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Cytochrome *c* Oxidase: Evidence for Interaction of Water Molecules with Cytochrome *a*

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ABSTRACT: The resonance Raman spectra of cytochrome *c* oxidase in protonated buffer compared to that in deuterated buffer indicate that water molecules are near the heme of cytochrome *a*. Differences in widths of the heme line at 1610 cm^{-1}, after short exposure to D_2O, and, additionally, of the heme line at 1625 cm^{-1}, after long exposure, can be accounted for by changes in resonance vibrational energy transfer between modes of cytochrome a^{2+} and the bending mode of water molecules in the heme pocket. On the basis of the assignment of these modes, we place one water molecule near the vinyl group and one water molecule near the formyl group of the cytochrome *a* heme. These water molecules may play several possible functional roles.

Cytochrome *c* oxidase is the terminal enzyme in the electron transport chain and serves as the catalytic site for the reduction of O_2 to H_2O (Wikström et al., 1981). Each monomeric unit contains two heme groups (cytochrome *a* and cytochrome a_3) and at least two copper atoms. Cytochrome a_3 and an associated copper atom (Cu_B) form the dioxygen binding site, whereas cytochrome *a* and Cu_A are the primary electron-acceptor sites from cytochrome *c*. An additional function of the multicenter enzyme is to translocate protons across the inner mitochondrial membrane. Despite the fact that this is now well established (Wikström et al., 1981; Wikström & Krab, 1979; Wikström, 1984; Thelen et al., 1985; Puettner et al., 1985; Sarti et al., 1985), the mechanism of the translocation activity is not yet understood and it is even uncertain as to which centers are involved, although some specific hypotheses have been proposed (Babcock & Callahan, 1983; Gelles et al., 1986).

To illucidate the key features of proton translocation, we have studied the effect of deuterated buffers on the enzyme by monitoring the resonance Raman spectra of the heme groups (Argade et al., 1986b). This technique identifies the vibrational modes of the heme that are sensitive to deuteration. This sensitivity may result from exchange of atoms on the heme or from vibrational coupling between heme atoms and exchangeable atoms on nearby groups. The effect of deuteration on the heme modes has been reported earlier, although neither the data nor their interpretation was in agreement (Argade et al., 1986b; Copeland & Spiro, 1986). We have now made a series of high signal-to-noise resonance Raman scattering measurements and carried out a detailed analysis to determine accurately the frequencies and widths of the deuteration sensitive lines. The results indicate that spectral lines corresponding to heme vibrational modes of cytochrome a^{2+} are broader in buffered H_2O than in buffered D_2O. On the basis of a model of vibrational coupling, we conclude from these new observations that water molecules are present near

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Table I: Frequencies and Widths (in cm^{-1}) of Raman Lines from Cytochrome a^{2+} As Determined from an Eight-Line Fitting Procedure[a]

short exposure (2 h)				long exposure (10 days)			
H_2O		D_2O		H_2O		D_2O	
freq	width	freq	width	freq	width	freq	width
1518	10.1	1518	10.3	1518	11.6	1518	11.4
1546	11.5	1546	11.4	1546	12.7	1546	12.2
1568	13.4	1568	13.0	1568	13.2	1568	13.2
1586	15.0	1587	15.1	1586	14.9	1586	15.1
1610	17.0	1610	15.3	1610	16.0	1609	13.8
1625	13.0	1625	12.6	1625	13.8	1625	12.0
1641	12.8	1642	7.8	1641	9.7	1642	8.9
1672	7.2	1673	6.1	1672	15.6	1673	9.3

[a] The main differences between the Raman spectrum of the enzyme in H_2O and that in D_2O are a consequence of the line width changes in the lines that are underscored.

the cytochrome *a* heme and interact with it.

