, measurements were in a solution of HAO (0.9−1.5 μM heme P460 of the active site) at 25 °C in 50 mM potassium phosphate buffer, pH 7.5. A Hewlett-Packard 8452A diode array spectrophotometer, equipped with a 7 cell multitransporter (thermojacketed and magnetically stirred), was used for difference spectra and kinetic measurements (where k_{obs} was less than 2 min^{-1}). The UV cutoff filter no. 3, supplied with the instrument, and a glass plate in front of the deuterium light source were employed to prevent photoreduction of HAO promoted by light at wavelengths less than 300 nm. The open sample compartment of the HP-8452A was shielded from room light. Small amounts of reduction of *c*-hemes of HAO did not affect formation of ligand−HAO complexes but did hinder analysis of spectra. Difference spectroscopy was necessary in order to observe the subtle effects of organohydrazines on HAO because of the large background absorbancy of the 7 *c*-hemes in each subunit of HAO. Reactions were typically performed in 4 mL quartz cuvettes (1.0 cm path length) covered with Parafilm or Teflon and containing 2 mL of solution stirred by a magnetic flea. Reactants were added in volumes of less than 2 μL by microsyringe, and scans were taken over the range 300−

820 nm. Scans were stored and difference spectra were produced by the kinetics software of the HP 8452a. Values of k_{obs} were calculated from time traces using Marquardt's algorithm as implemented on the Hewlett-Packard software.

Measurement of Activity and Steady-State Kinetics of HAO. Activity of HAO was routinely assayed under steady-state conditions as the rate of reduction (the change absorbancy at 550 nm *minus* the change in absorbancy at 744 nm) of 25 μM horse heart cytochrome *c* in the presence of 1−20 nM of HAO and 1−50 μM hydrazine or hydroxylamine in 50 mM potassium phosphate buffer, pH 7.5. Rates were corrected for a low rate of reduction by substrates in the absence of enzyme.

Kinetics of Inactivation of Organohydrazines. Typically, 0.5 mL of 1−5 μM HAO was placed in a conical microcentrifuge tube and stirred by a magnetic flea, and 2 μL of organohydrazine solution was added at time zero; 10 μL aliquots of the HAO solution were removed and diluted into a mixture containing 20 μM N_2H_4 or NH_2OH and 25 μM horse heart cytochrome *c* in 50 mM potassium phosphate buffer, pH 7.5, and the steady-state activity was measured. The final concentration of ethyl- or phenylhydrazine was always less than 0.5 μM since these compounds reduced horse heart cytochrome *c* directly.

Inactivation of Ferric HAO by Hydrogen Peroxide. HAO was inactivated with hydrogen peroxide by incubating the ferric enzyme (1−10 μM) with 100 μM H_2O_2 for various times, quenching the reaction with 100 μM NH_2OH, and dialyzing (membrane pore size 12 000−14 000 MW) with three changes (3 × 400 mL) of buffer for 12 h. A few units of catalase were added to the dialysis buffer.

RESULTS

Based on precedent with other hemoproteins, the reaction of RN_2H_3 compounds with HAO might be as follows:

$$RN_2H_3 + HAO \underset{K_d}{\rightleftharpoons} \{RN_2H_3{\cdot}HAO\} \xrightarrow{-4e^- - 3H^+} \{R^{\bullet+}N_2{\cdot}HAO\} \xrightarrow[k_{inac}]{-N_2} \{R{\cdot}HAO\}$$

RN_2H_3 may form a reversible complex with HAO and then be oxidized at the active site of HAO (most probably heme P460) with *c*-hemes of HAO as terminal electron acceptors. After release of N_2, the $R^{\bullet+}$ group may bind to the iron of heme P460 and react further.

