Tight Divalent Metal Binding to *Escherichia coli* F_1-Adenosinetriphosphatase. Complete Substitution of Intrinsic Magnesium by Manganese or Cobalt and Studies of Metal Binding Sites†

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ABSTRACT: Tight divalent metal binding sites in *Escherichia coli* F_1-adenosinetriphosphatase (F_1-ATPase) were studied. Native enzyme contained two Mg per F_1, confirming previous results. All of the Mg may be replaced by Co or Mn using a dissociation–repolymerization procedure. The substituted enzymes are homogeneous and contain two Mn per F_1 or two Co per F_1. They are fully active as ATPases, they rebind to F_1-depleted membranes, and they catalyze ATP-driven proton pumping. *N,N'*-Dicyclohexylcarbodiimide- (DCCD) inactivated F_1 retains all of the intrinsic tightly bound Mg. Evidence is presented that DCCD affects at least two β subunits in *E. coli* F_1, and therefore, the tightly bound metals appear not to be bound at the DCCD-reactive glutamate residue on the β subunit. However, the nature of the tightly bound metal (Mg, Mn, or Co) as well as the presence of added (2 mM) $MgSO_4$, $MnSO_4$, or $CoSO_4$ affected the rate of DCCD inactivation, showing that metal binding changes the β-subunit conformation. Isolated F_1 α subunit bound Mg, Mn, or Co stoichiometrically and independently of ATP binding. Isolated F_1 β subunit bound only small amounts of Mg, and no Co or Mn. Therefore, it is possible, although not conclusively shown, that the α subunit is the site of tight metal binding in the intact F_1.

We showed previously that purified soluble F_1-adenosinetriphosphatase (F_1-ATPase) from beef heart mitochondria contained intrinsic Mg (1 mol/mol of enzyme protein) which is tightly bound (estimated $K_d < 10^{-11}$ M). This Mg could not be exchanged, and removal of it was associated with irreversible depolymerization of the enzyme and loss of activity. The purified soluble beef heart F_1 could take up additional Mg, Mn, or Co to give an enzyme containing 2 mol of divalent metal per mole. The second site was a tight site like the first, but it exchanged during catalysis and thus could be substituted and characterized in more detail. Studies showed both divalent metal sites were present on membrane-bound beef heart F_1, and we proposed that the physiological form of the enzyme is 2Mg F_1 (Senior, 1979, 1981; Senior et al., 1980). Soluble purified *Escherichia coli* F_1-ATPase was shown to contain 2 mol of Mg per mole of enzyme protein as prepared (Senior et al., 1980).

The *E. coli* F_1-ATPase may be dissociated, separated into its component subunits, and then repolymerized to a fully active F_1 aggregate (Dunn & Futai, 1980). This opened up two avenues of experimentation which were not possible with beef heart F_1. First, it permitted analysis of bound metal and assay of metal binding properties in isolated, native subunits, and this could be of value in localizing the metal binding sites in intact F_1. Second, it suggested that by the method of dissociation–repolymerization, substitution of all of the bound Mg by Mn or Co might be attempted. This would demonstrate whether the specificity at the first site was for Mg only or whether other metals could bind there. Also, preparation of fully substituted enzyme could be valuable in application of spectroscopic techniques to probe the geometry of the metal binding sites, to follow changes in the environments of the sites during substrate binding, proton gradient formation, and catalysis, and to locate the sites by use of substitution-inert Co^{3+}.

In this paper, we report the preparation and characterization of *E. coli* F_1 in which the Mg is completely replaced by either Co or Mn, and we show these enzymes are catalytically active. Assays of bound metal and metal binding in purified F_1 α and β subunits are also reported, and the effect of *N,N'*-dicyclohexylcarbodiimide (DCCD)[1] inactivation on the metal binding sites is described.

