# Structural Changes in Cytochrome *c* upon Hydrogen–Deuterium Exchange†

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ABSTRACT: The resonance Raman spectra of yeast ferri- and ferro-iso-1-cytochrome *c* dissolved in $H_2O$ and $D_2O$ are reported. Hydrogen exchange in the protein leads to distinct spectral changes of heme vibrational bands, particularly in the region between 670 and 710 $cm^{-1}$ and at ~443 and ~450 $cm^{-1}$. The latter two bands, which have previously been assigned to porphyrin modes including bending vibrations of the propionate side chains [Hildebrandt, P. (1991) *J. Mol. Struct. 242*, 379–395], reveal frequency shifts by up to 4 $cm^{-1}$. These shifts are attributed to structural changes of the propionate groups caused by the energetic differences of the hydrogen and deuterium bonds between these substituents and the adjacent amino acid residues. The frequency shifts of the bands between 670 and 710 $cm^{-1}$ most likely reflect structural differences of the tetrapyrrole macrocycle itself. Time-dependent experiments revealed that the hydrogen exchange processes associated with the changes in the resonance Raman spectra are complete in less than 15 min. The protons which are involved are those in the interior of the heme pocket as concluded by comparison with the exchange rate constants previously determined by NMR spectroscopy [Mayne, L., Paterson, Y., Cerasoli, D., & Englander, S. W. (1992) *Biochemistry 31*, 10678–10685]. These protons are part of a hydrogen bonding network including the amide protons of Asn-52, Met-80, and Lys-79, the side chain protons of Asn-52, Tyr-67, Thr-78, Trp-59, and Thr-49, and the water molecules 121 and 166. The subtle alterations of the hydrogen bonding interactions which are induced by hydrogen–deuterium exchange are apparently sufficient to cause structural changes in the heme which are detected by the resonance Raman spectrum. The present results demonstrate that the hydrogen bonding network in the heme pocket of cytochrome *c* sensitively controls the conformation of the porphyrin.

Cytochrome *c* is an electron-transfer protein in the terminal part of the mitochondrial respiratory chain (Pettigrew & Moore, 1987). The three-dimensional structure of this heme protein has been well characterized by X-ray diffraction analysis of the protein crystals as well as by NMR spectroscopy in solution (Takano & Dickerson, 1981a,b; Bushnell et al., 1990; Louie & Brayer, 1990; Berghuis & Brayer, 1992; Gao et al., 1990). However, the mechanism of the electron-transfer reactions of cytochrome *c* with its physiological redox partners such as cytochrome *c* oxidase is still not understood in detail (Pettigrew & Moore, 1987). In this context, resonance Raman (RR)[1] spectroscopy promises to contribute to the understanding of the structure–function relationships of this protein (Hildebrandt et al., 1990). A prerequisite for extracting all structural information from the RR spectra is a complete vibrational assignment, which, unfortunately, is not yet available for cytochrome *c*. The RR spectra of cytochrome *c* are much more complex than those of other heme proteins or simple metalloporphyrins for which well-established vibrational assignments are available [see Procyk and Bocian (1992) and references cited therein]. In particular, the low-frequency region below 500 $cm^{-1}$ exhibits a complex band pattern. These bands sensitively reflect structural perturbations of the heme pocket such as those induced upon alterations of the pH (Hildebrandt, 1990, 1991). This spectral region also responds to binding of cytochrome *c* to cytochrome *c* oxidase. Thus, an unambiguous assignment of these bands is of utmost importance to understand the structural changes caused by binding to cytochrome *c* oxidase which are of functional relevance for the electron-transfer process.

Among these bands, there is a doublet at ~445 $cm^{-1}$ which exhibits a considerably lower RR intensity when cytochrome *c* is bound to cytochrome *c* oxidase (Hildebrandt et al., 1990). The disappearance of these bands has been shown to be a sensitive marker for a conformational state of cytochrome *c* in which the heme pocket assumes an open structure (Hildebrandt, 1991). On the basis of a variety of indirect evidence, this band has been assigned to a porphyrin mode including a major contribution from the bending vibrations of the propionate side chains of the heme (Hildebrandt, 1991; Hildebrandt et al., 1991, 1992).

The original goal of the present study was to reinforce this assignment. For this purpose, we have carefully measured the RR spectra of cytochrome *c* dissolved in $H_2O$ and $D_2O$ to determine the frequency shifts induced upon H/D exchange. In a previous RR study, Lewis et al. (1985) could not reliably detect isotopic effects. However, the accuracy of the polychromatic detection used by these authors was limited to ±2 $cm^{-1}$. The experimental equipment and techniques employed in the present work exhibit a significantly higher spectral resolution and accuracy and allow for the detection of frequency shifts even smaller than 1 $cm^{-1}$.

We have used iso-1-cytochrome *c* (Cyt) from yeast for which the 445-$cm^{-1}$ doublet is much better resolved than in the commonly used horse heart protein so that the analysis of the spectrum is greatly facilitated (Hildebrandt et al., 1992). In

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[1] Abbreviations: Cyt, iso-1-cytochrome *c* (the superscripts "3+" and "2+" denote the oxidized and the reduced form, respectively); RR, resonance Raman.

