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Cooperative Binding of Manganese(II) to Chloroplast Coupling Factor 1 Detected by NMR Proton Relaxation Enhancement†

Alice E. Haddy,‡ Wayne D. Frasch,*,§ and Robert R. Sharp‡
Department of Chemistry and Department of Cellular and Molecular Biology, The University of Michigan, Ann Arbor, Michigan 48109
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ABSTRACT: The binding of divalent manganese to soluble latent spinach chloroplast coupling factor 1 (CF_1) was examined by measurements of the water proton spin–lattice relaxation enhancement (PRE), revealing positive cooperativity between high-affinity sites. A method that used only a single enhancement parameter, ϵ_b, for the quantitation of cooperative PRE data was derived. Application to the high-affinity sites yielded a value of 9.01 ± 0.11 for ϵ_b. Two high-affinity sites participated in cooperative binding, although the possibility that a third site was present was not eliminated. The apparent binding constant to site ratio, K/n, was found to be 3.4–3.7 μM, giving a value for K of approximately 7 μM.

The soluble chloroplast coupling factor 1, CF_1,[1] which is the photosynthetic adenosinetriphosphatase, is composed of five distinguishable peptides with the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ (Merchant et al., 1983; Moroney et al., 1983). Evidence that the active site is on the β subunit (Carlier et al., 1979; Bruist & Hammes, 1981) suggests that there are three active sites. In both CF_1 and its mitochondrial counterpart, MF_1, the sites appear to be strongly coupled during the catalytic cycle (Boyer, 1979). This conclusion is supported by the observation that rapid turnover of the enzyme requires the binding of substrate to two of the three active sites (Grubmeyer & Penefsky, 1981; Cross et al., 1982). X-ray diffraction analysis does not support a simple 3-fold symmetry of subunits, suggesting structural inequivalence of the β subunits (Amzel et al., 1982).

Any of several divalent metal ions, including Mg(II), Mn(II), and Ca(II), is required for enzymatic activity (Nelson et al., 1972; Hochman et al., 1976). The metal cofactor coordinates to the phosphates of the nucleotide substrate at the active site with a specific stereochemistry to enable catalysis (Frasch & Selman, 1982). The most effective cofactor depends on the method of activation of the CF_1 ATPase. The thiol-activated ATPase has the highest rate of activity in the presence of Ca(II) (Farron & Racker, 1970); Mn(II) and Mg(II) are more effective at submillimolar concentrations but become inhibitory at higher concentrations (Hochman et al., 1976). Recently, it has been found (Pick & Bassilian, 1981) that octyl glucoside activates a Mg(II)- or Mn(II)-ATPase of soluble CF_1 that is not inhibited by high concentrations of these metals and, thus, more closely resembles the membrane-bound ATP synthase.

A recent study of the binding of Mn(II) to latent lettuce CF_1 was carried out with ESR to measure free Mn(II) (Hiller & Carmeli, 1985). In this study, CF_1 exhibited three cooperating Mn(II) binding sites with an apparent dissociation constant of 15 μM and a Hill coefficient of 2.9. Three low-affinity Mn(II) binding sites with a dissociation constant of 47 μM were also found, as well as several lower affinity nonspecific sites. However, the degree of cooperativity and the binding constants were found to depend on the previous history of the enzyme.

We have studied the binding of Mn(II) to latent CF_1 by using proton relaxation enhancement (PRE) of the solvent (Mildvan & Cohn, 1970). This technique provides a direct, sensitive probe of enzyme-bound Mn(II) and is particularly useful for characterizing metal binding at low mole ratios of metal to enzyme, where sigmoidicity due to cooperative interactions is most pronounced. The PRE data show clear evidence of positive cooperativity of Mn(II) binding to CF_1.

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‡ Department of Chemistry.
§ Department of Cellular and Molecular Biology.

