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Properties of the Manganese(II) Binding Site in Ternary Complexes of Mn·ADP and Mn·ATP with Chloroplast Coupling Factor 1: Magnetic Field Dependence of Solvent ^1H and ^2H NMR Relaxation Rates†

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ABSTRACT: The influence of the binding of ADP and ATP on the high-affinity Mn(II) binding site of chloroplast coupling factor 1 (CF_1) was studied by analysis of field-dependent solvent proton and deuteron spin–lattice relaxation data. In order to characterize metal–nucleotide complexes of CF_1 under conditions similar to those of the NMR experiments, the enzyme was analyzed for bound nucleotides and Mn(II) after incubation with AdN and $MnCl_2$ and removal of labile ligands by extensive gel filtration chromatography. As isolated, the enzyme contained 1.3–1.4 mol of adenine nucleotide (mostly ADP) and 1.3 mol of Mg(II) per mole of CF_1. After incubation with added Mn(II) and nucleotide, a total of three binding sites with high affinity for divalent metal ions (Mg^{2+} + Mn^{2+}) and/or nucleotide (ATP or ADP) were found. In the field-dependent NMR experiments, the Mn(II) binding site of CF_1 was studied for three mole ratios of added Mn(II) to CF_1, 0.5, 1.0, and 1.5, in the presence of an excess of either ADP or ATP. The results were extrapolated to zero Mn(II) concentration to characterize the environment of the first Mn(II) binding site of CF_1. In the presence of both adenine nucleotides, pronounced changes in the Mn(II) environment relative to that in Mn(II)–CF_1 were evident; the local relaxation rate maxima were more pronounced and shifted to higher field strengths, and the relaxation rate per bound Mn(II) increased at all field strengths. Analysis of the data revealed that the number of exchangeable water molecules liganded to bound Mn(II) increased from one in the binary Mn(II)–CF_1 complex to three and two in the ternary Mn(II)–ADP–CF_1 and Mn(II)–ATP–CF_1 complexes, respectively; these results suggest that a water ligand to bound Mn(II) in the Mn(II)–ADP–CF_1 complex is replaced by the γ-phosphate of ATP in the Mn(II)–ATP–CF_1 complex. The residence time of water within the inner coordination sphere was found to be 430 ns in the presence of ADP and 260 ns in the presence of ATP. The low-field electron spin relaxation time decreased in the ternary Mn(II)–AdN–CF_1 complexes relative to binary Mn(II)–CF_1, reflecting a lower Mn(II) site symmetry in the latter complex. A binding model is presented to account for these observations.

The requirement for a divalent metal in the synthesis and hydrolysis of ATP by chloroplast coupling factor 1 (CF_1)[1] suggests that the presence of adenine nucleotides should have a marked effect on the environment of enzyme-bound metal. The metal cofactor participates as a metal–nucleotide complex in the ATPase reaction of CF_1 (Hochman & Carmeli, 1981; Leckband & Hammes, 1987). Studies with exchange-inert Cr(III)–nucleotides have shown that the substrate for ATP hydrolysis is a tridentate metal–ATP complex while the substrate for ATP synthesis is the Λ-bidentate metal–ADP complex (Frasch & Selman, 1982); these results suggest that the metal ion remains coordinated to the phosphate oxygens throughout the hydrolysis reaction.

We have previously analyzed the magnetic field dependence of NMR relaxation rates of solvent protons and deuterons as a probe of the coordination environment of CF_1-bound Mn(II) (Haddy & Sharp, 1989). The present study extends this investigation from the binary Mn(II)–CF_1 complex to ternary complexes of Mn(II)–ADP–CF_1 and Mn(II)–ATP–CF_1. In each case, attention has been focused on the high-affinity Mn(II)-containing complexes in the given binary or ternary system. For this purpose, relaxation data have been collected as a function of added Mn(II) in the presence of excess nucleotide and the results of the analysis extrapolated to zero Mn(II)/CF_1 binding ratio. Measurements of the magnetic field dependence (the field dispersion profile) of the NMR spin–lattice relaxation rate, R_1, of solvent protons permit a determination of the metal ion hydration number and of the residence time of solvent molecules which exchange between the bulk aqueous phase and ligand sites in the Mn(II) coordination sphere. The analysis also characterizes magnetic interactions within the Mn(II) coordination sphere, specifically the spectral density function describing the dipolar interaction between electron and proton spins.

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; AdN, adenine nucleotide.

