Spectroscopic and Rapid Kinetic Studies of Reduction of Cytochrome *c*554 by Hydroxylamine Oxidoreductase from *Nitrosomonas europaea*†

David M. Arciero,‡ Claude Balny,§ and Alan B. Hooper*,‡

Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota 55108, and Institut National de la Santé et de la Recherche Médicale, Unité 128, 34033 Montpellier Cedex 1, France

Received July 5, 1991; Revised Manuscript Received September 9, 1991

ABSTRACT: During oxidation of hydroxylamine, hydroxylamine oxidoreductase (HAO) transfers two electrons to tetraheme cytochrome *c*554 at rates sufficient to account for physiological rates of oxidation of ammonia to nitrite in *Nitrosomonas europaea*. Spectroscopic changes indicate that the two electrons are taken up by a high-potential pair of hemes ($E^{\circ\prime}$ = +47 mV) (one apparently high spin and one low spin). During single-turnover experiments, in which the reduction of oxidized cytochrome *c*554 by NH_2OH-reduced HAO is monitored, one electron is taken up by the high-spin heme at a rate too fast to monitor directly (>100 s^{-1}) but which is inferred either by a loss of amplitude (relative to that observed under multiple-turnover conditions) or is slowed down by increasing ionic strength (≥300 mM KCl). The second electron is taken up by the low-spin heme at a 10–30-fold slower rate. The latter kinetics appear multiphasic and may be complicated by a transient oxidation of HAO due to the rapid transfer of the first electron into the high-spin heme of cytochrome *c*554. Under multiple-turnover conditions, a "slower" rate of reduction is observed for the high-spin heme of cytochrome *c*554 with a maximum rate constant of ~30 s^{-1}, a value also obtained for the reduction, by NH_2OH, of the cytochrome *c*554 high-spin heme within an oxidized HAO/*c*554 complex. Under these conditions, the maximum rate of reduction of the low-spin heme was ~11.0 s^{-1}. Both rates decreased as the concentration of cytochrome *c*554 was increased above the concentration of HAO. This phenomenon suggests either tight complex formation or the existence of several conformational states for HAO. Dependence of the rate constant for each heme under both single- and multiple-turnover conditions as a function of temperature, pH, ionic strength, and concentration of cytochrome *c*554 and experiments employing mammalian cytochrome *c* as the electron acceptor instead of cytochrome *c*554 suggest that a strong electrostatic interaction exists between HAO and cytochrome *c*554 within the HAO/*c*554 complex and that little, if any, energetic barrier exists to affect intramolecular electron transfer within the complex. The rapid rates of electron transfer, the high affinity of these two proteins, and the capacity for facile two-electron transfer make cytochrome *c*544 an excellent candidate as the physiological electron acceptor from HAO in *N. europaea*.

A puzzling feature of the ammonia oxidation pathway of the nitrifying bacterium *Nitrosomonas europaea* is the mechanism by which the organism appropriates electrons from the reaction catalyzed by hydroxylamine oxidoreductase (HAO)[1] ($NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$). It is thought that the organism utilizes the four electrons in pairs with two electrons directed to the ammonia monooxygenase (AMO) reaction ($NH_3 + O_2 + 2e^- + 2H^+ \rightarrow NH_2OH + H_2O$) and the other two passed through an electron transport chain to the terminal oxidase. This sorting of electrons presumably requires the presence of additional electron transport components capable of mediating electron flow into the AMO branch. A variety of soluble and membrane-bound redox-active molecules have been identified: the *c*-type cytochromes *c*554, *c*553, *c*552, $c_{CO}552$, $c_{CO}550$, c_m553, and c_m552 as well as ubiquinone, cytochrome aa_3, and cytochrome P460, an unusual cytochrome containing a unique heme-like moiety of unknown structure (Erickson & Hooper, 1972; Tronson et al., 1973; Yamanaka & Shinra, 1974; Miller & Wood, 1983; DiSpirito et al., 1985). However, existing evidence has not yet established how these components function in the overall flow of electrons in vivo.

Cytochrome *c*554 is hypothesized to be the primary electron acceptor from HAO, although the hypothesis is based more on the molecular properties of cytochrome *c*554 than on any significant direct evidence of electron transfer. As a tetraheme cytochrome (Anderson et al., 1986), cytochrome *c*554 is capable of accepting more than one electron at a time and has been shown to catalytically transfer electrons from HAO to a cytochrome *c*552 from *N. europaea* (Yamanaka & Shinra, 1974). In the absence of cytochrome *c*554, electron transfer from HAO to cytochrome *c*552 is slow with mammalian cytochrome *c*'s being more efficient. The isoelectric points of HAO (4.7) and cytochrome *c*554 (10.8) are complementary as one would expect for physiological partners in an electron transfer complex and both proteins are localized in the periplasmic space of *Nitrosomonas* (DiSpirito et al., 1985). Finally, electrochemical analysis of the various hemes in the cytochrome, as determined in the preceding paper (Arciero et al., 1991), shows that two of the hemes have a midpoint potential ($E^{\circ\prime}$ = +47 mV) which is in the same range as the NH_2OH/NO_2^- and NH_2OH/NO couples ($E^{\circ\prime}$ = +66 mV and −36 mV, respectively) (Aleem, 1970; Hyman & Wood, 1984).

As isolated, HAO is a large (M_r = 200000) and very complex hemoprotein (Terry & Hooper, 1981). It apparently consists of a heptaheme (M_r = 63000) subunit (Arciero &

† This work was supported by Grant NSF/INT-8914758 and La Fondation pour la Recherche Médicale Française.

* Author to whom correspondence should be addressed.

‡ University of Minnesota.

§ Institut National de la Santé et de la Recherche Médicale.

[1] Abbreviations: AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase.