[1] Abbreviations: PRE, proton relaxation enhancement; CF_1, chloroplast coupling factor 1; MF_1, mitochondrial coupling factor 1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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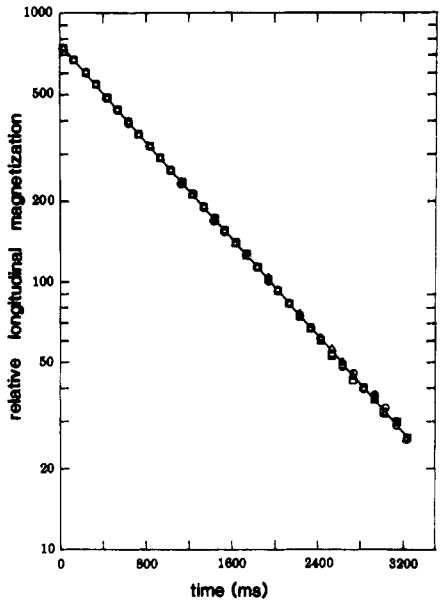

FIGURE 1: Decay of longitudinal magnetization, measured as described under Materials and Methods, for a sample of Mn(II) in distilled water. Three separate determinations are shown here, for which the R_1 values were 1.043 (circles), 1.048 (squares), and 1.042 s^{-1} (triangles).

An analysis of the data has been developed to describe cooperative interactions among sites. These experiments support the presence of two cooperative high-affinity binding sites with an apparent dissociation constant to site ratio, K/n, of 3.4–3.7 μM.

Materials and Methods

Spinach chloroplast coupling factor 1 (CF_1) was prepared as described previously (Frasch & Selman, 1982) and stored at 4 °C as an ammonium sulfate precipitate. It was found to contain all five subunits at >95% purity as determined by SDS–PAGE. Following heat activation (Farron & Racker, 1970), the enzyme hydrolyzed ATP at a rate of about 25 μmol of PO_4 (mg of CF_1)$^{-1}$ min^{-1}, as measured by the ammonium molybdate colorimetric method for free phosphate (Chen et al., 1956). Protein was quantitated (Bradford, 1976) by comparison with a bovine serum albumin standard. A molecular weight for CF_1 of 400 000 was used in all calculations (Moroney et al., 1983).

Titrations of CF_1 with Mn(II) were performed with 100 μL of 5–40 μM latent CF_1 in 40 mM Tris–SO_4 or Tris–HCl buffer, pH 8.0. Prior to each experiment, the protein was desalted by centrifuge chromatography through Sephadex G-50 (Penefsky, 1977), which effectively removed all traces of EDTA. The PRE measurements were not changed when the protein was passed through a second column. Titrations of CF_1 were conducted at 25 °C by adding microliter volumes of 0.20 to 5.00 mM $MnSO_4$ or $MnCl_2$.

Spin–lattice relaxation rates, $R_1 = 1/T_1$, were measured at 20.7 MHz with the modified triplet sequence $[180_0\text{–}\tau_1\text{–}(90_0\text{–}\tau_2\text{–}180_\pi\text{–}\tau_2\text{–}90_0\text{–}\tau_1)_n]$ as described previously by Sharp & Yocum (1980). This sequence gives superior speed and accuracy relative to the inversion–recovery method. Data acquisition and least-squares analysis were under microcomputer control. The accuracy of measurement was checked with distilled, degassed water (Simpson & Carr, 1958) and found to be better than ±3%. Repetitions on a given sample typically contained scatter less than ±0.75% (see Figure 1). Reported R_1 values, except for those of Figure 1, are averages of five separate determinations.

