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^{17}O, ^{1}H, and ^{2}H Electron Nuclear Double Resonance Characterization of Solvent, Substrate, and Inhibitor Binding to the [4Fe-4S]$^{+}$ Cluster of Aconitase†

Melanie M. Werst,‡ Mary Claire Kennedy,§ Helmut Beinert,*,§,‖ and Brian M. Hoffman*,‡
Department of Chemistry, Northwestern University, Evanston, Illinois 60208, and Department of Biochemistry and National Biomedical ESR Center, Medical College of Wisconsin, Milwaukee, Wisconsin 53226
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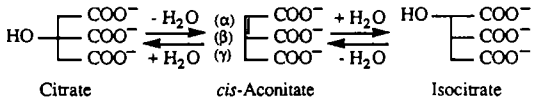
ABSTRACT: ^{17}O electron nuclear double resonance (ENDOR) studies at X-band (9-GHz) and Q-band (35-GHz) microwave frequencies reveal that the [4Fe-4S]$^{+}$ cluster of substrate-free aconitase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3] binds solvent, H_xO (x = 1, 2). Previous ^{17}O ENDOR studies [Telser et al. (1986) *J. Biol. Chem. 261*, 4840–4846] had disclosed that $H_x{}^{17}O$ binds to the enzyme–substrate complex and also to complexes of enzyme with the substrate analogues *trans*-aconitate and nitroisocitrate (1-hydroxy-2-nitro-1,3-propanedicarboxylate). We have used ^{1}H and ^{2}H ENDOR to characterize these solvent species. We propose that the fourth ligand of Fe_a in substrate-free enzyme is a hydroxyl ion from the solvent; upon binding of substrate or substrate analogues at this Fe_a site, the solvent species becomes protonated to form a water molecule. Previous ^{17}O and ^{13}C ENDOR studies [Kennedy et al. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8854–8858] showed that only a single carboxyl, at C-2 of the propane backbone of *cis*-aconitate or at C-1 of the inhibitor nitroisocitrate, coordinates to the cluster. Together, these results imply that enzyme-catalyzed interconversion of citrate and isocitrate does not involve displacement of an endogenous fourth ligand, but rather addition of the anionic carboxylate ligand and a change in protonation state of a solvent species bound to Fe_a. We further report the ^{17}O hyperfine tensor parameters of the C-2 carboxyl oxygen of substrate bound to the cluster as determined by the field dependence of the ^{17}O ENDOR signals. ^{17}O ENDOR studies also show that the carboxyl group of the inhibitor *trans*-aconitate binds similarly to that of substrate.

The enzyme aconitase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3] catalyzes the stereospecific interconversion of citrate and isocitrate via the dehydrated intermediate *cis*-aconitate.



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‡ Northwestern University.
§ Department of Biochemistry, Medical College of Wisconsin.
‖ National Biomedical ESR Center, Medical College of Wisconsin.

The active site contains a diamagnetic [4Fe-4S]$^{2+}$ cluster which can be reduced to give the paramagnetic [4Fe-4S]$^{+}$ ($S = {}^{1}/_{2}$) rhombic EPR state ($g_{1,2,3}$ = 2.06, 1.93, 1.86) that binds substrate strongly with 30% retention of activity (Emptage et al., 1983a). Mössbauer spectroscopy shows that a single labile iron site, Fe_a, changes its coordination number upon addition of substrate to enzyme in either oxidation state (Kent et al., 1985). Pronounced shifts in the g value of the [4Fe-4S]$^{+}$ species are observed by EPR upon binding of the substrate and the substrate analogues *trans*-aconitate and nitroisocitrate. With bound substrate the paramagnetic form of the enzyme has the g values 2.04, 1.85, 1.78; with bound nitroisocitrate and with *trans*-aconitate the g values are 2.04, 1.87, 1.77 and

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2.01, 1.88, and 1.80, respectively. No shift in the g values are observed in the presence of the weak inhibitor tricarballylate.

Considerations of the possible catalytic role of the cluster raise two major questions about the possible binding of exogenous ligands. The first is whether solvent H_xO (x = 1,2) and/or the OH of substrate can bind to the [4Fe-4S]$^+$ cluster. Hyperfine broadening (0.5 mT) of the EPR signal of reduced enzyme in the presence of substrate and solvent water containing ^{17}O indicated that such binding occurs (Emptage et al., 1983b) and subsequent ^{17}O ENDOR studies of samples equilibrated with substrate in $H_2{}^{17}O$ showed ENDOR signals from H_xO bound to the cluster. (A solution of active enzyme prepared with any one of the substrate forms, namely, citrate, isocitrate, or *cis*-aconitate, contains an equilibrium mixture.) No ^{17}O signal was seen that could be assigned to the exchangeable OH of the substrate, which suggested that substrate is bound to the enzyme largely in the form of *cis*-aconitate. However, the observation of ^{17}O ENDOR resonances from the active enzyme to which ^{17}OH-labeled nitroisocitrate, a substrate analogue, was bound strongly showed that the cluster can bind the OH group of a substrate (Telser et al., 1986). This complex of enzyme and inhibitor also gave ^{17}O ENDOR signals in $H_2{}^{17}O$-enriched solvent, which suggests that the cluster might simultaneously coordinate OH of the substrate and solvent, H_xO (Telser et al., 1986).

Hyperfine broadening of the EPR signal had also been observed for enzyme that had the inhibitor *trans*-aconitate bound (0.3 mT) in a solution containing $H_2{}^{17}O$. Subsequent ^{17}O ENDOR studies confirmed that a solvent species H_xO does bind to the complex of enzyme and inhibitor. The ^{17}O hyperfine interaction and quadrupolar coupling of the bound solvent species in the cases of *trans*-aconitate and nitroisocitrate are very similar to those for H_xO in the enzyme–substrate complex. A broadening of 0.16 mT that was observed for the weak inhibitor, tricarballylate, in $H_2{}^{17}O$ could be simulated with a hyperfine coupling constant of $A(g_1) \sim 8$ MHz. However, no ^{17}O ENDOR signal arising from the solvent had been observed in previous studies at X-band for this case (Telser et al., 1986).