Reduction of c-Hemes of HAO by Organohydrazines. Following incubation with phenylhydrazine, methylhydrazine, (hydroxyethyl)hydrazine, 1,1-dimethylhydrazine, or 1,1-diphenylhydrazine, approximately 1/4 of the *c*-hemes of HAO were reduced (25% of the possible ferrous α-absorbancy of HAO was observed). Hence the hydrazine compounds were oxidized. Heme P460 was not reduced (an absorbancy maximum at 460 nm did not appear in the substrate reduced *minus* oxidized spectrum). By the same criteria, heme P460 is also not reduced during reaction of HAO with hydrazine or hydroxylamine (Hooper et al., 1984). This is in keeping with the low value of the oxidation reduction potential (−260 mV; Collins et al., 1993) of heme P460 of HAO. Stopped-flow measurements (data not shown) determined a value of k_{obs} of 1.5 s^{-1} for reduction of *c*-hemes of HAO by 50 mM methylhydrazine (a concentration which was twice the K_d for inactivation). The value of k_{obs} for the reduction of *c*-hemes of HAO by hydroxylamine or hydrazine under these conditions is 27 s^{-1} (Hooper et al. 1984).

Table 1: Kinetic Data for the Inactivation of HAO by Organohydrazines

substrate	K_d or K_m^{app} [a] (μM)	k_{inac} (s^{-1})	k_{inac}/K_d ($s^{-1}\,\mu M^{-1}$)
NH_2OH	2		
NH_2NH_2	4		
CH_3NHOH	14		
CH_3NHNH_2	21	0.0095	0.45×10^{-3}
$HOCH_2CH_2NHNH_2$	60	0.027	0.45×10^{-3}
$C_6H_5NHNH_2$	180	0.018	0.11×10^{-3}

[a] The values of K_m^{app} for the three steady-state substrates, determined by a simple Michaelis−Menten treatment, are included for purposes of comparison.

Kinetics of Inactivation of HAO by RN_2H_3's. The time- and concentration-dependent inactivation of HAO were determined with (methyl-, (phenyl-, and (hydroxyethyl)-hydrazine (Figure 1). When HAO was incubated with phenylhydrazine in the presence of 0.5 mM N_2H_4, a decrease in activity was not seen; thus substrate apparently protected against inactivation. Rate constants (k_{obs}) at various fixed concentrations of RN_2H_3 were determined from semilogarithmic plots of the loss of activity as a function of time (Figure 1). Plots of k_{obs} versus the concentration of RN_2H_3 were hyperbolic (Figure 2) and were analyzed according to a pseudo first order, saturation scheme:

$$RN_2H_3 + HAO \underset{}{\overset{K_d}{\rightleftharpoons}} [RN_2H_3{\cdot}HAO] \xrightarrow{k_{inac}} \text{inactivated HAO}$$

described by the equation:

$$k_{obs} = \frac{k_{inac}[RN_2H_3]}{K_d + [RN_2H_3]} \quad (1)$$

Data of Figure 2 fitted to eq 1 are given in Table 1. Since this scheme represents the inactivation process in only two steps, the deduced value for k_{inac} represents the overall rate for the inactivation process. For all the compounds tested, the reduction of *c*-hemes of HAO by RN_2H_3 was at least 100-fold faster than the inactivation event.

Optical Spectra of the Products of Reaction of HAO with RN_2H_3. After complete inactivation of HAO with an alkyl- or arylhydrazine, the enzyme was reoxidized with ferricyanide and the optical difference spectrum determined, (RN_2H_3-treated HAO *minus* native HAO) (Figure 3A). The changes in the ferric optical spectra included a decrease in absorbancy at about 412 nm and a broad increase over the region from 440 to 620 nm. This optical change was similar, though not identical, for the methyl- or phenylhydrazine-treated HAO as well as the peroxide-treated HAO. The difference extinction coefficient for the loss of absorbancy in the Soret was ~15 $mM^{-1}\ cm^{-1}$ assuming one affected moiety per subunit of HAO. The changes in the ferrous optical spectra (Figure 3B) included (i) a major decrease in absorbancy at the 464 nm maximum of heme P460 ($\Delta\epsilon$ of 65 $mM^{-1}\ cm^{-1}$ for A_{464nm} *minus* $A_{744\ nm}$) and (ii) a smaller increase in absorbancy at 528 nm ($\Delta\epsilon$ of 24 $mM^{-1}\ cm^{-1}$ for A_{528nm} *minus* A_{744nm}) for phenylhydrazine, 520 nm ($\Delta\epsilon$ of 18 $mM^{-1}\ cm^{-1}$ for A_{520nm} *minus* A_{744nm}) for methylhydrazine, and 510 nm ($\Delta\epsilon$ of approximately 18 $mM^{-1}\ cm^{-1}$ for A_{510nm} *minus* A_{744nm}) for (hydroxyethyl)hydrazine. Inactivation of heme P460 of

