Characterization of the Interaction between PQQ and Heme *c* in the Quinohemoprotein Ethanol Dehydrogenase from *Comamonas testosteroni*†

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ABSTRACT: Quinohemoprotein ethanol dehydrogenase from *Comamonas testosteroni* (QH-EDH) contains two cofactors, 2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dione (PQQ) and heme *c*. Since previous studies on the kinetics of this enzyme suggested that both participate in electron transfer, spectroscopic investigations were performed of the oxidized and reduced holo- and apoenzyme (without PQQ but with heme *c*) to reveal the nature of the interaction between the two redox centers. From this it appears that the properties of the heme in the enzyme are affected by the presence of PQQ, as judged from the shift of the maxima in the ultraviolet/visible absorption spectra of the heme moiety in both reduced and oxidized QH-EDH and the 60-mV increase of the heme midpoint redox potential caused by PQQ addition. Also ^{1}H-NMR spectroscopy was indicative for interaction since binding of PQQ induced shifts in the resonances of the methyl groups of the porphyrin ring in the oxidized form of the apoenzyme and a shift in the methionine heme ligand resonance of the reduced form of the apoenzyme. On the other hand, resonance Raman spectra of the heme in the different enzyme forms were nearly similar. These results suggest that a major effect of PQQ binding to apo-QH-EDH is a rotation of the methionine ligand of heme *c*. Since no intermediate ^{1}H-NMR spectra were observed upon titration of apoenzyme with PQQ, apparently no exchange occurs of PQQ between (oxidized) holo- and apoenzyme at the NMR time scale and at that of the experiment. This is in agreement with the view that PQQ becomes tightly bound, the event leading to a compact enzyme conformation which is able to catalyze rapid intramolecular electron transfer.

When grown on ethanol, *Comamonas testosteroni* produces quinohemoprotein ethanol dehydrogenase (QH-EDH).[1] Curiously, the enzyme is in its apo form; that is, it contains heme *c* but not PQQ because the latter compound is not produced by the organism. However, active holoenzyme can be obtained by adding PQQ to the apoenzyme in the presence of Ca^{2+} (Groen et al., 1986). QH-EDH oxidizes primary alcohols as well as aldehydes. Kinetic studies indicate that, upon oxidation of an alcohol to its corresponding acid, the intermediate aldehyde formed is released from the enzyme, after which it competes with alcohol for the enzyme (Geerlof et al., 1994a,b). These studies also suggest that substrate oxidation occurs at the PQQ site, after which the electrons are transferred one by one from $PQQH_2$ to heme (Geerlof et al., 1994b). The intermediate required in this mechanism appears to be stable since the semiquinone form of PQQ ($PQQH^•$) has been detected in this enzyme (de Jong et al., 1995). The question whether electron transfer from $PQQH_2$ or $PQQH^•$ to ferriheme *c* occurs *via* a long-distance mechanism through the protein or *via* a short one because the cofactors are close together has not been answered yet. For that purpose, at least the nature of the interaction between the two cofactors should be known. Taking advantage of the fact that QH-EDH is obtained in its apo form when cultivation is carried out in the absence of PQQ and reconstitution with PQQ to holoenzyme occurs easily, this should be feasible.

Recently some indications are obtained that the presence of PQQ indeed affects the properties of the heme since EPR spectroscopy revealed that a shift in the three *g*-values and a narrowing of the peaks of the low-spin heme *c* in the enzyme occur upon PQQ binding (de Jong et al., 1995). To investigate the interaction further, several other spectroscopic techniques were applied to reduced and oxidized forms of the holo- and the apoenzyme.

MATERIALS AND METHODS

Apo-QH-EDH Isolation. Apoenzyme was isolated as described (de Jong et al., 1995) from *Comamonas testosteroni* LMD 26.36 grown in a mineral medium on 1% (w/v) ethanol. Enzyme activities were measured using *n*-butanol as a substrate and potassium ferricyanide as an electron acceptor (Groen et al., 1986).

Reconstitution to Holoenzyme. Reconstitution experiments were performed by adding 1 equiv of PQQ to the apoenzyme

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[1] Abbreviations: EPR, electron paramagnetic resonance; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; NMR, nuclear magnetic resonance; ppm, parts per million; PQQ, 2,7,9-tricarboxy-1*H*-pyrrolo-[2,3-*f*]quinoline-4,5-dione: $PQQH^•$, semiquinone form of PQQ; $PQQH_2$, quinol form of PQQ; QH-EDH, quinohemoprotein ethanol dehydrogenase.

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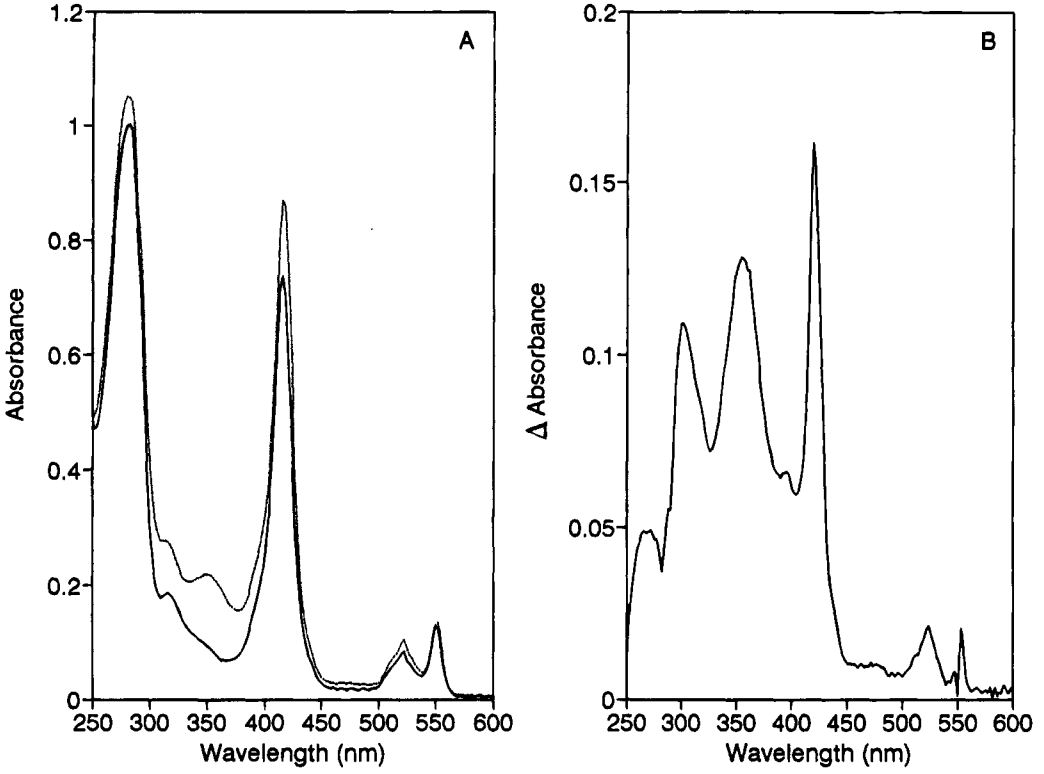

