Resolution of Multiple Heme Centers of Hydroxylamine Oxidoreductase from *Nitrosomonas*. 2. Mössbauer Spectroscopy†

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ABSTRACT: Hydroxylamine oxidoreductase (HAO) isolated from *Nitrosomas europaea* is a complex protein of M_r 220 000 with an $(\alpha\beta)_3$ subunit structure. Each $\alpha\beta$ subunit contains seven *c*-type hemes and approximately one unusual prosthetic group termed P-460. We have studied this enzyme in the oxidized and reduced states by using Mössbauer spectroscopy. In the fully reduced enzyme, approximately seven hemes per $\alpha\beta$ subunit contributed to one spectrum characteristic of low-spin ferrous heme. The remainder of the iron (10–15% of the total) yielded an ill-defined absorption pattern. Carbon monoxide binds to the P-460 as shown by optical spectra. The Mössbauer spectra of reduced hydroxylamine oxidoreductase which had been exposed to CO showed a new spectral component, corresponding to one iron site, with parameters characteristic of a low-spin ferrous heme–carbonyl complex. It appears that this component is derived from the ill-defined spectrum observed in the reduced enzyme. This is the first direct evidence that the P-460 moiety amounts to at least one Fe per $\alpha\beta$ subunit. Together the Mössbauer results and the optical spectra suggest that the P-460 moiety is a heme. The Mössbauer spectra of the oxidized (as isolated) enzyme suggest the presence of one or two low-spin ferric hemes which might be EPR undetectable because of either fast electronic spin relaxation or participation in a spin-coupled pair. The spectra gave no evidence for the presence of a ferrous site in oxidized HAO.

The multiheme enyme hydroxylamine oxidoreductase (HAO) from *Nitrosomonas europaea* [M_r 220 000, subunit structure of $(\alpha\beta)_3$, each $\alpha\beta$ subunit populated with seven *c*-type hemes and approximately one iron-containing P-460 prosthetic group] catalyzes the four-electron oxidation of hydroxylamine to nitrite (Erickson & Hooper, 1972; Hooper et al., 1978; Terry & Hooper, 1981). Earlier studies with electron paramagnetic resonance spectroscopy (EPR) showed that the *c*-type hemes comprise several spectrally distinct classes (Vickery & Hooper, 1981). In the preceding paper of this series (Lipscomb & Hooper, 1982), we have continued this effort to extensively characterize and quantify the EPR active iron centers of the oxidized and partially reduced enzyme. Since EPR spectroscopy is sensitive only to species with half-integer electronic spin, no information could be obtained for the catalytically significant reduced enzyme. Furthermore, quantitation of the EPR spectra of oxidized enzyme suggested that some of the heme iron is EPR silent. The Mössbauer technique complements EPR spectrscopy because it detects iron regardless of its spin and oxidation state. In many cases, the iron in proteins must be enriched with the isotope ^{57}Fe to obtain Mössbauer spectra with an adequate signal to noise ratio. In the case of HAO, this is not possible at present because the large number of *c*-type hemes obviates direct isotopic replacement, and because the low yield of cells (0.1 g/L of culture media) renders enrichment of the culture media impractical. Since so little is known about the iron environments in HAO, we have undertaken a Mössbauer study utilizing the ^{57}Fe present in natural abundance. Although the low isotopic abundance (2.2%) severely limits such a study, one can obtain some useful information not obtainable by any other method.

The catalytically essential P-460 (Hooper & Terry, 1977) unique to this enzyme has been particularly elusive in chemical and spectroscopic investigations conducted in the past. The intense optical absorption at 463 nm in the spectrum of the reduced moiety and the sensitivity of the spectrum to typical heme ligands suggest that P-460 is a heme (Erickson & Hooper, 1972). So far, no treatment of the enzyme has revealed the optical spectrum of the oxidized P-460, and all attempts to chemically remove it from the protein apparently result in its decomposition (Hooper et al., 1978). A rough quantitation has been achieved from a difference between the total iron and pyridine ferrohemochrome detectable heme iron in the enzyme (Hooper et al., 1978). By using a combination of optical and Mössbauer spectroscopy, we are able to show that CO binds specifically to reduced P-460 to afford a species with a Mössbauer spectrum typical of reduced heme–CO complexes. Quantitation of this spectrum provides the first direct demonstration that P-460 is present at a concentration of approximately one unit per $\alpha\beta$ dimer.

