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P460 OF HYDROXYLAMINE OXIDOREDUCTASE OF *NITROSOMONAS EUROPAEA*: SORET RESONANCE RAMAN EVIDENCE FOR A NOVEL HEME-LIKE STRUCTURE

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P460, an iron-containing chromophore at the active site of Hydroxylamine Oxidoreductase of the ammonia-oxidizing bacterium *Nitrosomonas europaea*, is a macrocycle of unknown structure with a Soret-like 460-nm absorption band in the ferrous form. The pigment can also be isolated in a peptide, "P460-Fragment". Resonance Raman spectroscopy (λ_{ex} = 457.9 nm) suggests that P460 is a new type of heme with symmetry properties lower than those of protophorphyrin IX or chlorins and similar to those of chlorophylls and isobacteriochlorins. Some of the resonance Raman vibrations of P460 are shifted in HAO as compared to those of P460-Fragment.

The conversion of ammonia to nitrite with hydroxylamine as intermediate is the sole source of energy for the bacterium, *Nitrosomonas europaea* (1). A chromophore, P460, is at the active site of the enzyme hydroxylamine oxidoreductase (HAO) which catalyzes the reaction: $NH_2OH + H_2O \rightarrow 4e^- + 5H^+ + NO_2^-$ (2). Selective destruction of the P460 chromophore in ferric HAO by an excess of hydrogen peroxide abolishes the enzymatic activity of HAO (3), indicating the important role of P460 in catalysis. P460 is characterized by a 463-nm Soret-like maximum observed in the ferrous form of a small polypetide of unknown function called P460-Fragment (4) and in the 63 kD subunit of the enzyme, HAO. The latter contains 7 *c*-type hemes per P460 chromophore (5).

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Abbreviations : HAO , Hydroxylamine Oxidoreductase; EPR, electron paramagnetic resonance.

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The structure of P460 is unknown. The magnitude of the extinction coefficient of the putative Soret maximum of the Fe(II) form (80 mM^{-1} cm^{-1} (6)) and the reactivity of P460 with CO and hydrogen peroxide are similar to those of known heme proteins (7,8) and suggest that P460 has a heme-like structure (3,6,7). Mössbauer spectroscopy shows P460 of ferrous HAO and P460-Fragment to have an unusually large quadrupole splitting (Δ Eq = 4.2 mm/cm, (6,7)), similar to the value reported for heme model compounds of 5-coordinate Fe(II) which contain a strongly anionic fifth ligand such as carboxylate, phenolate or chloride (9,10). Reduced P460 in the green-colored preparations of P460-Fragment has two absorption bands at 650 and 688 nm which are not found in HAO (6). The oxidized Fe(III) forms of P460 of HAO are also different in optical and magnetic properties from the ferric form in P460-Fragment, for example P460 in P460-Fragment exhibits a light absorption maximum at 435 nm and a nearly axial high spin (S= 5/2) type of Fe(III) EPR signal (3,6) whereas both these properties are absent in HAO (5,6,11,12). Soret resonance Raman, which has been useful in the determination of the structure of chromophores in green hemoproteins (13-15), has been employed here with P460.

MATERIALS AND METHODS

Purification of HAO (4) and P460-Fragment (6) from *Nitrosomonas europaea* was performed as before. Selective destruction of the P460 center in HAO, was achieved by 30 min incubation of ferric HAO at room temperature with a 5-fold excess of hydrogen peroxide (5,6). The irons of HAO or P460-Fragment were reduced by incubation for 30 min with an excess of $Na_2S_2O_4$. Resonance Raman measurements were obtained as before (13) at 4 °C. A 3 ml fluorescence cell was irradiated from below with the 457.9 nm line of an Argon ion laser (Spectra Physics 165); scattered light was collected in a plane perpendicular to the laser beam.

RESULTS

Soret resonance Raman Spectra of the P460 chromophore of Ferrous Hydroxylamine Oxidoreductase and P460-Fragment.