0006-2960/91/0430-11466$02.50/0

Hooper, 1988) in an α_n oligomeric structure where the value of n is probably 2 or 3 (Masson et al., 1990). The α subunit also contains a heme-like iron-containing macrocycle of unknown structure which has been named P460 (Erickson & Hooper, 1972) due to an absorption maximum observed in dithionite-reduced HAO (Hooper & Nason, 1965). P460 is considered to be part of the catalytic site of HAO since selective destruction of the chromophore by hydrogen peroxide results in an inactive enzyme (Hooper & Terry, 1977). The c-hemes of reduced HAO are distinguished spectrally by α-band absorbancy maxima at either 553 or 559 nm (in an absorbancy ratio of 5:2 for the α subunit) or potentiometrically with oxidation–reduction midpoint potentials ranging from +295 to −390 mV (Prince et al., 1983; Prince & Hooper, 1987). During steady-state turnover of hydroxylamine, the equivalent of ~2.5 of the seven c-hemes in HAO become reduced, in keeping with the calculated potential of the NH_2OH/NO_2^- couple.

As one might expect, kinetics of the reduction of the multiple hemes of either HAO (Hooper et al., 1984) or cytochrome c554 (DiSpirito et al., 1987) are complex. Reaction of HAO with dithionite reduces the c-hemes, as well as the P460 chromophore, with rate constants ranging from 7 s^{-1} to 0.01 s^{-1}. Reduction of HAO by its substrate, NH_2OH, is somewhat simpler. Of the ~2.5 heme equivalents reduced, the electrons are more equally divided between the 553- and 559-nm hemes but reduction of the 553-nm c-hemes probably precedes reduction of the 559-nm c-hemes. Reduction of cytochrome c554 by dithionite is biphasic with a very rapid first phase (140 s^{-1}) during which two (or possibly three) electrons are taken up (DiSpirito et al., 1987; Arciero et al., 1991).

In this report, the transfer of electrons from HAO to cytochrome c554 during hydroxylamine turnover is investigated. Some of the issues addressed include whether electron transfer is rapid enough to account for physiological rates of electron transfer, what number of electrons are accommodated by cytochrome c554, and which hemes in cytochrome c554 are involved. To minimize spectral changes, at 418 nm, associated with the reduction of hemes of HAO during turnover of NH_2OH, HAO was preincubated with excess NH_2OH under anaerobic conditions before being mixed with oxidized cytochrome c554. This preincubation poised HAO at its equilibrium redox level and facilitated selective monitoring of the hemes of cytochrome c554. The same strategy was employed to determine the kinetics of reduction of horse heart cytochrome c by NH_2OH-reduced HAO.

Materials and Methods

Enzymes and Chemicals. Cytochrome c554 was prepared from *N. europaea* by the procedure described in the preceding paper (Arciero et al., 1991). HAO was purified from *N. europaea* by a modification of a previously described procedure (Hooper et al., 1978) as follows. After the first ammonium sulfate fractionation step, the resuspended and dialyzed 60–80% ammonium sulfate precipitate was chromatographed on a Sephadex G-100 column (5.0 cm × 110 cm) equilibrated with 50 mM KPO_4 buffer, pH 7.5, containing 0.2 M KCl. The resulting HAO fraction was concentrated by ultrafiltration (Amicon YM-5 filter) and then subjected to chromatography on a Sepharose CL-6B column (2.5 cm × 110 cm) equilibrated with the same buffer. In a final step, HAO was purified to homogeneity either by ion-exchange chromatography on a DEAE-Sephacel (Pharmacia) column (2.5 cm × 17 cm) equilibrated with 20 mM Tris-HCl, pH 8.1, and developed with a 0.0–0.3 M NaCl gradient (500 mL × 500 mL) or by hydrophobic interaction chromatography on an octyl-Sepharose (Pharmacia) column (4.0 cm × 17 cm) equilibrated with 25 mM KPO_4, pH 7.5, buffer containing 2.0 M $(NH_4)_2SO_4$ and developed with a 2.0–0.0 M $(NH_4)_2SO_4$ gradient (600 mL × 600 mL).

Glucose oxidase (*Aspergillus niger* type II) and cytochrome c (horse heart type III) were obtained from Sigma and used without further purification. Glucose and hydroxylamine were from Merck and Aldrich, respectively.

Spectra. Spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer utilizing filters to absorb all UV radiation below 300 nm to prevent photoreduction of HAO. A dual-chamber cuvette was used for obtaining difference spectra. HAO and cytochrome c554 were each at a protein concentration of 2 μM before mixing. All solutions contained 20 mM KPO_4 buffer, pH 7.5, containing 0.1 M KCl. To record the NH_2OH-reduced cytochrome c554-*minus*-oxidized cytochrome c554 spectrum, 2 mM NH_2OH was added to the chamber containing HAO prior to recording the initial spectrum of NH_2OH-reduced HAO and oxidized cytochrome c554. The combined spectrum of the individual proteins was recorded and the data were stored with a Hewlett Packard 9000 Series 300 computer using Hewlett Packard Chemstation software. The two proteins were then mixed by inversion of the cuvette several times. The combined spectrum of the mixed proteins was then recorded, and the data were stored. Subtractions were carried out using the same Chemstation software.

Kinetic Measurements. Rapid-scan absorption spectra were recorded using a Union Giken (Osaka) Model RA 415–RA 401 fast-response spectrophotometer (Hooper et al., 1983). Kinetic traces were collected using an Aminco DW-2 spectrophotometer (Hooper & Balny, 1982). Rapid mixing of solutions was achieved utilizing a thermostated, anaerobic stopped-flow mixing device (Markley et al., 1981) that could be outfitted to either spectrophotometer. Solutions were deaerated by being flushed with N_2 for 20 m. In some early experiments using NH_2OH-reduced HAO, solutions also contained 2 nM glucose oxidase and 60 mM glucose to remove residual oxygen. However, in control experiments, while the presence of oxygen was not found to interfere with the kinetics of reduction of the cytochromes, the presence of the glucose/glucose oxidase oxygen-scavenging system occasionally did cause problems. Thus, flushing with N_2 was the only routine step taken to remove oxygen from the solutions in the majority of experiments. Heme concentrations of HAO, cytochrome c554, and cytochrome c were calculated using ϵ_{553} = 27.8 mM^{-1} cm^{-1} (Hooper et al., 1978), ϵ_{554} = 24.6 mM^{-1} cm^{-1} (Andersson et al., 1986), and ϵ_{550} = 27.5 mM^{-1} cm^{-1}, respectively. Because of some uncertainty in the oligomeric structure of HAO, the concentration of HAO is given per putative active site assuming seven c-type hemes per P460 center and is designated HAO(α). This is also a convenient unit of concentration to employ initially until the stoichiometry of complex formation between HAO and cytochrome c554 can be satisfactorily determined. The concentration of cytochrome c554 is calculated on the basis of four c-hemes per molecule.