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the heme group itself, there are no exchangeable protons, and even the propionate side chains are deprotonated and hydrogen-bonded to nearby amino acid side chains at neutral pH. Hence, one would expect that possible isotopic shifts in the RR spectra should originate exclusively from modes including internal coordinates of those heme substituents that are involved in hydrogen bonding interactions. However, in the course of these studies, we have found unexpected and even more pronounced isotopic effects also for porphyrin modes in other frequency regions that are described and discussed in the present paper.

## MATERIALS AND METHODS

*Sample Preparation.* Wild-type yeast iso-1-cytochrome *c* in which the cysteine at amino acid position 102 is replaced by a threonine was isolated and purified as described previously (Rafferty et al., 1990). For the RR measurements, a concentrated stock solution of the protein was diluted by a factor of ~100 to yield a 15 μM solution in 66 mM phosphate buffer at a pH or pD of 7.0. In the case of $D_2O$, the delay time between preparation of the solutions and the RR experiments was about 1 day unless otherwise indicated. No further spectroscopic changes were detected after equilibration for several days, confirming that the H/D exchange is complete for those groups which affect the structure of the heme pocket within less than 24 h. As will be discussed in the following section, ionic strength was varied in some experiments to influence the rate of hydrogen exchange. Ferricytochrome *c* was measured in the presence of a catalytic amount of cytochrome *c* oxidase (0.5%) in a rotating cell to avoid photoreduction. Ferrocytochrome *c* was obtained by titrating with dithionite and monitoring the oxidation-state marker band $\nu_4$ in the RR spectrum.

*Resonance Raman Measurements.* The RR spectra were measured with 413-nm excitation using a scanning double monochromator with a spectral resolution of 2.8 $cm^{-1}$ and a step width of 0.2 $cm^{-1}$. Details of the equipment and the band-fitting procedure are described elsewhere (Heibel et al., 1993). All spectra were recorded by repetitive scanning at room temperature. Comparison of the individual scans verified the integrity of the sample during the RR experiment. The principles of RR experiments using the dual-channel detection technique for quasi-simultaneous measurements have been described in detail elsewhere (Hildebrandt et al., 1990).

*Data Analysis.* The RR spectra were analyzed by a band-fitting program as described elsewhere (Heibel et al., 1993). Typical values of the standard deviations for the frequencies, half-widths, and intensities of most of the peaks were about 0.1 $cm^{-1}$, 0.4 $cm^{-1}$, and 3%, respectively. The accuracy was less for weak and broad bands adjacent to strong bands. To determine possible frequency shifts caused by H/D exchange reliably, the same frequencies and half-widths were used as initial parameters for the band-fitting of the spectra of $Cyt^{3+}(H_2O)$ and $Cyt^{3+}(D_2O)$ and kept constant for the first iteration cycles, allowing only the relative intensities to vary. In subsequent iteration cycles, the half-widths and frequencies were progressively released to optimize the fits.

## RESULTS AND DISCUSSION

The RR spectrum of iso-1-ferrocytochrome *c* ($Cyt^{2+}$) in $H_2O$ in the region between 240 and 500 $cm^{-1}$ is shown in Figure 1A. It displays a large number of sharp and well-resolved bands. Thus, the band-fitting analysis is straightforward and yields the spectral parameters as listed in Table 1. The assignment of these bands has been discussed elsewhere (Hildebrandt et al., 1991). At this point, we focus on the bands at 442.1 and 448.8 $cm^{-1}$ which we have attributed to porphyrin modes, including major contributions from the propionate bending vibrations. This spectral region is expanded in Figure 1C. The corresponding spectrum of $Cyt^{2+}$ in $D_2O$ is displayed in Figure 1B,D. Visual inspection of both spectra already reveals far-reaching similarities except for the asymmetric peak at ~446 $cm^{-1}$ ($D_2O$). The band-fitting yields two components at 440.6 and 446.4 $cm^{-1}$ in $D_2O$ which are downshifted compared to the corresponding bands in $Cyt^{2+}(H_2O)$. Although determination of the spectral parameters of the low-frequency component is less certain due to the weak intensity of this band, it is evident that both components of the composite peak are affected upon H/D exchange. Even if these bands arise solely from a pure propionate bending vibration and the carboxylate groups are protonated, the isotopic mass effect should lead to a frequency shift of less than 1 $cm^{-1}$. Moreover, at pH 7 the propionate groups are hydrogen-bonded to adjacent amino acids. Hence, an alternative explanation must hold for the observed frequency shifts in this region. Taking into account that the strengths of the hydrogen and deuterium bonds are different, we conclude that H/D exchange leads to an alteration in the structure of the hydrogen-bonded propionate groups which in turn is reflected by the frequency shifts, the changes of the half-widths, and the changes in relative intensities.

