

# The role of $Mg^{2+}$ in the light activation process of the $H^+$ -ATPase in intact chloroplasts

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Received 2 July 1982

*Photosynthesis*

*Light-activation*

*$H^+$ -ATPase*

*Intact-chloroplast*

*Thiol-modulation*

*$Mg^{2+}$ -dependence*

## 1. INTRODUCTION

The enzyme ATP synthase undergoes light activation in intact chloroplasts, which exposes its ATPase activity [1–4]. The physiological ATPase modulator has been suggested to be the dithiol protein thioredoxin which is reduced by ferredoxin via FTR in the light. This was based on the effect of the isolated thioredoxin system on ATPase activity of the isolated  $CF_1$  [5] or of broken chloroplasts [3]. The idea was recently strengthened by the findings that exogenous electron acceptors of photosystem I inhibit the activation process in intact chloroplasts [6,7], in a way which indicates the involvement of the ferredoxin site [7].

Studies of the structure of the thylakoid membrane (see [8] for review) revealed that the ATP synthase complex coincides with PS I in the thylakoid membrane and the  $CF_1$  is located in a close vicinity to FNR, both protruding into the stroma space. Fd is thought to be a soluble component which is loosely bound to the membrane, probably at the FNR site [9]. Recently we have shown that intact chloroplasts which are osmotically shocked in the presence of  $MgCl_2$  maintain their capacity to catalyze cyclic electron flow around PS I [10], suggesting that the binding of Fd to the thylakoids requires  $Mg^{2+}$ . In the work described here it is

shown that such a treatment of intact chloroplasts also maintains their capacity to activate the  $H^+$ -ATPase in the light. Similarly, it is suggested that the thiol modulator of the ATPase is loosely bound to the membrane in a  $Mg^{2+}$ -dependent mode.

## 2. MATERIALS AND METHODS

Spinach growth conditions and isolation of intact chloroplasts were described in [7]. Chloroplasts were 65–75% intact as assayed by ferricyanide reduction [11]. Light activation of chloroplasts was routinely done by 3 min of illumination with 100 000 lux white light intensity in a thermostatted bath at 22°C. The activation mix contained 0.35 M sorbitol, 40 mM Na-Tricine (pH 8), 0.25 mM  $P_i$ , 2100 units/ml catalase and intact chloroplasts containing 0.3–0.4 mg Chl/ml. Chloroplasts were either illuminated intact and then osmotically shocked and assayed, or shocked before illumination as described in the legends to fig.1 and tables 1–4.

ATPase activity was assayed under partially uncoupled conditions by the release of  $^{32}P_i$  from  $[\gamma\text{-}^{32}P]\text{ATP}$  as previously described [7].  $\text{ATP-P}_i$  exchange was assayed by the incorporation of  $^{32}P_i$  into ATP and determined according to [12]. Light induced 9AA fluorescence quenching was measured in an Eppendorf fluorimeter model 1100 according to [13]; illumination was provided by a 250 W halogen lamp filtered through 4 cm water and a Schott RG-5 glass filter.

ATP, NADP, MV and DTT were purchased from Sigma and  $^{32}P_i$  from Nuclear Research Centre, Negev. Fd (isolated from *Spirulina platensis* as

**Abbreviations:**  $CF_1$ , chloroplast coupling factor 1; Chl, chlorophyll; DTT, dithiothreitol; Fd, ferredoxin; FNR, Fd-NADP reductase; FTR, Fd-thioredoxin reductase; MV, methylviologen; 9AA, 9-aminoacridine; Th, thioredoxin; PS I, photosystem I;  $\Delta pH$ , transthylakoid pH gradient