In reduced HAO the P460 chromophore has a light absorption maximum at 463 nm, whereas the reduced *c*-hemes have their Soret maximum around 420 nm (4). This made possible the use of 457.9 nm as the excitation wavelength to obtain a resonance Raman spectrum (Figure 1A) of the ferrous P460 chromophore with minimal interference from the spectrum of the seven *c*-hemes which are present for each P460 center. As shown in Fig. 1B many of the features of the spectrum are no longer present in the spectrum of ferrous HAO which had been pretreated with hydrogen peroxide to selectively destroy the P460 center. Vibrations at 1592, 1535, 1492, 1401, 1358, 1309, 1174, 972, 848, 748, 720 and 681 cm^{-1}, which were not affected by treatment with H_2O_2, are associated with the low spin Fe(II) *c*- hemes of HAO. By difference, the vibrations attributed to P460 in HAO are: 1580, 1444, 1434, 1250, 1232, 1216, 1177, 1112, 1045, 1001, 977, 951, 815, 784, 758 and 709 cm^{-1}. Some vibrations at values below 550 cm^{-1} (Fig. 1A) are probably due to Fe(II)-ligand modes. All vibrations at wave numbers greater than 1100 cm^{-1} were strongly polarized (data not shown). Due to overlap of the absorption band of P460 and of the *c*-hemes it is difficult to obtain spectra from the P460 center utilizing other excitation wavelengths. It was not possible to obtain resonance

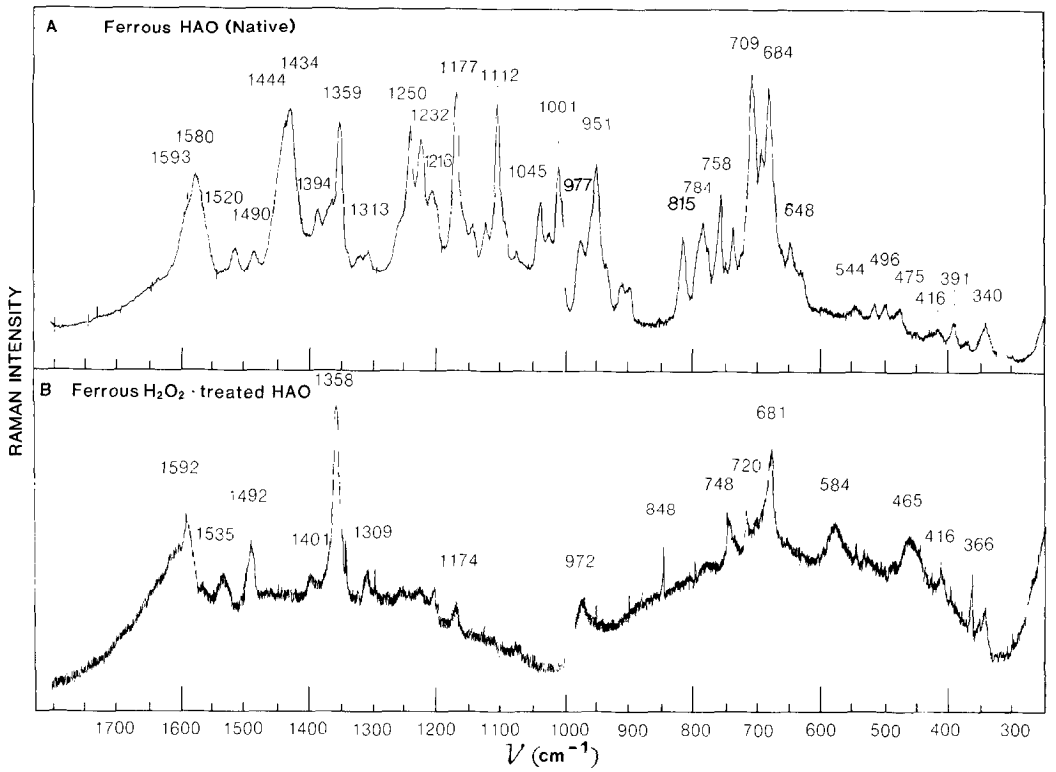

Figure 1. **Soret resonance Raman spectra of (A) ferrous native HAO and (B) ferrous P460-depleted (H_2O_2- treated) HAO.** Spectra were obtained at 4°C. Excitation was at 457.9 nm. Raman intensity is in arbitrary units. (A) Dithionite-reduced HAO (7 μM; A_{463nm} ~ 1.6) in 50 mM K/PO_4, pH 7.5; (B) Dithionite-reduced P460-depleted HAO (7 μM). Raman sensitivity in (B) was 4x sensitivity in (A).

Raman spectra of the P460 chromophore in ferric HAO because the spectra were completely dominated by vibrations of the *c*-hemes.

