Reaction with Cyanide of Hydroxylamine Oxidoreductase of *Nitrosomonas europaea*†

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ABSTRACT: Hydroxylamine oxidoreductase (HAO) catalyzes the reaction $NH_2OH + H_2O \rightarrow HNO_2 + 4e^- + 4H^+$, a step in the energy-generating oxidation of ammonia to nitrite by the bacterium *Nitrosomonas europaea*. Each subunit of HAO contains 7 *c*-hemes and 1 heme P460. The latter, a *c*-heme cross-linked from a methylene carbon to the ring of a protein tyrosine, forms part of the active site. The iron of heme P460 is probably linked by a bridging ligand to the iron of a *c*-heme. Here, the reaction of cyanide with ferric HAO was studied by optical, transient, and steady state kinetic techniques. The molecules, F^-, Cl^-, Br^-, N_3^-, SCN^-, and OCN^- did not react with HAO. A single molecule of cyanide bound with high affinity to heme P460 of HAO. The optical and kinetic characteristics of formation of the monocyano complex of HAO resembled those of cyanide derivatives of other heme proteins. Cyanide, in the monocyano complex, was a noncompetitive inhibitor and remained bound during turnover. HAO was found in two forms. The most common form, HAO-A, formed only the monocyano derivative of heme P460, whereas the other, HAO-B, formed a mono- and dicyano complex. The optical properties and kinetics of formation of the mono- and dicyano complexes were different enough to easily allow independent analysis. The optical and kinetic characteristics of formation of the monocyano complex of heme P460 of HAO A and B were very similar. The dicyano complex of HAO-B appeared to result from the addition of a second molecule of cyanide to heme P460. The rate of conversion of the monocyano to the dicyano complex was stimulated 100-fold by the binding of substrate. Formation of the mono-heme complex inhibited enzyme activity. The kinetic constants for the first-order formation of the monocyano derivative and the inhibition of substrate oxidation (under either transient or steady-state conditions) were different. The apparent discrepancy could be resolved by the hypothesis that HAO is functionally a dimer in which electrons rapidly equilibrate between the *c*-hemes of each subunit but not between oligomers. The results form the basis for the use of cyanide as a probe of the active site of HAO.

Nitrosomonas europaea, a nitrifying bacterium, obtains all of its energy for growth by the oxidation of ammonia to nitrite, an integral reaction in the cycling of nitrogen in nature. Hydroxylamine, an intermediate in the process (Hollocher *et al.*, 1981), is oxidized to nitrite by hydroxylamine oxidoreductase (HAO):[1] $NH_2OH + H_2O \rightarrow HNO_2 + 4e^- + 4H^+$ (Andersson & Hooper, 1983). HAO may be a dimer or trimer of a 63-kDa subunit (Rees, 1968;Yamanaka *et al.*, 1979; Terry & Hooper, 1981; Masson *et al.*, 1990). Each subunit, MW 63 kDa (Terry & Hooper, 1981), contains 7 *c*-hemes and one heme P460 (Arciero & Hooper, 1993), a *c*-heme covalently connected from a methylene carbon to a ring carbon of a tyrosine (Arciero *et al.*, 1993); the bridging is from Cys 229 and 232 to Tyr 467 (Sayavedra-Soto *et al.*, 1994). Ferrous heme P460 in HAO has an absorbance maximum at 463 nm. There is no clearly identified optical feature for heme P460 in the ferric state of HAO (Collins *et al.*, 1993).

Several lines of evidence implicate heme P460 in the active site of HAO. Incubation of ferric HAO with peroxide results in loss of activity concomitant with the loss of the absorbancy characteristic of heme P460 in ferrous HAO, although the *c*-hemes are unaffected (Hooper & Terry, 1977). Organohydrazines, which are suicide substrates for HAO, covalently derivatize heme P460 (Logan & Hooper, 1995). Further, ferrous heme P460 is the only heme of HAO which reacts with exogenous small molecules; it is readily oxidized to the the ferric form by dioxygen or hydrogen peroxide and binds carbon monoxide (Hooper & Balny, 1982; Hooper *et al.*, 1983). Optical, EPR, and Mössbauer spectra indicate that the iron of ferric heme P460 in HAO is coupled to a *c*-heme (Andersson *et al.*, 1984; Prince & Hooper, 1987; Hendrich *et al.*, 1994). The chemical models which most closely mimic the Mossbauer and optical properties of ferrous heme P460 are pentacordinate hemes with strongly anionic axial ligands (Nasri *et al.*, 1987).

HAO catalyzes the oxidation of NH_2OH (or NH_2NH_2 to N_2; Anderson, 1964) and the reduction of electron acceptors such as phenanzine methosulfate or mammalian cytochrome *c* (Hooper & Nason, 1965; Yamanaka *et al.*, 1979) or

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[1] Abbreviation: HAO, hydroxylamine oxidoreductase.

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cytochrome *c*-554 from *Nitrosomonas* (Yamanaka & Shinra, 1974; Arciero *et al.*, 1993). Approximately 35% of the *c*-heme of HAO is reduced in the presence of substrate, indicating that electrons can be transferred from the active site to *c*-heme (Hooper *et al.*, 1984). Catalysis may include deprotonation and removal of electrons from substrate and transfer of electrons from the active site to *c*-hemes of HAO and/or to the exogenous physiological electron acceptor.

Analysis of heme P460 in HAO has focused on the ferrous state, and little is known about reactions of HAO in the catalytically active ferric state. Other than substrate, the only compound known to react with the active site of ferric HAO is hydrogen peroxide, yet peroxide irreversibly destroys the activity and heme P460 of HAO. Cyanide binds reversibly and with high affinity to the iron of ferric hemoproteins in a reaction readily observed by spectroscopy. Here we describe the interaction of cyanide with ferric HAO.

EXPERIMENTAL PROCEDURES

Chemicals. All chemicals were reagent grade or better. Double-distilled or Millipore Super Q water was used throughout. Myoglobin, hemoglobin, horse heart cytochrome *c*, type III, bovine liver catalase, Sephadex, octyl-Sepharose, and DEAE-Sephacel resins were obtained from Sigma Chemical Co., St. Louis, MO. Sephadex and ampholines for isoelectric focusing were obtained from either Sigma or Pharmacia Inc., Piscataway, NJ. Potassium cyanide solutions of 100 mM or less were prepared from 2 M stock solutions and were used within 10 min of preparation and under low illumination.

Growth of Bacteria and Preparation of Enzyme. Nitrosomonas europaea (Schmidt strain) was grown either in 15-L carboys (Hooper *et al.*, 1972) or in continuous culture (Logan & Hooper, 1995). Crude extracts and the 60−80% ammonium sulfate precipitate was prepared as in Hooper *et al.* (1978). To determine whether the relative amounts of HAO-A or -B were dependent on methods of purification, the enzyme was prepared by the following four separate methods. For preparation I, the 60−80% ammonium sulfate fraction was redissolved, dialyzed, and subjected to preparative isoelectric focusing (LKB Multiphor) as previously described (Hooper *et al.*, 1978). The enzyme was then chromatographed on a Sephadex G-100 column (equilibrated with 50 mM potassium phosphate buffer, pH 7.5, containing 200 mM KCl and 10 mM EDTA). HAO-containing fractions were pooled, diluted 10-fold, and adsorbed onto a DEAE Sephacel column (2.5 × 10 cm) equilibrated with 10 mM Tris, pH 8.5. HAO was eluted in the fractions containing 100−300 mM KCl of a 300-mL 0−500 mM KCl gradient. Fractions were pooled; concentrated by ultrafiltration to about 1.2 mM heme; dialyzed against 50 mM potassium phosphate buffer, pH 7.5, containing 200 mM KCl; dispensed into aliquots; and stored at −20 ° C. The final 408/280 nm ratio for this preparation was 3.4. Preparation II omitted the IEF step, but included an additional pass through a Sephadex G-100 column after the DEAE Sephacel step. Preparation III employed the same procedure as I except that the 30−40% ammonium sulfate precipitate was the starting material. Preparation IV was based on Arciero *et al.* (1991). The 60−80% ammonium sulfate fraction was applied to a Sephadex G-100 column, and the HAO fractions were pooled, 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 25 and 20% ammonium sulfate in a 40 to 10% gradient (in 100 mM KCl and 50 mM potassium phosphate, pH 7.5). The final 408/280 nm ratio for this preparation was 3.8. No differences in the properties described in this paper were seen in the products of the four methods. Most of the work reported here employed enzyme made by means of preparation IV.

