View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

MODULATION OF COUPLING FACTOR ATPase ACTIVITY IN INTACT CHLOROPLASTS

The role of the thioredoxin system

John D. MILLS, Peter MITCHELL and Peter SCHÜRMANN*

Glynn Research Institute, Bodmin, Cornwall, PL30 4AU, England and **Laboratoire de Physiologie végétale et Biochimie, Université de Neuchâtel, Chemin de Chantemerle 20, CH-2000, Neuchâtel, Switzerland*

Received 5 February 1980

1. Introduction

Photophosphorylation has mostly been studied using washed, broken chloroplasts which have lost their outer envelopes and stromal contents [1]. These studies suggest that the enzyme which catalyses photophosphorylation (coupling factor, or CF_0–CF_1) can exist in several states of differing activity depending on the experimental conditions used. In dark-adapted chloroplasts, CF_0–CF_1 exists in a catalytically inactive state, but is converted into an active state on impressing $\Delta\bar{\mu}(H^+)$ across the thylakoid membrane, either in the form of ΔpH [2,3] or as $\Delta\psi$ [4,5]. The presence of an H^+-specific channel, or proton-well, through CF_0 effectively converts a $\Delta\psi$ into a ΔpH across CF_1 [6]. Thus the active state of the anisotropic coupling factor complex may only be induced near a dual pH optimum, when the stromal side of CF_1 is close to pH 8 and the CF_0 side is near pH 5. This situation corresponds to the normal environmental condition in illuminated intact chloroplasts (i.e., those that retain their outer envelopes and stromal contents [7]).

In broken chloroplasts, the catalytic properties of CF_0–CF_1 near the dual pH optimum depend on the presence or absence of dithiols. In the absence of dithiols, CF_0–CF_1 is observed to catalyse net synthesis, but not net hydrolysis of ATP, even though in both cases bond formation between the β and γ phosphoryl groups of ATP in the active site is intrinsically reversible [8]. If dithiols are included in the medium, incubation of CF_0–CF_1 near its dual pH optimum permits expression of MgATPase activity [2,9]. It has been proposed that dithiols modify CF_0–CF_1, converting it from a form that does not catalyse net hydrolysis of ATP to one that does [10], though the mechanism by which this occurs, or why ATPase activity is not expressed in the unmodified enzyme, are largely matters of conjecture.

It is of some importance to establish whether or not dithiol modification of coupling factor has any physiological significance. Buchanan and coworkers [11] have shown the existence in the stroma of Th, a dithiol protein widely distributed in nature. Thioredoxin is photoreduced in the chloroplast by ferredoxin and the enzyme Fd-Th reductase [11]. Reduced Th is thought to activate certain soluble enzymes of the stroma, and in addition has been shown to stimulate the appearance of ATPase activity that is observed on heating solubilised CF_1 in the presence of DTT [12].

In this letter, we show that stromal proteins in an unfractionated stromal extract are able to mimic the effects of dithiols in respect of their ability to unmask ATPase activity in washed broken chloroplasts maintained near the dual pH optimum by illumination. Similar results are obtained using purified preparations of Fd-Th reductase and Th_f in place of the crude stromal extract. The results suggest that dithiol modification of CF_0–CF_1 is of physiological significance,

Abbreviations: $\Delta\bar{\mu}(H^+)$, ΔpH and $\Delta\psi$, difference in electrochemical potential of protons, pH or electrical potential, respectively, between the aqueous phases separated by the thylakoid membrane; CF_0–CF_1, chloroplast coupling factor; DCCD, *N*-*N*′-dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; DTT, dithiothreitol; Fd, ferredoxin; MalNEt, *N*-ethylmaleimide; MV, methyl viologen; Th or Th_f, thioredoxin or thioredoxin$_f$; tricine, *N*-tris(hydroxymethyl)-methylglycine

and may reflect a means by which the enzyme is regulated in vivo.

2. Materials and methods

Intact chloroplasts were isolated from 60–80 g of 9–11 day old *Pisum sativum* seedlings (variety Feltham First) ground in 160 ml of a medium containing 0.35 M sorbitol, 5 mM ascorbic acid and 20 mM 2-(*N*-morpholino)ethanesulphonic acid brought to pH 6.5 with KOH. Chloroplasts were centrifuged at 3000 × *g* for 60 s, washed once in 40 ml of grinding medium and resuspended in a minimal volume of 0.35 M sorbitol, 2 mM ethylenediaminetetraacetic acid, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid brought to pH 7.6 with KOH. Chloroplasts were routinely 50–70% intact as judged by their impermeability to ferricyanide, but fixed CO_2 at rates <10 μmol O_2 evolved/(mg chlorophyll.h). These preparations were thus intermediate between types A and B as defined in [14].

For preparation of broken chloroplasts (type C of [14]) and stromal protein, the washed pellet as above was resuspended in 2 ml of 5 mM $MgCl_2$, 15 mM tricine–KOH (pH 8.0) which caused the organelles to lyse. Broken chloroplasts were collected by centrifugation and the supernatant used without further purification as the source of stromal protein.