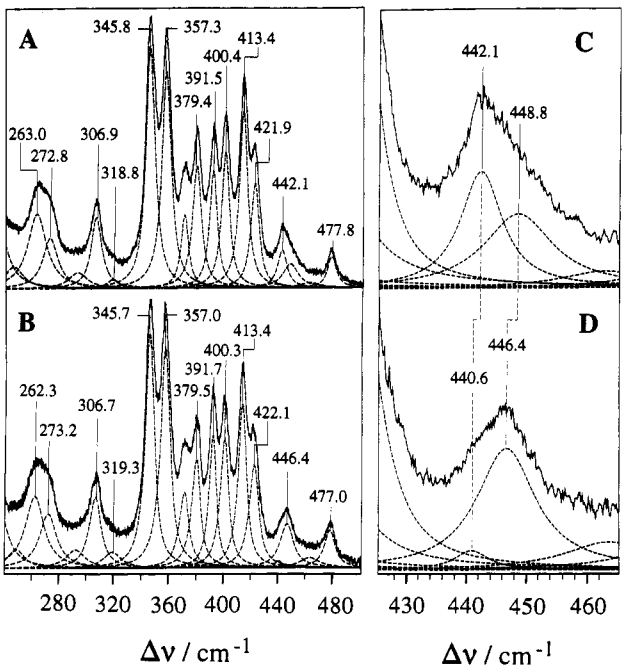


FIGURE 1: RR spectra of $Cyt^{2+}$ dissolved in $H_2O$ (A) and $D_2O$ (B) in the region between 240 and 500 $cm^{-1}$. Panels C and D are expanded views of the region between 425 and 465 $cm^{-1}$. The dashed lines represent the fitted Lorentzian line shapes.

For $Cyt^{3+}$, we also note distinct isotopic effects for these modes in $H_2O$ and $D_2O$ (Figure 2). Band-fitting yields two components at 443.8 and 452.4 $cm^{-1}$ in $H_2O$ and at 443.4 and 448.2 $cm^{-1}$ in $D_2O$. Apparently, in contrast to the reduced form, only the high-frequency component is shifted upon H/D exchange. An alternative interpretation may relate the 443.8-$cm^{-1}$ ($H_2O$) and 448.2-$cm^{-1}$ ($D_2O$) bands and those at 452.4 ($H_2O$) and 443.4 $cm^{-1}$ ($D_2O$) because the relative intensities and the band widths of the conjugate bands are very similar. This would imply that the low-frequency component shifts up to higher frequencies in $D_2O$ and vice versa.

Table 1: Spectral Parameters[a] of Cytochrome *c* in the Frequency between 260 and 500 $cm^{-1}$

| oxidized | | | | reduced | | | |
|---|---|---|---|---|---|---|---|
| $H_2O$ | | $D_2O$ | | $H_2O$ | | $D_2O$ | |
| $\nu$ ($\Delta\nu$) | $I_{rel}$ | $\nu$ ($\Delta\nu$) | $I_{rel}$ | $\nu$ ($\Delta\nu$) | $I_{rel}$ | $\nu$ ($\Delta\nu$) | $I_{rel}$ |
| | | | | 263.0 (16.7) | 0.42 | 262.3 (16.9) | 0.44 |
| 274.4 (11.1) | 0.24 | 274.4 (14.6) | 0.23 | 272.8 (13.6) | 0.28 | 273.2 (15.6) | 0.34 |
| 288.3 (6.2) | 0.09 | | | 293.3 (16.8) | 0.09 | 292.6 (17.3) | 0.11 |
| 305.5 (10.2) | 0.18 | 302.9 (16.1) | 0.16 | 306.9 (9.5) | 0.40 | 306.7 (10.5) | 0.43 |
| | | | | 318.8 (10.5) | 0.05 | 319.3 (16.0) | 0.10 |
| 348.9 (11.3) | 1.98 | 348.8 (10.2) | 1.91 | 345.8 (7.9) | 1.36 | 345.7 (7.9) | 1.43 |
| 361.2 (10.4) | 0.74 | 360.1 (10.8) | 0.83 | 357.3 (8.3) | 1.22 | 357.0 (8.5) | 1.34 |
| 375.0 (9.7) | 0.36 | 374.7 (7.5) | 0.28 | 370.6 (6.7) | 0.41 | 371.0 (8.5) | 0.46 |
| 382.2 (8.0) | 0.48 | 381.9 (7.1) | 0.43 | 379.4 (7.9) | 0.69 | 379.5 (7.8) | 0.66 |
| | | | | 391.5 (6.9) | 0.66 | 391.7 (6.5) | 0.80 |
| 398.1 (10.8) | 1.23 | 397.1 (11.8) | 1.08 | 400.4 (7.0) | 0.77 | 400.3 (7.5) | 0.79 |
| 413.3 (10.6) | 1.00 | 413.7 (11.4) | 1.00 | 413.4 (7.3) | 1.00 | 413.4 (7.3) | 1.00 |
| 419.9 (8.2) | 0.60 | 420.4 (7.1) | 0.49 | 421.9 (7.3) | 0.59 | 422.1 (8.7) | 0.62 |
| 443.8 (13.0) | 0.34 | 443.4 (7.5) | 0.14 | 442.1 (8.4) | 0.21 | 440.6 (6.9) | 0.04 |
| 452.4 (8.1) | 0.12 | 448.2 (10.5) | 0.25 | 448.8 (13.8) | 0.13 | 446.4 (11.8) | 0.27 |
| 464.1 (11.4) | 0.06 | | | 462.6 (17.2) | 0.03 | 463.4 (17.1) | 0.06 |
| 479.7 (8.2) | 0.15 | 478.8 (6.6) | 0.07 | 477.8 (7.6) | 0.18 | 477.4 (9.4) | 0.23 |
| 485.9 (15.2) | 0.07 | 483.9 (14.8) | 0.08 | | | | |