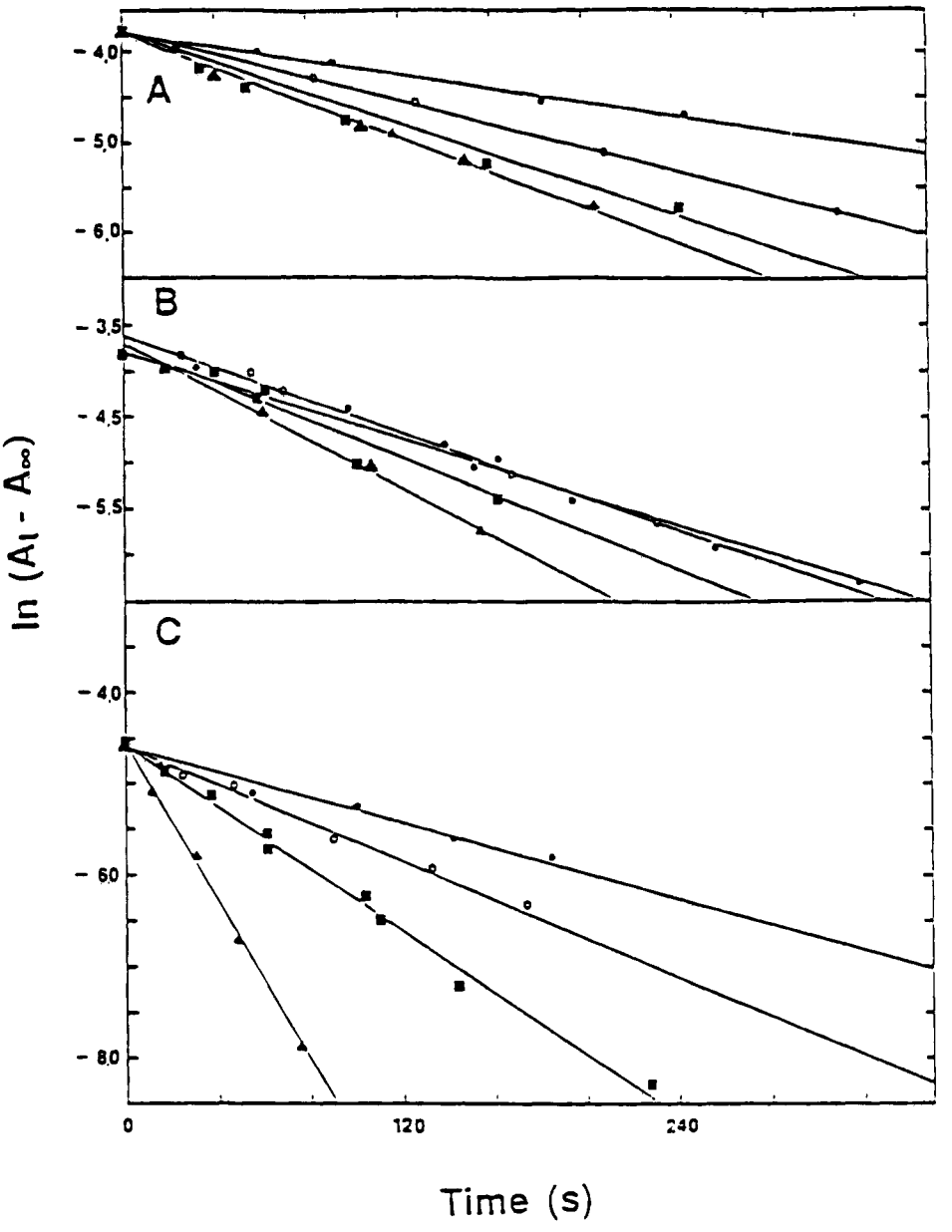

FIGURE 1: Inactivation of HAO by organohydrazines as a function of time and concentration of inactivator. Activity of HAO (assayed as turnover in the presence of 50 μM hydrazine and 27 μM horse heart cytochrome *c* as substrates) was determined with an aliquot taken at the indicated time of incubation in the presence of the indicated initial concentration of organohydrazine (A_t, the activity of HAO at time *t*; A_∞, the activity of HAO at infinite time at the concentration of organohydrazine tested). (A) Methylhydrazine: 10 mM (solid circles), 50 mM (open circles), 100 mM (solid squares), and 400 mM (solid triangles). (B) Phenylhydrazine: 50 mM (solid circles), 100 mM (open circles), 200 mM (solid squares), and 400 mM (solid triangles). (C) (Hydroxylethyl)hydrazine: 20 mM (solid circles), 40 mM (open circles), 110 mM (solid squares), and 800 mM (solid triangles).

ferric HAO by H_2O_2 resulted in changes in absorbancy of ferrous HAO consisting of only a decrease in the 464 nm band and lacking the increases in the 520 nm region seen consistently with organohydrazines.