Activation of CF_0–CF_1 was carried out by illuminating chloroplasts (white light, 1500 W/m^2) in a magnetically stirred test-tube maintained at 23°C. The activation medium consisted of:

(i) 0.25 ml of 1.2 M sorbitol, 1500 units of catalase, 100 mM tricine–KOH (pH 8.0);
(ii) 0.65 ml of 5 mM $MgCl_2$, 15 mM tricine–KOH (pH 8.0);
(iii) 0.1 ml of chloroplasts containing ~0.3 mg chlorophyll.

The order of mixing (i), (ii) and (iii) determined whether any intact chloroplasts present remained intact, or were lysed immediately prior to activation.

For assay of ATPase activity, 0.1 ml samples were periodically removed and added to 0.9 ml of a medium consisting of 2 mM $MgCl_2$, 1.1 mM NH_4Cl, 2.2 mM ATP and 15 mM tricine–KOH (pH 8.0) contained in centrifuge tubes in a shaking water bath (25°C) maintained in darkness. After 4 min, ATPase activity was stopped with 0.5 ml 20% trichloroacetic acid, the precipitate removed by centrifugation and 0.5 ml aliquots taken for determination of P_i [15]. All experiments were carried out at Glynn Research Institute. Spinach Fd-Th reductase and Th_f were purified at the University of Neuchâtel as in [16].

3. Results

Illumination of intact spinach chloroplasts has been shown to trigger the protonmotive ATPase activity of CF_0–CF_1 [17,18]. Fig.1(a) shows that similar results are observed using intact chloroplasts from pea seedlings. The figure depicts ATPase activity observed as a function of illumination time given during the activation period. In all cases, the final assay (but not the activation medium) contained 1 mM NH_4Cl which partly dissipates any ATP-dependent ΔpH across the thylakoid membrane and thereby accelerates the observed ATPase activity to near maximal rates [19]. In addition the assay medium contained no sorbitol so that any intact chloroplasts transferred from the activation stage became lysed, allowing the ATPase unrestricted access to its substrate.

As shown in fig.1(a), ATPase activity in intact chloroplasts is induced relatively rapidly by illumination, the rate being 70% maximal after 30 s in the light. In contrast, induction of ATPase activity was much slower when chloroplasts were lysed in the activation vessel (type D chloroplasts of [14]), and was incomplete even after 9 min in the light. Illumination alone was virtually ineffective using broken chloroplasts that had been lysed prior to activation then removed from the supernatant and resuspended in fresh medium (fig.1(a)).

These results suggest that the chloroplast stroma contains endogenous factors that function in the modulation of CF_0–CF_1 ATPase activity by light and which are diluted or inactivated on lysing the organelles. To investigate this idea further, we studied the effect of adding back unfractionated stromal protein to broken chloroplasts. As shown in fig.1(b), this procedure resulted in the observation of considerable ATPase activity after illumination of the chloroplasts, in marked contrast to the almost complete lack of effect of light seen in the absence of the stromal protein. Several observations suggest that the ATPase activity can be ascribed to CF_0–CF_1:

(i) It requires preillumination and the final activity observed corresponds closely to that seen in the

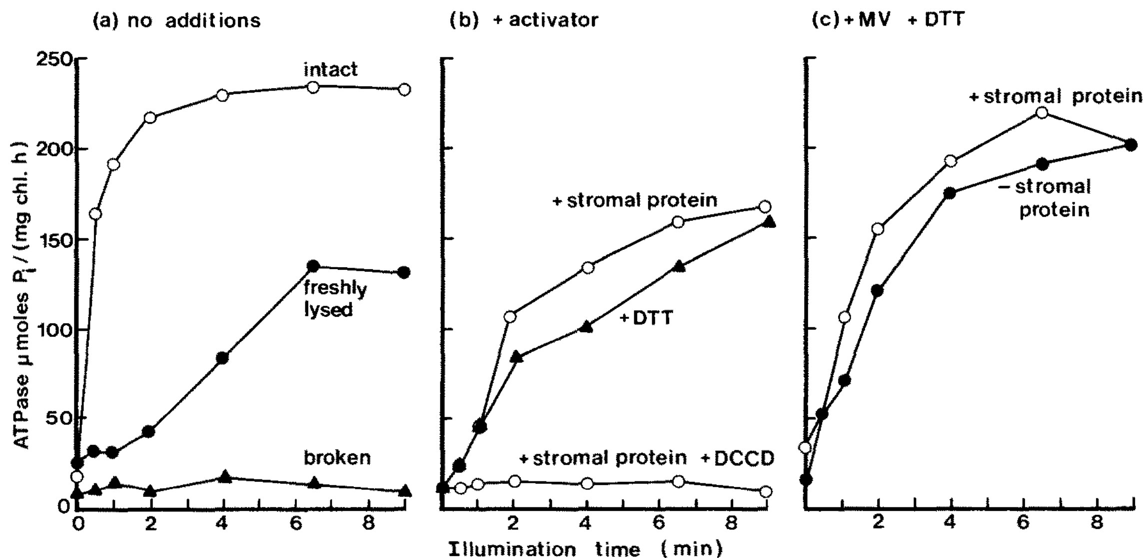

Fig.1 Light-triggered MgATPase activity of CF_0-CF_1 in pea chloroplasts. The final concentration of additions (where indicated) to the activation stage was: ~20 mg stromal protein/ml, 5 mM DTT, 0.1 mM DCCD, 50 µM MV. Asays commenced after 5 min preincubation in the activation stage. All chloroplast samples were dark-adapted for at least 1 h.

