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Resolution of Multiple Heme Centers of Hydroxylamine Oxidoreductase from *Nitrosomonas*. 1. Electron Paramagnetic Resonance Spectroscopy†

John D. Lipscomb and Alan B. Hooper*

ABSTRACT: Hydroxylamine oxidoreductase (HAO) from *Nitrosomonas europeae* [M_r 220 000, subunit structure of $(\alpha\beta)_3$ with seven *c*-type hemes and one P-460-type heme per $\alpha\beta$ subunit] catalyzes the oxidative conversion of NH_2OH to NO_2^-. We have used electron paramagnetic resonance (EPR) spectroscopy to monitor a reductive titration of the enzyme. The spectra show that the *c*-type hemes can be placed into at least four groups with different oxidation–reduction potentials and protein environments. Since the hemes are reduced sequentially, *g*-value assignments can be made for three of the major species (g = 3.38, 1.95, 0.7; g = 3.06, 2.14, 1.35; and g = 2.98, 2.24, 1.44), and the spectrum of each can be isolated from the composite spectrum of the protein. Quantitation of these three spectra suggests that approximately one-third of the heme in the enzyme resides in other species. Some of the unquantitated heme may contribute to one or more novel EPR active species with *g* values at g = 2.7, g = 1.85, and g = 1.66. A second fraction of this heme is probably EPR silent since high- and low-spin heme signals absent from the spectrum of resting HAO appear during the final half of the reductive titration. One of these newly EPR active species, with characteristic high-spin heme *g* values at 6.45 and 5.6, reduces concomitantly with the appearance of the optical spectrum of the reduced P-460 heme. On this basis, P-460 is tentatively assigned as the high-spin heme. Since no high-spin heme signal is observed in the resting enzyme, P-460 must either undergo a spin conversion as the other hemes are reduced or be EPR silent due to spin coupling or fast electronic spin relaxation. EPR spectra also show that NH_2OH reduces approximately 45% of the hemes when complexed anaerobically with HAO. A new low-spin heme (g = 2.86, 2.3) becomes EPR active in this complex, and the *g* values of most of the other EPR active hemes shift slightly.

Ammonia in aerobic soils or waters is rapidly oxidized to nitrite as the sole source of energy by the autotrophic nitrifying bacterium *Nitrosomonas europaea*. Hydroxylamine, or a closely related enzyme-bound chemical species, is an intermediate in the process (Hooper, 1978). The enzyme hydroxylamine oxidoreductase (HAO)[1] isolated from the bacterium catalyzes the rapid aerobic oxidation of hydroxylamine to nitrite in the presence of phenazine methosulfate as an artificial electron acceptor. HAO (M_r 220 000) has been shown to contain approximately 21 *c*-type hemes and 3 residues of an unusual prosthetic group termed P-460 (Hooper et al., 1978). The active enzyme is composed of three M_r 11 000 α subunits each containing one *c*-type heme and three M_r 63 000 β subunits each containing approximately six *c*-type hemes as well as the P-460 (Terry et al., 1979; Terry & Hooper, 1981). No evidence for the presence of other essential metal centers

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[1] Abbreviations: EPR, electron paramagnetic resonance; HAO, hydroxylamine oxidoreductase; EDTA, ethylenediaminetetraacetic acid.

0006-2960/82/0421-3965$01.25/0

has been obtained. The P-460 moiety is thought to be a heme because it has a Soret-like absorption maximum [ϵ = 68 (mM cm)$^{-1}$] at 463 nm when reduced and because it binds typical heme ligands such as CO and CN (Erickson & Hooper, 1972). The Mössbauer spectra of HAO and the reduced HAO–CO complex presented in the following paper (Lipscomb et al., 1982) also support this conclusion.

Part of the reaction cycle of HAO appears to proceed in the absence of O_2. When exposed to NH_2OH in anaerobic solution, HAO becomes partially reduced. It is estimated from optical measurements that ~30% of the *c*-type hemes are reduced, but the characteristic absorbance of reduced P-460 does not appear, suggesting that it either remains oxidized or is rapidly reoxidized during the reaction. The extent of reduction of the *c*-type hemes changes when alternate substrates such as hydrazine are substituted for NH_2OH. All of the *c*-type hemes and P-460 are reduced by sodium dithionite.

There are several indications that the P-460 and *c*-type hemes perform different roles during catalysis. Most significantly, the optical spectrum of P-460 disappears when the enzyme is treated with low concentrations of H_2O_2 (Hooper & Terry, 1977). Concurrently, enzymatic activity and substrate-coupled reducibility of the *c*-type hemes are lost. In contrast, exposure of the resting enzyme to H_2O_2 has no effect on the optical spectrum of the *c*-type hemes, and they retain their reducibility by dithionite. The small heme ligands such as CO which bind to the P-460 have almost no effect on the *c*-type heme optical spectrum, suggesting these hemes do not have a readily displaceable axial ligand.

The optical spectra of the *c*-type hemes (and presumably also the oxidized P-460) are so similar that it is impossible to further differentiate their roles in catalysis. Electron paramagnetic resonance (EPR) spectroscopy, on the other hand, is very sensitive to the axial ligand structure and protein environment of ferric heme. In previous studies, EPR was used to establish the presence of several different classes of *c*-type heme centers in the enzyme (Vickery & Hooper, 1979, 1981). It was shown that at least four resonances occurred in the spectral region between g = 2.7 and 3.4 which is typical for low-spin ferric heme. No evidence for high-spin ferric heme was detected. The complexity of the spectra, however, limited more detailed analysis. Further interpretation of the HAO EPR spectrum requires some sort of perturbation which affects the individual hemes differently.

The objectives of the current study are to distinguish the *c*-type hemes on the basis of their oxidation–reduction properties and to establish the relationship between P-460 and the *c*-type hemes. The *c*-type hemes are shown to have markedly different oxidation–reduction potentials, resulting in a stepwise reductive titration. This allows the assignment of g values of individual heme centers and the estimation of the concentration of these hemes within HAO. The results are discussed in terms of a model in which electrons enter HAO during a catalyzed oxidation of NH_2OH at the P-460 center but are transferred to external acceptors by the *c*-type hemes.