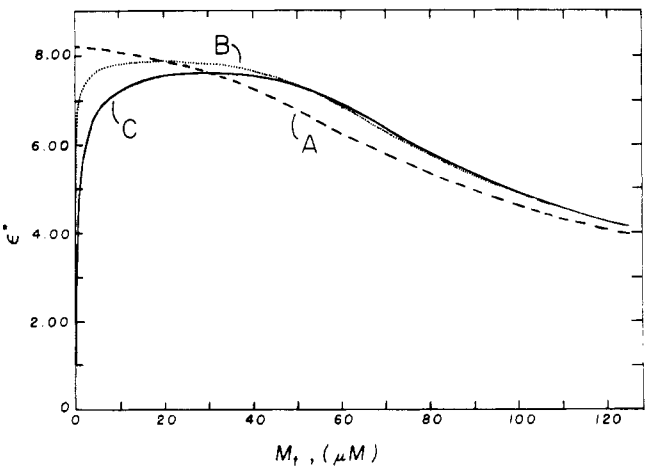

FIGURE 2: Theoretical plots of the bulk relaxation enhancement, ϵ^*, as a function of total Mn(II) concentration. All curves represent 25 μM enzyme with two binding sites per molecule. For curves B and C, the fraction of metal bound, x_b, was calculated from eq 2 with a successive approximation program. Parameters for curves B and C were chosen such that the maxima, at 19.2 and 30 μM M_t, respectively, passed through curve A. Bulk enhancements were then calculated from eq 1 by setting ϵ_b equal to 8.91: (curve A) $\alpha = 1$ and $K = 5$ μM; (curve B) $\alpha = 1.5$ and $K = 3.97$ μM; (curve C) $\alpha = 2$ and $K = 5$ μM.

Electron spin resonance (ESR) measurements of free Mn(II) were carried out on a Varian ER 200 spectrometer with center field at 3500 G, microwave power at 22 mW, and modulation amplitude at 25 G ($p-p$). Calibration curves were constructed for each gain setting from $MnSO_4$ standards in 40 mM Tris–SO_4, pH 8.0, by taking the height of the two center peaks of the six-line spectrum.

Proton Relaxation Enhancement Analysis. The Mn(II)–protein interaction was analyzed in terms of the bulk relaxation enhancement, ϵ^*, defined by eq 1 (Eisinger et al., 1962). The

$$\epsilon^* \equiv \frac{\Delta R_1^*}{\Delta R_1} = x_b(\epsilon_b - 1) + 1 \qquad (1)$$

right-hand side of the equation, which assumes a single class of binding sites, shows that ϵ^* is a linear function of the fraction of bound Mn(II), x_b (Mildvan & Cohn, 1970; Dwek, 1973). In eq 1, ΔR_1^* = increase in R_1 of the protein solution due to added Mn(II), ΔR_1 = increase in R_1 of the buffer due to added Mn(II), and ϵ_b = enhancement associated with bound Mn(II). As long as ϵ_b is greater than 1, as is frequently the case for Mn(II) binding to a protein (Mildvan & Cohn, 1970), ϵ^* is at a maximum when x_b is greatest.

For a protein with n sites that can participate in positive cooperativity, the association between metal and protein can be represented by

$$K^\alpha = nE_fM_f^\alpha/M_b \qquad (2)$$

where K is the apparent dissociation constant, E_f, M_f, and M_b are the concentrations of free enzyme, free metal, and bound metal (or filled sites), respectively, and α is the interaction parameter, analogous to the Hill constant (Schreier & Schimmel, 1974). If $\alpha = 1$, eq 2 reduces to the usual expression for equal and independent binding sites in which K is the true dissociation constant.

Theoretical plots of the bulk enhancement as a function of total Mn(II) concentration (Figure 2) show how the maximum enhancement, $\epsilon^{*,max}$, shifts with changes in α. For two equal and independent sites (curve A), ϵ^* is at a maximum at infinitely dilute Mn(II) and decreases with increasing Mn(II) concentration, reaching a theoretical limit of 1 at infinite

Mn(II). In the case of cooperative binding (curves B and C), ϵ^* is 1 when Mn(II) is infinitely dilute, indicating that no Mn(II) is bound initially, and increases to a maximum at some value of M_t greater than 0.

