Light-Stimulated Incorporation of *N*-Ethylmaleimide into Coupling Factor 1 in Spinach Chloroplasts†

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ABSTRACT: The inhibition of photosynthetic phosphorylation in spinach chloroplasts by *N*-ethylmaleimide (MalNEt) was previously found to be light dependent. Under conditions where MalNEt inhibited photophosphorylation, light markedly enhanced the incorporation of [^{3}H]MalNEt into coupling factor one (CF_1) in chloroplasts previously incubated with unlabeled MalNEt in the dark. Only one of the five subunits of CF_1, as detected by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, contained significant amounts of radioactive MalNEt. Uncouplers, such as ammonium chloride and carbonyl cyanide *m*-chlorophenylhydrazone, abolished the light-dependent inhibition of phosphorylation by MalNEt and largely prevented the light-dependent incorporation of MalNEt into the CF_1 subunit. Adenosine diphosphate in the presence of inorganic phosphate was also effective. The effect of MalNEt concentration on incorporation into CF_1 and inhibition of phosphorylation was similar. Both inhibition and incorporation were complete in 2 min of illumination. Although several groups in chloroplast-bound CF_1 react with MalNEt in the dark, these groups are apparently not required for phosphorylation since phosphorylation is not inhibited by MalNEt under these conditions. These results support the possibility that CF_1 undergoes a conformational change on illumination of chloroplasts which exposes a group or groups in one subunit of the enzyme to reaction with MalNEt.

Photophosphorylation in spinach chloroplasts is inhibited by *N*-ethylmaleimide (MalNEt)[1] only under certain conditions. A partial inhibition was observed when the chloroplasts were incubated with MalNEt in the light prior to the assay of phosphorylation, but no inhibition was detected when the chloroplasts were previously treated with MalNEt in the dark (McCarty *et al.*, 1972). Because MalNEt had no direct effect on electron flow or on light-dependent H^+ uptake (Neumann and Jagendorf, 1964), it was suggested that MalNEt inhibits phosphorylation at a terminal site. Furthermore, the Ca^{2+}-dependent ATPase activity of coupling factor one (CF_1) (Vambutas and Racker, 1965) was partially inhibited by incubation of chloroplasts with MalNEt in the light, but not in the dark.

In this paper, we report that MalNEt incorporation into chloroplast-bound CF_1 is markedly stimulated by light. Only one of the five subunits of bound CF_1 reacts with MalNEt in the light and this reaction appears to be responsible for the light-dependent inhibition of phosphorylation by MalNEt. Our results provide further support for the possibility that CF_1 undergoes a conformational change in the light, as suggested by Ryrie and Jagendorf (1971).

Materials and Methods

Materials. *N*-Ethyl[2-^{3}H]maleimide (75 Ci/mol) was purchased from New England Nuclear Corp. The pentane was evaporated with a stream of argon and the residue was dissolved in water to give a solution which contained 1 mCi of [^{3}H]MalNEt/ml. The solution was stored at −20°.

A monospecific antiserum to CF_1 was the generous gift of Dr. R. Berzborn. Spinach was purchased at local markets.

Methods. Chloroplasts were prepared as described previously (McCarty and Racker, 1967). Photophosphorylation (McCarty and Racker, 1967), protein (Sutherland *et al.*, 1949), and chlorophyll (Arnon, 1949) were determined by reported procedures. Trypsin-activated Ca^{2+}-ATPase activity of CF_1 was assayed according to Vambutas and Racker (1965).

Treatment with [^{3}H]MalNEt. In most experiments chloroplasts were exposed to unlabeled MalNEt in the dark to react readily accessible groups prior to incubation with [^{3}H]MalNEt. This procedure did not affect the light-dependent inhibition of phosphorylation by MalNEt. Chloroplasts (1 mg of chlorophyll/ml), suspended in a solution which contained 0.4 M sucrose, 0.02 M Tricine–NaOH (pH 8.0), and 0.01 M NaCl, were incubated for 5 min in the dark room temperature with 2 mM MalNEt. Dithiothreitol was then added to a final concentration of 1.1 mM to remove the unreacted MalNEt and the resulting mixture was centrifuged at 10,000*g* for 10 min. The chloroplasts were resuspended in the buffered sucrose solution to give a chlorophyll concentration of 0.3–0.5 mg/ml. The chloroplasts were collected by centrifugation as described above and finally were resuspended in a small volume of the sucrose solution.