Materials and Methods

Enzyme Preparation. HAO was isolated from cultures of *Nitrosomonas europaea* and assayed for catalytic activity as previously described (Hooper et al., 1978). The preparations used were greater than 99% homogeneous as judged by analytical discontinuous polyacrylamide gel electrophoresis. The enzyme was stored at −20 °C after lyophilization. Potassium phosphate buffer, 0.05 M at pH 7.5, was used for all experiments unless otherwise stated. The enzyme concentration was estimated by the protein method of Lowry et al. (1951) or by the composite heme extinction coefficient, ϵ = 2350 (mM cm)$^{-1}$ at 408 nm (Hooper et al., 1978), assuming a molecular weight of 220000 (Terry & Hooper, 1981). Total heme concentration was determined by the extinction coefficient, ϵ = 99 (mM cm)$^{-1}$ at 408 nm, based on the pyridine ferrohemochrome quantitation of the *c*-type hemes (Hooper et al., 1978) and corrected for the P-460 content of HAO (~12%). The correction is required because P-460 is not quantitated by the ferrohemochrome procedure. Water was glass distilled, and all chemicals were reagent grade.

Instrumentation, Data Collection, and Analysis. The EPR spectra were recorded at X band on a Varian E-109 spectrometer equipped with an Oxford ESR-10 liquid helium cryostat. Temperature measurements and g-value calibrations were achieved as previouly described (Lipscomb, 1980). Samples were frozen by slow immersion in liquid nitrogen. Data were recorded for integration and spectral subtraction procedures by using a digital computer interfaced directly to the spectrometer.

Spectra were integrated by using the procedure suggested by Aasa & Vänngård (1975) in which the first integral of a peak in the EPR first-derivative spectrum is related to the integral of an analogous peak in a standard. The measured integrals depend on both spin concentration and the anisotropy of the EPR spectrum. A correction factor for the anisotropy can be calculated if all three g values are known. A sample of cytochrome P-450$_{cam}$–imidazole complex was used as an integration standard. The concentration of this sample was determined by optical extinction (Gunsalus & Wagner, 1978) as well as double integration techniques vs. a 1 mM copper perchlorate solution. The low-spin ferric heme spectra were analyzed by using the model proposed by Griffith (1957). This model states that in a strong crystal field the five 3d electrons are localized in the d_{yz}, d_{xz}, and d_{xy} orbitals. These are made nondegenerate by the orthorhombic symmetry of the crystal field and mixed by spin–orbit coupling with the ensuing eigenstates determining the g values. Any two g values can be used to determine the third. Small deviation from the crystal field model can be compensated for by invoking an orbital reduction factor, k. In most cases, only small corrections are needed, and the value of k is close to 1. The crystal field parameters can be related to the axial ligands of the heme group by using the convention introduced by Blumberg & Peisach (1971). In this formalism, two parameters determined from the g values called tetragonality, Δ/λ, and rhombicity, V/Δ (where V and Δ are the energies which describe the separation of the t_{2g} orbitals and λ is the spin–orbit coupling constant), are used to place heme centers in groups of known ligand structure. Fits were made by using a digital computer to calculate the g values for given values of Δ, V, and λ. Values for k were chosen as close to 1 as possible.

Reductive Titration Procedures. Reductions were carried out under an argon atmosphere from which O_2 has been removed by passage over BASF Inc. copper catalyst at 150 °C. The gas was cooled and rehydrated by bubbling through water prior to exposure to the enzyme solutions. Solutions were degassed by six cycles of evacuation and exposure to Ar gas. The solutions were protected against minor amounts of O_2 remaining in the Ar gas or introduced during transfer by including 0.01 mg/mL *Pseudomonas aeruginosa* protocatechuate 3,4-dioxygenase and 50 μM protocatechuate (Sigma).

Photoreduction was carried out in an EPR tube fitted with a serum stopper for degassing. EDTA (2 mM) and proflavin (4 μM) (Sigma) were included in the enzyme solution to act

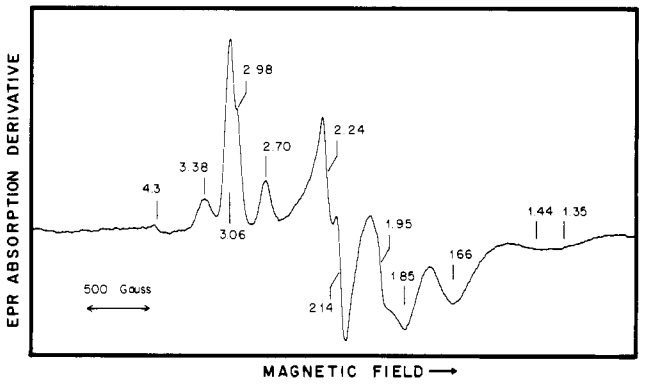

FIGURE 1: Resting hydroxylamine oxidoreductase. 146 μM enzyme in 50 mM potassium phosphate buffer, pH 7.5 at 10.5 K. Instrumental conditions were the following: microwave frequency = 9.220 GHz, modulation amplitude = 10 G, modulation frequency = 100 kHz, microwave power = 1 mW, scan rate = 400 G/min. The g values are indicated on the figure. Under these conditions, none of the signals were saturated.

as an electron source and photoreceptor, respectively (Tyson et al., 1972). The EPR tube was suspended in a water bath and exposed to light from a focused 500-W projector lamp for 10-s intervals.