The analysis for binding parameters for PRE measurements is based on a plot of $1/(\epsilon^* - 1)$ vs. $1/E_f$ (Dwek, 1973). The intercept gives ϵ_b, from which the fraction of bound Mn(II), x_b, is determined. For noncooperative systems, the analysis can be carried out by extrapolating ϵ^* to zero Mn(II) where $E_f = E_t$ and $\epsilon^* = \epsilon^{*,max}$. However, in the case of cooperative binding, ϵ^* approaches unity as the Mn(II) concentration decreases (see Figure 2), rendering this method impractical. A parallel analysis is based on measurements of the maximum enhancements, $\epsilon^{*,max}$, for various concentrations of total enzyme, E_t. It can be shown from eq 2 that when ϵ^* is maximal, and hence x_b is maximal (x_b^{max}), then

$$M_b(x_b=x_b^{max}) = n(1 - 1/\alpha)E_t \quad (3)$$

Equation 3 is equivalent to an equation derived by Dahlquist (1978) by combining differentiated Scatchard and Hill equations. Equations 2 and 3 give an expression for the free metal at x_b^{max}

$$M_f(x_b=x_b^{max}) = K(\alpha - 1)^{1/\alpha} \quad (4)$$

which then gives

$$x_b^{max} = \left(1 + \frac{\alpha K}{nE_t}(\alpha - 1)^{1/\alpha - 1}\right)^{-1} \quad (5)$$

In the limit of strong cooperativity between two binding sites (Figure 2, curve C), $\alpha = 2$ and eq 3 and 4 predict that the maximum fraction of ligand bound, and hence $\epsilon^{*,max}$, occurs when $M_t = E_t + K$. Similarly, if there is a lesser degree of cooperativity such that $\alpha = 1.5$ (curve B), $\epsilon^{*,max}$ occurs when $M_t = (2/3)E_t + (1/2)^{2/3}K$.

The enhancement, ϵ_b, for the high-affinity Mn(II) binding sites was determined by plotting $1/(\epsilon^{*,max} - 1)$ against $1/E_t$. Since $M_b(x_b=x_b^{max}) = n(1 - 1/\alpha)E_t$ when the enhancement is greatest ($\epsilon^{*,max}$), then $nE_f = nE_t - M_b = nE_t/\alpha$ and the modified analytical equation is

$$\frac{1}{\epsilon^{*,max} - 1} = \frac{\alpha C}{n(\epsilon_b - 1)}\frac{1}{E_t} + \frac{1}{\epsilon_b - 1} \quad (6)$$

where C is a constant that can be shown through eq 5 to be equal to $K(\alpha - 1)^{1/\alpha - 1}$. Thus, if n and α are known, the apparent binding constant, K, can be found. If $\alpha = 1$, eq 6 reduces to the form used for equal and independent sites. By use of the value of ϵ_b, the fraction of metal bound was calculated from eq 1 for the Mn(II) titrations, allowing Scatchard and Hill analyses.

Results

Figure 3 shows a titration of 15 μM CF_1 with Mn(II) and a corresponding titration of the buffer solution. The spin–lattice relaxation rate (R_1) of the buffer solution increased linearly with added Mn(II). The R_1 of the protein solution increased in two phases with a break in slope near 2–3 mol of Mn(II) added per mole of CF_1. The presence of two classes of Mn(II) binding sites has been observed previously (Hochman et al., 1976; Hochman & Carmeli, 1981; Hiller & Carmeli, 1985).

