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Activity and Conformational Changes in Chloroplast Coupling Factor Induced by Ion Binding: Formation of a Magnesium–Enzyme–Phosphate Complex†

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ABSTRACT: The effects of ions on the conformation and activity of chloroplast coupling factor (CF_1) were studied by using tryptophan as an intrinsic fluorescence probe in CF_1. The fluorescence of tryptophan decreased when $MgCl_2$ or sodium phosphate was added to the protein. The decrease indicated that Mg^{2+} and inorganic phosphate (P_i) bound directly to the protein. The decrease saturated at 1.2 mM Mg^{2+} and at 0.8 mM P_i, although P_i showed evidence of a higher affinity site saturating around 80 μM. The decrease in fluorescence could also be observed when P_i was added after addition of Mg^{2+}, which indicated that a ternary complex of Mg^{2+}–CF_1–P_i formed. If the reverse addition sequence was used, a ternary complex was not observed. The free energy of dissociation for each ion added singly was 4.6, 6.2, and 4.8 kcal/mol for Mg^{2+}, P_i (high-affinity site), and P_i (low-affinity site), respectively. The magnitude of these free energies and the presence of a ternary complex of Mg^{2+}, P_i, and CF_1 may be significant for the mechanism of photophosphorylation. The incubation of CF_1 in Mg^{2+} above 5 mM increased the polarization of CF_1 (tryptophan) fluorescence and changed the circular dichroism spectrum. These spectroscopic changes show that Mg^{2+} binds further at concentrations above the initial saturating concentration of 1.2 mM and that CF_1 changes conformation in response to these higher concentrations. Chloroplasts lost phosphorylating activity and CF_1 lost Ca^{2+}-ATPase activity when the preparations were preincubated at these high Mg^{2+} concentrations but then removed from the preincubating media and assayed under uniform conditions. Thus, the additional binding and altered conformation were inhibitory for CF_1 and chloroplast activities. The results indicate that Mg^{2+} binds directly to CF_1 and may have two roles: a catalytic one associated with the formation of a Mg^{2+}–CF_1–P_i complex and a regulatory one at high Mg^{2+} concentrations. The regulatory one resembles the effects in vivo caused by dehydration of leaf tissue, which increases the concentration of cellular ions, and this suggests that both the catalytic and the regulatory roles of Mg^{2+} could be important in vivo.

The synthesis of ATP, which is central to metabolic energy conversion in biological organisms, is associated with a transmembrane gradient in electrochemical potential created by proton movement. Although the dissipation of the gradient results in ATP synthesis, the molecular mechanism is poorly understood and of great interest. Several theories have been advanced (Weber, 1972; Boyer, 1977; Racker, 1977; Penefsky, 1977; Kasahara & Penefsky, 1978), all of which require the binding of ADP and inorganic phosphate (P_i)[1] to the coupling protein (ATP synthetase) complex in the membrane. In addition, certain theories suggest that other ions, particularly Mg^{2+}, may be required to bind to the protein (Weber, 1972; Racker, 1977).

Weber (1972) pointed out that, on a theoretical basis, the binding of ions to the coupling protein could provide sufficient free energy for the transfer of P_i to ADP. Racker (1977) postulated that, by analogy with the Na^+–K^+–ATPases and Ca^{2+}-ATPases, the ATP synthetases may form a magnesium acyl phosphate intermediate and that the role of protons may be to dissociate Mg^{2+} from the coupling protein with the release of ATP. Evidence for the formation of a phosphoenzyme intermediate in ADP–ATP interconversions was first found with Na^+–K^+- and Ca^{2+}-ATPases when the enzymes

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[1] Abbreviations: CF_1, chloroplast coupling factor protein (ATP synthetase when attached to the membrane); PMS, phenazine methosulfate; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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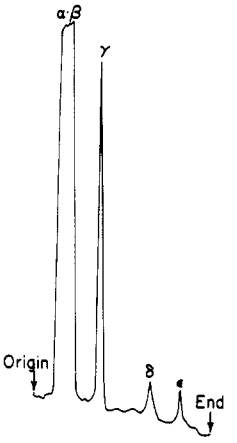

FIGURE 1: Densitometric trace of a polyacrylamide gel showing the subunit structure of purified CF_1 prepared by the chloroform method as described in the text.

were incubated with ATP (Dagani & Boyer, 1973; Bastida et al., 1973) or with P_i (Lindenmayer et al., 1968; Masuda & Meis, 1973). Penefsky (1977) and Kasahara & Penefsky (1978) subsequently demonstrated one or two binding sites for P_i on the coupling protein (F_1 ATPase) from beef heart mitochondria. Furthermore, the Na^+-K^+-ATPase of the plasma membrane (Taniguchi & Post, 1975) and the Ca^{2+}-ATPase of sarcoplasmic reticulum (Knowles & Racker, 1975) were phosphorylated by P_i to form an acylphosphoprotein that mediated the stoichiometric transfer of P_i to ADP to form ATP. Mg^{2+} was required for this activity, and kinetic analysis suggested that a ternary complex of ATPase, P_i, and Mg^{2+} formed (Kuriki et al., 1976). Recently, Feldman & Sigman (1982) showed a similar formation of ATP on soluble CF_1 although high concentrations of Mg^{2+} and P_i were employed.

Central to these hypotheses is the binding of P_i and Mg^{2+} to the coupling protein with a resultant change in the activity of the protein. Nucleotides readily bind to ATPases (Strotmann et al., 1977; Harris, 1978) but, with the exception of P_i binding to beef heart ATPase (Penefsky, 1977; Kasahara & Penefsky, 1978) and a structural Mg^{2+} tightly bound to beef heart ATPase (Senior, 1979), there is little direct evidence that P_i or Mg^{2+} binds or that ligand binding alters the activity of the protein. One of the difficulties may lie in the lability of the ligand–protein complex when equilibrium conditions are disturbed during attempts to isolate the complex. The recent demonstration (Beliveau et al., 1982; Zurawski et al., 1982) of tryptophan in CF_1 provides an intrinsic fluorescence probe for detecting ligand binding without disturbing the ligand–protein equilibrium. Furthermore, the recent demonstration (Younis et al., 1979) that prior exposure of CF_1 to Mg^{2+} can alter the subsequent activity of the protein provides a means to test whether the binding is biologically significant. The following work was therefore undertaken to determine whether P_i and Mg^{2+} bind to CF_1 and how the binding alters the activity of the protein.

