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PHOTOSYNTHETIC PHOSPHORYLATION IN *CHLAMYDOMONAS REINHARDI*

EFFECTS OF A MUTATION ALTERING AN ATP-SYNTHESIZING ENZYME

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SUMMARY

A mutant strain of *Chlamydomonas reinhardi* with impaired photosynthesis is described. The inability of this mutant to fix CO_2 is attributed to a genetic lesion affecting primarily the mechanism of photosynthetic phosphorylation. Evidence is presented to suggest that only the terminal stages of ATP synthesis have been affected in this mutant, and that this metabolic change may be correlated to specific structural alterations. Studies with uncouplers of photosynthetic phosphorylation demonstrate that the alteration in the terminal stages of ATP synthesis has also resulted in a differential sensitivity to uncoupling agents. Implications of such observations to the reaction sequence leading to ATP synthesis are discussed.

INTRODUCTION

The fixation of CO_2 by the enzymes of the Calvin cycle depends upon the reducing power and energy produced by the light-driven electron transport chain of photosynthesis. Insufficient levels of either NADPH or ATP, the primary products of the light reactions, will prevent the reduction of CO_2 into sugars. Because of the interdependence between these light and dark reactions, the inability to fix CO_2 has been used successfully as a criterion for detecting mutations affecting the photosynthetic apparatus at both the electron transport stage and at the CO_2 fixation stage.

Several mutant strains of *Chlamydomonas reinhardi* and other green algae which have impaired photosynthesis have been reported and characterized. With the exception of F-60, a mutant strain of *C. reinhardi* deficient in phosphoribulokinase activity[1], all of the mutant strains described so far have been shown to be blocked in the path of electron transport[2]. These strains, therefore, can produce neither the necessary NADPH nor the ATP required for CO_2 fixation.

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; PMS, phenazine methosulfate.

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It was believed that a similar approach to the study of photosynthetic phosphorylation would prove to be as fruitful in helping to define the biochemical events responsible for the conversion of oxidation–reduction energy into ATP. It was expected that such mutants would be unable to grow under phototrophic conditions but able to grow under mixotrophic or heterophic conditions where a fixed carbon source has been provided. In this paper, we describe a photosynthetically-deficient mutant strain of *C. reinhardi* which has normal electron transport activities but which is unable to mediate the light-dependent synthesis of ATP.

MATERIALS AND METHODS

Two strains of *C. reinhardi* were used in the experiments described: the wild type (strain 137c), and the mutant, designated F-54 and obtained by treatment of the wild type with the chemical mutagen methylmethanesulfonate[3]. F-54 was one of a large class of high fluorescent mutant strains isolated by Mr. PIERRE BENNOUN[3].

Both the wild-type and mutant strains were cultured in 300 ml of Tris–acetate–phosphate medium under conditions previously described[4]. Cells were harvested during the logarithmic phase of growth, and chloroplast fragments were prepared by either the sonic disruption of cells or by the disruption of cells by grinding in sand[5].

Photoreduction of $NADP^+$ and 2,6-dichlorophenolindophenol (DCIP) were followed on a Model 14 Cary recording spectrophotometer[5,6] using chloroplast fragments prepared by either of the methods stated above. An Amino–Chance dual-wavelength spectrophotometer was used to measure light-induced absorbance changes associated with cytochromes 553 and 559 in sonicated chloroplast preparations[4]. The light-induced pH changes were assayed by the method of NEUMANN AND LEVINE[7] using chloroplast fragments washed free of buffer and resuspended in 0.01 M KCl supplemented with bovine serum albumin. The chloroplast fragments were prepared from cells that were disrupted by grinding in sand.

Cyclic and noncyclic photosynthetic phosphorylation, with phenazine methosulfate (PMS) and potassium ferricyanide as cofactors, respectively, were assayed by the procedures of GORMAN AND LEVINE[5]. Photosystem I-mediated photosynthetic phosphorylation, using the autoxidizable electron acceptor methyl viologen and diaminodurol as electron donor, was assayed according to the procedure of IZAWA *et al.*[8]. Chloroplast fragments from cells disrupted by grinding in sand were employed for the measurement of both the O_2 uptake and the ATP synthesis associated with methyl viologen reduction. For the O_2 uptake portion of the assay, the components of the reaction mixture (see Table IV) were pipetted into a test tube, mixed briefly, and kept on ice in semidarkness. A 1.5-ml aliquot of the reaction mixture was introduced into a lucite cell fitted with a Clark-type oxygen electrode attached to a Yellow Springs Instrument Co. Oxygen Monitor (Model 53). The reaction was started by turning on the light, which was provided by a 1000-W tungsten projection lamp, and passed through water to remove the infrared component. Light intensity at the level of the vessel was 40000 lux. The reaction was stopped by turning off the light.

