ENDOR and ESEEM Studies of Cytochrome *c* Oxidase: Evidence for Exchangeable Protons at the Cu_A Site†

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ABSTRACT: Electron nuclear double resonance (ENDOR) and electron spin echo envelope modulation (ESEEM) spectroscopies were used to study whether protons in the immediate protein environment around Cu_A in cytochrome *c* oxidase are susceptible to solvent exchange. The enzyme was incubated in buffered D_2O under resting or turnover conditions for 90 min and then frozen to quench the hydrogen/deuterium-exchange process. ENDOR spectra of the deuterated sample were essentially identical to those of control samples. The ESEEM spectra, however, provided a clear indication of the introduction of deuterium into the Cu_A environment following incubation in buffered D_2O. The extent of deuterium incorporation was not affected by enzyme turnover. An analysis of the ESEEM data indicated that water is in reasonably close proximity to the Cu_A site, but not in the immediate coordination sphere of the metal(s). We estimate a minimum distance of 5.4 Å between the Cu_A center and the protein/water interface. This relatively short surface separation distance is consistent with the role of Cu_A as the immediate oxidant of cytochrome *c* in the cytochrome oxidase (Hill, B. C. (1991) *J. Biol. Chem. 266,* 2219–2226).

Cytochrome *c* oxidase is the terminal protein in the mitochondrial respiratory chain and catalyzes the reaction in which molecular oxygen is reduced to water in a four-electron reduction. There are four redox-active metal centers in cytochrome *c* oxidase, two copper centers, Cu_A and Cu_B, and two iron atoms bound as heme *a* chromophores that are designated cytochromes *a* and a_3. Reduction of oxygen takes place at the binuclear metal center consisting of the cytochrome a_3 iron and the Cu_B center. Cu_A is the immediate oxidant of cytochrome *c* and is involved in a rapidly established redox equilibrium with cytochrome *a*; the latter metal transfers these reducing equivalents to the binuclear center. The protein spans the inner mitochondrial membrane and actively pumps protons out of the matrix by using the redox free energy made available during the catalytic cycle (Wikström, 1977; Chan & Li, 1990). The proton translocation activity contributes to the electrochemical gradient across the inner membrane that is used ultimately in the synthesis of ATP. A number of reviews of this important protein have appeared (Wikström et al., 1981; Naqui, et al., 1986; Babcock & Wikström, 1992; Malmström, 1993).

Proton currents in the enzyme are crucial to two aspects of its catalytic cycle. First, protons must be delivered in a controlled fashion to the binuclear center during the dioxygen reduction and bond cleavage process. Recent work by the Göteborg group (Oliveberg et al., 1991; Hallen & Nilsson, 1992) has begun to address this issue. Second, the proton-pumping function of the enzyme requires proton translocation through the enzyme. Both of these activities require that solvent protons exchange into the protein structure in an orderly fashion during catalysis. In addition, redox-linked changes in hydrogen-bond strength or protonation state changes upon oxidation reduction provide means by which to fine-tune the free energy changes associated with electron-transfer chemistry. The protons involved in all of these processes are expected to be exchangeable; several spectroscopic probes have the capability to detect these exchange processes. For magnetic techniques, in particular, relatively long-range interactions influence the spectral response and provide an opportunity to detect proton exchange remote from the magnetic center.

Although molecular models for proton pumps based on both cytochrome *a* (Babcock and Callahan, 1983) and Cu_A (Gells et al., 1986) have been postulated, recent evidence (Puustinen et al., 1989, Wikström, 1989) supports the binuclear center and specifically, Cu_B, as the site of the proton pump (see discussion in Chan and Li (1990) and Babcock and Wikström, (1992)). The midpoint potential of Cu_A is independent of pH, which suggests that redox-state-dependent protonation changes are unlikely to occur for this center (Wikström et al., 1981). Nonetheless, establishing proton accessibility and exchange processes at the metal centers of cytochrome oxidase remains an important component in achieving a molecular-level understanding of its function. The occurrence of exchangeable protons has been established for cytochrome *a* by Raman spectroscopy (Copeland & Spiro, 1986; Sassaroli et al., 1989; Schnoover et al., 1988). The Cu_B/a_3 sites in the binuclear center are accessible to proton exchange by their nature and function in O_2 reduction. Exchangeable protons

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at the Cu_A site have not previously been observed.