Reduction by sodium dithionite was carried out in an EPR tube closed with a stopcock at the top of the tube. Above the stopcock was fitted an antechamber equipped with a serum stopper and a separate stopcock. The antechamber allowed the serum stopper to be replaced frequently, a positive Ar pressure to be maintained during dithionite additions, and a small amount of O_2-contaminated dithionite solution to be expelled from the gas-tight transfer syringe prior to introduction into the enzyme solution. After each addition of dithionite, the optical spectrum of the HAO solution in the EPR tube was scanned repeatedly until no further change was observed. Dithionite solutions were adjusted to pH 7.5 and calibrated by optical titration of horse heart cytochrome c (Sigma). Cytochrome P-450$_{cam}$ was the generous gift of Dr. I. C. Gunsalus, and protocatechuate 3,4-dioxygenase was prepared as described by Que et al. (1976).

Results

Resting Enzyme. The EPR spectrum of HAO at 10.5 K is shown in Figure 1. The spectrum was unchanged in the presence of O_2 or potassium ferricyanide, suggesting that reduced metal centers, if present, were not readily oxidized. A Mössbauer spectrum of HAO shown in Figure 3 of the following paper (Lipscomb et al., 1982) clearly shows that both high- and low-spin ferrous hemes are absent. The preparation of HAO used to obtain the spectrum in Figure 1 contained a small amount of contaminating Fe^{3+} as shown by the characteristic signal near $g = 4.3$ but little contaminating Cu^{2+} which would have produced a signal near $g = 2$. Other preparations contained more of these contaminants (see, for example, Figure 2) but had the same specific enzyme activity and stability.

The EPR resonances at low field ($g = 3.38$, $g = 3.06$, $g = 2.98$, and $g = 2.7$)[2] are in reasonable positions to be the g_z components of the many low-spin ferric ($S = {}^1/_2$) hemes present in HAO. The number and separation of these resonances suggest that there are many different pools of hemes present even though they are all fundamentally c type. Assignment of the g_y and g_x resonances which are associated with specific g_z resonances cannot be made directly because of the complexity and degree of overlap in the spectrum at higher field. However, zero-crossing resonances near $g = 2.2$ and negative-going resonances near $g = 1.4$ are in appropriate spectral regions. Many of the resonances can be specifically assigned on the basis of shifts in their intensity and position upon reduction, substrate complexation, or pH change as shown below.

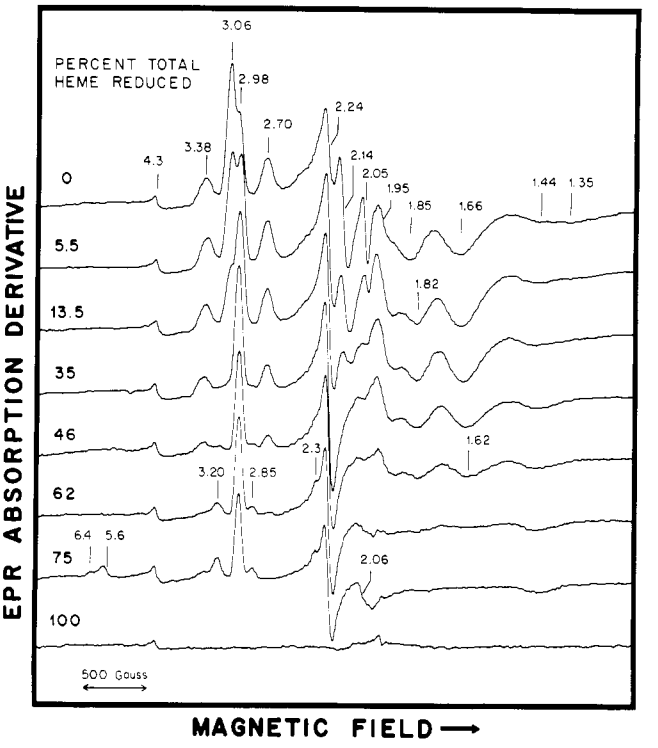

FIGURE 2: Titration of HAO with sodium dithionite. An anaerobic sample of HAO (508 μM heme) was mixed with small aliquots of 2 mM dithionite solution at pH 7.5. The percentage of total heme reduced shown on the figure is calculated from 2 times the final concentration of dithionite divided by 508 μM. Temperature = 10 K. Instrumental conditions were the same as described in Figure 1. A small signal from Cu^{2+} which remained in the presence of excess dithionite has been subtracted from each spectrum to simplify the $g = 2$ region. This signal was not observed in most other HAO preparations.

Reductive Titration. HAO was reduced in small increments under anaerobic conditions by using either sodium dithionite or light in the presence of EDTA and proflavin as the reducing agent. There was no significant difference in the spectra derived from these two techniques, so the more quantitative dithionite titration is shown in Figure 2. At early stages of the reduction, there was a pronounced decrease in the signals at $g = 3.06$, $g = 2.14$, and $g = 1.35$, whereas the signals at $g = 2.98$, $g = 2.24$, and $g = 1.44$ increased significantly (also see Figure 3A). This unusual behavior strongly suggests that $g = 3.06$, 2.14, 1.35 and $g = 2.98$, 2.24, 1.44 are the correct g-value assignments for two of the major heme species. Part of the contaminating Cu^{2+} in this preparation ($g = 2.05$) was also reduced at low reductant concentrations.

When dithionite was supplied in concentration equal to approximately 30% of the total heme in the sample, the $g = 2.98$, 2.24, 1.44 spectrum reached a maximum intensity. Above 30% reduction, this species was reduced but did not disappear from the EPR spectrum entirely until nearly 100% of the total heme in HAO had been reduced. The $g = 3.06$, 2.14, 1.35 species disappeared entirely from the spectrum between 0% and 35% reduction. Between 35% and 62% re-

[2] In this report, g values which are assigned as the g_z, g_y, and g_x components of a single g tensor will be separated by commas whereas unassigned, perhaps unrelated, resonances will each be preceded by "g".