The phosphorylation coupled to methyl viologen reduction was assayed in

25-ml erlenmeyer flasks containing magnetic stirring bars. Following the introduction of the reaction mixture, each flask was placed upon a magnetic stirrer which allowed continuous stirring to be provided throughout the experiment. The reaction was begun by turning on the light (40000 lux from a 1000 W tungsten lamp). At the end of the illumination period, 0.2 ml of 20 % trichloroacetic acid was added to stop the reaction. The contents of each flask were then transferred to a conical centrifuge tube and subjected to centrifugation at 6500 × *g* for 5 min to pellet the trichloroacetic acid-precipitable material. The supernatant was decanted and kept on ice. Dark controls were run for all reaction conditions investigated.

Extraction and assay for labeled organic phosphate was conducted on all phosphorylation reaction mixtures by the modification of GORMAN AND LEVINE[5] of the method of AVRON[9].

Whole cell preparations of the wild-type and mutant strains were used for studies of CO_2 fixation. The fixation of $^{14}CO_2$ by photosynthesis and photoreduction followed the methods of TOGASAKI as described by GOODENOUGH *et al.*[10].

Assays of the latent Ca^{+2}-dependent ATPase utilized the method of VAMBUTAS AND RACKER[11], except that the heat activation of the Ca^{2+}-dependent ATPase was conducted at 58° instead of 65°.

The concentration of inorganic phosphate in the resulting supernatant was determined by the method of TAUSKY AND SHORR[12].

Chlorophyll concentrations were determined by a modification[13] of the method of MACKINNEY[14].

All chemicals employed were of the highest purity commercially available. Carrier-free $Na_2H^{32}PO_4$ was purchased from Tracerlab, Waltham, Mass. FCCP, nigericin, and diaminodurol were the generous gifts of Dr. P. G. Heytler, Dr. R. Harned, and Dr. N. E. Good, respectively.

RESULTS

CO_2 fixation

Wild-type cells of the green alga *C. reinhardi* are capable of CO_2 fixation by both photosynthesis and photoreduction[15]. Typical rates for these reactions ob-

TABLE I

RATES OF CO_2 FIXATION BY PHOTOSYNTHESIS AND PHOTOREDUCTION IN THE WILD-TYPE AND F-54 STRAINS OF *C. reinhardi*

Photosynthetic CO_2 fixation was measured at 25° in reaction mixtures containing 10 μmoles $NaH^{14}CO_3$ (specific activity, 0.5 μC/μmole), cells equivalent to 0.1 mg of chlorophyll, and minimal medium to a total volume of 2.2 ml. The cells were incubated in air for 5 min in the light before introduction of $NaH^{14}CO_3$. Samples were taken at 1-min intervals for 5 min. The fixation of CO_2 by photoreduction was also measured at 25° in reaction mixtures containing 0.02 μmoles of DCMU in addition to the above components. The cells were incubated in a H_2 atmosphere for 30 min in the dark and then for 10 min in the light before introduction of the $NaH^{14}CO_3$. Samples were taken at 1-min intervals for 5 min.

Strain	*μmoles CO_2 fixed per mg chlorophyll·h*	
	Photosynthesis	*Photoreduction*
Wild type	95	28
F-54	4	5

tained from mixotrophically grown wild-type cells of *C. reinhardi* are given in Table I. Comparison of these rates with those found for mixotrophically grown F-54 cells (Table I) reveals that the mutant strain is incapable of normal rates of CO_2 fixation under either an air or a H_2 atmosphere.

Photosynthetic electron transport reactions

The photosynthetic electron transport chain of *C. reinhardi* can be examined by a series of reactions which require proper functioning of either the entire path of electron flow from water to $NADP^+$, or a defined portion of the entire pathway[16–19].