FIGURE 1: Ultraviolet/visible absorption spectra of reduced apo- and holo-QH-EDH in 20 mM MOPS/KOH, pH 7.5, and 5 mM $CaCl_2$. Reduction of apo-QH-EDH is performed by addition of 1 mM DL-dithiothreitol. Spectra were compared at equal concentrations using the specific absorption coefficients $A^{0.1\%}_{280nm} = 2.41$ (apo-QH-EDH) and $A^{0.1\%}_{280nm} = 2.52$ (holo-QH-EDH) (de Jong et al., 1995). (A) Ultraviolet/visible spectra of (—) apo-QH-EDH and (- - -) holo-QH-EDH. (B) Difference spectrum of the reduced holo-QH-EDH spectrum minus the reduced apo-QH-EDH spectrum.

in 20 mM MOPS/KOH, pH 7.5, containing 5 mM $CaCl_2$. After reconstitution, the preparation was chromatographed on a Mono-S column to separate holo-QH-EDH from non-reconstituted, inactive apo-QH-EDH (de Jong et al., 1995).

Ultraviolet/Visible Spectroscopy. Ultraviolet/visible absorption spectra were recorded on a Hewlett Packard HP 8452A, a SLM Aminco DW 2000, or a Shimadzu spectrophotometer (Model 265FS). To obtain difference spectra of the oxidized or reduced enzyme, with and without PQQ, samples were oxidized by titration with potassium ferricyanide or reduced by addition of dithiothreitol or sodium ascorbate.

Midpoint Redox Potentials. Midpoint redox potentials were determined by means of potentiometric titrations, monitored optically, as described by Dutton et al. (1971). The solution potential was measured with a Crison potentiometer equipped with platinum (P1312 Radiometer) and Ag/AgCl (K8040 Radiometer) electrodes and is quoted relative to the normal hydrogen electrode. The following redox mediators were present at final concentrations of 5 μM: 1,4-naphtoquinone, methylene blue, triquat, phenosaphranine, benzyl viologen, methyl viologen, dichlorophenolindophenol, benzoquinone, anthraquinone-2-sulfonic acid, phenazine methosulfate, dimethyltriquat, indigo tetrasulfonate, 2 hydroxy-1,4-naphtoquinone, 5-hydroxy-1,4 naphtoquinone, duroquinone, phenazil, and safranine. Solution redox potentials (in equilibrium) were varied by adding appropriate volumes of deaerated dithionite as reductant. All experiments were performed under an argon atmosphere (the argon was passed through an Oxygen Trap from Chemical Research Supplies).

^{1}H-NMR Spectroscopy. High-resolution ^{1}H-NMR spectra were recorded in the Fourier transform mode on a Bruker AMX-300 spectrometer (300 MHz) equipped with a temperature control unit. The spectra were obtained by an exponential multiplication by 10 Hz line broadening of free induction decays prior to Fourier transformation to improve the signal to noise ratio. All chemical shift values are quoted in parts per million (ppm) from internal 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]propionate, positive values referring to low-field shifts. The samples in 20 mM MOPS/KOH, pH 7.5, containing 5 mM $CaCl_2$ were exchanged several times with the required buffer (prepared with 99.8% ^2H_2O) by centrifugation in a Centricon 10 microconcentrator (Amicon Co.). The enzyme concentrations varied from 0.5 to 1.7 mM. Oxidation of the enzyme was achieved by addition of potassium ferricyanide; reduction, by addition of dithionite. Titration of apo-QH-EDH with PQQ was monitored at 293 K. The pH dependence of the heme resonances was studied over the range 5.6—9.4 at 293 K.

Resonance Raman Spectroscopy. Resonance Raman spectra were recorded as described previously (Hurst et al., 1991; Loehr & Sanders-Loehr, 1993). The samples of the reduced apo- and holoenzyme were maintained near 0 °C in an ice—water Dewar, whereas the oxidized samples were maintained at ~90 K in a liquid nitrogen Dewar during data collection (Loehr & Sanders-Loehr, 1993). The reduced samples were measured using 413.3-nm excitation; the oxidized samples, using 514.5-nm excitation.

