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ENERGY TRANSDUCTION IN PHOTOSYNTHETIC BACTERIA

VII. INHIBITION OF THE COUPLING ATPase BY *N*-ETHYLMALEIMIDE RELATED TO THE ENERGIZED STATE OF THE MEMBRANE

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SUMMARY

N-Ethylmaleimide, at millimolar concentrations, irreversibily inhibits photophosphorylation and ATPase activity of photosynthetic membranes from *Rhodopseudomonas capsulata*. The inhibitory effect of *N*-ethylmaleimide is evident only if the membranes are preincubated with the inhibitor in the light and in the absence of phosphorylation substrates. ADP and orthophosphate (or arsenate) exert a protective effect against the inhibition if they are present during the preillumination stage. The energization of the membrane by ATP hydrolysis, measured as ATP-induced quenching of 9-aminoacridine fluorescence, also is inhibited irreversibly by *N*-ethylmaleimide.

Uncouplers protect the ATPase from inhibition by *N*-ethylmaleimide at concentrations at which they inhibit photophosphorylation. The ATPase, as measured either in the dark or in the light, is also inhibited by carbonylcyanide *p*-trifluoromethoxyphenylhydrazone in parallel with photophosphorylation.

These results are interpreted as evidence that the high-energy state of the membrane induces a conformational change of the ATPase, making it sensitive to attack by *N*-ethylmaleimide; this conformational change might be related to the active state of the ATPase.

INTRODUCTION

ATP synthesis coupled to photosynthetic or respiratory electron flow in membranes from facultative photosynthetic bacteria is catalyzed by an ATPase whose molecular properties are similar to those of the mitochondrial or chloroplast coupling ATPases [1, 2]. In *Rhodopseudomonas capsulata*, the ATPase activity of the membrane-bound enzyme can be enhanced by light under conditions optimal for cyclic electron flow [3]. ADP, if added before exposing the membranes to light, completely inhibits the effect promoted by illumination. This behaviour shows strong similarities with that of the ATPase of spinach chloroplasts, whose activated state is

triggered by light [4] and is destabilized by ADP [5]. Mainly on this basis we have previously suggested that the light activation of ATPase in *Rps. capsulata* could reflect a conformational change of the enzyme, promoted by the energization of the membrane in the absence of ADP. More direct evidence of energy-linked conformational changes have been obtained for the chloroplast ATPase by Ryrie and Jagendorf [6] by measuring the light-induced incorporation of non-exchangeable ^3H into the enzyme. In addition, McCarty and Fagan [7], have succeeded in labelling specifically the γ-subunit of the ATPase with radioactive *N*-ethylmaleimide in a reaction that depends on a high-energy state of the membrane. Evidence for a relationship between conformational changes of the ATPase from *Rps. capsulata* and the activity of the enzyme is presented in this paper. We show that inhibition of photophosphorylation and the ATPase can be obtained by preincubation of the membrane with *N*-ethylmaleimide in the light. Furthermore, the oligomycin-sensitive ATPase, measured in the light or in the dark, is inhibited by concentrations of uncouplers which protect the enzyme from photoinhibition by *N*-ethylmaleimide.

Some of the data reported here have been presented at the International Symposium on Membrane Proteins in Transport and Phosphorylation, Bressanone, Italy, 1974.

MATERIALS AND METHODS

Rps. capsulata, strain St. Louis (American Type Culture Collection n. 23782) was grown anaerobically in the light at 30 °C in the medium described by Ormerod et al. [8]. Chromatophores were prepared in glycylglycine buffer, 0.05 M, pH 7.2, containing 5 mM $MgCl_2$ as previously reported [9]. ATPase activity and light-dependent phosphorylation were assayed at pH 8 by procedures outlined in refs 3 and 9.

The ATP-dependent quenching of fluorescence of 9-aminoacridine was measured using a filter fluorimeter of conventional design [10]. The assay contained in a final volume of 2.5 ml: glycylglycine, pH 7.8, 80 μmoles; $MgCl_2$, 25 μmoles; KCl, 250 μmoles; ATP, 5 μmoles; valinomycin, 2 μg; and 9-aminoacridine, 10 nmoles.