Results and Discussion

Equilibrium Level of Reduction of Cytochrome c554 by HAO in the Presence of NH_2OH. Interpretation of the kinetic data presented in this work is dependent on knowledge of the number of electrons passed into cytochrome c554 from HAO during oxidation of NH_2OH. A reductive titration of cytochrome c554 by NH_2OH in the presence of substoichiometric amounts of HAO indicated that not all hemes in cytochrome c554 are reducible by NH_2OH (Yamanaka & Shinra, 1974).

Table I: Reduction of Hemes of HAO and Cytochrome *c*554 by Hydroxylamine

wavelength (nm)	k_{obs} (s^{-1}) HAO + *c*554[a]	HAO[b]
432	21.3 ± 0.2	nd[c]
437	28.5 ± 0.3	nd
553.5	18.2 ± 0.7	30 ± 3
560	13.5 ± 0.6	19 ± 2

[a] Reaction of 2.0 mM NH_2OH (syringe 1) with 1.0 μM oxidized HAO(α) (syringe 2) and 1.0 μM oxidized cytochrome *c*554 (syringe 2) in 20 mM KPO_4 buffer, pH 7.5, containing 0.1 M NaCl at 20 °C. [b] Reaction of 0.2 mM NH_2OH (syringe 1) with 2.1 μM oxidized HAO(α) (0.7 μM HAO) (syringe 2) in 0.1 M phosphate buffer, pH 7.0, at 2 °C. From Hooper et al. (1984). [c] nd, not determined.

However, because of the complexity of the optical changes in HAO and cytochrome *c*554 and uncertainty at that time regarding the heme content of cytochrome *c*554, it was not possible to determine the number of hemes reduced in cytochrome *c*554 by HAO in the presence of NH_2OH.

Because the electrochemical titration of cytochrome *c*554 described in the preceding paper (Arciero et al., 1991) clearly discriminates between the hemes present in the cytochrome, reduced-*minus*-oxidized spectra generated in that titration can be used to obtain a good estimate of the equilibrium level of reduction of cytochrome *c*554 by HAO during turnover of NH_2OH. By comparing both the absolute spectrum of NH_2OH-reduced cytochrome *c*554 as well as the NH_2OH-reduced-*minus*-oxidized difference spectrum of cytochrome *c*554 to corresponding absolute and reduced-*minus*-oxidized difference spectra generated in the electrochemical titration, it was determined that NH_2OH-reduced cytochrome *c*554 is poised at a potential somewhere in the plateau region between the the high-potential and intermediate-potential couples of cytochrome 554. Because the absorbance is relatively insensitive to the applied potential in this region of the voltammogram, the potential at which NH_2OH-reduced cytochrome *c*554 is poised can only be narrowed to the range between −60 and −110 mV, assuming, of course, that HAO does not alter the midpoint potentials of any of the hemes in cytochrome *c*554. Since coulometric evidence indicates a pair of high-potential hemes (+47 mV) in cytochrome *c*554 which spectroscopically resemble a low-spin/high-spin pair with absorption maxima for the reduced-*minus*-oxidized spectrum near 422 and 432 nm, respectively, it is concluded that it is these two hemes in cytochrome *c*554 that become reduced after equilibration of the cytochrome 554/HAO/NH_2OH system.

Kinetics of Reduction of the Oxidized HAO/Cytochrome c554 Complex by Hydroxylamine. As a test of the assumption that cytochrome *c*554 has no effect on the reduction of any of the hemes of HAO by NH_2OH (and vice versa), reduction of hemes within the oxidized HAO/*c*554 complex by NH_2OH was investigated. These results are shown in Table I. When monitored at 560 nm, cytochrome *c*554 contributes a negligible amount to the absorbance changes observed during the reduction. An isosbestic point in the reduction of HAO by NH_2OH is present at 437 nm. Thus, the rate of reduction of 559-nm hemes in HAO were followed at 560 nm, and the reduction of the high-spin heme of cytochrome *c*554 is followed at 437 nm. Rate constants measured at 432 and 553.5 nm are actually multiphasic, consisting of contributions of reduction of hemes in both HAO and cytochrome *c*554 but, since rates of reduction of hemes in both proteins are somewhat similar, pseudomonophasic kinetic traces are observed. Importantly, lags were not observed in any of the kinetic traces, thus implying a free flow of electrons in both HAO and cytochrome *c*554. In addition, as seen in Table I, rate constants measured for the reduction of the HAO/*c*554 complex by NH_2OH are in agreement with those measured for the reduction of HAO by NH_2OH in the absence of cytochrome *c*554 (Hooper et al., 1984).

