Metal Ligation by Walker Homology B Aspartate βD262 at Site 3 of the Latent but Not Activated Form of the Chloroplast F₁-ATPase from Chlamydomonas reinhardtii*

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Chia-Yuan Hu‡‡, Wei Chen‡‡, and Wayne D. Frasch§

From the ‡Department of Chemistry and Biochemistry, the §Graduate Program in Molecular and Cellular Biology, and †The Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, Arizona 85287-1601

Site-directed mutations D262C, D262H, D262N, and D262T were made to the β subunit Walker Homology B aspartate of chloroplast F₁-ATPase in Chlamydomonas. Photoautotrophic growth and photophosphorylation rates were 3–14% of wild type as were ATPase activities of purified chloroplast F₁, indicating that βD262 is an essential residue for catalysis. The EPR spectrum of vanadyl bound to Site 3 of chloroplast F₁, as VO²⁺-ATP gave rise to two EPR species designated B and C in wild type and mutants. ⁵¹V-hyperfine parameters of species C, present exclusively in the activated enzyme state, did not change significantly by the mutations examined indicating that it is not an equatorial ligand to VO²⁺ nor is it hydrogen-bonded to a coordinated water at an equatorial position. Every mutation changed the ratio of EPR species C/B and/or the ⁵¹V-hyperfine parameters of species B, the predominant conformation of VO²⁺-nucleotide bound to Site 3 in the latent (down-regulated) state. The results indicate that the Walker Homology B aspartate coordinates the metal of the predominant metal-nucleotide conformation at Site 3 in the latent state but not in the conformation present exclusively upon activation and elucidates one of the specific changes in metal ligation involved with activation.

F₀F₁-ATP synthases are found in the plasma membrane of bacteria, the inner membrane of mitochondria, and the thylakoid membrane of chloroplasts where they catalyze ATP synthesis driven by an electrochemical gradient (1). The F₀ portion contains membrane-spanning subunits and is responsible for translocating protons across the membrane. The F₁ portion is an extrinsic membrane complex, composed of five different subunits α, β, γ, δ, and ε, and retains the ability to hydrolyze ATP after purification from F₀. In the crystal structure of F₁ from bovine heart mitochondria (2), the catalytic sites are located on each of the three β subunits with some contribution from the proximal α subunit. The enzyme crystallized with one catalytic site that contained Mg²⁺-AMPPNP, one that contained Mg²⁺-ADP, and one that was empty. Three noncatalytic sites, each located primarily on an α subunit, contained bound Mg²⁺-AMPPNP in this structure.

Chloroplast F₁ has about four metal-nucleotides tightly bound upon purification from F₀ (6). The metal-nucleotide bound to the site designated Site 3 can be removed by gel filtration chromatography, whereas depletion of Site 2 requires partial unfolding of F₁ by precipitation in ammonium sulfate and EDTA (7), and depletion of Sites 1 and 4 require the removal of the ε subunit (8). Recent evidence indicates that Site 3 is catalytic (9). Fluorescence resonance energy transfer (FRET) measurements using TNP nucleotides enabled the mapping of the positions of Sites 1–3 relative to each other and to locations of fluorescent groups covalently modified to unique locations on CF₁ (10). This FRET map shows a close correspondence to the locations of the metal-nucleotides in the crystal structure of F₁ from bovine mitochondria. From this correspondence, Site 2 is a noncatalytic site and Site 1, like Site 3, is catalytic (7). The observation of unique identifiable locations for Sites 1–3 and the correspondence between the FRET map and the crystal structure indicate that each of the metal-nucleotide binding Sites 1–3 can be selectively filled with metal-nucleotide complex (7).

The F₁ portion binds substrate with high affinity in a manner that allows rapid interconversion of ADP and phosphate with bound ATP in the absence of the proton-motive force (3). The proton-motive force drives two sequential conformational changes of the catalytic site that decreases the affinity of the enzyme for ATP relative to ADP that facilitates the selective dissociation of ATP. This generates a chemical gradient in which the cellular concentration of ATP is much higher relative to ADP and phosphate than it would be at equilibrium. The conformation of each of three catalytic sites on the enzyme is staggered such that the enzyme contains a catalytic site in each of the three sequential conformations at any instant as supported by the structure of F₁ (2).