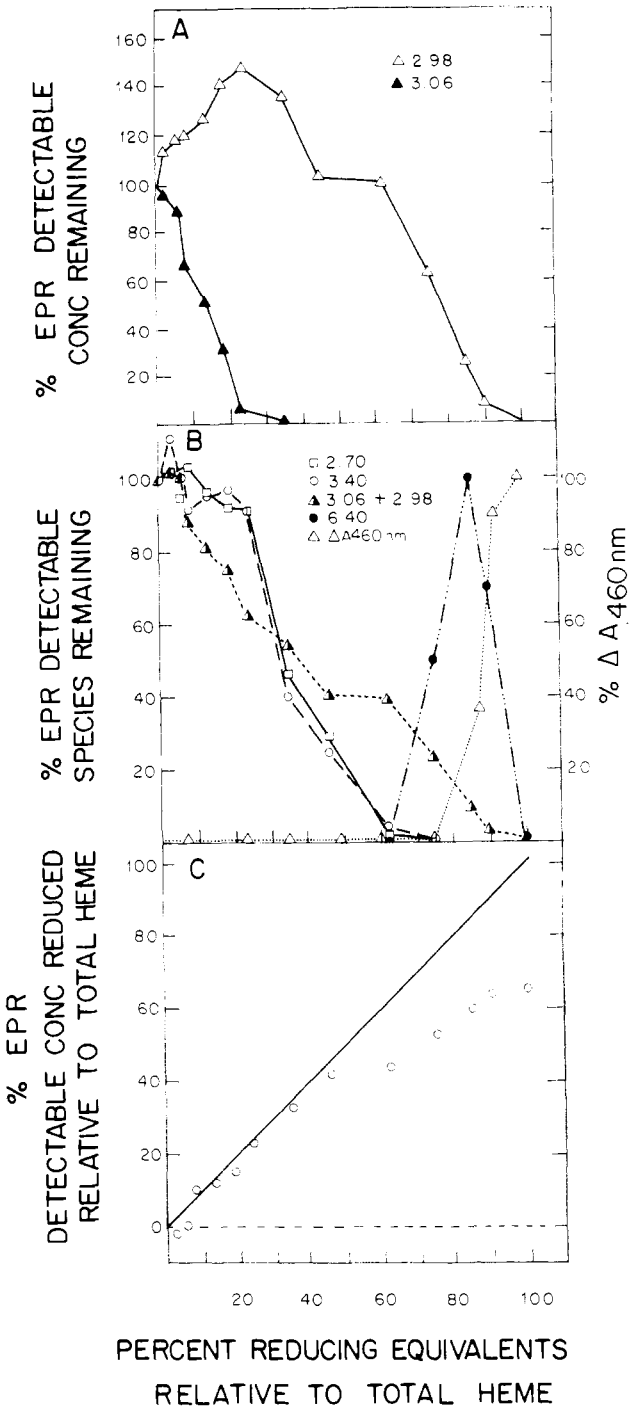

FIGURE 3: Reductive titration of individual EPR-detectable species. (A) The concentrations of g = 2.98, 2.24, 1.44 and g = 3.06, 2.14, 1.35 species determined by integration of the EPR spectra are expressed as a percentage of the concentration present in the resting enzyme and plotted vs. the percentage of 508 μM reducing equivalents added. The primary data are from Figure 2 and other spectra from the same series. (B) As in (A) except the two species in (A) are expressed as a sum and the other EPR-detectable species are also shown. The species with low-field g values at g = 2.7 and g = 6.4 species are plotted as a function of normalized integrated signal area rather than concentration. The percentage of change in the optical absorption spectrum at 463 nm as a function of dithionite added is also shown as a measure of P-460 reduction. (C) The decrease in the sum of the concentrations of the g = 3.38, 1.95, 0.7, g = 2.98, 2.24, 1.44, and g = 3.06, 2.14, 1.35 species expressed as a percentage of 508 μM total heme concentration plotted vs. the percentage of 508 μM reducing equivalents added. The 45° solid line is the expected result if only the EPR-quantitated hemes in the resting enzyme were reduced.

duction, the signals at g = 3.38, g = 2.7, g = 1.85, and g = 1.66 disappeared concurrently, suggesting that they arose either from the same species or from several species with similar redox potentials. Above 62% reduction, new signals at g = 6.45, g = 5.6, g = 3.20, g = 2.85, and g = 2.06 appeared.

Signals appearing in pairs slightly above and below g = 6 commonly arise from g_y and g_x of high-spin Fe^{3+} heme centers with a small rhombic distortion in the axial ligand field symmetry. The signals of this type in HAO at g = 6.45 and 5.6 maximized at about 85% reduction and then disappeared. The signals at g = 3.2, g = 2.85, g = 2.3, and g = 2.06 may belong to two or more new low-spin Fe^{3+} hemes. These signals appeared and disappeared at slightly lower stages of reduction than the high-spin heme signals. Throughout the titration, slight changes in the line shape and g value were observed for several resonances, particularly those at g = 1.85 and g = 1.66.

Integration of the HAO Spectra. Integration of the complex EPR spectra such as those shown in Figure 1 or 2 is difficult but, nevertheless, useful in the case of HAO because the unusual appearance and disappearance of signals during the course of the titration suggest that some of the heme is not seen in the EPR spectrum of the resting enzyme. The integration method suggested by Aasa & Vänngård (1975) avoids many of the problems requiring only accurate single integration of the low-field, g_z resonances and correct assignment of the other g values. In the following sections, it is shown that data from the reductive titration of HAO could be used to deconvolute and assign g values for the majority of the heme spins in HAO represented by the g values near g = 3.