The chloroplasts (1–3 mg of chlorophyll) were then incubated at 20° with [^{3}H]MalNEt at a final chlorophyll concentration of 0.5 mg/ml. The stirred reaction mixture contained 50 mM Tricine–NaOH (pH 8.0), 50 mM NaCl, 0.05 mM pyocyanine, and, usually, 1 mM MalNEt containing the amounts of [^{3}H]MalNEt indicated for each experiment. After 90–120 sec in the light (2.5×10^6 ergs/(cm^2 sec)) or dark, a slight excess of dithiothreitol was added and the mixture was diluted to 50 μg of chlorophyll/ml with cold 10 mM NaCl. An incubation time of 90–120 sec was selected because the light-dependent inhibition of phosphorylation reached completion within this time. After centrifugation at 10,000*g*

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[1] Abbreviations used are: CF_1, chloroplast coupling factor 1; MalNEt *N*-ethylmaleimide; Tricine, tris(hydroxymethyl)methylglycine.

TABLE I: Precipitation of [^{3}H]MalNEt in the CF_1 Fraction by an Antiserum to CF_1.[a]

Source of CF_1 Fraction	[^{3}H]MalNEt in Fractions (cpm)				% of [^{3}H]MalNEt Pptd (from Supernatant Data)
	Control Serum		Antiserum		
	Supernatant	Precipitates	Supernatant	Precipitates	
Chloroplasts treated with MalNEt in the light	310	0	78	129	75
Chloroplasts treated with MalNEt in the dark	134	6	128	14	5

[a] Aliquots of dialyzed CF_1 fractions from chloroplasts treated in the light with [^{3}H]MalNEt (41 μg of protein, 320 cpm) and from those incubated in the dark with [^{3}H]MalNEt (40 μg of protein, 140 cpm) were incubated 30 min at room temperature with either 13 mg of control serum or 12 mg of antiserum in a final volume of 1.0 ml. Isotonic NaCl was used as the diluent. The samples were centrifuged at 12,000*g* for 15 min and the supernatants were withdrawn and saved. The pellets were resuspended in 1 ml of 0.9% NaCl and the precipitates were recollected by centrifugation as described above. The pellets were resuspended in 0.5 ml of H_2O and the [^{3}H]MalNEt contained in the pellets and supernatant fluids was determined. Because some loss of the precipitates unavoidably occurred during washing, the measurements of the radioactivity in the supernatant fluids are more reliable.

for 10 min, the chloroplasts were resuspended in the buffered sucrose solution to give a chlorophyll concentration of 2 mg/ml.

Isolation of CF_1. The chloroplasts were added to 20 volumes of acetone at −20° as described by Vambutas and Racker (1965). CF_1 was solubilized from the acetone-precipitated chloroplasts by extraction with a solution (1–1.5 ml/mg of chlorophyll) which contained 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 4 mM ATP. Solid $(NH_4)_2SO_4$ was added to the chilled extracts to 25% of saturation. After 30–60 min at 0–4°, the mixtures were centrifuged at 10,000*g* and the precipitates were discarded. Solid ammonium sulfate was then added to the supernatant fluids to 45% of saturation and the resulting mixtures were stored at least overnight at 4°. Prior to assay, aliquots of the fractions were centrifuged at 10,000*g* for 10 min and the precipitates, which contained the CF_1, were dissolved in a small volume of either the Tris–EDTA–ATP buffer or, for acrylamide gel electrophoresis, in the solution described below.