The resonance Raman spectrum (excitation at 457.9 nm) of ferrous P460-Fragment is shown in Fig. 2. The preparation of P460-Fragment exhibited significant fluorescence relative to HAO. Some of the vibrations (at 1573 and 1357 cm^{-1}, for example) in the P460-Fragment may be associated with the slight contamination with *c*-cytochrome (6). Bands associated with the P460 chromophore in P460-Fragment appear to include : 1565, 1458, 1443, 1250, 1228, 1214, 1170, 1124, 1020, 1001, 971, 955, 943, 836, 788, 741, 718 and 700 cm^{-1}. In general the Soret Raman spectrum of the chromophore in the P460-Fragment is similar to the spectrum obtained of the P460 center in HAO; in particular, unusually intense vibrations are observed in the 1400 to 1500 cm^{-1} region for both preparations. Thus we conclude that the type of macrocycle appears to be the same. Nonetheless, many of the lines in the P460-Fragment are shifted relative to HAO; thus it appears that there are small differences in either the macrocycle itself or the protein environment. Because of the limited availability of P460-Fragment and interfering fluorescence observed when the P460-Fragment was excited at 406 or 413 nm, excitation with other wavelengths and determination of the Raman spectrum of the ferric form were not carried out.

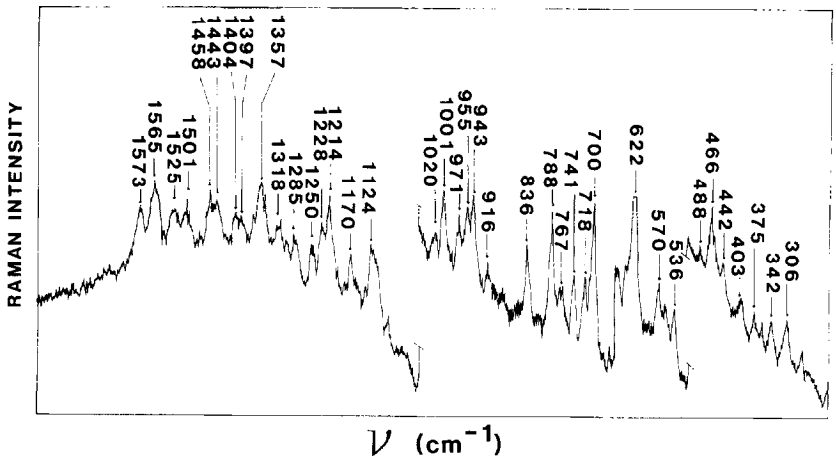

Figure 2. **Soret resonance Raman spectrum of ferrous P460-Fragment.** Dithionite-reduced P460-Fragment (A_{463nm} ~ 1.0). Conditions as in Fig. 1B.

DISCUSSION

The resonance Raman spectrum of the *c*-hemes in hydrogen peroxide-treated HAO (Fig. 1B) is typical of 6-coordinate low spin ferrous *c*-hemes and of a tetrapyrrolic macrocycle in which none of the four pyrrole rings are reduced. The spectrum attributed, by difference, to the P460 center in HAO is obviously qualitatively different from a typical protoheme spectra. The latter spectrum can be explained by normal coordinate analysis assuming a D_{4h} symmetry group. In contrast, the presence of a large number of vibration bands strongly suggests a lower symmetry group for the P460 macrocycle. The Raman spectrum of the P460 macrocycle is clearly different from the corresponding spectrum of any other known iron-containing chromophores (see recent review (15), for heme like structures) including protophorphyrin IX derivatives, heme *d* and d_1(16), siroheme (17), hemes found in myeloperoxidase (13), sulfoheme (18) or green hemoprotein (13,14), iron sulphur clusters (19) or iron-tyrosinate-proteins (20). The Raman spectrum of P460 is clearly different from the metal-containing chromophores which, thus far, have not been observed to have an iron-containing form in nature; the highly reduced (hydro)porphyrin Cofactor F430 (21), a corrin (22), or a metal-pyrroloquinoline quinone or 3,4,6-trihydroxyphenylalanine complex (23). The previous identification of P460 as a heme-like macrocycle is based on the fact that the intensity of its ferrous Soret-like light absorption band (80 mM^{-1} cm^{-1}) is an order of magnitude greater than structures which are not heme-like (6,7). Excitation of hemes at their B-band (near or on their Soret maximum) gives rise to polarized Raman vibrations from the extended porphyrin macrocycle. Structures with lower symmetry (such as chlorophylls) also exhibit this property. The many relatively intense vibrations in the resonance Raman spectrum of P460 indicate that the chromophore has an electronic structure in which the excited states are delocalized over an extended macrocycle. Thus the basic features of the Soret Raman spectra of P460, in particular the large number of vibrational modes that are enhanced and the polarized character of the observed spectrum may be taken as additional evidence that the absorption maximum at 463 nm is a Soret band.