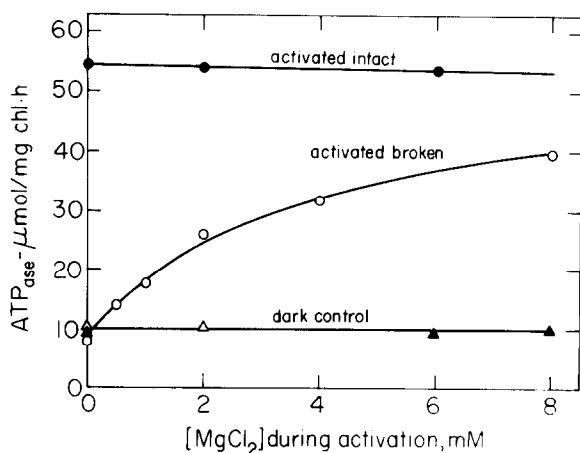


Fig.1.  $\text{MgCl}_2$  concentration dependence of the light activation of the  $\text{H}^+$ -ATPase in intact and broken chloroplasts. Reaction conditions were as described for table 1 except for the presence of  $\text{MgCl}_2$  at the indicated concentration during the pre-activation. Assay mixture was identical for all samples. Closed symbols — chloroplasts pretreated intact. Open symbols — pretreated after lysis.

described in [14]) was kindly provided by Mr M. Sheffer.

### 3. RESULTS

The light-activated ATP hydrolysis has been assayed in intact chloroplasts either indirectly [1,4] or directly after an osmotic shock of the chloroplasts [1,3,6,7]. The latter is required to overcome the impermeability of the envelope to nucleotides. We have found that intact spinach chloroplasts which were osmotically shocked in 20 mM Na-Tricine prior to illumination, lost their capacity to undergo light activation. Most of this capacity was nevertheless, preserved, if  $\text{MgCl}_2$  was present in the shocking medium. Table 1 demonstrates the results. ATPase activity of chloroplasts which had been broken in the absence of  $\text{MgCl}_2$  was about as low as that of dark incubated chloroplasts. With 6 mM  $\text{MgCl}_2$  present in the shocking medium, the rate of the light induced ATPase activity was 58% as compared with chloroplasts which were pre-illuminated while intact. The chloroplasts shocked as above (either with or without  $\text{MgCl}_2$ ) were fully broken before illumination, as judged by the ferri-

Table 1

$\text{Mg}^{2+}$  requirement of the light activation of the ATPase in freshly broken chloroplasts

| Pretreatment                    | ATPase activity                                          |                   |     |
|---------------------------------|----------------------------------------------------------|-------------------|-----|
|                                 | Rate<br>( $\mu\text{mol}/\text{mg Chl} \cdot \text{h}$ ) | Light-dark<br>(%) | (%) |
| Light, intact                   | 66.7                                                     | 53.7              | 100 |
| broken                          | 16.1                                                     | 3                 | 6   |
| broken + EDTA, 1 mM             | 16.0                                                     | 3                 | 6   |
| broken + $\text{MgCl}_2$ , 6 mM | 44.0                                                     | 31                | 58  |
| Dark, intact                    | 14.5                                                     | —                 | —   |
| broken                          | 11.1                                                     | —                 | —   |
| broken + EDTA, 1 mM             | 12.2                                                     | —                 | —   |
| broken + $\text{MgCl}_2$ , 6 mM | 14.2                                                     | —                 | —   |

Chloroplasts were either illuminated intact (0.1 ml containing 40  $\mu\text{g}$  Chl) and then broken by a 10-fold dilution in the assay mixture, or broken about 2 min before illumination in 20 mM Na-Tricine and the indicated addition. Assay mixture was added at the end of 3 min illumination or a dark period, as indicated, containing in 1 ml the following (in final concentrations): 20 mM Na-Tricine (pH 8), 5 mM  $\text{MgCl}_2$ , 3 mM ATP containing  $4 \times 10^5$  cpm [ $^{32}\text{P}$ ]ATP, 0.5 mM  $\text{NH}_4\text{Cl}$  and chloroplasts containing 40  $\mu\text{g}$  Chl. Reaction was stopped after 4 min by trichloroacetic acid