Deconvolution of Resonances Near g = 3. It is apparent from a qualitative appraisal of Figure 2 as well as the integration results presented in Figure 3A that the g = 2.98 resonance did not reduce as a single species. After an initial increase in EPR-detectable spin concentration, the g = 2.98 species started to reduce at approximately the same dithionite concentration as the several magnetic species represented by g values at g = 3.38, g = 2.7, g = 1.85, and g = 1.66. However, the titration of the g = 2.98 species continued well beyond the concentration of reductant (~60%) required to fully reduce these other species (Figure 3A,B). Since all the EPR active centers are hemes, and thus one-electron acceptors, this type of non-Nernstian titration is impossible unless either there are two types of hemes with the same g values at g = 2.98, 2.24, and 1.44 or a conformational change occurs which modifies the redox potential during the titration [see, for example, Babcock et al. (1978)]. We propose that the correct explanation contains elements of both of these alternatives. Specifically, the species with g values at g = 3.06, 2.14, 1.35 is first converted to a species with g values nearly identical with those of the g = 2.98, 2.24, 1.44 species, presumably via a conformational change in the protein coincident with the onset of reduction. Then it is reduced independently of the original g = 2.98, 2.24, 1.44 species. Three lines of evidence support this conclusion. (A) As shown in Figure 3A, a pronounced plateau appears in the titration of the g = 2.98, 2.24, and 1.44 species between 45 and 62% reduction. In other experiments (not shown), this plateau was always observed although it extended in some cases to approximately 70% reduction. Since the spectrum of the 62% reduced sample contains essentially only the g = 2.98, 2.24, 1.44 signal, it can be subtracted from the spectrum of the resting enzyme to isolate the g = 3.06, 2.14, 1.35 spectrum and the spectra of the other species which had undergone reduction. Subtraction of the 62% reduced spectrum (unscaled) from the 0% reduced

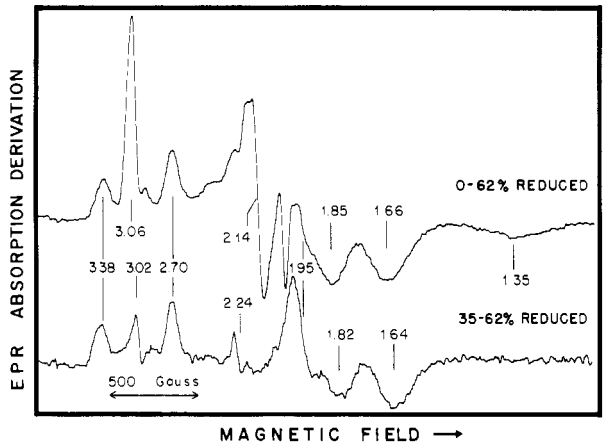

FIGURE 4: Spectral subtractions to isolate resonances of low concentration species. (Top) The 62% reduced spectrum from the data shown in Figure 2 is subtracted from the spectrum of the resting enzyme. (Bottom) 1.25 times the 62% reduced spectrum is subtracted from the 35% reduced spectrum.

spectrum eliminated the $g = 2.98, 2.24, 1.44$ species entirely (Figure 4, top spectrum). Thus, the concentration of this species which remains oxidized at the plateau in the titration is essentially the same as that present in the resting enzyme. (B) The resolved spectra with assigned g values could readily be integrated to give the spin contribution of each species to the native spectrum. Also, the spectra could be summed in various ratios to empirically determine the ratio of $g = 3.06$ to $g = 2.98$ type species at each stage of the reduction (Figure 3A). If the net reductions of the $g = 3.06$ and 2.98 type species are considered together, as shown in Figure 3B, then the titration appears to be a series of independent centers titrating in a Nernstian fashion. That is, the first phase of the $g = 2.98$ plus 3.06 type species titration begins before the titration of the other magnetic species and also finishes first. Thus, the titration results are explained simply if the $g = 3.06, 2.14, 1.35$ species and the new portion of the $g = 2.98, 2.24, 1.44$ species are considered as one entity. (C) During the first 40% of the reductive titration, the decrease in total observed spin concentration correlated very well with the reducing equivalents added (Figure 3C). Thus, no previously EPR-silent spins are unmasked in the $g = 3$ region by reduction of the $g = 3.06$, 2.14, 1.35 species.

On the basis of these results, the EPR-detectable species in resting HAO can be divided into groups of centers with different reductive potentials. The $g = 3.06, 2.14, 1.35$ species and its putative shifted form have the highest potentials followed by the two or more species with g values at $g = 3.38$, $g = 2.7$, $g = 1.85$, and $g = 1.66$. The $g = 2.98, 2.24$, and 1.44 species observed in resting HAO must have the lowest potential by more than 60 mV since it begins to reduce after the other EPR-detectable low-spin hemes are completely reduced.

Assignment of g Values for the Species with $g_z = 3.38$ and 2.70. Hemes with a g_z value near 3.38 usually display a g_y value close to 2. In the spectra shown in Figures 1 and 2, there is a shoulder at $g = 1.95$ on the $g = 1.85$ resonance which may, in fact, be the zero-crossing g_y resonance of the $g = 3.38$ magnetic species. This resonance is shown in Figure 2 to titrate concurrently with the $g = 3.38$ signal. The g values 3.38 and 1.95 can be fit (see Materials and Methods) with the Griffith (1957) model ($k = 1.01$) to predict $g_x = 0.7$. No signal was observed in this region, but it would be very broad, and signals of this type are often not seen in first-derivative EPR spectra.

Many types of hemes exhibit g values near 2.7 with typical g_y values of 2.2–2.5 and g_x values of 1.6–1.9. Both of these g-value regions are complex in spectra of HAO, so appropriate g_x and g_y assignments cannot be made directly. However, the sequential rather than simultaneous reduction of EPR-active species of HAO allows considerable simplification of the spectrum of resting enzyme by subtraction of the spectra of the species with assigned g values. In the top trace of Figure 4, the spectrum of the $g = 2.98, 2.24$, and 1.44 species has been removed. The hyperfine pattern from the Cu^{2+} resonance at $g = 2.05$ is clearly visible on the low-field side of the $g = 2.14$ peak, but there is no evidence for a zero-crossing g_y resonance above $g = 2.2$ which could be assigned with the $g_z = 2.7$ resonance. In the lower trace of Figure 4, the spectra of both of the major resonances in the $g = 2.2$ region have been removed. Small residual signals at $g = 3$ and 2.24 cannot be removed by this subtraction process. This is probably an indication that the putative shifted form of the $g = 3.06, 2.14$, 1.35 species does not assume the exact g values and line widths of the $g = 2.98, 2.24$, and 1.44 species present in resting HAO. The remainder of the $g = 2$–2.5 spectral region is devoid of signals, suggesting that the assignment of other g values to accompany the $g = 2.7$ resonance is not straightforward. The g_y signal at 1.95 assigned with the $g_z = 3.38$ signal was well isolated by this procedure. It is possible that the $g = 1.95$ resonance contains g_y for both the $g = 3.38$ and 2.7 resonances. However, this interpretation would require a value of k of about 0.65 for the species with $g = 2.7$, which is well outside the usual range.