Nucleotides bind the catalytic sites as a complex with Mg²⁺ (4), which serves as a cofactor for the reaction. The decrease in affinity for ATP that results from the sequential conformational changes is directly dependent on the presence of Mg²⁺ (5). These differences in affinity, which can be as much as 5 orders of magnitude for the F₁ from Escherichia coli, strongly suggest that the selective release of ATP results from changes in the metal ligands that are a consequence of the different conformations of the catalytic site.

Thylakoids maintain high rates of photophosphorylation by diverting some of the reducing equivalents derived from the light-driven electron transfer reactions to thioredoxin that in turn keeps a disulfide bond on the γ subunit of CF₁ reduced. Formation of this disulfide in darkness converts the enzyme from the activated to the latent state that has very low ATPase activity. Addition of ADP accelerates the dark decay of ATPase.

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‡ To whom correspondence should be addressed. Tel.: 480-965-8663; Fax: 480-965-8699; E-mail: frasch@asu.edu.

§ The abbreviations used are: F₁, the extrinsic membrane portion of the F₀F₁-ATP synthase; CF₁, chloroplast F₁; FRET, fluorescence resonance energy transfer; P-loop, phosphate-binding loop also known as the Walker Homology A sequence; WHB, Walker Homology B sequence; TNP-nucleotides, 2′(3′)-trinitrophenyl-nucleotides; AMPPNP, 5′-adenosyl-β,γ-imidodiphosphate.
activity, suggesting that tightly bound ADP in a catalytic site serves a regulatory function. This is required as part of the mechanism to maintain the enzyme in its latent state in the dark (14, 15). Conversion of this ADP from loosely to tightly bound correlates with formation of latent CF1 (14). This regulatory interconversion only occurs upon subsequent addition of Mg$^{2+}$ (15, 16). Thus, the metal that serves as a cofactor by binding as a complex with the nucleotide can also serve in a regulatory role by forming a nonfunctional conformation.

Vanadyl (V(IV)-O$^{2+}$) has been used as a direct probe to identify the types of groups that serve as metal ligands at Sites 2 and 3 of CF$_1$ (11, 13, 27). The A and g tensors of $^{51}$V hyperfine couplings from the EPR spectrum of the bound VO$^{2+}$ are a direct measure of the nature of the equatorial metal binding ligands (28). In a mixed ligand environment, each type of ligand contributes independently to the observed $^{51}$V-hyperfine coupling (28, 29). As a result, the $^{51}$V-hyperfine parameters can provide information concerning the type of groups coordinated to the enzyme-bound metal.

When Site 2 of latent CF$_1$ is filled with VO$^{2+}$-nucleotide, the bound VO$^{2+}$-ATP gives rise to EPR species A (11). At Site 3, the majority of VO$^{2+}$-nucleotide binds in a ligand environment that gives rise to EPR species B, whereas a smaller fraction binds to Site 3 in a form that gives rise to EPR species C (11, 12). Upon activation of the enzyme, all of the signal intensity of species B converts to species C, suggesting that the latter results from the metal ligands when the enzyme is catalytically active. Titration of VO$^{2+}$-nucleotide to CF$_1$ that had been depleted of metal-nucleotide only from Site 3 showed that the VO$^{2+}$ binds selectively to a single site (11, 13).

It is possible for the Mg$^{2+}$ bound at each catalytic site to have up to six ligands. However, in the two catalytic sites in the crystal structure of F$_1$ from bovine mitochondria that contain Mg$^{2+}$-nucleotide, only the oxygens of the phosphates and the hydroxyl of Thr-156 were within the 2.5 Å distance that would suggest that they were ligands. This threonine is a residue in a motif composed of GXXXXGKT known as Walker Homology A or phosphate-binding loop (P-loop) conserved among several enzymes that catalyze ATP hydrolysis.