cyanide assay [11] (not shown). The relative rate of light induced ATPase activity of chloroplasts shocked in the presence of  $\text{MgCl}_2$  as above varied in different experiments between 40–75% as compared with chloroplasts which were pre-illuminated intact. The presence of EDTA in the shocking medium had no effect (table 1) indicating that the dilution of the endogenous  $\text{Mg}^{2+}$  is sufficient to cause maximal loss of the light-activation capacity. It should be mentioned that the time course of the activation stage was similar for all treatments (intact chloroplasts or broken with or without  $\text{Mg}^{2+}$ ), being saturated within about 20 s of illumination, and stable during at least 5 min (not shown). Mills et al. have previously reported that a partial light activation of the ATPase occurs in freshly lysed pea chloroplasts but it was far slower than the activation of intact chloroplasts [3]. The different results might be due to the different experimental details or the different plants used.

ATPase activity was assayed under partially uncoupled conditions to enhance the light induced vs

Table 2

Effect of the breaking conditions on the light activation of ATP-P<sub>i</sub> exchange

| Breakage conditions                       | ATP-P <sub>i</sub> exchange               |     |
|-------------------------------------------|-------------------------------------------|-----|
|                                           | ( $\mu\text{mol/mg Chl} \cdot \text{h}$ ) | (%) |
| A. None (intact chloroplasts)             | 9.43                                      | 100 |
| + MgCl <sub>2</sub> , no Vortex           | 8.25                                      | 87  |
| B. None                                   | 13.68                                     | 100 |
| + MgCl <sub>2</sub> , 3 s Vortex          | 5.90                                      | 43  |
| 10 s Vortex                               | 2.24                                      | 16  |
| 30 s Vortex                               | 0.30                                      | 2   |
| No salt, 3 s Vortex                       | 0.14                                      | 1   |
| 30 s Vortex                               | 0.12                                      | 1   |
| 30 s Vortex, then MgCl <sub>2</sub> added | 0.08                                      | 1   |
| Dark control                              | 0.09                                      | 1   |

Chloroplasts were illuminated either intact or broken in the presence or absence of 10 mM MgCl<sub>2</sub>, as indicated. In exp.A breaking was done by gentle shaking by hand. In exp.B chloroplasts were mixed on a Vortex (medium speed) for the indicated duration. Other details were as described for table 1, except for the addition of 2 mM P<sub>i</sub> (containing  $4 \times 10^6$  cpm <sup>32</sup>P<sub>i</sub>) and the omission of [<sup>32</sup>P]ATP and NH<sub>4</sub>Cl. Assay was stopped after 8 min.

dark activity ratio as discussed before [7]. The so called dark Mg-ATPase activity does not originate in CF<sub>1</sub> since it is neither affected by energy transfer inhibitors nor by uncouplers [1,7]. Table 1 shows that it is also essentially unaffected by an osmotic shock in the presence or absence of MgCl<sub>2</sub> done prior to the dark pre-incubation.

The dependence of the light induced H<sup>+</sup>-ATPase activity of chloroplasts which were shocked before illumination on the concentration of MgCl<sub>2</sub> in the shocking medium is demonstrated in fig.1. It can be seen that mM range of concentrations was required to maintain the light activation capacity, with 3–4 mM MgCl<sub>2</sub> giving 50% activity. Intact chloroplasts, which are impermeable to Mg<sup>2+</sup> [15], were hardly affected by the presence of MgCl<sub>2</sub> during light activation. It should be mentioned that the concentration of MgCl<sub>2</sub> as well as the other ingredients present in the final assay mixture were the same for all the different samples.