Materials and Methods

Plant Material, Chloroplast Isolation, and Protein Extraction. Chloroplasts were isolated as described by Lien & Racker (1971) from commercial spinach (*Spinacia oleracea* L.), obtained from a local market. CF_1 was extracted with chloroform followed by column chromatography on a Sephadex G200 superfine gel (Younis et al., 1977). This purified protein contained the five subunits characteristic of CF_1, was unaccompanied by any other proteins detectable by polyacrylamide gel electrophoresis (Figure 1), and had high specific activity [20–21 μmol of ATP hydrolyzed·(mg of protein)$^{-1}$·h^{-1}]. The protein was stored at 1 °C in 2 M $(NH_4)_2SO_4$, 2 mM ATP, 1 mM EDTA, and 20 mM Tricine/OH^-, pH 8, until needed for measurements. Before use, the suspension was centrifuged at 14000*g*, dissolved in 40 mM Tris-HCl, pH 8, buffer, recentrifuged at 14000*g*, and desalted on a Sephadex G-50 column (20 × 1 cm) equilibrated with the same buffer. Molar concentrations of the protein were based on a molecular weight of 325 000 (Farron, 1970).

Assays. Cyclic photophosphorylation by the chloroplasts was measured potentiometrically in the presence of 50 μM phenazine methosulfate (PMS), 3 mM sodium phosphate, pH 7.8, 17 mM KCl, 1.5 mM ADP, and 3 mM $MgCl_2$ according to Dilley (1972) by using a recording pH meter. Illumination was with saturating red light (180 W m^{-2}, 400–700 nm, maximum at 668 nm) passed through a heat filter (Mohanty & Boyer, 1976). Ca^{2+}-dependent ATPase activity of the isolated CF_1 was measured before heat activation (latent enzyme) and after heat activation of the protein (60–63 °C for 4 min in the presence of 40 mM Tricine/NaOH, pH 8, 20 mM ATP, and 5 mM dithiothreitol) according to Lien & Racker (1971). Chlorophyll concentrations were determined spectrophotometrically in 80% acetone extracts (MacKinney, 1941; Arnon, 1949). Protein concentrations of CF_1 were measured spectrophotometrically according to Warburg & Christian (1941), and the concentrations were multiplied by 1.85 to give the dry weight of protein (Farron & Racker, 1970).

Fluorescence Measurements. Fluorescence intensities for CF_1 (tryptophan) were measured with a T-format (Weber, 1952a) photon counting polarization photometer described by Jameson et al. (1978). The excitation wavelength was 295 nm, and the excitation bandwidth was 1.67 nm. The emitted fluorescence was filtered through Corning glass C.S. 7-51 filters. The total intensity of fluorescence passed by the filters was obtained by adding the parallel intensity to 2× the perpendicular intensity ($I_\parallel + 2I_\perp$) while the sample was excited by the parallel polarizer. Conditions were adjusted so that the total counts were (2–3) × 10^5. A correction for the scattered parasitic light was made by introducing into the path of the exciting beam a filter similar to that used to separate excitation from fluorescence (Weber, 1960). In all cases, this correction was small.

The binding of Mg^{2+} and P_i was measured by determining the decrease, i.e., quenching, of the fluorescence of tryptophan in the protein by the ion. All binding experiments were conducted with the protein at 2 μM concentration in 1 mL of 40 mM Tris-HCl, pH 8, and $MgCl_2$ or sodium phosphate was added from 1 M stock. After each addition of the solution, 2 min elapsed before the fluorescence emission was measured.

Fluorescence polarization measurements of CF_1 (tryptophan) were also made by using a polarization photometer (Weber & Bablouzian, 1966; Jameson et al., 1978). This instrument allows simultaneous monitoring of the parallel ($I_\parallel$) and perpendicular ($I_\perp$) components of polarized fluorescence by using two photomultipliers at right angles to the exciting beam. It also allows for subtraction of scattering at low signal to noise ratios. The polarization of fluorescence is defined as that fraction of the total fluorescence that is polarized according to $(I_\parallel - I_\perp)/(I_\parallel + I_\perp)$.

Fluoresence lifetimes were measured with a cross-correlation phase fluorometer described by Spencer & Weber (1969) with light modulated at a single frequency of 18 MHz.

Circular Dichroism Measurements. The circular dichroism of CF_1 was measured between 200 and 250 nm by using a JASCO Model J40A automatic recording spectropolarimeter.

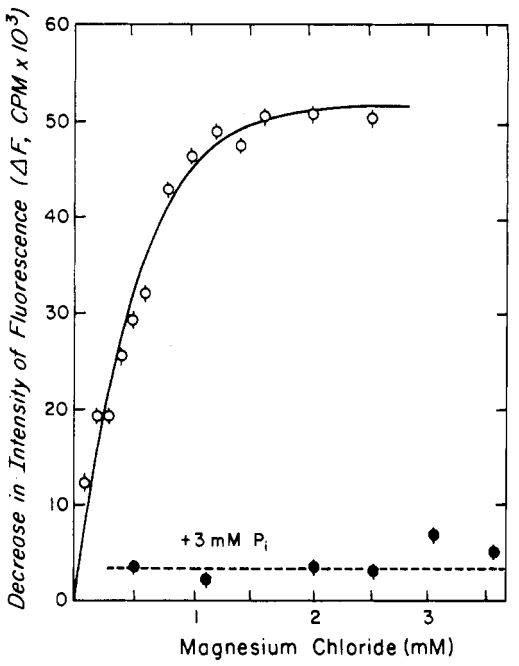

FIGURE 2: Fluorometric titration of chloroplast coupling factor (latent CF_1 ATPase) with $MgCl_2$. The protein concentration was 2 μM in 40 mM Tris-HCl, pH 8 (upper curve), or the same buffer plus 3 mM sodium phosphate (lower curve). The titration was carried out at 25 °C. Vertical bars are the square root of the decrease in counts per minute.