The rates obtained for the reactions of the electron transport chain in the wild-type and mutant strains are compared in Table II. It is apparent that F-54 is incapable of the photoreduction of $NADP^+$ when electrons are supplied from water, a reaction which requires the overall operation of the chain. In contrast, examination of the rates obtained from F-54 for the two partial reactions which require either Photosystem I or II activity, demonstrate that the mutant is capable of the Photosystem II-dependent reduction of DCIP and the Photosystem I-dependent reduction of $NADP^+$ from the DCIP–ascorbate couple. Thus, the ability of F-54 to perform the two partial reactions of the electron transport chain but not the reaction involving the entire chain suggests that any block in the path of electron flow occurring in the mutant must lie somewhere in a region bypassed by the two reactions, possibly at the level of cytochrome 553 or the unidentified pool component M believed to be located between cytochromes 559 and 553 (ref. 16).

TABLE II

PHOTOSYNTHETIC ELECTRON TRANSPORT REACTION IN THE WILD-TYPE AND F-54 STRAINS OF *C. reinhardi*

For the Hill reaction with DCIP, the cuvette in the sample compartment of the spectrophotometer contained chloroplast fragments (10 μg chlorophyll) prepared by the sonic disruption of whole cells, and the following in μmoles: potassium phosphate buffer (pH 7.0), 20; KCl, 40; $MgCl_2$, 5; and DCIP, 0.1. The final volume was adjusted to 2.0 ml with distilled water. The DCIP was omitted from the control cuvette in the reference compartment. For the Hill reaction with $NADP^+$, the cuvette contained chloroplast fragments (10 μg chlorophyll) prepared by the sonic disruption of cells, and the following in μmoles: potassium phosphate buffer (pH 7.0), 20; KCl, 40; $MgCl_2$, 5; $NADP^+$, 0.5; and ferredoxin prepared from wild-type *C. reinhardi*, 0.005. Half a unit of ferredoxin-$NADP^+$ reductase, prepared from wild-type *C. reinhardi* was also added. The final volume was adjusted to 2.0 ml with distilled water. Ferredoxin, ferredoxin–$NADP^+$ reductase, and $NADP^+$ were omitted from the control cuvette. For the photoreduction of $NADP^+$ from the DCIP–ascorbate couple, the reaction contained, in addition to the components for the $NADP^+$–Hill reaction, the following in μmoles: DCIP, 0.1; sodium ascorbate buffer (pH 7.0), 10; and DCMU, 0.2. The control cuvette contained everything but ferredoxin, ferredoxin–$NADP^+$ reductase, and $NADP^+$. All reactions were run at 25°.

Strain	*μmoles reduced per mg chlorophyll·h*		
	Hill reaction		*$NADP^+$ reduction with DCIP–ascorbate couple*
	DCIP	*$NADP^+$*	
Wild type	120–175	80–120	150–175
F-54	95–105	0–15	80–105

Light-induced absorbance changes

Light-induced absorbance changes associated with cytochromes 553 and 559 provide a means by which the region of the electron transport chain between the two photosystems can be examined[4]. Inhibition of electron transport by mutation or chemical inhibitors results in distinct changes in the pattern of light-induced oxidation and reduction. The light-induced absorbance changes at 553 and 559 nm observed in mutant strains lacking cytochrome 559 (ac-141)*, the pool component M (ac-21), and cytochrome 553 (ac-206) are tabulated in Table III, together with the absorbance changes observed in F-54 and the wild type. In contrast to these mutant strains, F-54 exhibits light-induced absorbance changes at 553 and 559 nm that are indistinguishable from those of the wild type, strongly suggesting that the region of the electron transport chain between the two photochemical systems is intact.

TABLE III

LIGHT-INDUCED CYTOCHROME CHANGES IN THE WILD-TYPE AND MUTANT STRAINS OF *C. reinhardi*

Light-induced absorbance changes were monitored in an Aminco–Chance dual-wavelength spectrophotometer. The measuring wavelengths for cytochromes 553 and 559 were 553 and 559 nm, respectively. The reference wavelength was 575 nm, and the wavelengths of the actinic light were as indicated.