We now report that ENDOR measurements at Q-band demonstrate that a solvent species, H_xO, binds to the [4Fe-4S]$^+$ cluster in all states of reduced active aconitase; in particular, we discuss the nature of H_xO bound to the cluster of substrate-free enzyme and enzyme in the presence of tricarballylate and fluorocitrate. ^1H and ^2H ENDOR measurements have been used to characterize the H_xO of substrate-free enzyme as well as that in the complexes with substrate and the substrate analogues *trans*-aconitate and nitroisocitrate. The observation of H_xO bound to substrate-free enzyme is consistent with the recently reported crystal structure of the [4Fe-4S] cluster which indicates that the fourth ligand of Fe_a in the substrate-free enzyme is a solvent species, H_2O or OH^- (Robbins & Stout, 1989).

The second question is the possible role of carboxyl group binding to the cluster as a means of positioning the substrate. Previous ^{17}O and ^{13}C ENDOR studies showed that only the carboxyl at C-2 of the propane backbone of substrate (*cis*-aconitate) is strongly bound to the [4Fe-4S]$^+$ cluster of aconitase but that the C-1 carboxyl group of nitroisocitrate also can bind (Kennedy et al., 1987). These findings led to a substantial revision in proposals for the enzyme mechanism. We now report the full ^{17}O hyperfine coupling tensor for the bound oxygen of the C-2 carboxyl of substrate. ^{17}O ENDOR studies also show that the C-1 carboxyl group of nitroisocitrate and a carboxyl of *trans*-aconitate have properties similar to those of substrate. The accompanying paper (Werst et al., 1990) reports on the characterization of the [4Fe-4S]$^+$ cluster of substrate-free and substrate-bound aconitase by ^{57}Fe, ^{33}S and ^{14}N ENDOR spectroscopy.

Procedures and Theory

Sample Preparation. In general, the materials and methods were those used previously (Kennedy et al., 1983; Emptage et al., 1983a). Photoreduction of Q-band samples was carried out essentially as described for the corresponding X-band experiments (Emptage et al., 1983b; Telser et al., 1986) with the exception that all samples contained ~3 mM DTT. Because of the difficulty of adding microliter amounts to samples in an anaerobic chamber, the concentrations of oxalate and deazaflavin varied between 10–15 mM and 7–13 μM, respectively. Dithionite reductions were done as described (Kennedy et al., 1987). However, the incubation time of dithionite-reducted enzyme in ^2H_2O with nitroisocitrate was reduced to 5–10 min. Enzyme concentrations varied between 0.4 and 1.0 mM, and when present, substrate or substrate analogues were 5–10 times the enzyme concentration. Samples in ^2H_2O were prepared by desalting concentrated enzyme solutions by centrifugation on columns equilibrated with the appropriate buffer in ^2H_2O. The eluates were then diluted to the desired enzyme concentration with the same buffer in ^2H_2O. Samples in $H_2{}^{17}O$ were prepared by diluting concentrated enzyme solution with buffer prepared in $H_2{}^{17}O$. This solution was then further concentrated on an Amicon Centricon-10 filtration unit. The resulting enzyme solution was again diluted with buffer in $H_2{}^{17}O$ to the desired amount in order to achieve an optimal $H_2{}^{17}O$ concentration. The above procedures were performed anaerobically whenever active enzyme was used. *cis*-Aconitate labeled with ^2H in carbon-bound hydrogens was prepared from *cis*-aconitic anhydride. Two grams was dissolved in 6 mL of ^2H_2O, brought to pH 0.8–1 with concentrated ^2HCl, sealed into a thick-walled glass tube, and heated to ~75 °C in a sand bath for 10 days. The sample was lyophilized. Deuteration of the *trans*-aconitic acid thus formed was 92%. The percent deuteration was assessed by ^1H NMR by comparison to a sample of ^1H *trans*-aconitate (or *cis*-aconitate, see below) of equal concentration. The concentrations of these solutions were determined by weight and for *cis*-aconitate also by enzymatic assay with aconitase. The powder obtained was dissolved in a minimal volume of hot ^2H_2O, and the solution was briefly boiled with charcoal and filtered. Then ^2HCl was added and the sample was slowly cooled and kept cold until the acid had crystallized out. The crystals were filtered, washed, and then dissolved in ^2H_2O. The solution was lyophilized and the remaining white powder weighed. It proved to be important not to use the crystals from the recrystallization directly since they do not have the proper consistency for subsequent treatment with acetic anhydride. The desired amount of powder was converted to a mixture of *cis*- and *trans*-aconitic anhydride by using acetic anhydride (Malachowski & Maslowski, 1928). The *cis*-anhydride was extracted from the mixture selectively by boiling benzene as described by these authors. The *cis*-anhydride crystallized out immediately and was filtered, washed with cold benzene, dried, and used as such. The deuteration at this point was ~75%.

Nitroisocitrate (3-hydroxy-2-nitro-1,3-propanedicarboxylate) was the kind gift of Dr. J. V. Schloss (E. I. du Pont de Nemours and Co.). (−)-*erythro*-2-Fluorocitrate was the kind gift of Dr. E. Kun (University of California, San Francisco).