The Cu_A site has generated renewed interest owing to the demonstration by Hill (1991) that this center is the site of cytochrome *c* oxidation and to the possibility that the Cu_A species is a binuclear copper center (Kroneck et al., 1988; Farrar et al., 1991; Antholine et al., 1992). Recent discussions of the nuclearity of the Cu_A site and proposals as to its structure have not emphasized issues of its solvent accessibility. Kelly et al. (1993) and van der Oost et al. (1992), in developing a model in which the Cu_A site is composed of a dimer of copper ions, have provided data that indicate that the binding domain for Cu_A is located in a region of the subunit II sequence that is exposed to the aqueous milieu. Such a location is consistent with a role for this region in cytochrome *c* binding (Bisson & Montecucco, 1982; Millet et al., 1983; Taha & Ferguson-Miller, 1992) and oxidation (Hill, 1991).

In the experiments reported here, we have returned to the question of whether the Cu_A site is accessible to proton exchange by looking for the presence of magnetic hyperfine interaction between hydrogen and deuterium nuclei and the unpaired electron of Cu_A^{2+}. We used electron nuclear double resonance (ENDOR[1]) and electron spin echo envelope modulation (ESEEM) spectroscopies in this study and carried out the exchange reaction both under static conditions and with the enzyme following catalytic turnover. Our ENDOR results did not show any change upon deuterium exchange; our pulsed-EPR data, however, provide evidence for weakly coupled, solvent-exchangeable protons in proximity of Cu_A. Redox turnover of the enzyme did not increase the amount of deuterium at the Cu_A site.

MATERIALS AND METHODS

Sample Preparation. Beef-heart cytochrome oxidase was isolated as previously described (Babcock et al., 1976). Protein concentration was determined by measuring the absorbance of the reduced minus that of the oxidized enzyme at 605 nm. Half of the cytochrome oxidase preparation was dissolved in 6 mL of HEPES/lauryl maltoside buffer to an enzyme concentration of 70 μM, pH 7.4 in H_2O. Two milliliters of this solution was subjected to enzymatic turnover at 4 °C. Reduced cytochrome *c* (10 mM) was used as the reducing agent, enough for 35 redox cycles of the protein. Tetramethylphenylenediamine (TMPD) (20 μM) was added to mediate electron transfer. The enzyme was allowed to turn over for 50 min in a small beaker with oxygen gas blown over the surface to maintain O_2 available for reduction as the reaction proceeded. An additional 2 mL of the protein solution was incubated in the identical buffer for the same amount of time in the absence of reducing agent.

The other half of the protein preparation was dissolved in 6 mL of HEPES/lauryl maltoside, pH 7.4 in D_2O (pH corrected for deuterium isotope effect), also to an enzyme concentration of 70 μM. Two milliliters of this solution in deuterated buffer was subjected to enzymatic turnover in a manner identical to that for the sample in H_2O. A nonturnover sample was also prepared by exposing an identical protein sample to the deuterated buffer for the same time period.

After incubation or turnover, all samples were concentrated by precipitation with saturated ammonium sulfate. The precipitate was collected by centrifugation at 19 000 rpm and then redissolved in a minimal amount (200 μL) of appropriate buffer (HEPES/lauryl maltoside in H_2O or D_2O) (final enzyme concentration 450 μM). Each sample was immediately placed in a precision EPR tube and frozen in liquid N_2. The total time each sample was exposed to deuterated buffer before freezing was 90 min.