Materials and Methods

Mammalian cytochrome *c* oxidase was isolated from beef heart by the methods of Yonetani (1960) and Yoshikawa et al. (1977) and frozen at liquid nitrogen temperature until ready for use. Samples were diluted to 100 μM (heme) in 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) at pH 7.4 with 1% dodecyl β-D-maltoside. Minimal amounts of sodium dithionite were used to generate the reduced samples. In the deuterated samples, the deuterium enrichment was about 90%. Samples with prolonged exposure to D_2O were prepared by incubating the resting enzyme in D_2O at 4 °C. Mixed valence cyanide-bound preparations were made by adding limited amounts of sodium dithionite or ascorbate–TMPD (tetramethylphenylenediamine) to an aerobic preparation of the cyanide-bound resting enzyme. The final cyanide concentration was 20 mM. Turnover conditions were generated by exposing a mixture of cytochrome *c* oxidase, TMPD, and ascorbate to oxygen and stirring for 10 min.

Resonance Raman spectra were obtained on previously described Raman difference instrumentation with 441.6-nm excitation (Rousseau, 1981). To analyze the resonance Raman data and deconvolute the complex spectra, a curve-fitting routine was utilized. In this procedure the spectra were deconvoluted on a computer by using a nonlinear least-squares fitting procedure based on Marquardt's algorithm (Marquardt, 1963; Nash, 1979). The best fit was found with a 1:1 Gaussian:Lorentzian mixture. In the fitting protocol a specific number of lines were specified, and the intensity, frequency, and width of each were varied until a good fit was obtained.

Results

Figure 1 shows new data comparing the resonance Raman spectra of cytochrome *c* oxidase in H_2O versus D_2O. These data resulted from extended exposure ($\approx$10 days) of the enzyme in the resting state to the solvent, followed by the formation of the mixed valence $[a^{2+}, a_3{}^{3+}$ $(CN^-)]$ complex. The high signal-to-noise ratio in the data allowed us to determine accurately the frequencies, widths, and intensities of the eight lines observed in the spectra by using the nonlinear least-squares fitting procedure. The results of the fits, illustrated by the solid line in Figure 1, are tabulated in Table I. The main differences in the spectrum in the 1600–1650-cm^{-1} region based on this eight-line fit are clear: the lines at 1610 and 1625 cm^{-1} each narrow by $\sim$2 cm^{-1} in the deuterated solvent. No frequency shifts greater than 1 cm^{-1} are detected in any of the lines. The change in width of the line at 1672 cm^{-1} is not considered significant because the line is so weak.