RESULTS

Ultraviolet/Visible Absorption Spectra. To study the effect of binding of PQQ on the optical spectrum of the heme *c*

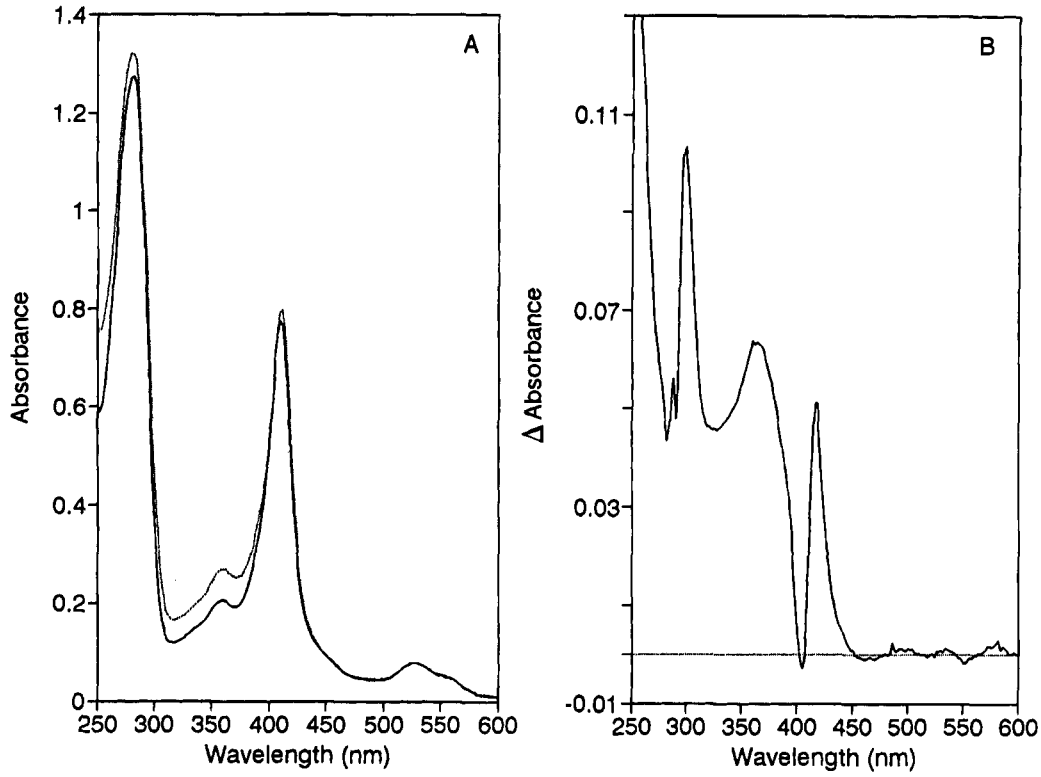

FIGURE 2: Ultraviolet/visible absorption spectra of oxidized apo- and holo-QH-EDH in 20 mM MOPS/KOH, pH 7.5, and 5 mM $CaCl_2$. Oxidation of holo-QH-EDH is performed by addition of ferricyanide. Spectra were compared at equal concentrations using the specific absorption coefficients $A^{0.1\%}_{280nm}$ (apo-QH-EDH) and $A^{0.1\%}_{280nm} = 2.52$ (holo-QH-EDH) (de Jong et al., 1995). (A) Ultraviolet/visible spectra of (—) apo-QH-EDH and (- - -) holo-QH-EDH. (B) Difference spectrum of the oxidized holo-QH-EDH spectrum minus the oxidized apo-QH-EDH spectrum.

moiety in QH-EDH, the redox state of the heme must be the same in the absence as well as in the presence of PQQ. This was achieved by addition of ferricyanide or dithiothreitol. Complete oxidation of the holo-QH-EDH with potassium ferricyanide required 20−100 equiv depending on the preparation, caused by the presence of endogenous substrate (de Jong et al., 1995). Comparison of the reduced apo- and holoenzyme (Figure 1) revealed small shifts in the maxima and small increases in the intensity of all major (i.e., α, β, γ, and δ) cytochrome absorption bands upon binding of PQQ. In addition to changes in the peaks of the cytochrome, new broad absorption bands appeared at 354 nm (Figure 1b) and 362 nm (Figure 2b) which are ascribed to bound $PQQH_2$ and bound PQQ, respectively. Evidence for these assignments is provided by the observation that further addition of dithionite, ascorbate, or dithiothreitol to the substrate-reduced holo-QH-EDH (Figure 1a), or further addition of ferricyanide to oxidized holo-QH-EDH (Figure 2a), did not change the optical spectra. The difference spectra of the holo- minus the apo-QH-EDH in the reduced and oxidized states were compared with the absorbance of free PQQ or $PQQH_2$ in the same buffer. No similarity was observed with either PQQ or $PQQH_2$, suggesting that a change of the optical properties of the cofactor occurs upon binding to the enzyme. However, the larger molar absorption coefficient for bound $PQQH_2$ as compared to that of bound PQQ and the blue shift occurring on reduction are properties similar to those of free PQQ (Duine & Frank, 1980). Significant differences between the optical spectra of bound PQQ and free PQQ have also been reported in the case of the PQQ-containing methanol dehydrogenases (Duine et al., 1981) and glucose dehydrogenases (Dokter et al., 1986).

The α band is shifted from 550.6 (apo) to 552.0 nm (holo), the γ band is shifted from 416.3 nm (apo) to 417.6 nm (holo), and the δ band is shifted from 316 nm (apo) to 314 nm (holo). Comparison of the cytochrome spectra in oxidized apo- and holoenzyme due to PQQ binding (Figure 2) mainly showed a shift in the γ band, from 410 (apo) to 412 nm (holo).

Midpoint Redox Potentials. The midpoint oxidation−reduction potential of the heme *c* in QH-EDH at pH 7.5 was obtained by following the absorbance of the α band during titration (Figure 3). Midpoint potentials of +80 mV for the heme *c* in the apoenzyme and +140 mV for the holoenzyme were determined. In both cases heme *c* titrated with a value of $n = 1$.