Magnetic Resonance Measurements. EPR and ENDOR spectra at X-band (9 GHz) and Q-band (35 GHz) were re-

corded as described elsewhere (Venters et al., 1986; Gurbiel et al., 1989). In the first approximation, a single-crystal-like ENDOR pattern for a ^1H nucleus ($I = {}^1/_2$) has frequencies given by

$$\nu_\pm = |\nu(^1H) \pm A(^1H)/2| \quad (1)$$

Because $\nu(^1H) > A(^1H)/2$, $\nu_\pm$ forms a hyperfine-split doublet centered at $\nu(^1H) \sim 14$ MHz at X-band (9 GHz) or $\nu(^1H) \sim 53$ MHz at Q-band (35 GHz) with $\Delta\nu = \nu_+ - \nu_- = A(^1H)$. The single-crystal ($\Delta m = \pm 1$) ENDOR pattern of a nucleus, N, with $I \geq 1$, namely, ^{17}O ($I = {}^5/_2$) and ^2H ($I = 1$), has frequencies given by

$$\nu_\pm(m) = |A^N/2 \pm \nu_N + 3P^N(2m - 1)| \quad (2)$$

where $-I + 1 \leq m \leq I$, A^N and P^N are the orientation-dependent hyperfine and quadrupole coupling constants, and ν_N is the nuclear Larmor frequency, $\nu_N = g_N\beta_N H/h$ (Abragam & Bleaney, 1970; Atherton, 1973). All parameters are taken as positive absolute values. For an ^{17}O pattern, such as the X-band spectrum shown here, $A^O/2 > \nu_O > 3P^O(2m - 1)/2$, the ENDOR pattern consists of a Larmor-split doublet of quintets ($2I = 5$) centered at $A^O/2$, with the $\nu_+(m)$ and $\nu_-(m)$ separated by $2\nu_O$; within each group the lines are separated by $3P^O$. When, as is true for Q-band spectra, $\nu_O > A^O/2$, a hyperfine-split doublet of quintets centered at ν_O is observed. In some cases the individual lines of the quintet, due to quadrupolar interaction are not resolved.

For deuterium ENDOR ($I = 1$), when $\nu(^2H) > A(^2H)/2 > P(^2H)$, eq 2 in principle describes a four-line pattern, consisting of a hyperfine-split doublet centered at $\nu(^2H)$, with $\nu_\pm$ further split by the quadrupolar term. However, although a four-line ^2H ENDOR pattern that can be described by eq 2 might indeed arise from a single type of deuteron with resolved quadrupole couplings, it might also represent the signal from two inequivalent deuterons, neither of which exhibits resolved quadrupolar splittings. This ambiguity can be resolved by comparison of ^1H and ^2H single-crystal-like ENDOR patterns. The magnetic parameters of the ^1H and ^2H nuclei are related by fundamental nuclear properties:

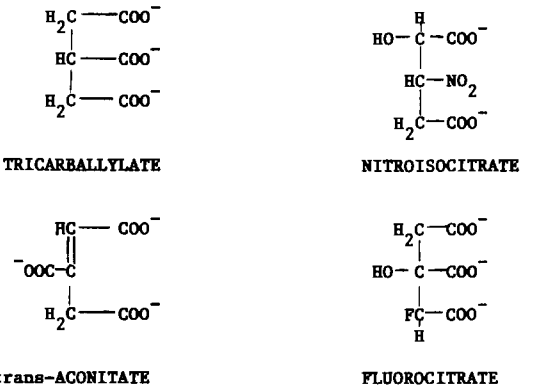
$$A(^1H)/A(^2H) = \nu(^1H)/\nu(^2H) = g(^1H)/g(^2H) = 6.5 \quad (3)$$

and thus assignment of an ENDOR spectrum for one isotopic species directly predicts the features of the other. ^2H resonances cannot easily be seen at X-band because for a $g \sim 2$ EPR signal the ^2H ENDOR pattern would extend from ca. 0.5 to 3.5 MHz, and it is difficult to obtain ENDOR signals at such low radio frequencies. However, at Q-band (35 GHz), the ^2H ENDOR pattern is centered at $\nu(^2H) \sim 8$ MHz and extends from ca. 6.5 to 9.5 MHz. Resonances in this range are readily detected, and all the ^2H ENDOR spectra shown here have been taken at Q-band microwave frequency.

The samples employed in this study are frozen solutions and thus contain a random distribution of all protein orientations. However, ENDOR spectra taken with the magnetic field set at the extreme edge of the EPR spectrum, near g_1 or g_3, give single-crystal-like patterns from the subset of molecules for which the magnetic field happens to be directed along a g-tensor axis (Rist & Hyde, 1970). The ^1H and corresponding ^2H ENDOR spectra shown here have all been obtained by monitoring the g_3 EPR signal. ^{17}O ENDOR signals have been taken at selected g values across the entire EPR envelope and analyzed by procedures previously described (Hoffman et al., 1985; Hoffman, 1984; True et al., 1988; Gurbiel et al., 1989) to completely determine the hyperfine coupling tensors.

The recorded ENDOR pattern often shifts in the direction of the radiofrequency sweep, and the ^{17}O hyperfine parameters reported here are the average from measurements with positive and negative sweeps. This is necessary because for ^{17}O ENDOR X-band signals ($A^O/2 > \nu_O$) the hyperfine coupling constant is determined by the frequency of the center of the pattern (eq 2); for ^{17}O Q-band ENDOR ($A^O/2 < \nu_O$) where only the ν_+ feature is seen because of hyperfine enhancement effects, the hyperfine coupling is determined from ν_+ and the calculated value of ν_O through use of eq 2. For ^1H and ^2H spectra, A^N is obtained from the magnitude of the hyperfine splittings of the spectrum (eqs 1 and 2) and is independent of sweep directions. The ^2H ENDOR spectra presented here have all been obtained by scanning from low to high radiofrequency because the pattern obtained by scanning the opposite direction is highly asymmetric; in each case the center shifts ca. 0.4 MHz in the direction of the scan. For ^1H ENDOR, the pattern shifts 0.1 MHz in the direction of the scan.

