Redox-Dependent Hydration of Cytochrome *c* and Cytochrome b_5 Studied through ^{17}O NMRD

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Electrochemical studies have shown that in cytochromes *c* and b_5 the enthalpy of reduction is negative and is the major contribution to the free energy of the process.[1] There is also an entropic contribution, which is however smaller. It is the sum of several contributions, like redox-dependent mobility of the protein and variations in protein hydration. The definition of hydration is complex, as it involves water population of specific cavities, accessibility of specific protein regions to solvent molecules, or aspecific hydration.[2] In every case, it is difficult to directly monitor hydration. In some cases, high-resolution X-ray structures provide a number of ordered water molecules interacting with the protein, but no information on their lifetime is available nor, sometimes, on the occupancy of the interaction sites.[3] High-resolution NMR experiments based on the observation of NOEs between water and protein protons also provide information on the presence of long-lived water molecules.[4] With this in mind, we decided to use the ^{17}O nuclear magnetic relaxation dispersion (NMRD) technique[5] to study the hydration of one specimen each of the cytochrome *c* and b_5 classes in the two accessible oxidation states. Calculations show that the paramagnetism of the oxidized state contributes negligibly to ^{17}O relaxation.

Water ^{17}O NMR measurements on 10% ^{17}O-enriched water samples were performed between 4.0 and 108.5 MHz (for ^{17}O, corresponding to 29.5 and 800 MHz for protons) with commercially available instruments and with a Bruker CXP 90 electromagnet with variable field capability. The solutions contained horse heart cytochrome *c* (Sigma, St. Louis, MO) 6.1 mM in 100 mM phosphate buffer, pH 5.6, and 2 and 1.4 mM oxidized and reduced rabbit liver cytochrome b_5 in 100 mM phosphate buffer at pH 5.6 and 6.5. The reduced proteins were obtained from the native oxidized species using a slight molar excess of ascorbate or dithionite and stored anaerobically.[6]

Figure 1 shows the four NMRD profiles of water ^{17}O T_1^{-1} versus B_0 (expressed as ^{17}O Larmor frequency) at 300 K, where the bulk water relaxation has been subtracted and the data have been normalized to a water:protein molar ratio of 1. Data at lower temperature (not shown) indicate that the relaxation values are not limited by exchange. Under these conditions, the longitudinal quadrupolar relaxation rate enhancement of ^{17}O due to the interaction with the protein, $R_1 - R_{bulk}$, is given by the sum of two contributions (eq 1):

$$R_1 - R_{bulk} = \alpha + \beta\tau_c\left(\frac{0.2}{1+\omega_I^2\tau_c^2} + \frac{0.8}{1+4\omega_I^2\tau_c^2}\right) \quad (1)$$

$$\alpha = (N_S/N_T)(\langle R_S\rangle - R_{bulk}) \qquad \beta = (N_I^*/N_T)(\omega_Q S_I)^2$$

where ω_I is the ^{17}O Larmor frequency in rad s^{-1},[7] N_S, N_I^*, and N_T are the number of surface water molecules, the apparent number of long-lived water molecules, and the total number of water molecules per protein molecule in the solution, respectively, $\langle R_S\rangle$ is the average intrinsic spin relaxation rate of surface water, $\omega_Q = 7.61 \times 10^6$ rad s^{-1} is the rigid-lattice nuclear quadrupole frequency,[8] and S_I is the average (rms) orientational order parameter for the ^{17}O of long-lived water molecules.

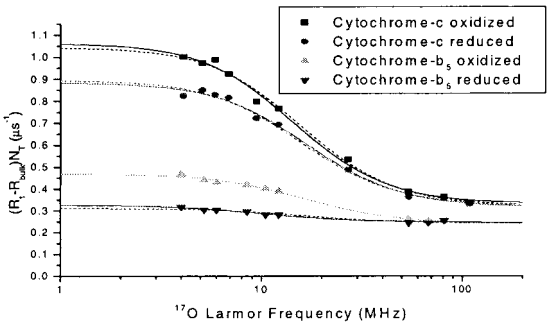

Figure 1. 300 K NMRD profiles (expressed as relaxation enhancements times the total number of water molecules per protein molecule, N_T) of water solutions containing oxidized/reduced cytochrome *c* or b_5. The estimated error bars are 3 times the size of the points.

In eq 1, the parameter α represents the high-field plateau, $\beta\tau_c$ is the difference between the low- and the high-field plateaus, and τ_c is the correlation time for the quadrupolar relaxation of ^{17}O and is given by $\tau_c^{-1} = \tau_R^{-1} + \tau_M^{-1}$, where τ_R is the rotational correlation time of the protein and τ_M is the lifetime of the water molecule in the bound state.

For the water molecules that interact with the protein with a residence time much shorter than τ_R, their lifetime modulates ^{17}O relaxation, and if the lifetime becomes shorter than the reciprocal of the highest accessible Larmor frequency (i.e., shorter than about 2 ns), no field dependence is observed. The contribution of these water molecules is thus represented by the α parameter. The β parameter is related to orientationally ordered and long-lived water molecules, i.e., water molecules with a residence time longer than, or of the order of, τ_R. Equation 1 qualitatively predicts that the difference between the low- and the high-field plateau, $\beta\tau_c$, is proportional to the number of long-lived protein-bound waters. For proteins of the size of the present cytochromes, one orientationally ordered long-lived water should contribute about 0.25 μs^{-1} to the normalized relaxation enhancement at low field.