RESULTS

Preincubation of photosynthetic membranes from *Rps. capsulata* in the presence of *N*-ethylmaleimide causes an inhibition of photophosphorylation and Mg^{2+}-dependent ATPase (measured in light), if the preincubation is performed in the light and in the absence of substrates (Fig. 1). With increasing concentrations of *N*-ethylmaleimide, the inhibition levels off at about 50 % for both photophosphorylation and light-ATPase. It is completed in about 1 min of illumination. In contrast, no effect of *N*-ethylmaleimide on photophosphorylation or the ATPase (measured in dark) is observed when the membranes are kept in the dark in the presence of *N*-ethylmaleimide. Illumination and incubation with *N*-ethylmaleimide must be simultaneous in order to cause the inhibition; no effect can be detected if *N*-ethylmaleimide is added in the dark to membranes that have been illuminated previously in the absence of phosphorylation substrates. If, however, the membranes are preilluminated in presence of *N*-ethylmaleimide, an inhibition of dark-ATPase activity can

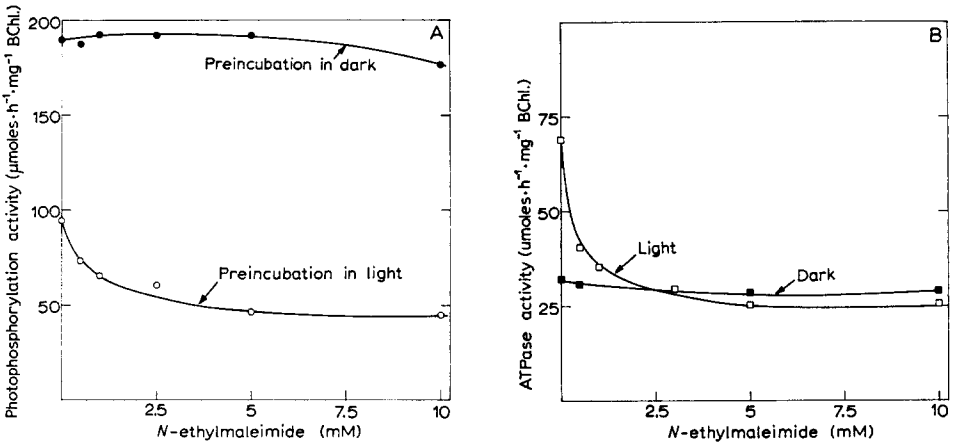

Fig. 1. Effect of *N*-ethylmaleimide on photophosphorylation (Fig. 1A) and ATPase activity (Fig. 1B). The assays were preincubated in the light or in the dark for 5 min at 30 °C in the presence of *N*-ethylmaleimide and in the absence of substrates; after this preincubation excess dithiothreitol was added to completely titrate *N*-ethylmaleimide. ATPase activity was then measured in the light for preilluminated samples or in the dark for the dark-preincubated assays. Photophosphorylation was measured according to standard procedures in assays preincubated in the light or in the dark. It was normally found that lower rates of photophosphorylation were obtained if the particles were preincubated in the light in the absence of substrates. (see also ref. 3)

be observed. This effect is shown in Table I, where results of *N*-ethylmaleimide addition on light- and dark-ATPases are compared in assays in which the inhibitor was preincubated with the membranes in the dark or in the light.

N-Ethylmaleimide also inhibits photophosphorylation (an assay necessarily performed in the light), but only if a preincubation is performed in the absence of substrates. The requirement for the preincubation can be explained on the basis of the experiments shown in Table II. Both orthophosphate and ADP, if present during preillumination, exert a protective effect against inhibition of photophosphorylation

TABLE I

EFFECT OF *N*-ETHYLMALEIMIDE ON ATPase ACTIVITY OF PHOTOSYNTHETIC MEMBRANES FROM *RHODOPSEUDOMONAS CAPSULATA*

Photosynthetic membrane fragments, corresponding to 38 μg of bacteriochlorophyll in 1 ml, were preincubated for 5 min at 30 °C in the dark or in the light in the presence of 5 mM *N*-ethylmaleimide (NEM) or without any addition. After this treatment ATPase activity was measured. As described in a previous paper [3], preillumination produces a substantial increase of the activity measured in the light. Activity is expressed as μmoles of ATP hydrolyzed per h per mg of bacteriochlorophyll.