The ability to maintain the endogenous light-activation of the ATPase during rupture of the chloroplast envelope did not depend only on the

presence of MgCl<sub>2</sub>. An additional factor was found to be the harshness of the mixing during the osmotic shock. In the experiments summarized in table 2, intact chloroplasts were osmotically shocked either by a gentle mixing of the test tube by hand, or on a Vortex for the indicated duration. Breaking was completed 2–4 min before light activation. In these experiments ATP-P<sub>i</sub> exchange activity was assayed after the illumination. As can be seen in table 2, gently broken chloroplasts in the presence of 10 mM MgCl<sub>2</sub> maintained 87% of the light activation capacity (table 2, exp.A) while a more vigorous mixing reduced the activity. After 30 s of Vortex mixing the ATPase was not light activated even though MgCl<sub>2</sub> was present in the shocking medium (table 2, exp.B). It is also clear from table 2 that osmotic shock in the absence of MgCl<sub>2</sub> did not depend on the mixing conditions since a short mixing was already sufficient to prevent the light activation. The addition of MgCl<sub>2</sub> after the 30 s Vortex mixing and before illumination, did not restore the activity.

The data presented so far may suggest that the factors which are involved in the light-activation of the H<sup>+</sup>-ATPase are loosely bound to the thylakoid membrane. The relatively high concentration of MgCl<sub>2</sub> which is required to maintain the activation capacity during the rupture of the envelope might suggest that the binding occurs via electrostatic bonds. If this is true, then it is expected not to be unique for Mg<sup>2+</sup>. Table 3 shows that this indeed is the case. Ca<sup>2+</sup> was found to be about as effective as Mg<sup>2+</sup> in preserving the light-activation capacity of broken chloroplasts. Mn<sup>2+</sup> or high concentration of KCl or NaCl were slightly less effective. In control experiments, the different salts were added to the assay medium of chloroplasts which had been pre-illuminated intact. As can be seen (table 3, column (2)), the extra Me<sup>2+</sup> (2 mM on top of the 5 mM MgCl<sub>2</sub> routinely present in the assay medium) was inhibitory, in the following order: Mg<sup>2+</sup> < Ca<sup>2+</sup> < Mn<sup>2+</sup>. The monovalent salts, on the other hand, slightly increased the activity when added after activation. The cause of the above inhibitory effects is not clear yet.

The presence of Zn<sup>2+</sup> or Co<sup>2+</sup> did not maintain the light-activation capacity of broken chloroplasts (table 3). This might be due to an inhibitory effect rather than their inability to maintain the ATPase modulating system attached to the membrane,

Table 3

Dependence of ATP-P<sub>i</sub> exchange activity of freshly broken chloroplasts on the cation present during breakage

| Salt added                                           | ATP-P <sub>i</sub> Exchange                             |            |                      |
|------------------------------------------------------|---------------------------------------------------------|------------|----------------------|
|                                                      | (1) broken<br>( $\mu\text{mol/mg Chl} \cdot \text{h}$ ) | (2) intact | (1) $\times$ 100/(2) |
| A. None                                              | 0.12                                                    | 11.26      | 1                    |
| MgCl <sub>2</sub> , 10 mM                            | 5.68                                                    | 9.58       | 59                   |
| CaCl <sub>2</sub> , 10 mM                            | 4.60                                                    | 8.95       | 51                   |
| MnCl <sub>2</sub> , 10 mM                            | 1.88                                                    | 4.99       | 38                   |
| ZnCl <sub>2</sub> , 10 mM                            | 0.06                                                    | 3.99       | 2                    |
| CoCl <sub>2</sub> , 10 mM                            | 0.07                                                    | 8.16       | 1                    |
| MgCl <sub>2</sub> , 10 mM + ZnCl <sub>2</sub> , 5 mM | 0.01                                                    | 6.73       | 0                    |
| MgCl <sub>2</sub> , 10 mM + CoCl <sub>2</sub> , 5 mM | 0.82                                                    | 8.47       | 10                   |
| B. MgCl <sub>2</sub> , 10 mM                         | 5.90                                                    | 13.68      | 43                   |
| NaCl, 200 mM                                         | 5.58                                                    | 15.74      | 35                   |
| KCl, 200 mM                                          | 5.50                                                    | 15.25      | 36                   |

