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Isolation and Properties of a Protein from Chloroplasts Required for Phosphorylation and H^+ Uptake*

W. S. Lynn and K. D. Straub

ABSTRACT: A soluble protein, which is obligatorily required for both H^+ uptake and photophosphorylation in chloroplasts but which contains no trypsin-activated adenosine triphosphatase activity is readily extracted from chloroplasts by ethylenediaminetetraacetic acid. Purification of this protein, CF_e, has been achieved and it appears that a single protein is required both for H^+ uptake and phosphorylation. Addition of the purified factor to ethylenediaminetetraacetic acid treated chloroplasts under hypotonic conditions results in 100% restoration of phosphorylation, complete restoration of the ability of the ethylenediaminetetraacetic acid chloroplasts to accumulate H^+ in light as well as restores the ability of trypsin to activate adenosine triphosphatase in the reconstituted ethylenediaminetetraacetic acid chloroplasts. The factor has been identified as CF_1, but which probably contains bound Mg^{2+} or other divalent cations.

Extraction of spinach chloroplasts with EDTA removes a protein which is required both for H^+ uptake and for photophosphorylation. Addition of a single protein, isolated from the soluble EDTA extract of the chloroplasts, completely restores both phosphorylation and the ability of the chloroplasts to accumulate H^+ during electron flow. Purification of this protein, CF_e, has been achieved. Previous studies (McCarty and Racker, 1965) have indicated that the factor responsible for restoring H^+ uptake to EDTA-treated chloroplasts is CF_1. However, an antibody prepared against CF_1 and Dio 9, agents which inhibit phosphorylation, does not inhibit H^+ uptake (McCarty and Racker, 1965). Treatment of CF_1 with trypsin is known to activate a Ca^{2+}-requiring ATPase (Vambutas and Racker, 1965). Treatment of the purified protein herein reported with trypsin does not activate any ATPase activity. However, CF_e can be converted by removal of cations into a protein which appears to be identical with CF_1, as obtained from Dr. E. Racker. A description of some of the chemical, enzymatic, and functional properties of CF_e is the subject of this report.

Methods

Preparation of Chloroplasts. Intact spinach chloroplasts were prepared as before (Lynn, 1968). EDTA-treated chloroplasts were prepared from 150 g of commercial spinach using the method of McCarty and Racker (1968) except that the pH was 7.2 and the chloroplasts were extracted for only 5 min at room temperature. The treated chloroplasts, after centrifugation, 12,000*g* for 10 min, were resuspended in 0.15 M sucrose.

Isolation of Coupling Factor. All of the following steps were performed at 0–5°.

* From the Department of Medicine and Biochemistry, Duke University Medical Center, Durham, North Carolina 27706. *Received August 13, 1969.* This research was supported by Grant GM14022-03 from the National Institutes of Health.

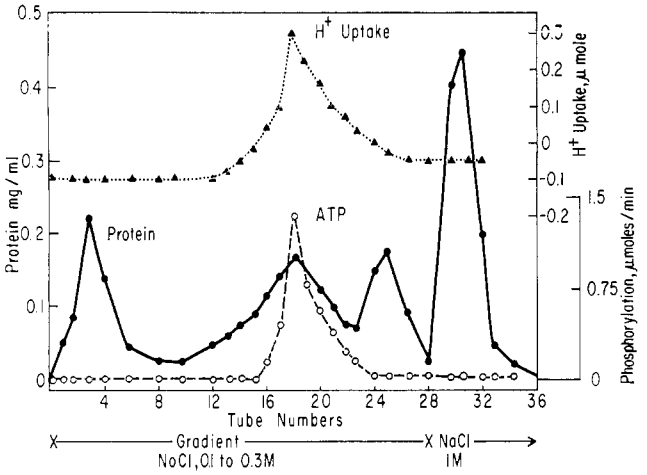

FIGURE 1: Fractionation of CF_c on DEAE-cellulose. pH 5 enzyme (see Methods and Table I) was eluted from DEAE-cellulose by a 0.1–0.3 M NaCl linear gradient. Aliquots (3 ml) were obtained. Proteins and enzyme activity (H^+ uptake and photophosphorylation) were assayed, using 0.4 ml of each fraction and EDTA chloroplasts (see Methods). A similar elution pattern was obtained using a 0.05–0.2 M Na_2SO_4 gradient.