Materials and Methods

HAO was prepared from cell extracts of *Nitrosomonas europaea* as previously described (Hooper et al., 1978). The enzyme from several preparations was pooled and used for the EPR studies as described in the preceding paper (Lipscomb & Hooper, 1982) as well as the Mössbauer study described here. The enzyme was stored at −20 °C as a lyophilized powder and resuspended in a minimum volume of 50 mM potassium phosphate buffer, pH 7.5, for the spectroscopic studies. The concentration of HAO in the Mössbauer sample estimated from the optical absorption spectrum was approximately 0.3 mM (7.2 mM heme). Approximately 6 months was required to prepare the amount of purified enzyme required for a single Mössbauer sample. Consequently, all of the results refer to measurements made on the same sample. Reduction of the sample was performed under an argon atmosphere by adding an aliquot of 1 M sodium dithionite solution in 1 M potassium phosphate buffer adjusted to pH 7.5. The final concentration of dithionite was 100 mM. The sample was frozen in liquid nitrogen after a 20-min incubation period. A small aliquot of the reduced Mössbauer sample was

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transferred anaerobically to degassed buffer for EPR and optical spectroscopy. The CO complex for optical spectroscopy was prepared by bubbling CO through the reduced sample for 30 s and then allowing the sample to stand for 15 min. The CO complex of the Mössbauer sample was prepared by thawing the reduced sample in an atmosphere of CO, gently stirring, and then allowing the sample to stand for 20 min before refreezing. The entire procedure was carried out in the Mössbauer sample cell to prevent loss of sample. Several attempts were made to remove CO from the reduced Mössbauer sample without loss of enzyme from the sample. Incubation of the thawed sample under an argon atmosphere to remove dissolved CO from the sample solution failed to remove any CO from the enzyme complex as shown by both Mössbauer and optical spectroscopy. Partial oxidation of the sample by short-term (5-h) dialysis against 10 volumes of oxygenated buffer also failed. The CO was finally dissociated from the enzyme by exhaustive dialysis 2 times against 400 volumes of oxygenated buffer for 20 h. The buffer contained 0.1 mg of catalase/L to protect against peroxide inactivation. The enzyme solution was reconcentrated by packing the dialysis bag in dry Sephadex beads. The overall loss of enzyme by these handling procedures was about 30%, but the enzyme retained high specific activity, and the optical and EPR spectra of native, oxidized enzyme were restored. Following reduction, the characteristic optical spectrum of P-460 was also observed.

Extinction coefficients for the reduced P-460 of HAO were calculated by utilizing the following values and assumptions: (a) the heme P-460:heme *c* ratio is equal to the CO-binding Fe:other Fe ratio as determined by Mössbauer spectroscopy (see Results); (b) the extinction coefficient for the reduced *c*-type hemes has an average value of 160 mM^{-1} cm^{-1} per heme at 418 nm as previously determined (Hooper et al., 1978); (c) the absorbancy values and hence the extinction coefficients apply to HAO taken from the sample of which the Mössbauer spectra were recorded. The contribution of P-460 to the composite absorption spectrum of HAO was estimated by specifically destroying the P-460 with H_2O_2 (Hooper & Terry, 1977). After H_2O_2 treatment, the ratio of the absorbance at 463 nm to that at 553 nm for the reduced *c*-type hemes (which are unaffected by H_2O_2) was found to be 0.228.

The Mössbauer spectrometer was of the constant acceleration type. All isomer shifts, δ_{Fe}, are quoted relative to Fe metal at room temperature. EPR measurements were made as described in the preceding paper (Lipscomb & Hooper, 1982). Optical measurements were made with an Aminco DW-2 spectrophotometer.

Results

A Mössbauer spectrum of dithionite-reduced HAO taken at 4.2 K is shown in Figure 1A. The spectrum consists mainly of a symmetric quadrupole doublet, with very narrow absorption lines. By least-squares fitting two Lorentzian lines (0.27 mm/s full width) to the doublet, we obtained values for the quadrupole splitting, $\Delta E_Q = 1.06 \pm 0.02$ mm/s and $\delta_{Fe} = 0.45 \pm 0.02$ mm/s. These values are, within the uncertainties, the same as those observed for reduced (low-spin ferrous) cytochrome b_5 from calf liver (P. Strittmatter and E. Münck, unpublished results). This latter protein is known from X-ray diffraction studies to have two histidyl residues coordinated to the heme iron (Mathews et al., 1971).