Strain	*Cytochrome 559*		*Cytochrome 553*	
Actinic light	*650 nm*	*720 nm*	*650 nm*	*720 nm*
Wild type	Reduced	Oxidized	Reduced	Oxidized
ac-141	No spectral change	No spectral change	Oxidized *	Oxidized
ac-21	Reduced	No spectral change	Oxidized *	Oxidized
ac-206	Reduced	No spectral change	No spectral change	No spectral change
F-54	Reduced	Oxidized	Reduced	Oxidized

* This oxidation is attributed to the activation of Photosystem I by 650-nm light, as discussed in the text.

Photosynthetic phosphorylation

ATP synthesis coupled to three paths of electron flow were also examined in the wild-type and mutant strains. The rates for these reactions obtained from the two strains are compared in Table IV, and it can be seen that F-54 is deficient in all three types of photosynthetic phosphorylation. Although capable of wild-type levels of ferricyanide reduction, F-54 was unable to mediate the noncyclic photosynthetic phosphorylation normally coupled to this path of electron flow. Similarly, the mutant strain was not able to support the ATP formation associated with the auto-oxidizable Photosystem I acceptor methyl viologen, although rates of methyl viologen reduction are also comparable to those found in the wild type. This reaction is a Photosystem I-dependent phenomenon[8], relying upon a diamino-

* Recent studies by B. L. EPEL AND W. L. BUTLER[33] reveal that this mutant is deficient in C 550 and the ascorbate-reducible cytochrome b_{559}.

TABLE IV

PHOTOSYNTHETIC PHOSPHORYLATION IN WILD-TYPE AND F-54 STRAINS OF *C. reinhardi*

For measurement of ATP synthesis coupled to ferricyanide reduction, the reaction mixture contained chloroplast fragments (40–60 μg chlorophyll) prepared from whole cells disrupted by grinding in sand, and the following in μmoles: glycylglycine buffer (pH 8.0), 40; potassium phosphate (pH 8.0), 10, containing 1 μC $^{32}P_i$; $MgCl_2$, 5; bovine serum albumin, 4 mg; ADP (pH 7.5), 5; AMP (pH 7.5), 5; potassium ferricyanide, 2. For cyclic photosynthetic phosphorylation, 0.067 μmoles of PMS was substituted for the potassium ferricyanide and 0.02 μmoles DCMU added to block electron flow from Photosystem II. For ATP synthesis coupled to methyl viologen reduction, the reaction mixture contained in addition to the chloroplast fragments described above, the following in μmoles: tricine buffer (pH 8.0), 100; $MgCl_2$, 5; diaminodurol, 1.2; sodium ascorbate, (pH 7.0), 5; methyl viologen, 0.2; ADP (pH 7.5), 5; AMP (pH 7.5), 5; potassium phosphate (pH 8.0), 10, containing 1 μC $^{32}P_i$; DCMU, 0.02.

Strain	*Ferricyanide reduction* *	*ATP formation* **	*Methyl viologen reduction* *	*ATP formation* **	*Cyclic ATP (PMS) formation* **
Wild type	425–520	40–55	1170–1700	90–130	200–2300
F-54	320–395	Nil	1700–2000	Nil	Nil

* μmoles reduced per mg chlorophyll·h.
** μmoles ATP synthesized per mg chlorophyll·h.

duro–ascorbate electron donor couple, and insensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)[8]. Cyclic photosynthetic phosphorylation, mediated by PMS[20], was also absent in F-54, and although no net reduction of an electron carrier could be assayed under these conditions, the PMS-catalyzed light-induced pH change, believed to be a measure of cyclic electron flow[21], was intact in the mutant strain.

Light-induced pH changes

With the localization of the genetic block in F-54 to some stage in photosynthetic ATP synthesis, it became necessary to attempt a more precise identification of the stage in the phosphorylation reaction sequence which was affected. Studies of light-induced pH changes in the wild-type and mutant strains provided a means of examination for early reactions leading to ATP synthesis; reactions that are perhaps closely related to the generation of a high-energy intermediate[22–27].

Two distinct pH changes have been characterized in *C. reinhardi*[21], one associated with cyclic electron flow around Photosystem I and mediated by PMS, the other associated with noncyclic electron flow from Photosystem II and dependent on *p*-benzoquinone. As indicated in Table V, both types of changes are catalyzed by the mutant strain, with both rates and extents of proton movement comparable to those observed in the wild type.