ENDOR Spectrometer Design and Data Acquisition. ENDOR spectra were obtained at X-band by using a Bruker ER200D spectrometer equipped with an ENDOR/triple accessory (Bruker ER250). The radio-frequency circuit was driven by an ENI 3100L power amplifier at frequencies generated by a Wavetek 3000-446 synthesizer. The microwave cavity resonator was of a TM type (Bruker 250ENB) and was fitted with a 17-turn coil of a free-standing design, a general description of which has been published previously (Bender et al., 1989). In order to maximize the rf field at the sample, the axial length of the coil was limited to approximately 2 cm (vs the 4-cm total cavity length). This configuration is compatible with recommended sample positioning within an Oxford ESR900 flow cryostat. Finally, the impedance of the rf circuit was matched to the 50-Ω output of the amplifier by using a Pi-network capacitive tank circuit (i.e., a two-variable capacitor shunt to ground that is spanned by the ENDOR coil). This high-*Q* matching technique was satisfactory for these experiments because of the narrow range of swept rf frequency that was used (5 MHz). The impedance match was achieved at the frequency corresponding to the midrange of the sweep. All experiments were performed at 4.2 K.

ESEEM Spectrometer Design and Data Acquisition. The electron spin echo experiments were performed with a home-built, pulsed-EPR spectrometer providing a 1-kW pulses of 12-ns duration (Britt et al., 1989). All ESEEM experiments were performed at 4.2 K with samples in quartz EPR tubes inserted into a loop-gap resonator probe immersed in liquid He (Britt & Klein, 1987). All ESEEM experiments on the EPR-detectable Cu^{2+} signal were performed with a spectrometer frequency of 9.1718 GHz at a magnetic field of 3200 G. The cytochrome a_3 EPR signal presents minimal background at this field. The two-pulse time domain ESEEM patterns were obtained by recording the amplitude of the electron spin echo with a boxcar averager as the interpulse time τ was varied in 10-ns increments. The three-pulse ESEEM patterns were obtained in similar fashion with the time T between microwave pulses II and III varied in 20-ns increments and the time between pulses I and II held fixed at 221 ns. Frequency domain ESEEM results were obtained by calculating the Fourier cosine transforms of the time domain data after reconstruction of the instrumental dead times with the Fourier backfill method described by Mims (1984).

RESULTS AND DISCUSSION

ENDOR and ESEEM spectroscopies provide high-resolution techniques by which to resolve nuclear hyperfine interactions that contribute to the broad lines that are characteristic of radicals and metal centers in immobilized biological samples. Both Scholes and co-workers (Stevens et al., 1982; Martin et al., 1988) and Hoffman and his colleagues (Hoffman et al., 1980) have used ENDOR to study nuclei that are strongly coupled to the unpaired electron in Cu_A^{2+}. A model for the ligation environment that involves two cysteine sulfurs and two nitrogens, at least one of which arises from a histidine residue, has emerged from this work. More recently, metal quantitative, spectroscopic analysis, and molecular genetic experiments forced a reconsideration of the nuclearity

[1] Abbreviations: ENDOR, electron nuclear double resonance; ESEEM, electron spin echo envelope modulation; EPR, electron paramagnetic resonance.

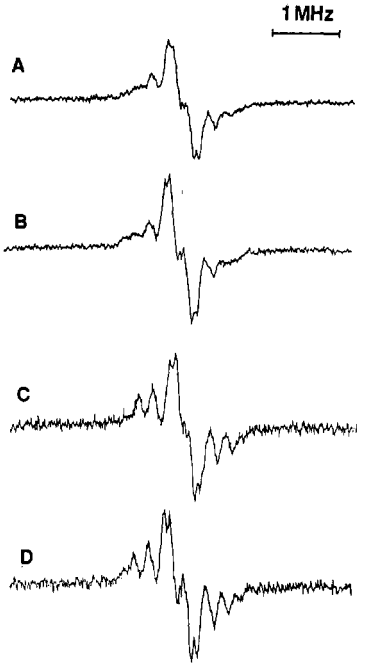

FIGURE 1: ENDOR spectra of cytochrome *c* oxidase: (A) distant powder ENDOR spectrum of native resting enzyme obtained at $g = 2.01$ ($g_\perp$); (B) distant powder ENDOR spectrum ($g = 2.01$) of enzyme turned over in D_2O buffer; (C) distant single, crystal-like ENDOR spectrum obtained by *g*-orientation selection at $g = 2.18$ ($g_\parallel$) of native resting enzyme; (D) distant single, crystal-like ENDOR spectrum at $g = 2.18$ of enzyme turned over in D_2O. Spectrometer conditions: microwave powder, 0.25 mW; rf power, 20 W; frequency modulation amplitude, ±15 kHz; temperature, 4.2 K.

and structure of the Cu_A center; the weight of the evidence now appears to favor a copper dimer structure with cysteine (two), histidine (two), and methionine (one) ligands (see Kelly et al. (1993)).