Unless otherwise stated, the concentration of HAO is based on the concentration of the subunit (and therefore of heme P460) using an extinction coefficient of 700 mM^{-1} cm^{-1} at the *c*-heme Soret peak (408 nm) in the ferric state, or a value of 140 mM^{-1} cm^{-1} at 552 nm in the dithionite-reduced state.

Optical Measurements of Ligand Binding. Unless otherwise stated, all measurements were at 25 °C, in 50 mM potassium phosphate buffer, pH 7.5. A Cary 15 or a Aminco DW-2 dual beam spectrophotometer was used for some measurements of the (HAO + cyanide *minus* HAO) difference spectra. Typically, the sample and reference cuvettes (3 mL total volume, 1 cm path length) contained 1.5 mL of HAO solution . A 10-μL aliquot of test solution was added to the sample cuvette, and an equal volume of water was added to the reference cuvette. The absorption spectrum was then scanned in the range 300−700 nm.

A Hewlett-Packard 8452A spectrophotometer, equipped with a 7-cell multitransporter (thermojacketed and magnetically stirred), was also used for difference spectra and in kinetic measurements in cases where k_{obs} was less than 2 min^{-1}. All wavelengths were accurate to 2 nm. The 8452A is a single-beam, diode-array instrument utilizing a deuterium light source. At wavelengths of less than 300 nm it was necessary to use the UV cutoff filter no. 3 supplied with the instrument and a glass plate in front of the deuterium light source to avoid photoreduction of a small fraction of the *c*-hemes of HAO. The open sample compartment of the HP-8452A was shielded from light to minimize photoreduction. Small amounts of reduction did not affect formation of cyano-HAO complexes but did hinder analysis of spectra because of the relatively large optical changes associated with reduction of *c*-heme. Reactions were typically performed in 4-mL quartz cuvettes (1.0 cm path length), stirred by a magnetic flea, containing 2 mL of solution and covered with parafilm or Teflon. Reactants were added in volumes of less than 2 μL with microsyringes, and scans were taken over the range 300−820 nm. Values of k_{obs} were calculated by first-order log plots or directly from time traces using Marquhardt's algorithm as implemented on the software provided by Hewlett-Packard.

Steady-State Kinetic Measurements of Activity of HAO. Routine assays of rates of HAO activity under steady-state conditions took place in a reaction mixture containing 25 μM horse heart cytochrome *c*, 1−20 nM HAO, and 1−50 μM hydrazine or hydroxylamine, in 50 mM potassium phosphate buffer, pH 7.5. The reduction of horse heart cytochrome *c* was followed as the change in the value of the absorbancy at 550 nm *minus* the absorbancy at 744 nm. Turnover was the reduction of ~430 nmol of cytochrome *c* per min per nmol of HAO at 50 μM hydrazine. For studies of inhbition by cyanide of HAO in the steady state, HAO, cytochrome *c*, and cyanide were preincubated for 15 min,

Table 1: Optical and Kinetic Properties of Cyanide Complexes of HAO

cyanide complex of HAO[a]	Soret peak[b] (nm) $\Delta\epsilon$ (cm^{-1} mM−1)	no. of *c* heme per cyanide bound[c] mol/mol	k_{on}[d] (M^{-1} s^{-1})	k_{off}[e] (s^{-1})	K_d^k[f] (μM)	K_d^{eq}[g] (μM)
A-I	434−436 (3 ± 0.3)	6.6 ± 0.6	200 ± 20	(1.5 ± 0.5) × 10^{-3}	8 ± 3	9 ± 2
B-I	434 (1.8 ± 0.3)	6.4 ± 0.6	200 ± 8	(2.0 ± 0.5) × 10^{-3}	10 ± 3	8 ± 2
B-II	506 (4 ± 0.4)	ND[h]	7.3 ± 0.4	~4 × 10^{-5}	ND	40 ± 10

[a] I or II are the mono- or dicyano derivatives of HAO. A and B refer to forms of the enzyme described in the text. [b] Soret peak positions and difference extinction coefficients ($\Delta\epsilon$) were determined from the difference spectra: (HAO + cyanide *minus* HAO) (Figure 1). Values of $\Delta\epsilon$ are per heme P460 of HAO. [c] The ratio of *c* hemes of HAO per cyanide bound was determined by titration (Figure 3). [d,e] k_{on} and k_{off} are the rate constants for the pseudo-first-order addition of cyanide to HAO. The latter correspond to the reaction depicted in eq 1 for the rate of formation of the monocyano complexes of HAO-A and -B, determined from plots of k_{obs} vs. [KCN]. For complex II of HAO-B, k_{on} is the value determined by a pseudo-first-order treatment of the data in Figure 4 and is also consistent with the value for k_2 in the reaction depicted in eq 2. k_{off} (corresponding to k_{-2} in eq 2) for complex II of HAO-B was calculated using the values determined for K_d^{eq}, k_1, k_{-1}, and k_2, and $K_{d_{B\text{-}II}} = (k_{-1}k_{-2})/(k_1k_2)$, where the rate constants correspond to the reaction depicted in eq 2. [f] K_d^k is the K_d as determined kinetically, using the values in columns 4 and 5, and $K_d^k = k_{-1}/k_1$, for the reaction depicted in eq 1. [g] K_d^{eq} is the K_d as determined at equilibrium from a hyperbolic fitting of the amplitudes of the data at completion of the reaction between HAO and cyanide. [h] ND, not determined.

and the reaction was started by addition of hydrazine. Preincubation for 30 min of hydrazine, cytochrome *c*, and cyanide at concentrations greater than 90 μM resulted in inhibition of the subsequent rate of enzymic reduction of horse heart cytochrome *c* , possibly due to the formation of a cyanide−mammalian cytochrome *c* adduct or the reaction of cyanide with hydrazine. Hence cyanide concentrations of less than 90 μM were used in steady-state studies.

Kinetic Measurements in the Millisecond Range. A thermostated, stopped-flow mixing apparatus fitted to an Aminco DW-2 spectrophotometer (Markley *et al.*, 1981; Hooper & Balny, 1982) was employed. Values of k_{obs} were calculated by the program Curfit written for an Apple II computer interfaced to the spectrophotometer with a Datalab data accumulation instrument.

Titration of Complex I of HAO-A and HAO-B with KCN. An optical cell (1 × 1 cm, path length 0.11 cm) was constructed with glass microscope slides sealed with silicone caulking. Typically, 2−3 nmol of enzyme (absorbancy at 408 nm ~1.4) was contained in a total volume of 100 μL. KCN solution was added in volumes of less than 0.5 μL by microsyringe. The solution was mixed by gently moving the needle of the syringe from side to side 20 times. The solution was scanned until the formation of complex I of HAO-A or -B was complete (determined as the increase in the absorption difference at 434 nm minus that at 470 nm).

Inactivation of Ferric HAO by Hydrogen Peroxide. Ferric HAO (1−10 μM) was incubated with 100 μM H_2O_2 for various times. Inactivation was quenched with 100 μM NH_2-OH and dialysis (membrane pore size 12 000−14 000 MW) with three changes (3 × 400 mL) of buffer for 12 h.

RESULTS

The data shown below include optical spectra of putative mono- and dicyano complexes of heme P460 of HAO, initially designated complex I and II, respectively. The optical properties, cyanide affinity, and kinetics of formation of the mono- and dicyano complexes were different enough to easily allow independent analysis. HAO was found to exist in two forms, designated HAO-A and HAO-B. The prevalent form, HAO-A, was able to bind only one cyanide, whereas HAO-B formed mono- and then dicyano complexes. HAO-A and HAO-B were not interconvertible under the usual conditions of storage or assay.