The instrument was calibrated with (+)-camphorsulfonic acid, the path length was 1 mm, the protein concentration was 0.5 mg mL^{-1}, and the temperature was 25 °C. The mean residue molecular weight was taken to be 114.

Results

Fluorescence Quenching. $MgCl_2$ decreased the fluorescence of tryptophan in CF_1 (Figure 2). The decrease was saturated at about 1.2 mM $MgCl_2$. Sodium phosphate also decreased the fluorescence of CF_1 (Figure 3), but the initial approach to saturation at 80 μM (Figure 3, inset) was followed by a further decrease in fluorescence that saturated at 0.8 mM (Figure 3). No further change occurred in the fluorescence at saturation concentrations for either salt when observations were extended to 15 min. The saturation concentrations for each salt were highly reproducible, but the maximum quenching of fluorescence at saturation varied between protein preparations.

The effect of these salts was altered if they were added sequentially to CF_1. After saturation with $MgCl_2$, addition of sodium phosphate caused the two-phase decreased noted with sodium phosphate alone, but the decrease was in addition to that already caused by $MgCl_2$ (Figure 4). The saturation concentrations were essentially unaltered.

In contrast, in short time experiments after exposure to 0.6 mM sodium phosphate, addition of $MgCl_2$ to CF_1 did not cause a decrease in fluorescence beyond that observed with sodium phosphate alone (Figure 5). A similar result was observed when $MgCl_2$ was added to CF_1 in the presence of 3 mM sodium phosphate (Figure 2).

Fluorescence Polarization. We tested whether the effects of $MgCl_2$ and sodium phosphate could also be detected by changes in the polarization of the tryptophan fluorescence in CF_1. The relatively slow rotational relaxation time of proteins causes a portion of the amino acid fluorescence to be polarized, since the rotational relaxation time of the macromolecule is on the order of the fluorescence lifetime for the amino acids

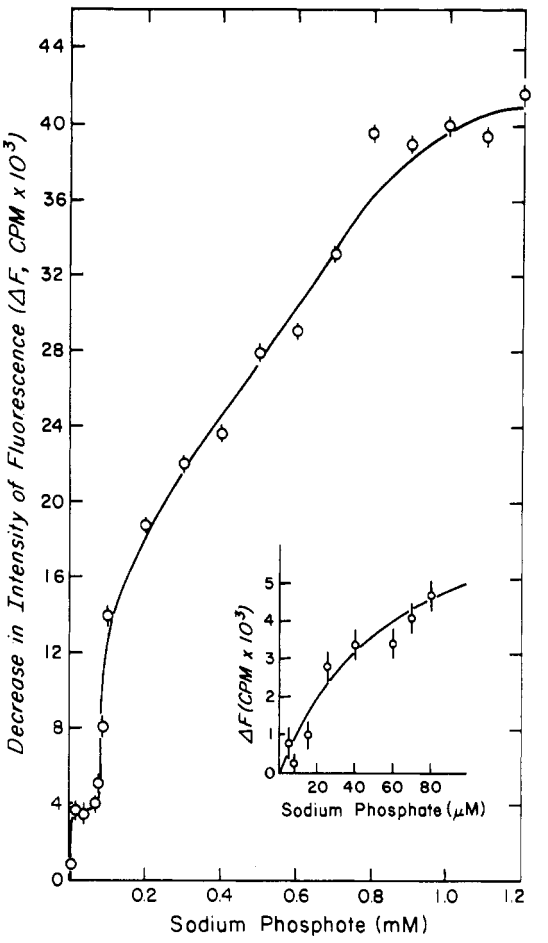

FIGURE 3: Fluorometric titration of chloroplast coupling factor (latent CF_1 ATPase) with sodium phosphate. The inset shows titration at low concentrations of sodium phosphate. Experimental conditions were as in Figure 1.

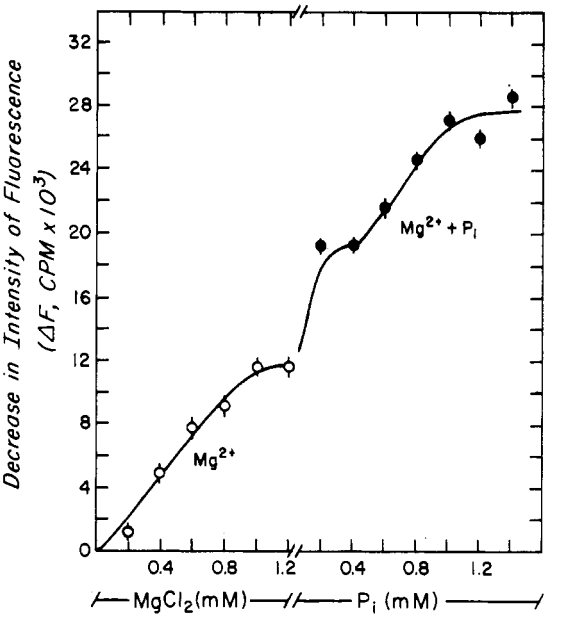

FIGURE 4: Fluorometric titration of chloroplast coupling factor (latent CF_1 ATPase) with $MgCl_2$ followed by sodium phosphate. Experimental conditions were as in Figure 1.