Scholes and co-workers have looked for more weakly coupled protons in the Cu_A site that were accessible to H_2O/D_2O solvent exchange, but they observed no changes in the ENDOR spectrum that could be attributed to deuterium incorporation (personal communication). The derivative mode of recording ENDOR spectra is superior to the absorption mode in resolving couplings in congested spectral regions. We have used this approach to obtain distant ENDOR spectra in the matrix region where hyperfine coupling to weakly interacting protons contributes. Figure 1A,B illustrates the matrix ENDOR line obtained at a field setting that corresponds to $g = 2.01$. The spectrum is characterized by two well-resolved features and a broad shoulder. We can further resolve the matrix spectrum by using *g*-orientation selection, and these data, recorded at $g = 2.18$ in the $g_\parallel$ region, are illustrated in Figure 1C,D. Three well-resolved features corresponding to weak dipolar couplings of less than 1 MHz are observed. In these experiments, the incident powers and modulation amplitude were deliberately kept minimal in order to obtain optimal conditions for resolving the distant ENDOR features, thus allowing us to use the technique to look further out from the Cu-coordination sphere into the protein (Lambe et al., 1961).

We have not identified the location of the nuclei responsible for the transitions observed in Figure 1; our principal interest in these ENDOR experiments has been to determine whether qualitative changes could be observed in the spectrum of the resting enzyme and of the enzyme following deuterium incorporation during turnover, thus indicating whether the region near the Cu_A site is accessible to solvent exchange. As can be seen from Figure 1, there are no changes in the relative intensities of the distant ENDOR peaks following deuterium incorporation by way of solvent exchange.

With the failure of ENDOR spectroscopy to reveal any hyperfine coupling to exchangeable protons in the Cu_A site, we utilized a second magnetic resonance technique, ESEEM, to study the hyperfine interactions. The two-pulse ESEEM data are similar to those previously published for the Cu_A site of beef-heart cytochrome oxidase (Mims et al., 1980). The two-pulse pattern shows a phase-memory decay of 1.5 μs. The decay pattern is modulated at the nuclear sublevel frequencies of nuclei magnetically coupled to the unpaired electron spin of the Cu^{2+} ion. The frequencies are revealed in the Fourier cosine transform of the ESEEM pattern shown in Figure 2A. The Fourier component at 13.7 MHz arises from weakly coupled protons resonating at or near the proton Larmor frequency corresponding to the 3200-G applied magnetic field. The lower-frequency transitions presumably arise from protein ^{14}N nuclei in the vicinity of the Cu_A^{2+} site.

Figure 2B displays the corresponding two-pulse ESEEM data for the cytochrome *c* oxidase sample incubated in D_2O for 90 min before freezing at 77 K. A new modulation component is observed at the 2.1-MHz Larmor frequency of the deuteron nucleus. This arises from deuterons introduced into exchangeable sites within a distance of several Å of the Cu_A^{2+} ion. The ESEEM data for the sample subjected to enzymatic turnover in D_2O are shown in Figure 2C. A 2.1-MHz feature is present in these data as well. We wish to distinguish between deuterons introduced by the enzymatic turnover process and those introduced by passive exchange processes. However, the short-phase memory, inherent in the two-pulse ESEEM experiment, limits the resolution and accuracy of quantification of this low-frequency transition.