The doublet shown in Figure 1A accounts for 85–90% of the total Fe in the sample. Closer inspection (see Figure 3C) reveals additional absorption, most conspicuously appearing at Doppler velocities of −1 and +3.2 mm/s. Presently we do not understand the nature of this species; we will refer to it as component X. We have made an attempt to characterize component X further by recording spectra under different conditions. Spectra taken at 4.2 K in zero field and in an applied field of 600 G are, within the counting statistics, indistinguishable (the spectra of both runs were added, and the sum is displayed in Figure 3C). The spectrum of component X is also unchanged within the limits of the signal to noise ratio when recorded at 125 K. Finally, we have studied this sample with EPR spectroscopy; the reduced sample gave no evidence for the presence of any EPR active species.

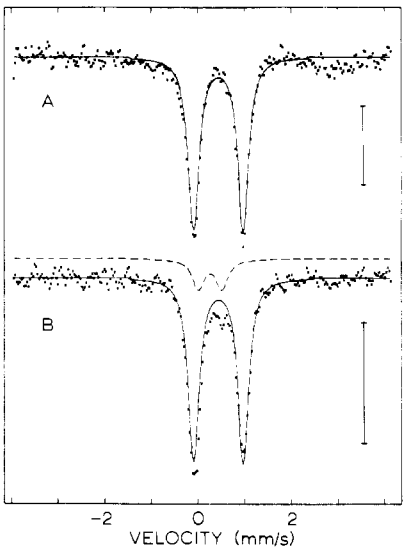

FIGURE 1: Mössbauer spectra of hydroxylamine oxidoreductase recorded at 4.2 K in zero applied magnetic field. The enzyme contained ^{57}Fe in natural abundance, and the vertical bars indicate 0.5% absorption. (A) Spectrum of reduced HAO. The solid line is a quadrupole doublet with $\Delta E_Q = 1.60$ mm/s, $\delta_{Fe} = 0.45$ mm/s, full width $\Gamma = 0.27$ mm/s, and area 85% that of the data. Besides this doublet, due to the low-spin ferrous *c*-type hemes, the spectra give evidence for another species, component X. This species is more conspicuous (see Figure 3C) when the spectrum is recorded with a larger velocity sweep. (B) Spectrum of reduced HAO after the sample had been exposed to CO. So that the effects of CO binding would be emphasized, the theoretical curve from (A) is redrawn in (B), again with an area 85% that of the data. The quadrupole doublet associated with the P-460–CO complex (12% of total Fe) is indicated by the dashed curve. The sum of the absorptions represented by the sum of the dashed and solid curves gives an excellent representation to the data.

As pointed out above, reduced HAO binds carbon monoxide. The optical difference spectrum of the reduced HAO–CO complex *minus* reduced HAO is shown in Figure 2. The major spectral feature in the difference spectrum is a large decrease at 463 nm where the P-460 moiety absorbs and a concomitant increase at 444 nm. The changes seen around 420 nm are too small to be attributable to any significant formation of a CO complex with one of the *c*-type hemes.

A Mössbauer spectrum of the CO-complexed, reduced HAO is shown in Figure 1B. A comparison of the two spectra in Figure 1 reveals that two features have changed as the result of exposing the sample to CO: First, a new doublet appeared with intensity corresponding to about 12% of total Fe or one Fe per $\alpha\beta$ subunit. Second, component X has vanished. The solid line drawn through the data points in Figure 1B is the same curve as that shown in Figure 1A. It can be seen that exposure to carbon monoxide has increased the absorption at 0 and +0.5 mm/s. By addition of a quadrupole doublet to the simulation (indicated in Figure 1B) with $\Delta E_Q = 0.5$ mm/s and $\delta_{Fe} = 0.26$ mm/s, a good fit to the data is obtained. The Mössbauer parameters of the new doublet clearly indicate the presence of a ferrous heme–CO complex.

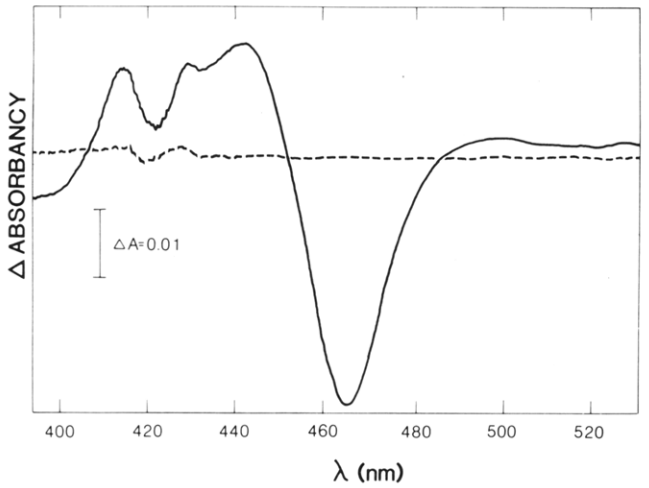

FIGURE 2: Optical absorption spectrum of reduced HAO plus CO *minus* reduced HAO. The CO complex of reduced HAO taken from the Mössbauer sample and diluted to 6.25 μM heme *c* was formed as described under Materials and Methods. The dashed line represents the reduced HAO *minus* reduced HAO spectrum for this experiment.