Materials and Methods

Preparation of Purified Soluble E. coli F_1 and E. coli Cell Membranes. Normal (*unc*$^+$) soluble F_1 and F_1-depleted membranes were prepared as described previously (Wise et al., 1981; Perlin et al., 1983). The F_1-depleted membranes were depleted of metal ions by suspending in 10 mM Tris–sulfate and 5 mM EDTA, pH 8.0, at 4 °C, centrifuging 150000*g* for 60 min, and repeating this procedure 4 times.

Analysis of Metals. Methods for the preparation of metal-free buffers and equipment and minimization of contamination by extraneous metals have been described previously (Senior, 1979; Senior et al., 1980). Direct metal analyses by atomic absorption spectrophotometry were done as before (Senior, 1979); ^{60}Co and ^{54}Mn were counted in a Beckman γ 8000 counter. For analysis of bound metal, F_1 samples (0.1 mL) were passed through centrifuge columns (Sephadex G-50F) (Penefsky, 1977) equilibrated with 50 mM Tris–sulfate, pH 8.0 (Chelex-100 treated), and diluted with the same buffer as required.

Dissociation of F_1. F_1 was dissociated in a buffer identical with the dissociation buffer of Dunn & Futai (1980) except

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[1] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; AMPPNP, 5'-adenylyl imidodiphosphate.

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that 1 mM CDTA was substituted for 1 mM EDTA. Aliquots of 100–150 μL of F_1 at a concentration of 18–20 mg/mL were first equilibrated with this buffer on 1-mL centrifuge columns (Sephadex G-50F) (Penefsky, 1977). Eluates were diluted with 3 volumes of dissociation buffer and dialyzed against dissociation buffer at 4 °C for 24–48 h. Dialysis was discontinued when the Mg/F_1 ratio had been reduced to <0.08 mol/mol.

Repolymerization of F_1. Dissociated F_1 was equilibrated on 1-mL Sephadex G-50F centrifuge columns with repolymerization buffer [50 mM Tris–sulfate, pH 7.2 (Chelex-100 treated), 0.1 mM dithiothreitol, and 10% (v/v) glycerol] to which ATP and $MgSO_4$, $CoSO_4$, or $MnSO_4$ were added to give final concentrations of 2 mM. Eluates (≥0.5 mg of protein/mL) were then incubated at 20 °C to allow repolymerization to occur.

Measurement of pH Gradient Formation. The formation of pH gradients was monitored by using acridine orange, essentially as described by Perlin et al. (1983). The standard 2-mL reaction medium contained 10 mM Tris-HCl, pH 7.4, 200 mM KCl, 2 mM $MgSO_4$, $MnSO_4$, or $CoSO_4$, 200 μg of membrane protein, varied amounts of F_1, and 2.5 μM acridine orange. The reaction was initiated by the addition of 2 mM ATP and was terminated with 38 mM NH_4Cl.

Purification of F_1 Subunits. The procedure of Dunn & Futai (1980) was followed.

Inhibition of F_1-ATPase Activity by DCCD. Inhibition was initiated by the addition of DCCD (final concentration 100 μM) to 95 μL of a solution of 19 μg of F_1 in 40 mM Tris–sulfate, pH 7.4, and 8% (v/v) glycerol at 30 °C. Aliquots of 2.5 μg of F_1 were removed periodically and assayed for ATPase activity. [^{14}C]DCCD was used for DCCD binding studies.

Routine Procedures. ATPase activity was assayed as described previously (Senior, 1979). Protein concentrations were determined by the method of Miller (1959). All incubations were done in plastic tubes.

Chemicals. [^{14}C]DCCD was obtained from Research Products International; CDTA was from Aldrich Chemical; $^{54}MnCl_2$ and $^{60}CoCl_2$ were from Amersham Corp. Nonradioactive Co and Mn salts were "Specpure" grade, obtained as described in Senior et al. (1980).

RESULTS

Intrinsic Metal Content of Purified E. coli F_1. Since our earlier work (Senior et al., 1980) in which we reported that *E. coli* F_1 contained approximately 2 mol of Mg/mol of F_1, a further four F_1 preparations were analyzed and had an average Mg content of 1.89 mol/mol (range = 1.80–2.05). No Mn or Co was detected. Two preparations of enzyme were used for the work reported here, with Mg contents of 1.80 and 1.93 mol/mol of F_1, respectively.