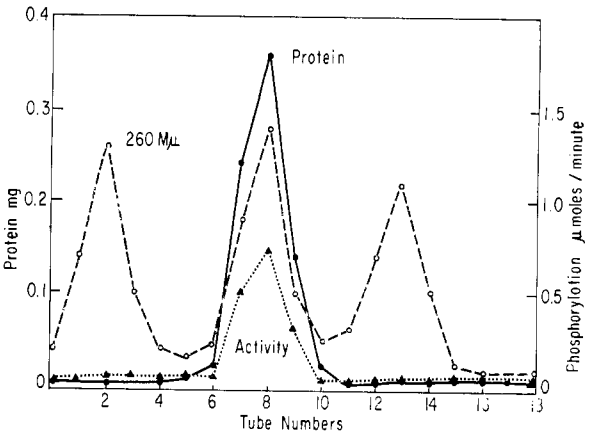

FIGURE 2: Fractionation of CF_c by electrophoresis. Fractions, 16–22 (Figure 3) were combined and concentrated by dialysis under pressure to 3 ml. Protein (1.0 mg) of this material was electrophoresed on Sephadex G-25 for 10 hr (see Methods) and then eluted from the column into 2-ml aliquots. Absorbance at 260 and 280 mμ and activity were measured on 0.4 ml of each fraction (see Methods). The 280:260 mμ ratio of fraction 3 was 0.21. The ratio for fraction 8 was 1.58.

STEP 1. Either $MgCl_2$ (0.75 mM), $CaCl_2$ (0.75 mM), or $CdCl_2$ (3.0 mM) and mercaptoethanol (0.01 M) was added to the above EDTA supernatant. The pH was adjusted to 5.8 with dilute HCl and the resultant precipitate was removed by centrifugation at 20,000*g* for 10 min.

STEP 2. The pH of the above slightly green supernatants was adjusted to pH 5.0 with dilute HCl. The resulting precipitate, which contained 80% of the protein, 90% of the lipid, and all the chlorophyll, was removed by centrifugation at 15,000*g* for 5 min. This precipitate was dissolved in 6 ml of sodium phosphate (0.5 mM, pH 7.2) and the final pH was carefully adjusted to 7.4.

STEP 3. The soluble pH 5 proteins were then applied to a column (1 × 6 cm) of coarse DEAE-cellulose, which had previously been washed with 1 M NaCl followed by 20 bed volumes of water. The protein was then eluted either with NaCl, using a 0.1–0.3 M gradient, or with Na_2SO_4, using a 0.05–0.20 M gradient. This gradient removed all the active protein; however, 6 M urea was required to remove all the protein. Much of the lipid and the chlorophyll remained on the column, but could be eluted with 100% ethanol. The original pH 5 enzyme contained about 30% lipid by weight. After elution from DEAE, the protein is no longer precipitable at pH 5.

STEP 4. The active preparation of the DEAE-cellulose column (Figure 1) was electrophoresed for 10 hr at 300 V on a column of Sephadex G-25 (2 × 50 cm) which had been equilibrated with 0.6 mM sodium phosphate (pH 7.2). After electrophoresis, the protein was fractionally eluted from the Sephadex column. After elution, the enzyme is stable and can be stored at −15°. As indicated in Figure 2, electrophoresis removes material which absorbs light at 260 mμ, but only one fraction of protein is observed.