Our prior experience with Mössbauer spectra of proteins suggested to us that the fuzzy absorption features of component X result from a distribution of species, often associated with aggregates of impurity iron. To our surprise, component X vanished when the sample was exposed to CO. This suggests that the spectral component associated with the P-460–CO complex (the doublet with $\Delta E_Q = 0.5$ mm/s) originates from component X; i.e., component X might represent reduced P-460. After the experiments on CO-complexed HAO were completed, the carbon monoxide was removed. Unfortunately, exhaustive procedures were required to remove the ligand (see Materials and Methods). These procedures resulted in appreciable loss of material which made a further detailed Mössbauer study impractical. Although we could confirm that the quadrupole doublet attributed to a P-460–CO complex had vanished, the signals were too weak for observation of component X.

Mössbauer spectra taken at 4.2 K on oxidized HAO are shown in Figure 3A,B. In this state, the enzyme shows a rich EPR spectrum with evidence for several types of low-spin ferric heme. Low-spin ferric hemes which are EPR active, i.e., which have sufficiently slow electronic spin relaxation rates, yield broad spectra exhibiting paramagnetic hyperfine structure [for a discussion, see, for example, Münck (1978)]. The absorption pattern associated with these species is spread out over a velocity range of about 10 mm/s, resulting in a small signal amplitude. It was thus clear from the beginning that a Mössbauer study of the Fe centers in unenriched HAO had to focus on detecting iron species which give rise to quadrupole doublets. The spectrum in Figure 3B contains a broad magnetic component with an overall splitting as found in cytochrome b_5 (P. Strittmatter and E. Münck, unpublished results); this observation is in agreement with the EPR results (Lipscomb & Hooper, 1982) which have suggested the presence of five to six hemes per $\alpha\beta$ dimer with nitrogenous ligands.

The spectrum in Figure 3A was recorded in zero applied field. Under these conditions, the effects of transferred hyperfine interactions of ligand ^{14}N-labeled nuclei tend to blur sharper features which might be present in the spectra recorded in applied fields. This allows one to search with better sensitivity for the presence of species yielding quadrupole doublets. The spectrum in Figure 3A contains two sharp features which appear to belong to a quadrupole doublet. This interpretation is strengthened by the observation that the Mössbauer pa-

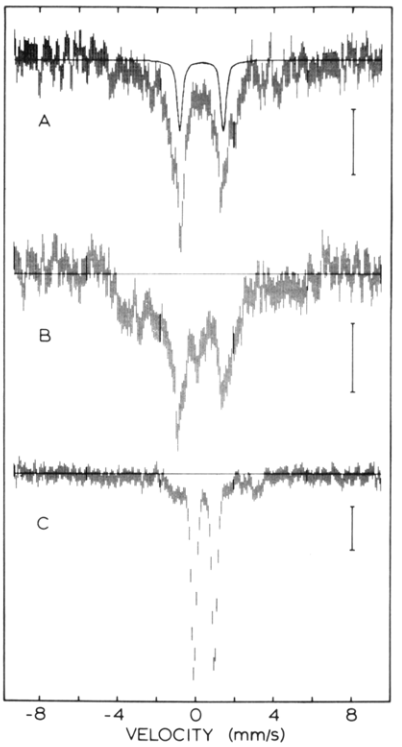

FIGURE 3: Mössbauer spectra of hydroxylamine oxidoreductase recorded at 4.2 K. The vertical bars indicate 0.2% absorption. (A) Spectrum of oxidized HAO taken in zero applied field. The position of a quadrupole doublet is indicated by the solid line. (B) Spectrum of oxidized HAO taken in a magnetic field of 600 G applied parallel to the observed Mössbauer radiation. (C) Spectrum of reduced HAO taken at 4.2 K in zero field (see Figure 1A). The ill-defined absorption features extending from a Doppler velocity of −1.5 to +3 mm/s belong to component X, a spectrum of as yet unidentified nature.

rameters, $\Delta E_Q = 2.1$ mm/s and $\delta_{Fe} = 0.24$ mm/s, correspond almost exactly to those observed for the cytochromes *c* (Lang et al., 1968), c_2 (Huynh et al., 1978), and b_5 (P. Strittmatter and E. Münck, unpublished results). At least one, but probably two, iron site per $\alpha\beta$ dimer contributes to this doublet. The doublet reflects either low-spin ferric hemes with fast electronic spin relaxation or a pair of spin-coupled hemes. It is also possible that the doublet is connected with the species which yields unusual, and as yet unexplained, EPR resonances at $g = 2.7$, $g = 1.85$, and $g = 1.66$ [see Figure 1 of the preceding paper (Lipscomb & Hooper, 1982)].