Acrylamide Gel Electrophoresis. Acrylamide gels (50 × 750 mm) were polymerized with ammonium persulfate in the presence of 0.1 M sodium phosphate buffer (pH 7.2), 10% acrylamide, 0.135% methylenebisacrylamide, 0.15% *N,N,N',N'*-tetramethylenediamine, and 0.1% sodium dodecyl sulfate (Shapiro *et al.*, 1967). The ammonium sulfate precipitates of the partially purified CF_1 fractions or of more highly purified CF_1 (Farron, 1970) were dissolved in 0.1 ml of a solution which contained 1% sodium dodecyl sulfate and 0.2% β-mercaptoethanol. After 1 hr at room temperature, sucrose was added to 10% and Bromothymol Blue to 0.005%. Aliquots of these samples (25–65 μg of protein) were placed on the gels and electrophoresis was carried out for 5–7 hr at room temperature in a buffer consisting of 0.05 M sodium phosphate buffer (pH 7.2) and 0.05% sodium dodecyl sulfate. The gels were either fixed and stained or were frozen and sliced into 2-mm sections. Radioactivity in the gel slices was determined as described by Zaitlin and Hariharasubramanian (1970). A Beckman Model LS-230 scintillation counter was used. For the determination of radioactivity in samples other than gel slices, 9 ml of a scintillation fluid was used which contained 1.5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(4-methyl-5-phenyloxaxolyl)]benzene in 3 l. of toluene–Triton X-100 (2:1, v/v).

Results

[^{3}H]MalNEt Incorporation into Bound CF_1. Significant amounts of radioactivity were found in the partially purified CF_1 fractions from chloroplasts incubated for 2 min with 1 mM [^{3}H]MalNEt, even though the chloroplasts had been previously exposed to unlabeled MalNEt in the dark. In an average of ten experiments, MalNEt incorporation in the light was 1.32 nmol/mg of protein in the fraction whereas in the dark, this value was 0.60. The light stimulation of MalNEt incorporation into the CF_1 fraction was of interest since it occurred under conditions in which MalNEt inhibited phosphorylation.

To test the extent of the incorporation of [^{3}H]MalNEt into CF_1, the amount of radioactivity in the CF_1 fractions which could be precipitated by a monospecific antiserum to CF_1 was determined. About 75% of the radioactivity in the CF_1 fraction from chloroplasts illuminated in the presence of [^{3}H]MalNEt was precipitated by the antibody (Table I). In contrast, after correction for the small amount of radioactivity precipitated by the control serum, only 5% of the radioactivity in the CF_1 fraction from chloroplasts incubated with [^{3}H]-MalNEt in the dark was precipitated. The addition of twice as much antibody did not result in the precipitation of more radioactivity. Furthermore, the reaction of MalNEt with CF_1 did not appear to markedly affect the precipitation of the enzyme by the antibody. Similar amounts of protein were precipitated by the antibody from extracts which contained unmodified CF_1.

Thus, although the stimulation by light of MalNEt incorporation into the CF_1 fraction in chloroplasts previously treated with unlabeled MalNEt was only twofold, very little of the MalNEt incorporated in the dark was present in CF_1. On the basis of the antibody results, MalNEt incorporation into CF_1 in the light was about 20 times that in the dark.

Location of the Incorporated MalNEt in the Subunits of CF_1. CF_1 has been purified to homogeneity as judged by analytical acrylamide gel electrophoresis (Farron, 1970; Racker *et al.*, 1971) and sedimentation in the ultracentrifuge (Farron, 1970). Homogeneous CF_1, dissociated with sodium dodecyl sulfate, showed 5 components after acrylamide gel electrophoresis in the presence of this detergent (Racker *et al.*, 1971). In view of the homogeneity of the CF_1, these com-

TABLE II: Effect of NH_4Cl, Carbonyl Cyanide *m*-Chlorophenylhydrazone (CCP) and ADP and P_i on the Incorporation of [^{3}H]MalNEt into CF_1.[a]

Treatment Conditions	MalNEt Incorp into CF_1 Fraction: pmol/mg of Chlorophyll	MalNEt Incorp into CF_1 Fraction: cpm	MalNEt Incorp into the γ Subunit (pmol/mg of Chlorophyll)
Dark	38	67	4
Light	115	1219	94
Light + 5 mM NH_4Cl	40	123	6
Light + 5 μM CCP	53	249	13
Light + 3 mM ADP + 3 mM P_i	35	86	4