Conditions of preincubation	ATPase activity	
	Dark	Light
Dark	35	—
Light	34	79
Dark in the presence of NEM (5 mM)	33,5	—
Light in the presence of NEM (5 mM)	12	37

TABLE II

PROTECTION OF PHOTOPHOSPHORYLATION FROM *N*-ETHYLMALEIMIDE INHIBITION BY ADP AND PHOSPHATE

Photosynthetic membrane fragments (33 μg bacteriochlorophyll in 1.5 ml) were preilluminated for 5 min at 30 °C in the presence of 5 mM *N*-ethylmaleimide (NEM) and of photophosphorylation substrates, as indicated; in control experiments NEM was omitted. After this treatment the remaining substrates were added and photophosphorylation was measured by standard procedures. Activity is expressed as μmoles of $^{32}P_i$ incorporated per h per mg of bacteriochlorophyll.

Additions	Photophosphorylation		
	Preilluminated in absence of NEM	Preilluminated in presence of NEM (5 mM)	
	Activity	Activity	Inhibition (%)
None	89	42.3	52.4
ADP (2 mM)	100	67	33
Phosphate (2 mM)	100	56	44
ADP (2 mM) and phosphate (2 mM)	82.5	73.5	11

by *N*-ethylmaleimide; the protection is virtually complete if both substrates are present together. An analogous effect can be observed also for the light-ATPase, as Table III shows. Addition of low concentrations of ADP ($5 \cdot 10^{-5}$ M) during the preincubation reduces the inhibition by *N*-ethylmaleimide from 74 to 43 %; 2 mM arsenate causes a similar protection. In these experiments, arsenate substitutes for phosphate in order to avoid interference between the measurements of the light-ATPase and photophosphorylation. When both arsenate and ADP are added simul-

TABLE III

PROTECTION OF LIGHT DEPENDENT ATPase FROM *N*-ETHYLAMLEIMIDE INHIBITION BY ADP AND ARSENATE PRESENT DURING THE PREILLUMINATION STAGE

Photosynthetic membrane fragments (33 μg bacteriochlorophyll in 1.0 ml) were preincubated for 5 min at 30 °C in the presence of 5 mM *N*-ethylmaleimide (NEM) and of ADP or (and) arsenate as indicated in the Table; in control experiments NEM was omitted. In agreement with previously described results, addition of low concentrations of ADP during the preillumination stage inhibited nearly completely the stimulation of the activity by light: this inhibition is evident also in the presence of 2 mM arsenate. Activity is expressed as μmoles of ATP hydrolyzed per h per mg of bacteriochlorophyll.

Additions	Light-dependent ATPase activity		
	Preilluminated in the absence of NEM	Preilluminated in the presence of NEM (5 mM)	
	Activity	Activity	% Inhibition
None	49.6	13.0	73.6
$5 \cdot 10^{-5}$ M ADP	31.8	17.8	42.8
2 mM arsenate	68.5	32.4	51.0
$5 \cdot 10^{-5}$ M ADP and 2 mM arsenate	39.6	39.8	0.0

taneously, no effect of *N*-ethylmaleimide can be observed.

In addition to the protective action of these substrates against *N*-ethylmaleimide inhibition, additional effects of these compounds on the control activities are apparent from the results in Table III. The first is the inhibitory effect of ADP on light activation of ATPase, which has been described previously in detail [3], the second is a stimulatory effect of arsenate on the ATPase, which is the topic of the accompanying paper [11].

N-Ethylmaleimide also inhibits the rate and extent of energization of the membrane by ATP. In the experiments described below the energization of the membranes was evaluated measuring the attenuation of the fluorescence of 9-aminoacridine, which, according to a widely accepted model, is related to the pH difference across the membrane [12]. In these experiments (Table IV) the quenching of the fluorescence of 9-aminoacridine, induced in the dark by ATP hydrolysis, was measured in membranes that had been preincubated with 5 mM *N*-ethylmaleimide. Control experiments were performed by addition of a slight excess of dithiothreitol before the preillumination, in order to titrate the inhibitor completely. In a second series of experiments, also described in Table IV, 4 mM phosphate was added during or after preillumination with *N*-ethylmaleimide; this anion has been shown to be an activator of ATP-dependent membrane energization [11] in analogy with the effect of arsenate on the light-ATPase. Again, phosphate protected the enzyme from the action of *N*-ethylmaleimide only if it was present during the preillumination. The stimulatory effect of phosphate on the level of quenching was observed both in control and *N*-ethylmaleimide-treated membranes.