Optical Difference Spectroscopy of the Reaction of HAO with Cyanide. Reaction of cyanide with HAO was followed by difference spectroscopy since the resulting absorbancy changes were small relative to the high background of the 7 *c*-hemes of HAO. Formation of cyano complex I of HAO-A was characterized by an increase in absorbancy of the Soret peak at 434 nm and a decrease at 400 nm (Figure 1A). Smaller increases or decreases were observed in the 560−750 or 470− 560 nm range, respectively. Isosbestic points were 418, 466, and 550 nm. Formation of cyano complex I of HAO-B (Figure 1B) was also characterized by a pronounced increase in absorbancy at 434 nm and a decrease at 400 nm. As compared with complex I of HAO-A, somewhat smaller and broader increases or decreases occurred in the 500−600- or 600−700-nm range, respectively. Isosbestic points were 410, 460, 600, and 700 nm. The difference extinction coefficient at 434 nm for complex I of HAO-B was ~60% of the value with HAO-A (see Table 1).

Formation of cyano complex II of HAO-B (Figure 1C) was characterized by a pronounced increase in absorbancy at 506 nm, a broad increase in the 600−700-nm region, and a pronounced decrease at 566 nm. In the lower wavelength region, the data indicate either a pronounced decrease at 340 and 400 nm or a broad decrease in the 300−460-nm range accompanied by a pronounced increase at 376 nm. Isosbestic points were 460, 550, and 610 nm.

The difference spectra observed during the formation of the three cyano complexes described above do not resemble those seen during the formation of either cyano mammalian cytochrome *c* (Horecker & Kornberg, 1946; Schejter & Aviram, 1969) or the cyano derivative of *c*-heme of HAO formed at high pH values as described below. This suggested that heme P460, the only other metal center in HAO, may be the moiety that reacted with cyanide. The progressive disappearance of the 434-nm peak of complex I of HAO-B concommitant with the appearance of the 506 nm peak of cyano complex II suggested that complex -I was converted to complex -II by the binding of a second cyanide molecule to the same heme center.

Reaction of HAO with Other Ligands of Ferric Heme. Mixing of HAO with 10 mM F^-, Cl^-, Br^-, N_3^- , SCN^-, or OCN^- did not result in changes in optical difference spectra or inhibition of activity.

Formation of Cyano c-Heme at High Values of pH. An additional cyano-heme derivative of HAO-A or -B was observed at pH values greater than 9.6 or 9.0, respectively (data not shown). At a fixed concentration of cyanide the

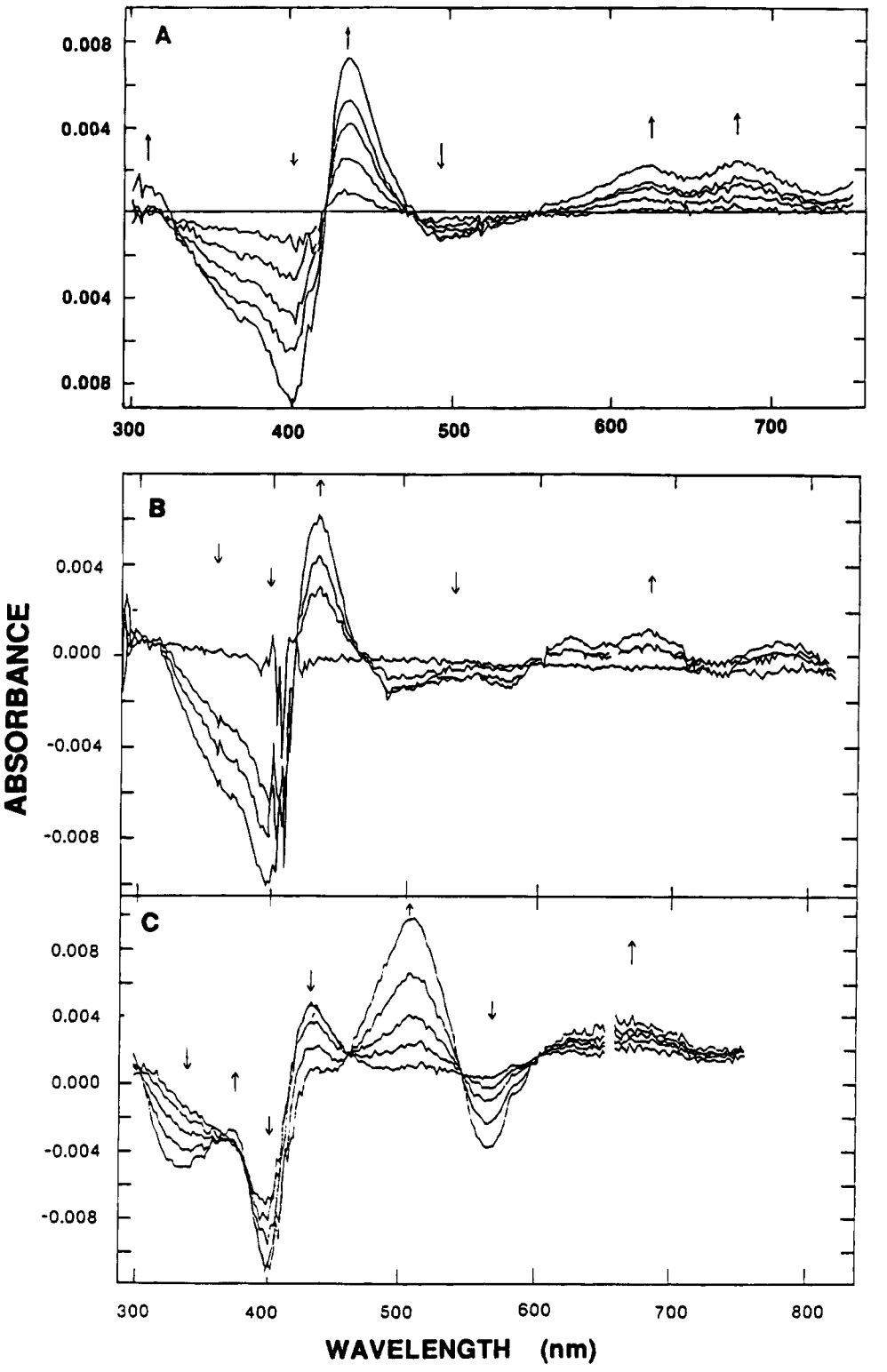

FIGURE 1: Optical difference spectra (ferric HAO + KCN minus ferric HAO) of the reaction of cyanide with HAO. Arrows indicate the direction of absorbancy changes at the indicated wavelength. (A) Formation of complex I by HAO-A. Spectra shown are 12, 36, 72, 120, and 408 s after addition of 50 μM KCN (final concentration) to a solution containing 1.8 μM HAO-A. (B) Formation of complex I by HAO-B. Spectra shown are prior to and 60, 120, and 300 s after addition of 40 μM KCN (final concentration) to a solution containing 2.6 μM HAO-B. (C) Formation of complex II by HAO-B. Spectra shown are 20, 40, 70, 140, and 340 s after addition of 1 mM KCN (final concentration) to a solution containing 1.8 μM HAO-B.

rate of formation of the cyano complex at high pH values was approximately twice that of complex I. The optical changes accompanying the formation at high pH of the cyano complexes were similar with HAO-A or -B. For HAO-A, maxima were at 356, 420, 450–456, and 548 nm; a minimum was at 404 nm; and isosbestic points were at 368, 413, 504, 526, and 564 nm. These features are similar to those seen in the difference spectra for the reaction of cyanide with mammalian cytochrome *c* (Horecker & Kornberg, 1946; Schejter & Aviram, 1969). Although the enzyme was still active at the high pH values, it was not possible to determine whether cyano complex I was also formed because of the

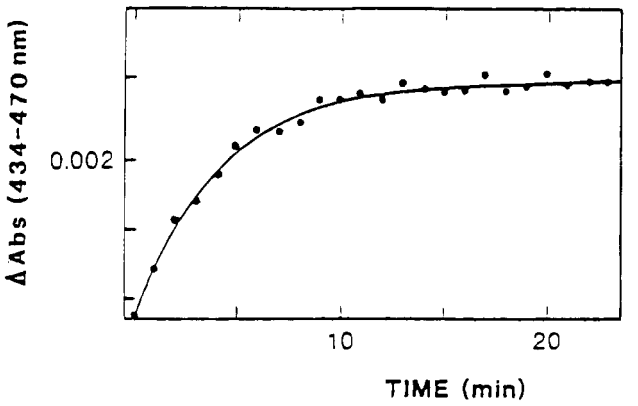

FIGURE 2: Typical time trace of formation of cyano complex I of HAO and an exponential fitting to the data points. The reaction mixture contained 7.5 μM KCN and 1.7 μM HAO-B.

greater intensity of the optical signals for formation of the cyano-heme *c* complex (the difference extinction coefficent was 5 mM^{-1} cm^{-1} at 420 nm per subunit of HAO).