^{1}H-NMR Spectroscopy of the Reduced Apo- and Holo-QH-EDH at 293 K. ^{1}H-NMR spectra were measured of reduced apo- and holo-QH-EDH at 293 K to investigate the nature of the axial ligands of the heme and the effect of PQQ binding on them. The high-field part of the spectra are shown in Figure 4. Apo-QH-EDH has resonances at −1.89 and −2.87 ppm, and the holoenzyme has resonances at −1.52 and −2.72 ppm. The resonances observed outside the main absorption envelope in this region are assigned to the γ-methylene and ε-methyl protons of the heme-bound methionine [cf. Senn and Wütrich (1983)].

^{1}H-NMR Spectroscopy of the Oxidized Apo- and Holo-QH-EDH at 303 K. ^{1}H-NMR spectra were measured of oxidized apo- and holo-QH-EDH at 303 K to investigate the effect of PQQ binding on the heme resonances (Figure 5). The low-field part of the spectrum of oxidized apo-QH-EDH clearly shows all four resonances, each of three-proton intensity, from the protons belonging to the methyl groups

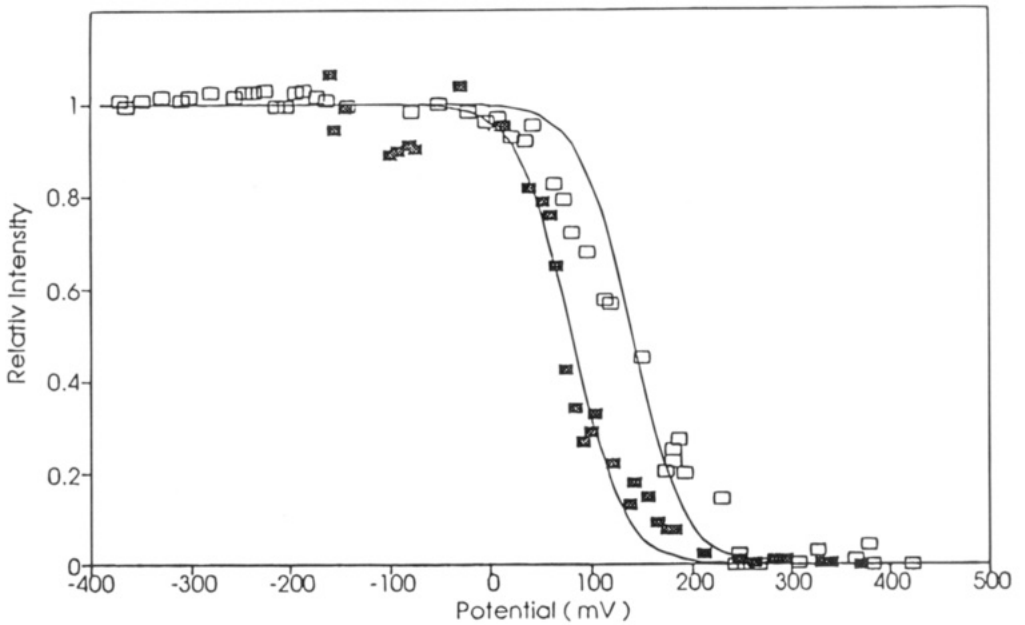

FIGURE 3: Determination of the midpoint redox potentials of apo- and holo-QH-EDH. Measurements were performed as described in Materials and Methods: (□) apoenzyme; (■) holoenzyme. The relative intensity projected on the *y*-axis is the increase in absorbance of the α band divided by the maximal increase of the α band upon total reduction of the heme *c*.

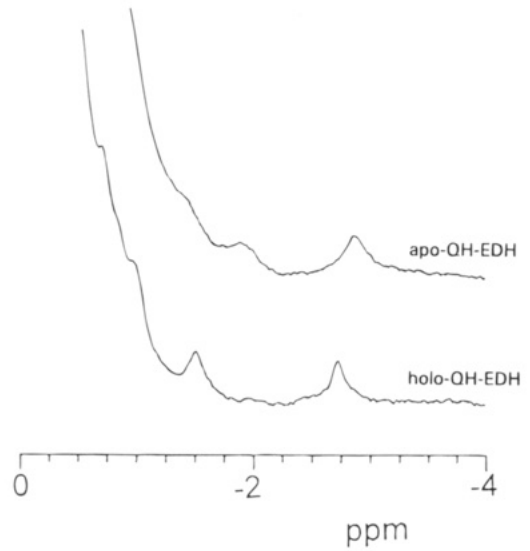

FIGURE 4: ^{1}H-NMR spectroscopy of the reduced apo- and holo-QH-EDH at 303 K. Samples of apo- and holo-QH-EDH in 20 mM MOPS/KOH, pH* 7.5, and 5 mM $CaCl_2$ were reduced by titration with dithionite. Holo-QH-EDH was separated by non-reconstitutable apo-QH-EDH by Mono-S column chromatography (de Jong et al., 1995). *Quoted pH values are meter readings uncorrected for the isotope effect.

on the porphyrin ring [cf. La Mar (1979)]. The spectrum of oxidized holo-QH-EDH shows only three resonances due to overlap of two of the heme methyl resonances, leading to a single peak whose intensity accounts for six protons. All resonances shift substantially as a result of PQQ binding (Table 1), the shifts varying between 2.3 ppm upfield to 7.5 ppm downfield. Comparison of the spectra of apo- and holo-QH-EDH reveals much smaller line widths for the latter. The resonance belonging to the three ϵ-methyl protons of the methionine ligand is located at −12.6 ppm for the apoenzyme and at −13.5 ppm for the holoenzyme.