The g value set at 3.38, 1.95, and 0.7 was used to scale the integrated area of the $g = 3.38$ peak at each stage of the reductive titration shown in Figure 2. The result is reported in Figure 3B. The $g = 2.7$ resonance was also integrated, but no correlation to spin concentration was made. For this signal, the loss in peak area as a function of reducing equivalents added is shown in Figure 3B. Both of these species appeared to reduce at the same point in the titration, indicating similar oxidation–reduction potentials.

Correlation of the High-Spin Species with P-460. The high-spin species which first appears in the reductive titration after approximately 70% of the total heme has been reduced (Figure 2) is quite broad and difficult to quantitate. It maximizes at about 85% total heme reduction. The EPR-detectable fraction of the species at this stage accounts for at most 4% of the total heme (0.3 high-spin heme per $\alpha\beta$ subunit; see below). Subsequent disappearance of the high-spin signal between 85 and 100% total reduction correlates with the appearance of the 463-nm optical absorption band of ferrous P-460 heme as shown in Figure 3B. On this basis, it seems likely that the high-spin heme signal derives from P-460, but it is not known why this signal is absent from resting HAO or why little EPR-detectable high-spin species is observed. It appears that the high-species has a lower oxidation–reduction potential than all or most of the c-type hemes.

Reversibility. On exposure to air, the 100% reduced HAO sample was oxidized within about 1 min. The optical and EPR spectra of the resting enzyme were restored. Likewise, greater than 95% of the original catalytic activity for conversion of NH_2OH was restored. Rereduction of the same sample with dithionite gave an identical set of titration spectra including the appearance and disappearance of the high-spin signals near $g = 6$. The signal near $g = 4.3$ was increased about 10% in the reoxidized spectrum.

Number of Hemes of Each Type Present in HAO. The magnetic properties of the hemes for which g values have been assigned and the fraction of total heme which they represent are summarized in Table I. The absolute number of hemes

Table I

signal			crystal field parameters				integration		
g_z	g_y	g_x	k	V/Δ	Δ/λ	ligands[b]	% total heme[c]	rel ratio[d]	no. per enzyme[e]
3.38	1.95	0.7[a]	1.01	0.37	2.30	Lys, His	11.0	1.0	3.0
3.06	2.14	1.35	0.98	0.44	3.40	His, His	33.5	3.04	9.12
2.98	2.24	1.44	1.0	0.54	3.15	His, His	21.5	1.95	5.95
						total	66.0		18.07

[a] Estimated from crystal field parameters shown. [b] See Brautigan et al. (1977). [c] Based on total concentration determined for the sample from optical extinction (see Materials and Methods). [d] Relative to the $g = 3.38, 1.95, 0.7$ species. [e] A heme is assumed to occur at least 3 times in the trimeric enzyme molecule.

of each type present in a molecule of HAO was difficult to determine directly from the EPR spectral integration because the total heme concentration is based on optical measurements rather than directly comparable EPR data. However, the EPR spectral integration did directly provide an independent measure of the concentration of heme present in each species as well as ratios of the concentrations of the species. Since the species occurred in nearly integer ratios (Table I) and the hemes being quantitated were covalently linked *c* type, it is likely that all of the enzyme molecules contained their full complement of each heme type. The least populous heme must occur at least 3 times in the native enzyme, assuming that the enzyme is fundamentally a trimer of heterogeneous dimers (Terry & Hooper, 1981). If the $g = 3.38, 1.95, 0.7$ type heme is assumed to occur 3 times, then, using the ratios shown in Table I, the EPR-quantitated fraction represents approximately 18 hemes with assigned resonances per resting native enzyme. Furthermore, this quantitation accounts for 66% of the total heme, so the total number of hemes, per enzyme molecule, is approximately 27. This number is in qualitative agreement with that determined by chemical procedures (Terry & Hooper, 1981) which suggested that 24 hemes were present. Due to the integer ratios of hemes, the quantitation is essentially unchanged if based on one of the other heme types for which the g values are known with greater certainty. The hemes not directly accounted for by the EPR quantitation may give rise to the unassigned resonances of resting HAO, or they may be EPR silent.

Axial Ligand Assignments. The assignment of ligands shown in Table I is based entirely on the extensive study of cytochrome *c* and derivatives by Brautigan et al. (1977). It is likely that the $g = 3.06, 2.14, 1.35$ and $g = 2.98, 2.24, 1.44$ species have the same axial ligands, suggesting that their different spectral and redox properties arise from factors such as the geometry and polarity of their binding sites in the protein. This analysis suggests that the proposed shift in the g values of the $g = 3.06, 2.14, 1.35$ species could occur without exchange of axial ligands.