Kinetics and Stoichiometry of Formation of Cyano Complex I. The time course of formation of cyano complex I of HAO-B (Figure 2) was consistent with a monophasic (single exponential) reaction between cyanide and HAO. Values of k_{obs} were the same at all wavelengths at which significant absorbancy change occurred. The dependence on the concentration of cyanide of the rate of formation of cyano complex I of HAO-B (data not shown) was consistent with a pseudo-first order reaction:

$$\text{HAO} \underset{k_{-1}}{\overset{k_1[\text{KCN}]}{\rightleftharpoons}} \{\text{HAO·CN}\} \qquad (1)$$

The kinetic data for formation of complex I of HAO-A were virtually identical to the values for HAO-B. The data was analyzed according to the equation $k_{obs} = k_1[\text{KCN}] + k_{-1}$, where $k_{obs}$was the rate observed at a fixed concentration of cyanide. The dissociation constant for complex I of HAO-B was determined according to the equation $\Delta A^{434-470nm} = \Delta A_{max}{}^{434-470nm} - K_d(\Delta A^{434-470nm}/[\text{KCN}])$. The derived values for k_1 and k_{-1} (denoted k_{on} and k_{of}) are in Table 1. Values for the dissociation constants (K_d) were in good agreement whether they had been determined kinetically or at equilibrium. A direct titration of cyanide binding in complex I of either HAO-A or -B (Figure 3; Table 1) was consistent with the binding of one cyanide per ~7 *c*-hemes (i.e., per subunit). Further, the kinetics of formation of cyano complex I was consistent with a simple first-order process involving one cyanide molecule per subunit of HAO. We conclude that complex I is a monocyano derivative of HAO.

Kinetics and Stoichiometry of Formation of Complex II of HAO-B. Time traces of formation of complex II of HAO-B (Figure 4A) were consistent with a single-exponential reaction with cyanide. At all wavelengths at which significant change occurred, values of k_{obs} were the same and exhibited the same dependence on the concentration of cyanide. Pseudo-first-order treatment of the data (eq 1) gave a reliable value for k_1 (Table 1) but not for k_{-1}. Because the optical spectra in Figure 1C suggested a precursor–product relationship between complexes I and II of HAO-B, the kinetics of formation of complex II were analyzed as two consecutive additions of cyanide:

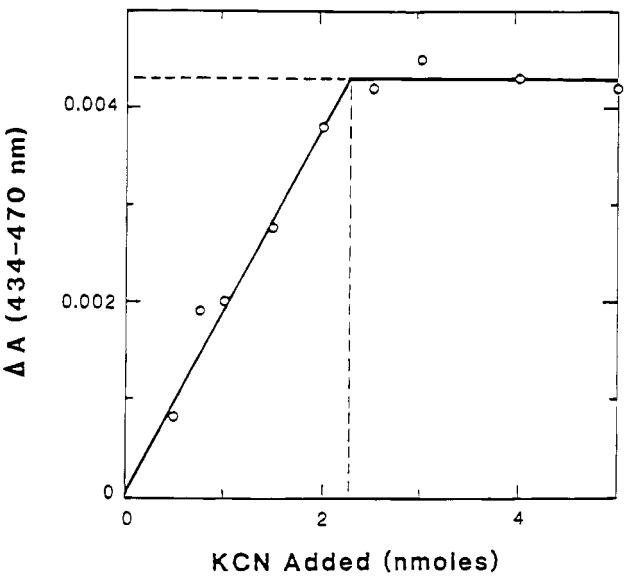

FIGURE 3: Stoichiometry of formation of cyanide complex I of HAO, shown as absorbancy at 434 nm (formation of monocyano complex I) as a function of concentration of cyanide. A 1.1 mm path length cell contained 2.2 nmol of HAO-B. Assuming a K_d of 10 μM (Table 1), the total amount of cyanide or HAO remaining unbound was calculated to be less than 5%.

$$\text{HAO} \underset{k_{-1}}{\overset{k_1[\text{KCN}]}{\rightleftharpoons}} \underset{\text{complex I}}{\{\text{HAO·CN}\}} \underset{k_{-2}}{\overset{k_2[\text{KCN}]}{\rightleftharpoons}} \underset{\text{complex II}}{\{\text{HAO·(CN)}_2\}} \qquad (2)$$

The curves in Figure 4B,C, drawn according to the simultaneous linear differential equations for eq 2, were consistent with the binding of cyanide in two consecutive steps. The combination of rate constants resulted in a virtually linear curve, and the consecutive additions of cyanide were kinetically isolated from each other. We conclude that complex II is a dicyano complex of a metal center of HAO-B. The dissociation constant for complex II was computed using the final amplitudes of the kinetic data of Figure 4 B,C and the relationship $\Delta A^{504-538nm} = \Delta A_{max}{}^{504-538nm} - K_d(\Delta A^{504-538nm}/[\text{KCN}])$.

Interestingly, the rate of formation of the dicyano complex from the monocyano complex was stimulated 100-fold in the presence of hydroxylamine (data not shown). The multiple-turnover substrates NH_2NH_2 and CH_3NHOH as well as the suicide substrates CH_3NHNH_2 and $C_6H_5N_2H_3$ also stimulated the rate of addition of the second cyanide. All of these substrates are known to react at the heme P460-containing center with the concomitant reduction of one or more *c*-hemes of HAO. However, reduction of HAO by dithionite under conditions where most *c*-hemes were reduced but heme P460 remained ferric had no effect on the rate of formation of the dicyano derivative. Thus the substrate stimulation of the binding of the second cyanide seems to be mediated at the active site.

Following addition of substrate to the dicyano complex of HAO-B, the slow rate of reduction of *c*-hemes of HAO was the same as for the monocyano derivative (see below). Optical evidence was not seen for putative intermediates {HAO CN NH_2OH} or {HAO $(CN)_2$ NH_2OH}.

Temperature Dependence of Formation of Cyano Complexes I and II. Activation parameters for the formation of cyanide complexes were determined from Arrenhius plots of data (not shown) measured over the range 3–25 °C. For