[a] Chloroplasts were incubated in the dark with 2 mM MalNEt for 5 min and were collected by centrifugation as described in Materials and Methods. Aliquots of the chloroplast suspension (1 mg of chlorophyll) were treated with 1 mM [^{3}H]MalNEt containing 25 μCi of [^{3}H]MalNEt/ml for 90 sec and CF_1 fractions were prepared. After electrophoresis and staining, the bands corresponding to CF_1 subunits were cut and the radioactivity in the slices was determined.

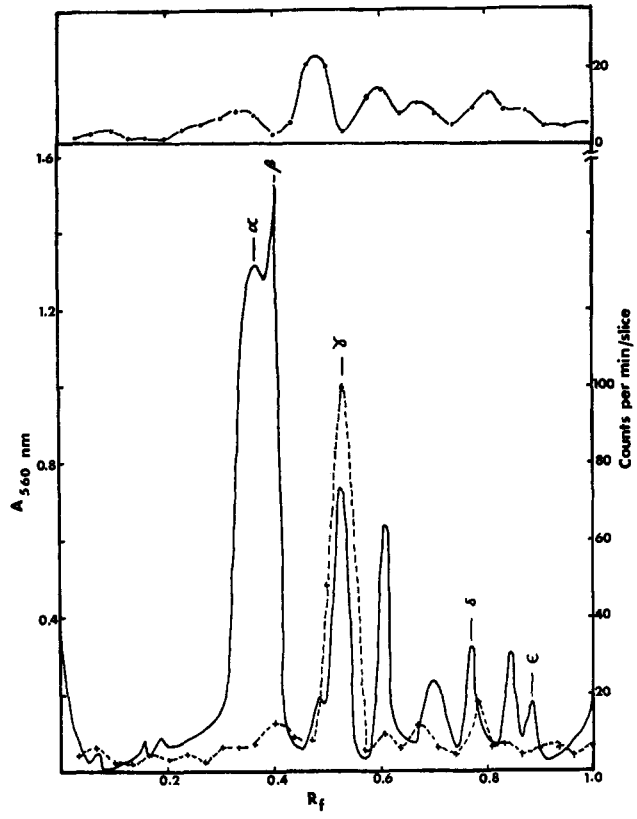

FIGURE 1: Acrylamide gel electrophoresis of [^{3}H]MalNEt-labeled CF_1 fractions. CF_1 extracts were prepared from [^{3}H]MalNEt-treated chloroplasts and aliquots were subjected to electrophoresis on acrylamide gel in the presence of sodium dodecyl sulfate. Gels were either sliced into 2-mm sections or were fixed and stained. The stained gels were scanned with a Gilford Model 240 spectrophotometer equipped with a linear transporter. α, β, γ, δ, and ϵ refer to the subunits of CF_1. (—) Densitometer tracing, (×) distribution of radioactivity in CF_1 fraction from chloroplasts treated with [^{3}H]MalNEt in the light and (●) (the upper curve) distribution of [^{3}H]MalNEt in CF_1 fraction from chloroplasts incubated with MalNEt in the dark. R_F is mobility relative to that of the tracking dye.

ponents, denoted α, β, γ, δ, and ϵ in order of increasing mobility, are probably subunits of the enzyme.

To find which of the subunits of the enzyme reacted with MalNEt, extracts containing [^{3}H]MalNEt–CF_1 were subjected to gel electrophoresis under dissociating conditions. Radioactivity in the gel slices was determined and the mobilities of the radioactive zones was compared to those of the stained proteins in duplicate gels. Most of the radioactivity in the CF_1 fraction from chloroplasts treated with radioactive MalNEt in the light was present in one area of the gel (Figure 1). This zone corresponded well with one of the protein components present in the same extract. From electrophoresis of purified CF_1 in the same run, this component was found to have a mobility identical with that of the γ subunit of CF_1. Varying the gel concentration between 8 and 12% did not result in a separation of this component from the radioactivity.