An absolute requirement of illumination for obtaining inhibition by *N*-ethylmaleimide of photophosphorylation and ATPase activities can be interpreted by assuming that energization of the membrane is necessary in order to expose one (or more) sulphydryl groups, essential for these activities, to the action of the inhibitor. This view is supported by the observation that uncouplers can protect the enzyme if they are added together with *N*-ethylmaleimide during the preincubation. As well

TABLE IV

INHIBITION OF ATP-PROMOTED QUENCHING OF 9-NH_2 ACRIDINE FLUORESCENCE BY *N*-ETHYLMALEIMIDE

Photosynthetic membrane fragments (36 μg bacteriochlorophyll in 2.5 ml) were preilluminated for 3 min at 30 °C under the conditions described in the first column of this table; after preillumination 2.6 mM dithiothreitol and 4 mM P_i were added in the dark as specified in the second column of the table and ATP-induced quenching of the fluorescence of 9-amino acridine was measured. The results are presented as per cent quenching of fluorescence under steady state conditions in the dark. NEM = *N*-ethylmaleimide.

Additions		Quenching (%)
Before preillumination	After preillumination	
5 mM NEM, 2.6 mM dithiothreitol	—	22.6
5 mM NEM	2.6 mM dithiothreitol	8.0
5 mM NEM, 2.6 mM dithiothreitol, 4 mM P_i	—	44.0
5 mM NEM, 4 mM P_i	2.6 mM dithiothreitol	42.5
5 mM NEM	2.6 mM dithiothreitol, 4 mM P_i	20.2

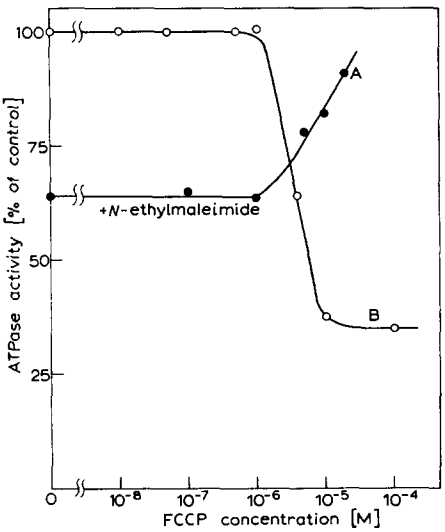

Fig. 2. Protection by FCCP of light ATPase from photoinhibition by *N*-ethylmaleimide. Curve A: photosynthetic membranes (40 μg bacteriochlorophyll (BChl.) in 1.0 ml) were preilluminated for 3 min at 30 °C in the presence of 5 mM *N*-ethylmaleimide and increasing concentrations of FCCP; after preillumination, but before the measurement, in the light, of ATPase activity, FCCP and dithiothreitol to a final concentration of 20 μM and 5 mM, respectively, were added to all samples. Curve B: shows the inhibitory effect of FCCP on light-ATPase measured under standard conditions. Control activities were 56 and 42 μmoles ATP hydrolyzed per h per mg BChl. for Curves A and B, respectively.

known, the uncoupling effect of carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) can be reversed by thiol reagents. We have taken advantage of this fact for the experiment shown in Fig. 2 (curve A), in which increasing concentrations of FCCP were added to assays containing 5 mM *N*-ethylmaleimide. Before the ATPase assay, but after the 5 min preincubation in the light, FCCP and dithiothreitol were added to a final concentration of 20 μM and 5 mM, respectively. Under these conditions, dithiothreitol was found to prevent any further inhibitory effects of *N*-ethylmaleimide and to block completely the uncoupling effect of FCCP. FCCP at concentrations higher than 5 μM was found to protect the light-ATPase from inactivation by *N*-ethylmaleimide, the protection being nearly complete at 20 μM. At these concentrations, complete inhibition of photophosphorylation is usually obtained in *Rps. capsulata* chromatophores. Curve B in the same figure shows the effect of increasing concentrations of FCCP on light-ATPase. The uncoupling of the membrane causes a drastic inhibition of light-ATPase, which is decreased nearly to the level of the oligomycin-insensitive activity if an excess of FCCP (10^{-4} M) is added. The concentration span in which this inhibition occurs corresponds exactly to that in which FCCP protects against inhibition by *N*-ethylmaleimide, and corresponds also to the concentration range of inhibition of photophosphorylation.