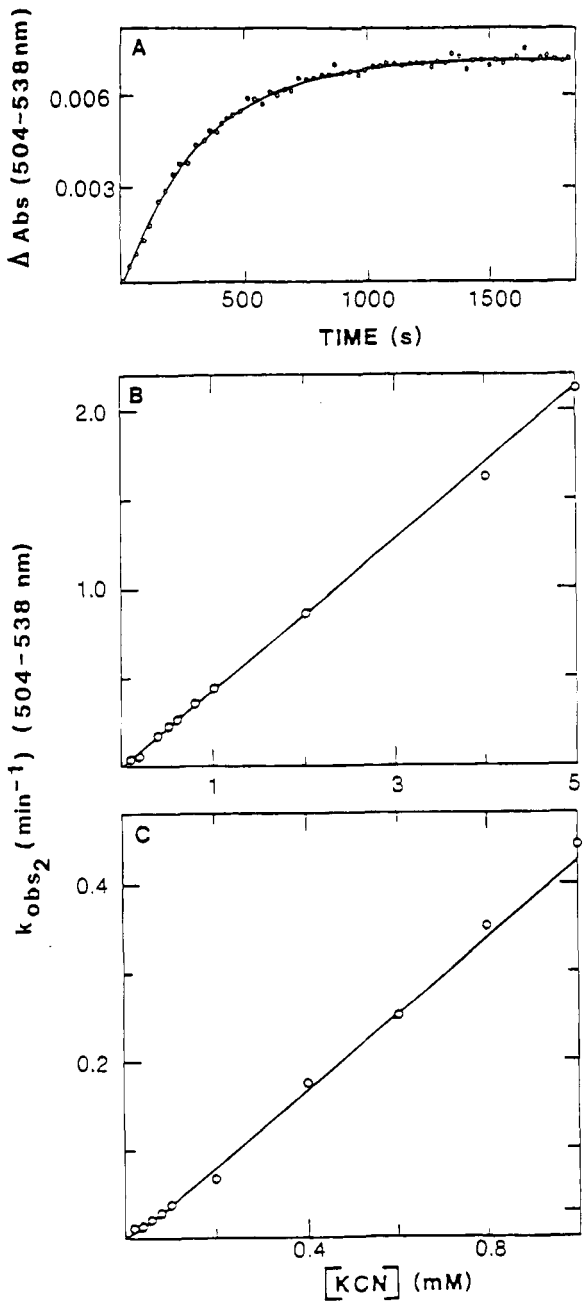

FIGURE 4: Kinetics of formation of complex II of HAO-B. (A) Typical time trace of formation of complex II with an exponential fitting to the data points. The reaction mixture contained 2.3 μM HAO-B and 500 μM KCN. (B and C) Dependence of concentration of cyanide of the rate of formation of complex II. The curve drawn through the experimental points was calculated for the sequential addition of two cyanide molecules to HAO-B, to form complexes I and then II: $k_{obsB\text{-}II} = (p - q)/2$, where $p = [KCN]k_1 + k_{-1} + [KCN]k_2 + k_{-2}$ and $q = (p^2 - 4([KCN]^2 k_1 k_2 + k_{-1} k_{-2} + [KCN]k_1 k_{-2}))^{1/2}$. $k_{obsB\text{-}II}$ is the observed rate at a fixed concentration of cyanide. The values for k_1 and k_{-1} were from Table 1. The value for k_2 was determined by an initial pseudo-first-order approximation to the data in panels B and C, and the value of k_{-2} was estimated as described in Table 1.

HAO-B, values were $\Delta H^{act} = 61 \pm 5$ kJ mol^{-1} and $\Delta S^{act} = -40 \pm 20$ J K mol^{-1} for complex I and $\Delta H^{act} = 79 \pm 2$ kJ mol^{-1} and $\Delta S^{act} = -6 \pm 4$ J K mol^{-1} for complex II. For HAO-A values were $\Delta H^{act} = 68 \pm 2$ kJ mol^{-1} and ΔS^{ac} -60 ± 20 J K mol^{-1}.

Dissociation of the Mono- or Dicyano Complexes. Cyanide dissociated very slowly from the mono- or dicyano complexes of HAO; dialysis for 16 h at 4 °C or passage over a Sephadex G-25 desalting column was required. After dissociation of cyanide, the enzyme was fully active and reactive with cyanide. Cyanide was more rapidly removed from the monocyano complex of HAO-A after complete reduction of all hemes (*c* and P460) of the enzyme with dithionite and passage over a desalting column, presumably due to the low affinity of cyanide for ferrous hemes.

Significantly, dissociation of cyanide from heme P460 of HAO-A complex I or HAO-B complex II was not stimulated by reaction of the enzyme with 0.5 mM hydrazine followed immediately by rapid passage through a desalting column. Hence cyanide appears to remain bound to the enzyme during catalysis. During the reaction with hydrazine ~40% of the *c*-heme was reduced, but heme P460 was not. Since cyanide binds ferric iron, this is consistent with cyanide binding to heme P460.

Properties of HAO-A and HAO-B. HAO-A was the form of the enzyme usually isolated. HAO-B was isolated from one batch of cells. The form isolated did not appear to depend on the several methods of isolation employed (see Experimental Procedures). There was no significant difference between HAO-A and -B in the amount of absorbancy due to heme P460, turnover number or resting EPR spectrum. However, the apparent K_m values for N_2H_4 of HAO-A and HAO-B differed (3.0 ± 0.5 and 5.0 ± 0.5 μM, respectively).

HAO-B was converted to HAO-A when the enzyme was fully reduced by dithionite and reoxidized during passage over a gel filtration column. (Ferrous HAO is oxidized by O_2; Hooper & Balny, 1982). HAO-B was sometimes converted to HAO-A by aging at 4 or 25 °C, especially under illumination by room light. Incubation of ferric HAO-A for 12 h with increasing amounts of hydrogen peroxide led to the progressive conversion to HAO-B (Figure 5). As expected, the resulting enzyme preparation had a lower content of heme P460, a lower reducibility of *c*-hemes due to a transformation of ferric heme P460 by peroxide (Hooper & Terry, 1977). In addition, the treated enzyme had a higher K_m and a lower V_{max} for hydrazine (Figure 5).

The following treatments caused no apparent interconversion of HAO-A and -B: (a) gel filtration (in the presence or absence of EDTA); anion-exchange, cation-exchange, or hydrophobic interaction chromatography; preparative isoelectric focusing in which the enzyme was at pH values ranging from 4.7 to 8.5 for several hours; (b) freezing and thawing three times, in the presence or absence of 30% glycerol or with exposure to 2 M urea; (c) reduction by substrate, followed by reoxidation; (d) incubation of 50% HAO-A and HAO-B or HAO-A with cytochrome *c*-554, the biological electron acceptor from HAO (Yamanaka & Shinra, 1974; Arciero *et al.*, 1993).

Cyanide Binds at or near Heme P460. Cyanide is known to decrease the rate of inactivation of ferric P460 of HAO by peroxide (Hooper & Terry, 1977). Preliminary data (not shown) indicate that the reaction of hydrogen peroxide with ferric HAO involves formation of at least three possible intermediates detected by optical difference spectroscopy. Preincubation of HAO-A or -B with cyanide so as to form the monocyano complex reduced the rate of formation of the putative peroxide-HAO intermediates by at least 100-fold (data not shown). Correspondingly, destruction of all heme P460 of HAO-A or HAO-B with an excess of hydrogen peroxide resulted in the loss of the ability of HAO to form

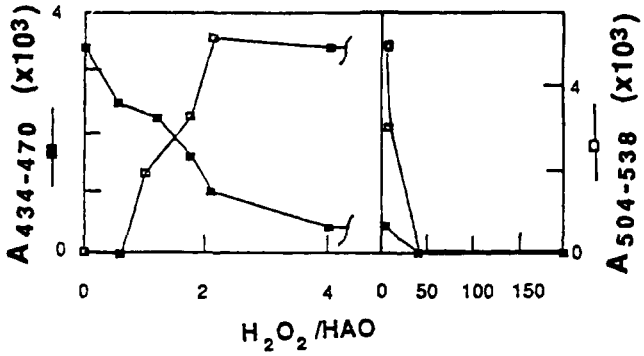

FIGURE 5: Conversion of HAO-A to HAO-B as a function of the concentration of hydrogen peroxide. HAO-A (100 μM by active site) was incubated with the indicated amount of H_2O_2 for 12 h, and properties of the treated enzyme were determined. The relative amounts of HAO-A or HAO-B were determined as the absorbance change at 434−470 nm or 504−538 nm, respectively, in the presence of 400 μM KCN in a 1 μM sample of HAO. The amount of heme P460 present was determined as the difference in absorbancy between 442 and 464 nm in the difference spectrum (HAO + dithionite + CO minus HAO + dithionite). At peroxide: HAO ratios of 2 or 50, the amount of heme P460 had decreased by 17 or 90%, respectively. The reduction by hydrazine was determined as the difference in absorbancy at 552 nm minus 744 nm of 0.5 μM HAO in the presence of 200 μM hydrazine. At peroxide:HAO ratios of 2 or 50, the amount of substrate-reducible *c*-heme had decreased by 50 or 90%, respectively. Values of V_{max} and K_m were determined in steady-state assays in the presence of 12 μM horse heart cytochrome *c* . At peroxide:HAO ratios of 2 or 50, values of V_{max} had decreased by 50 and 100%, respectively. At a peroxide:HAO ratio of 2 the value of K_m had increased by 60%.

the mono- or dicyano complexes (Figure 5). Thus cyanide and peroxide react at or near the same center on HAO, probably heme P460, a component of the active site.