Superior results may be obtained with the three-pulse "stimulated" ESEEM technique. The overall decay of the three-pulse echo envelope is much slower than that for the two-pulse echo envelope. Thus, low-frequency modulation may persist to higher values of the Fourier time variable ($T + \tau$) than can be observed in the corresponding two-pulse envelope. In addition, the large 13.7-MHz proton modulation can be suppressed by working at a value of the fixed time τ equal to a multiple of the proton precession period. This eliminates any interference from Fourier side lobes resulting from imperfect reconstruction of the instrumental dead time. Finally, we note that the modulation in the ESE envelope from multiple nuclei can be expressed approximately as the product of the modulation patterns from the individual-nuclei. Thus, we can isolate the contribution of deuterons introduced into the vicinity of the site by dividing the ESEEM pattern obtained for samples prepared with deuterated buffer by the pattern obtained from samples prepared with nondeuterated buffer. We obtain three-pulse ESEEM patterns for the nondeuterated samples that are similar to those previously published (Mims et al., 1980). The modulation from protein ^{14}N nuclei is shallow, with small Fourier peaks at 1.6, 1.9, and 3.1 MHz. The modulation due to deuterons can readily be extracted by using the ratio method described above, and we present only the ratioed data in this paper.

Figure 3A shows the three-pulse ratioed ESEEM data for the sample incubated in D_2O for 90 min. The corresponding data for the sample subjected to enzymatic turnover in D_2O are displayed in Figure 3B. The ESEEM pattern obtained from a sample subjected to enzymatic turnover in normal protonated buffer is used as the divisor for both sets of data. The peak at the deuteron Larmor frequency of 2.1 MHz is well isolated in both data sets. There are no other features