The optical spectra of *c*-hemes were not affected by derivatization with organohydrazines (Figure 3) except in the case of (hydroxyethyl)hydrazine where absorbancy of ferrous HAO decreased at 418, 424, and 552 nm (data not shown). Based on the optical change observed, ~0.5 mol of *c*-heme was affected per mole of heme P460 modified.

The difference spectra (ferrous HAO + RN_2H_3 + CO *minus* ferrous HAO + RN_2H_3) (Figure 3) were used to quantitate the amount of native heme P460 remaining in the samples (seen by the size of the shift in absorbancy from 464 to 442 nm upon binding CO). With methyl- or phenylhydrazine a maximum of ~80% of the heme P460 was modified (see Figure 3). Oxidation of phenylhydrazine-treated HAO with ferricyanide followed by passage over a gel filtration column and reaction with fresh phenylhydrazine did not cause modification of additional heme P460.

RN_2H_3-modified HAO still bound CO as indicated by the disappearance of the positive features of the ferrous HAO + RN_2H_3 *minus* ferrous HAO difference spectra (Figure 3B,C). There were no reproducible positive features at wavelengths above 520 nm in the ferrous CO difference spectrum of RN_2H_3-treated HAO.

Stoichiometry of Reaction with Methyl- or Phenylhydrazine. As shown in Figure 4, a 1:1 ratio of phenylhydrazine to active site of HAO resulted in maximum inactivation (~80%) as measured by the rate of turnover. As determined