Inhibition by Cyanide of Substrate Reduction of c-Hemes of HAO. Stopped-flow rapid-scan kinetic measurement of the reaction of HAO with hydroxylamine or hydrazine demonstrate several phases in the expected reduction of ~33% or ~40% of the *c*-hemes of HAO, respectively. Within ~0.5 s ~73% of the process is complete (Hooper *et al.*, 1984). To observe the time course of inhibition by cyanide of the reduction of c-hemes of HAO by substrate, HAO was preincubated for various times with KCN in one reservoir of the stopped-flow apparatus before mixing with substrate. The subsequent time course of reduction of the enzyme, followed as the increase in absorbance at the α peak of the *c*-hemes (552 nm), is in Figure 6. Significantly, in the presence of cyanide, the total amount of reduction by NH_2OH of *c*-hemes of HAO-A or HAO-B was diminished by only 0 or 15%, respectively. As seen by measurements on the millisecond time scale, the rate (k_{obs}) of the fast phase was unchanged (data not shown); however, the amount of *c*-heme reduced in the fast phase progressively decreased with the time of preincubation with cyanide. The rate of reduction of the remaining substrate-reducible heme was ~400-fold slower than the fast phase of the uninhibited enzyme and similar to the rate during the slow phase of the uninhibited enzyme. In contrast, cyanide did not change the kinetics of reduction of *c*-hemes of HAO by dithionite (data not shown).

Kinetics of the Loss of the Fast Phase. The data suggest that the remaining fast phase of reduction of *c*-hemes was catalyzed by active site which had not yet bound cyanide to form the monocyano complex. Hence the kinetics of elimination of the fast phase of activity was expected to resemble the first-order kinetics of binding of cyanide seen by a change in optical spectra (Figure 2). An initial treatment of the data with the assumption that the loss of the fast phase of activity as a function of time and concentration of cyanide also followed pseudo-first-order kinetics resulted in values for the rate of formation of the putative inhibiting species and for an apparent dissociation constant ($k^{f\phi}_{onapp}$ = 140 ± 10 M^{-1} s^{-1} and $K^{f\phi}_{dapp}$ = 25 ± 7 μM Table 1) which appeared to differ from the values obtained for k_1 (k_{on}) = 200 ± 20 M^{-1} s^{-1} and K_d (K^{kin}_d = 8 ± 3 and K^{eq}_d) = 9 ± 2 μM) for the formation of the monocyano complexes (seen

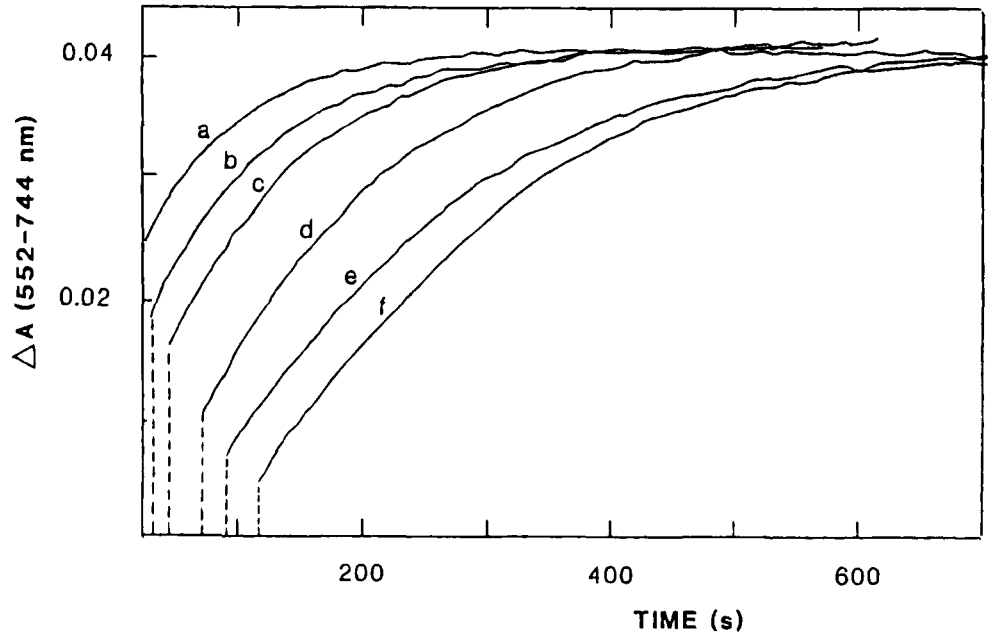

FIGURE 6: Time course of reduction of *c*-hemes of HAO-B (1.1 μM) by NH_2OH (50 μM) as a function of time of preincubation in the presence of 100 μM KCN: (a) control in the absence of KCN; (b−f) substrate added 10, 26, 57, 90, or 137 s after mixing with cyanide. The length of each dashed line indicates the amount of reduction of *c*-heme that occurred within 10 s (the fast phase).

as the 434-nm absorption band). The disagreement between these two pairs of kinetic constants could be resolved if electrons from substrate rapidly equilibrated between subunits of oligomeric HAO. If HAO is functionally a multimer and the active site of only one subunit is occupied by cyanide, substrate may still bind to the unoccupied site(s) and be rapidly oxidized. If electrons are rapidly transferred to hemes of all functional subunits, the extent of reduction of hemes by substrate in the fast phase would be unaffected until all substrate sites of the oligomer were occupied by cyanide.

It was possible to demonstrate that electron transfer between oligomers (or possible exchange of monomers between oligomers) is extremely slow and need not be considered in models of catalysis. A sample of HAO was inactivated by peroxide so that reducibility of the *c*-heme of HAO by substrate was diminished by 50% (although the same amount of *c*-heme was reducible by dithionite). When a 20-fold excess of this HAO was incubated with native HAO and substrate, the total reduction of *c*-heme was no greater than the sum of *c*-heme reduced when the active and inactive enzyme were separately incubated with substrate.

To test the "functional dimer" hypothesis by an analysis of the kinetic data, it was assumed that cyanide forms the high-affinity monocyano complex with heme P460 of HAO in a pseudo-first-order process as indicated by the optical data of Figure 2. An expression for the rate of formation of the cyanide-saturated intermediate for the cases where HAO was a monomer, dimer, trimer, or tetramer was derived as a simplified case of the general solution for an *n*-step series of reactions (Rodiguin & Rodiguina, 1964). All microscopic binding rates (k_1 and k_{-1} values) were assumed to be the same and were multiplied by the appropriate statistical factors [see Cantor and Schimmel (1980)]. The observed time courses at three different concentrations of cyanide were found to be most consistent for the case where HAO-A is a dimer (Logan, 1991).

Kinetics of Inhibition by Cyanide of the Steady-State Turnover of HAO. Inhibition by cyanide (at concentrations resulting in the formation of the monocyano derivative) of the steady-state turnover of HAO was investigated at a fixed concentration of mammalian cytochrome *c* (Figure 7). Cyanide altered both the slope and the intercept of hydrazine-variable reciprocal plots. The data fit the simple classic noncompetitive inhibition by cyanide of the reaction of hydrazine with HAO. The values of dissociation constants for cyanide based in inhibition of enzyme activity were 25 ± 7 or 24 ± 7 μM for HAO A-I and B-I, respectively. These values are different from the values of 9 ± 2 or 8 ± 2 μM for K_d based on direct observation of optical changes accompanying the formation of the monocyano complex of heme P460 of HAO. According to the functional dimer hypothesis, the value for the K_d for cyanide calculated from measurement of enzyme inhibition under steady-state conditions should, in effect, be the macroscopic K_d of cyanide binding to the second active site in dimeric HAO, i.e., double the microscopic K_d for the second addition of cyanide to dimeric HAO, or ~18 μM. Hence the data are supportive of the functional dimer hypothesis.

DISCUSSION

The present work provides the background data for the reaction of cyanide with HAO that is necessary to and has been used in (Hendrich *et al.*, 1994) analysis of the active site and mechanism of turnover of HAO.