Ca^{2+}-dependent ATPase

These results strongly suggested that the inability of F-54 to synthesize ATP might be the result of a mutation affecting the terminal stages of phosphorylation, past the proposed site of the proton pump. Chloroplast ATPase activity was therefore investigated. The rationale for this approach is based on work in higher plants which suggests that the Ca^{2+}-dependent ATPase is the same enzyme which in intact cells and under normal conditions is involved in ATP synthesis[27].

TABLE V

LIGHT-INDUCED pH CHANGES IN WILD-TYPE AND F-54 STRAINS OF *C. reinhardi*

The standard reaction mixture for measurements of pH changes contained chloroplast fragments (150–200 μg chlorophyll) prepared from whole cells disrupted by grinding in sand, 40 μmoles KCl, 0.2 μmoles *p*-benzoquinone or 0.1 μmole PMS, and distilled water to 2.0 ml.

Strain	*Rate (μequiv H^+ per mg chlorophyll · h)*	*Extent (mμequiv H^+ per mg chlorophyll)*
p-Benzoquinone-stimulated		
Wild type	110	250
F-54	123	300
PMS-stimulated		
Wild type	89	173
F-54	100	244

TABLE VI

Ca^{2+}-DEPENDENT ATPASE ACTIVITY IN THE WILD-TYPE AND F-54 STRAINS OF *C. reinhardi*

Assays of the latent Ca^{2+}-dependent ATPase were conducted in two stages using chloroplast fragments prepared by the sonic disruption of whole cells. The reaction mixture for the activation stage contained chloroplast fragments equivalent to 50 μg chlorophyll, and the following in μmoles: Tris–HCl buffer (pH 7.8), 20; sodium EDTA (pH 7.8), 2; ATP (pH 7.5), 1; and distilled water to a final volume of 0.7 ml. After incubation at either 22 or 58° for 2 min, the following components were added for the reaction stage: ATP (pH 7.5), 3 μmoles; $CaCl_2$, 15 μmoles. The final volume was 2.0 ml. $CaCl_2$ was omitted from the control flask.

Strain	*μmoles P_i released per mg chlorophyll · h*	
	22°	*58°*
Wild type	0	47
F-54	45	48

As is the case in higher plants, the Ca^{2+}-dependent ATPase activity associated with the coupling factor in chloroplasts of wild-type *C. reinhardi* was found to be latent, in that hydrolytic activity appeared only after a heat activation step at 58°. Examination of F-54 for the Ca^{2+}-dependent ATPase revealed that the mutant was also capable of Ca^{2+}-dependent ATP hydrolysis, but unlike the wild-type strain, heat activation was not required for expression of the ATPase activity. The data are summarized in Table VI, and it is apparent that F-54 can catalyze a Ca^{2+}-dependent ATP hydrolysis without benefit of heat treatment. It can alo be seen that the rates of ATPase activity attained by F-54 are equivalent to those achieved by wild type only after heat activation. The enzyme from the wild-type and the mutant strains demonstrated strong similarities in all other respects investigated: both exhibit a pH optimum of 8.2, a strong dependence on Ca^{2+} which reached an optimum at 7.5 mM, oligomycin insensitivity, and inhibition by ADP. It thus

appears that the non-latent Ca^{2+}-dependent ATPase that characterizes F-54 is in fact the analog of the latent Ca^{2+}-dependent ATPase seen in the wild type.

When phosphorylation studies were done in the presence of a glucose and hexokinase trap for ATP, no significant increase in ATP synthesis was observed. This result suggests that the "non-latent" character of the Ca^{2+}-dependent ATPase does not bring about a rapid hydrolysis of any ATP synthesized. Thus, we would suggest that the inability to synthesize ATP and the loss of latency are both functional expressions of the structural change of the synthesizing enzyme.

Effect of uncouplers

With the tentative localization of the genetic lesion in F-54 to the terminal stages of ATP synthesis, it became possible to attribute the mutant's inability to photoreduce $NADP^+$ from water not to a missing electron carrier, but rather to a back pressure exerted on the electron transport chain by an accumulation of some intermediate of the phosphorylation sequence. This would be analogous to the sort of situation believed to be created by energy transfer inhibitors like DIO-9 (refs. 28 and 29) or phlorizin[30]. These two inhibitors of the pathway of energy transfer from electron transport to ATP formation are known to inhibit both phosphorylation and coupled electron transport[28,30]. Addition of uncouplers to systems blocked by energy transfer inhibitors results in restoration of electron transport but not of phosphorylation[29].