As expected, little radioactivity was found to be associated with the CF_1 subunits in extracts from chloroplasts incubated with [^{3}H]MalNEt in the dark.

To more clearly establish that MalNET is incorporated into the γ subunit of CF_1, labeled CF_1 in the chloroplast extracts was precipitated by the antibody to the enzyme. The precipitates were dissociated with sodium dodecyl sulfate and the components were separated by electrophoresis. The CF_1 subunit bands were clearly resolved from those of the antiserum. Furthermore, the components present in the partially purified CF_1 extracts which are not attributable to CF_1 were almost undetectable in the gels of the precipitates. Seventy-seven per cent (720 cpm) of the total radioactivity in the CF_1 fraction from chloroplasts illuminated in the presence of [^{3}H]MalNEt was found in subunits of the enzyme. Of the radioactivity in CF_1, only 5% (34 cpm) was present in the α and β subunits together whereas 95% (692 cpm) was in the γ component. Although 17 cpm was found in the α and β subunits, no radioactivity was found in the other subunits of CF_1 from chloroplasts incubated in the dark with [^{3}H]MalNEt.

Thus, it seems clear that light in some manner promotes the reaction between a group (or groups) in the γ subunit of bound CF_1 and MalNEt under conditions where MalNEt inhibited phosphorylation.

Relation between the Incorporation of MalNEt into CF_1 and the Inhibition of Phosphorylation by MalNEt. In view of the fact that MalNEt is incorporated into the γ subunit of CF_1 under conditions where phosphorylation is inhibited by this reagent, it was of interest to examine more closely the relationship between these phenomena. Uncouplers, such as NH_4Cl and carbonyl cyanide *m*-chlorophenylhydrazone, prevent the inhibition which occurred when chloroplasts were illuminated in the presence of MalNEt. ADP and P_i were also effective (McCarty *et al.*, 1972). If the light-stimulated reaction of MalNEt with CF_1 is related to the inhibition of phosphorylation by this reagent, uncouplers as well as ADP and P_i should also prevent the incorporation. As may be seen in Table II, this was the case. The effects of MalNEt concentration on incorporation into the γ subunit and on inhibition of phosphorylation were similar (Figure 2). Half-maximal incorporation and inhibition was observed at 0.3 mM MalNET and saturation was approached at 1 mM. Further-

TABLE III: Incorporation of [^{3}H]MalNEt into Chloroplasts not Pretreated with [^{2}H]MalNEt in the Dark.[a]

CF_1 Fraction Source	Total [^{3}H]MalNEt Incorp (nmol/mg of Protein)	[^{3}H]MalNEt Incorp into CF_1 Subunits (nmol/mg of Protein in CF_1 Fraction)			
		$\alpha + \beta$	γ	δ	ϵ
Chloroplasts incubated with [^{3}H]MalNEt in the light	11.8	0.5 ± 0.05	3.8 ± 0.25	0.06 ± 0.02	1.8 ± 0.10
Chloroplasts incubated with [^{3}H]MalNEt in the dark	7.2	0.4 ± 0.05	2.5 ± 0.23	0.03 ± 0.02	1.6 ± 0.16

[a] Chloroplasts (1 mg of chlorophyll) were treated in the light or dark for 2 min with 1 mM MalNEt containing 0.05 mCi of [^{3}H]MalNEt in 2 ml of the standard incubation mixtures. The isolated CF_1 fractions equivalent to about 60 μg of protein were dissociated with sodium dodecyl sulfate and electrophoresed as usual. After staining, the bands corresponding to the CF_1 subunits were cut out and radioactivity was determined. The standard deviations are calculated from quadruplicate determinations.