Chart I



Results and Discussion

^{17}O, ^1H, and ^2H ENDOR measurements have been performed on the following specifically labeled species: E, reduced active enzyme (natural isotopic abundance); ES, the enzyme–substrate complex (natural isotopic abundance) [we concluded (Telser et al., 1986) that the substrate is bound largely in the form of the dehydrated intermediate, *cis*-aconitate]; ES[^{17}O], enzyme–substrate complex in which the substrate has been specifically labeled with ^{17}O at the carboxyl at C-2 of the propane backbone; ES[^2H], enzyme–substrate complex with perdeuterated substrate; EN, complex of enzyme and the substrate analogue nitroisocitrate (see Chart I); ETn, complex of enzyme and the substrate analogue *trans*-aconitate; ETb, complex of enzyme and the weak inhibitor tricarballylate; EF, complex of enzyme and inhibitor fluorocitrate. These species have been studied in solvents $^1H_2{}^{17}O$, $^1H_2{}^{16}O$, and $^2H_2{}^{16}O$. Abbreviation such as ES[^{17}O;$H_2{}^{17}O$] will be used to further represent the isotopic composition of the substrate and solvent.

^{17}O ENDOR of Enzyme in $H_2{}^{17}O$, E[$H_2{}^{17}O$]. Results of ^{17}O Q-band ENDOR measurements made on E[$H_2{}^{17}O$] reveal that the [4Fe-4S]$^+$ cluster binds solvent, H_xO, in the absence of substrate. Figure 1A shows the ^{17}O resonances obtained by monitoring the $g_2 = 1.93$ EPR signal; these resonances are not seen in corresponding measurements of E[$H_2{}^{16}O$]. The inset of Figure 1 shows the X-band (9.5-GHz) ^{17}O ENDOR spectrum of ES[$H_2{}^{17}O$]. We assign the intense feature in the spectrum in Figure 1A at ca. 12.7 MHz to the ν_+ feature of a pattern described by eq 2, centered at $\nu_O = 7.5$ MHz and with $A(g_2) = 10.4$ MHz. According to eq 1, the ν_- feature would fall at $\sim$2.3 MHz, but it is very difficult to see ENDOR signals at radiofrequencies less than ca. 3 MHz. The signal at 12.7 MHz cannot be assigned as the ν_- feature of a pattern centered at $A^O/2 \sim 20$ MHz. In this case the ν_+ feature

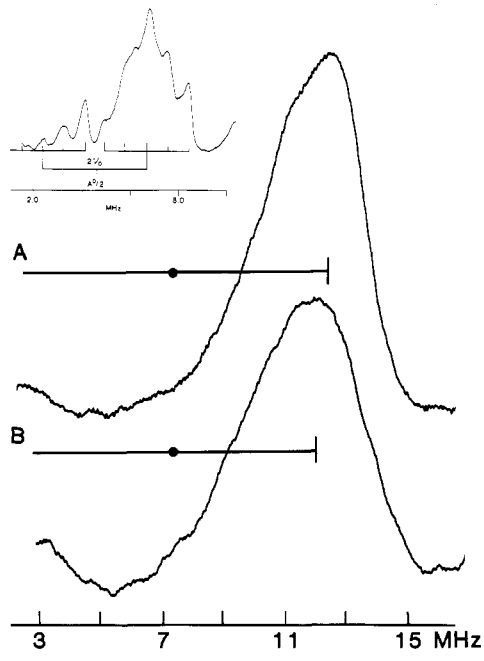


FIGURE 1: ^{17}O Q-band (34.68-GHz) ENDOR spectrum of (A) enzyme in $H_2{}^{17}O$ and (B) enzyme plus tricarballylate in $H_2{}^{17}O$. Conditions: $g_2 = 1.93$; magnetic field $H = 1.284$ T; temperature = 2 K; microwave power, 0.08 mW; field modulation, 100 kHz; modulation amplitude, 0.6 mT; radio-frequency scan rate, 6 MHz/s; time constant, 0.032 s; radio-frequency power, 10 W. The assignment of A^H (|—|) and the Larmor frequency (●) are indicated. Inset: ^{17}O X-band (9.53-GHz) ENDOR spectrum of enzyme plus substrate in $H_2{}^{17}O$. Conditions are as described above except as follows: $g = 1.88$; $H = 0.3650$ T; modulation amplitude, 0.3 mT; microwave power, 0.1 mW; scan rate, 3 MHz/s.

Table I: ^{17}O Hyperfine (A) and Quadrupole (P) Tensors for $H_x{}^{17}O$ Bound to the [4Fe-4S]$^+$ Cluster of Aconitase

	species[a]	$A_{1,2,3}$			Euler angles[b]	$P_{1,2,3}$		
E[$H_2{}^{17}O$]	OH^-	9	12	10	c		d	
ES[$H_2{}^{17}O$][d]	H_2O	9.0	8.5	8.4	$\alpha \sim 35$	−0.5	0.35	0.15
ETn[$H_2{}^{17}O$][e]	H_2O	9.5	9.3	9.2	$\alpha \sim 30$	−0.5	0.35	0.15
EN[$H_2{}^{17}O$][f]	H_2O	9.0	8.5	8.4	$\alpha \sim 35$	−0.5	0.35	0.15

[a] Assignments of the protonation states of cluster-bound H_xO are discussed in the text. [b] Angles defined in True et al. (1989) give the orientations of the A tensor in the g frame. [c] A tensor is coaxial with g tensor. [d] Resolved quadrupole splitting is not observed for this ^{17}O species. [e] Telser et al. (1986). [f] The hydroxyl group of nitroisocitrate also binds to the cluster, but its ^{17}O parameters are different.

would fall at ca. 27.7 MHz and would be much more intense than that at 12.7 MHz because of hyperfine enhancement effects (Abragam & Bleaney, 1970), but no such signal is seen. Furthermore, such an assignment would give $A(g_2) \sim 40$ MHz, which is inconsistent with the minimal ^{17}O broadening of the EPR signal (Emptage et al., 1983b).

Analysis of the field dependence of the ^{17}O signal (Gurbiel et al., 1989; True et al., 1988) gives a hyperfine tensor that is coaxial with the g tensor and whose principal values are given in Table I. The observation of an ^{17}O signal with such substantial hyperfine interaction shows that the ^{17}O-containing species coordinates directly to the [4Fe-4S]$^+$ cluster.