(10^{-8}–10^{-9} s; Weber, 1952a; Laurence, 1969). A change in the amount of polarization reflects a change in the conformation of the macromolecule or a quenching of the fluorescence of the amino acids if the fluorescence lifetime is constant (Weber, 1952a,b). If the change is brought about by a ligand,

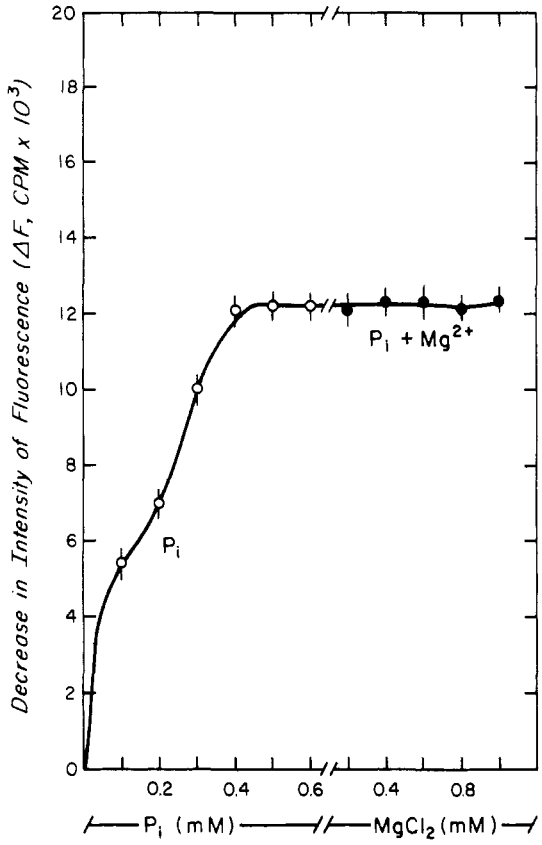

FIGURE 5: Fluorometric titration of chloroplast coupling factor (latent CF_1 ATPase) with sodium phosphate followed by $MgCl_2$. Experimental conditions were as in Figure 1.

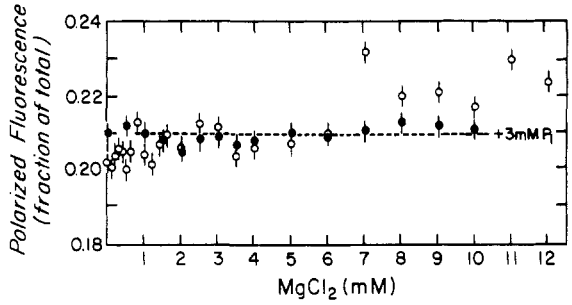

FIGURE 6: Polarization of CF_1 (tryptophan) fluorescence at various Mg^{2+} concentrations in the presence (●) and absence (○) of 3 mM sodium phosphate. The enzyme was not heat activated. The temperature was 25 °C. The protein (3 μM) was excited by a wavelength of 295 nm, and polarization was measured from the fluorescence emission passed by a Corning CS7-51 filter. The exciting light was unfiltered. The vertical bars represent the mean polarization ± 1 standard deviation.

the change is a measure of the binding of the ligand.

$MgCl_2$ slightly increased the polarization of CF_1 (tryptophan) fluorescence (Figure 6) at concentrations above 6 mM. When 3 mM sodium phosphate was present initially, this increase no longer occurred (Figure 6). There was no effect of $MgCl_2$ on the lifetime of tryptophan fluorescence, which was 5.5 ns for the modulation lifetime and 4.3 ns for the phase lifetime (Spencer & Weber, 1969).

Circular Dichroism Spectra. The possibility that the change in polarization could have been caused by a change in CF_1 conformation led us to test further whether a conformational change occurred. We measured the circular dichroism spectrum of CF_1 in the presence of a range of $MgCl_2$ concentrations. At a concentration (2 mM) of $MgCl_2$ approximating that present during assays of photophosphorylation, there was

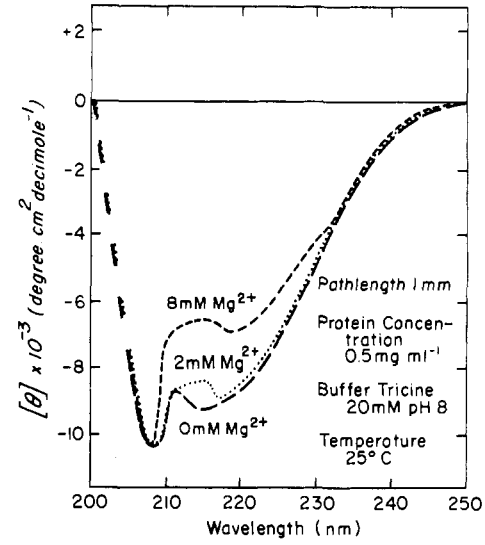

FIGURE 7: Circular dichroism spectra for chloroplast coupling factor without $MgCl_2$, with 2 mM $MgCl_2$, and with 8 mM $MgCl_2$. The enzyme was not heat activated. The temperature was 25 °C, the path length was 1 mm, the buffer was 20 mM Tricine/NaOH (pH 8.0), and the protein concentration was 0.5 mg mL^{-1}.

little change in the molecular ellipticity (θ) of the protein (Figure 7). At a concentration (8 mM) which was above the usual assay concentrations, θ showed an increase in the region between 208 and 230 nm. These spectral alterations indicate that $MgCl_2$ influenced the orientation of protein subunits (β structure) and/or the arrangement of "randomly" coiled regions of the protein (Greenfield & Fasman, 1969). The lack of a $MgCl_2$ effect at 208 nm shows that $MgCl_2$ did not change the quantity of α helix in CF_1 (Greenfield & Fasman, 1969).

Biological Activity. Both the polarization and θ data indicated that CF_1 was altered by $MgCl_2$ concentrations above 6 mM. A similar alteration was observed previously in θ of CF_1 isolated from dehydrated leaf tissue (Younis et al., 1979). Since dehydration concentrates ionic constituents of cells and since dehydration altered the activity of CF_1 (Younis et al., 1979), it was possible to test whether prior exposure to ions affected subsequent assays of CF_1 activity. If the assays were carried out in the absence of the ion or at uniform concentrations of ion, the complicating effects of ion interactions with substrates could be avoided or held constant, and the effects could be attributed to ion–enzyme interactions.