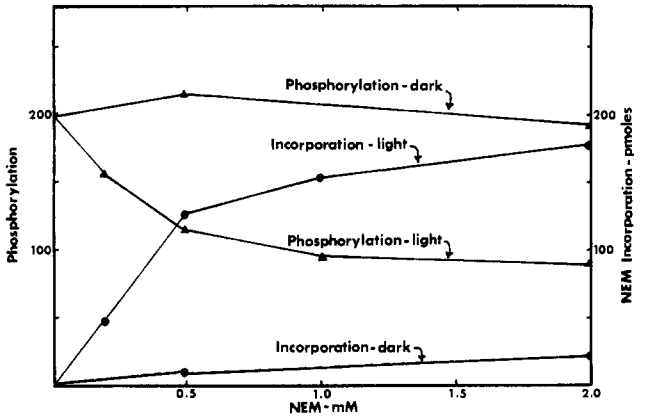

FIGURE 2: MalNEt concentration dependence of inhibition of phosphorylation and incorporation into the γ subunit of CF_1. Chloroplasts were incubated with MalNEt as described in the legend to Table IV, except that the MalNEt concentration was varied. Pyocyanine-dependent phosphorylation was assayed and CF_1 was prepared from the remaining chloroplasts. CF_1 subunits were separated by electrophoresis and the [^{3}H]MalNEt in the γ subunit determined after fixing or staining of the gels. Phosphorylation is given as micromoles of P_i esterified per hour per milligram of chlorophyll.

more, the light-dependent incorporation of MalNEt into the γ subunit of bound CF_1 reached completion in 2 min.[2] A similar period of illumination was required to elicit maximal inhibition of phosphorylation by MalNEt (McCarty *et al.*, 1972).

[^{3}H]MalNEt Incorporation into CF_1 in Chloroplasts not Previously Treated with Unlabeled MalNEt. In the experiments described above, the chloroplasts were incubated with 2 mM MalNEt in the dark for 5 min prior to their exposure to [^{3}H]MalNEt. Therefore, any groups in CF_1 which react with MalNEt in the dark would not have been detected. To test for such groups, chloroplasts were incubated directly with radioactive MalNEt for 2 min in the light or the dark. The extent of incorporation of MalNEt into the CF_1 fractions was much higher in both the light and the dark as compared to that in chloroplasts which were incubated with unlabeled MalNEt prior to their exposure to [^{3}H]MalNEt (Table III). Furthermore, only 53% of the radioactivity in the CF_1 fraction from chloroplasts illuminated in the presence of [^{3}H]MalNEt was present in the CF_1 subunits, indicating that other proteins have reacted with MalNEt under these conditions. A stimulation of MalNEt incorporation into the total CF_1 fraction by light was evident. Although all of the subunits of the enzyme except the δ subunit were labeled in both the light and the dark, only incorporation into the γ subunit was stimulated by light. The extent of the light-stimulated incorporation into the γ subunit (1.3 nmol of MalNEt/mg of protein) was similar to that observed in chloroplasts treated with unlabeled MalNEt in the dark prior to exposure to [^{3}H]MalNEt in the light. Light may also enhance the rate at which other chloroplast proteins react with MalNEt since the light-stimulated portion of MalNEt incorporation into the total CF_1 fraction was greater than that into CF_1 itself.

Finally, it is of interest that two to three groups in chloroplast-bound CF_1 are readily attacked by MalNEt in the dark. Since the incubation of chloroplasts in the dark with MalNEt did not affect phosphorylation, these groups probably do not have a direct function in the phosphorylation mechanism.

Discussion

The demonstration that light enhanced the reactivity of a group (or groups) in the γ subunit of CF_1 in chloroplasts to MalNEt suggests that the state of CF_1 in illuminated chloroplasts differs from that in darkened ones. Illumination may alter the conformation of CF_1 such that a group, which is inaccessible to MalNEt in the dark, becomes accessible. This possibility is supported by the observation that most of the groups in CF_1 are inaccessible to sulfhydryl reagents under nondenaturing conditions (Farron, 1970). Thus, even a small conformational change could expose a group in the γ subunit to reaction with MalNEt. Ryrie and Jagendorf (1971) first proposed that illumination alters the conformation of CF_1 in chloroplasts on the basis that ^{3}H could be detected in CF_1 from chloroplasts illuminated in the presence of ^3H_2O whereas none was found in CF_1 from chloroplasts incubated with ^3H_2O in the dark. There are some similarities between incorporation of MalNEt into CF_1 and hydrogen exchange into the enzyme. Both processes are greatly enhanced by light and are sensitive to uncouplers.