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Chloroplast CF_1 contains three high-affinity nucleotide binding sites, termed sites 1, 2, and 3, the properties of which have been described previously (Leckband & Hammes, 1987). These sites reportedly differ in their requirement for divalent cation. Site 1 is specific for ADP, possibly as Mg·ADP, with extremely high affinity and in most studies has been found to be occupied in the isolated enzyme (Carlier & Hammes, 1979; Hiller & Carmeli, 1985). Site 2 binds Mg(II)–ATP with an obligatory requirement for the divalent metal cation; this site also binds ADP in either the presence of the absence of metal (Bruist & Hammes, 1981). Mg·ATP bound to site 2 is not labile but is removed by $(NH_4)_2SO_4$ precipitation of the enzyme. Site 3 also binds both ADP and metal–ATP, but with lower affinity than site 2.

In addition to these nucleotide and metal–nucleotide binding sites, CF_1 contains two to three high-affinity binding sites for Mn(II) in the absence of accompanying nucleotide (Haddy et al., 1985; Hiller & Carmeli, 1985). These metal sites have properties supporting their identification with unoccupied nucleotide binding sites 2 and 3.

Materials and Methods

Spinach chloroplast coupling factor 1 was prepared as described previously (Haddy & Sharp, 1989) and stored as an $(NH_4)_2SO_4$ precipitate. For NMR experiments, latent CF_1 was desalted over a 1 × 28 cm Sephadex G-50 column, then passed over a Chelex-100 column (1 × 3 cm) to remove labile divalent metal cations, and reconcentrated by pressure dialysis. This preparation was divided into two aliquots to which ADP (Sigma, grade IX) or ATP (Sigma, equine muscle, "vanadium free") was added to a final concentration of 240 μM. These samples were again split into three aliquots each and placed in NMR tubes to which the indicated concentrations of $MnCl_2$ were added. One aliquot was also prepared without added nucleotide or $MnCl_2$ as a diamagnetic control.

For binding studies, the desalted enzyme (15–45 μM) was then incubated with the indicated concentrations of ATP or ADP plus $MnCl_2$ for 50–60 min at 22–23 °C; the nucleotide was added first. Labile nucleotide and Mn^{2+} were then removed by passage over a second Sephadex G-50 column (1 × 28 cm). The reconcentrated enzyme was denatured in 0.67 M HCl and analyzed for bound nucleotides and Mn(II) as described previously (Haddy & Sharp, 1989).

Spin–Lattice Relaxation Rate Measurements. The magnetic field dependence of solvent proton spin–lattice relaxation rates, $R_1 = 1/T_1$, was measured as described previously (Haddy & Sharp, 1989). The data analysis relied on comparative ^1H and ^2H R_1 measurements, which were made at 12.63 MHz using solutions prepared in 75 mol % ^2H_2O. The CF_1 solutions showed a small but significant instability. Over the 12-h measurement period, values of R_{1p} varied by at most 13% for the Mn·ADP·CF_1 complex and at most 5% for the Mn·ATP·CF_1 complex.

Paramagnetic relaxation rates due to the ternary Mn·ANP·CF_1 complexes were calculated by subtracting from the measured R_1 the diamagnetic contribution of the apoenzyme and the paramagnetic contribution from low molecular weight Mn(II)–nucleotide complexes. Uncomplexed Mn^{2+} was assayed by ESR and found to be negligible (<0.5 μM). The diamagnetic correction ranged from 13% to 33% of the observed relaxation rates at 15 MHz and from 30% to 60% of the observed relaxation rates at 62 MHz.

Paramagnetic contributions from Mn(II)–nucleotide complexes were calculated as for hexaaquo-Mn(II) as described previously (Haddy & Sharp, 1989) using the appropriate hydration numbers and a rotational correlation time based on the size of these complexes. The concentrations of Mn(II) not bound to CF_1 were calculated from the dissociation constants determined by Hochman and Carmeli (1981) for Mn(II) binding to CF_1 in the presence of 2–4 equiv of ATP and 2–5 equiv of ADP; unbound Mn(II) calculated this way was assumed to exist as Mn(II)–nucleotide complexes since the free Mn^{2+} was negligible. Overall corrections for effects of low molecular weight complexes accounted for not more than 7.5% and in most cases less than 2% of the observed relaxation rates.