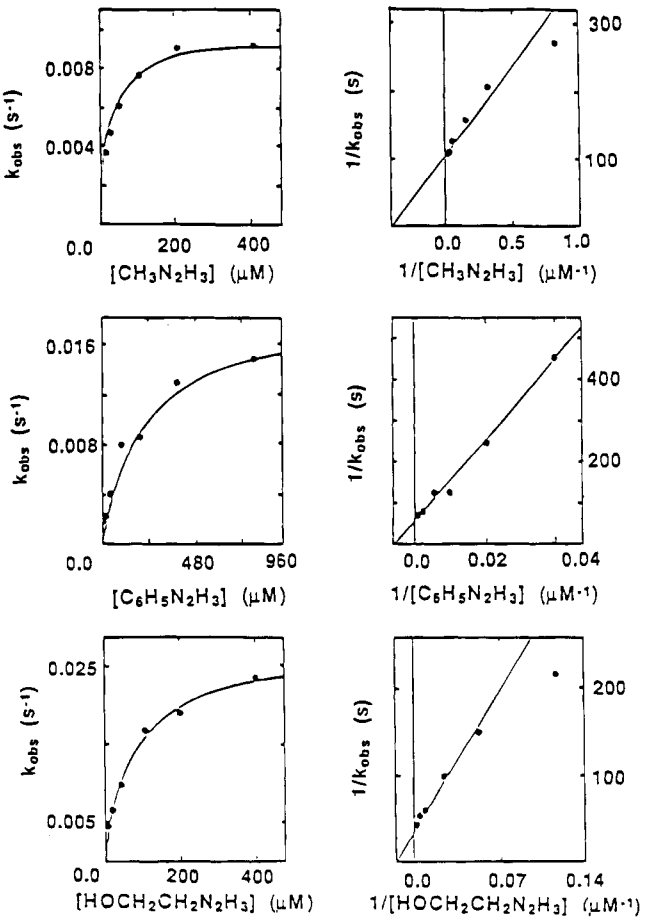

FIGURE 2: Values of k_{obs} for the inactivation of HAO by organohydrazines as a function of concentration of inactivator. Values of k_{obs}, calculated from Figure 1, are plotted in linear and semilogarithmic form as a function of concentration of the indicated organohydrazine.

Table 2: Incorporation of ^{14}C from $^{14}C_6H_5N_2H_3$ into HAO

treatment[a]	% P460 destroyed by $C_6H_5N_2H_3$	mol of ^{14}C label/ mol of HAO
HAO + $^{14}C_6H_5N_2H_3$	70 ± 5	2.0 ± 0.1
HAO + KCN + $^{14}C_6H_5N_2H_3$[b]	60 ± 10	1.6 ± 0.2
HAO + H_2O_2 + $^{14}C_6H_5N_2H_3$	0	1.0 ± 0.2

[a] HAO (2 mL of 1.6 μM in 50 mM potassium phosphate buffer, pH 7.5) was incubated with 12 μM $^{14}C_6H_5N_2H_3$ for 2 h. Excess phenylhydrazine was removed by concentration of the solution in a Centricon microconcentrator (molecular weight cutoff 10 000) and rediluting with buffer. The process was carried out four times. The amount of heme P460 remaining was estimated as the difference in absorbance between 442 and 464 nm in the difference spectra: (ferrous HAO + CO *minus* ferrous HAO). [b] Before addition of phenylhydrazine, HAO was preincubated with 1 mM cyanide. [c] Before addition of phenylhydrazine, HAO was pretreated with 100 μM hydrogen peroxide (see Materials and Methods) so as to completely destroy heme P460.

optically, the same (~80%) maximum modification of heme P460 by phenylhydrazine was observed at either a 1:1 or a 200:1 mole ratio of phenylhydrazine to active site. In contrast, ~5 molecules of methylhydrazine per active site were required for maximum destruction of heme P460 of HAO.

After reaction with a 7.5-fold excess of phenylhydrazine, HAO bound 2 phenyl groups per subunit (Table 2). Interestingly, peroxide-inactivated HAO incorporated one phenyl group per subunit. The peroxide-treated enzyme showed no significant changes in optical difference spectra after reaction with phenylhydrazine (data not shown), indicating that the phenyl group was probably not incorporated into a heme of HAO. [^{14}C]$C_6H_5N_2H_3$-labeled HAO was analyzed on SDS–PAGE gels in which protein was stained by Coomassie blue and radioactivity visualized by fluorography. The protein bands due to HAO were radioactive (data not shown). Breakage of the thioether linkage between *c*-hemes and cysteines of the protein with sulfenyl chloride by the method of Fontana et al. (1973) results in single band of apparent molecular mass 63 kDa on PAGE (Terry & Hooper, 1981). Importantly, this proecedure did not remove radioactivity from the latter band (data not shown). These observations indicate that the phenyl group remains bound to HAO under protein-denaturing conditions and is thus covalently attached to HAO. Further, the phenyl group is bound to a residue whose attachment to the protein does not depend on a thioether bond.