Chloroplasts were light activated either after (1) or before (2) an osmotic shock. In (1) chloroplasts were delicately broken in 0.4 ml of 20 mM Na-Tricine (pH 8) plus the indicated salts. After light activation 1.6 ml assay medium was added containing (in final concentrations) 20 mM Na-Tricine (pH 8), 5 mM MgCl<sub>2</sub>, 2 mM NaP<sub>i</sub> (containing  $6 \times 10^6$  cpm <sup>32</sup>P<sub>i</sub>), 3 mM ATP, chloroplasts containing 30  $\mu\text{g}$  Chl and 1/5 of the indicated salt, which was present in the shocking medium. Intact chloroplasts (2) were illuminated in a volume of 0.1 ml with no additions, then osmotically shocked by the addition of 1.9 ml of assay medium containing the same final concentrations as in (1). In this case the indicated salts were present only in the assay medium, and not during light activation.

since when present together with Mg<sup>2+</sup> in the shocking medium, these ions prevented the light activation (table 3).

It has been shown that the activation of the H<sup>+</sup>-ATPase in intact chloroplasts [3,7,16] is similar to the non-physiological system of broken and washed chloroplasts [17] being dependent on both  $\Delta\text{pH}$  and thiol modulation. The components which are lost during the osmotic shock treatments described here might be involved in either  $\Delta\text{pH}$  formation or thiol modulation or both. To check these possibilities the effects of Fd, NADP and DTT on the light activation in chloroplasts freshly broken in the presence or absence of Mg<sup>2+</sup> were tested and the results are summarized in table 4. Clearly the addition of Fd and/or NADP to chloroplasts which were broken without Mg<sup>2+</sup> (MgCl<sub>2</sub> was added after the osmotic shock and before Fd and/or NADP) did not restore the light activation.

Measurements of light induced 9AA fluorescence quenching indicated that there was a substantial  $\Delta\text{pH}$  formed in chloroplasts freshly shocked without Mg<sup>2+</sup>, although somewhat smaller than that formed in chloroplasts shocked + Mg<sup>2+</sup> or in the presence of the artificial electron carrier MV. The addition of DTT to these chloroplasts, on the other hand, did restore the H<sup>+</sup>-ATPase light-activation, suggesting that the loss of the light activation capacity upon rupture without Mg<sup>2+</sup> is mostly due to the loss of the thiol modulating system rather than the inability to form  $\Delta\text{pH}$ .

In chloroplasts which were broken in the presence of Mg<sup>2+</sup> the addition of NADP before illumination was inhibitory (table 4). This result agrees well with that reported by Mills et al. [16] for broken chloroplasts supplied with Fd, Th, and FTR and is compatible with the explanation of a competition between NADP and the CF<sub>1</sub> activator

Table 4

The effect of Fd, NADP and DTT on the light activation in freshly broken chloroplasts

| Addition<br>before activation | Broken — $Mg^{2+}$            |                         |                                  | Broken + $Mg^{2+}$            |                         |                                  |
|-------------------------------|-------------------------------|-------------------------|----------------------------------|-------------------------------|-------------------------|----------------------------------|
|                               | ATP— $P_i$ exchange<br>(Rate) | 9AA fluorescence<br>(%) | 9AA fluorescence<br>(% quenched) | ATP— $P_i$ exchange<br>(Rate) | 9AA fluorescence<br>(%) | 9AA fluorescence<br>(% quenched) |
| None                          | 0.07                          | 1                       | 37                               | 2.95                          | 35                      | 51                               |
| Fd                            | 0.08                          | 1                       | —                                | 2.64                          | 32                      | —                                |
| NADP                          | 0.11                          | 1                       | —                                | 1.73                          | 21                      | —                                |
| Fd + NADP                     | 0.08                          | 1                       | —                                | 0.86                          | 10                      | —                                |
| DTT                           | 4.13                          | 51                      | 36                               | 5.94                          | 71                      | —                                |
| Fd + DTT                      | 5.88                          | 73                      | —                                | 6.37                          | 76                      | —                                |
| NADP + DTT                    | 5.46                          | 68                      | —                                | 6.72                          | 81                      | —                                |
| Fd + NADP + DTT               | 6.13                          | 76                      | —                                | 6.37                          | 81                      | —                                |
| MV + DTT                      | 8.04                          | 100                     | 54                               | 8.33                          | 100                     | 52                               |