[a] Frequencies ($\nu$) and half-widths ($\Delta\nu$) are given in $cm^{-1}$, relative intensities ($I_{rel}$) refer to the 413-$cm^{-1}$ band.

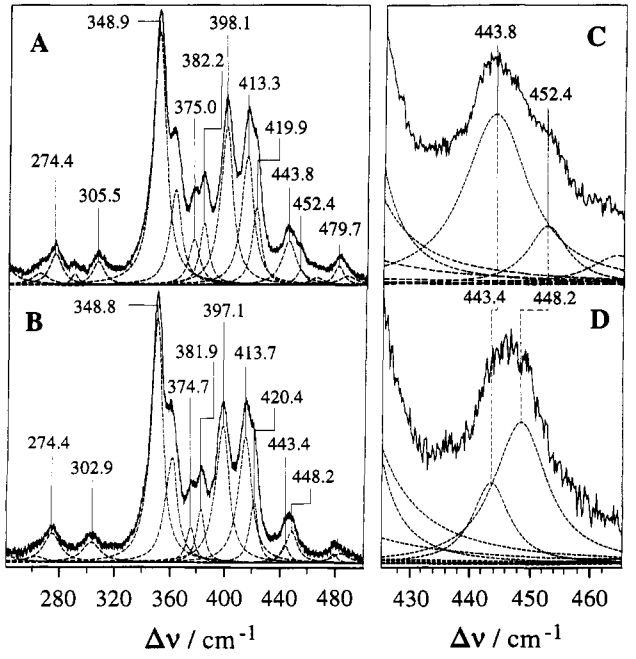


FIGURE 2: RR spectra of $Cyt^{3+}$ dissolved in $H_2O$ (A) and $D_2O$ (B) in the region between 240 and 500 $cm^{-1}$. Panels C and D are expanded views of the region between 425 and 465 $cm^{-1}$. The dashed lines represent the fitted Lorentzian line shapes.

Aside from the isotopic effects of these bands, only minor spectral changes occur in this frequency region. The only exception is a downshift and broadening of the 305.5-$cm^{-1}$ band to 302.9 $cm^{-1}$ in $Cyt^{3+}$; the corresponding band in $Cyt^{2+}$ at 306 $cm^{-1}$ was not affected upon H/D exchange. On the basis of a comparison of the RR spectra of a variety of heme proteins, Debois et al. (1984) proposed that this mode contains substantial deformations of the propionates. Thus, it may be that vibrations of these side chains contribute to both the 445- and the 305-$cm^{-1}$ bands. In fact, conformational changes in Cyt which are reflected by the doublet at ~445 $cm^{-1}$ are often paralleled by spectral changes of the 305-$cm^{-1}$ band, for example, in state II of Cyt bound to charged surfaces or in the acid form of $Cyt^{3+}$ (Hildebrandt, 1990).

An alternative assignment for the propionate bending in $Cyt^{2+}$ has been suggested by Hu and Spiro (unpublished results), who attributed these modes to the doublet at 371 and 379 $cm^{-1}$ while the (unresolved) peak at ~422 $cm^{-1}$ was ascribed to an out-of-plane mode (pyrrole swivel). The present data show that in contrast to the ~442/450-$cm^{-1}$ doublet the bands at 371 and 379 $cm^{-1}$ are not affected upon H/D exchange (except for a slight narrowing of the 371-$cm^{-1}$ band). These findings as well as the arguments given in our previous work (Hildebrandt, 1990, 1991; Hildebrandt et al., 1991, 1992) led us to retain the original assignment of the two bands between 440 and 450 $cm^{-1}$. However, a definite assignment will only be possible based on RR data of Cyt with isotopically labeled heme propionates.