The ν_+ feature in Figure 1A is quite broad, with a width at half-height of ca. 4 MHz; this breadth is attriuted to unresolved quadrupolar splittings of the $I = {}^5/_2$ ^{17}O nucleus by analogy with the X-band ^{17}O ENDOR signal for the solvent $H_x{}^{17}O$ bound to ES. This latter signal is shown in the inset to Figure 1; its ν_+ and ν_- features both clearly show a resolved quadrupolar quintet with a net width of ca. 3 MHz. Resolved

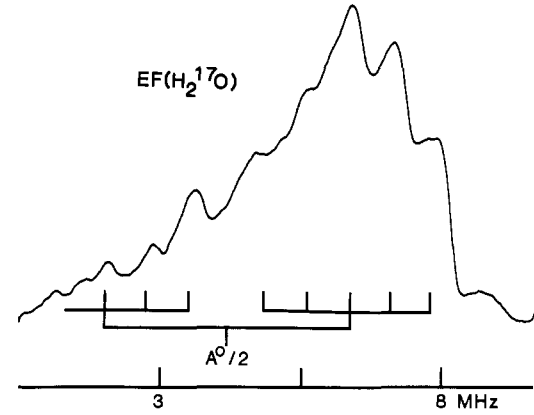


FIGURE 2: ^{17}O X-band (9.9-GHz) ENDOR spectrum at $g = 1.88$ of enzyme plus fluorocitrate in $H_2{}^{17}O$. Conditions as described for Figure 1 inset except as follows: $H = 0.377$ T; modulation amplitude, 0.16 mT.

quadrupolar splittings of similar magnitude also are observed in the ^{17}O ENDOR X-band spectrum of ES[$H_2{}^{17}O$], ETn-[$H_2{}^{17}O$], and EN[$H_2{}^{17}O$] (Telser et al., 1986).

The ^{17}O hyperfine and quadrupole tensor parameters for $H_x{}^{17}O$ bound to E, ES, ETn, and EN are summarized in Table I. The parameters for E[$H_2{}^{17}O$] differ from those for the other three, which agree among themselves. This supports the assignment made below that $(OH)^-$ is bound to the cluster of E, but H_2O is bound in the other three cases.

^{17}O ENDOR of Enzyme plus Tricarballylate in $H_2{}^{17}O$, ETb[$H_2{}^{17}O$]. Reduced enzyme in the presence of the weak inhibitor, tricarballylate, has g values identical with those of the substrate-free enzyme; this indicates that tricarballylate binding to the enzyme causes negligible perturbations of the electronic structure of the [4Fe-4S]$^+$ cluster (Emptage et al., 1983b). The ^{17}O Q-band ENDOR spectrum of ETb[$H_2{}^{17}O$] (Figure 1B) exhibits a signal not present in the corresponding spectrum of ETb[$H_2{}^{16}O$], thus arising from a solvent species, $H_x{}^{17}O$, bound to the cluster. This feature (Figure 1B) is assigned to the ν_+ feature of a signal centered at ν_O corresponding to $A(g_2) = 10$ MHz. This ^{17}O ENDOR signal was not clearly seen in earlier X-band studies (Telser et al., 1986) because the sample was lower in concentration. As with H_xO bound to the [4Fe-4S]$^+$ cluster in E[$H_2{}^{17}O$], the breadth of the ν_+ signal (4 MHz at half-height) of the ^{17}O g_2 spectrum of ETb[$H_2{}^{17}O$] (Figure 1B) is attributed to unresolved quadrupolar splittings. EPR simulations show that the hyperfine broadening of the EPR signal in $H_2{}^{17}O$ (0.16 mT) is consistent with a single bound solvent species, $H_x{}^{17}O$, characterized by the ENDOR-determined hyperfine coupling (Telser et al., 1986).

^{17}O ENDOR of Enzyme plus Fluorocitrate in $H_2{}^{17}O$, EF-[$H_2{}^{17}O$]. The X-band ENDOR signal at $g = 1.88$ from EF-[$H_2{}^{17}O$] (Figure 2) shows an ^{17}O pattern that consists of a Larmor-split doublet of quintets that is centered at $A^O/2 = 4.3$ MHz, with the $\nu_-(m)$ partner greatly reduced in intensity. It is believed that inhibition by fluorocitrate involves elimination of hydrogen fluoride and reaction with solvent ($H_2{}^{17}O$) to form a complex of enzyme and 4-^{17}OH-*trans*-aconitate (Kent et al., 1985). However, the characteristics of the ^{17}O signals observed closely match those of solvent, H_xO, bound to [4Fe-4S]$^+$ cluster in ES; we have been unable to distinguish any separate signal due to ^{17}OH of the putative product, hydroxy-*trans*-aconitate, as opposed to the case with the ^{17}OH of nitroisocitrate (Telser et al., 1986) bound to the enzyme. The mechanism of inhibition of fluorocitrate will be the subject of a later study.

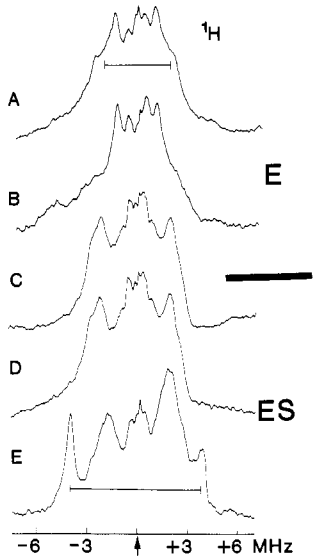


FIGURE 3: Single-crystal-like ^{1}H Q-band (35.4-GHz) ENDOR spectra at g_3 [arrow indicates $\nu(^1H)$]. Enzyme: (A) in ^1H_2O; (B) in ^2H_2O. Enzyme plus substrate: (C) perdeuterated substrate, ^2H_2O; (D) substrate, ^2H_2O; (E) substrate, ^1H_2O. Conditions for panels A and B as in Figure 1 except for the following: g_3 = 1.86; H = 1.359 T; microwave power, 0.315 mW; modulation amplitude, 0.2 mT; radio-frequency scan rate, 0.5 MHz/s; time constant, 0.128 s; $\nu(^1H)$ = 57.85 MHz. Conditions for panels C–E are the same except for the following: g_3 = 1.78; H = 1.4185 T; $\nu(^1H)$ = 60.38 MHz; modulation amplitude, 0.16 mT.