The Walker Homology B (WHB) motif is also conserved among several Mg$^{2+}$-nucleotide binding proteins including adenylyl kinase, phosphofructokinase, human mdrl protein, ATP/ADP translocase, elongation factor Tu, as well as the a and b subunits of the F$_1$-ATPases (17–22). This motif, with the consensus sequence of four hydrophobic residues followed by an aspartate, terminates a β-strand with the carboxyl group facing the binding pocket for metal-nucleotide (2, 20). The aspartate carboxyl of WHB has been suggested to hydrogen bond to a water that is coordinated to the metal, or to coordinate to a metal directly in several proteins including the b subunit of the F$_1$-ATPase (8, 23–26). The crystal structure of the bovine mitochondrial F$_1$-ATPase shows the closest carboxyl-oxygen of this residue to be 3.9 Å to 4.3 Å from the metal at catalytic sites that contain bound Mg$^{2+}$-AMP-PNP and Mg$^{2+}$-ADP, respectively (2).

Recently, site-directed mutations of the P-loop threonine of the b subunit in CF$_1$ from Chlamydomonas (bT168) were compared to determine whether changes in the EPR spectra of VO$^{2+}$ bound to catalytic Site 3 could be detected (9). The mutations were found to cause changes in both the signal intensity and $^{51}$V hyperfine parameters of the bound VO$^{2+}$ that gave rise to EPR species C in a manner that indicated that this residue was a metal ligand in the activated conformation. The fact that changes in EPR species B in the mutant CF$_1$ indicated that the P-loop threonine is not a ligand in the form that predominates in the latent state of the enzyme.

We now report an analysis of site-directed mutants of the WHB-aspartate (βD262) of Chlamydomonas CF$_1$ by EPR spectroscopy of VO$^{2+}$ bound to catalytic Site 3. The results presented here indicate that βD262 participates in metal binding at Site 3 in the metal-nucleotide complex that predominates in the latent form, but not in the complex that occurs in the activated form of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids and Cell Strains Containing Mutations—**

Chlamydomonas reinhardtii strains (CC-125 and CC-373) were obtained from the C. reinhardtii Culture Collection at Duke University. The plasmid pWT-373 (8) was used as a template for double-stranded, oligonucleotide-mediated, site-directed mutagenesis following the protocol described in Stratagene Chameleon double-stranded site-directed mutagenesis manual. Each mutagenesis reaction requires a selection primer and a mutagenic primer. The sequence of the selection primer is the same for every mutagenesis reaction, 5′-CCG CCC GAA GAA CGG ATC CCA ATG ATG AC-3′. The sequences of the mutagenic primers for different mutated plasmids are listed as follows. 1) pD262C, 5′-TTA TTC TCC ATT TGT AAC ATT TTC CGG TTC GTA CAA GCT G; 2) pD262H, 5′-TTA TTC TCC ATT CAT AAC ATT TTC CGT TCT; 3) pD262N, 5′-TTA TTC TCC ATT AAC ATT TTC CGT TCT; 4) pD262T, 5′-TAT TCT TCA TTA CAA ACA TTT TCC GGT TCG TAC AAG CFG G.

Mutated plasmid DNA was transformed into the C. reinhardtii chloroplast genome using biolistic transformation following procedures as described previously (8, 30, 31). Southern blot analyses and double-stranded DNA sequencing (32) were used to verify the presence of homoplasmic cell lines with desired mutations.

**Biochemical Characterization of C. reinhardtii Mutants—**

Cell cultures and photoautotrophic growth curves of each C. reinhardtii strain were maintained and measured as per Hu et al. (8). Thylakoid membranes in which electron transfer and ATP synthesis were tightly coupled were prepared, and photophosphorylation assays were measured as per Hu et al. (8). The ability of purified thylakoid membranes to generate a light-driven proton gradient was monitored using 9-aminoacridine fluorescence quenching as per Chen et al. (9).