Dissociation and Repolymerization of F_1. After dialysis in the dissociation buffer (which contained 1 mM CDTA, 1 M NaCl, 250 mM $NaNO_3$, 0.1 mM dithiothreitol, and 50 mM succinate–Tris, pH 6.0 at 4 °C), the activity of the enzyme fell to near zero, and the Mg content of the dissociated subunits was ≤0.08 mol/mol of F_1.

The repolymerization of F_1 had to be done under conditions which allowed control of the divalent cation concentration, and so the dialysis procedure of Dunn & Futai (1980) was modified to the simpler incubation procedure as described under Materials and Methods. Dissociated subunits were first equilibrated with the repolymerization buffer (see Materials and Methods) and then allowed to incubate at 20 °C. Regain of ATPase activity was maximal after 90 min and was equal to that reported previously (Dunn & Futai, 1980). Indeed, the specific activity of the repolymerized F_1 was usually higher than that of the starting enzyme, presumably due to partial failure to reincorporate ε subunit into the F_1 aggregate. As measured by the ability to catalyze ATP-driven proton gradient formation when rebound to F_1-depleted membranes, repolymerization reached a maximum level after 40-min incubation, and the extent of pH gradient formation by repolymerized 2Mg F_1 was similar to that seen with native F_1.

Table I: Metal Content of Repolymerized F_1 Preparations[a]

metal	expt	separation method	metal/F_1 (mol/mol)
^{54}Mn	1	S300	1.94
	2	S300	2.02
	3	CC	1.90
	4	CC	2.01
^{60}Co	1	S300	1.92
	2	CC	2.10
	3	CC	2.10
	4	CC	2.48
Mg	1	CC	1.91
	2	CC	1.91

[a] F_1 was dissociated and repolymerized in the presence of $MgSO_4$, $^{54}MnSO_4$, or $^{60}CoSO_4$ as described under Materials and Methods. Samples for metal analysis were separated from loosely bound ligands by elution at room temperature through 1-mL centrifuge columns (CC) containing Sephadex-G50F in 50 mM Tris–sulfate, pH 8.0 (Chelex-100 treated), or by passage through a column of Sephacryl S300 (S300) (0.9 × 55 cm) in the same buffer at room temperature. Each experiment represents one repolymerization mixture, which was analyzed in duplicate for metal content.

The Mg, Mn, and Co contents of repolymerized F_1 samples are reported in Table I. The Mg, Mn, or Co content was typically close to 2 mol of metal/mol of F_1. Inclusion of 1 mM EDTA and/or 10% (v/v) glycerol in the buffer used in Table I did not affect the results. Further treatment of the F_1 by passage over second centrifuge columns did not reduce the metal content significantly. Occasionally repolymerized F_1 preparations contained higher than 2 mol of Co or Mn per F_1 after passage through centrifuge columns as in Table I, but repeated passage of enzyme through centrifuge columns always reduced the metal content to values similar to those of Table I. The Mg content of the repolymerized 2Mn F_1 and 2Co F_1 preparations was close to zero (analyzed in parallel experiments using nonradioactive Co and Mn). The ATPase activity of repolymerized 2Mg F_1, 2Mn F_1, and 2Co F_1, measured with $MnSO_4$, $CoSO_4$, or $MgSO_4$ in the assay medium, was similar to that of native F_1.

Homogeneity of 2Mn F_1 and 2Co F_1 Preparations. Repolymerized ^{54}Mn- and ^{60}Co-containing F_1 preparations were examined for homogeneity by chromatography on a 0.9 × 55 cm column of Sephacryl S300. The column was eluted with 50 mM Tris–sulfate, pH 8.0, and 1 mM EDTA at 20 °C. Both enzymes emerged in a symmetrical peak in which protein, radioactivity, and ATPase activity coincided. Preparations also contained a small, low molecular weight protein peak which contained no radioactivity or ATPase activity and presumably contained dissociated subunits. Since the repolymerized enzyme mixture consisted mostly of intact F_1 aggregate, the S-300 chromatography was not routinely employed as a preparative step, the centrifuge column procedure being much more rapid. Also, δ and ε subunits were partially lost during the S-300 chromatography.