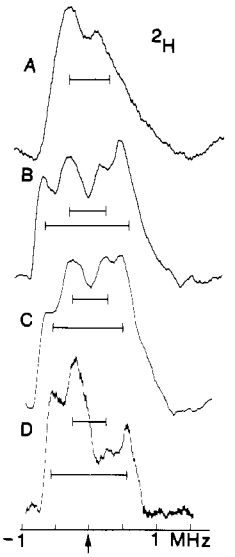


FIGURE 4: Single-crystal-like ^{2}H Q-band ENDOR spectrum at g_3; arrow indicates $\nu(^2H)$. (A) Enzyme without substrate in ^2H_2O: g = 1.86; H = 1.356 T; $\nu(^2H)$ = 8.86 MHz. (B) Enzyme plus substrate in ^2H_2O: H = 1.4188 T; g = 1.78; $\nu(^2H)$ = 9.27 MHz. (C) Enzyme plus *trans*-aconitate in ^2H_2O: g = 1.80; H = 1.407 T; $\nu(^2H)$ = 9.19 MHz. (D) Enzyme plus nitroisocitrate in ^2H_2O: g = 1.78; H = 1.386 T; $\nu(^2H)$ = 9.06 MHz. Conditions as for Figure 1 except for the following: modulation amplitude, 0.063 mT; radio-frequency scan rate, 3 MHz/s; time constant, 0.016 s.

^1H and ^2H ENDOR of H_2O Bound to the $[4Fe\text{-}4S]^+$ Cluster of Substrate-Free Enzyme, E. ^{1}H and ^{2}H ENDOR measurements of E have been used to further characterize the cluster-bound H_xO solvent species. Comparison of the g_3 = 1.86 single-crystal-like ^{1}H ENDOR spectra of E[^1H_2O] and E[^2H_2O] (panels A and B, respectively, of Figure 3) discloses at least one ^{1}H Larmor doublet that is lost in ^2H_2O solvent. This doublet, whose splitting corresponds to $A(^1H)$ = 4 MHz, is in all probability associated with the H_xO species detected by ^{17}O ENDOR. The nonexchangeable protons correspond to doublets with A = 1, 2, 5, and 8 MHz; under different experimental conditions (not shown; Werst, 1990), the signals from protons with the larger hyperfine splittings (A = 5, 8 MHz) are enhanced. We cannot assign these resonances to specific protons, but they likely arise from protons of cysteine or other amino acid residues that interact with the paramagnetic Fe-S cluster.

The ENDOR intensity associated with the exchangeable proton is poorly resolved because of overlapping resonances from the many nonexchangeable protons. However, there is no interference in the corresponding ^{2}H ENDOR measurements because they show only deuterons that replace protons upon solvent exchange. The single-crystal-like ^{2}H ENDOR spectrum at g_3 for E[^2H_2O] shows a single hyperfine-split doublet, $A(^2H)$ = 0.6 MHz, without resolved quadrupolar splitting by the I = 1 deuterium nucleus (Figure 4A). Through eq 3, this result corresponds to $A(^1H)$ = 3.9 MHz, the coupling observed for the exchangeable proton of Figure 3A. The observation of a single type of exchangeable proton in the resolved deuterium spectrum (as well as in the ^{1}H spectrum), along with considerations of charge balance, leads us to suggest that the solvent species detected by ^{17}O ENDOR in the substrate-free enzyme is a hydroxyl ion, not a water molecule. This interpretation is supported presently by the data for ES.

^1H and ^2H ENDOR of H_xO Bound to $[4Fe\text{-}4S]^+$ of the Enzyme plus Substrate or trans-Aconitate, ES and ETn. Addition of substrate causes pronounced changes in the ^{1}H ENDOR of the nonexchangeable protons of the enzyme. This can be seen by comparing the spectra of E[^2H_2O] (Figure 3B) and ES[^2H;^2H_2O] (Figure 3C). Because the substrate is perdeuterated (~70%) and the solvent is ^2H_2O, the differences in these spectra reflect the changes in the cluster's interaction with the nonexchangeable protons of the protein itself. The ^{1}H spectrum of the enzyme–substrate complex in ^2H_2O, ES-[^2H_2O] (Figure 3D), is indistinguishable from the ^{1}H ENDOR spectrum of the complex of enzyme and perdeuterated substrate in ^2H_2O, ES[^2H;^2H_2O] (Figure 3C). Also, the ^{2}H ENDOR spectra of these two samples are identical (see below). Thus, there are no detectable hyperfine interactions with the protons of the substrate.