Freezing and thawing or prolonged storage at 0 or 25° inactivates the biological activity of the pH 5 protein. Storage of the pH 5 protein, in the presence of dithiothreitol or cysteine, does not protect against loss of activity. Elution of the enzyme from DEAE-cellulose with NaCl also results in a preparation which rapidly loses biological activity and precipitates after 2 hr at 25°. Elution of the enzyme with Na_2SO_4 and mercaptoethanol yields a much more stable preparation. Approximately 2 mg of the active protein is obtained per 150 g of spinach.

The most stable preparations have been obtained, using $CdCl_2$ in step 1. Estimates of the minimum molecular weights of CF_c, CF_1, as prepared with Na_2SO_4 as above, and CF_1, supplied by Dr. E. Racker (McCarty and Racker, 1965), using the gel exclusion method of Davison (1968) revealed that the minimal molecular weight of all three proteins is approximately 57,000. Dextran Blue 2000, serum albumin, yeast hexokinase, trypsin, and cytochrome *c* were used as standards. Estimates of the molecular weight of the native enzyme, using Sepharose 4B and the method of Marrink and Gruber (1969), were also obtained. Dextran 2000, catalase (Boehringer), urease, serum albumin, and hexokinase were used as standards. The calculated molecular weights of all three preparations of native CF_1 were approximately 250,000.

Assays. Rate and extent of H^+ uptake, using chloroplasts containing 0.4 mg of chlorophyll, was assayed titrimetrically in 12 ml of 0.12 M NaCl, 0.2 mM P_i and $MgCl_2$, and 0.005 mM PMS^{+1} as before (Lynn, 1968). Rates of phosphorylation and electron flow were also measured titrimetrically, as before (Lynn, 1968). Protein was assayed turbidimetrically, using trichloroacetic acid, and spectrally at 280 mμ. Lipid was measured gravimetrically, after elution of the lipid from the DEAE-cellulose column with 100% ethanol. ATPase activity of CF_1 was assayed titrimetrically in 10 ml of 1.0 mM Tris-sulfate (pH 8.0) containing 5 mM $CaCl_2$ and 1.0 mM ATP at 25°. The reaction was started by addition of 10 μg of L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin (Worthington). Soybean trypsin inhibitor (30 μg) was added at 1 min. P_i was measured colorimetrically, as before (Lynn, 1968), on aliquots of the reaction mixtures.

[1] PMS^+ = phenazine methosulfate.

TABLE I: Fractionation of CF_p.[a]

Purification	Protein (mg)	Lipid (mg)	Act., Program Required for Maximal Act. (mg)	
			H^+ Uptake	ATP Formation
EDTA extract	13.8	4.1	1.65	1.90
pH 5 precipitation	11.9	3.6	0.98	1.01
DEAE	3.2	0	0.10	0.12
Electrophoresis	2.6	0	0.085	0.095

[a] Assays of H^+ uptake and photophosphorylation were assayed, using EDTA chloroplasts (0.47 mg of chlorophyll) and varying amounts of fractionated protein. The data are expressed, as the amount of protein required to restore 90–100% of the original activity (H^+ uptake and photophosphorylation) to the EDTA chloroplasts.

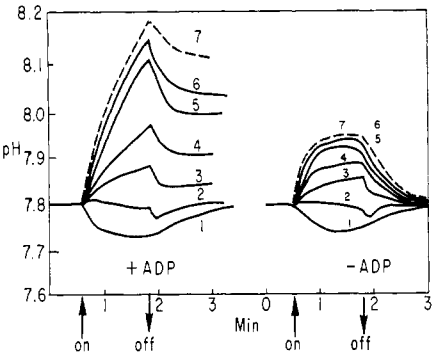

FIGURE 3: Effect of concentration of CF_c. EDTA chloroplasts (0.45 mg of chlorophyll) were reconstituted with varying amounts of purified CF_c under hypotonic conditions (see Methods). Assay of H^+ uptake (absence of ADP) and photophosphorylation (presence of ADP, 0.25 mM) were recorded titrimetrically in the presence and absence of CF_c (see Methods) in 13 ml of buffer (see Methods), using saturating white light. Trace 1 = no CF_c, trace 2 = 5 μg of CF_c, trace 3 = 15 μg of CF_c, trace 4 = 30 μg of CF_c, trace 5 = 60 μg of CF_c, trace 6 = 100 μg of CF_c, and trace 7 = control chloroplasts (not treated with EDTA). P_i was measured at the end of each experiment and found to correlate well with the pH trace.