While different hydrogen bonding and deuterium bonding interactions of the propionate side chains provide a plausible

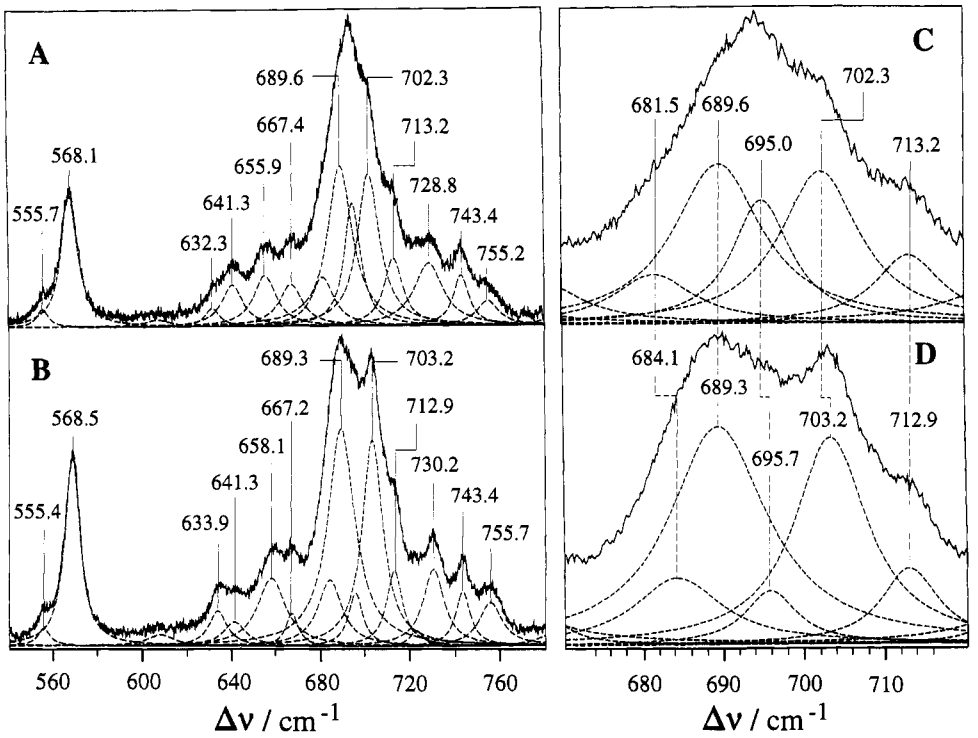


FIGURE 3: RR spectra of $Cyt^{3+}$ dissolved in $H_2O$ (A) and $D_2O$ (B) in the region between 540 and 780 $cm^{-1}$. Panels C and D are expanded views of the region between 670 and 720 $cm^{-1}$. The dashed lines represent the fitted Lorentzian line shapes.

explanation for the spectral changes in the low-frequency range of $Cyt^{2+}$ and $Cyt^{3+}$, surprising isotopic effects are noted in the region between 540 and 780 $cm^{-1}$. The corresponding spectra of $Cyt^{3+}$ in $H_2O$ and $D_2O$ are shown in Figure 3A,B. In $H_2O$, the spectrum is dominated by a broad and asymmetric peak at ~685 $cm^{-1}$ which exhibits several shoulders and smaller bands on both wings of the envelope. In $D_2O$, the central peak is replaced by a doublet constituted by two similarly strong peaks. The band-fitting analysis was straightforward below 670 and above 720 $cm^{-1}$ because the number of bands and their approximate positions could be determined by visual inspection of the spectra, thereby providing good starting values for the fitting procedure. Between 670 and 720 $cm^{-1}$, we initially tried fitting these spectra with four components, but no satisfactory fit was possible with this assumption. Including a fifth component, however, allows for excellent simulation of the spectra as demonstrated by comparison of the residuals of the five- and four-component fits (Figure 4A,B).

The RR spectra of the reduced Cyt reveal similar behavior with distinct spectral changes induced by H/D exchange in the region between 670 and 720 $cm^{-1}$ (Figure 5). These spectra were analyzed in the same manner as described above for the oxidized cytochrome *c*. Again, the residuals of the fits demonstrate that five bands are required to simulate this region adequately (Figure 4C,D). The results of the fitting analysis of both the oxidized and the reduced proteins are listed in Table 2.

The assignment of the bands in this region is not yet established. The number of bands between 540 and 780 $cm^{-1}$ is much greater than that observed for other heme proteins and even exceeds the number of all the in-plane and out-of-plane porphyrin modes calculated for metalloporphyrins such as nickel octaethylporphyrin (Li et al., 1989, 1990a,b). The most plausible explanation for this large number of bands is that the admixture of internal vibrations of the heme substituents to those of the porphyrin cannot be neglected in this region. This conclusion implies that symmetry selection rules that are helpful for analyzing the vibrational pattern of more symmetric porphyrin systems break down in the analysis

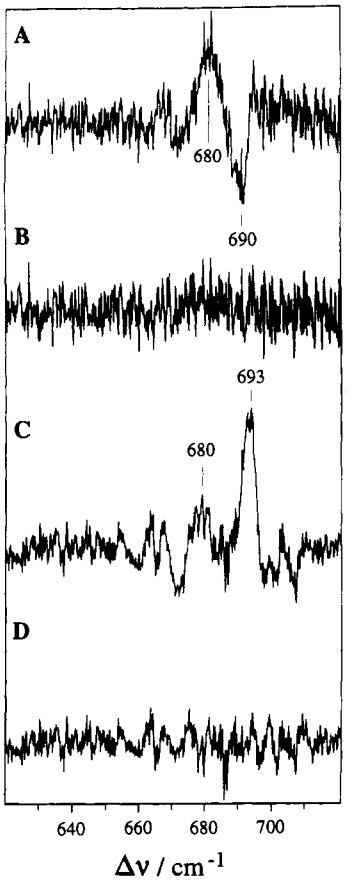


FIGURE 4: Residuals of the fitted spectra of $Cyt^{3+}$ and $Cyt^{2+}$ in the region between 620 and 720 $cm^{-1}$. (A) $Cyt^{3+}$ dissolved in $H_2O$ using four components. (B) $Cyt^{3+}$ dissolved in $H_2O$ using five components. (C) $Cyt^{2+}$ dissolved in $D_2O$ using four components. (D) $Cyt^{2+}$ dissolved in $D_2O$ using five components.