Isolation of soluble CF$_1$-ATPase from C. reinhardtii and the selective filling of VO$^{2+}$-ATP into Site 3 were carried out as per Chen et al. (9). The ATPase activity was determined using the coupled ATPase assay including lactic dehydrogenase, and pyruvate kinase as described by Harris and Bashford (33). To activate the CF$_1$, final concentrations of 50 mM dithiothreitol and 20% ethanol were incubated with purified CF$_1$ for more than one h at room temperature. The reaction rates were determined from the initial slopes, typically in the first 20–30 s after adding the metal to the reaction mixture.

**EPR Analyses—**

EPR experiments were carried out at X-band (9 GHz) using a Bruker 580E spectrometer with a TE$_{102}$ standard cavity and a liquid nitrogen flow cryostat operating at 125 K. Simulations of the CW-EPR spectra employed the program QPOWA (12, 34).

**RESULTS**

**Effects of Mutations on Yield and Composition of CF$_1$ and Photoautotrophic Growth—**

Growth curves of wild type and mutant Chlamydomonas cultures were obtained under photoautotrophic conditions at 25 °C at a light intensity of 80 microEinstein·m$^{-2}$·s$^{-1}$. As shown in Table I, all of the mutations of βD262 caused dramatic decreases in the ability to grow photoautotrophically compared with wild type. The D262N, C, and T mutants grew at 5–10% of the wild type rate. The D262H mutant was completely incapable of photoautotrophic growth.

The effects of these mutations on rates of phenazine methosulphate-dependent photophosphorylation of isolated thylakoids, and ATPase activity of purified CF$_1$ preparations are summarized in Table I. The results are consistent with the relative ability of the mutants to grow photoautotrophically. In all cases, the activities of the D262N, C, and T mutants were about 10% of wild type, whereas those of the D262H mutant were negligible.

The subunit composition of CF$_1$ isolated from wild type and mutants are compared by SDS-polyacrylamide gel electrophoresis in Fig. 1. All mutants were found to contain α, β, γ,
and ε subunits as does the wild type. The abundance of the δ subunit relative to the other subunits was variable among preparations. This subunit is known to be weakly associated with the Chlamydomonas CF1 and is easily lost when the enzyme preparation is stored (8, 35). The variability of the abundance of the δ subunit among preparations did not differ from that of wild-type CF1. Other bands visible in these preparations are polypeptides that have been reported previously to copurify with CF1 from Chlamydomonas. A 12% acrylamide, Coomassie-stained gel was used to visualize polypeptides from about 15 μg of protein purified from wild type (WT) and the mutant cells as indicated.

The low rates of photophosphorylation were not the result of the inability of the thylakoids to form a light-driven proton gradient. Fig. 2 shows the relative fluorescence quenching of 9-aminoacridine fluorescence quenching. The rate and extent of fluorescence quenching was about the same in each of the mutants as in the wild type.

Thus, none of the mutations caused the membranes to become uncoupled. Combined with the observations that the yield and subunit composition of the mutant proteins are the same as wild type, it is unlikely that any of the mutations has caused a large conformational change that interferes with folding and assembly of the CF1F0 complex.

Effects of Mutations on the EPR spectrum of VO2+ bound at Site 3 of CF1—Fig. 3 shows parallel transitions of EPR spectra of VO2+ bound to Site 3 of CF1 from wild type Chlamydomonas. Because of the anisotropy that results from the oxo group of VO2+, the single unpaired electron and the nuclear spin I = 7/2 result in an EPR spectrum that consists of 8 transitions from that of wild-type CF1. Other bands visible in these preparations are polypeptides that have been reported previously to copurify with CF1 from Chlamydomonas and with thylakoids from wild type. Based on the rate of 250 μmol of ATP (mg of chlorophyll·h)−1 using Mg2+-ADP and phosphate concentrations of 2 and 3 mM, respectively, with thylakoids from wild type.