DISCUSSION

Organohydrazine Compounds Are Suicide Substrates of HAO. As shown here, the reaction of phenylhydrazine with HAO satisfies the criteria for inactivation by a suicide substrate (Walsh, 1982): (1) Kinetics of inactivation are consistent with a hyperbolic (saturating) first order reaction. (2) The substrate covalently labels the active site, heme P460. (3) Inactivation was prevented in the presence of an excess of the substrate, hydrazine. Because of their similar reactivity with HAO, methyl- and (hydroxyethyl)hydrazine are also presumed to be suicide substrates.

Phenylhydrazine Binds at Two Sites on HAO. The phenyl group of $^{14}C_6H_5N_2H_3$ was incorporated into two sites on HAO. The site of primary significance to this paper is heme P460. Based upon the nearly complete inactivation of enzyme and modification of heme P460 observed at a 1:1 ratio of phenylhydrazine to active site, this site reacted with high affinity and efficiency. A second site was still reactive with phenylhydrazine even when the heme P460 and catalytic activity of HAO had been destroyed by peroxide and thus is probably not at or near the active site. Although the first site was preferentially modified if phenylhydrazine was limiting, phenylhydrazine in excess also labeled the second site. Hence activity of the first site was apparently not required for reaction of phenylhydrazine with the second. The second site of labeling, which is not a heme, is possibly an amino acid residue as seen with horseradish peroxidase (Ator & Ortiz de Montellano, 1987).

Heme P460 Is Derivatized by Organohydrazines. In the high-affinity reaction of phenylhydrazine with HAO, the data are consistent with attachment of the phenyl group to either an amino acid residue or heme P460 but not a *c*-heme. The [^{14}C]phenyl group remained attached to the protein even when *c*-hemes were removed by cleavage of their thioether linkage by treatment with sulfenyl chloride. Even after cleavage of the thioether bonds between the protein and heme P460, the latter would have remained attached to the protein by its linkage to tyrosine (Arciero et al., 1993).

Changes of the optical spectra of ferrous HAO after derivatizing HAO with organohydrazines indicate that heme P460 was covalently modified by the R group. The Soret peak shifted to the red (near 520 nm) and decreased greatly in intensity (typically about 75% for the compounds tested