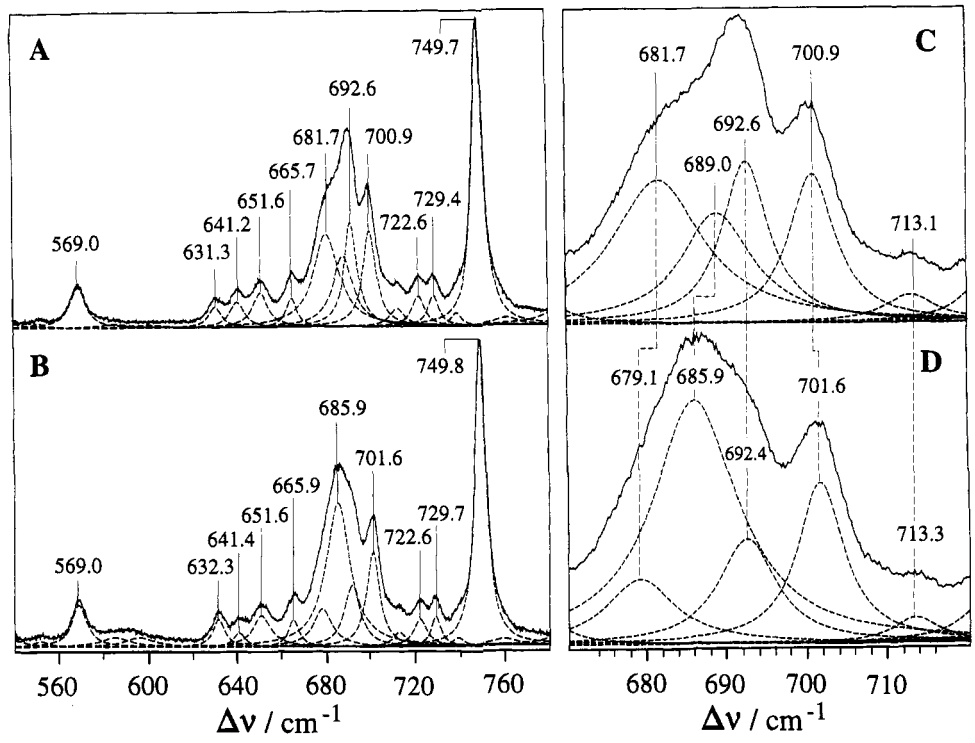


FIGURE 5: RR spectra of $Cyt^{2+}$ dissolved in $H_2O$ (A) and $D_2O$ (B) in the region between 540 and 780 $cm^{-1}$. Panels C and D are expanded views of the region between 670 and 720 $cm^{-1}$. The dashed lines represent the fitted Lorentzian line shapes.

of cytochrome *c* owing to the greater asymmetry in heme substituents. In the limiting case, modes of such asymmetric porphyrins should be localized in the individual pyrrole rings as found for linear tetrapyrroles (Smit et al., 1993). Consequently, the number of RR-active bands increases. For example, in highly symmetric porphyrins, the RR spectra display a single band between 710 and 670 $cm^{-1}$ which is readily assigned to the mode $\nu_7$ calculated for $D_{4h}$ symmetry (Czernuszewicz et al., 1989). In contrast, the RR spectra of $Cyt^{2+}$ and $Cyt^{3+}$ display four bands of relatively strong intensity in this region (Figures 5C, 5D, 3C, and 3D). Alternatively, one may envisage contributions from C–S stretching vibrations of the thioether bonds of the heme as suggested by Hu and Spiro (unpublished results). However, we note that structural changes in the region of the thioether bridges which are brought about by substitution of Phe-82 by Gly or Ser (Louie et al., 1988; Gao et al., 1991; Louie & Brayer, 1989) are not reflected by changes in the RR spectrum between 670 and 710 $cm^{-1}$ (unpublished results).

Comparison of the RR spectra in Figures 5C, 5D, 3C, and 3D reveals distinct frequency shifts upon H/D exchange for some of the bands. In $Cyt^{3+}$, the band at 681.5 $cm^{-1}$ is upshifted by 2.6 $cm^{-1}$ in $D_2O$. In the reduced form, both bands at 681.7 and 689.0 $cm^{-1}$ are downshifted by 1.6 and 3.1 $cm^{-1}$ in $D_2O$. In addition, the intensity distribution between both bands is drastically altered although the sum of the relative intensities of both bands is the same in $H_2O$ and $D_2O$. Also, other bands in the vicinity of 680 $cm^{-1}$ exhibit frequency shifts >1 $cm^{-1}$ [e.g., bands at 632.3 (631.3), 655.9, and 728.8 $cm^{-1}$ of $Cyt^{3+}$ ($Cyt^{2+}$)]. In addition, some of these bands exhibit substantial changes in their relative intensities.