Table I

| Genotype     | Photautotroph growth ratea | ATP synthase activityb | ATPase activityc |
|--------------|---------------------------|----------------------|------------------|
| Wild-type    | 100                       | 100                  | 100              |
| βD262H       | 0                         | 3                    | 0                |
| βD262N       | 9                         | 10                   | 4                |
| βD262C       | 5                         | 14                   | 5                |
| βD262T       | 4                         | 10                   | 10               |

a Measured as the rate of increase in optical density (cell scattering) of the liquid culture at 720 nm in log phase at 25 °C with a light intensity of 80 microEinsteins·m−2·s−1.
b Based on the rate of 250 μmol of ATP (mg of chlorophyll·h)−1 using Mg2+-ADP and phosphate concentrations of 2 and 3 mM, respectively, with thylakoids from wild type.
c Based on the rate of 0.6 μmol of ATP hydrolyzed (mg of CF1·min)−1 using 10 mM Mg2+-ATP with CF1 purified from wild-type Chlamydomonas.

Fig. 1. Analysis by SDS-polyacrylamide gel electrophoresis of the polypeptide constituents of chloroplast F1 preparations purified from wild type and mutant Chlamydomonas. A 12% acrylamide, Coomassie-stained gel was used to visualize polypeptides from about 15 μg of protein purified from wild type (WT) and the mutant cells as indicated.

Fig. 2. Light-driven proton gradient formation in thylakoids purified from: a, wild type; b, D262C; c, D262N; d, D262T; and e, D262H mutants of Chlamydomonas measured by 9-aminoacridine fluorescence quenching.

Fig. 3. The parallel regions of the VO2+-EPR spectrum when VO2+-ATP is bound at Site 3 of wild type Chlamydomonas CF1 (a). 1 mol equivalent of VO2+ was added as a complex with ATP to 57.5 mg of CF1 that had been depleted of metal-nucleotide from Site 3. EPR conditions were as follows: field modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; sweep rate, 0.95 mT/s; time constant, 82 ms; microwave power, 1.0 mW, temperature, 100 K; microwave frequency, 9.66278 GHz; number of scans, 200. Simulated spectra for species B (b) and species C (c) were generated using the program QPOWA with the experimental conditions above and the 51V-hyperfine parameters from Table II.
serve as equatorial ligands. These changes will be evident as differences in the spacing between the $-7/2$, $-5/2$, $+3/2$, and $+5/2$, and $+7/2$ transitions shown in Fig. 3, which do not overlap with perpendicular transitions.

Addition of an equivalent of VO$^{2+}$-ATP to CF$_1$ under conditions in which all other higher affinity binding sites for metal-nucleotides were filled with Mg$^{2+}$-nucleotide complexes resulted in two sets of parallel transitions for VO$^{2+}$. The simulated spectrum for each set (Fig. 3, spectra b and c) and the values of $A_i$ and $g_i$ used to generate these spectra are given in Table II. Spectra b and c correspond to EPR species B and C that result from the two specific binding environments for VO$^{2+}$ as a complex with nucleotide in Site 3 each with its own set of equatorial ligands. In the latent form of the enzyme, species B predominates, but activation induces the conversion of species B into species C. The ratios of the amplitudes of the simulated spectra for EPR species B and C from each mutant that, when summed, reproduced the experimental spectra are also shown in Table II. These data provide the ratio of the amount of vanadyl bound in the form that gives rise to species B versus species C.

The parallel transitions of the EPR spectrum from VO$^{2+}$-ATP bound to Site 3 of CF$_1$ with the D262H mutation is shown in Fig. 4 along with the simulations of EPR species B and C that best fit the experimental data. The value of $A_i$ for EPR species B in this mutant decreased 4.4 MHz from that of the wild type enzyme to a value of 493.8 MHz, whereas the $^{51}$V-hyperfine components of species C remained unchanged (Table II). This mutation also caused an increase in the species C:B ratio by more than 3-fold of that observed with wild type CF$_1$. Because $A_i$ and $g_i$ are not affected by the mutations, these changes are indicative of changes in the affinity of the VO$^{2+}$-ATP complex for the conformation of Site 3 that gives rise to species B.

**DISCUSSION**

The results presented here indicate that D262 serves as an essential residue of the chloroplast F$_1$F$_0$-ATP synthase in both a catalytic and regulatory capacity. Catalytic function of the enzyme was significantly affected by every mutation examined. In addition, every mutation changed the ratio of EPR species C:B and/or the $^{51}$V-hyperfine parameters of EPR species B, the predominant conformation of VO$^{2+}$-nucleotide bound to Site 3 of latent CF$_1$. None of the crystal structures of F$_1$ determined to date (2, 38, 39) has provided any information concerning the conformation of the metal-nucleotide bound to CF$_1$ in the latent state.