ATP-Driven Proton Pumping by 2Mn F_1 and 2Co F_1 Reconstituted with F_1-Depleted Membranes. First, it was necessary to deplete the F_1-depleted membranes of endogenous Mg by extensive washing. F_1-depleted membranes, as normally prepared in this laboratory (Perlin et al., 1983), con-

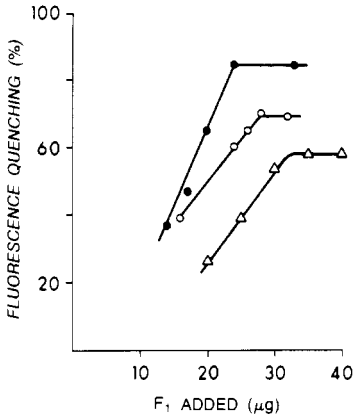

FIGURE 1: ATP-driven proton pumping by Co- and Mn-substituted F_1F_0. F_1-depleted membranes were thoroughly washed to deplete them of bound metal (see text), then native (2Mg) F_1, 2Mn F_1, or 2Co F_1 was rebound, and the ATP-driven quenching of acridine orange fluorescence was measured (see Materials and Methods). For each determination, 200 μg of membrane protein was used, and the metal salt ($MgSO_4$, $MnSO_4$, or $CoSO_4$) in the medium corresponded to that bound on the F_1. (●) Native 2Mg F_1; (○) 2Mn F_1; (△) 2Co F_1.

tained ca. 30 nmol of Mg/mg of protein. This was reduced to ca. 2 nmol of Mg/mg of protein after five washes in 10 mM Tris–sulfate and 5 mM EDTA, pH 8.0, with retention of ability to rebind native F_1 and show normal ATP-driven pH gradient formation. Further washes impaired pH gradient formation and did not substantially reduce the Mg content below 2 nmol/mg of protein. Tightly bound Mg, therefore, seems to be a natural constituent of the *E. coli* cell membrane, in amounts similar to that seen in beef heart mitochondrial inner membrane (Senior, 1981).

The 5 times washed membranes were used in further studies. Native enzyme (2Mg F_1) or repolymerized 2Mn F_1 or 2Co F_1 was rebound to the membranes, and ATP-driven pH gradient formation was measured. The assay was carried out in a 2 mM concentration of added divalent cation (Mg, Mn, or Co) corresponding to the metal bound on the F_1. It was evident (Figure 1) that *E. coli* F_1 in which all the intrinsic Mg was replaced by Mn or Co retained the ability to bind to F_1-depleted membranes and to catalyze ATP-driven proton pumping. The maximum acridine orange fluorescence quenching seen at saturating levels of F_1 was 84% for native (2Mg) F_1, 71% for 2Mn F_1, and 58% for 2Co F_1. Native F_1 showed equal maximal levels of fluorescence quenching in the presence of added 2 mM $MgSO_4$, $MnSO_4$, or $CoSO_4$ (data not shown). Therefore, the lowered responses for 2Mn F_1 and 2Co F_1 were due to the bound metals. Figure 1 shows that 2Mn F_1 and 2Co F_1 must be present at higher concentration than native F_1 in order to reach the maximum fluorescence quenching response. No significance can be attached to this finding, however, since it may simply reflect partial failure to incorporate δ and/or ε subunit in the repolymerized oligomer.

Effect of DCCD on Bound Metal in F_1. It is well established that DCCD inhibits the ATPase activity of native *E. coli* F_1 and that several divalent cations, added in the millimolar range of concentration, protect the enzyme from inhibition (Satre et al., 1979; Yoshida et al., 1982). We investigated first the inhibition of 2Mn F_1 and 2Co F_1 by DCCD and found that they were inhibited more slowly than native 2Mg F_1 (Table II). Free Mn or Co at 2 mM further protected each enzyme from DCCD inactivation.