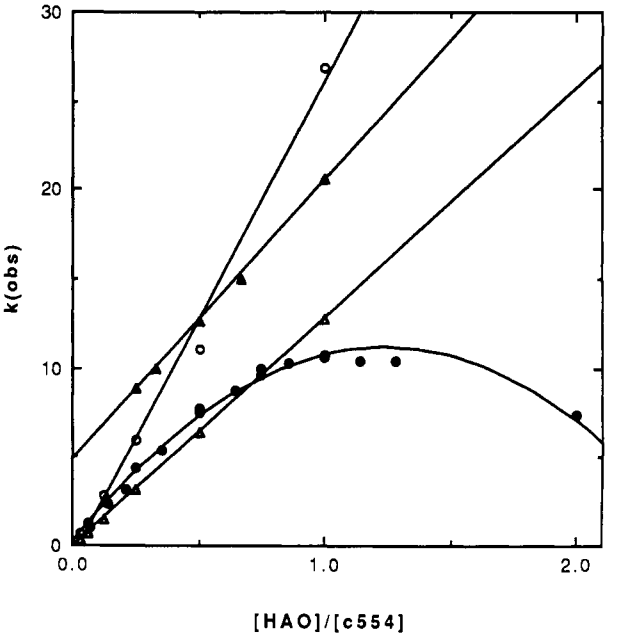

FIGURE 1: Concentration dependence of reduction of cytochrome *c*554 by HAO during turnover of hydroxylamine or hydrazine. The temperature was 20 °C in 20 mM KPO_4 buffer, pH 7.5, containing 0.1 M KCl; $[NH_2OH] = [NH_2NH_2] = 2.0$ mM. Reactions: various concentrations of substrate-reduced HAO(α) (syringe 1) with 2.0 μM oxidized cytochrome *c*554 (syringe 2) monitored at 436 nm with NH_2OH (○) or NH_2NH_2 (△) as substrate or at 418 nm with NH_2OH (●) as substrate; 2.0 μM NH_2OH-reduced HAO(α) (syringe 1) with 2, 3, 4, 6, or 8 μM oxidized cytochrome *c*554 (syringe 2) monitored at 436 nm (▲). Rate constants are obtained from kinetic traces shown in Figure 2 and are averages of three separate mixing experiments. The theoretical curve fitting the data at 418 nm is a second-order polynomial of the type $k_{obs} = a + b([HAO(\alpha)]/[c554]) + c([HAO(\alpha)]/[c554])^2$ where *a*, *b*, and *c* equal 0.08, 17.8, and −7.2, respectively ($R^2 = 0.985$).

Kinetics of Reduction of Cytochrome c554 by NH_2OH-Reduced HAO. To simplify the study of electron transfer from HAO to cytochrome *c*554, spectral interference arising from reduction of HAO hemes by NH_2OH was minimized by preincubating anaerobic HAO with NH_2OH and then mixing it with oxidized cytochrome *c*554. The assumption is that prior reduction of HAO with NH_2OH serves to poise HAO at its equilibrium redox level so that any absorbance changes observed upon mixing of the contents from the two syringes should be due solely to the reduction of cytochrome *c*554. As discussed below, this assumption may not be strictly valid in some experiments in which the concentration of HAO is greater than the concentration of cytochrome *c*554. Nevertheless, prior reduction of HAO by NH_2OH apparently does not introduce any additional complications in the analyses but most certainly allows abstraction of information from the system which would not otherwise be obtained.

Since the two hemes of cytochrome *c*554 reduced by HAO during NH_2OH turnover are presumably a low-spin/high-spin pair, it should be possible to follow the individual reduction of these two hemes by monitoring at 418 and 436 nm, respectively. At either of these two wavelengths, the kinetics should be dominated by contributions from only a single heme. This is precisely what was observed; the reduction of these two hemes occurred at different rates and showed distinct behavior. Shown in Figure 1 is the dependence of the ratio [HAO-

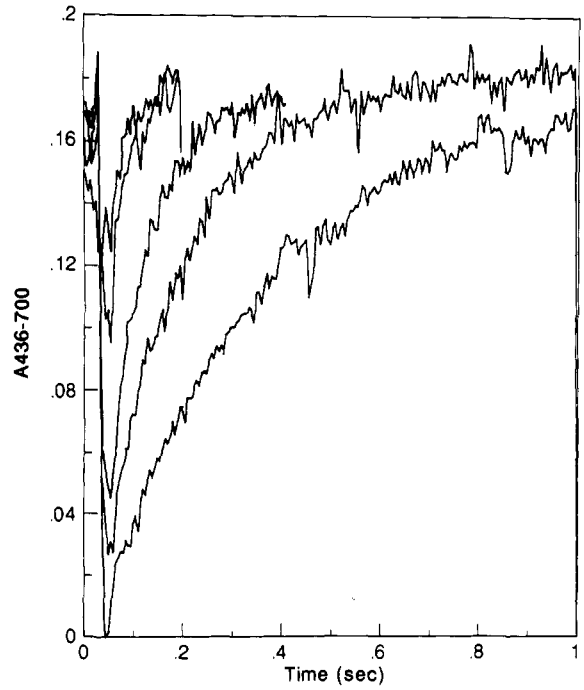

FIGURE 2: Kinetic traces obtained for reduction of cytochrome c554 by HAO during NH_2OH turnover revealing amplitude loss occurring during transition from multiple- to single-turnover conditions. Reaction of 2.0 μM oxidized cytochrome c554 (syringe 1) with 0.25, 0.50, 1.0, 2.0, or 4.0 μM NH_2OH-reduced HAO(α) (syringe 2). The slowest kinetics are observed for 0.25 mM HAO(α) (bottom trace) while the fastest kinetics are observed for 4.0 mM HAO(α) (top trace). Other conditions are as in the legend to Figure 1.

(α)]/[c554] on the rate constant. The experiment was carried out by holding the concentration of cytochrome c554 constant while varying the concentration of HAO(α).

At 418 nm, k_{obs} increased linearly at low [HAO(α)]/[c554], reached a plateau of approximately 10.5–11.0 s^{-1} at approximately stoichiometric amounts of HAO(α) and cytochrome c554, and then decreased at higher values. Kinetic traces appeared monophasic at [HAO(α)]/[c554] < 1 but were multiphasic (2–3 phases) at [HAO(α)]/[c554] > 1 and could not be fitted precisely. No attempt was made to fit the data to a second-order rate expression, as demanded by the experimental conditions, because of some uncertainties regarding origin of electrons as discussed below. The rate constants at [HAO(α)]/[c554] > 1 used in the figure represent the "best" average rate constant extracted from the data assuming monophasic kinetics. The amplitude of the absorbance change remains constant at all concentrations of HAO(α).