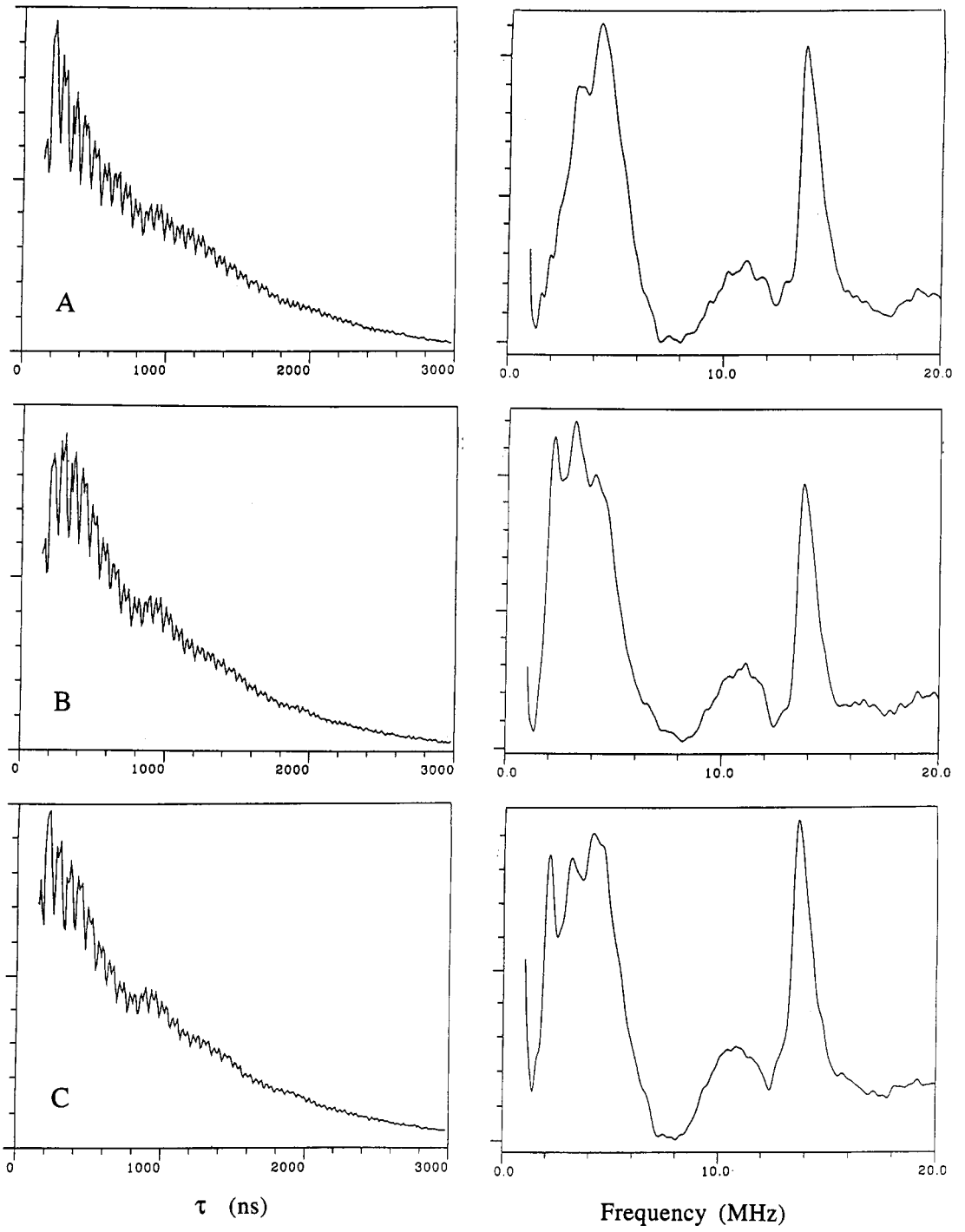

FIGURE 2: Time domain and Fourier cosine transform of two-pulse ESEEM data for the Cu_A site of cytochrome *c* oxidase: (A) following enzymatic turnover in H_2O; (B) following 90-min incuation in D_2O; (C) following enzymatic turnover in D_2O. All data were obtained at 4.2 K. The spectrometer frequency was 9.1718 GHz, and the magnetic field was set at 3200 G (g = 2.05).

present above the level of the background noise. The deuteron modulation is of similar amplitude for both samples. In order to compare the two data sets more rigorously, we divide the ESEEM spectrum of the sample subjected to enzymatic turnover in D_2O by the spectrum of the sample incubated in D_2O for the same time period (Figure 3C). There is no observed deuteron modulation present at a level above the background noise.

The initial modulation due to weakly coupled deuterons is approximately 10% of the overall echo amplitude in each of the samples prepared in the D_2O-enriched buffer. The signal-to-noise ratio of each isolated 2.1-MHz Fourier peak, corresponding to 10% modulation in the time-domain data, is approximately 6/1. Therefore, we estimate that a modulation greater than 0.10/6 = 0.016 would be detectable in Figure 3C. The absence of an observable 2.1-MHz peak in the ratioed data of Figure 3C therefore demonstrates a maximum modulation contribution from deuterons introduced by catalytic turnover but not by simple exchange of approximately 1.6%.

We can estimate the distance from the Cu^{2+} site at which a single deuteron would cause a 1.6% modulation in the ESE envelope. This allows us to set a limit on the distance a deuteron may be approaching in the turnover sample without being detected. We first assume a classical dipole–dipole magnetic interaction between the electronic and nuclear spins. As a first approximation, we ignore the small quadrupole moment