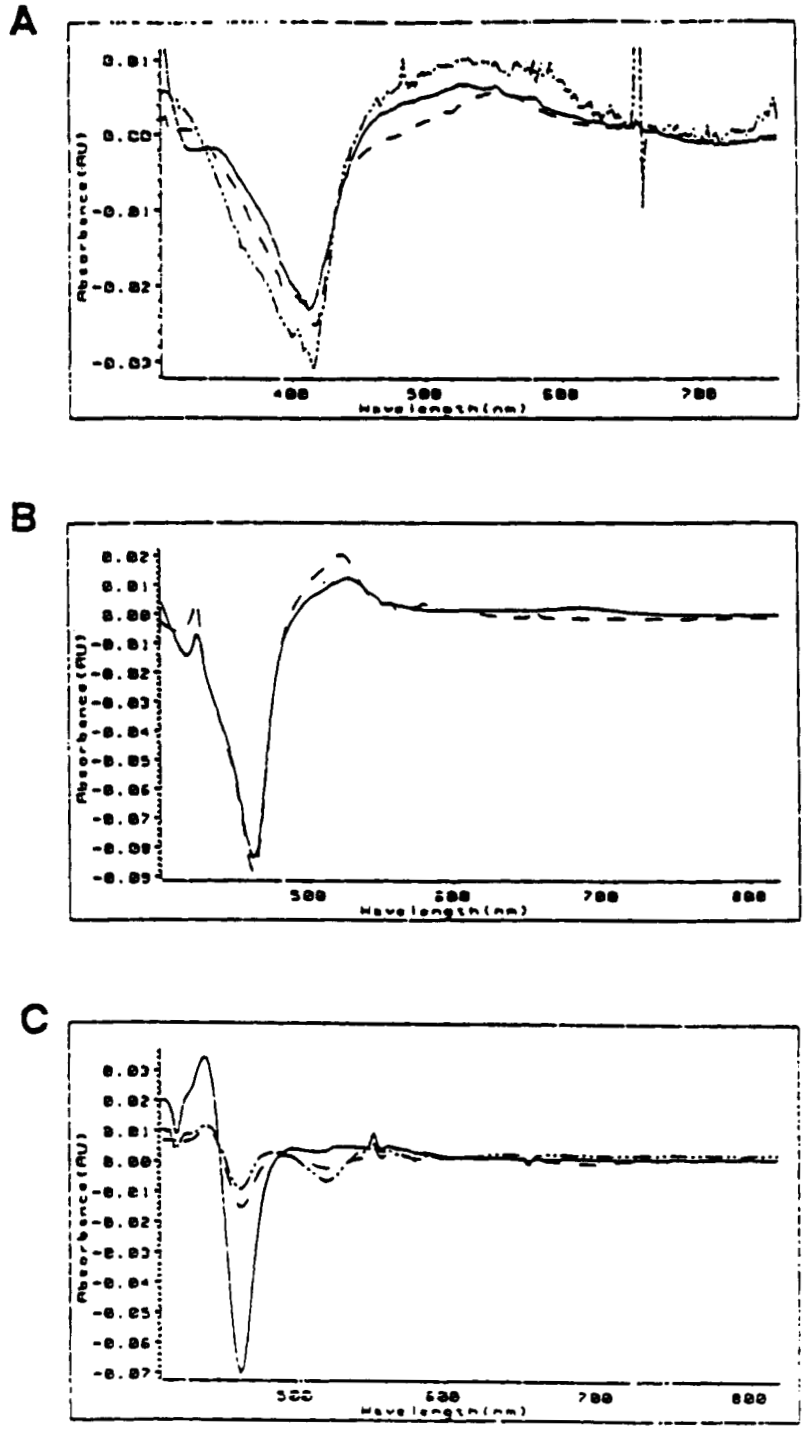

FIGURE 3: Changes in optical spectra following reaction of HAO with methylhydrazine, phenylhydrazine, or hydrogen peroxide. (A) Ferric optical difference spectra (HAO + reactant *minus* untreated HAO). Incubation of HAO was with phenylhydrazine (solid trace), methylhydrazine (dashed trace), or hydrogen peroxide (alternating dotted and dashed trace). At the end of a 30 min incubation, a small amount of ferricyanide was added to reoxidize the samples. The HAO concentration was 1.7 μM for treatment with methyl- or phenylhydrazine and 2 μM for treatment with hydrogen peroxide. Hydrogen peroxide, methylhydrazine, and phenylhydrazine were at a concentration of 100 μM. (B) Ferrous optical difference spectra obtained by reduction of the samples used in part A: (HAO + x + dithionite *minus* untreated HAO + dithionite). Methylhydrazine (solid trace), phenylhydrazine (dashed trace). (C) Ferrous CO optical difference spectra of the samples of part B: (HAO + RN_2H_3 + dithionite + CO *minus* HAO + RN_2H_3 + dithionite). Methylhydrazine-treated HAO (dashed trace), phenylhydrazine-treated HAO (alternating dotted and dashed trace), untreated HAO, *i.e.*, the native ferrous CO difference spectrum (solid trace).

here: ΔA_{464} *minus* ΔA_{744} = 65 mM^{-1} cm^{-1}). Similar features were observed in the ferrous difference spectra of N- or meso-derivatized protoheme IX-containing hemoproteins (e.g., Ator et al., 1987; Augusto et al., 1982; Ortiz de Montellano & Kerr, 1983). As one would expect different electronic effects from different R groups, the varied putative Soret absorption maxima in the 520 nm region depending on the nature of the hydrazine derivatives are also consistent with the covalent modification of heme P460 by the R group. The identification of heme P460 as the moiety derivatized by the hydrazine compound further confirms that heme P460 is at the active site of HAO.