In all the experiments using EDTA-treated chloroplasts, to obtain maximal reactivation it was necessary to add CF_c or CF_1 to the chloroplasts suspended in 10 mM NaCl (pH 6.0) and equilibrate for 2 min. After the equilibration, NaCl, $MgCl_2$, P_i, ADP, and PMS^+ were then added as above and the pH was adjusted to 7.8 with dilute NaOH. Addition of CF_c to EDTA chloroplasts suspended in the complete isotonic buffer restored only about 10% of the phosphorylation activity.

Results and Discussion

As indicated in Figure 3, essentially complete reactivation of both phosphorylation and H^+ uptake could be obtained in EDTA-treated chloroplasts by addition of the purified CF_c or CF_1, prepared with Na_2SO_4, under hypotonic conditions. In the absence of CF_c, H^+ ions are produced during electron transport (Figure 3) when PMS^+, pyocyanine, or methylene blue is the electron acceptor. The rate of H^+ production is slow and the maximal extent of H^+ production is only one-third of the H^+ uptake observed after addition of CF_c. The amounts of these cationic electron acceptors required are stoichiometric with the amounts of H^+ production. With neutral electron acceptors, such as menadione, FMN, methyl red, or benzyl viologen, H^+ uptake is observed. The maximal extent of H^+ uptake is 15% of that observed after addition of CF_c. Only catalytic amounts of the neutral electron acceptors are required for H^+ uptake. High redox potential acceptors, such as benzoquinone or ferricyanide, in excess amounts, completely inhibit both H^+ uptake and H^+ production with the above electron acceptors. H^+ production is stimulated (20%) by uncoupling agents, *i.e.*, NH_4^+, salicylanilides, or trifluorotetrachlorobenzimidazole; however, H^+ uptake is completely abolished. Thus, it is concluded that the observed H^+ production is a result of the fact that the cationic electron acceptors accept one H^+ per two electrons when reduced (Lynn, 1967). The small residual H^+ uptake observed in these EDTA chloroplasts with neutral electron acceptors is probably the result of the fact that the EDTA treatment does not remove all of the CF_1 (McCarty and Racker, 1967). Rate of electron flow, per milligram of chlorophyll, as measured with ferricyanide, was unaffected by the EDTA treatment, nor was any effect of addition of CF_c or CF_1 to the EDTA chloroplasts on rate of electron flow observed.

Purification of CF_c or CF_1 has been achieved (Table I). Spectral analysis of the purified protein reveals the usual spectrum of a typical protein with a 280 mμ:260 mμ ratio of 1.58. The factor contains no extractable lipid. The coupling activity of CF_c is not inactivated by treatment with RNase or by a 30-sec treatment with trypsin. Prolonged treatment with trypsin inactivates CF_c and CF_1 (Vambutas and Racker, 1965). As indicated below (Figure 4) the ATPase activity of CF_1 is fully activated by trypsin in less than 30 sec. CF_c contains no assayable ATPase activity, with or without trypsin treatment, when isolated in the presence of Mg^{2+} or with and without dithiothreitol and calcium (McCarty and Racker, 1968). However, isolation of the enzyme in the presence of Ca^{2+} or Cd^{2+} and elution of the enzyme with Na_2SO_4 (step 3) yields a preparation which contains a very active trypsin-activated ATPase (Figure 4). The factor is inactivated by alkali (10 min at pH 10.5) or by heat (80° for 5 min). CF_c is bound by the EDTA chloroplasts and is not removed by repeated washing of the chloroplasts with 0.4 M sucrose.