Chloroplasts (containing 35  $\mu$ g Chl) were broken in 0.5 ml of 20 mM Na-Tricine (pH 8) either in the absence (left part) or the presence (right part) of 10 mM  $MgCl_2$  by 10 s or 2 s Vortex mixing, respectively. In the former case  $MgCl_2$  was added after the osmotic shock and before the indicated additions. Chloroplasts were then light activated, followed by the addition of 0.5 ml of double strength assay mix containing (in final concentrations): 20 mM Na-Tricine (pH 8), 2 mM  $NaP_i$  (plus  $3 \times 10^6$  cpm  $^{32}P_i$ ) and 4 mM ATP. The concentrations of Fd, NADP, DTT and MV were 0.012, 0.2, 5 and 0.2 mM, respectively. Rates are given in  $\mu$ mol/mg Chl  $\cdot$  min. For light induced 9AA fluorescence quenching chloroplasts were broken as above and then 1.5 ml of 20 mM Na-Tricine (pH 8) + 5 mM  $MgCl_2$  + 2  $\mu$ M 9AA (final concentrations) was added. The rate ATP— $P_i$  exchange of pre-illuminated intact chloroplasts was 9.95 and 9AA response was 58% (— MV) or 65% (+ MV)

for reduced Fd. It is further strengthened by the lack of inhibition by NADP when added together with DTT which can directly interact with  $CF_1$  (table 4). The addition of Fd (alone or together with NADP or DTT) to chloroplasts broken in the presence of  $Mg^{2+}$  had only slight effects, indicating that under these conditions the endogenous Fd remains partly attached to the thylakoids, as previously suggested [10].

#### 4. DISCUSSION

The activation by light of a few key enzymes of  $CO_2$  fixation has been suggested by Buchanan et al. and strongly supported by experimental results, to involve thiol modulation of these enzymes which is catalyzed by thioredoxin. The latter is photoreduced by PS I via ferredoxin and FTR. According to this review these three soluble proteins link between the light induced electron transport which takes place in the thylakoid membrane and

the modulatable enzymes of the carbon-cycle which are located in the stroma (see [18] and [19] for review). An alternative proposed mechanism for light-activation involves membranal thiol modulators [20]. The light induced modulation of the thylakoid ATP synthase which exposes its latent ATPase activity [1] is probably also mediated by the ferredoxin—thioredoxin system [3,6,7,16]. In the present work the interaction of the  $H^+$ -ATPase activating system with the thylakoid membrane has been studied. The results strongly suggest that the activating system is loosely bound to the thylakoid membrane rather than freely soluble in the stroma, with  $Mg^{2+}$  playing an important role in the binding. This is indicated by the loss of the light activation capacity upon osmotic rupture of the chloroplasts envelope in the absence of  $Mg^{2+}$ . This loss can be prevented by the presence of a few mM  $MgCl_2$  (table 1, fig.1). Maximal protecting effect required 10–12 mM  $Mg^{2+}$ , a concentration which falls within the physiological range. The

total  $Mg^{2+}$  content of the stroma has been estimated to range between 16–24 mM [15,21]. It might be higher, though in the vicinity of the thylakoid surface and lower in the bulk stromal space [22].  $Mg^{2+}$  was suggested to be the major cation present in the thylakoid surface diffuse layer in vivo [22].