We conducted such an experiment by incubating chloroplasts in resuspension media containing various $MgCl_2$ concentrations, diluting a small aliquot of the mixture in assay medium, and assaying photophosphorylating activity. Photophosphorylation occurred rapidly after preincubation at 0 mM $MgCl_2$ but was inhibited 50% after preincubation at 10 mM $MgCl_2$ and above (Figure 8, open circles). When the $MgCl_2$ concentration was varied directly in the assay solution (without prior incubation), the inhibition was not as severe (Figure 8, closed circles), which suggests that the assay substrates may have been protected somewhat against the inhibitory effects of high $MgCl_2$ concentrations.

Heat-activated CF_1, when similarly preincubated in various $MgCl_2$ concentrations prior to assay, showed a large inhibition of Ca^{2+}-ATPase activity (Figure 9). As with photophosphorylation, the inhibitory effect was saturated at 10 mM $MgCl_2$. If $MgCl_2$ was supplied before heat activation, inhibition of Ca^{2+}-ATPase activity again occurred but to a lesser extent (Table I). It is important that the Ca^{2+}-ATPase assay contained no Mg^{2+} other than the small amount carried over when the enzyme was transferred from the preincubation

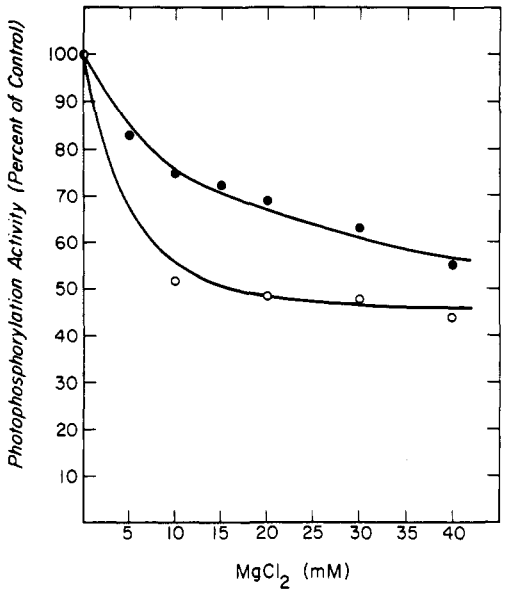

FIGURE 8: Effect of $MgCl_2$ preincubation of chloroplasts on subsequently assayed photophosphorylation. (○) Photophosphorylation at various preincubation concentrations of Mg^{2+}. Chloroplasts (1 mg of chloroplasts mL^{-1}) were divided into several 0.2-mL aliquots and incubated for 15 min at room temperature in 0.4 M sucrose, 20 mM Tricine/NaOH, pH 7.8, 10 mM NaCl, and the concentrations of $MgCl_2$ shown in the figure. For subsequent assay of cyclic (PMS-mediated) photophosphorylation, 20 μL of this mixture was added to 2 mL of the assay medium, which contained 3 mM $MgCl_2$. (●) Photophosphorylation assayed directly (no preincubation) at the $MgCl_2$ concentrations shown in addition to 3 mM Mg^{2+} already present in the assay solution. Chloroplasts (20 μL) and the amounts of $MgCl_2$ shown were added directly to the assay solution (2 mL). The photophosphorylation rate was 1500 μmol of ATP·(mg of chlorophyll)$^{-1}$·h^{-1} in the controls.

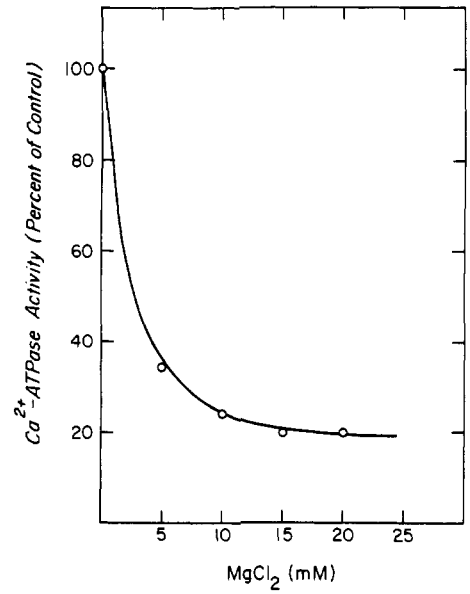

FIGURE 9: Effect of $MgCl_2$ preincubation of heat-activated coupling factor on the subsequently assayed Ca^{2+}-ATPase activity of the protein. Aliquots of the heat-activated coupling factor were incubated for 20 min at room temperature in 20 mM ATP, 5 mM DTT, 40 mM Tricine/NaOH, pH 8.0, and the concentrations of $MgCl_2$ shown in the figure. Sample volumes were corrected with distilled water to keep the protein concentration constant. The assay (1 mL) was started by adding aliquots (10 μL) of the preincubation mixture containing the enzyme to the assay solution. Control activity was 20 μmol of ATP hydrolyzed·(mg of protein)$^{-1}$·min^{-1}.

Table I: Ca^{2+}-ATPase Activity of Coupling Factor Preincubated with $MgCl_2$ before or after Heat Activation[a]

	incubation with $MgCl_2$	
$[MgCl_2]$ (mM)	before heat activation	after heat activation
0	21	21
5	23	7
10	14	5

[a] Activity is given in units of micromoles of inorganic phosphate per minute per milligram of protein. For preincubation before heat activation, $MgCl_2$ was added from a 1 M stock solution to a solution containing coupling factor (1 mg mL^{-1}), 40 mM Tricine/NaOH (pH 8), 20 mM ATP, and 5 mM dithiothreitol. After preincubation for 10 min, the protein was activated by heating in a water bath at 60–63 °C for 4 min. Ten microliters of the preincubation mixture was then added to 1 mL of medium for assay of Ca^{2+}-ATPase. When preincubation was done after heat activation, the procedure was the same except the sequence of events was heat activation, addition of $MgCl_2$, preincubation, transfer of enzyme aliquot, and assay.

solution to the assay solution (Mg^{2+} concentration during the assay was 1% of the preincubation concentration). Therefore, the effects during assay represented persistent effects of the preincubation.