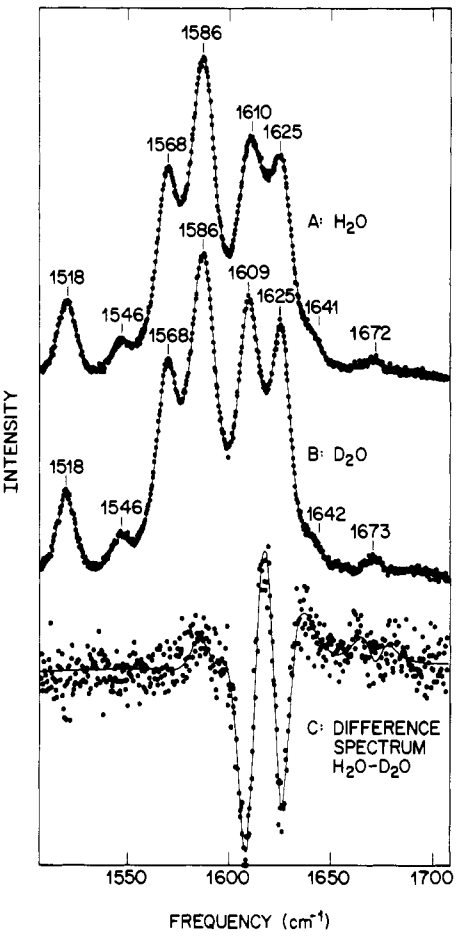

FIGURE 1: Comparison of the resonance Raman spectra of cytochrome a^{2+} in H_2O (A) and after a 10-day exchange in D_2O (B). The samples were prepared by incubating resting cytochrome *c* oxidase in H_2O or D_2O for 10 days and then forming the mixed valence cyanide adduct $[a^{2+}, a_3{}^{3+}$ $(CN^-)]$. The spectra were obtained on 100 μM samples solubilized in HEPES (100 mM) at pH 7.4 with 1% dodecyl β-D-maltoside excited with a He–Cd laser (441.6 nm). The points are unsmoothed data points to which a linear background correction has been applied. The solid lines are a result of the fitting procedure described in the text. To fit the difference spectrum (C), the parameters for fitting spectra A and B were used without further adjustment.

We have also made measurements of the enzyme after short incubation periods but under conditions of enzyme turnover. Following the incubation period, the mixed valence cyanide adduct was formed. We find spectral changes in these samples as well, but these changes are not as pronounced as those obtained with incubation for several days. These data were analyzed by the same procedures described above, yielding fits of comparable quality to those shown in Figure 1. As with the long incubation time, we find no change in position of these lines upon changing the solvent to D_2O. In both data sets (H_2O and D_2O) lines are observed at 1610 and 1625 cm^{-1}. When the sample is placed in D_2O, the line at 1610 cm^{-1} narrows by $\sim$2 cm^{-1} whereas the line at 1625 cm^{-1} is unchanged. The frequencies and widths of all the lines in this region are also listed in Table I.

To test the effects of D_2O on a reference compound, we have examined the effect of solvent on the carbonyl stretching mode of acetone by diluting it in H_2O, D_2O, CH_3OH, and $(CH_3)_2O$ (ether). The frequencies and widths of this mode in each of these solvents are listed in Table II. The width of the carbonyl line is wider in H_2O than in all the other solvents including those that can form hydrogen bonds and those that cannot. The center frequency of the line depends on its molecular

Table II: Frequency and Width (in cm^{-1}) of the Carbonyl Stretching Mode of Acetone in Various Solvents[a]

solvent	freq	width	comments
H_2O	1697	20	H-bonding; resonance transfer
D_2O	1695	17	H-bonding; no resonance transfer
CH_3OH	1707	18	H-bonding; no resonance transfer
CH_3OCH_3	1716	13	no H-bonding; no resonance transfer

[a] Hydrogen bonding may occur when the solvent is H_2O, D_2O, or CH_3OH but not when the solvent is CH_3OCH_3 (ether). Vibrational resonance energy transfer can only occur when the solvent is H_2O.

environment but is relatively insensitive to the changes from H_2O to D_2O.

Discussion

The resonance Raman spectrum of cytochrome *c* oxidase obtained with visible excitation contains contributions from both cytochromes *a* and a_3, which makes its interpretation difficult. However, by proper selection of oxidation and ligand binding states, and laser excitation frequencies, separation of the spectra has been achieved (Ching et al., 1985; Argade et al., 1986a). Indeed, with 441.6-nm excitation, the mixed valence cyanide-bound enzyme [a^{2+}, a_3^{3+} (CN^-)] displays only the cytochrome a^{2+} spectrum (Ching et al., 1985). Thus, the data, which we report in Figure 1 and which are summarized in Table I, are spectra of cytochrome *a* and indicate that the modes of cytochrome *a* change in width upon solvent exchange from H_2O to D_2O.

In previous work in which curve deconvolution was not used, Argade et al. (1986b) studied the effect of deuteration on the enzyme and concluded that, in the 1400–1800-cm^{-1} region of the Raman spectrum of cytochrome *a*, there were intensity differences but no frequency changes in comparison to the spectrum in protonated solvent. In a later study, Copeland and Spiro (1986) reported that upon prolonged exposure (3–5 days) to D_2O more extensive spectral changes are observed than those originally described by Argade et al. (1986b). Most significant was a reported shift of a line from 1611 to 1607 cm^{-1}. This was argued as support for the assignment of this line to the C=O stretching mode associated with the formyl periferral substituent of cytochrome *a* (Copeland & Spiro, 1986). By using the very careful curve-fitting procedure described here, we find no evidence for any shifts in peak positions (within 1 cm^{-1}) of any lines in the high-frequency region of the spectrum. Under all conditions that we have used, only the width and peak intensity changes could be detected, so we consider in detail possible mechanisms of line broadening.