Analysis of the Field-Dependent Relaxation Data. Analysis of the magnetic field dependent ^1H and ^2H R_1 data utilized the Solomon–Bloembergen–Morgan (SBM) theory with the modifications described previously (Haddy & Sharp, 1989). The analysis involves four parameters: q, the hydration number of bound Mn(II); τ_m, the mean residence time of solvent in the manganese coordination sphere; and two motional correlation times, $\tau_{s,v}$ and $\tau_{s,d}^{o}$, which describe fluctuations of the Mn(II) ligand field. Approximate values of the first two parameters were calculated from ^1H and ^2H R_1 data at 12.63 MHz, using the relations:

$$\tau_m = \frac{r^6}{B}\left(\frac{2\omega_{I,m}}{3}\right)\left[\frac{(\gamma^p/\gamma^d)^2 T_{1p}^{p} - T_{1p}^{d}}{T_{1p}^{d} - T_{1p}^{p}}\right] \quad (1)$$

$$q = \frac{r^6}{B}\left(\frac{2\omega_{I,m}}{3}\right)\left\{\frac{55.5[(\gamma^p/\gamma^d)^2 - 1]}{E(T_{1p}^{d} - T_{1p}^{p})}\right\} \quad (2)$$

where γ is the magnetogyric ratio, I and S, respectively, $\omega_{I,m}$ is the proton Larmor frequency at the local maximum in $1/T_{up}$, p and d designate proton and deuteron, respectively, and $B = 2(\hbar\gamma_s\gamma_I)^2 S(S+1)/15$. r, the separation of nuclear and electronic spins, was taken to be 2.77 Å, the crystallographic value for $Mn(H_2O)_6^{2+}$. These initial values were refined by an iterative nonlinear least-squares fit involving four parameters, q, τ_m, $\tau_{s,v}$, and $\tau_{s,d}^{o}$, to the ^2H and variable-field ^1H R_1 data.

Results

Quantitation of Bound Manganese and Nucleotides. Complicating the interpretations of metal ion binding to CF_1 are slow binding processes with half-lives on a time scale of several tens of minutes. These effects are pronounced for latent CF_1 and have been attributed to slow conformational changes of the enzyme (Carmeli et al., 1987; Schumann, 1987). This longer time scale is appropriate to the present NMR experiments, which involved an incubation period of 0.5 h or more at 22–23 °C followed by a measurement period of several hours at 25 °C. We therefore tested predictions of the three-site high-affinity nucleotide binding model of CF_1 (Leckband & Hammes, 1987) under conditions that more closely simulate the NMR experiments, namely, prolonged incubation (50–60 min at 22 °C) of the enzyme with nucleotide in the presence of a Mn(II) cofactor. Following incubation, labile low molecular weight species were removed by gel filtration chromatography, a step requiring about 20 min. Metal and nucleotide contents that were nonlabile over this time scale are summarized in Table I.

As isolated, the enzyme preparation contained endogenous nucleotide at a level of 1.1–1.3 mol of ADP/mol of CF_1 and 0.1–0.2 mol of ATP/mol of CF_1 plus endogenous Mg at a level of 1.3 mol of Mg/mol of CF_1. When the enzyme was incubated with 1 molar equiv of $MnCl_2$ in the presence of 1–2.5 molar equiv of ATP, approximately 0.7 mol of Mn(II) and 0.8–1.1 mol of ATP per mole of CF_1 remained bound (rows

Table I: Binding of Mn(II) and Nucleotides That Could Not Be Removed by Extensive Gel Filtration[a]

	incubation medium			bound (mol/mol of CF_1)		
[CF_1] (μM)	[Mn^{2+}] (μM)	[ATP] (μM)	[ADP] (μM)	ADP	ATP	Mn(II)
30	31	33	0	1.4	0.8	0.72
20	19	50	0	1.5	1.1	0.75
24	51	52	0	1.7	1.3	1.7
41	29	275	0	1.6	1.4	0.46
35	52	385	0	nd	1.9	1.1
39	19	0	97	1.3	nd	0.37

[a] CF_1 pretreated by gel filtration as incubated with the indicated concentrations of $MnCl_2$ and nucleotide, then freed of dissociable metal and nucleotide, and analyzed as described under Materials and Methods. Errors for nucleotide determinations were 10% or less; errors for Mn(II) determinations were about 5%. nd = not determined.