The heme at 436 nm exhibited fundamentally different behavior. The increase in the measured k_{obs} was linear at all values of [HAO(α)]/[c554] up to stoichiometric amounts. Hidden, however, in the data was the presence of a very fast phase that revealed itself only by a decrease in total observable amplitude as the concentration of HAO(α) was increased. This is shown in Figure 2 where a series of kinetic traces, recorded at the same concentration of cytochrome c554 but at different concentrations of HAO(α), are overlaid. The maximum amplitude for the series of kinetic traces (measured at approximately 5 ms after mixing; i.e., the dead time for the instrument) is clearly dependent on the concentration of HAO(α). Simple extrapolation to $t = 0$ does not account for the missing amplitude. A plot of the missing amplitude of the fast phase as a function of the concentration of HAO(α) [approximated by the relationship $\Delta A = A(\text{final}) - A(t \approx 5\text{ms})$] exhibited the expected sigmoidal relationship (data not shown). Ultimately, under single-turnover conditions, where the concentration of HAO is twice the concentration of cytochrome c554, only a very fast phase is present. Attempts to fit the residual data directly gives a rate constant of 100–150 s^{-1}. However, calculation of the rate constant based on the residual amplitude observed (0.04 AU vs 0.17–0.18 AU expected) and a knowledge of the dead time of the instrument suggests a minimum value of 250–300 s^{-1}.

The slower phase observed at 436 nm under multiple-turnover conditions showed a maximum value of between 25 and 30 s^{-1}. This is comparable to the value obtained when the oxidized HAO/c554 complex is reduced by hydroxylamine (Table I). It is believed that this slower phase is only observed under multiple-turnover conditions because HAO needs to abstract additional electrons from the substrate before it can reduce subsequent molecules of cytochrome c554. Presumably the maximum rate at which it can do this would be the rate at which electrons are fed into cytochrome c554 during reduction of the oxidized HAO/c554 complex by NH_2OH.

This interpretation was further borne out by the use of NH_2NH_2 as substrate instead of NH_2OH. When the reduction was followed, at 436 nm, of the high-spin heme of cytochrome c554 by HAO during NH_2NH_2 turnover, similar behavior was observed. Again, there was evidence for a very fast phase that was inferred only because of a decrease in amplitude as the concentration of HAO was increased at a fixed concentration of cytochrome c554. A slower phase was present as well (Figure 1) that showed a maximum value of about 13 s^{-1}, a value that was also obtained for the reduction, by NH_2NH_2, of cytochrome c554 within the oxidized HAO/c554 complex.

Spectroscopic Changes. Spectroscopic changes recorded during the pre-steady-state reduction of cytochrome c554 were consistent with the observed kinetics and are shown in Figure 3. With substoichiometric amounts of cytochrome c554 to HAO(α), a relatively narrow band was observed centered at 424 nm (Figure 3, bottom spectra) whereas at excess amounts of cytochrome c554 to HAO(α) the oxidized-*minus*-reduced difference absorption band was broader with significant changes seen at 432 nm (Figure 3, top spectra). Under single-turnover conditions, reduction of the high-spin heme occurred during the time of mixing and the spectroscopic changes were dominated by the slower reduction of the low-spin heme. When the concentration of cytochrome c554 was 4-fold greater than the concentration of HAO(α), the difference spectra included spectral contributions from multiple turnovers. Hence, spectral changes associated with reduction of the high-spin heme are observed as well.

Effect of Extrinsic Variables on Kinetics of Reduction of Cytochrome c554 Hemes under Single-Turnover Conditions. Reduction of cytochrome c554 under single-turnover conditions (defined as [HAO(α)] ≥ 2[c554]) was studied as a function of temperature, ionic strength, and pH at both 436 and 418 nm. The very fast phase inferred at 436 nm could not be slowed down by decreasing the temperature to 2 °C or by varying the pH between 6.0 and 8.5 but could be affected by high ionic strength, thus providing direct evidence for its existence. Results obtained as a function of ionic strength at pH 7.5 and 7.0 are shown in Figure 4. At pH 7.5, monophasic kinetics were observed when the concentration of KCl was 300 mM or higher. At pH 7.0 and lower, a rate constant could only be determined when the concentration of KCl was 400 mM or higher. At pH 6.5 and 6.0, the kinetic analyses became complicated by the presence of multiple phases. As the kinetics became slower due to the increase in ionic strength, there was

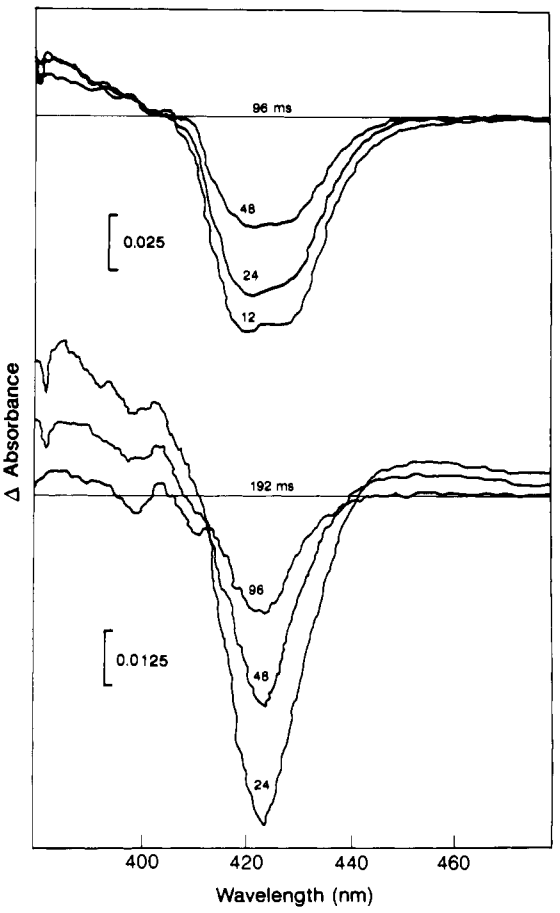

FIGURE 3: Rapid-scan difference spectra recorded during reduction of cytochrome *c*554 by HAO during hydroxylamine turnover. Spectra were collected at the indicated times following mixing, subtracted from the absorption spectrum recorded at the time indicated by the flat base line, and then plotted as the difference spectra. Reaction of 0.70 μM oxidized cytochrome *c*554 (syringe 1) with 1.4 μM NH_2OH-reduced HAO(α) (syringe 2) (bottom), and 2.0 μM oxidized cytochrome *c*554 (syringe 1) with 0.5 μM NH_2OH-reduced HAO(α) (syringe 2) (top). Scanning velocity was 50 nm/ms. Other conditions are as in the legend to Figure 1.