Mechanisms of vibrational line broadening in condensed media have been extensively studied, and several relaxation processes have been found to contribute to the measured line width. Although theories have been written explicitly for simple liquids (Gordon, 1965; Perchard et al., 1972; Bartoli & Litovitz, 1972; Davis & Oppenheim, 1972; Rothschild et al., 1975; Tokuhiro & Rothchild, 1975; DeZwaan et al., 1975; Döge et al., 1977; Wang & McHale, 1980) or for solids (Diestler, 1974; Lin et al., 1976; Young & Moore, 1984), the general concepts may be qualitatively applied to our system as well. Factors that may contribute to the line width of the vibrational modes of the heme in a protein matrix are (1) inhomogeneous contributions from heterogeneous environments of the vibrational oscillator (for example, due to different protein substates); (2) relaxation to other vibrational, translational, or librational states of the heme; (3) relaxation transfer to the amino acid groups of the protein that are in van der Waals contact with the heme; and (4) resonance vibrational energy transfer to a group that is in contact with the heme and has vibrational frequencies close to that of the mode being considered.

The latter process, resonance vibrational energy transfer, can make a significant contribution to the line width in liquids (Tokuhiro & Rothschild, 1975; DeZwaan et al., 1975; Döge et al., 1977; Wang & McHale, 1980; Lin et al., 1976). In addition to requiring coincidence of the vibrational frequencies, there must be a coupling interaction that can cause a vibrational energy transfer between the two groups that are involved and are separated by some distance, *R*. Dipole–dipole coupling and short-range repulsive potential interactions have been shown to be possible mechanisms for resonance vibrational energy transfer in liquids. Tokuhiro and Rothschild (1975) could account for the narrowing of the C–H deformation mode of chloroform by ~ 2 cm^{-1} upon dilution with deuteriochloroform, where there is no longer any mode at the resonance frequency, by both of these coupling interactions. In the case of the coupling interaction through the repulsive potential, the two interacting groups must be in van der Waals contact. For the dipolar interaction the intensity falls off as $1/R^3$, so again the effect would be largest if the acceptor group is in close proximity to the atoms of the mode that is broadened.

There have been other reports of changes in line width occurring in a Raman active mode when a sample was placed in D_2O, and the data have been accounted for by resonance vibrational energy transfer. Hashimoto et al. (1986) accounted for a D_2O-induced narrowing of the Fe=O stretching mode at 767 cm^{-1} in compound ES of cytochrome *c* peroxidase by coupling to a rotation–vibration mode of water molecules. Similarly, a 4-cm^{-1} narrowing of the C=N stretching mode at 1641 cm^{-1} in the Schiff base linkage to retinal in bacteriorhodopsin upon placing the protein in D_2O was shown by Hildebrandt and Stockburger (1984) to result from a coupling between the C=N stretching mode and a broad bending mode of an H_2O molecule near the chromophore. The data we obtained, and report in Table II, on the carbonyl mode in acetone are also consistent with resonant vibrational energy transfer to an associated H_2O molecule.

We invoke vibrational resonance energy transfer to account for our data in cytochrome *c* oxidase since the other mechanisms of line broadening would not be expected to be sensitive to deuteration. The broad bending mode of H_2O is in the 1600-cm^{-1} region so energetically, it is able to couple to the heme vibrational modes. Upon short exposure of the enzyme to D_2O the line at 1610 cm^{-1} narrows. We infer that this is due to immediate replacement of an H_2O molecule with D_2O in which the bending mode occurs at ~ 1180 cm^{-1}, out of resonance with the 1610-cm^{-1} mode. After longer exposure the line at 1625 cm^{-1} also narrows. This is due to the presence of additional molecules of H_2O in the heme pocket after the longer exposure.