The great complexity of the Raman spectra of P460 suggests that the symmetry group of the structure is even lower than is found with chlorin moieties which have D_2 or C_2 symmetry (15) and that P460 may have C_1 symmetry as is found with chlorophylls (15,24). Interestingly, the degree of perturbation appears to be similar in P460 and the chlorophylls where the Raman spectrum of the tetrapyrrole macrocycle is perturbed by the addition of an extra isocyclic ring (though we do not necessarily take this as indication of the presence of an isocyclic structure in P460[1]). The difference between the tetrapyrrole structure of P460 and protoporphyrin IX is probably large. This is suggested by the fact that 21-thiatetra-p-tolylporphyrin (protoporphyrin IX with a sulphur atom substituted for one of the pyrrole nitrogens), which has ferrous Mössbauer and optical absorption properties similar to those of P460 (25), nevertheless has a Raman spectrum which is not nearly as different from the spectrum of protoporphyrin IX as is the Raman spectrum of P460 (Babcock, unpublished).

The absence of vibrations in the range 1620 - 1780 cm^{-1} suggest the absence of carbonyl groups on the P460 macrocycle. We do not know the nature of the group(s) or ring structure(s) which may be bound to the tetrapyrrole ring of P460 and which are responsible for the perturbations in symmetry properties. Consequently, it is not possible to assign the Raman modes of P460 based on normal coordinate analysis as used for chlorins, isobacteriochlorins or chlorophylls. However we note that the ferrous Raman spectra of P460 are quite similar to spectra of the ferrous isobacteriochlorins siroheme (17, 26) and heme d_1 (21) as well as chlorophylls (27,28). For example the intense vibrations from P460 at 1444, 1434 cm^{-1} , may correspond to vibrations at 1473, 1463 cm^{-1} in siroheme, 1459 cm^{-1} in heme d_1, 1440 cm^{-1} in chlorophyll *a* or 1470, 1447, 1420 cm^{-1} in bacteriochlorphyll *a*. The triplet at 1250, 1233 and 1216 cm^{-1} in the Raman spectrum of P460 in HAO may correspond to similar modes (which have been shifted to higher or lower energies) as compared with values of 1321, 1301 and 1281 cm^{-1} for siroheme, 1222 (and 1282) cm^{-1} for heme d_1, 1292, 1270 and 1230 cm^{-1} for chlorophyll *a* and 1289 and 1257 cm^{-1} for bacteriochlorophyll *a*. Although some of the intense modes in the spectrum of P460 are close to modes in the spectra of isobacteriochlorins and chlorophylls, as illustrated above, other modes of the spectrum are absent or shifted by 10 to 100 cm^{-1}, indicating that the structure of P460 is unique.

Two of the four pyrroles are reduced in bacteriochlorophyll *a* whereas one of the four pyrroles are reduced in chlorophyll *a* or *b*. Comparison of the Raman spectra (27,28) of chlorophyll *a* and bacteriochlorophyll *a* suggest that, in contrast to the major increased complexity going from a chlorin to a isobacteriochlorin, in the C_1 symmetry group, the progression from one to two reduced pyrroles, does not drastically alter the Raman spectra since the symmetry is already low. Thus the number of reduced pyrroles of P460 cannot be deduced from the present Raman data, though a chlorin structure can be excluded. The presence of 3 reduced rings

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[1] The perturbation of the Raman modes of P460 is large compared to the corresponding perturbation going from chlorin to isobacterchlorin. The latter perturbation results from redistribution of the symmetric modes. The present Raman spectra suggest that each of the four pyrroles of P460 may have unique Raman modes arising from covalently bound group(s) and/or an additional ring structure (which also should have specific modes).

appears unlikely since the Raman spectrum of P460 is different from Cofactor F430 or corrin. To summarize, in keeping with the unusual optical spectrum of P460 (3,6), the Raman evidence suggests that P460 is a unique tetrapyrrole in which either two of the pyrroles are reduced or an additional ring is present and one or two pyrroles are reduced.

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