Weber et al. (5) measured the binding affinities of nucleotides to the catalytic sites of the $\beta\gamma$Y331W mutant of E. coli F$_1$ by the fluorescence quenching that results from a direct interaction between the adenine ring of the nucleotide and the tryptophan residue. These studies revealed that Mg$^{2+}$ was responsible for the large differences in affinities of nucleotide among the three catalytic sites. When the WHB-aspartate was mutated to create a double mutant $\beta\gamma$Y331W/βD242N of E. coli F$_1$, the nucleotide binding affinities of the three catalytic sites were not increased by Mg$^{2+}$ but became closely similar to the lower affinity observed when nucleotide binds alone (26). Based on these results Weber et al. (26) concluded that there must be a water molecule not visible in the crystal structure that is both hydrogen-bonded to the WHB carboxyl and coordinated to the

| Strain     | Ratio of C:B | Species B | Species C |
|------------|--------------|-----------|-----------|
| Wild-type  | 0.87         | 498.2     | 456.5     | 1.957     |
| βD262H     | 0.28         | 493.8     | 458.0     | 1.957     |
| βD262T     | 0.51         | 502.2     | 456.5     | 1.957     |
| βD262N     | 0.68         | 498.2     | 456.5     | 1.957     |
| βD262C     | 0.17         | 498.2     | 457.5     | 1.957     |

**Table II**

Experimental $^{51}$V-hyperfine parameters derived from VO$^{2+}$ bound as a complex with ATP at Site 3 in CF$_1$ from wild-type and βD262 mutants.

Experimental parameters were determined by simulation of entire spectrum using QPOWA.
WHB-Carboxyl Mutants Change EPR Spectra of VO$^{2+}$ Bound to CF$_1$

**Fig. 5.** The parallel regions of the VO$^{2+}$-EPR spectrum when VO$^{2+}$-ATP is bound at Site 3 of the βD262H mutant of Chlamydomonas CF$_1$ (a). 1 mol equivalent of VO$^{2+}$ was added as a complex with ATP to 20 mg of CF$_1$ that had been depleted of metal-nucleotide from Site 3. EPR conditions were as follows: field modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; sweep rate, 0.95 mT/s; time constant, 82 ms; microwave power, 1.0 mW; temperature, 100 K; microwave frequency, 9.66437 GHz; number of scans, 180. Simulated spectra for species B (b) and species C (c) were generated using the program QPOWA with the experimental conditions above and the $^{51}$V-hyperfine parameters from Table II.

**Fig. 6.** The parallel regions of the VO$^{2+}$-EPR spectrum when VO$^{2+}$-ATP is bound at Site 3 of the βD262N mutant of Chlamydomonas CF$_1$ (a). 1 mol equivalent of VO$^{2+}$ was added as a complex with ATP to 36 mg of CF$_1$ that had been depleted of metal-nucleotide from Site 3. EPR conditions were as follows: field modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; sweep rate, 0.95 mT/s; time constant, 82 ms; microwave power, 1.0 mW; temperature, 100 K; microwave frequency, 9.66434 GHz; number of scans, 660. Simulated spectra for species B (b) and species C (c) were generated using the program QPOWA with the experimental conditions above and the $^{51}$V-hyperfine parameters from Table II.

metal in all three catalytic sites.

The results presented here for EPR species C show that, in this conformation, the WHB carboxyl is neither an equatorial ligand to VO$^{2+}$ nor is it hydrogen-bonded to a water molecule coordinated at an equatorial position at Site 3. The $^{51}$V-hyperfine parameters of EPR species C, the species present exclusively in the activated enzyme, were not changed significantly by any of the mutations examined. These mutations did inhibit catalytic function as observed for the D242N mutant in *E. coli* F$_1$ (26). Therefore, it is possible that the WHB carboxyl has hydrogen bonded to the vanadyl-oxo or to a water molecule coordinated at the axial position of the VO$^{2+}$ in Site 3. The loss of activity in these mutants could also be explained if this residue were to serve as a direct ligand in one of the other catalytic sites. This will be resolved as more of the residues that serve as metal ligands at each catalytic site are identified.