One site of DCCD reaction has been identified as a particular glutamic acid residue (E-192) in the β subunit of *E. coli* F_1 (Yoshida et al., 1982). Because the rate of inactivation by DCCD is significantly reduced in the presence of added Mg, it was speculated that E-192 is a site for Mg binding (Yoshida et al., 1982). If such were the case, an F_1 molecule which carries bound dicyclohexylurea (the adduct from the DCCD reaction) might exhibit a reduced capacity to bind metals. We therefore determined the Mg content of native F_1 which had been inactivated with DCCD. F_1 which was 98% inhibited with respect to ATPase activity contained 1.76 Mg/F_1; a control sample, carried through the same procedure but without DCCD, contained 1.74 Mg/F_1. It therefore appears that the DCCD-reactive glutamate residue is not a site of tight Mg binding. A possible flaw in this conclusion is that only one of the three β subunits per F_1 may have reacted with DCCD at the labile glutamate, leaving the other two available for ligation with Mg.

When we used [^{14}C]DCCD and inactivated the F_1-ATPase activity by 98%, we did find that the F_1 retained approximately only 1 mol of ^{14}C label per mole of F_1. However, Yoshida et al. (1982) point out that, due to the decomposition of the *O*-acylisourea product that is formed initially by reaction of DCCD with the carboxyl side chain, the measured degree of labeling by [^{14}C]DCCD may not reflect the true degree of reaction. Therefore, it was possible that the enzyme which was 98% inactivated contained one, two, or even all three β subunits per F_1 which had reacted with DCCD. The experiment in Table III was performed to test which of these

Table II: Inhibition of F_1 Preparations by DCCD[a]

enzyme preparation	added metal	$t_{1/2}$ of inactivation (min)
2Mg F_1	none	5.4
	2 mM $MgSO_4$	11.9
2Mn F_1	none	8.8
	2 mM $MnSO_4$	18.2
2Co F_1	none	19.1
	2 mM $CoSO_4$	26.5

[a] Native 2Mg F_1, 2Mn F_1, and 2Co F_1 were treated with DCCD (100 μM) as described under Materials and Methods in the presence or absence of 2 mM added divalent metal. ATPase activity was assayed at intervals as described under Materials and Methods. Results shown are triplicate determinations.

Table III: DCCD Reaction with β Subunits of *E. coli* F_1-ATPase[a]

re-polymerization mixture	% regain of ATPase act. after repolymerization: expected for model β_3^i	$\beta_2^i\beta_1$	$\beta_1^i\beta_2$	actual seen
(A) no added β	0	4.0	30.2	4.2
(B) 1× added β	13.0	30.2	58.4	15.7
(C) 2× added β	30.2	47.6	70.6	28.3

[a] Native F_1 was inactivated by [^{14}C]DCCD (100 μM) for 105 min at 30 °C as described under Materials and Methods. The enzyme was 98.4% inactivated; it was then dissociated by dialysis against dissociation buffer for 42 h. The ^{14}C/F_1 ratio at this stage was 1.09, and the ATPase activity was zero. The dissociated subunits (1 mg/mL) were then repolymerized with (A) no addition, (B) addition of 1 equiv of native β subunits (0.39 mg/mL), and (C) addition of 2 equiv of native β subunits (0.79 mg/mL). The expected percent regain of ATPase activity was calculated for three models of inactivation by DCCD: (i) β_3^i in which three of the three β subunits per F_1 had reacted with DCCD; (ii) $\beta_2^i\beta_1$ in which two of the three β subunits per F_1 had reacted with DCCD; (iii) $\beta_1^i\beta_2$ in which one of the three β subunits per F_1 had reacted with DCCD. For each of the three models, the expected percent regain of ATPase activity after repolymerization was calculated as the fraction of total unreacted β subunit raised to the third power. The control for the actual inactivated F_1 was an F_1 sample carried through the entire procedure except that ethanol alone replaced DCCD. It reconstituted to a specific activity of 19.8 μmol of ATP hydrolyzed per minute per milligram at 30 °C, pH 8.0, which was similar to the activity of native F_1 under identical assay conditions. Results shown in column 5 are means of triplicate experiments.