A more complete picture of the action of FCCP is presented in Fig. 3, which compares the effects of the uncoupler on the light-ATPase (Fig. 2, curve B) with those on the dark-ATPase and on photophosphorylation. The results show that all of the ATPase activity that is sensitive to oligomycin, is inhibited by an excess of

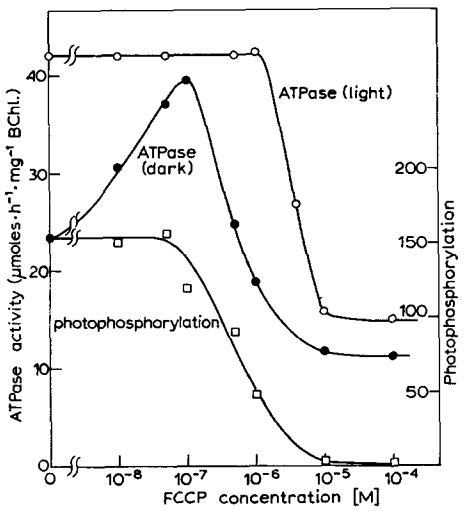

Fig. 3. Effect of FCCP on photophosphorylation and ATPase activity measured in the light or in the dark. The conditions of the assay were standard. BChl., bacteriochlorophyll. The oligomycin-insensitive activity was 9.7 μmoles per h per mg BChl. and was not affected by light or FCCP.

FCCP (10 μM or higher) both in the dark and in the light, whereas oligomycin-insensitive activity is completely unaffected by this uncoupler. It is also apparent that FCCP is a more effective inhibitor of ATPase in the dark (apparent $K_i = 0.5$ μM) than in the light (apparent $K_i = 5$ μM), as would be expected if the energization by photosynthetic electron flow were to counteract the dissipatory effect of FCCP. The difference between the effects of FCCP on the ATPase in the dark or in the light is most evident at suboptimal concentrations of uncoupler, since a conspicuous

TABLE V

EFFECT OF VALINOMYCIN AND NIGERICIN IN PRESENCE OF K^+ ON LIGHT AND DARK ATPase

Photosynthetic membrane fragments (35 μg bacteriochlorophyll in 1.0 ml) were preincubated in the light or in the dark for 5 min at 30 °C in a standard mixture for the ATPase assay to which 50 mM KCl had been added and in the presence of the antibiotics indicated. ATPase activity was measured in the light or in the dark as described. Activity is expressed as μmoles of ATP hydrolyzed per h per mg of bacteriochlorophyll.

Additions	ATPase activity	
	Light	Dark
None	64.5	32.2
Nigericin (4 μg)	51.5	46.5
Valinomycin (4 μg)	32.2	33.4
Nigericin (4 μg) and valinomycin (4 μg)	22.5	19.5
Oligomycin (5 μg)	10.2	10.0
Valinomycin, nigericin and oligomycin	8.2	8.3

stimulation of dark activity is obtained at FCCP 10^{-7} M or lower. These concentrations have no effect on the light-ATPase. The conclusion that these effects of FCCP are due to its action as an uncoupler is supported by similar observations which we have described previously for other uncoupling molecules such as DNP [2] and oxidized 2,6-dichloroindophenol (DCIP) [3], but the general significance of these inhibitions had previously escaped our attention.

It is well established that chromatophore preparations can be uncoupled synergistically by the simultaneous addition of valinomycin and nigericin in the presence of K^+, whereas addition of either one of these two ionophores exerts only minor effects on photophosphorylation [13]. We have taken advantage of the highly specific mechanism of uncoupling by these two antibiotics for testing the general validity of the response of oligomycin-sensitive ATPase to uncoupling conditions. Results of such experiments are summarized in Table V; in the dark, addition of nigericin plus valinomycin produces a substantial inhibition of the dark-ATPase while nigericin alone stimulates the enzyme considerably and valinomycin alone has no effect. In the light, nigericin and valinomycin added alone have a clear inhibitor effect, which is more marked for valinomycin and which brings the activity to the level observed in the dark. If these antibiotics are added together, the activity in the light is reduced to the level measured in the dark under the same uncoupling conditions. In the same Table it is shown that oligomycin-insensitive activity is unaffected either by light or by uncouplers.