As indicated in Figure 3, the extent of restoration of H^+ uptake always parallels the extent of restoration of phosphorylation. Phosphorylation has never been observed under conditions in which no H^+ uptake occurs. However, either prolonged treatment of chloroplasts with EDTA (20 min) or aging (24 hr at 0°) of the EDTA chloroplasts results in preparations in which CF_c will partialy restore H^+ uptake but will not restore phosphorylation. As noted previously (Lynn, 1968; Lynn and Straub, 1969), the above observations seems to indicate that phosphorylation is an event secondary to that of H^+ uptake.

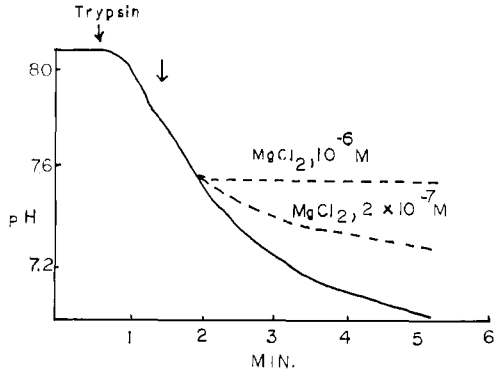

FIGURE 4: Activation of ATPase of CF_1 by trypsin. CF_1 (Na_2SO_4 enzyme; 40 μg) was incubated at 25° in 10 ml of 1 mM Tris-sulfate, 2 mM $CaCl_2$, and 1 mM ATP. L-(1-Tosylamido-2-phenyl) ethyl chloromethyl ketone trypsin (10 μg) was added where indicated. Soybean trypsin inhibitor (30 μg) was added at 1.5 min and $MgCl_2$ at the concentrations indicated was added at 2m in to duplicate experiments. The activity of the enzyme was 39 μmoles of ATP hydrolyzed/mg of protein per min. ATPase was assayed titrimetrically (see Methods.

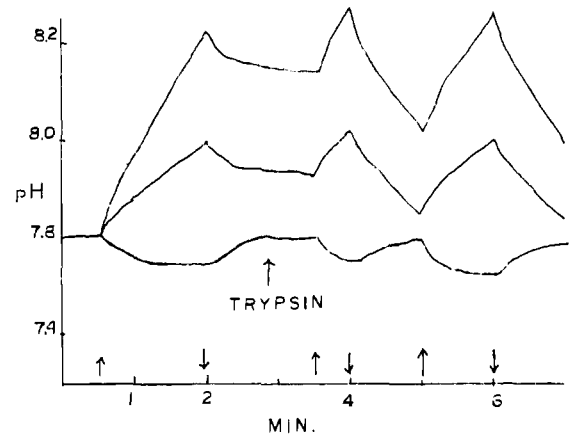

FIGURE 6: Activation of ATPase by trypsin in reconstituted EDTA chloroplasts. Conditions as in Figure 3. Lower trace, EDTA chloroplasts; middle trace, 30 μg of CF_c plus EDTA chloroplasts; top trace, 60 μg of CF_c plus EDTA chloroplasts. Trypsin (25 μg) was added where indicated. ATP synthesis and ATPase were assayed titrimetrically (see Methods).

The activity of CF_c coincides precisely with the protein peak following electrophoresis (step 4, Table I), and only one protein is observed chromatographically, using Sepharose 4B or Agarose (Figure 2). Also, microelectrophoresis on polyacrylamide gels of the active fractions of CF_c or CF_1 from step 3 and step 4 at pH 7.4 reveals one single band of protein. It is concluded that a single protein is capable of fully restoring both phosphorylation and H^+ uptake in EDTA-treated chloroplasts.