Table IV: Binding of Mg, Co, and Mn to Purified α and β Subunits of *E. coli* F_1[a]

additions	metal bound (mol/mol of F_1)			
	Mg	Co	Mn	total
α subunit				
+$MgSO_4$	0.97			0.97
+$MnSO_4$	0.37		0.49	0.86
+$CoSO_4$	0.37	0.54		0.91
+$MgSO_4$ + ATP	1.05			1.05
+$MnSO_4$ + ATP	0.32		0.63	0.95
+$CoSO_4$ + ATP	0.23	0.79		1.02
β subunit				
+$MgSO_4$	0.26			0.26
+$MnSO_4$	0.00		0.057	0.057
+$CoSO_4$	0.00	0.036		0.036
+$MgSO_4$ + ATP	0.24			0.24
+$MnSO_4$ + ATP	0.09		0.019	0.109
+$CoSO_4$ + ATP	0.27	0.033		0.303

[a] Purified subunits were equilibrated in 50 mM Tris–sulfate, pH 8.0 (Chelex-100 treated), on centrifuge columns containing Sephadex G-50F. To the subunit solution (approximately 5 μM) were added $MgSO_4$, $^{54}MnSO_4$, or $^{60}CoSO_4$ (250 μM) and nucleotides (250 μM) as required. Incubations were carried out for 2 h at 20 °C (shown to be optimal in preliminary experiments). Subunits were then eluted from centrifuge columns in 50 mM Tris–sulfate, pH 8.0 (Chelex-100 treated), and analyzed for bound metal. Results shown are averages of at least quadruplicate experiments. It should be noted that the α subunit contained Mg initially (see text) which was not fully displaced by Mn or Co.

possibilities was correct. Native F_1 was inactivated by DCCD and dissociated into its subunits. Then the enzyme was repolymerized in the presence of (a) no additional β subunits, (b) one equivalent amount of extra, native, purified β subunits, or (c) two equivalent amounts of extra, native, purified β subunits. The actual measured regain of ATPase activity is shown in Table III, column 5, expressed as a percent of the regain of activity measured in control enzyme which was not treated with DCCD but was dissociated and repolymerized. The expected percent regain of activity predicted by each of three models, in which 98% inactivation of F_1-ATPase correlates respectively with reaction of one, two, or three of the three β subunits per F_1 with DCCD, is shown in Table III, columns 2–4. The data are not consistent with the idea that 98% inactivation correlated with reaction of one of the three β subunits per F_1 despite the fact that the measured $^{14}C/F_1$ ratio after inactivation was 1.09. Rather, the data suggest that at least two and probably three β subunits per F_1 had reacted with DCCD. Therefore, this experiment substantiates the conclusion that the DCCD-reactive E-192 of the β subunit is not a site for the tight Mg binding in native F_1.

Binding of Mg, Mn, and Co to Purified α and β Subunits of E. coli F_1. Three different preparations of purified α subunit were found to contain an average of 0.54 Mg/α subunit (mol/mol) (range 0.34–0.85). Since the dissociated subunits analyzed immediately after dialysis against dissociation buffer contained very little Mg (above), the α subunits must have bound the Mg during the chromatography steps involved in α purification. This suggested that the α subunit contained a metal binding site.

Table IV shows purified α subunit could bind Mg, Mn, or Co when incubated with these metal ions. The maximum binding seen was 1 mol of divalent metal per mole of α subunit, even when concentrations up to 5 mM metal ion were tested (not shown). α subunit binds adenine nucleotide to the extent of 1 mol/mol (Dunn & Futai, 1980) in either the presence or the absence of added divalent cation (Perlin et al., 1984). In the presence of ATP, the total metal bound to α subunit was still 1 mol/mol (Table IV).