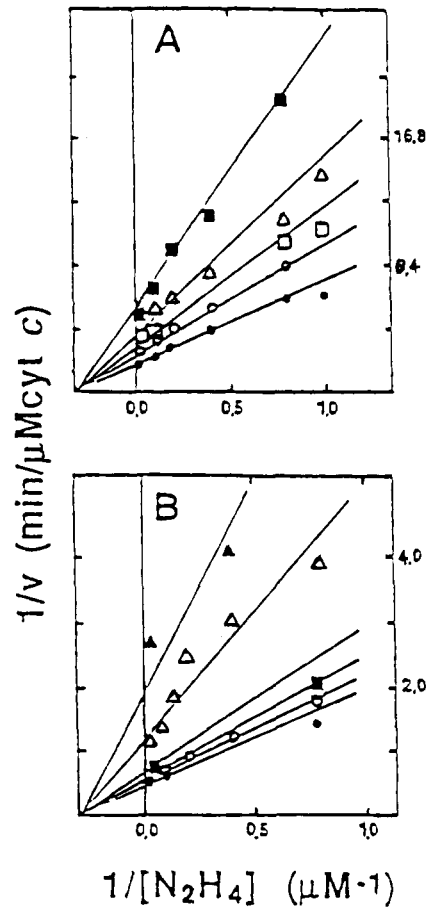

FIGURE 7: Steady-state kinetics of inhibition of HAO-A (A) and HAO-B (B) by cyanide. The fit to the Lineweaver–Burke plots was for classic noncompetitive inhibition as analyzed using Marquhardt's algorithm. (A) HAO-A (6 nM by active site) without KCN (●) or with 6 (○), 12 (□), 20 (△), or 80 μM KCN (■). (B) HAO-B (20 nM by active site) without KCN (●), or with (○), 6 (■), 12 (□), 40 (△), or 80 μM KCN (▲). HAO was preincubated with the indicated concentration of KCN. The reaction mixtures contained 27 μM horse heart cytochrome *c* and the indicated concentrations of hydrazine.

Cyanide Binds to a Ferric Heme of HAO. Many properties of the reaction of cyanide with HAO are similar to the properties of formation of cyano derivatives of other hemoproteins (Table 2). For example, values are in the low micromolar range for dissociation constants of the cyanide complexes with HAO observed here (at pH values below 9) and for other cyanohemoproteins. The low values are in keeping with covalent binding of an anionic ligand to a ferric heme iron in HAO rather than to an adjacent "anion pocket" for which values of dissociation constants are in the low millimolar range [e.g., thiocyanate binding to lactoperoxidase (Modi *et al.*, 1989) or nitrate binding to chloroperoxidase (Lambeir *et al.*, 1983a)]. The fact that cyanide is dissociated from the monocyano or dicyano complex by reduction of all irons of HAO with dithionite is consistent with binding to a ferric heme iron (Antonini & Brunori, 1971).

In contrast to the heme of mammalian cytochrome *c* (Horecker & Kornberg, 1946; Schejter Aviram, 1969), the *c*-hemes of HAO, which are also hexacoordinate, do not bind cyanide at pH values below 9. In more alkaline solution the equivalent of less than one *c*-heme of HAO binds cyanide.

Table 2: Properties of Cyanide Derivatives of HAO and Other Hemoproteins

cyanohemoprotein	k_1[a] ($M^{-1}\,s^{-1}$)	K_d (μM)	position of Soret peak[b] (nm)	$\Delta\epsilon$[c] ($cm^{-1}\,mM^{-1}$)	activation enthalpy (kcal mol^{-1})	reference
Met hemoglobin	200	5	424	43		*d*
Met myoglobin	400	10	426	63		*d*
mitochondrial *aa* $_3$ oxidase	2	1	432	28	14	*e*
chloro-peroxidase	5×10^4	100			6	*f*
lactoperoxidase	1×10^6	30	435	52		*g*
catalase	1×10^6	3	428	40		*h*
mammalian cytochrome *c*	9	2.5	418	14	17	*i*
HAO complex A-I	200	9	434	3	16	*j*
HAO complex B-I	200	9	434	2	15	*j*
HAO complex B-II	7	40	506	4	18	*j*

[a] For all cited hemoproteins, the reaction with cyanide was reported to be consistent with pseudo-first-order kinetics. [b] Wavelength maximum or [c] millimolar extinction coefficient of the cyano derivative is from the difference spectrum: (cyanide + ferric hemoprotein) *minus* (ferric hemoprotein). [d] Kinetic data of Antonini and Brunori (1971). Optical data of equine myoglobin and human hemoglobin. [e] van Buuren *et al.* (1972). Two forms of cytochrome aa_3 oxidase differ in the rate of reaction with cyanide (Baker *et al.*, 1987); values of van Buuren *et al.* (1972) are for the slowly reacting form. [f] Dolman *et al.* (1968) and Lambeir et al. (1983b). [g] Dolman *et al.* (1968). [h] Kinetic data of Palcic and Dunford, 1981, for human catalase. Optical data for bovine catalase was measured in this work. [i] Kinetic data of Horecker and Kornberg, 1946. Optical data measured here on horse heart cytochrome *c*. [j] This work.

Cyanide Binds to Heme P460 of HAO. HAO contains 7 *c*-hemes and 1 heme P460 per monomer. Complex I of HAO contains 1 cyanide per 7 *c*-hemes, suggesting binding to heme P460. Further, the optical difference spectra of complex I of HAO-A or -B are not those typically observed with the binding of cyanide to a *c*-heme. A puzzling aspect of the binding of cyanide to ferric heme P460 of HAO is that the difference extinction coefficients are smaller by a factor of 4−20-fold as compared with other hemoproteins (Table 2). The unusually small difference extinction coefficent for cyanide binding may be related to the unusually low value of the extinction coefficient of ferric heme P460 of HAO (Collins *et al.*, 1993). In contrast, the value for the ferrous form is similar to other hemes (64 $mM^{-1}\,cm^{-1}$ in the Soret).

Heme P460 is a component of the substrate binding site and is the peroxide-reactive moeity of HAO. Hence inhibition by cyanide of HAO activity, inhibition by cyanide of peroxide inactivation of HAO, and loss of cyanide binding following peroxide inactivation are consistent with the binding of cyanide to ferric heme P460. Correspondingly, the rate of dissociation of the monocyano complex of HAO was unaffected by reduction with substrate (which does not reduce heme P460), whereas the rate of dissociation of complex A-I greatly increased when all hemes (including heme P460) were reduced by dithionite.

The conclusion that cyanide binds heme P460 of HAO has been used to identify heme P460 as a component of a complex within HAO which has an integer spin EPR signal (Hendrich *et al.*, 1994).

Nature of Interaction of Cyanide with the Active Site of HAO: The Monocyano Compound. A reasonable model for catalysis by HAO involves binding of hydroxylamine to ferric heme P460 followed by oxidation to NO^+ by removal of four electrons and three protons and hydrolysis to nitrous acid. The enzyme may facilitate deprotonation of substrate or intermediates. Transfer of electrons to the acceptor cytochrome *c*-554 of *Nitrosomonas* may occur by way of *c*-hemes of HAO or directly from the active site. Inhibition of substrate oxidation by formation of the monocyano complex of HAO is shown here to be noncompetitive; the affinity of substrate was unaffected, but the rate of reduction of *c*-hemes of HAO decreased by a factor of at least 100. It appears that cyanide does not inhibit the rate of binding of substrate, but rather catalysis and/or electron transfer to *c*-hemes of HAO or cytochrome *c*-554.

Cyanide is shown to remain bound during catalysis, suggesting that the active site has two ligand binding sites. The active site of HAO is thought to contain heme P460 bridged by an anionic bridging group to a *c*-heme (Hendrich *et al.*, 1995). It is possible that cyanide displaces the bridging ligand and the substrate binds from the other side of heme P460 or *visa versa*.

Binding of the Second Cyanide to the Active Site of HAO-B. The optical absorbancy features of the monocyano complex of HAO-B disappear as the features of the dicyano complex appear. Further, the kinetics of formation of the dicyano complex are pseudo-first-order with respect to cyanide. This suggests that the dicyano complex of HAO-B forms by the binding of a second molecule of cyanide to a monocyano heme. The product may be a bis-dicyano heme complex (which has no precedent in biology). Interestingly, the formation of the bis-dicyano derivative of heme model compounds is accompanied by two successive 15-nm red shifts in the optical spectrum, and the second cyanide binds with approximately 30 fold lower affinity than the first (Scheidt *et al.*, 1980). These properties are similar to those of the successive formation of the monocyano and dicyano complexes of HAO-B, although the extinction coefficient of the latter is smaller.