^{1}H-NMR Measurement of a Titration of Oxidized Apo-QH-EDH with PQQ. The effect of PQQ on the heme-methyl protons was also studied by titrating oxidized apo-QH-EDH with aliquots of PQQ. The enzyme was kept in the oxidized state during titration by the addition of excess potassium ferricyanide. ^{1}H-NMR measurements were performed at 293 K because the apo-QH-EDH slowly denatured at 303 K. During titration, the broad peaks of the oxidized apo-QH-EDH are converted into the sharper peaks of the oxidized holo-QH-EDH without the appearance of intermediate peaks, suggesting that there is no exchange of PQQ in the NMR time scale between the apo- and holoform, in agreement with column chromatography (de Jong et al., 1995). It was further observed that, after addition of about 0.6−0.7 PQQ/apoenzyme, the NMR spectrum no longer changes; see, e.g., the persistent presence of the residual peak at 37.0 ppm in Figure 6. This finding is in agreement with our previous results (de Jong et al., 1995) showing that the apoenzyme contains a non-reconstitutable fraction of about 30%. Indeed, the ^{1}H-NMR spectrum of holo-QH-EDH prepared by means of Mono-S chromatography to remove this fraction does not show the peak at 37.0 ppm (Figure 5). In contrast to the measurement at 303 K discussed above, the resonances of the four methyl groups of the porphyrin in the oxidized holo-QH-EDH are now all well resolved. The chemical shift values are summarized in Table 1.

^{1}H-NMR Measurement of a pH Titration of Oxidized Holo-QH-EDH. To exclude interference of pH effects on the comparisons (a slight drop in pH occurs upon removal of endogenous substrate when oxidized holo-QH-EDH is prepared), ^{1}H-NMR spectra were taken of oxidized holo-QH-EDH at various pH values. Examination of the ^{1}H-NMR spectra of the oxidized holo-QH-EDH at different pH values did not show the drastic changes in chemical shift values compared to the changes observed upon binding of PQQ to the apoenzyme. Chemical shifts as a function of the pH* are shown in Figure 7 for the two middle resonances. The changes of the chemical shifts of the heme methyl resonances were fitted by a one-proton titration curve. This yielded pK_a

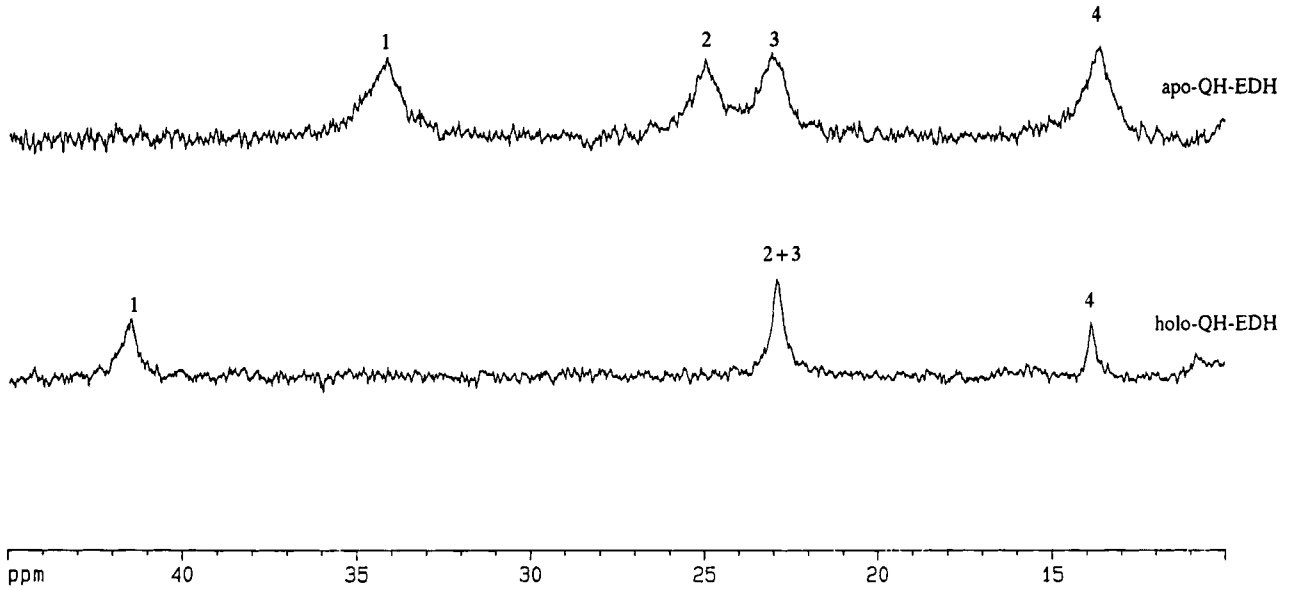

FIGURE 5: ^{1}H-NMR of the oxidized apo- and holo-QH-EDH at 303 K. Samples of apo- and holo-QH-EDH in 20 mM MOPS/KOH, pH* 7.5, and 5 mM $CaCl_2$ were oxidized by titration with potassium ferricyanide. Holo-QH-EDH was separated from non-reconstitutable apo-QH-EDH by Mono-S column chromatography (de Jong et al., 1995). *Quoted pH values are meter readings uncorrected for the isotope effect.

Table 1: ^{1}H-NMR Resonances from the Heme Methyl Groups of Oxidized Apo- and Holo-QH-EDH[a]

	apo-QH-EDH		holo-QH-EDH	
	293 K	303 K	293 K	303 K
	peak position (ppm)			
peak 1	37.0	34.1	43.7	41.6
peak 2	26.0	25.2	23.0	22.9
peak 3	23.0	23.1	22.7	22.9
peak 4	15.0	13.6	13.4	13.9

[a] Samples of apo- and holo-QH-EDH in 20 mM MOPS/KOH, pH* 7.5, and 5 mM $CaCl_2$ were oxidized by titration with potassium ferricyanide. Chemical shift values are quoted in ppm from internal 3-trimethylsilyl-[2,2,3,3-$^2H_4$]propionate. Assignments of peaks 1−4 are based on Figure 5.

values of 7.9 ± 0.2 (resonance 2) and 8.2 ± 0.4 (resonance 3).