We have carefully investigated the entire range of the porphyrin fundamentals from 120 to 1700 $cm^{-1}$. However, above 800 $cm^{-1}$, the spectral differences between the $H_2O$ and $D_2O$ solutions are very small and generally do not involve frequency or half-width changes greater than ±1 $cm^{-1}$ or intensity changes exceeding ±10%. The only exception to this generalization is the mode $\nu_{10}$ in $Cyt^{3+}$ that is located at 1636.5 $cm^{-1}$. The frequency and the relative intensity of this band are unchanged, but the half-width is decreased from 10.5 $cm^{-1}$ in $H_2O$ to 8.7 $cm^{-1}$ in $D_2O$.

We also attempted to determine the time dependence of the spectral changes induced by H/D exchange. The most pronounced spectral changes (i.e., in the region between 670 and 720 $cm^{-1}$) were complete within the time required for acquisition of one spectrum (~15 min). Also, the frequency shifts in the propionate bending region occur faster than 15 min. Increasing the ionic strengths of the protein solutions to 0.1 M did not reduce the exchange rates sufficiently to permit its observation with either the reduced or the oxidized cytochrome *c* (Liu et al., 1989).

Rate constants for the H/D exchange of many of the amide protons have been determined by Englander and co-workers using NMR spectroscopic techniques (Wand et al., 1986; Mayne et al., 1992). In general, the values are substantially smaller for the reduced than for the oxidized cytochrome *c*, and they decrease significantly with increasing ionic strength. In any case, the exchange rates of most of the amide protons are much slower than the time resolution of the present experiment. Thus, we conclude that the RR spectral changes observed in this study do not result from alterations of the hydrogen bonding interactions of the amide backbone. The only exchange rate constants of amide protons that are of the same order of magnitude as the time resolution of the RR experiment are those of Asn-52, Met-80, and Lys-79. These residues are located in the heme pocket and are hydrogen-bonded to the side chains of Thr-49 and Thr-78 and to heme propionate group D (Louie & Brayer, 1990; Berghuis & Brayer, 1992). This observation implies that the side chain protons of Tyr-67, Thr-78, Asn-52, Trp-59, and Thr-49 as well as the water molecules 121 and 166 (which are part of an extended hydrogen bonding network on the Met-80 side of the heme and the deeper part of the heme pocket) are also exchanged on the same time scale or even faster. Since these

Table 2: Spectral Parameters[a] of Cytochrome *c* in the Frequency between 550 and 770 $cm^{-1}$

| oxidized | | | | reduced | | | |
|---|---|---|---|---|---|---|---|
| $H_2O$ | | $D_2O$ | | $H_2O$ | | $D_2O$ | |
| $\nu$ ($\Delta\nu$) | $I_{rel}$ | $\nu$ ($\Delta\nu$) | $I_{rel}$ | $\nu$ ($\Delta\nu$) | $I_{rel}$ | $\nu$ ($\Delta\nu$) | $I_{rel}$ |
| 555.7 (6.5) | 0.13 | 555.4 (6.5) | 0.09 | 551.1 (6.6) | 0.11 | 551.6 (8.4) | 0.12 |
| 568.1 (8.8) | 1.00 | 568.5 (8.2) | 1.00 | 569.0 (8.6) | 1.00 | 569.0 (8.2) | 1.00 |
| | | | | 585.8 (15.2) | 0.09 | 585.3 (16.1) | 0.22 |
| 607.4 (14.5) | 0.05 | 608.0 (15.3) | 0.06 | 600.7 (15.4) | 0.07 | 596.2 (16.1) | 0.22 |
| 632.3 (8.1) | 0.14 | 633.9 (9.7) | 0.18 | 631.3 (6.9) | 0.5 | 632.3 (6.5) | 0.67 |
| 641.3 (11.6) | 0.31 | 641.3 (12.3) | 0.13 | 641.2 (7.9) | 0.62 | 641.4 (6.2) | 0.34 |
| 655.9 (12.2) | 0.38 | 658.1 (15.1) | 0.35 | 651.6 (8.5) | 0.84 | 651.6 (9.9) | 0.75 |
| 667.4 (12.7) | 0.31 | 667.2 (6.8) | 0.17 | 665.7 (5.9) | 0.70 | 665.9 (5.8) | 0.64 |
| 681.5 (12.1) | 0.37 | 684.1 (13.2) | 0.35 | 681.7 (13.8) | 2.27 | 679.1 (10.5) | 0.91 |
| 689.6 (13.2) | 1.19 | 689.3 (14.6) | 1.14 | 689.0 (10.8) | 1.74 | 685.9 (12.8) | 3.43 |
| 695.0 (8.8) | 0.91 | 695.7 (7.8) | 0.28 | 692.6 (7.0) | 2.55 | 692.4 (8.6) | 1.46 |
| 702.3 (11.9) | 1.13 | 703.2 (11.0) | 1.08 | 700.9 (7.1) | 2.35 | 701.6 (7.0) | 2.25 |
| 713.2 (10.2) | 0.50 | 712.9 (8.6) | 0.39 | 713.1 (8.0) | 0.44 | 713.3 (7.1) | 0.35 |
| | | 722.6 (6.8) | 0.08 | 722.6 (7.0) | 0.74 | 722.6 (7.8) | 0.66 |
| 728.8 (15.6) | 0.46 | 730.2 (11.5) | 0.40 | 729.4 (6.1) | 0.74 | 729.7 (5.2) | 0.72 |
| 743.4 (8.6) | 0.37 | 743.4 (8.0) | 0.28 | 739.6 (5.7) | 0.32 | 739.6 (5.9) | 0.21 |
| 755.2 (11.7) | 0.19 | 755.7 (12.3) | 0.22 | 749.7 (5.8) | 7.42 | 749.8 (5.7) | 7.36 |
| | | | | 761.7 (13.7) | 0.19 | 759.8 (15.0) | 0.15 |