The line at 1625 cm^{-1} in heme proteins has been assigned as a stretching mode of the vinyl group by several isotopic substitution studies (Rousseau et al., 1983; Choi et al., 1982a,b), and hence its sensitivity to H_2O places a water molecule near the vinyl group (Figure 2). There is no general agreement as to the assignment of the line at 1610 cm^{-1}. Babcock and Callahan (1983) and Callahan and Babcock (1983) have argued that it is the carbonyl stretching mode of the formyl group of cytochrome *a* although this mode is normally detected at higher frequencies (1640–1680 cm^{-1}). They interpreted the very low frequency in the reduced state to be a consequence of strong hydrogen bonding. Others have argued that this frequency is too low for a carbonyl stretching mode even in the presence of strong hydrogen bonding and

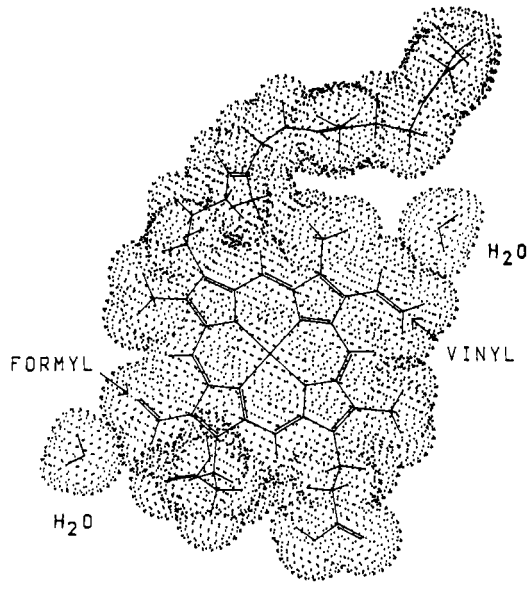

FIGURE 2: A model of proposed positions of water molecules near cytochrome *a*. The spectral broadening in the Raman spectra suggests that one water molecule is near the formyl group and the other is near the vinyl group of cytochrome *a*.

that, therefore, this line may originate from a mode of the porphyrin macrocycle, such as ν_{10} (Argade et al., 1986b; Choi et al., 1983; Ogura et al., 1984). In reviewing other cases where resonance vibrational energy transfer has been implicated (Hashimoto et al., 1986; Hildebrandt & Stockburger, 1984) as well as reported cases of vibrational coupling between heterogeneous molecules (Bajdor et al., 1984; Kincaid et al., 1985), we note that these effects occur in oscillators in which the principle motion is limited to a few atoms. On this basis, our data are more consistent with the 1610-cm^{-1} line in cytochrome a^{2+} originating from the carbonyl stretching mode of the formyl group which can couple to a water molecule to become broadened, rather than from a heme mode such as ν_{10} in which several atoms contribute to the normal coordinate (Abe et al., 1980). This interpretation is consistent with the observation (Copeland & Spiro, 1986) that, in the oxidized state of the enzyme, the cytochrome a^{3+} mode at $\sim$1650 cm^{-1}, assigned as the carbonyl stretching mode, is sensitive to exposure to D_2O. On the basis of these assignments we place at least two water molecules next to the heme of cytochrome *a*: one near the vinyl group and one near the formyl group as depicted in Figure 2.