The deep trough in the middle of the Mössbauer spectrum of the resting HAO (Figure 3A) strongly suggests the absence of ferrous material, either high spin or low spin. Doublets with $|\Delta E_Q| < 1.3$ mm/s and $\delta_{Fe} = 0.45$ mm/s due to high-spin ferric hemes with either fast electronic spin relaxation or spin coupled to another ferric species are apparently not contained in the spectra; their presence would be discernible in the velocity region of the trough. On the other hand, the presence of at least one species with a quadrupole splitting close to the upper range of high-spin ferric hemes ($\Delta E_Q = 1.6$ mm/s) cannot be excluded since its absorption lines would not be sufficiently resolved.

Discussion

We have shown above that carbon monoxide binding to reduced HAO yields a species with $\Delta E_Q = 0.5$ mm/s and $\delta_{Fe} = 0.26$ mm/s. These parameters, in particular the isomeric shift, compare very favorably with those reported for other heme–CO complexes. For example, values of $\Delta E_Q = 0.3$ mm/s and $\delta_{Fe} = 0.29$ mm/s have been reported for both cytochrome P-450 (Sharrock et al., 1973) and myoglobin

(Lang & Marshall, 1966). A very similar value for δ_{Fe} was found recently (J. A. Christner, E. Münck, P. Janick, and L. M. Siegel, unpublished results) for a complex of CO with the isobacteriochlorin (siroheme) moiety in *Escherichia coli* sulfite reductase; ΔE_Q = 0.80 mm/s and δ_{Fe} = 0.23 mm/s. Thus, the Mössbauer data support earlier suggestions that the P-460–carbon monoxide complex quantitates to about 12% of total Fe or one Fe per $\alpha\beta$ dimer (Hooper et al., 1978; Hooper & Terry, 1977). With this information, we obtain (see Materials and Methods) ϵ = 68 mM^{-1} cm^{-1} at 463 nm for the absorption spectrum of reduced P-460 and ϵ = 46 mM^{-1} cm^{-1} at 465 nm for the spectrum of reduced P-460 *minus* reduced P-460 + CO. Among the iron-containing prosthetic groups, only hemes have extinction coefficients of such magnitude. Thus, both the Mössbauer parameters and the value for the extinction coefficients near 460 nm are consistent with the identification of P-460 as a heme. This heme does not necessarily contain protoporphyrin IX.

Mössbauer spectra of low-spin ferrous hemes are not very sensitive to the nature of the axial endogenous ligands. For instance, the isomer shifts are, within the uncertainties, the same (δ_{Fe} = 0.45 mm/s) for His–His, His–Met, or His–Lys ligations. The quadrupole splittings of reduced cytochrome *c* isolated from different species vary from 1.14 to 1.18 mm/s. Cytochrome b_5 isolated from calf liver displays a splitting of ΔE_Q = 1.04 ± 0.03 mm/s. Quite recently (B. H. Huynh, unpublished results), the heme *c* of the heme *cd* nitrite reductase from *Thiobacillus dentrificans* was found to have ΔE_Q = 1.17 ± 0.04 mm/s; EPR data from this enzyme strongly suggest one histidyl residue and one lysinyl residue as axial ligands for the heme *c* moiety. In the preceding paper, we have used data from EPR spectroscopy to argue that at least five hemes per dimer have two histidyl ligands. This is supported by the Mössbauer data of reduced HAO; the observed quadrupole splitting of ΔE_Q = 1.06 mm/s is suggestive of a ligand coordination (His–His) as observed for cytochrome b_5. The EPR resonance at g = 3.38 is indicative of a histidine–lysine coordination; one heme per $\alpha\beta$ dimer with ΔE_Q = 1.17 mm/s could easily be accommodated in the sharp doublet of Figure 1A. Since all the *c*-type hemes have nearly the same values for ΔE_Q and δ_{Fe}, one might deplore the lack of spectral resolution. On the other hand, since the *c*-type hemes behave nearly as one species in reduced HAO, one can expect to get valuable information about component X when ^{57}Fe-enriched material becomes available.

Acknowledgments

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