Resonance Raman Spectroscopy. Figures 8 and 9 show the resonance Raman spectra of the reduced forms of the apo- and holo-QH-EDH, respectively, using 413.1-nm excitation. The two spectra are very similar. The peak positions of various bands are all within 3 cm^{-1}, indicating little or no changes in bond lengths and/or force constants within the porphyrin system. The spectra are very similar to those of cytochrome *c* [cf. Hu et al. (1993)]. The finding that the 688- (apo) and 689-cm^{-1} (holo) bands which is predominantly a C_a−S stretching mode, differ by only 1 cm^{-1} indicates that the binding of PQQ does not affect the C_a−S bond strength. Figure 10 shows the resonance Raman spectra of the oxidized forms of the apo- and holo-QH-EDH in the high-frequency region using 514.5-nm excitation. Measurement with Soret excitations (413.1 and 406.7 nm) was not possible since excitations in this region caused photoreduction of the oxidized samples, as was indicated by the oxidation state marker ν_4 at 1375 (oxidized) and 1360 cm^{-1} (reduced). Bands in the high-frequency region are most characteristic of the heme group being in a low-spin, hexacoordinate state in both forms of the protein. The porphyrin skeletal modes observed with Q excitation (ν_{10}, ν_{11}, ν_{19}), which reflect the bond strengths around the porphyrin ring, show no significant frequency shifts due to PQQ binding. All other porphyrin skeletal modes observed are typical of oxidized cytochromes *c*.

DISCUSSION

As previously reported (de Jong et al., 1995), the 695-nm band observed in the ultraviolet/visible absorption spectrum of oxidized apo-QH-EDH suggests that the heme has a histidinyl-methionyl-Fe coordination. The ^{1}H-NMR spectra of reduced apo- and holo-QH-EDH (Figure 4) clearly confirm that the methionyl residue is the sixth ligand. Furthermore, the relatively sharp signals and narrow isotropic shifts in the spectra indicate that Fe^{3+} is in the low-spin state (Bertini & Luchinat, 1986). The resonance Raman spectra of reduced and oxidized enzyme also confirm the low-spin, hexacoordination state of the heme.

The broad signals between 15 and 37 ppm downfield of the internal standard in the ^{1}H-NMR spectra of the oxidized apoenzyme are attributed to the four heme methyl groups (La Mar, 1979). Upon titration with PQQ these are transformed into the relatively sharp resonances of the heme methyl groups in the oxidized holo-QH-EDH. Since no intermediate forms are observed, apparently no exchange of PQQ occurs at the NMR time scale and at that of the experiment.

The pK_a value observed for the methyl resonances in ^{1}H-NMR spectra of heme *c* containing proteins is ascribed to that of the most buried propionic side chain of the porphyrin ring (Chao et al., 1979). Since from the spectra of holo-QH-EDH at varying pH a pK_a value of 8 is deduced for the two middle heme methyl resonances, this extremely high value must be explained by assuming that the side chain is buried in a very hydrophobic environment. Resonance peak 1 (Figures 5 and 6) shifts substantially upon binding of PQQ, suggesting an increase of the paramagnetic contribution of the heme. However, this is in contradiction with the decrease in line width, suggesting a decrease of this contribution. Two possibilities are proposed to explain the discrepancy. First,

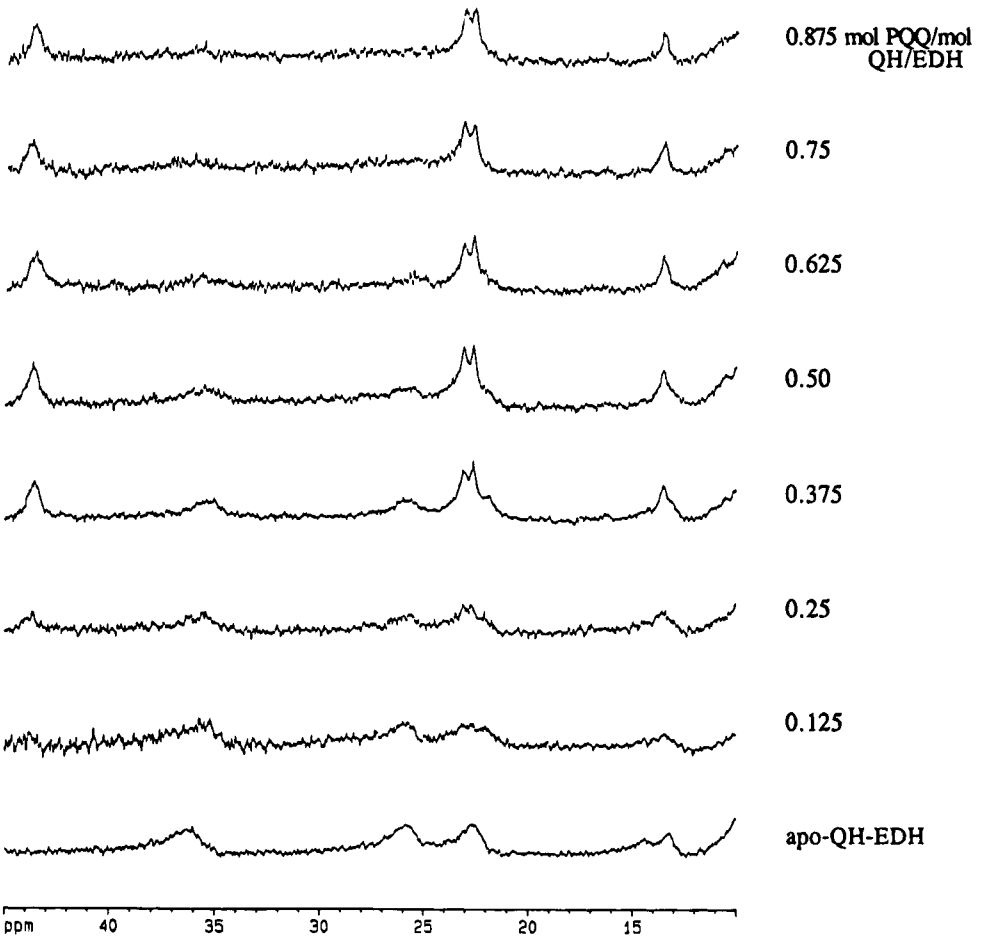

FIGURE 6: ^{1}H-NMR spectroscopy of the titration of the oxidized apoenzyme with PQQ at 293 K. Apo-QH-EDH in 20 mM MOPS/KOH, pH* 7.5, and 5 mM $CaCl_2$ was oxidized by titration with potassium ferricyanide. Every addition of PQQ corresponds to 0.125 equiv based on the concentration of the apoenzyme. *Quoted pH values are meter readings uncorrected for the isotope effect.