The importance of  $Mg^{2+}$  for the light activation of the  $H^+$ -ATPase was also indicated in intact chloroplasts by the use of the divalent ionophore A-23187. Preliminary results showed that the depletion of  $Mg^{2+}$  by A-23187+EDTA prevented the ATPase light activation in intact chloroplasts, while the addition of  $Mg^{2+}$  before illumination partially restored the activity (data not shown). Further indications for the electrostatic nature of the binding of the modulating system to the stromal face of the thylakoid were:

- (i) The ease of detachment, even in the presence of  $Mg^{2+}$ , by vigorous mixing (i.e., by friction forces, table 2);
- (ii) the low cation specificity and the substitution by monovalent cations at high concentrations (table 3).

We have previously reported that the endogenous cyclic electron flow around PS I can be preserved in chloroplasts which are freshly broken in the presence of  $Mg^{2+}$ . The activity is lost when  $Mg^{2+}$  is absent, but can then be restored by the addition of  $Mg^{2+}$  and Fd [10]. In the activation process of the ATPase, however, Fd is not the only

essential factor which is detached from the membrane upon an osmotic shock in the absence of  $Mg^{2+}$ . As indicated in table 4 the addition of Fd to such treated chloroplasts did not restore the activity while DTT, on the other hand, did. It is thus suggested that the thiol modulating system as well as Fd are loosely attached to the thylakoids of intact chloroplasts. Upon rupture without  $Mg^{2+}$  the whole complex is solubilized, while a gentle rupture of the envelope in the presence of  $Mg^{2+}$  (or other cations) can maintain the modulating complex mostly bound to the membrane. It can be further visualized that the complex of Fd, FTR and Th is bound to FNR which is located in a close vicinity to the  $CF_1$  knob-like structure (fig.2), making the regulation of  $CF_1$  by thiol modulation most efficient. Moreover, recent reports suggest that some of the light-activated carbon cycle enzymes previously thought to be soluble in the stroma space are, at least in part, bound to the thylakoid membrane in a  $Mg^{2+}$  dependent mode [23–26]. The ATPase modulating system is probably more loosely attached to the membrane as judged by the ease of its removal (table 2) as compared with [23–26] and the failure to maintain the ATPase activation capacity of broken and washed chloroplasts (not shown). Likewise, it has been shown that the activator of NADP-dependent malate dehydrogenase is more loosely bound to the thylakoids than the enzyme itself [24].

We would like to further hypothesize, that the mechanism of light induced activation of chloroplast enzymes physically occurs on the stromal surface of the thylakoids, close to the acceptor side of PS I. The interaction of the modulating systems and the target enzymes with the membrane and with each other depends on  $Mg^{2+}$  ions and might thus be regulated by changes in local surface charge.

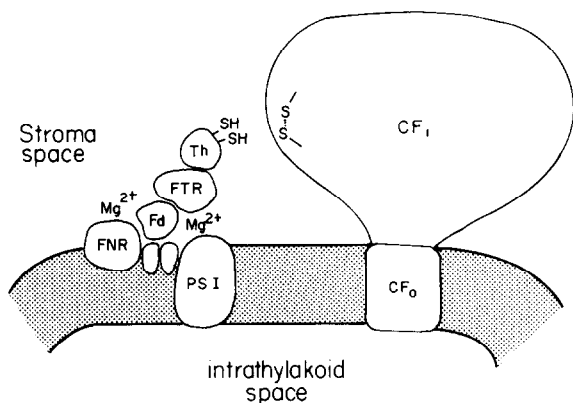


Fig.2. Schematic representation of a possible orientation of the ATP synthase complex and its thiol-modulating system in chloroplasts.

#### ACKNOWLEDGEMENT

Mrs. Drora Nadav is greatly acknowledged for technical assistance and Dr. U. Pick for helpful discussions. This research was supported by the Israel Academy of Sciences and Humanities, Basic Research Foundation.

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