In order to confirm that the enhancement of Mn(II) bound to the high-affinity sites was large enough to dominate the increase in R_1 at low Mn(II) to CF_1 ratios, complementary NMR and ESR measurements were undertaken (Table I). These experiments compared the increase in R_1 produced by added Mn(II) to the amount of Mn(II) bound to the enzyme

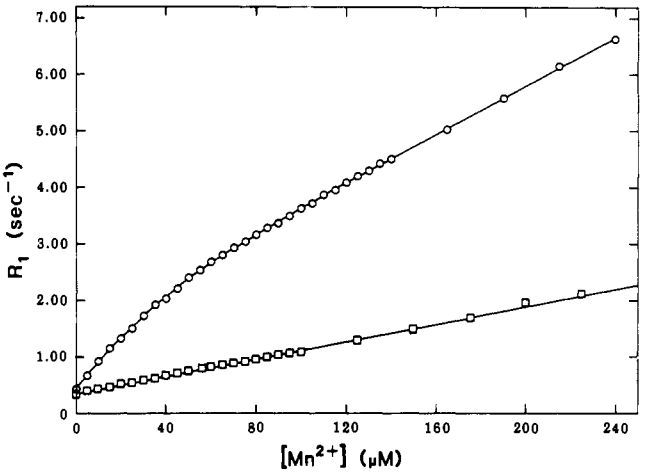

FIGURE 3: Increase in relaxation rate (R_1) of water protons as a function of added Mn(II) concentration. The 100-μL samples contained 40 mM Tris buffer, pH 8.0 (squares), or buffer with 15 μM latent CF_1 (circles).

Table I: Comparison of R_1 Measurements with the Extent of Binding of Mn(II) to Latent CF_1[a]

Mn_b/CF_1 (±10%)	$[Mn_b]$ (±10%) (μM)	ΔR_1^* (±2%) (s^{-1})	$\Delta R_1^*/[Mn_b]$ (±10%) (s^{-1} μM^{-1})
0.73	8[b]	0.453	0.057
1.5	17[c]	0.92	0.054
2.0	48[c]	2.35	0.049
3.1	34[b]	1.82	0.054
3.3	78[c]	3.80	0.049
4.2	100[c]	5.1	0.051
5.5	60[b]	2.82	0.047

[a] After measurement of the relaxation rates (R_1) before and after Mn(II) addition, each sample was transferred to a flat cell for measurement of the free Mn(II) by ESR. [b] 11 μM CF_1 was used. [c] 24 μM CF_1 was used.

for different mole ratios of bound Mn(II) to CF_1. Enzyme-bound Mn(II) was calculated from the free Mn(II) measured directly by ESR. The increase in R_1 (ΔR_1^*) produced per micromolar bound Mn(II) (Mn_b) remained fairly constant over the range of concentrations tested. The lack of a significant change in $\Delta R_1^*/[Mn_b]$ as Mn_b/CF_1 increased indicated that ϵ_b is approximately equal for both types of sites.

CF_1 solutions of various concentrations between 5 and 40 μM were titrated with Mn(II), such that the maximum stoichiometries of bound Mn(II) to CF_1 were less than 2. In each experiment, the bulk enhancement typically passed through a maximum, as in Figure 4, when the concentration of added Mn(II) was close to the concentration of CF_1. Comparison of the experimental data to the theoretical curves of Figure 2 indicates that the binding of Mn(II) to the high-affinity sites was cooperative.

The enhancement, ϵ_b, for Mn(II) bound to the high-affinity sites was determined with eq 6 (see Materials and Methods). For each titration experiment, between 5 and 15 observed enhancement values that defined the maximum were averaged to find the maximum bulk enhancement, $\epsilon^{*,max}$. With data from 10 titrations, the quantity $1/(\epsilon^{*,max} - 1)$ was plotted against $1/E_t$, as shown in Figure 5. From the y intercept, ϵ_b was found to be 9.01 ± 0.11; the slope of the line yielded a value of 6.4 ± 0.2 μM for $\alpha C/n$.