1 and 2 of Table I). Thus, the total bound divalent cation [Mg(II) + Mn(II)] was 2.0–2.1 mol/mol of CF_1. In addition, the bound ADP increased from an initial value of 1.1 molar equiv to 1.4–1.5 molar equiv; this increase apparently reflects dephosphorylation of added ATP. Total concentrations of bound metal [Mg(II) + Mn(II)] were close to that of bound nucleotide (ADP + ATP), which was 2.2–2.6 mol/mol of CF_1. Bruist and Hammes (1981) have reported that latent CF_1, like heat-activated CF_1, binds a single molar equivalent of Mg(II)–ATP in a nonlabile manner in site 2. The binding stoichiometries of Table I, rows 1 and 2, are consistent with the reported properties of sites 1, 2, and 3 if site 1 is occupied by ADP [probably as a Mg(II)–ADP complex] and site 2 is occupied by a mixture of Mn(II)–ATP and Mg(II)–ATP. Site 3 also appears to be partially occupied by ADP or metal–ADP.

When CF_1 was incubated with about 2 molar equiv each of $MnCl_2$ and ATP, the total bound divalent cation [Mg(II) + Mn(II)] and the total bound adenine nucleotide (ADP + ATP) both increased to about 3 molar equiv (Table I, row 3). Both ADP and ATP were bound at levels significantly in excess of 1 molar equiv. The three nucleotide site model (Bruist & Hammes, 1981; Leckband & Hammes, 1987) suggests an interpretation in terms of which site 1 is occupied by divalent metal–ADP, site 2 by metal–ATP, and site 3 by a mixture of metal–ADP plus metal–ATP. While this assignment explains the measured metal and nucleotide stoichiometries, the finding of a third nonlabile nucleotide binding site is somewhat unexpected in terms of the reported lability of site 3. This difference may relate to the use of Mn(II), as opposed to Mg, in our experiments. More similar to our results perhaps are those of Girault and co-workers (Girault et al., 1982), who reported three bound nucleotides (ATP + ADP) per CF_1 after a 1-h incubation of the enzyme with excess ATP and Mg^{2+}, followed by rapid removal of unbound substances by centrifuge chromatography.

In a fourth experiment, CF_1 was incubated with excess ATP (6.7 molar equiv) plus sufficient Mn(II) to fill site 2 but not site 3. Under these conditions, 3.0 molar equiv of nucleotide was bound to CF_1 along with 1.8 molar equiv of divalent metal ion [Mn(II) + Mg(II)]. This result indicates that nucleotide, in the form of either ADP or ATP, can occupy a third site without accompanying metal ion. As described above, however, the third site, in the presence of more than 1 molar equiv of added Mn^{2+}, preferentially binds the Mn(II)–AdN complex rather than AdN alone.

This finding was tested by incubating CF_1 with a large excess of ATP (11 molar equiv) and 1.5 molar equiv of Mn(II), which should have been just enough divalent cation [2.8 molar equiv of Mg(II) + Mn(II)] to fill three sites (Table I, row 5). Under these conditions, about 3 molar equiv of adenine nucleotide and 2.4 molar equiv of divalent cation were bound to CF_1, again suggesting that high-affinity binding of nucleotide to a third site can occur both with and without accompanying metal ion. These properties are in general accord with the reported properties of nucleotide site 3, particularly with respect to its substantial affinity for adenine nucleotides and their metal complexes. Summarizing the results of experiments involving added Mn(II) and ATP, three nonlabile binding sites were found for both divalent metal and adenine nucleotide.

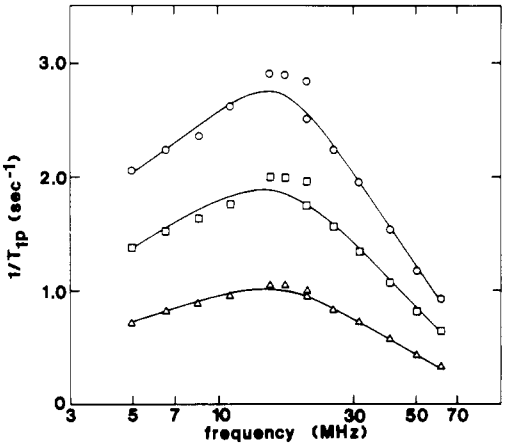

FIGURE 1: Magnetic field dependence of the paramagnetic spin–lattice relaxation rate ($1/T_{1p}$) of water protons for Mn(II)–CF_1 in the presence of added ADP at 25 °C. NMR samples contained 20 μM latent CF_1, 10 μM added ADP, and 10 (triangles), 20 (squares), or 30 (circles) μM $MnCl_2$. The plotted data are corrected for relaxation contributions due to the apoenzyme and for binary Mn·ADP as described under Materials and Methods. Solid curves represent the least-squares fits to the data.