Three different preparations of purified β subunit were found to contain an average of 0.07 Mg/β subunit (mol/mol) (range 0.01–0.133). The isolated β subunit did not bind Mg, Co, or Mn stoichiometrically (Table IV), in either the presence or the absence of adenine nucleotide. Increasing the concentration of added metal or nucleotide did not give increased binding over that seen in Table IV (data not shown). No significant binding of ATP, GTP, or AMPPNP to the β subunit was noted. Even when radioactive nucleotide was used at a concentration of 1 mM, only 0.15 mol of ATP/mol of β subunit was bound. GTP and AMPPNP bound to a similar or a lower extent.

In our hands, the yield of purified γ subunit obtained by the Dunn & Futai (1980) procedure was very low. This prohibited us from obtained information regarding metal content or metal binding capacity of the γ subunit.

Discussion

In the introduction, we summarized our previous work on the tight metal binding sites in beef heart mitochondrial F_1-ATPase. Results presented in this paper show that the *E. coli* enzyme is generally similar to the beef heart enzyme in its binding of divalent metals but that there are differences which render the *E. coli* enzyme preferable for further studies.

Like the beef heart mitochondrial F_1, the *E. coli* enzyme has two tight binding sites for Mg, Mn, and Co. This was shown here by analyses of the native enzyme and by dissociation–repolymerization of the enzyme. In the beef heart enzyme, one of the two sites could not be exchanged, and attempts to remove Mg from it lead to irreversible depolymerization. For this reason, it proved not possible to replace this tightly bound Mg by any other metal in the beef heart mitochondrial F_1. Here, it was shown that in the *E. coli* F_1 both of the intrinsic Mg could be removed by depolymerization and that by repolymerization, enzyme containing two Co or two Mn could be generated. These fully substituted enzymes were active as ATPases and were able to rebind to F_1-depleted membranes and catalyze ATP-driven proton pumping across membranes. It is hoped this will allow spectroscopic analyses of the environments of tightly bound Mn and Co on F_1 during proton pumping and ATP hydrolysis, which may yield valuable information about conformational changes during catalysis.

The location of the tight divalent metal sites on F_1 is not yet determined; however, our experiments are relevant to this question. The isolated α subunit bound one Mg, Co, or Mn (mol/mol), apparently independently of whether nucleotide was present or not. Therefore, α subunits are candidates for the binding of the tight metals in intact F_1. Tight binding of Mg at two sites in beef heart F_1 also occurred independently of nucleotide binding (Senior et al., 1980). Recent evidence suggests that α subunits are the sites of nonexchangeable nucleotide binding in F_1 [discussed in Perlin et al. (1984)].

On the other hand, the fact that isolated β subunits did not bind metal stoichiometrically need not disqualify them as metal binding sites in intact F_1, since in this work, and as previously described (Dunn & Futai, 1980), isolated β subunit from *E. coli* F_1 did not bind nucleotides either, yet there is good evidence that the β subunits in intact F_1 carry all or part of the catalytic sites [reviewed in Senior & Wise (1983)]. Cooperative subunit interactions seem involved for both metal binding (Senior, 1979) and nucleotide binding. Our data show that the DCCD-reactive glutamate residue in the β subunit is not the site of tight metal binding. However, occupancy of the tight metal sites by different metals changed the rate of inactivation by DCCD, and the influence of looser metal site(s) on this inactivation was apparent from the fact that

added metal ion at 2 mM concentration gave further protection. Thus, both tightly bound and loosely bound metals affect the β-subunit conformation and influence the accessibility of the DCCD-labile glutamate to attack by the inactivator indirectly.

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Registry No. ATPase, 9000-83-3; DCCD, 538-75-0; Co, 7440-48-4; Mn, 7439-96-5; Mg, 7439-95-4; H^+, 12408-02-5.

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