a concomitant increase in the the amplitude of the optical changes such that at very high salt concentrations the amplitude of the absorption change due to reduction of cytochrome *c*554 was the same as that observed only at low ratios of HAO/*c*554.

Temperature and pH dependencies were also investigated at 418 nm. Under all conditions, multiphasic kinetics were observed and optical changes had the expected amplitudes. Little, if any, dependence of either temperature or pH on rate constants or amplitudes was evident; similar traces were obtained between 25 and 5 °C and between pH 8.0 and 6.0. Ionic strength dependence at 418 nm was not investigated.

Effect of Extrinsic Variables on Kinetics of Reduction of Cytochrome c554 Hemes under Multiple-Turnover Conditions. In order to define the slower phase observed at 436 nm, the kinetics of reduction were also investigated under multiple-turnover conditions (defined as [*c*554] ≥ 2[HAO(α)]) as a function of the concentration of cytochrome *c*554, temperature, pH, and ionic strength. The concentration dependence was determined at a fixed concentration of HAO and is included in Figure 1. As the concentration of cytochrome *c*554 was increased, the rate constant decreased. The temperature dependence was measured at the ratio of [*c*554]/[HAO] = 4 between 2 and 25 °C. Both NH_2OH and NH_2NH_2 were tested as substrates. The Arrhenius plots are shown in Figure

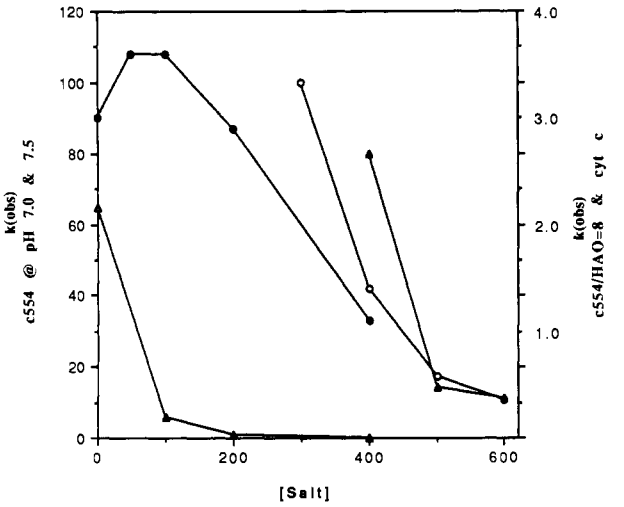

FIGURE 4: Effect of ionic strength on rate of reduction of hemes of cytochrome *c*554 and cytochrome *c* by HAO during hydroxylamine turnover. The temperature was 20 °C in 20 mM KPO_4 buffer containing KCl (K^+) or NaCl (Na^+) at the indicated concentrations; [NH_2OH] = 2.0 mM. Reactions: 1.5 μM oxidized cytochrome *c*554 (syringe 1) at pH 7.0 (▲) (K^+) or pH 7.5 (○) (K^+) with 3.0 μM NH_2OH-reduced HAO(α) (syringe 2); 3.2 μM oxidized cytochrome *c*554 (syringe 1) (●) (Na^+) with 0.4 μM NH_2OH-reduced HAO(α) (syringe 2); and 1.75 μM cytochrome *c* (syringe 1) (△) (Na^+) with 0.53 μM NH_2OH-reduced HAO(α) (syringe 2).

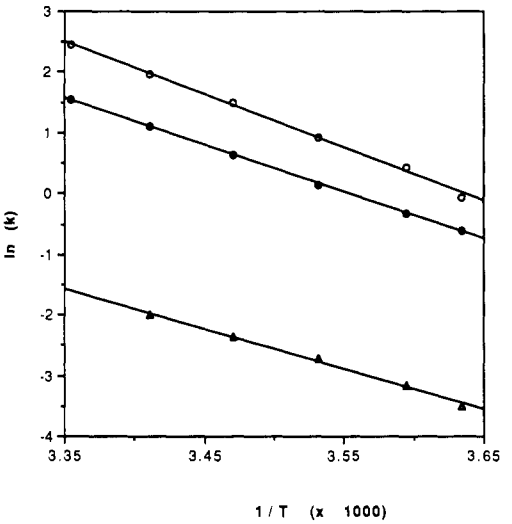

FIGURE 5: Effect of temperature on rate of reduction of hemes of oxidized cytochrome *c*554 or cytochrome *c* by HAO during substrate turnover. Reaction of 1.5 μM oxidized cytochrome *c*554 (syringe 1) with 0.38 μM NH_2OH-reduced (○) or NH_2NH_2-reduced (●) HAO(α) (syringe 2) in 20 mM KPO_4 buffer, pH 7.5, containing 0.1 M KCl at the indicated temperatures. Reaction of 2.0 mM oxidized cytochrome *c* (syringe 1) with 0.53 μM NH_2OH-reduced HAO(α) (△) (syringe 2) in 20 mM KPO_4 buffer containing 0.1 M NaCl at the indicated temperatures.