The greatly accelerated formation of the dicyano complex upon addition of substrate to the monocyano complex cannot be explained. The complex {HAO CN NH_2OH} or the equivalent complex with an *N*-oxide intermediate in the oxidation of hydroxylamine would appear to further activate heme P460 for the binding of cyanide. This implies that the complex {HAO $(CN)_2$ NH_2OH} may be formed. Reduction of one or more of the *c*-hemes of HAO is known to result in changes in the EPR parameters of other ferric hemes, possibly including heme P460 (Lipscomb & Hooper, 1982; Prince & Hooper, 1987). Analogus heme−heme interactions may stimulate binding of the second cyanide.

Formation of the dicyano complex has no apparent additional effect upon the oxidation of substrate by HAO under steady-state or transient conditions; i.e., the catalytic step that was made rate-limiting by the binding of the first cyanide remains rate-limiting. It is, of course, possible that

the rate of dissociation of cyanide is also stimulated by binding of substrate to the monocyano derivative so that inhibition is, in effect, due to the first cyanide.

Functional Oligomeric Structure of HAO. The subunit molecular mass of HAO is approximately 63 kDa (Terry & Hooper, 1981); hence dimeric HAO should have a molecular mass of 126 kDa. HAO apparently has physical properties that complicate conventional methods of determining the molecular mass. The value by most physical determinations suggests a trimer: 180 000 from gel filtration and SDS−PAGE (Yamanaka *et al.*, 1979) and 200 000 from ultracentrifugation (Rees, 1968) and electrophoresis (Terry & Hooper, 1981). The value of 150 000 determined by electrophoresis (Masson *et al.*, 1990) most closely approximates that of a dimer. In all of these studies, native HAO behaved as a single species that did not undergo a monomer−oligomer equilibrium on a slow time scale. As reported here, the kinetics of formation of the monocyano complex taken together with the kinetics of inhibition by cyanide of steady-state turnover of HAO and reduction of *c*-hemes of HAO by substrate led to the hypothesis that the enzyme functions as a dimer in which electrons rapidly equilibrate between different subunits. Further, rapid exchange of electrons or subunits between oligomers of HAO is shown here not to occur.

Multiple Forms of HAO. The reaction of cyanide with HAO distinguishes forms HAO-A and HAO-B, which are apparently interconvertible under some circumstances. The properties of the monocyano complexes of HAO-A and -B are very similar, although a second cyanide can add to the latter. Multiple forms of hemoproteins are not uncommon. For example, cytochrome *c* peroxidase (Bosshard *et al.*, 1991) and cytochrome oxidase (Schoonover *et al.*, 1988; Baker *et al.*, 1987) have forms which differ in reactivity with substrate or ligands.

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REFERENCES

Anderson, J. H. (1964) *Biochem. J. 91*, 8−17.
Andersson, K. K., & Hooper A. B. (1983) *FEBS Lett. 164*, 236−240.
Andersson, K. K., Kent, T. A., Lipscomb, J. D., Hooper, A. B., & Munck, E. (1984) *J. Biol. Chem. 259*, 6833−6840.
Antonini, E., & M. Brunori (1971) *Hemoglobin and myoglobin in their reactions with ligands*, North Holland Publishing Company, Amsterdam.
Arciero, D. M., & Hooper, A. B. (1993) *J. Biol. Chem. 268*, 14645−14654.
Arciero, D. M., Balny, C., & Hooper, A. B. (1991) *Biochemistry 30*, 11466−11472.
Arciero, D. M., Hooper, A. B., Cai, M., & Timkovich, R. (1993) *Biochemistry 32*, 9370−9378.
Baker, G. M., Noguchi, M., & Palmer, G. (1987) *J. Biol. Chem. 262*, 595−604.
Bosshard, H. R., Anni, H., & Yonetani, T. (1991) in *Peroxidases in Chemistry and Biology*, (Everse, J., Everse, K. E., & Grisham, M. B., Eds.) CRC Press, Boca Raton, FL.
Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry*, W. H. Freeman and Co., San Francisco.
Collins, M., Arciero, D. M., & Hooper, A. B. (1993) *J. Biol. Chem. 268*, 14655−14662.
Dolman, D., Dunford, H. B., Chowdhury, D. M., & Morrison, M. (1968) *Biochemistry 7*, 3991−3996.
Hendrich, M. P., Logan, M., Andersson, K. K., Arciero, D. M., Lipscomb, J. D., & Hooper, A. B. (1994) *J. Am. Chem. Soc. 116*, 11961−11968.
Hollocher, T. C., Tate, M. E., & Nicholas, D. J. D. (1981) *J. Biol. Chem. 256*, 10834−10836.
Hooper, A. B., & Balny, C. (1982) *FEBS Lett. 144*, 299−303.
Hooper, A. B., Erickson, R. H., & Terry, K. R. (1972) *J. Bacteriol. 110*, 430−438.
Hooper, A. B., Debye, P., Andersson, K. K., & Balny, C. (1983) *Eur. J. Biochem. 134*, 83−87.
Hooper, A. B., Maxwell, P. C., & Terry, K. (1978) *Biochemistry 17*, 2984−2989.
Hooper, A. B., Tran, V. M., & Balny, C. (1984) *Eur. J. Biochem. 141*, 565−571.
Hooper, A. B., & Terry, K. (1977) *Biochemistry 16*, 455−459.
Horecker, B. L., & Kornberg, A. (1946) *J. Biol. Chem. 165*, 11−20.
Lambeir, A. M., Dunford, H. B., & Pickard, M. A. (1983a.) *J. Inorganic Biochem. 19*, 291−300.
Lambeir, A. M., Heremans, K., & Dunford, H. B. (1983b) *Biophys. Chem. 18*, 195−201.
Lipscomb, J. D., & Hooper, A. B. (1982). *Biochemistry 21*, 3965−3972.
Logan, M. S. P. (1991) Ph.D. Thesis, University of Minnesota.
Logan, M. S. P. , & Hooper, A. B. (1994) *Biochemistry* (submitted).
Markley, J. L., Travers, F., & Balny, C. (1981) *Eur. J. Biochem. 120*, 477−485.
Modi, S., Behere, D. V., & Mitra, S. (1989) *Biochemistry 28*, 4689−4694.
Masson, P., Arciero, D. M., Hooper, A. B., & Balny, C. (1990) *Electrophoresis 11*, 128−133.
Nasri, H., Fischer, J., Weiss, R., Bill, E., & Trautwein, A. (1987) *J. Am. Chem. Soc. 109*, 2549−2550.
Palcic, M. M., & Dunford, H. B. (1981) *Arch. Biochem. Biophys. 211*, 245−252.
Prince, R. C., & Hooper, A. B. (1987) *Biochemistry 26*, 970−974.
Rees, M. K. (1968) *Biochemistry 7*, 366−372.
Rodiguin, N. M., & Rodiguina, E. N. (1964) *Consecutive Chemical Reactions*, Van Nostrand, New York.
Sayavedra-Soto, L. A., Hommes, N. G., & Arp, D. J. (1994) *J. Bacteriol. 176*, 504−510.
Scheidt, W. R., Haller, K. J., & Hatano, K. (1980) *J. Am. Chem. Soc. 102*, 3017−3021.
Schejter, A., & Aviram, B. (1969) *Biochemistry 8*, 149−153.
Schoonover, J. R., Dyer, R. B., Woodruff, W. H., Baker, G. M., Noguchi, M., & Palmer, G. (1988) *Biochemistry 27*, 5433−5440.
Segel, I. H. (1975) *Enzyme Kinetics*, John Wiley and Sons, New York.
Terry, K., & Hooper, A. B. (1981) *Biochemistry 20*, 7026−7032.
Van Buuren, K. J. H., Nicholls, P., & van Gelder, B. F. (1972) *Biochem. Biophys. Acta* 256, 258−276.
Yamanaka, T., & Shinra, M. (1974) *J. Biochem. 75*, 1265−1273.
Yamanaka, T., Shinra, M., Takahashi, K., & Shibasaka, M. (1979) *J. Biochem. 86*, 1101−1108.

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