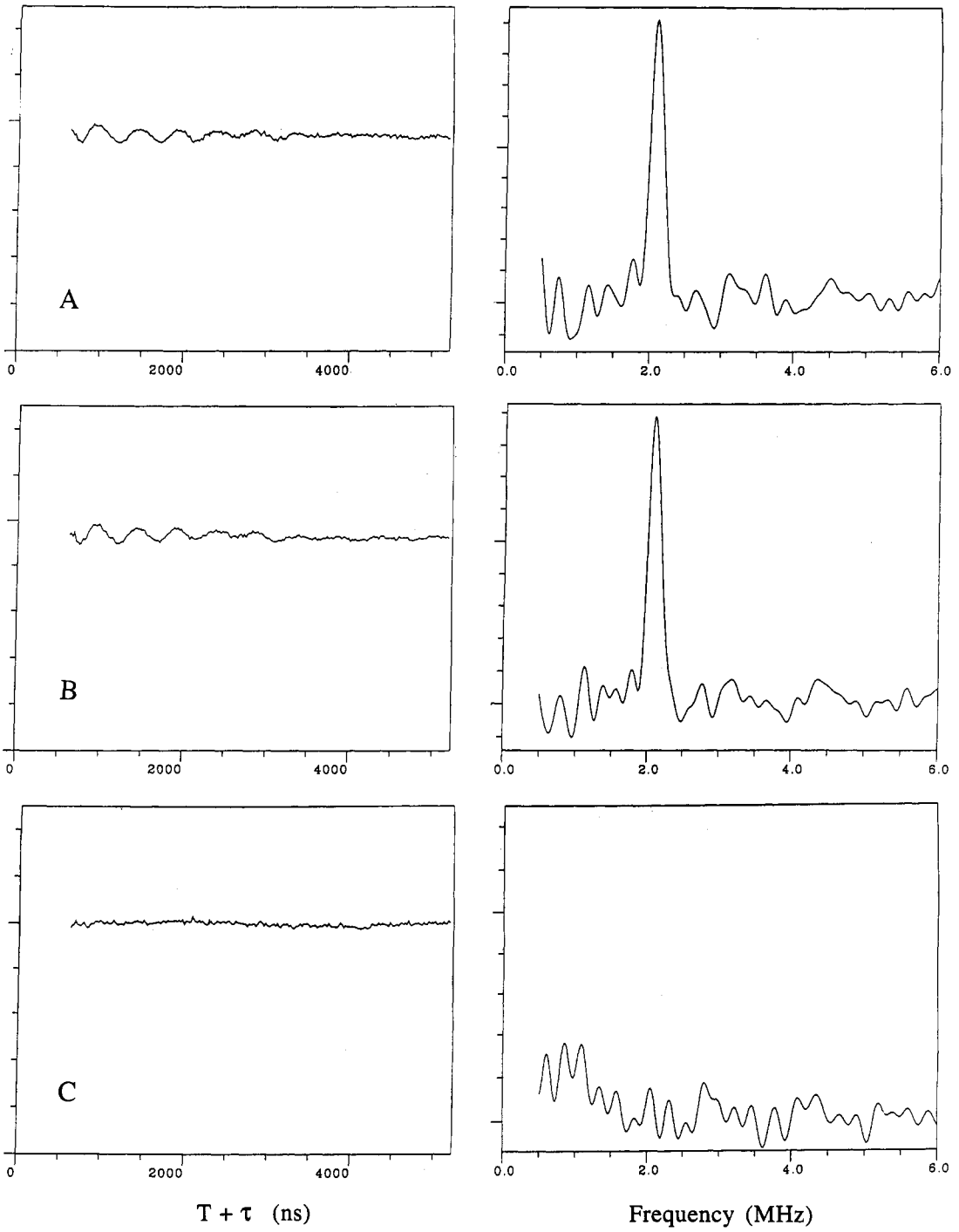

FIGURE 3: Time domain and Fourier cosine transform of ratioed three-pulse ESEEM data: (A) data from sample incubated in D_2O divided by data from sample subjected to enzymatic turnover in H_2O; (B) data from sample subjected to enzymatic turnover in D_2O divided by data from sample subjected to enzymatic turnover in H_2O; (C) data from sample subjected to enzymatic turnover in D_2O divided by data from sample subjected to incubation in D_2O. The time τ between pulses I and II was held fixed at 221 ns. All other spectrometer conditions are as in Figure 2.

of the spin $I = 1$ deuteron. We can write an expression for the three-pulse ESE modulation function to first order in the depth parameter k_D:

$$E_{mod}(\tau,T) = 1 - \frac{1}{2}k_D\left[\left(\sin^2\left(\frac{1}{2}\omega_\alpha\tau\right)\right)(1 - \cos(\omega_\beta(\tau + T))) + \left(\sin^2\left(\frac{1}{2}\omega_\beta\tau\right)\right)(1 - \cos(\omega_\alpha(\tau + T)))\right]$$