Discussion

Ligand binding to protein is readily detectable from changes in protein (tryptophan) fluorescence. The decrease in fluorescence caused by $MgCl_2$ or sodium phosphate shows that at least one ion from each salt was binding to CF_1. For $MgCl_2$, the ion must have been Mg^{2+} since the protein–buffer solution already contained excess Cl^-. For sodium phosphate, the ion was likely to have been phosphate since sodium ion has little effect on CF_1 at the concentrations we used [e.g., see Shahak (1982) for the Na^+ effect on the ATP–P_i exchange activity of CF_1]. The binding must have been to CF_1 since our preparations were highly purified.

Fluorescence methods for detecting ion binding to protein have the advantage that the protein–ion complex remains in the presence of free ion during the measurements, thus assuring the maintenance of equilibrium between the protein and free ion. Penefsky (1977) showed P_i binding to beef heart mitochondrial ATPase by using a gel column technique to separate the P_i–ATPase complex from free P_i. The binding was Mg^{2+} dependent, which differs from our results showing P_i binding in the absence of Mg^{2+}. The difference may be associated with the difference in equilibrium conditions during the measurement. Thus, P_i binding could have been detected in our experiments because of the continual presence of free P_i but could have gone undetected by Penefsky (1977) due to reversal of P_i binding as free P_i was separated from bound P_i during passage through the gel column.

Penefsky (1977) suggested that the Mg^{2+} dependence of P_i binding might involve Mg^{2+} binding to the ATPase rather than the formation of a Mg^{2+}–P_i complex. Our observations are in support of this suggestion because Mg^{2+} was able to bind directly to CF_1 in the absence of P_i. Furthermore, a ternary complex between Mg^{2+}, CF_1, and P_i appeared to be formed when Mg^{2+} was added to CF_1 prior to the addition of P_i. It is clear that P_i did not displace Mg^{2+} during this addition, since the magnitude of the fluorescence decrease was similar to that for each ion supplied singly and additive when each ion was supplied sequentially.

On the other hand, the lack of demonstrated binding of Mg^{2+} when the order of ion addition was reversed could have resulted from a slow approach to equilibrium. Penefsky (1977) observed that P_i binding required as long as 15 min in his experiments. A second possibility is that higher Mg^{2+} con-

Table II: Dissociation Constants and Standard Free Energies of Dissociation for P_i and Mg^{2+} Complexes with Chloroplast Coupling Factor[a]

reaction	dissociation constant	standard free energy (kcal/mol)
$E + Mg^{2+} \rightleftarrows E\text{-}Mg^{2+}$	K_1 = 0.4 mM	4.6
$E + P_i \rightleftarrows E\text{-}P_i$ (high affinity)	K_2 = 25 μM	6.2
$E + P_i \rightleftarrows E\text{-}P_i$ (low affinity)	K'_2 = 0.28 mM	4.8

[a] Calculations assume a stoichiometry of 1 for all reactions.

centrations are required for Mg^{2+} binding in the presence of P_i than in its absence. A third possibility, that Mg^{2+} binding released previously bound P_i, seems unlikely because CF_1 would be placed in a Mg^{2+}–CF_1 complex that binds P_i, as when Mg^{2+} addition is followed by P_i addition (Figure 4).

Regardless of this point, however, the formation of a ternary complex could be an important aspect of the mechanism of photophosphorylation. Weber (1972) suggested that the free energy of ion binding could be involved in the transfer of free energy from that stored in the proton gradient to that in the terminal phosphate of ATP during phosphorylation. Racker (1977) has also proposed that two ion ligands, Mg^{2+} and P_i, may be involved in the phosphorylation mechanism.

The concentration dependence of P_i and Mg^{2+} binding is an important aspect of these hypotheses. Saturation binding occurred at 1.2 and 0.8 mM for Mg^{2+} and P_i, respectively, with evidence for an initial saturation of a high-affinity site at 80 μM for P_i. These concentrations give maximum rates of photophosphorylation in vitro [1.5–3 mM Mg^{2+}, 2–3 mM P_i; see Whatley & Arnon (1963), Jagendorf & Uribe (1966), and Dilley (1972)] and are similar to stromal concentrations for Mg^{2+} in vivo [1–3 mM; see Portis & Heldt (1976), Krause (1977), and Portis (1981)].

It is likely that equilibrium binding was achieved with Mg^{2+} and P_i added singly to CF_1, since the fluorescence change was stable at saturation. Assuming the following equilibria apply:

$$E + Mg^{2+} \rightleftarrows E\text{-}Mg^{2+} \quad (1)$$

$$E + P_i \rightleftarrows E\text{-}P_i \quad (2)$$

the apparent dissociation constants (K_D) for Mg^{2+} and P_i binding were calculated from $K_D = [E][ion]/[E\text{-}ion]$ at half-saturation. For Mg^{2+}, the K_D was 0.4 mM, and for P_i, the K_D was 25 μM (high-affinity site) and 0.28 mM (low-affinity site). Kasahara & Penefsky (1978) observed a pH-dependent K_D for P_i that was similar, ranging from 27 μM to 0.29 mM. Accordingly, the standard free energy of binding was 4.6, 6.3, and 4.8 kcal/mol for Mg^{2+}, P_i (high-affinity site), and P_i (low-affinity site), respectively, in our experiments (Table II). These binding energies are large enough to be significant for the formation of the terminal phosphate bond of ATP, particularly if a ternary complex is involved in photophosphorylation so that the free energy of binding of both ions contributes to the reaction.