Scatchard analyses of the binding of Mn(II) to CF_1 were carried out, as shown in Figure 6. The data passed through a maximum in $\tau/[Mn_f]$ (where $\tau = Mn_b/CF_1$), indicating positive cooperativity of binding. The maximum occurred when τ was equal to 0.68 ± 0.03 as an average of four experiments. Binding to the low-affinity sites was evident at

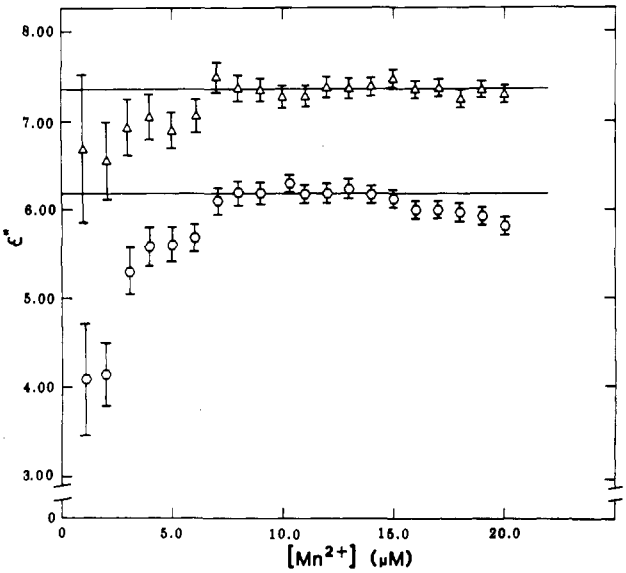

FIGURE 4: Observed relaxation enhancement, ϵ^*, as a function of added Mn(II) concentration. Titrations were carried out as in Figure 3. Bulk enhancements were calculated from R_1 values according to eq 1. The solid lines indicate the average maximum enhancements observed: (hexagons) 12 μM latent CF_1; (triangles) 23 μM latent CF_1.

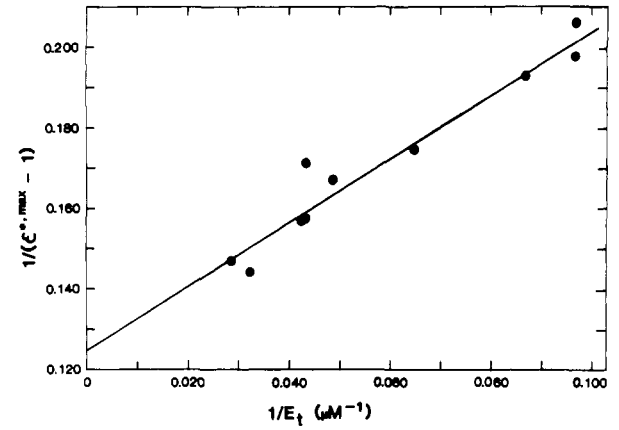

FIGURE 5: Determination of the enhancement, ϵ_b, for Mn(II) bound to the high-affinity sites. The average maximum enhancements ($\epsilon^{*,max}$) from 10 titration experiments were related to the total enzyme concentrations according to eq 6. From the y intercept, $\epsilon_b = 9.01 \pm 0.11$; from the slope, $\alpha C/n = 6.4 \pm 0.2$ μM.

values of τ greater than approximately 2.

By use of a saturation level of two Mn(II) bound per CF_1 for the high-affinity sites (see Discussion), a Hill analysis was calculated from the data of Figure 6, as shown in Figure 7. For two sites, $[CF_{1b}] = (1/2)[Mn_b]$ and $[CF_{1f}] = [CF_{1t}] - (1/2)[Mn_b]$. The data were linear until the CF_{1b}/CF_{1f} ratio was greater than 2, where the slope increased due to low-affinity Mn(II) binding. Hill coefficients, α_H, were 1.4 and 1.5 for the 12 and 10 μM CF_1 samples, respectively, in agreement with α_H calculated from eq 7 of the Discussion. Plots constructed assuming three total sites also gave Hill constants consistent with eq 7; however, deviations from linearity due to low-affinity binding occurred close to half-saturation.