Finally, the binding of a substoichiometric concentration of Mn(II) was measured in the presence of excess ADP (2.5 molar equiv) without added ATP. The major portion of added Mn(II) bound tightly to the enzyme without a significant increase in bound ADP (Table I, row 6), indicating that a nonlabile Mn(II)–ADP site is not formed under these circumstances. However, the NMR studies described below clearly show that a Mn(II)–ADP site is present on CF_1 prior to removal of unbound ADP. Evidently, this site is labile during gel filtration. In contrast, the Mn(II)–ADP site observed following ATP addition was nonlabile during gel filtration, as described above. Thus, it appears that the lability of bound Mn(II)–ADP depended on the experimental conditons; ADP formed after the addition of ATP was nonlabile, while ADP bound from medium ADP was labile. The formation of bound ADP from solution ATP has been demonstrated previously (Girault et al., 1982); in that study, it was concluded that the bound ADP arose directly from hydrolysis of bound ATP rather than from ADP which had been released and rebound.

Field Dispersion R_1 Profiles of Mn(II)–AdN–CF_1 Complexes. Magnetic field dispersion profiles of Mn(II)–ADP–CF_1 and of Mn(II)–ATP–CF_1 are shown in Figures 1 and 2, respectively. The presence of added adenine nucleotide caused

Table II: Nonlinear Least-Squares Fit Parameters for Mn(II)–CF_1 Solutions in the Presence of Added Nucleotide[a]

addition[b] (AdN, [$MnCl_2$])	τ_d (ps)	$1/\tau_{s,v} + 1/\tau_r$ (s^{-1})	$\tau_{s,d}^{\circ}$ (ns)	τ_m (ns)	q
ADP, 0	100			434	3.25
10		0.62×10^8	0.151 (±7.5%)	426 (±5.0%)	3.14 (±2.6%)
20		0.62	0.154 (±9.9%)	427 (±6.6%)	2.94 (±3.4%)
30		0.66	0.169 (±12.0%)	414 (±7.5%)	2.87 (±3.9%)
ATP, 0	100			259	1.92
10		0.78×10^8	0.234 (±10.6%)	261 (±6.7%)	1.88 (±2.9%)
20		0.86	0.251 (±7.8%)	266 (±4.7%)	1.79 (±2.1%)
30		0.93	0.275 (±9.3%)	267 (±5.2%)	1.77 (±2.4%)

[a] These parameters correspond to data shown in Figures 1 and 2 for ADP and ATP, respectively. Values of $1/\tau_{s,v} + 1/\tau_r$ were assigned by using the best fit to the deuteron data of Table III. Errors represent standard deviations generated by the least-squares fitting procedure assuming $r = 2.77$ Å; absolute values of $\tau_{s,d}^{\circ}$ contain large absolute error (see text). The first value of each set represents extrapolations of q and τ_m to zero Mn(II) concentration. [b] The nucleotide concentration was 240 μM; that of $MnCl_2$ (in micromolar) is tabulated.

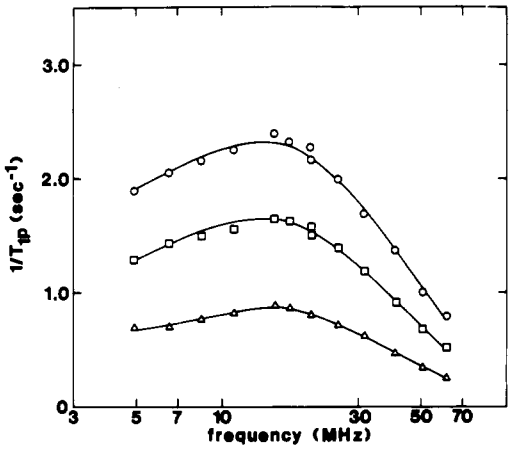

FIGURE 2: Magnetic field dependence of the paramagnetic spin–lattice relaxation rate ($1/T_{1p}$) of water protons for Mn(II)–CF_1 in the presence of added ATP. Samples contained 20 μM latent CF_1, 240 μM added ATP, and 10 (triangles), 20 (squares), or 30 (circles) μM $MnCl_2$. Data were corrected for effects of the apoenzyme and for binary Mn·ATP as described under Materials and Methods. Solid curves represent the least-squares fits to the data.