Changes in other regions of the resonance Raman spectrum of cytochrome *a* have also been detected upon deuteration of both reduced (Argade et al., 1986b) and oxidized (Copeland & Spiro, 1986) cytochrome *c* oxidase. In the reduced enzyme a complex change was detected at 1236 cm^{-1} and a 7-cm^{-1} line shift at 436 cm^{-1} (Argade et al., 1986b). The deuteration changes at 1236 cm^{-1} could result from changes in the formyl group environment since in this spectral region the C_b–CHO stretching mode has been assigned (Choi et al., 1983). It was argued by Argade et al. (1986b) that the line at 436 cm^{-1} is the same mode as that seen at 488 cm^{-1} in model heme *a* complexes and which displays a significant change upon deuteration of the formyl group (Choi et al., 1983). Thus, both of these other changes in the reduced enzyme could be additional manifestations of water molecules interacting with the formyl group, but additional studies appear warranted. The changes reported by Copeland and Spiro (1986) in the formyl carbonyl stretching mode at 1650 cm^{-1} of the oxidized enzyme are also very complex owing to the presence of other lines near that frequency from various forms of cytochrome a_3 and possible coupling to ν_{10} of cytochrome *a*, which occurs at 1640 cm^{-1}. Thus, the relationship between changes we observe and those in the oxidized preparation cannot be assessed at present.

The presence of H_2O molecules near heme groups is not unprecedented, but is quite common. In many ferric heme proteins such as the globins, as shown by crystal structure determinations, a water molecule coordinates directly to the heme iron or is very close to it (Ladner et al., 1977; Finzel et al., 1984; Takano, 1977; Poulos et al., 1986). In other heme proteins water molecules are near the hemes, though not covalently linked to the iron atom (Finzel et al., 1984; Poulos et al., 1986; Fermi, 1975; Fita & Rossmann, 1985; Bhatia et al., 1983). For many of these proteins, peroxidases, for example, water molecules are expected to be present because they have enzymatic functions which require access to the active sites (Finzel et al., 1984; Poulos et al., 1986; Fita & Rossmann, 1985). These access channels are often lines with H_2O molecules (Finzel et al., 1984). In cytochrome *c* and cytochrome c_2, which are electron-transport enzymes and thereby function as does cytochrome *a*, water molecules are found near the heme in both internal and external regions (Bhatia et al., 1983; Takano & Dickerson, 1980). Furthermore, a recent report (Kornblatt et al., 1988) of a large volume change in cytochrome *c* oxidase at high pressure was interpreted as evidence for solvent (water) entry and expulsion. Similarly, the change in tryptophan fluorescence upon partial reduction of the enzyme is consistent with water molecule entry into the protein interior (Copeland et al., 1987). Thus, our finding based on spectroscopic evidence that water molecules are near cytochrome *a* is consistent with crystallographic studies of heme proteins and other studies of cytochrome *c* oxidase.

Until a high-resolution crystal structure of cytochrome *c* oxidase is available, we must rely on spectroscopic and biochemical studies to unravel the structural features of this membrane-bound enzyme and the corresponding functional significance. The presence of water molecules interacting with cytochrome *a* can have several possible functional implications. First, they could be a signature that the cytochrome *a* heme is exposed to the aqueous environment. Such a position of the heme in the membrane-bound enzyme would be important for the electron-transfer function of cytochrome *a*. Second, water molecules can act as structural elements that play a role in stabilizing the heme environment by forming part of a hydrogen-bonding network. In other heme proteins such networks have been found (Finzel et al., 1984; Fita & Rossmann, 1985). Third, the water molecules can play an electrostatic role since their polarity will tend to stabilize the ferric oxidation state of the heme (Poulos et al., 1986). Fourth, the water molecules may be part of the proton translocation pathway. Cytochrome *a* has been implicated as a possible center in the proton translocation pathway (Wikström et al., 1981; Wikström & Casey, 1985), so our evidence that water can reach the site indicates that molecular water may be a part of it. These findings are fully consistent with the recent report (Kornblatt et al., 1988) of a large volume change at high pressure which was interpreted as evidence for solvent entry and expulsion. Similarly, the change in tryptophan fluorescence upon partial reduction is consistent with water molecule entry into the protein interior (Copeland et al., 1987). At present, our data demonstrate that water molecules are near cytochrome *a* and interact with it. Further studies will refine our understanding of the functional role of the water.

Registry No. H_2O, 7732-18-5; cytochrome *c* oxidase, 9001-16-5; cytochrome *a*, 9035-34-1.

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