Discussion

Measurements of the proton relaxation rate as a function of added Mn(II) (Figure 3) indicated that Mn(II) bound to more than one class of sites on CF_1 and that there were two to three high-affinity sites. By comparison of the observed relaxation rates with measurements of bound Mn(II) by ESR (Table I), the enhancement for the high-affinity sites was

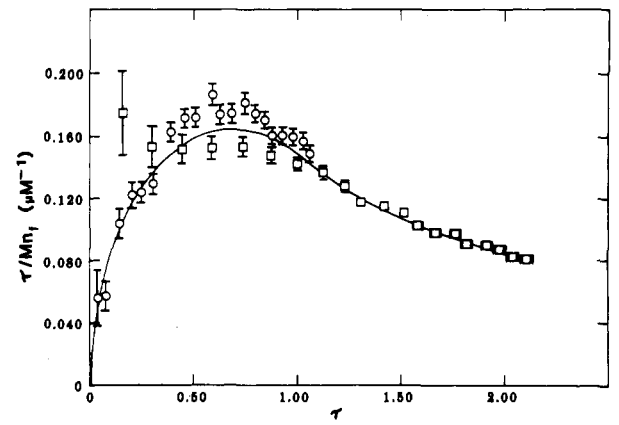

FIGURE 6: Scatchard plot of the binding of Mn(II) to latent CF_1. τ represents Mn(II) bound per CF_1 molecule (Mn_b/CF_1). The amount of bound Mn(II) was calculated from the R_1 data by using eq 1 and the value of ϵ_b determined from Figure 5: (squares) 10 μM CF_1; (circles) 12 μM CF_1.

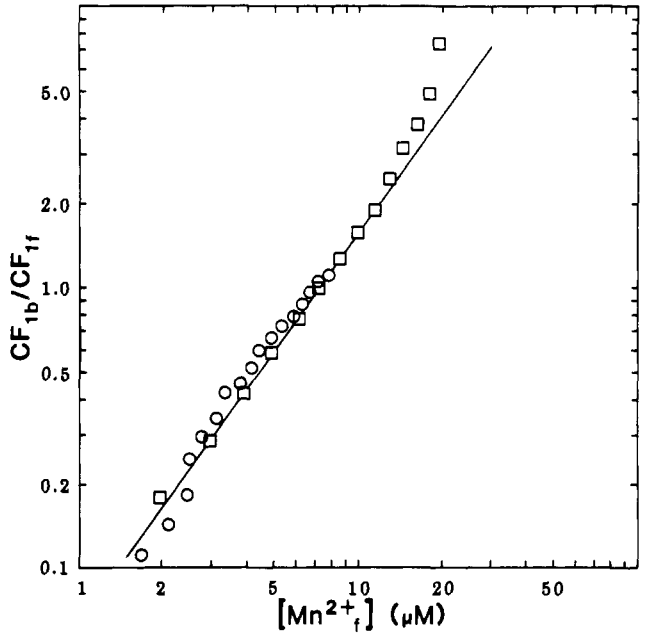

FIGURE 7: Hill plot of the binding of Mn(II) to latent CF_1. By use of the data of Figure 6, the fractional saturation was calculated for two high-affinity sites per CF_1 molecule, where $[CF_{1b}] = (1/2)[Mn_b]$ and $[CF_{1f}] = [CF_{1t}] - (1/2)[Mn_b]$: (squares) 10 μM CF_1; (circles) 12 μM CF_1. Slopes at half-saturation are 1.5 and 1.4 for the 10 and 12 μM samples, respectively.

found to dominate the relaxation behavior at low Mn_b/CF_1 ratios. The binding of Mn(II) to the high-affinity sites was then examined in detail by PRE. Positive cooperativity of binding was evident in measurements of the bulk enhancement as a function of added Mn(II) (Figure 4) as well as in Scatchard analyses of the data (Figure 6). Quantitation of the binding parameters according to eq 6 gave an enhancement, ϵ_b, of 9.01 ± 0.11 and a value for $\alpha C/n$ of 6.4 ± 0.2 μM for the high-affinity sites. The degree of binding cooperativity was assessed through a combination of Scatchard and Hill analyses.