Table III: Comparison of Experimental and Calculated $1/T_{1p}$ Values for Deuteron and Proton in the Presence of Added Nucleotide[a]

nucleotide	[Mn(II)] (μM)	nucleus	$1/T_{1p}$, exptl	$1/T_{1p}$, calcd
ADP	20	^1H	1.772 ± 0.019	1.772
		^2H	0.215 ± 0.036	0.195
	30	^1H	2.577 ± 0.025	2.577
		^2H	0.253 ± 0.048	0.281
ATP	20	^1H	1.360 ± 0.017	1.360
		^2H	0.098 ± 0.029	0.111
	30	^1H	1.980 ± 0.017	1.980
		^2H	0.180 ± 0.036	0.161

[a] Measurements of deuteron and proton relaxation rates were made at 12.63 MHz (25 °C) on 200-μL solutions containing 20 μM latent CF_1, 240 μM nucleotide, either 20 or 30 μM $MnCl_2$, and about 3/1 D_2O/H_2O solvent. Listed ^2H R_1's are averages of 75–80 measurements; ^1H R_1's are averages of 10 measurements. Error values were propagated from the standard deviations for the R_1 measurements in the absence and presence of added Mn(II). Parameters used for calculated values are in Table II. These data fits are those which most accurately reproduced an average of the proton–deuteron relaxation rate ratios of the 20 and 30 μM $MnCl_2$ samples for each nucleotide. For the calculations, the Mn(II)–enzyme concentration, E, was adjusted to that of the experiments of Table II by comparing proton relaxation rates at 12.63 MHz.

profound changes in the relaxation properties of the high-affinity Mn(II) binding site relative to those found for the binary Mn(II)–CF_1 complex; in general, the ternary adenine nucleotide containing complexes exhibited more pronounced relaxation rate maxima which were shifted to higher magnetic fields.

Nonlinear least-squares fits to the data were performed [see Haddy and Sharp (1989)] to obtain values for the residence time, τ_m, and the number, q, of exchangeable water molecules within the inner coordination sphere of bound Mn(II) (Table II). In order to provide reasonably accurate starting values for the fitting parameters and to avoid uncertainty due to the possible existence of false error minima, companion ^1H and ^2H data were taken at 12.63 MHz. Since the paramagnetic portions of R_1 due to two isotopes differ only due to the known difference in magnetogyric ratios, the deuteron data provide extra constraints on the overall consistency of the analysis as well as independent and reasonably accurate starting values for the fitting parameters. The success of the final parameter sets, obtained from fitting the proton data, in predicting the experimental ^2H R_1's is shown in Table III. Excellent agreement was obtained in all cases.

Dispersion profiles were determined as a function of added Mn(II) to permit extrapolation to low binding ratios of Mn(II) to CF_1. Extrapolated fitting parameters, which correspond to the first site filled by added Mn(II), are shown in Table II. The solvent hydration numbers increased from 1.09 ± 0.1, in the case of Mn(II)–CF_1, to about 1.92 ± 0.2 for Mn(II)–ATP–CF_1 and 3.25 ± 0.3 for Mn(II)–ADP–CF_1. (The uncertainty is estimated here to be ±10%, reflecting uncertainty both in the data fits and in values, such as r, which were set to perform the fits.) These differences in hydration number are reflected, in part, in the parallel increases in $1/T_{1p}$ per mole of added Mn(II) that was observed for the different complexes. Solvent exchange lifetimes also differed between the complexes, with Mn(II)–ADP–CF_1 showing a mean residence time that was about 1.7 times longer than that of Mn(II)–ATP–CF_1.

Important changes also occurred in the electron spin relaxation parameters of bound Mn(II). The most dramatic change appeared in the low-field electron spin relaxation time, $\tau_{s1}^{\circ} = (1/\tau_{s,d}^{\circ} + 1/\tau_{s,v}^{-1}$, which shortened by nearly an order of magnitude in the Mn(II)–ADP–CF_1 and Mn(II)–ATP–CF_1 complexes relative to Mn(II)–CF_1 (assuming no change in the slow distortion time τ_d). In the adenine nucleotide containing complexes, the electron spin relaxation rate, $1/\tau_{s1}$, at the lowest experimental field strengths was 4–6 times greater than that in the Mn(II)–CF_1 complex. This decrease in low-field electron spin relaxation time was caused by an increase in the static zero-field splitting and reflects a pronounced decrease in Mn(II) site symmetry relative to the binary Mn(II)–CF_1 complex (Haddy & Sharp, 1989).