[a] Frequencies ($\nu$) and half-widths ($\Delta\nu$) are given in $cm^{-1}$; relative intensities ($I_{rel}$) refer to the 569-$cm^{-1}$ band.

hydrogen bonding interactions also include both heme propionate groups, it is readily understood why the time dependence of the isotopic shifts of the propionate bending modes could not be resolved in the present RR experiments.

It should be noted that in previous UV RR experiments, Liu et al. (1989) found a much slower exchange rate for the indole proton of Trp-59 even at low ionic strengths. These earlier experiments were carried out in the absence of phosphate ions which are known to bind specifically to the lysine-rich domain on the front surface of Cyt. Whether or not this difference in experimental conditions, is the origin for this discrepancy remains to be clarified in further experiments.

The rapid H/D exchange of water molecules 121 and 166 in the heme pocket can also account for the band narrowing of $\nu_{10}$. In $H_2O$, vibrational energy transfer can take place from this delocalized porphyrin mode to the bending mode of the water molecule at $\sim$1635 $cm^{-1}$. The bending mode of $D_2O$ is at $\sim$1200 $cm^{-1}$ so that no vibrational energy transfer is possible, and the lifetime of the vibrationally excited state is increased as reflected by the narrowing of the band width of $\nu_{10}$ by 1.8 $cm^{-1}$. Similar effects have been observed in the RR spectra of other chromoproteins [cf. Hildebrandt and Stockburger (1984), Hashimoto et al. (1986), and Sassaroli et al. (1989)].

The isotopic effects in the RR spectra of Figures 3 and 5 are more difficult to understand. Changes of the relative RR intensities might be brought about by differences of the charge distribution or dielectric constant in the heme pocket at pH 7.0 and pD 7.0, due to alterations of the p$K_a$ values of amino acid side chains upon H/D exchange. However, this explanation can be ruled out since we have found that the RR spectra of $Cyt^{2+}(H_2O)$ are identical in the pH range between 6.0 and 8.0. The majority of the modes in the range between 600 and 700 $cm^{-1}$ which are affected upon H/D exchange are most likely dominated by $C_aC_mC_a$ and $C_aC_m$ bending vibrations of the porphyrin and pyrrole folding motions (Li et al., 1989, 1990a,b). Hence, the observed isotopic effects apparently do not reflect modified hydrogen bonding interactions of the propionate side chains but subtle conformational changes of the entire tetrapyrrole macrocycle. These changes may result from alterations of the heme pocket structure arising from displacement of amino acids or internal water molecules in the heme pocket which in turn would affect steric interactions with the porphyrin and its substituents.

The enthalpies of formation of hydrogen and deuterium bonds may differ by up to 20 kJ/M (Joesten & Schaad, 1974). Bond length differences between the donor and the acceptor atoms are of the order of a few hundredths of an angstrom (Olovsson & Jönsson, 1976). On first sight, it is surprising that such subtle differences can affect the geometry of the porphyrin macrocycle. However, taking into account the complex hydrogen bonding network in this part of the heme pocket, one may imagine that these effects combine in a cooperative manner. In addition, we are not able to interpret the observed spectral changes of the porphyrin modes quantitatively in terms of bond length and bond angle changes.

Evidently, the deuterium-induced effects are smaller than the redox-linked structural changes which are reflected by more pronounced differences in the RR spectra of $Cyt^{3+}$ and $Cyt^{2+}$. Nevertheless, the present results indicate that the hydrogen bonding network on the Met-80 side of the heme plane and in the vicinity of the heme propionates is crucial not only for controlling the electrostatic interactions in the heme pocket and the charge distribution in the heme (Cutler et al., 1989; Davies et al., 1993) but also for controlling the molecular geometry of the porphyrin.

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