Dahlquist (1978) has shown that the Hill constant, α_H, and the value of τ, τ_m, at which the maximum occurs in a Scatchard plot exhibiting positive cooperativity are related by

$$\alpha_H = (1 - \tau_m/n)^{-1} \quad (7)$$

where n is the number of sites. Values for α_H range from 1 for no cooperativity, when $\tau_m = 0$, up to n in the limit of strong

Table II: Binding Parameters of Mn(II) to CF_1 Given Two or Three Binding Sites[a]

n	τ_m	α_H	K (μM)	K/n (μM)
2	0.68	1.5	6.8	3.4
3	0.68	1.3	11.2	3.7

[a] The value of τ or τ_m at which the Scatchard maximum occurred was obtained directly from plots such as that shown in Figure 6. Considering cases of two and three high-affinity sites for n, Hill constants, α_H, were calculated from eq 7. The apparent binding constant, K, was then calculated for each n and α_H pair from eq 6 by using the slope of Figure 5.

cooperativity between sites, when $\tau_m = n - 1$. A Hill constant calculated from this equation is usually in close agreement with the value obtained from a Hill plot. Equation 7 is very similar to eq 3, in which τ_m is replaced by $M_b(x_b=x_b^{max})/E_t$. Comparison of these equations reveals that the Hill constant, α_H, and the interaction parameter, α, defined by eq 2 are mathematically identical.

Considering cases of both two and three high-affinity sites for CF_1, eq 7 was used to find possible interaction parameters. A value of 0.68 for τ_m implies that α_H was 1.5 for two sites or 1.3 for three sites, as given in Table II. Taking the integer above α_H as the maximum number of cooperative binding sites, the position of the Scatchard maximum indicates that coupling was between two high-affinity Mn(II) binding sites. Values for α (or α_H) allowed an estimate of the apparent dissociation constant, K, and the ratio K/n from eq 6 (Table II). The apparent binding constant, K, was 6.8 and 11.2 μM for two and three sites, respectively. Values for K/n were 3.4 and 3.7 μM.

The results presented here are in general agreement with those of Hiller & Carmeli (1985), who also observed positive cooperativity of Mn(II) binding with an apparent dissociation constant similar to those reported here. Those workers proposed that the degree of cooperativity depends on the previous history of the enzyme. Our results support the presence of cooperativity between two sites, while Hiller and Carmeli, using enzyme stored in glycerol at −80 °C, reported cooperativity between three sites. In earlier experiments using CF_1 stored under conditions similar to those used in the present study, Hochman & Carmeli (1981) found that the binding of Mn(II) to CF_1 did not exhibit positive cooperativity. The source of these differences in the binding of Mn(II) is not currently apparent.

The method employed here has been successful in describing the binding of Mn(II) to CF_1 by utilizing a single enhancement parameter for the two (or possibly three) cooperative binding sites. This need not imply that the intrinsic enhancements for the various high-affinity sites are identical. When coupling between sites is strong such that primarily a single form of the liganded enzyme is observed, the measured enhancements can be described by a single effective ϵ_b for the multiply bound ligand–enzyme complex rather than by several intrinsic ϵ_bs for the individual sites. Such an interpretation for these data is supported by the linearity of the Hill plot in Figure 7; if we had observed a significant fraction of singly bound ligand–protein complexes, a portion of the curve at low ligand concentrations would have had a slope approaching unity. This leads us to conclude tentatively that we have observed largely multiply bound ligand for the concentration range investigated here.

The interaction between two metal binding sites reported here might imply that divalent cations have a regulatory role in enzyme activity as well as the well-known role of orienting substrates for catalysis (Frasch & Selman, 1982). In studying latent CF_1, we have analyzed Mn(II) binding to a form of the isolated protein that most closely resembles the native membrane-bound ATP synthase. The success of the PRE technique in studying this cooperative system provides a basis for further studies involving the more complex situation of CF_1 in the presence of metal–nucleotide complexes.

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