Restoration of $NADP^+$ photoreduction with uncoupling agents was attempted in F-54. Using water as the electron donor, rates of $NADP^+$ photoreduction in the presence and absence of uncouplers were obtained. These rates are given in Table VII, and it is apparent that restoration of electron flow can be achieved in F-54 with both gramicidin and nigericin. This curative effect of uncouplers strongly suggests that the electron transport chain in F-54 is intact and that the metabolic block is localized to the sequence of reactions related to ATP synthesis.

The ineffectiveness of carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) in restoring electron transport suggested that the site of action for this uncoupler may be different from the target site of gramicidin and nigericin, both known to affect membrane permeability[31,32]. If this is the case, then the mutation

TABLE VII

EFFECT OF UNCOUPLERS ON $NADP^+$ REDUCTION IN F-54

These assays of the Hill reaction with $NADP^+$ utilized chloroplast fragments prepared from whole cells disrupted by grinding in sand. The chlorophyll concentration in the reaction mixture was 10 μg. All other components of the reaction mixture are identical to those described in Table II.

Conditions	*Rate of $NADP^+$ reduction (μmoles reduced per mg chlorophyll·h)*
No additions	0
2 μM FCCP	0
1 μM gramicidin D	57
2 μM nigericin	40

in F-54 may have rendered the FCCP target site insensitive to the uncoupler. Earlier studies with *C. reinhardi* (R. P. LEVINE, personal communication) had shown that ADP was necessary for optimal enhancement of electron transport with FCCP, suggesting that this uncoupler may act at a stage of ATP synthesis terminal to that involving ion movement.

It therefore became interesting to examine the effects of arsenate, a phosphate analog which demonstrates ADP-dependent uncoupling in chloroplasts[29,34,35]. Since photosynthetic phosphorylation cannot be assayed in F-54, the light-induced pH change was used to monitor the effect of arsenate. As is evident from Table VIII, arsenate does induce an ADP-dependent stimulation of proton movement in the wild type, but appears unable to induce a similar stimulation in the mutant. It therefore appears that F-54 is also insensitive to the action of this uncoupler.

TABLE VIII

EFFECT OF ARSENATE ON THE EXTENT OF THE LIGHT-INDUCED pH RISE IN THE WILD-TYPE AND F-54 STRAINS OF *C. reinhardi*

The reaction mixtures for determination of the effect of arsenate on the light-induced pH rise contained chloroplast fragments (prepared from whole cells by grinding in sand), equivalent to 100 μg chlorophyll, and the following in μmoles: KCl, 40; PMS, 0.1; $MgCl_2$, 4; Na_2AsO_4, 0.25; ADP, 1.0. The final volume was adjusted to 2.0 ml with distilled water.

Conditions	*Wild type (μequiv H^+ per mg chlorophyll)*	*F-54 (μequiv H^+ per mg chlorophyll)*
No additions	180	119
+ arsenate and Mg^{2+}	151	95
+ arsenate, Mg^{2+}, ADP	498	100

DISCUSSION

The results reported in this paper suggest that the mutant strain of *C. reinhardi*, F-54, is unable to fix CO_2 because of a genetic lesion affecting the ATP synthesizing apparatus associated with photosynthetic electron transport. Furthermore, it appears that the mutation in F-54 has resulted in an alteration of an enzyme involved in the terminal stages of phosphorylation, past the stage at which the preliminary "coupling" of oxidation–reduction energy to ATP synthesis occurs.