Table II: Effect of pH on the Rate of Reduction of Cytochrome *c*554 High-Spin Heme by HAO during Turnover of Hydroxylamine[a]

pH	6.0	6.5	7.0	7.5	8.0
k_{obs} (s^{-1})	2.5 ± 0.1	4.2 ± 0.1	6.4 ± 0.2	7.7 ± 0.2	8.7 ± 0.2

[a] Reaction of 2.0 μM oxidized cytochrome *c*554 (syringe 1) with 0.50 μM NH_2OH-reduced HAO(α) (syringe 2) at 20 °C in 20 mM KPO_4 buffer, containing 0.1 M KCl, at the indicated pH values.

5. Activation energies were calculated to be 71 ± 2 kJ/mol and 62 ± 1 kJ/mol for NH_2OH and NH_2NH_2, respectively.

Within the pH range 6.0–8.0, an increase in pH leads to a proportional increase in the rate of reduction of cytochrome *c*554 at a ratio of [*c*554]/[HAO(α)] = 4 (Table II). Mon-

ophasic kinetics were observed at all values of pH. The amplitude of the absorption change was constant between pH 6.5 and 8.0 but was decreased by approximately 25% at pH 6.0. The effect of ionic strength on the reduction of cytochrome $c554$ by NH_2OH was determined at a ratio of $[c554]/[HAO(\alpha)] = 8$. As seen in Figure 4, the rate of reduction at pH 7.5 was relatively insensitive to added salt until [NaCl] > 0.10 M. The rate of reduction was highest at 0.05–0.10 M NaCl.

Kinetics of Reduction of Mammalian Cytochrome c by NH_2OH-Reduced HAO. Mammalian cytochrome *c* is known to act as an electron acceptor in in vitro assays of HAO (Hopper & Nason, 1965). In addition, cytochrome *c* and cytochrome *c*554 each have a p*I* of 10.7–10.8. Reduction of cytochrome *c* by HAO during turnover of NH_2OH was investigated both to understand some of the factors influencing its reduction and to contrast with the reduction of cytochrome *c*554. The effect of cyt *c* concentration, temperature, and ionic strength on the kinetics of reduction was tested. For the range $0.25 \leq [\text{cyt } c]/[HAO(\alpha)] \leq 20$ and in the presence of 0.1 M NaCl, essentially identical rates were measured, indicating no concentration dependence on k_{obs} for the reduction of cytochrome *c*. The temperature dependence in the range between 2 and 20 °C is included in the Arrhenius plot of Figure 5. Activation energy was calculated to be 50 ± 1 kJ/mol. The dependence on ionic strength, at a ratio of $[\text{cyt } c]/[HAO] = 3.3$, followed the typical behavior expected for a simple electrostatic interaction between HAO and cytochrome *c* on the basis of polyelectrolyte theory (Figure 4). Implications and development of the theory have been described for the interaction between liposomal cytochrome c_1 and cytochrome *c* (Kim et al., 1989).

Complex Formation vs Conformational States. Whenever one observes a decrease in rate constant as the concentration of one protein (cytochrome *c*554) is increased under multiple-turnover conditions, there are two possible explanations for the result. One interpretation is that HAO and cytochrome *c*554 form a tight, electrostatically stabilized complex which influences the rate constant through product inhibition. The alternative explanation is that HAO exists in two or more conformational states having different binding and kinetic properties such that higher concentrations of cytochrome *c*554 effectively reduce the concentration of the kinetically competent conformer. The evidence from the present work seems to favor the first explanation. In the reduction of cytochrome *c*554 by HAO a very fast phase observed at 436 nm under single-turnover conditions converts to a slower phase under multiple-turnover conditions. The maximum rate constant of the slower phase is comparable to the maximum rate observed for the reduction of HAO by hydroxylamine, or the reduction of cytochrome *c*554 within an oxidized HAO/*c*554 complex. This is more consistent with the formation of a tight complex. If a tight complex was not being formed, the maximum value for the slower rate at 436 nm should be less than the rate at which HAO is itself reduced.

The dependence on ionic strength in both the single- and multiple-turnover experiments is also consistent with the model involving tight complex formation and can be attributed to an effect on an electrostatic interaction which would be expected to exist between proteins of such opposite isoelectric points (10.8 vs 4.7). In order to support the conformer model, one would have to argue that the ionic strength modulates the interconversion between conformations such that the kinetically competent conformer is favored at the higher ionic strength. Finally, the contrasting results with cytochrome *c* are also

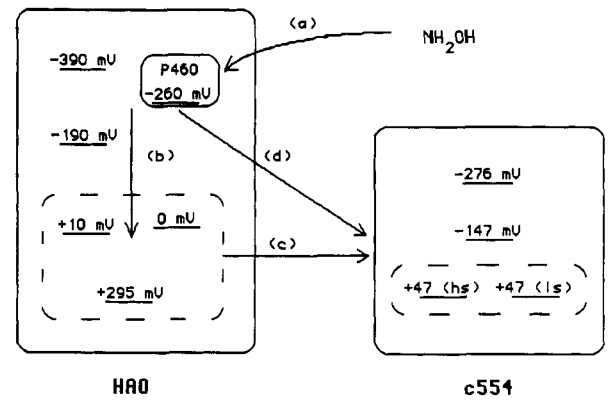

FIGURE 6: Model of HAO and cytochrome *c*554 depicting redox-active hemes and showing possible pathways for transfer of electrons both intramolecularly and intermolecularly. Hemes are identified by their midpoint potentials. Hemes reduced at equilibrium in the presence of excess hydroxylamine are enclosed in dashed boxes.

consistent with formation of a tight complex between HAO and cytochrome *554*; similar concentration and ionic strength dependencies would be expected for the two electron acceptors if HAO existed in different conformations.

Origin of Electrons Transferred to Cytochrome c554 under Single-Turnover Conditions. Being multi-heme cytochromes, HAO and cytochrome *c*554 represent such a complex system that attempting to describe, in detail, the origin of electrons or pathways of electron transfer is extremely difficult. Although these are not central points in this investigation, some of these issues are addressed here.