The trypsin-activated ATPase activity of CF_1, as isolated using $CaCl_2$ or $CdCl_2$ and Na_2SO_4 (see Methods), is strikingly inhibited by low concentrations of divalent cations, such as Mg^{2+}, Cd^{2+}, Zn^{2+}, or Hg^{2+} (Figures 4 and 5). In the presence of 5 mM $CaCl_2$ and 1 mM ATP, 50% inhibition of ATPase activity occurs on addition of 1.5×10^{-7} M $MgCl_2$. This inhibition is not reversed by 2 mM EDTA or 10 mM mercaptoethanol. Vambutas and Racker (1965) previously noted that Mg^{2+} inhibited the ATPase activity of CF_1; however, much larger concentrations of Mg^{2+} than those reported above were required. Cd^{2+}, Zn^{2+}, and Hg^{2+} (10^{-5} M) also completely inhibit the enzyme, however, complete reversal of the inhibition by the above cations is observed on addition of either EDTA or mercaptoethanol (Figures 4 and 5). The trypsin-activated ATPase is not inhibited by iodoacetamide, *N*-ethylmaleimide, *p*-mercurisulfonic acid (5 mM) or tributyl tin. The enzyme is inhibited by 50% by ADP (0.35 mM) under the conditions of Figure 4.

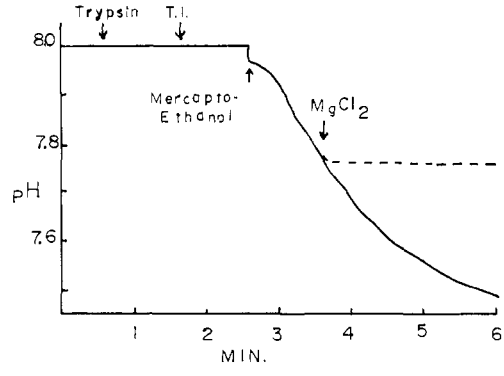

FIGURE 5: Effect of Cd^{2+}, mercaptoethanol, EDTA, and Mg^{2+} on trypsin-activated ATPase of CF_1. Conditions as in Figure 4 except that either $CdCl_2$ on $ZnCl_2$ (10^{-4} M) was added at time 0. The trace shown is in the presence of $CdCl_2$. A similar trace for $ZnCl_2$ was also observed. Trypsin and trypsin inhibitor were added where indicated. Either 5 mM mercaptoethanol or 1.5 mM EDTA was added where indicated. The trace for merceptoethanol is shown, but a similar trace was observed on addition of EDTA. $MgCl_2$ (10^{-5} M) was added where indicated to a duplicate experiment. Complete inhibition of the ATPase occurred whether the enzyme was activated with mercaptoethanol, EDTA, or both.

CF_c when isolated in the presence of Mg^{2+} can be converted into a form of the enzyme which contains trypsin-activated ATPase by precipitation of the enzyme at pH 5 in the presence of 5 mM $CaCl_2$ or $CdCl_2$, followed by elution of the enzyme from DEAE-cellulose using Na_2SO_4 and mercaptoethanol (see Methods). Since CF_c and CF_1 also have similar molecular weights, elute from DEAE-cellulose under the same conditions, electrophorese similarly, and restore phosphorylation, H^+ uptake activity, and ATPase activity to EDTA chloroplasts, it is concluded that CF_c is the same protein as CF_1. The activity of CF_c, as an ATPase, depends upon the kind of divalent cations present, but the coupling activity of the protein does not. The amount of Mg^{2+} or other cations bound by CF_c has not been assayed; however, since Mg^{2+} at 2×10^{-7} M inhibits the enzyme at a concentration of 1×10^{-7} M, assuming that the minimal molecular weight of the enzyme is 60,000 (Figure 4), it appears that only one Mg^{2+} per monomeric unit of the enzyme is bound.