Hydroxylamine Complex. Anaerobic addition of NH_2OH to HAO resulted in a rapid change in the EPR spectrum (Figure 5) which was most directly interpreted as partial reduction. During the course of titrations with NH_2OH (data not shown), the $g = 3.06, 2.14, 1.35$ signal disappeared entirely while the $g = 2.98, 2.24, 1.44$ signal first increased and then decreased in intensity very much as seen in Figures 2 and 3A for the dithionite titration. The maximum decrease in EPR signal intensity occurred with approximately a 2-fold excess of NH_2OH over total heme concentration. The remaining signal near $g = 3$ was shifted to $g = 2.99, 2.23, 1.40$ but had only slightly more integrated area than the $g = 2.98, 2.24, 1.44$ signal present in the substrate-free native enzyme. Comparison of Figure 5 with the titration shown in Figure 2 suggests that the EPR-detectable heme is approximately 45% reduced in

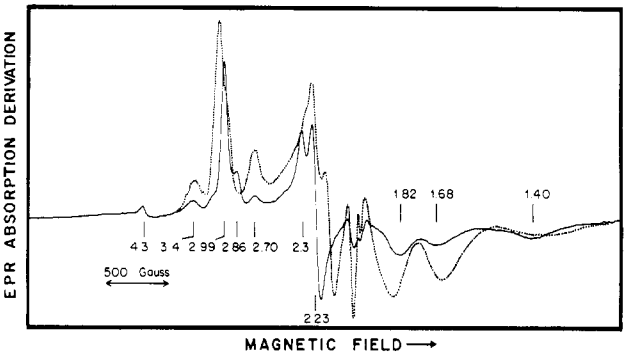

FIGURE 5: Complex of HAO with hydroxylamine (—). 1.0 mM NH_2OH was added to an anaerobic preparation of HAO containing 558 μM heme in 50 mM potassium phosphate buffer, pH 7.5. Instrumental conditions were as described in Figure 1. Temperature = 10 K. The spectrum of resting HAO (···) of the same concentration is included for comparison.

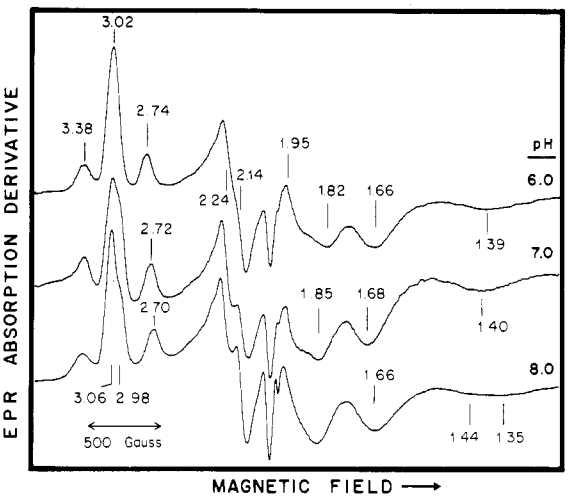

FIGURE 6: Effect of pH on HAO EPR spectra. A sample of HAO containing 558 μM heme was dialyzed vs. 5 mM potassium phosphate buffer, pH 7, plus 200 mM KCl. The pH was then lowered to pH 6 by adding aliquots of 1 M KH_2PO_4. After the EPR spectrum was recorded, the pH was raised again to pH 7 with 1 M K_2HPO_4 and finally to pH 8 in the same way. The final concentration of potassium phosphate added was approximately 75 mM. Temperature = 10 K. Instrumental conditions were as shown in Figure 1.

the substrate complex. The new low-spin species which appears at $g = 2.85$ at this stage of reduction is much more intense and sharper in the NH_2OH complex. Similarly, the new resonance at $g = 2.3$ is also sharp and well resolved, suggesting that these two signals are g_z and g_y, respectively, of a new low-spin heme spectrum.

Effects of pH Variation. The pH of the buffer in which HAO was frozen affected the observed EPR g values and line shape as shown in Figure 6. At pH 6, the $g = 3.06$ and 2.98 signals coalesced to give the appearance of one resonance at

$g = 3.02$, but the resonances near $g = 2.2$ remained resolved. When the pH was increased from 6.7 to 7.3 (only the spectrum at pH 7 is shown in Figure 6), a sharp transition occurred in which the $g = 3.06$ and 2.98 peaks became resolved while the other resonances sharpened. There also appeared to be some shift in spin population in favor of the $g = 3.06$, 2.14, 1.35 species as the pH was increased in this range. Above pH 7.5 and up to the catalytic pH optimum at pH 9 (Hooper & Nason, 1965), there appeared to be little change in the spectra. Throughout the pH titration, the resonances at $g = 2.7$, $g = 1.85$, and $g = 1.66$ shifted slightly, but the line widths remained approximately constant. Above pH 9, the resonance at $g = 3.38$ broadened significantly. The sensitivity of the EPR spectrum to relatively small changes in pH shown in Figure 6 as well as to very low levels of reduction shown in Figure 2 suggests that the protein structure in the vicinity of the hemes is quite easily changed. The species with g_y values close to $g = 3$ are particularly sensitive to these changes, supporting the contention presented above that at some levels of reduction they assume essentially the same g values.

Discussion

c-Type Hemes. EPR spectroscopy is the method of choice for resolution of the low-spin ferric hemes of HAO [compare, for example, optical (Hooper et al., 1978) or Mössbauer (Lipscomb et al., 1982) spectroscopic studies]. It is clear from the EPR spectrum of the resting enzyme shown here and in previous work (Vickery & Hooper, 1981) that there are several readily distinguishable groups of *c*-type heme, but the spectrum is too complex to make specific g-value assignments. Without such assignments, quantitation is not feasible. In order to make these assignments, we have perturbed the enzyme in two mechanistically relevant ways: a reductive titration and formation of the substrate complex. In combination, these procedures allowed resolution and integration of three of the major *c*-type heme groups which account for approximately two-thirds of the heme in the enzyme. These groups appear to have markedly different redox potentials and reduce sequentially when reducing equivalents are introduced by addition of dithionite or substrate or by photoreduction. The fact that the three titration methods gave very similar spectra at each stage of reduction and that all of the heme is oxidized rapidly by O_2 or phenazine methosulfate suggests that electrons can be transferred readily between heme groups. Thus, the elements of an electron-transport chain within HAO appear to be present.