As we have shown here (Figure 2, Table I), the high-spin heme of cytochrome *c*554 accepts the first electron from NH_2OH-reduced HAO at a rate 4–10 times faster than HAO is itself reduced by NH_2OH. This raises the question as to whether the electron is transferred to cytochrome *c*554 from one of the reduced hemes in HAO (Figure 6, pathways b and c) or directly from the HAO active site and bypassing the reduced hemes (Figure 6, pathway d). If the electron is originating in one of the reduced hemes of HAO, one would expect to see a transient oxidation of that heme. And, if there is a transient oxidation of one of the hemes of HAO it should be revealed in the kinetics at 418 nm (a wavelength at which hemes for both HAO and cytochrome *c*554 could contribute to absorption changes). The evidence is conflicting. When monitoring at 418 nm under single-turnover conditions, kinetic traces are multiphasic, thereby suggesting that several processes are occurring at this wavelength. However, amplitudes of the absorption changes under both single- and multiple-turnover conditions are the same at 418 nm. It is possible that once HAO is poised at its equilibrium level of reduction by NH_2OH, the enzyme itself is poised to abstract an electron from bound substrate (or intermediate) and transfer it to cytochrome *c*554 at a rate much faster than it can when HAO is fully oxidized. Testing this possibility is beyond the scope of the present investigation.

It seems unusual that, in the single-turnover experiments, the two hemes of cytochrome *c*554 are reduced at such different rates since all four hemes interact both electronically and magnetically (Andersson et al., 1986). This could be due to a restriction in the system that allows for the rapid transfer of only a single electron to cytochrome *c*554 while the transfer of the second electron requires a reorganization of the complex (either reequilibration of electrons within HAO or conformational changes accompanying the one-electron oxidation of HAO or reduction of cytochrome *c*554). However, since the two hemes of cytochrome *c*554 in question both have the

same midpoint potential yet appear to exhibit slight positive cooperativity during the electrochemical titration, it is very surprising that the intramolecular transfer of electrons within cytochrome *c*554 would be this slow. On the other hand, there is a possibility, which cannot be ruled out, that the slow rate measured at 418 nm may not be the true rate of reduction of the low-spin heme of cytochrome *c*554; the low-spin heme may be reduced just as rapidly as the high-spin heme so that, at 418 nm under single-turnover conditions, the rereduction of hemes of HAO is actually being monitored. If this second electron is transferred very rapidly from a reduced heme in HAO to the low-spin heme of cytochrome *c*554, the net absorption change during the transfer should be minimal.

Conclusions. Electron transfer from HAO to cytochrome *c*554 during NH_2OH turnover has been studied primarily in order to ascertain whether cytochrome *c*554 is kinetically qualified to act as the physiological electron acceptor from HAO in vivo. The evidence presented here strongly suggests that it is. Cytochrome *c*554 acts as a two-electron acceptor under these conditions. These electrons are taken up by the high-potential pair of hemes in cytochrome *c*554 designated heme $+47_{hs}$ and heme $+47_{ls}$. These electrons are transferred from HAO to cytochrome *c*554 at rates commensurate with or greater than rates of reduction of HAO by its substrate, NH_2OH. Furthermore, the kinetic evidence strongly suggests that HAO and cytochrome *c*554 form a very tight electrostatically stabilized complex.

References

Aleem, M. I. H. (1970) *Annu. Rev. Plant Physiol. 21*, 67–90.

Andersson, K. K., Lipscomb, J. D., Valentine, M., Münck, E., & Hooper, A. B. (1986) *J. Biol. Chem. 261*, 1126–1138.

Arciero, D. M., & Hooper, A. B. (1988) *J. Cell Biol. 107*, 619a.

Arciero, D. M., Collins, M. J., Haladjian, J., Bianco, P., & Hooper, A. B. (1991) *Biochemistry* (preceding paper in this issue).

DiSpirito, A. A., Taaffe, L. R., & Hooper, A. B. (1985) *Biochim. Biophys. Acta 806*, 320–330.

DiSpirito, A. A., Balny, C., & Hooper, A. B. (1987) *Eur. J. Biochem. 162*, 299–304.

Erickson, R. H., & Hooper, A. B. (1972) *Biochim. Biophys. Acta 275*, 231–244.

Hooper, A. B., & Nason, A. (1965) *J. Biol. Chem. 240*, 4404–4057.

Hooper, A. B., & Terry, K. R. (1977) *Biochemistry 16*, 455–459.

Hooper, A. B., & Balny, C. (1982) *FEBS Lett. 144*, 299–303.

Hooper, A. B., Maxwell, P. C., & Terry, K. R. (1978) *Biochemistry 17*, 2984–2989.

Hooper, A. B., Debey, P., Adnersson, K. K., & Balny, C. (1983) *Eur. J. Biochem. 134*, 83–87.

Hooper, A. B., Tran, V. M., & Balny, C. (1984) *Eur. J. Biochem. 141*, 565–571.

Hyman, M. R., & Wood, P. M. (1984) in *Microbial Growth on C1 Compounds* (Crawford, R. L., & Hanson, R. S., Eds.) pp 49–52, American Society for Microbiology, Washington, DC.

Kim, C. H., King, T. E., & Balny, C. (1989) *Biochem. Biophys. Res. Commun. 163*, 276–283.

Markley, J. L., Travers, F., & Balny, C. (1981) *Eur. J. Biochem. 120*, 477–485.

Masson, P., Arciero, D. M., Hooper, A. B., & Balny, C. (1990) *Electrophoresis 11*, 128–133.

Miller, D. J., & Wood, P. M. (1983) *Biochem. J. 211*, 503–506.

Prince, R. C., & Hooper, A. B. (1987) *Biochemistry 26*, 970–974.

Prince, R. C., Larroque, C., & Hooper, A. B. (1983) *FEBS Lett. 163*, 25–27.

Terry, K. R., & Hooper, A. B. (1981) *Biochemistry 20*, 7026–7032.

Tronson, D. A., Ritchie, G. A. F., & Nicholas, D. J. D. (1973) *Biochim. Biophys. Acta 310*, 331–343.

Yamanaka, T., & Shinra, M. (1974) *J. Biochem. 75*, 1265–1273.