Comparison of the single-crystal-like ^{1}H ENDOR spectrum at g_3 of ES[^2H_2O] and ES[^1H_2O] (Figures 3D and 3E) clearly shows a signal of an exchangeable proton with a hyperfine coupling of 7.8 MHz, twice that of the exchangeable proton found for E. We concluded earlier that substrate is bound largely in the form of the dehydrated intermediate *cis*-aconitate (Telser et al., 1986). Hence, this ^{1}H signal is assigned to the cluster-bound H_xO species previously detected by ^{17}O ENDOR. The ^{1}H ENDOR spectra of ETn in ^1H_2O and ^2H_2O (not shown; Werst, 1990) similarly show an exchangeable proton, $A1(^1H)$ = 6 MHz, which we assign to the cluster-bound solvent species, H_xO. The signals from the corresponding single-crystal-like g_3 ^{2}H ENDOR spectra of ES-[^2H_2O] and ETn[^2H_2O] (Figure 4, panels B and C, respectively), each can be assigned to a pair of hyperfine-split deuteron doublets without resolved quadrupolar splittings and centered at $\nu(^2H)$ = 9.54 MHz (eq 2). The resulting hyperfine couplings for ES, $A1^{ES}(^2H)$ = 1.2 MHz and $A2^{ES}(^2H)$ = 0.5 MHz, are equivalent to ^{1}H hyperfine coupling of $A1^{ES}(^1H)$ = 7.8 and $A2^{ES}(^1H)$ = 3.2 MHz, respectively, according to eq 3. For ETn, the hyperfine splittings, $A1^{ETn}(^1H)$ = 1 MHz and $A2^{ETn}(^2H)$ = 0.5 MHz, correspond to $A1^{ETn}(^1H)$ = 6 and $A2^{ETn}(^1H)$ = 3.2 MHz, respectively. In each case, the larger deuteron coupling, $A2(^2H)$, precisely predicts the ^{1}H hyperfine

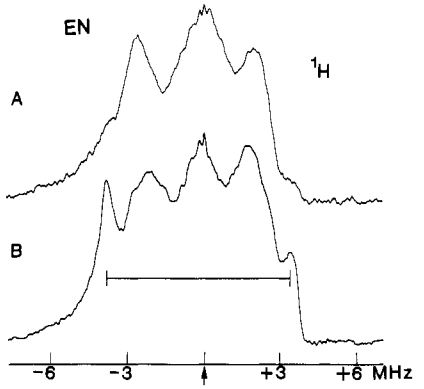


FIGURE 5: Single-crystal-like ^{1}H Q-band (34.5-GHz) ENDOR spectra of enzyme plus nitroisocitrate at $g_3 = 1.78$ [arrow indicates $\nu(^1H)$]. (A) ^2H_2O and (B) ^1H_2O. Conditions as for Figure 3 except for the following: $H = 1.383$ T; $\nu(^1H) = 57.85$ MHz.

coupling for the exchangeable proton, $A2(^1H)$, observed in the ^{1}H ENDOR; the smaller deuteron coupling $A2(^2H)$ predicts a proton signal that is not readily detected in the ^{1}H spectrum because it would strongly overlap with the resonances from nonexchangeable protons. This $^1H/^2H$ correspondence rules out the possibility that the four-line pattern in Figure 4B,C arises from a single deuteron whose $\nu_\pm$ lines are split by a quadrupole term, $3P$ (eq 2). Because the spectra of the [4Fe-4S]$^+$ clusters of ES (Figure 4B) and ETn (Figure 4C) reveal two distinct exchangeable protons, we assign the cluster-bound H_xO as an asymmetrically bound water molecule, H_2O. It should be noted that *trans*-aconitate has no hydroxyl group. Thus, in the presence of this inhibitor, any enzyme-bound oxygen species, other than a carboxyl, must originate from the solvent, and the close similarity of the ^{2}H ENDOR spectra of ETn[$H_2{}^{17}O$] with those of ES[$H_2{}^{17}O$] confirms that in both cases the solvent species bound is H_2O. The ^{17}O hyperfine tensors of hydroxyl bound to the cluster of E and of H_2O bound to ES (Table I) show significant differences, supporting the assignment of different protonation states of H_xO in the two cases.

^1H and ^2H ENDOR of H_xO Bound to the [4Fe-4S]$^+$ Cluster of Enzyme plus Nitroisocitrate, EN. The ^{1}H ENDOR spectra at g_3 of EN[^2H_2O] and EN[^1H_2O] indicate the presence of an exchangeable proton with $A1^{EN}(^1H) = 7$ MHz (Figure 5, panels A and B, respectively). The single-crystal-like ^{2}H ENDOR spectrum at g_3 of EN[^2H_2O] (Figure 4D) exhibits two double signals, $A1(^2H) = 1$ MHz and $A2(^2H) = 0.5$ MHz, as in the case of ES[^2H_2O] and ETn[^2H_2O] (Figure 4, panels B and C, respectively). Again, as with the solvent species, H_xO, bound to the complex of enzyme and substrate, *cis*-aconitate and inhibitor *trans*-aconitate, the deuteron splitting $A1(^2H)$ precisely corresponds to the ^{1}H hyperfine coupling for the exchangeable proton $A1(^1H)$ observed in the ^{1}H ENDOR and $A2(^2H)$ corresponds to a proton signal that would strongly overlap with the resonances of nonexchangeable protons. The ^{1}H and ^{2}H hyperfine parameters for the solvent species bound to the cluster in E, ES, ETn, and EN are summarized in Table II.

Previous ^{17}O ENDOR experiments of EN (Telser et al., 1986) disclosed that the nonexchangeable hydroxyl group (^{17}OH) of nitroisocitrate can bind to the cluster in addition to a solvent species, H_xO; hence one of the ^{1}H and ^{2}H resonances mentioned above might, in principle, have arisen from the exchangeable proton of the hydroxyl group of the substrate analogue. However, the hyperfine couplings of the two protons are so similar to those of H_2O bound to the cluster in ES and ETn that we do not favor such an assignment. We have not identified any separate resolved deuterium resonances from the OH group of nitroisocitrate.

Table II: ^{1}H and ^{2}H Hyperfine Splittings (A, MHz) of Solvent Species H_xO Bound to the [4Fe-4S]$^+$ Cluster of Aconitase[a]

	$A(^1H)$		$A(^2H)$	
	^{1}H(2)	^{1}H(2)	^{2}H(1)	^{2}H(2)
E	[b]	4	[b]	0.6
ES	8	[b]	1.2	0.5
ETn	6	[b]	1.0	0.5
EN	7	[b]	1.1	0.5

[a] All hyperfine splittings are obtained from single-crystal-like spectra taken at g_3. Assignments of H_xO are given in Table I. [b] Not detected.