DISCUSSION

The results described in this paper demonstrate that the coupling ATPase present in photosynthetic membranes of *Rps. capsulata* can be partially inactivated by *N*-ethylmaleimide if the membranes are illuminated in the presence of this inhibitor and in the absence of phosphorylation substrates. The enzyme is protected against inactivation by ADP and P_i (or As_i) and by the uncoupler FCCP. The inhibitory action of *N*-ethylmaleimide can be observed in three energy-transducing reactions mediated by the coupling ATPase, namely ATP hydrolysis, synthesis and ATP-driven proton translocation. The degree of inhibition does not normally exceed 50–60 % of the total activity in agreement with the results reported for spinach chloroplasts [7]; the reasons for this partial insensitivity to the inhibitor are at present unexplained.

In our opinion these observations have to be considered in connection with the activating effect that photosynthetic electron flow exerts on the ATPase [3]. The linkage between the two phenomena is suggested by several lines of evidence: a) light enhances ATPase activity, increasing the V of the enzyme and in parallel causes the enzyme to become sensitive to *N*-ethylmaleimide; b) both the activation by light and the sensitivity of the enzyme toward *N*-ethylmaleimide are promptly reversed when the assays are transferred from light to dark; c) ADP, at a concentration as low as 50 μM blocks the activating effect of light and at the same concentration protects, at least partially, the enzyme from attack by *N*-ethylmaleimide; d) the effect of light on inhibition by *N*-ethylmaleimide can be completely prevented by FCCP at a concentration which causes a complete uncoupling of the membrane; e) all uncouplers, irrespective of their mechanism of energy dissipation, inhibit the oligomycin-sensitive ATPase practically to levels corresponding to the oligomycin-insensitive activity.

This effect is immediate upon the addition of the uncoupler even if the enzyme is already hydrolyzing ATP (unpublished observations). These results are opposite to the expected response to uncouplers of an energy-transducing ATPase, but are consistent with similar data obtained in spinach chloroplasts, in which all uncouplers inhibit the ATPase in the light-triggered state [14].

On the basis of these results we suggest that an energized state of the membrane is an obligate condition for an active conformation of ATPase. One would then expect the ATPase to exhibit its maximum turnover rate in the light. In the dark, a low turnover rate of ATPase would be expected if the hydrolysis of ATP can sustain only a low level of energization. The observation that valinomycin and K^+ can block nearly completely the activation of ATPase by light (Table V) suggests that the effect of illumination on the enzyme is mainly due to an increase of the membrane potential.

Conditions which induce maximum ATPase activity (except for the effect of P_i) correspond to those in which a maximal inhibition by *N*-ethylmaleimide is observed. The maximum level of ATPase activity, which does not correspond to a state producing a maximum rate of photophosphorylation (cf. Fig. 1A and also ref. 3, Table III) should correspond therefore to a conformation of the enzyme in which sulfhydryl groups essential for energy transduction are exposed to the attack by *N*-ethylmaleimide. The nature of this "opening" of the ATPase molecule is unknown.

In spinach chloroplasts [7] light induces a specific labelling by *N*-[^{14}C]ethylmaleimide of the γ-subunit of chloroplast coupling factor I, a subunit which is essential for energy transduction but does not contain the binding site for ATP. In mitochondria the energized state of the membrane and the concentration ratio of ATP and ADP are important factors involved in the binding of ATPase inhibitor to mitochondrial coupling factor I [15]. The molecular characteristics of *Rps. capsulata* coupling ATPase are very similar to those of mitochondrial coupling factor I and chloroplast coupling factor I; preliminary experiments in this laboratory have also indicated the presence in the enzyme of at least the α, β and γ subunits. Moreover the low level of ATPase activity of the soluble enzyme, in contrast to the high activity of mitochondrial coupling factor I and of *Rhodospirillum rubrum* coupling factor [16], suggests the presence of an endogenous ATPase inhibitor, as has been demonstrated for chloroplast coupling factor I.

The true significance of these analogies in structure and function among the energy-transducing ATPases remain to be established by further investigation.

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