P-460. In this study, the P-460 prosthetic group has been correlated with the high-spin signal at $g = 6.45$ and 5.6 which appears late in the reductive titration. This is based on the fact that the EPR active species is reduced concomitantly with the appearance of the characteristic reduced P-460 absorbance spectrum. Two other experiments support this conclusion. First, the high-spin EPR spectrum does not appear in samples of HAO which have been treated with H_2O_2 to specifically destroy the P-460 (Hooper & Terry, 1977; Hooper & Vickery, 1981). Second, a heme-containing peptide has been isolated as a byproduct of the purification of HAO which shows the unique reduced absorbance maximum of P-460 near 463 nm, binds NH_2OH, and has EPR g values near 6.4 and 5.6 (Erickson & Hooper, 1972; Hooper & Vickery, 1981). This assignment, however, raises other questions. First, why is there no high-spin EPR signal in resting HAO? Second, why does the high-spin signal quantitate to much less than one spin per $\alpha\beta$ protein dimer, when Mössbauer spectroscopy [see Lipscomb et al. (1982)] strongly suggests that there is one P-460 per $\alpha\beta$ dimer? The appearance of EPR signals representing non-stoichiometric amounts of heme in partially reduced proteins is usually associated with spin coupling between the heme and another metal or free radical to produce an EPR-silent species with integer spin. The coupled spins are EPR silent until one member is reduced, thereby generating an EPR active half-integer spin complex [see, for example, Van Gelder & Beinert (1969)]. The appearance of new low-spin signals in addition to the high-spin signals at advanced stages of reduction of HAO suggests that the coupling moiety may be one of the low-spin *c*-type hemes. If the coupled low-spin heme and P-460 had similar electrode potentials, then either species might be the first to be reduced. Thus, the EPR spectra of both would appear, but quantitation of the spectra would show less than stoichiometric concentrations as observed. Two other possible explanations for the absence of a high-spin signal in resting HAO should be considered: the P-460 might be low spin and/or it might undergo unusually fast electronic spin relaxation and thus become EPR undetectable. Mössbauer spectroscopy provides some evidence in support of these explanations [see Lipscomb et al. (1982)]. Most notably, the Mössbauer spectrum of resting HAO at 4.2 K contains an unusual quadruple doublet which can be most readily associated with two low-spin Fe^{3+} hemes per $\alpha\beta$ dimer exhibiting fast electronic spin relaxation. The spectra contain no evidence for high-spin iron, but the existence of one such iron cannot be entirely discounted.

Unassigned EPR Active Hemes. The principal unassigned EPR resonances occur at $g = 2.7$, $g = 1.85$, and $g = 1.66$. Simplification of the resting HAO EPR spectrum by subtraction of the spectra of hemes with assigned g values shows that none of these unassigned resonances belong to typical low-spin heme spectra. Nevertheless, the novel signals are present in every preparation of HAO, titrate as ordinary oxidation–reduction centers, and shift in response to substrate binding, suggesting that they are an integral part of the spectrum of the enzyme. The only similar resonances which have been reported are seen in cytochrome *c* oxidase upon reoxidation by O_2 (Shaw et al., 1978). These resonances at $g = 1.78$ and 1.69 are transient with a $t_{1/2}$ of a few minutes and are associated with a derivative resonance at $g = 5$. The origin of these resonances is not clear, although it is possible that they arise from spin interaction between cytochrome a_3 and one of the other paramagnetic species. The possibility for similar interaction in HAO is clearly present.

Model for the Catalytic Roles of P-460 and the c-Type Hemes. The catalytic role of P-460 has been distinguished from that of the *c*-type hemes of HAO largely on the basis of the observation that selective destruction of P-460 by H_2O_2 interferes with both turnover of the enzyme and the substrate-coupled reduction of the *c*-type hemes. Reduction by compounds other than substrates and spectroscopic properties of the *c*-type hemes are unchanged. The simplest mechanistic interpretation of these observations is that the catalyzed oxidation of NH_2OH occurs at the P-460 center with either P-460 or perhaps one of the *c*-type hemes being the intial receptor of the electrons. Utilization of this energy by the cell to synthesize ATP and reduced pyridine nucleotide requires oxidation of the species reduced during the initial catalytic events. This could occur through direct interaction of an intracellular acceptor with the P-460 site. On this basis, however, the requirement for the large number of different types of hemes present in HAO is somewhat puzzling. One possible explanation is that the sites of NH_2OH oxidation and intracellular acceptor reduction are physically separated, so that electron transfer via the *c*-type hemes serves to connect

the two sites. One difficulty with this scheme results from the large increase in midpoint electrode potential observed between the P-460 moiety and the highest potential *c*-type hemes. If the electrons are transferred through all of the heme sites, then a substantial loss of free energy would occur prior to utilization of the energy by the cell. This loss could be circumvented in vivo in several ways including utilization of only a subset of the hemes in the putative electron-transport pathway. Another intriguing possibility is that HAO may be oriented across the cell membrane so that NH_2OH is oxidized outside of the cell but electrons are transported by the *c*-type hemes to a proton utilizing terminal oxidase inside the cell. The energy of NH_2OH oxidation would thus be stored directly in the form of a proton gradient which could be coupled to ATP formation. Such systems are generally regulated by factors such as pH and extent of reduction which, as shown here, also affect the heme environments of HAO.

While the studies reported here are consistent with this model, all the measurments were made under static, equilibrium conditions. The actual point of entry of electrons into HAO and the route of distribution must be approached through dynamic measurements. The spectra reported here form the basis for studies of this type which are in progress.

Acknowledgments

We acknowledge the assistance of C. Lyman, K. Terry, and V. M. Tran in growth of the bacteria, preparation of the enzyme, and titration of the enzyme by visible absorption spectra, respectively. We are grateful to Dr. E. Münck and Dr. L. Vickery for many stimulating discussions and criticism of the manuscript.

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