Other mechanisms for the light-induced incorporation of MalNEt into CF_1 are possible. For example, light could increase the reactivity of bound CF_1 by promoting the reduction of a disulfide bond in the enzyme. Light-dependent electron flow in chloroplasts generates a low potential reductant which could reduce a disulfide linkage. The newly

[2] J. Fagan and R. E. McCarty, unpublished observations.

formed SH groups may, then, react with MalNEt. Although CF_1 may contain a disulfide bond (Farron, 1970), this possibility is not too attractive since uncouplers abolish the incorporation of MalNEt into the enzyme whereas they stimulate electron flow.

It is possible that the light-induced change in CF_1 is involved in the activation of Mg^{2+}-ATPase (Petrack and Lipmann, 1961; Hoch and Martin, 1963), and [^{32}P]P_i-ATP (Carmeli and Avron, 1966; McCarty and Racker, 1968) exchange activities which occur when chloroplasts are illuminated in the presence of sulfhydryl compounds. Accompanying the activation of these reactions is a modification of CF_1 which may be detected since the CF_1 extracted from chloroplasts illuminated in the presence of dithiothreitol was an active, rather than latent, Ca^{2+}-ATPase (McCarty and Racker, 1968). Thus the light-induced change in CF_1 may enhance the reactivity of the enzyme to dithiothreitol as well as MalNEt. This hypothesis is consistent with the marked sensitivity of the induction of Mg^{2+}-ATPase activity to uncouplers. Furthermore, the light-induced modification of CF_1 may be essential to phosphorylation. For example, CF_1 as it exists in chloroplasts in the dark may not be able to participate in phosphorylation unless energy is supplied to allow the enzyme to assume an activated form.

The reaction of MalNEt with a group in the γ subunit of CF_1 is probably the cause of the inhibition of phosphorylation by this reagent. This conclusion is supported by the observations that both processes are light dependent, are sensitive to uncouplers, show a similar dependence on MalNEt concentration and have similar kinetics. How the reaction of MalNEt with the enzyme inhibits phosphorylation is not known. It should be emphasized that phosphorylation is only partially inhibited by MalNEt (McCarty *et al.*, 1972), even in chloroplasts sonicated in the presence of MalNEt prior to illumination.[2] Therefore, inaccessibility of CF_1 in chloroplasts to MalNEt is not likely to be the cause of the partial inhibition. As suggested previously, only half of the CF_1 in chloroplasts may undergo the light-induced modification which allows the inhibitory reaction of MalNEt with CF_1. However, if this was the case, repeated illumination of chloroplasts would be expected to elicit further inhibition, but this was not observed.[2] It is also possible that CF_1 has multiple active sites and that MalNEt reacts with only part of them. Alternately, the group with which MalNEt reacts may not be at the active site or sites and its reaction with MalNEt only modifies the activity of the enzyme. For example, the state of MalNEt-modified CF_1 in illuminated chloroplasts may not be as favorable for phosphorylation as that of native CF_1.

The number and identity of the groups which react with MalNEt are not known. However, it is probable that MalNEt reacts with a SH group in the γ subunit since dithiobis(nitrobenzoic acid) inhibits phosphorylation in a manner similar to MalNEt. Approximately 1 nmol of MalNEt/mg of protein in the CF_1 fraction from chloroplasts illuminated with MalNEt was precipitated by the antiserum to CF_1. Assuming that 50% of the protein in this fraction was CF_1, this incorporation is equivalent to about 0.6 nmol of MalNEt/nmol of CF_1. Although it is clear that only a small amount of MalNEt reacts specifically with CF_1 in the light, a more precise evaluation of the stoichiometry of the reaction must be made with a homogeneous preparation of the MalNEt-reacted CF_1.

Acknowledgments

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