Discussion

Mn(II) Binding Sites on Binary and Ternary Complexes of CF_1. Three chemically distinct high-affinity Mn(II) environments have been characterized corresponding to the bi-

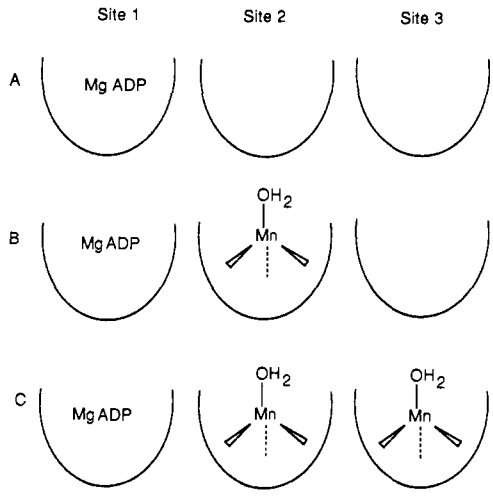

FIGURE 3: Proposed Mn(II) binding sites on latent CF_1 in relation to nucleotide binding sites 1, 2, and 3. The enzyme as isolated is pictured in (A) with Mg(II)–ADP in site 1. The first and second molar equivalents of bound Mn(II) are pictured in equivalent, approximately tetrahedral sites in (B) and (C).

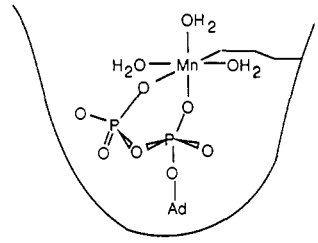

FIGURE 4: Proposed high-affinity binding site for Mn(II)–ADP on CF_1.

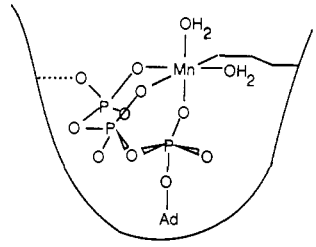

FIGURE 5: Proposed high-affinity binding site for Mn(II)–ATP on CF_1.

nary Mn·CF_1 complex and the ternary Mn·ADP·CF_1 and Mn·ATP·CF_1 complexes. The isolated enzyme used in these studies contained endogenous nonlabile nucleotide, predominantly in the form of ADP (Carlier & Hammes, 1979; Hiller & Carmeli, 1985; Haddy & Sharp, 1989), plus nonlabile endogenous Mg(II) at approximately equimolar levels [1.3 mol of Mg(II)/mol of CF_1]. We interpret the NMR results in terms of the model shown in Figure 3.

The enzyme as isolated has nucleotide site 1 occupied by ADP, while sites 2 and 3 are predominantly unoccupied (Figure 3A). Because the latent enzyme also contains endogenous Mg(II) at a level approximately equimolar to endogenous nucleotide, site 1 is shown containing an endogenous metal–nucleotide complex, although evidence concerning this point is ambiguous. ADP bound to site 1 is known to exchange with medium nucleotide in the presence or absence of EDTA (Carlier & Hammes, 1979; Bruist & Hammes, 1981; Girault et al., 1982); however, the association constant of Mg(II)–$EDTA^{2-}$ is relatively low, $K_m < 10^9\ M^{-1}$ (Anderegg, 1964), and it is possible that traces of Mg^{2+} may remain in solution, particularly if it is released very slowly by the enzyme.

The first molar equivalent of added Mn(II) enters a site where the metal ion is bound with high affinity without accompanying nucleotide (Figure 3B). This site evidently corresponds to the 4-coordinate site reported by Carmeli et al. (1986). Bound Mg(II) has a single exchangeable water with a residence time of 330 ns and electron spin relaxation properties that indicate an unusually high degree of site symmetry (Haddy & Sharp, 1989). The amount of added Mn(II) that can be accommodated in the population of binding sites having these properties is greater than a single molar equivalent. Thus, two high-affinity sites with properties that are indistinguishable in the NMR relaxation experiments appear to exist (Figure 3C).