Data Analysis. The dispersion curves of the four proteins investigated have been fitted to eq 1, and the results are shown in Table 1 and Figure 1. The fits were performed by either letting τ_c be an independent parameter for all four samples (solid curves) or assuming a single τ_c for each of the two proteins (dashed curves). In the latter case, an average best-fit value of τ_c was obtained for cytochrome *c*, while for cytochrome b_5 the same value obtained for the oxidized protein was imposed to the reduced one. The τ_c parameters obtained using the latter procedure are shown in the third column of Table 1. The last column of Table 1 reports the quantity $N_I^*S_I^2$, obtained from the low-field dispersions and the τ_c values. It represents the minimal number of long-lived waters, assuming they are orientationally ordered

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Table 1. Best-Fit Parameters Obtained from Fitting Eq 1 to the Data in Figure 1

protein	N_S ($\langle R_S\rangle/R_{bulk} - 1) \times 10^3$	τ_c (ns)	$N_I^* S_I^2$
ox. cyt-c	2.40 ± 0.12	5.7 ± 0.5	2.19 ± 0.16
red. cyt-c	2.40 ± 0.12	5.7 ± 0.5	1.74 ± 0.16
ox. cyt-b5	1.77 ± 0.06	5.5 ± 0.6	0.81 ± 0.09
red. cyt-b5	1.80 ± 0.03	5.0	0.22 ± 0.02

($S_I^2 = 1$). If the long-lived waters experience some librational motions ($S_I^2 < 1$) and/or their lifetime is of the order of their intrinsic ^{17}O relaxation time or longer, their number can be larger. As proposed by Halle et al.,[8] the high-field plateau is related to the number of waters significantly different from bulk.

Results and Discussion. From the above analysis, two or three water molecules are apparently bound in a long-lived fashion to oxidized cytochrome *c*. The high-field plateau is the same for oxidized and reduced cytochrome *c*, within the error. So, it appears that surface hydration is not affected by the redox state. On the other hand, a difference of about 0.15 μs^{-1} in the low-field value of $\beta\tau_c$ between oxidized and reduced species is observed. This would correspond to a water molecule that is less orientationally ordered and/or has a lower occupation of its binding site in the reduced form. This information becomes quite meaningful if compared with information from high-resolution NMR and X-ray. Cytochrome *c* has a water molecule in the so-called catalytic site, close to the iron in the distal cavity.[9,10] The presence of this water was confirmed by NMR in the reduced[11] and oxidized[12] states. Another water molecule in the oxidized species forms H bonds with Gln-42 N, Lys-39 O, and one oxygen of the heme propionate 7. This water molecule was detected by NMR[11,12] and is consistent with X-ray data.[10] However, the structure of the reduced species both in the solid state and in solution shows that propionate 7 changes its hydrogen bond network upon reduction.[10,13] Furthermore, the NH group of Gln-42 has been found to be more mobile in the reduced form in the subnanosecond to nanosecond time scales.[14] Therefore, it is conceivable that this binding site is less occupied and/or its water is less orientationally ordered. A third water binding site is present[10,11,15] which could also have a smaller population in the reduced species as a result of the decrease of the positive charge of iron, while the evidence for two or three additional long-lived waters[11] is less firm.[15] In any case, the chemical sense suggests that the water binding site most affected by reduction is the one involving propionate 7 and Gln-42.

Also in the case of cytochrome b_5, the low-field data show that the oxidized form interacts with a water molecule or a fraction of it with a residence time longer than τ_c, which apparently is not present in the reduced species. An analysis similar to that of cytochrome *c* cannot be performed because of the lack of independent data. However, it appears that in both cases the oxidized species exhibits a larger fraction and/or higher orientational order of long-lived water molecules than the reduced species. This could be due to the presence of a positive unit charge on the iron(III)−heme moiety, which is lacking in the iron(II)−heme moiety. We recall that proteins possess many positive and negative charges exposed on the surface, but their contribution to electrostatic interactions is quenched by the high solvent dielectric. Conversely, a single charge, when buried in the "low dieletric" interior of the protein as is the case for the iron(III)−heme moiety in these systems, can play a much stronger electrostatic role.

Liberation of one full water molecule from immobilized in the oxidized form to free in the bulk solvent in the reduced form could contribute up to several tens of joules per kelvin per mole to the entropy of reduction.[16] As the entropy of reduction for cytochromes *c* and b_5 has been found to be negative by a similar amount,[1] it appears that another redox-dependent change may occur which has a negative entropy and is only partially compensated by the liberation of a water molecule. Such a process could be the decrease in protein mobility in both the subnanosecond and micro- to millisecond time scales, which is a known property of the present cytochromes.[14,17]

A final comment is due to the difference in high-field relaxivity between cytochrome *c* and cytochrome b_5 in both redox forms. An estimate of solvent exposure of each of the crystallographically defined surface waters in the two proteins[10,18] suggests that there are more semi-buried waters in cytochrome *c* than in cytochrome b_5. Molecular dynamics calculations on cytochrome *c*[19] suggest that several of these waters may have lifetimes of the order of 0.2−0.3 ns.

^{2}H NMRD data are sometimes collected to complement ^{17}O data, especially when the latter are in the exchange-limited regime.[8] These measurements have not been attempted in this case also because of the existence of ^{1}H NMRD data for cytochrome *c*.[20] The latter indicate that the contribution of exchangeable protein protons is particularly large for this protein[20] and thus may not be negligible, even for the quadrupolar ^{2}H nucleus.

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