The observed shift in the spectra of ferric HAO from the Soret to a broad absorbance over the visible region is smaller

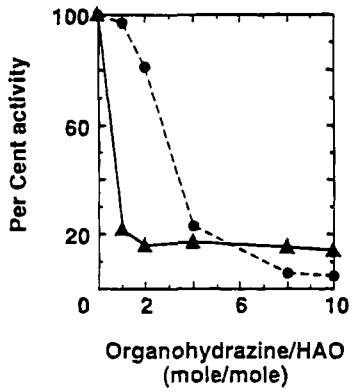

FIGURE 4: HAO activity as a function of ratio of organohydrazine: HAO. HAO was incubated with various amounts of phenylhydrazine (solid line) or methylhydrazine (dashed line) for 1 h and the remaining activity determined from the steady-state turnover of HAO with hydrazine (50 μM) and horse heart cytochrome *c* (25 μM).

in size and extent of red shift than changes observed in the reaction of other hemoproteins with organohydrazines. For several protoheme IX-containing proteins, the ferric Soret peak shifts to the red by about 20 nm and decreases by about 50% (about 50 mM^{-1} cm^{-1}) in Soret intensity (e.g., Ator et al., 1987; Augusto et al., 1982; Ortiz de Montellano & Kerr, 1983). Nevertheless, the ferric derivatized HAO *minus* ferric HAO difference extinction coefficients for HAO are large enough (15 mM^{-1} cm^{-1}) to implicate modification of a heme. The ferric (derivatized HAO *minus* native) difference extinction coefficients for HAO are a factor of 4 smaller than for the ferrous (derivatized HAO *minus* native) difference spectra. For protoheme IX proteins, there is usually a much smaller difference (0.5−2) between the ferric and ferrous forms. Hence ferric heme P460 of HAO may have an unusually small extinction coefficient as compared to its ferrous extinction coefficient and compared to values for other protein-bound hemes. In fact a ferric Soret maximum for heme P460 was not detected during redox titration of HAO (Collins et al., 1993).

Site of Attachment of R Group of RNH_2NH_2. The present data do not allow identification of the site of attachment of the R group to heme P460. With catalase and hemoglobin (Augusto et al., 1982; Ortiz de Montellano, 1983) or horseradish peroxidase (Ator & Ortiz de Montellano, 1987) the R group is attached to the pyrrolic N or the meso carbon, respectively. The iron of an *N*-phenyl heme can apparently not bind ligands on the same face of the heme as the phenyl moiety. The meso derivatization does not seem to affect the ability of small ligands to bind, since compound I of horseradish peroxidase can still form (Ator & Ortiz de Montellano, 1987; Ator et al., 1987). Derivatizing HAO with an organohydrazine resulted in a small but reproducible absorbancy change near 520 nm of the ferrous enzyme which disappeared upon reaction with CO (Figure 3C). This suggests that organohydrazine-derivatized heme P460 may still bind CO. If so, this suggests that either the R group had derivatized the heme P460 moiety at the periphery of the heme (e.g., at the *meso* carbon) so that ligand binding was not prevented or, if the R group has bound to the Fe or a pyrrolic N of heme P460, that CO binds to the opposite face.

Implications for the Mechanism of Oxidation of Substrate by HAO. A diazene is thought to be an intermediate in the reaction of organohydrazines with hemoproteins (Artaud et al., 1990; Battioni et al., 1983; Guilard et al., 1987; Lancon et al., 1984). Diazene intermediates are typical ferrous ligands, yet heme P460 was not observed to be reduced during the reaction. There is evidence that a group such as histidine is a bridging ligand between the irons of heme P460 and a *c*-heme at the active site of HAO (Hendrich et al., 1994). If a diazene intermediate is involved, we speculate that either heme P460 is reduced transiently in the time scale of the derivatizing reaction or the *c*-heme of the active site is reduced during catalysis and binds the diazene intermediate which then shuttles back to the P460 ring.

Reaction of (Hydroxyethyl)hydrazine with a c-Heme. The decrease in the absorbancy of *c*-heme of HAO induced by (hydroxyethyl)hydrazine is in keeping with derivatizing a *c*-heme or a strong perturbation of the environment of the *c*-heme. Since the reaction may be dependent on enzymatic generation of a reactive form of (hydroxyethyl)hydrazine, this *c*-heme may in close proximity to heme P460. Hence, reaction with (hydroxyethyl)hydrazine may provide a probe of an additional metal center of HAO.

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