Trypsin is unable to activate a Mg^{2+}-requiring ATPase in EDTA chloroplasts. However, addition of CF_c or CF_1 to EDTA chloroplasts restores the ability of trypsin to activate a Mg^{2+}-requiring ATPase in these chloroplasts (Figure 6). The rate of the reconstituted ATPase is dependent upon the amount of CF_1 added (Figure 6). As previously indicated (Lynn and Straub, 1969), the trypsin-activated ATPase of

intact or reconstituted chloroplasts shows an obligatory requirement for Mg^{2+}. Thus, although the isolated ATPase activity of CF_1 is completely inhibited by Mg^{2+}, chloroplasts from which CF_1 has been removed contain minimal ATPase and on reconstitution of the depleted chloroplasts by CF_1, the trypsin-activated ATPase now specifically requires Mg^{2+}.

It is concluded that CF_1 is a protein which for some reason is required by chloroplasts for the energetic accumulation and retention of H^+. CF_1 may function as a H^+ carrier, as proposed by Dilley and Shavit (1968), for CF_1 is anionic at pH 7.0. Or, CF_1 may function analogous to that of imidazole (Lynn, 1968) as an internal nonpermeable buffer which during electron flow causes the retention of H^+. Since CF_1 is also obligatorily required for phosphorylation, and since previous observations (Jagendorf and Uribe, 1967; Lynn, 1968; Lynn and Straub, 1969) suggest that phosphorylation is a consequence of the accumulation of H^+, it appears that CF_1 activates phosphorylation by promoting the accumulation of H^+ by the chloroplasts.

The above observations suggest, as previously proposed (Lynn and Straub, 1969), that electron transport through the alternating one- and two-electron carriers of chloroplasts may function in energy-transfer reactions by increasing the kinetic activity of the slower moving H^+ within the membranes. In the absence of one of the membrane components, *i.e.*, CF_1, the activated H^+ can readily equilibrate with the outside. In the presence of CF_1, which can bind H^+ as indicated by the fact that a high concentration of H^+ causes precipitation of magnesium or calcium CF_1, an increase of internal H^+ activity by electron transport results in the binding of H^+ to CF_1. An increase in H^+ activity on one side of the membrane may also occur as a result of charge separation and the resultant transmembrane potential, as observed by Witt (1967). In either case, the function of CF_1 appears to be to protect the H^+ side of the membrane from the exterior and thus allow the maintenance of a high activity of H^+, as measured by H^+ uptake and neutral red (Lynn, 1968).

CF_1 may also be the bound ADP kinase of chloroplasts, which as previously reported (Lynn and Straub, 1969), requires both protonation and reduction for it to function as a Mg^{2+} ATPase. However, the Ca^{2+}-requiring ATPase of isolated CF_1 does not require reduction for activity, nor is its activity affected by strong oxidants (unpublished observations) or by thiol inhibitors. Nor can the isolated Mg^{2+} form of CF_1 be converted into an ATPase by strong reductants. However, in the presence of oxidized Cd^2 , Zn^{2+}, or Hg^{2+}, CF_1 is inhibited and the inhibition is readily reversed by reductants. Chloroplasts are known to contain a Mg^{2+}-requiring ATPase (Vambutas and Racker, 1965). Preliminary studies from this laboratory indicate that a particulate Mg^{2+}–ATPase, obtained by treatment of chloroplasts with EDTA and mercaptoethanol, is inhibited by CF_1. This isolated Mg^{2+}–ATPase requires reductants for activity. The Mg^{2+}–ATPase which is observed in chloroplasts after treatment with light and trypsin (Lynn and Straub, 1969) or with light and dithiothreitol (McCarty and Racker, 1967, 1968), in contrast to CF_1 is inhibited by tributyl tin or dicyclohexylcarbodiimide. Antiserum specific for CF_1 inhibits both CF_1 and the light-activated bound Mg^{2+}–ATPase. It remains quite uncertain therefore whether CF_1 or the bound Mg^{2+}–ATPase is the catalytic site of ADP kinase. It is possible that CF_1 is required in some way for the reduction and protonation of the Mg^{2+}–ATPase and that the Mg^{2+}–ATPase is the observed ADP kinase. Further studies on Mg^{2+}–ATPase are in progress.

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