Several lines of evidence suggest that Mn(II) binds at empty nucleotide sites 2 and/or 3. These include the facts that Mn(II) is a competitive inhibitor of ATPase activity with a K_i in the low micromolar range (Hochman et al., 1976; Hochman & Carmeli, 1981); the number of high-affinity Mn(II) sites on latent CF_1 is two [Haddy et al., 1985; Haddy & Sharp, 1989; but see also Hiller and Carmeli (1985), who report three sites], which appears consistent with the number of unoccupied nucleotide sites; Mn(II) binds cooperatively (Haddy et al., 1985; Hiller & Carmeli, 1985), indicating a coupling between sites which is similar to the coupling between nucleotide sites (Boyer & Kohlbrenner, 1981); and Mg(II) inhibits the exchange of ADP bound to site 1 (Feldman & Boyer, 1985), a process that may involve blocked access to nucleotide site 3 (Schumann, 1987). The NMR experiments described here provide a more direct basis for assessing whether Mn(II) in the tight metal site can be displaced by nucleotide or by a Mn(II)–nucleotide complex.

When the first molar equivalent of Mn(II) binds in the presence of excess ADP, the Mn(II) environment changes dramatically. The known characteristics of ADP binding (Bruist & Hammes, 1981) suggest that this situation corresponds to occupancy of one of the two labile ADP sites (sites 2 and 3 in the absence of ATP) by Mn(II)–ADP. The hydration number increases to three, and the electron spin relaxation time in the low-field limit decreases by an order of magnitude relative to that of the binary complex, indicating greatly reduced site symmetry. The binding site pictured in Figure 4 contains six-coordinate Mn(II) (Carmeli et al., 1986), in which the Λ-bidentate isomer of Mn(II)–ADP (Frasch & Selman, 1982) coordinates with three exchangeable waters and a single protein ligand. The latter ligand may be the *N,N'*-dicyclohexylcarbodiimide (DCCD)-modifiable glutamate residue, the modification of which is protected by divalent metal ions (Arana & Vallejos, 1981) and causes the loss of a nucleotide binding site (Shoshon & Selman, 1980).

When up to 1 molar equiv of Mn(II) binds in the presence of excess ATP, the chemical properties of the site differ from those of either Mn(II)–CF_1 or Mn(II)–ADP–CF_1. The conditions used correspond to those in which site 2 is occupied. The hydration number is 2, the mean residence time has shortened to 260 ns, and the electron spin relaxation time is relatively short, similar to that of Mn(II)–ADP–CF_1. In Figure 5, we have depicted a six-coordinate (Carmeli et al., 1986), tridentate Mn(II)–ATP complex (Frasch & Selman, 1982) with two exchangeable waters and a single ligand from the protein.

The data presented here do not support a hypothesis that the high-affinity Mn(II) binding sites observed on binary Mn(II)–CF_1 is also present in the ternary Mn(II)–AdN–CF_1 complexes. Added nucleotide dramatically changes the properties of the tightest site with respect to hydration number, chemical exchange kinetics, and electron spin relaxation properties of the metal ion. This finding is consistent with the

notion that Mn(II)–ADP and Mn(II)–ATP compete with free Mn^{2+} for an unoccupied nucleotide binding site as pictured in Figure 3. Chemical analysis (Table I) suggests the presence of two tight Mn(II) binding sites in the ternary Mn(II)–ATP–CF_1 complex [see also Hochman and Carmeli (1981)], neither of which has NMR properties compatible with those observed for the high-affinity site in binary Mn(II)–CF_1. Thus, we find little evidence to support the existence of a high-affinity Mn(II) binding site that is independent of the three well-characterized binding sites for adenine nucleotide.

The proposed model suggests questions that are amenable to experimental test using the techniques developed here. Of particular interest is the nature of the interaction of bound P_i at the metal binding sites. Figures 4 and 5 suggest that a function of divalent cations in catalysis may be to bind and position P_i for the phosphorylation reaction. If so, bound phosphate should displace at least one coordinating water molecule in the Mn(II)–ADP–CF_1 ternary complex, a process that should be observable in NMR relaxation experiments. Another interesting observation concerns the role of P_i, which releases tightly bound Mg(II) that is thought to be responsible for the inactive state of CF_1 (Feldman & Boyer, 1986). The role of P_i at the high-affinity metal binding sites requires further investigation.

Registry No. ATP, 56-65-5; ATPase, 9000-83-3; ADP, 58-64-0; Mn, 7439-96-5; ATP synthase, 37205-63-3.

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