Senior (1979) has described a tightly bound Mg^{2+} in beef heart ATPase which is bound to the protein with a stoichiometry of 1:1. This ion appears to play a structural role since, in its absence, the ATPase dissociates to its subunits and its activity is lost. If there is a similar structural requirement for CF_1, Mg^{2+} is likely to have been present in our preparations prior to Mg^{2+} addition, and, thus, the effects we observed must have involved binding that exceeded the minimum for structural integrity of the protein.

Biological Significance. The significance of P_i binding to CF_1 is apparent from its involvement in ATP synthesis as a substrate. However, the significance of Mg^{2+} binding is not as apparent except for the theoretical involvement of Mg^{2+} in ATP synthesis (Weber, 1972, 1974; Racker, 1977). The saturation binding of these ions in the concentration range likely to be present in vivo (Portis & Heldt, 1976; Portis, 1981) is consistent with a possible biological role for this binding. Furthermore, the alteration of CF_1 to an inhibitory conformation at Mg^{2+} concentrations above those for saturation provided an opportunity to determine whether prior binding of Mg^{2+} to CF_1 caused effects on subsequent activity.

The inhibition of Ca^{2+}-ATPase activity following preincubation with Mg^{2+} but assay with negligible Mg^{2+} shows that Mg^{2+} binding has a biological effect. Mg^{2+} may or may not have remained bound to CF_1 during assay, but it clearly bound during preincubation, as shown by fluorescence quenching, fluorescence polarization, and circular dichroism. Thus, regardless of the fate of the Mg–CF_1 complex during assay, CF_1 was altered in a way that affected its activity.

The data from a similar experiment with chloroplasts also showed that high Mg^{2+} concentrations (above 5 mM) caused losses in photophosphorylation. Since no assay components were in the preincubation medium, the inhibition must be attributed to direct Mg^{2+}–chloroplast interactions.

Adolfsen & Moudrianakis (1973) indicated that Mg^{2+} forms a Mg^{2+}–protein complex rather than a Mg–ATP complex during ATP hydrolysis, and Hilborn & Hammes (1973) showed that ATP and Mg–ATP were bound to mitochondrial coupling factor with similar affinities. However, kinetic data suggested that Mg^{2+} interacted with the nucleotide substrates and Mg–ATP may be the substrate for mitochondrial (Kozlov & Skulachev, 1977) and chloroplast (Hochman et al., 1976) ATPase. Our assay conditions for photophosphorylation included Mg^{2+} (3 mM) so that any substrate requirements for Mg^{2+} should have been satisfied. Thus, although the Mg–ATP complex could have been involved in photophosphorylation, our results with preincubation show that a direct Mg^{2+}–chloroplast interaction was also involved. It is noteworthy that we observed effects of Mg^{2+} preincubation when CF_1 was assayed for Ca^{2+}-ATPase which has no substrate requirement for Mg^{2+}. Substrate quantities of Mg^{2+}, therefore, were not necessary to see effects on activity.

The conformational changes induced by high Mg^{2+} concentrations (above 6 mM) suggest two roles for this ion in addition to any structural role (Senior, 1979). The first may contribute directly to photophosphorylation, where the ternary complex Mg^{2+}–CF^1–P_i may be an intermediate. The second could be regulatory, where high Mg^{2+} concentration (6 mM and above) alters the conformation of the protein and decreases the rate of photophosphorylation (Younis et al., 1979; Pick & Bassilian, 1981). CF_1 and Mg^{2+} would then exist in three states. The first would contain only the Mg^{2+} accompanying the enzyme through the extraction procedure. The second would contain Mg^{2+} bound at low concentrations and likely to be involved in photophosphorylation. Phosphate could bind to CF_1–Mg^{2+} in this state. The third contains Mg^{2+} bound at high concentrations and leads to inhibitory effects.

Figure 10 shows a diagrammatic representation of these states wherein CF_1 (first state), Mg–CF_1 and Mg–CF_1–P_i (each an example of the second state), and CF_1–P_i represent active forms of the protein. At high Mg^{2+} concentration, the complex CF_1–Mg (third state) is inactive. Although the concentrations of Mg^{2+} necessary for transition between the second and third states seem clear for the isolated enzyme (above 5 mM in the absence of P_i), the effective concentrations could be different when CF_1 is situated in the membrane.

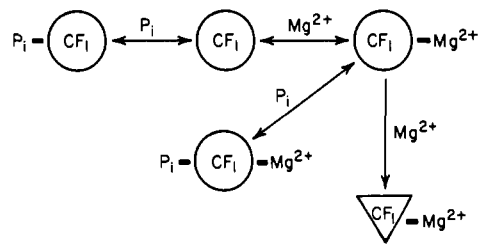

FIGURE 10: Hypothetical scheme for chloroplast coupling factor showing the role of Mg^{2+} ion and inorganic phosphate (P_i). Coupling factor (CF_1) shown prior to Mg^{2+} or P_i binding is in state 1 (see text) and represents the enzyme after isolation. The protein exhibits an active conformation (circles) after isolation and at low concentrations of Mg^{2+} and P_i (state 2) but an inactive conformation (triangle) at high concentrations of Mg^{2+} (state 3). As shown, P_i and Mg^{2+} bind to CF_1 when added singly and when added sequentially if Mg^{2+} is supplied before P_i. If the reverse sequence of addition occurs, Mg^{2+} binding is not observed.

Relationship of Mg^{2+} to Photophosphorylation in Vivo. Spectroscopic methods of detecting ligand binding and protein conformation have the advantage that the protein can remain in its native state without heat activation or other types of protein modification. Therefore, CF_1 should have remained close to its state in the biological membrane, although the membrane could have altered the effects (cf. Figures 8 and 9).