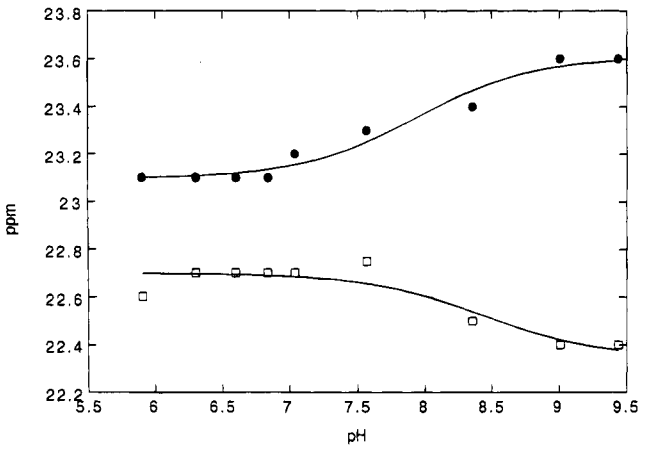

FIGURE 7: ^{1}H-NMR of oxidized holo-QH-EDH at varying pH and at 293 K. pH* titration of the heme methyl resonances (2 (□) and 3 (●) in Figure 5). Data points are indicated by symbols; the solid lines correspond to theoretical one-proton titration curves with pK_a = 7.9 ± 0.2 (resonance 2) or pK_a = 8.2 ± 0.4 (resonance 3). *Quoted pH values are meter readings uncorrected for the isotope effect.

binding of PQQ leads to additional binding interactions, resulting in a more compact structure of the enzyme molecule as a whole. Second, binding of PQQ makes the heme-containing domain more independent of other domains so that its rotation speed becomes faster. At present it is not possible to decide which hypothesis is correct. The tentative conclusion is that PQQ binding induces a conformational change, as was already suggested before from the differences between the chromatographic behavior and the EPR signals of apo- and holoenzyme (de Jong et al., 1995).

Normally, the resonances of the four methyl groups in low-spin ferricytochromes are grouped together in two distinct pairs separated by 10−20 ppm (Turner, 1993). The most downfield pair is situated between 38 and 27 ppm, and the relatively upfield pair, between 17.5 and 7.2 ppm (Timkovich et al., 1984). It has been suggested that such a situation originates from two diagonally opposite pyrrole rings having similar distributions of the unpaired electron but dissimilar from the other pair. However, although several suggestions have been made (Timkovich et al., 1994) no clear answer has been given as to what causes the shifts of the methyl group resonances. The more dispersed pattern of the methyl resonances of QH-EDH suggest a different pattern of electron distribution. The high value of the methyl group (43.7 ppm) observed at 293 K in the holoenzyme is lower in the apoenzyme (37.0 ppm, assuming that the four methyl resonances are lined up in the same order for both enzyme forms). This indicates that the spin density on the pyrrole ring closest to that methyl group increases upon PQQ binding.

Substantial effects on the heme properties resulting from reconstitution have also been observed in the case of *p*-cresol methyl hydroxylase (McLendon et al., 1991). This enzyme consists of two identical flavoprotein subunits and two identical cytochrome *c* subunits which can be separated from

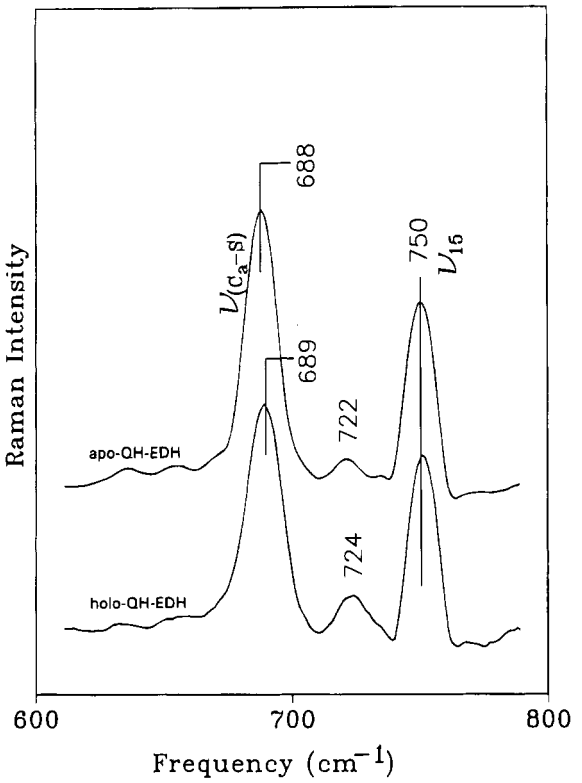

FIGURE 8: Resonance Raman spectra (600−800 cm^{-1}) of the reduced apo- and holo-QH-EDH, with laser excitation at 413.1 nm at $T = 273$ K.