This alteration in the terminal stages of ATP synthesis has also resulted in a differential sensitivity of the mutant strain to uncouplers. Although it is not possible to assay ATP synthesis in F-54, stimulation of electron transport activities provides a means for monitoring uncoupler action. Using this technique on the mutant strain it was possible to define two classes of uncouplers: one which could elicit stimulation of electron flow, another which could not. Of the uncouplers of the first kind, gramicidin and nigericin are known[31,32] to affect permeability changes in thylakoid membranes, and it is believed[29,31,32] that their uncoupling properties can be attributed to these effects on ion gradients. The intactness of the light-induced pH changes in F-54 implies that the proton pumping apparatus is functional and therefore susceptible to these agents. Arsenate, an uncoupler of the

second sort, requires ADP before stimulation of electron transport becomes evident, suggesting that the involvement of this compound in the phosphorylation reaction sequence may be further removed from the energy conservation step than either gramicidin or nigericin[29,34]. The ineffectiveness of arsenate in stimulating the pH change is consistent with this idea, and supports the hypothesis that the genetic lesion in this mutant has affected only the terminal stages of ATP synthesis. The failure of FCCP to stimulate electron flow in F-54 is more difficult to rationalize. Although there is some evidence* that FCCP requires ADP for maximum enhancement of electron transport in *C. reinhardi*, recent reports on the mode of action of this uncoupler[36] suggest that it effects changes in membrane permeability. More extensive examination of the effects of FCCP on *C. reinhardi* is required before firm conclusions can be drawn as to its primary site of action, but the insensitivity of F-54 to this uncoupler does suggest a possible involvement at the terminal stages of ATP synthesis.

Although some of the photosynthetic properties of this phosphorylation-deficient mutant strain have been defined, the exact nature of the alteration in the Ca^{2+}-dependent ATPase is yet to be characterized. The loss of the latent character normally associated with this enzyme suggests that the alteration may involve the sulfhydryl groups known to be associated with this enzyme[37]. FARRON AND RACKER[38] have recently reported that the number of titratable sulfhydryl groups on the purified spinach coupling factor changes after the heat activation step which is needed to elicit ATPase activity. It is therefore not unreasonable to suggest that the conformation of the Ca^{2+}-dependent ATPase in F-54 mimics the conformation of the normal enzyme after heat treatment. Extensive studies on purified coupling factor from both wild-type and mutant strains of *C. reinhardi* will be necessary, however, before conclusive answers can be provided.

It is apparent, however, that impairment of the Ca^{2+}-dependent ATPase has resulted in a total loss of ability to perform photosynthetic phosphorylation; neither cyclic nor noncyclic sites of ATP synthesis are functional in F-54. Genetic analysis of the mutant strain demonstrates that the mutation behaves in a Mendelian fashion as a single gene alteration (B. COSBEY, unpublished observations), suggesting that this alteration in a single gene has affected a single enzyme. Thus it would seem that the coupling enzymes involved at all sites of ATP synthesis in the chloroplast are either identical or similar enough to be equally affected by the same genetic lesion. The occurrence of a common coupling enzyme for all sites of ATP synthesis may indicate that the substrates involved in the transphosphorylation of ADP to ATP are also identical at all sites. If the final reaction in the phosphorylation sequence is represented as $ADP + X \sim P \rightleftarrows ATP$, it is highly probable that the high-energy intermediate, $X \sim P$, is the same at each coupling site. This would preclude X being a component of the electron transport chain, like a cytochrome or a plastoquinone, and supports the notion of a common high-energy phosphorylated intermediate into which site-specific non-phosphorylated intermediates are converted. Such a reaction sequence has been proposed by BOYER[39], and the evidence provided by strain F-54 lends strong genetic support to such a mechanism.

* R. P. LEVINE, personal communication.

Preliminary electron microscopic studies on chloroplast membranes from the wild type and F-54 indicate that the two strains may have significant morphological differences. The 100-Å particles believed to be associated with both coupling factor and ATPase activities[40] can be clearly distinguished on negatively-stained preparations of chloroplast membranes from the wild type. When prepared in a similar fashion, however, membranes of F-54 chloroplasts appear devoid of these particles. Although the precise relationship of these particles to the chloroplast membranes of the mutant F-54 is yet to be clarified, this preliminary examination suggests that the alteration in the Ca^{2+}-dependent ATPase may be correlated to significant structural changes.

It is apparent from these introductory studies with strain F-54 of *C. reinhardi*, however, that much information about the mechanism of ATP synthesis can be derived from examination of mutant organisms. Although extensive work on yeast has not yet resulted in the successful isolation of a mutant of oxidative phosphorylation[41], this preliminary study on a mutant with impaired photosynthetic phosphorylation confirms the feasibility of a genetic approach to the problem of ATP synthesis.

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