We have recently shown that cellular water loss sufficient to cause a 2–3-fold increase in cellular ion concentrations resulted in losses of photophosphorylation that could be detected during chloroplast assay in vitro (Younis et al., 1979). Therefore, this water loss experiment and the Mg^{2+} preincubation experiments reported here were similar except that preincubation was carried out in vivo in the former and in vitro in the latter case. The decrease in photophosphorylation caused by the water loss and the Mg preincubation experiments was 50–55% (Younis et al., 1979; Figure 8 of this paper). The decrease persisted after dilution of the ions in both cases. The decrease in activity was associated with the altered conformation of CF_1 in both cases [circular dichroism spectra, cf. Figure 7 and Younis et al. (1979)]. The conformational change involved similar alterations in subunit orientation and randomly coiled regions of the protein, but in neither case was the quantity of α helix altered (Younis et al., 1979). Therefore, many of the activity and conformational changes caused in vitro by Mg^{2+} concentrations above 5 mM were similar to those caused in vivo by cellular dehydration that would have raised cellular ion concentrations to this range.

It is unlikely that the in vitro pretreatments would completely duplicate the in vivo behavior of photophosphorylation, because there were molecular changes in addition to those involving photophosphorylation in the water loss experiments (Younis et al., 1979). However, the similarities of the in vitro and in vivo data suggest that (1) Mg^{2+} may interact directly with CF_1 during photophosphorylation in vivo and that (2) the concentrations of ions may be important regulators of photophosphorylation in cells.

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Registry No. ATPase, 9000-83-3; Mg, 7439-95-4; P_i, 14265-44-2.

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Separate Sites of Low and High Affinity for Agonists on *Torpedo californica* Acetylcholine Receptor†

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ABSTRACT: We have studied alkylation of the membrane-bound acetylcholine receptor (AcChR) from *Torpedo californica* electric organ by the cholinergic agonist bromoacetylcholine (BrAcCh). Following reduction of the AcChR with dithiothreitol (DTT) under strictly controlled conditions, a single class of binding sites was covalently labeled by BrAcCh. The extent of alkylation was dependent on the concentration of both DTT and BrAcCh and reached a maximum when a number of sites equivalent to the number of α-bungarotoxin (α-BTx) binding sites were labeled. The reaction with BrAcCh was completely inhibited by saturating concentrations of α-BTx. On the contrary, complete alkylation of the AcChR with [^{3}H]BrAcCh consistently inhibited only ~50% of α-BTx binding. The effects of DTT reduction and subsequent BrAcCh alkylation on the cation-gating properties of the AcChR were investigated in rapid kinetic experiments. DTT reduction resulted in a slight decrease in the maximum cation flux and a small shift in the effective dissociation constant to higher acetylcholine (AcCh) concentration. The flux response was completely inhibited by maximal alkylation of the membrane vesicles by BrAcCh. A low-affinity binding site for AcCh, which is likely to be important in AcChR activation, has been revealed for *T. californica* AcChR by studying the effects of cholinergic ligands on the fluorescence of a probe, 4-[(iodoacetoxy)ethylmethylamino]-7-nitro-2,1,3-benzoxadiazole (IANBD), covalently bound to the AcChR protein. Maximal labeling by BrAcCh did not affect the binding of AcCh to the low-affinity binding site, as monitored by changes in the fluorescence of this probe. This low-affinity binding site must therefore be distinct from the site labeled by BrAcCh. The results strongly support the notion that the nicotinic AcChR contains multiple binding sites for cholinergic ligands.

The nicotinic acetylcholine receptor (AcChR)[1] is the first neurotransmitter receptor to be identified as a molecular entity and purified to homogeneity after solubilization both in nondenaturing detergents and in the native membrane-bound form [reviewed in Conti-Tronconi & Raftery (1982)]. The function of the AcChR in the postsynaptic membrane is to mediate, in response to agonist binding, a rapid ion flux through a cation-selective channel contained within the AcChR molecule [reviewed in Conti-Tronconi & Raftery (1982)]. Channel activation occurs rapidly following neurotransmitter release, and the channel remains open for a few milliseconds. After exposure to agonist for longer periods, desensitization leads to channel closing and a loss of the permeability response (Katz & Thesleff, 1957). Quantitative in vitro studies of the ligand-binding and cation-gating properties of the membrane-bound *Torpedo* AcChR can be made since isolation of postsynaptic membrane fragments containing the AcChR as the only protein component is possible (Neubig et al., 1979; Elliott et al., 1979). Upon exposure to cholinergic agonists, the membrane-bound *Torpedo* AcChR undergoes a slow conformational change (time scale of seconds) to a state of higher affinity for these ligands (Weber et al., 1975; Weiland et al., 1976, 1977; Lee et al., 1977; Quast et al., 1978). This increase in affinity for agonists and the relatively slow time scale of the transition ($t_{1/2}$ ~80 s at 1 μM carbamylcholine) allowed its correlation with the phenomenon of desensitization observed in vivo.

Both muscle (Conti-Tronconi et al., 1982b) and electric organ (Raftery et al., 1980; Conti-Tronconi et al., 1982a) AcChRs are pseudosymmetric complexes of four structurally related subunits of molecular weights 40000, 50000, 60000, and 65000 in *Torpedo californica*, present in the AcChR molecule in the ratio 2:1:1:1. Affinity labeling techniques have shown that the subunit of lowest M_r (commonly referred to as "α") carries a high-affinity binding site(s) for cholinergic agonists and/or antagonists, since, following reduction of a reactive disulfide bond near this (these) site(s), it can be alkylated by the agonist bromoacetylcholine (Chang et al., 1977;

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[1] Abbreviations: AcChR, acetylcholine receptor; DTT, dithiothreitol; α-BTx, α-bungarotoxin; [^{125}I]-α-BTx, ^{125}I-labeled α-bungarotoxin; IANBD, 4-[(iodoacetoxy)ethylmethylamino]-7-nitro-2,1,3-benzoxadiazole; MBTA, (4-maleimidobenzyl)trimethylammonium chloride; BrAcCh, bromoacetylcholine; AcCh, acetylcholine; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; Tetram, *O,O'*-diethyl *S*-2-(diethylamino)ethylphosphorothioate; EDTA, ethylenediaminetetraacetic acid; ANTS, 8-amino-1,3,6-naphthalenetrisulfonate; HTX, histrionicotoxin; NBD, 7-nitro-2,1,3-benzoxadiazole; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

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