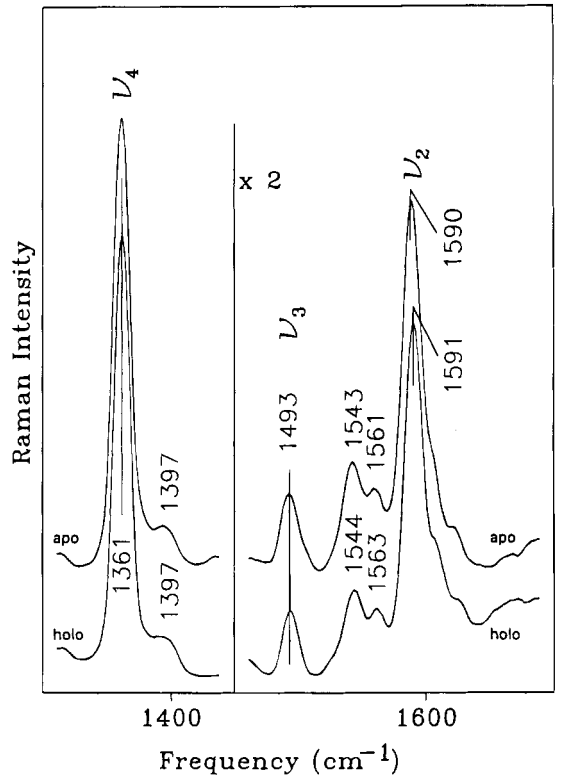

FIGURE 9: Resonance Raman spectra (1300−1700 cm^{-1}) of reduced apo- and holo-QH-EDH, with laser excitation at 413.1 nm at $T = 273$ K.

each other and isolated in intact form (Shamala et al., 1986). Binding of the flavoprotein to the cytochrome subunit causes a shift of the methyl resonances up to 8 ppm and a strong increase of the midpoint potential of the cytochrome. On

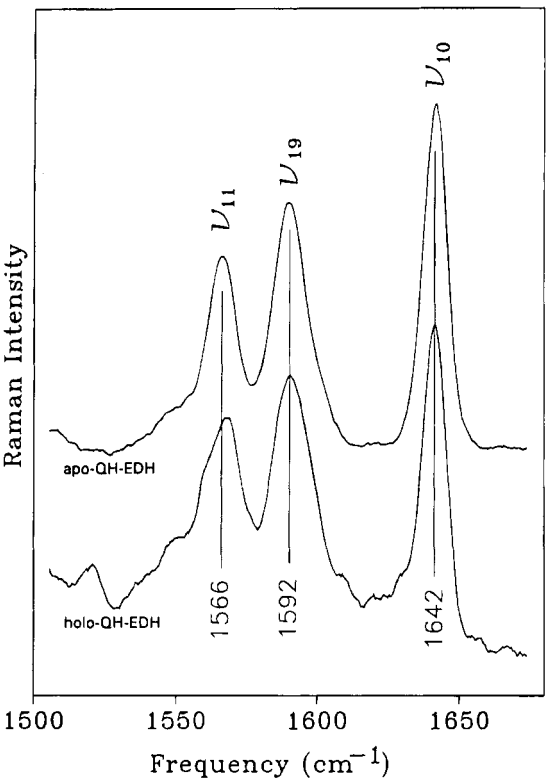

FIGURE 10: Resonance Raman spectra (1500−1700 cm^{-1}) of oxidized apo- and holo-QH-EDH, with laser excitation at 514.5 nm at $T = 90$ K.

the basis of X-ray crystallographic data for the intact protein and the NOESY spectral data of the reduced cytochrome *c* subunit, it has been suggested that a reorientation of the axial methionyl residue occurs by a rotation of ~180° around the Cγ−Sδ bond upon binding of the flavoprotein subunit to the cytochrome subunit (McLendon et al., 1991). The reorientation is accompanied by a redistribution of the electron density in order to accommodate the new bonding, reflected by the shifts in the methyl resonances. Since similar phenomena occur upon binding of PQQ to apo-QH-EDH, rotation of the methionyl residue may also take place in this enzyme.

The similar resonance Raman spectra of the different enzyme forms indicate that no significant changes in the porphyrin bonds and the C_a−S (cysteine) bonds occur (Hu et al., 1993). It also confirms that the shifts observed in the NMR spectra are not caused by ring current changes but possibly by a rotation of the methionine ligand. Resonance Raman spectroscopy does not readily detect changes of axial ligands to the heme, at least when they do not affect the spin state (Loehr & Loehr, 1973; Spiro, 1983). Furthermore, the axial ligand methionine makes no determinable contribution to the resonance Raman spectrum of cytochromes *c* in either oxidation state (Hu et al., 1993).

Our results indicate that binding of PQQ to apo-QH-EDH induces a conformational change of the protein, a reorientation of the methionine ligand of heme *c*, an increase of electron density on one of the pyrrole rings, and an increase of the midpoint redox potential of the heme. Although this clearly indicates that the presence of PQQ in the enzyme affects the properties of the heme, it is still unclear whether the interaction between the two cofactors is direct or indirect. Forthcoming information on the three-dimensional structure of the enzyme may shed light on this.

Undoubtedly, the phenomena observed are related to the function of the enzyme, that is, to catalyze efficient oxidation of alcohols and to transfer electrons rapidly, internally between PQQ and heme and externally from heme *c* to its unknown natural electron acceptor. Information on this may also be relevant for other quinoproteins, e.g., methanol dehydrogenase from methylotrophic Gram-negative bacteria, only containing PQQ and using cytochrome c_L as its (external) electron acceptor (Day & Anthony, 1990), and ethanol dehydrogenase from *Pseudomonas aeruginosa*, only containing PQQ and transferring its electrons to cytochrome c_{EDH} (Schrover et al., 1993). In this connection it should be mentioned that part of the amino acid sequence of QH-EDH is very similar to that of methanol dehydrogenase (including the two vicinal cysteines and the tryptophan sandwiched to PQQ; unpublished results) and that methanol dehydrogenase and ethanol dehydrogenase have similar properties. Thus, QH-EDH, probably being a fusion of the dehydrogenase and the electron-accepting cytochrome *c*, and obtainable in the apo as well as the holo form, seems an attractive model enzyme for the comparative studies on intramolecular and intermolecular electron transfer of similar systems.

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