The EPR data that result from VO$^{2+}$ bound to Site 3 of mutant CF$_1$ provide insight into the metal ligation responsible for the changes in EPR species B in each of these mutants. Based on the measured coupling constants of $A_i$ from model studies (28, 37), the hyperfine coupling for a given group of equatorial ligands can be calculated from Eq. 1:

$$A_{cal} = \sum n_i A_i / 4$$

(Eq. 1)

where $i$ counts the different types of equatorial ligand donor groups, $n_i (= 1–4)$ is the number of ligands of type $i$, and $A_i$ is the measured coupling constant for equatorial ligand donor group of type $i$ (28). Similar equations can be written for $g_i$ and for $A_{calc}$, though the changes in $A_i$ are the largest and most easily discerned.

Table III shows the values of $A_i$ and $g_i$ calculated from Eq. 1 that give the closest fit to the experimental data derived from simulation of the spectra (Table II) and summarizes the equatorial ligands used for these calculations. The best fit of the data for species B at Site 3 of wild type CF$_1$ includes a water, as well as carboxyl, hydroxyl, and phosphate groups as equatorial ligands to VO$^{2+}$ (9). One interpretation of the data presented here is that the putative carboxyl ligand is D262. In this case, we expect to observe a change in $^{51}$V-hyperfine parameters consistent with displacement of the carboxyl by the type of group substituted in the D262 mutation. Alternatively, D262 may be hydrogen-bonded to the putative water ligand derived from the best fit to EPR species B. No changes $^{51}$V-hyperfine parameters are typically expected except in the rare event that the mutation causes a ligand to be displaced. Instead, the differences in the ability of the mutated groups to hydrogen bond to water are anticipated to change the ratio of EPR species C:B.

If D262 acts as a direct metal ligand, the 4.4 MHz change in $A_i$ of species B observed with D262H can be easily explained as
a simple substitution of an imidazole nitrogen for a carboxyl oxygen as an equatorial ligand to the bound VO$_2^+$. This change in $A_1$ is more difficult to explain if D262 is only hydrogen-bonded to the coordinated water because this requires a double substitution of imidazole nitrogen for water and water for carboxyl.

The EPR data shown for the D262N mutation does not provide any information to distinguish between the possible roles of the carboxyl as a direct ligand, or as an indirect ligand hydrogen bonded to a coordinated water. An asparagine side chain coordinated to VO$_2^+$ shows changes in $A_1$ that can be resolved only at much higher signal-to-noise than that reported here (8). The ability of this side chain to hydrogen bond to water also allows for the latter possibility. It is noteworthy that the D262N mutation causes a small change in the ratio of EPR species C:B signal intensities.

If the D262T mutation were to result solely in the substitution of a carboxyl for a hydroxyl oxygen, a decrease in $A_1$ of about 22 MHz is expected. However, an increase in $A_1$ of 4 MHz was observed in this mutant to a value of 502.2 MHz. These data are best fit to a set of equatorial ligands where the carboxyl group and the hydroxyl group have been substituted for a simple substitution of an imidazole nitrogen for a carboxyl oxygen as an equatorial ligand to the bound VO$_2^+$. This change in $A_1$ is more difficult to explain if D262 is only hydrogen-bonded to the coordinated water because this requires a double substitution of imidazole nitrogen for water and water for carboxyl.

The EPR data shown for the D262N mutation does not provide any information to distinguish between the possible roles of the carboxyl as a direct ligand, or as an indirect ligand hydrogen bonded to a coordinated water. An asparagine side chain coordinated to VO$_2^+$ shows changes in $A_1$ that can be resolved only at much higher signal-to-noise than that reported here (8). The ability of this side chain to hydrogen bond to water also allows for the latter possibility. It is noteworthy that the D262N mutation causes a small change in the ratio of EPR species C:B signal intensities.