In this expression ω_α and ω_β are the angular frequencies of the $\Delta m_i = \pm 1$ nuclear sublevel transitions for the two electronic spin states. The depth parameter k_D is given by

$$k_D = \frac{8}{3}\left(\frac{\omega B}{\omega_\alpha\omega_\beta}\right)^2$$

where ω is the Larmor frequency of the deuteron and

$$B = \frac{gg_n\beta\beta_n}{\hbar r^3}(3\cos\theta\sin\theta)$$

is the angular frequency component that corresponds to the projection of the dipole-induced magnetic field that is

orthogonal to the applied magnetic field. In this expression, θ is the polar angle between the applied field and the vector of length $|r|$ between the electron and deuteron. In the weak coupling limit, $\omega_\alpha \approx \omega_\beta \approx \omega_I$, and the total modulation depth for the single component at angular frequency ω_I becomes approximately $(8/3)(B/\omega_I)^2$. The equation for the depth parameter can be modified by averaging over a sphere to account for all of the possible orientations of the electron–nucleus position vector with respect to the magnetic field, resulting in the following expression:

$$\overline{k_D} = \frac{16}{5}\left(\frac{g\beta}{H_0 r^3}\right)^2$$

(Mims et al., 1990). With our experimental parameters of $g = 2.05$ and $H = 3200$ G, this can be simplified to

$$\overline{k_D} = \frac{112}{r^6}$$

where r is expressed in angstrom units. The maximum modulation of 1.6% corresponds to a value of $k_D = 0.016/2 = 0.008$, which would arise from a deuteron 4.9 Å from the Cu(II).

A more rigorous approach to the analysis of the ESEEM data requires inclusion of the small electric quadrupolar coupling of the deuteron. The ESEEM amplitudes can be calculated numerically with the density matrix formalism presented by Mims (1972). The details of such calculations are described by Britt et al. (1989). We have used such a treatment to simulate the ESEEM powder patterns for deuterons weakly coupled to an electron. The results are relatively insensitive to the exact value of deuteron quadrupolar coupling parameters in the range of $e^2qQ \approx 0.1$–0.2 MHz expected for deuterons in biological environments. The simulations show a 1.6% modulation from a single deuteron at a distance of approximately 5.0 Å.

These results demonstrate that there are no deuterons introduced into the immediate vicinity of the Cu_A site (less than 5 Å) exclusively by enzymatic turnover in the 90-min time scale during which deuterons were allowed to exchange. However, exchangeable sites on the protein or in the buffer contribute 10% modulation after the 90-min incubation. Accessibility of exchangeable hydrogens in Cu enzymes has been previously studied with ESEEM (Mims et al., 1984). The modulation from deuterons exchanged into the vicinity of the Cu_A site is about 50% as deep as that observed for the Cu(II) site of the electron-transfer protein stellacyanin. The modulation is 58% as deep as that observed with azurin. If it is assumed that all of the modulation arises from deuterons in the buffer and exchangeable sites on the surface of the protein, the distance to the surface can be calculated for various geometric models. For example, the "hemispherical" distribution model, with a continuum of solvent deuterons occupying a 2π solid angle past a radial cutoff distance r_{min} (Figure 3C in Mims et al. (1984)), predicts the closest solvent deuterons to be 5.4 Å from the Cu_A site. However the true distance to the protein/water interface will be greater if interior hydrogen sites closer to the Cu center are exchangeable on the time scale of D_2O incubation. For example, in azurin, the hemispherical model predicts a distance of 4.6 Å to solvent deuterons, whereas the X-ray crystal structure shows a 7.8-Å distance from the Cu(II) to the protein/water interface (Adman et al., 1978). We therefore consider 5.4 Å to be an approximate minimum distance from the Cu_A site to the protein/water interface of the cytochrome c oxidase enzyme.

CONCLUSION

Our results clearly show the existence of exchangeable protons near the Cu_A metal center, although not in the inner coordination sphere. We are unable to detect any increase in deuterons in the immediate vicinity of the Cu_A site upon enzymatic turnover in the time scale of our experiments. This result is consistent with the growing evidence that the binuclear center is the site of proton pumping. The presence of exchangeable protons at the Cu_A center is an important finding that demonstrates accessibility of this metal center to solvent protons that may influence its role in electron transfer. The minimum distance from the Cu_A site to the protein/water interface of the cytochrome c oxidase enzyme is approximately 5.4 Å.

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