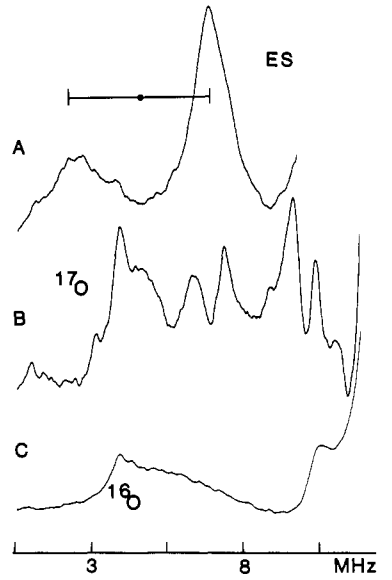


FIGURE 6: ^{17}O X-band (9.91-GHz) ENDOR spectra enzyme plus substrate: (A) With C-2 carboxyl group of substrate ^{17}O labeled, at $g_3 = 1.78$. (B) As in panel A except at $g_2 = 1.853$ (9.96 GHz). (C) As in panel B, but with unlabeled (^{16}O) citrate. Conditions as in Figure 1 (inset) except for the following: (A) $H = 0.399$ T; modulation amplitude, 0.1 mT; (B, C) $H = 0.384$ T; microwave power; 0.008 mW, modulation amplitude; 0.25 mT, scan rate, 3 MHz/s.

^{17}O ENDOR: Interaction of the C-2 Carboxyl of Substrate (cis-Aconitate) with the [4Fe-4S]$^+$ Cluster. Previous ^{17}O and ^{13}C ENDOR studies showed that only the carboxyl at C-2 of the propane backbone of substrate (presumably as *cis*-aconitate) binds to the [4Fe-4S]$^+$ cluster of aconitase (Kennedy et al., 1987). We have now studied in detail the field dependence of the ^{17}O ENDOR signal of this carboxyl. The single-crystal-like g_3 ENDOR spectrum of ES[$^{17}O;H_2O$] (Figure 6A) shows a Larmor-split ^{17}O doublet centered at $A(g_3)/2 = 4.5$ Hz; the spectrum's simplicity suggests that only one oxygen of the carboxyl group is bound. The half-height breadth of the ν_+ line ($\sim$1.5 MHz) is taken to result from the unresolved quadrupole splitting with $3P(g_3) \sim 0.4$ MHz. The ^{17}O signal at intermediate g values is complex, as seen at g_2 (Figure 6B), and is further complicated by a signal at $\sim$4 MHz that we attribute to an ^{14}N resonance ($A \sim 6$ MHz) and a ^{1}H signal at $\sim$10.5 MHz (compare panels A and B of Figure 6). This ^{14}N resonance is discussed in detail in the following paper in this issue. Such a complex spectrum can only be interpreted by analyzing the spectra obtained at many positions across the EPR envelope, using methods previously described. Preliminary results of this analysis suggest that the hyperfine tensor is not coaxial with the g tensor and has principle values, $A_{1,2,3} \approx$ [10, 15, 9] MHz. The quadrupole splittings cannot be fit by assuming coaxial hyperfine and quadrupole tensors; we estimate $P_{1,2,3} \approx$ [0.13, 0.26, −0.13]

MHz from spectra taken at $g_{1,2,3}$.

^{17}O ENDOR: Interaction of a Carboxyl Group of the Inhibitor (trans-Aconitate) with the [4Fe-4S]$^+$ Cluster. The ^{17}O ENDOR spectrum observed at g_2 for the complex of E and *trans*-aconitate that has been nonspecifically ^{17}O labeled in its carboxyl groups (spectrum not shown) gives a hyperfine splitting of $A(g_2)$ = 13 MHz. This value for a carboxyl of *trans*-aconitate is similar to that for the C-1 carboxyl of nitroisocitrate (13 MHz) (Kennedy et al., 1987) as well as that for the C-2 carboxyl of substrate, *cis*-aconitate (15 MHz). The similarity of these values indicates that the interaction of the carboxyl oxygen with the [4Fe-4S]$^+$ cluster is comparable for substrate and substrate analogues.

Conclusions

^{17}O, ^1H, and ^2H ENDOR measurements show that a solvent species, H_xO, is bound to the [4Fe-4S]$^+$ cluster of aconitase in *all* states of the enzyme. Mössbauer spectroscopy (Kent et al., 1985) reveals that the unique iron site, Fe_a, expands its ligand sphere from tetrahedral to a five- or six-coordinate environment when substrate binds. Previous ENDOR measurements (Kennedy et al., 1987) show that substrate and substrate analogues bind to Fe_a via a single carboxyl group and suggest that the hydroxyl group can also coordinate to Fe_a. We propose that in the absence of substrate a solvent hydroxyl ion is the exogenous fourth ligand of Fe_a, along with three sulfide ligands of the [4Fe-4S]$^+$ cluster. The substrate or substrate analogue binds to the iron site, Fe_a, of the [4Fe-4S]$^+$ cluster via addition of a negatively charged carboxyl group (and possibly a hydroxyl group) and concomitantly the solvent species, OH, bound to Fe_a becomes protonated to form a water molecule, H_2O. Thus, the enzyme-catalyzed interconversion of citrate and isocitrate does not involve the displacement of an endogenous ligand but rather the addition of ligands from substrate and change in the protonation state. This is consistent with the recently reported crystal structure of the [4Fe-4S] cluster which indicates that the fourth ligand of Fe_a in the substrate-free enzyme is a solvent species, H_2O or OH^- (Robbins & Stout, 1989).

Acknowledgments

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Registry No. OH^-, 3352-57-6; H_2O, 7732-18-5; aconitase, 9024-25-3; tricarballylate, 99-14-9; fluorocitrate, 357-89-1; *trans*-aconitate, 4023-65-8; nitroisocitrate, 73491-91-5; *cis*-aconitate, 585-84-2.

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