presence of DTT, which is known to modulate coupling factor (fig.1(b) and [10]);

(ii) ATPase activity that is induced by light is completely inhibited by 0.1 mM DCCD (fig.1(b));

(iii) 1 mM NH_4Cl stimulates the rate 2–3-fold;

(iv) Induction of 9-aminoacridine fluorescence quenching is also observed to parallel induction of ATPase activity (results not shown).

The latter two observations indicate that the ATPase is protonmotive [6,10,18,19]. Virtually no ATPase activity is displayed by stromal proteins in the absence of chloroplasts.

As depicted in fig.1(c), the presence of an electron acceptor (MV) in the activation stage only slightly increased the final rate observed in the presence of DTT. It seems therefore that under these conditions, endogenous electron flow is sufficient to generate the ΔpH required to bring CF_0-CF_1 close to its dual pH optimum.

The addition of stromal protein to chloroplasts treated with MV plus DTT generally enhanced the ATPase activity observed after illumination (fig.1(c)), particularly at shorter illumination times where stimulations in rate of up to 50% have been noted.

One other feature apparent in the data of fig.1 is the low level of ATPase activity observed in dark-adapted chloroplasts. As noted elsewhere [18], the ATPase responsible for this activity is not protonmotive, is resistant to lysis and washing of the membranes and is not much affected by a variety of treatments which modify the response of the CF_0-CF_1 ATPase, including most of those reported in this letter. At present, the source of the dark activity is not known.

The results presented so far suggest that an unidentified factor in the stromal fraction functions in the same manner as DTT in the modulation of coupling factor activity. Several observations (data not shown) suggest that the unidentified factor is a protein:

(i) Heating stromal protein to 60°C for 5 min destroys its ability to modulate ATPase activity;

(ii) Treatment of stromal protein with trypsin (1–2%, w/w) inactivates the factor by 50% (and higher concentrations of trypsin are even more inhibitory);

(iii) As shown in table 1, the factor does not pass through a dialysis membrane.

Table 1 also shows that the stromal protein is partly inactivated by treatment with MalNEt. The inhibitory effect of MalNEt was increased when the stromal fraction was pretreated with DTT. The latter result indicates that the active protein in the stromal fraction contains essential sulphydryl groups that are partially reducible by DTT and available for reaction

Table 1
Inactivation of stromal protein by treatment with MalNEt

Pretreatment of stromal protein[a]	ATPase activity [μmol P_i released/(mg chlorophyll . h)]	
	Dark[b]	Light[b]
1. Untreated	21	164
2. Dialysed	18	157
3. MalNEt	12	92
4. DTT, MalNEt	17	53

[a] Pretreatment of stromal protein was as follows: 1. No MalNEt, DTT or dialysis, 2. stromal protein (~40 mg protein/ml) treated with (5 mM DTT + 9 mM MalNEt) and dialysed for 4 h against 2 × 1000 ml 5 mM $MgCl_2$, 15 mM tricine–KOH (pH 8.0); 3. stromal protein treated with (2 mM DTT + 9 mM MalNEt) for 15 min, then 3 mM DTT added and dialysed as above; 4. stromal protein incubated with 2 mM DTT for 5 min, then 9 mM MalNEt added. After 15 min, 3 mM DTT was added and dialysed as above

[b] ATPase activity was assayed as in fig.1 except that the volume of activation stage was reduced to 0.4 ml. Dark samples were taken after 5 min preincubation in the dark immediately prior to illumination (8 min)

with MalNEt. A possible candidate for such a protein is Fd-Th reductase [11].

We have not yet isolated the active factor from the pea stromal protein used so far in these experiments. However, purified Fd-Th reductase and Th_f from spinach have been tested for their ability to modulate the ATPase activity of broken chloroplasts in the light. The results of these experiments are shown in table 2. Under the anaerobic conditions used, considerable ATPase activity was indeed observed following a period of illumination in the presence of these two proteins. The appearance of activity on illumination was dependent on the presence of both Fd and Fd-Th reductase but not Th_f. However, the presence of Th_f in the activation medium usually enhanced the final ATPase activity (by up to 25%, though the magnitude of this stimulation was somewhat variable). The experiments reported in table 2 were carried out in the presence of DCMU and an electron donor system (DCPIP/ascorbate) to prevent photosynthetic O_2 evolution. If these were omitted, or the experiment carried out under air instead of N_2, the light-induced ATPase activity was much decreased. The latter two observations demonstrate the remarkable sensitivity to O_2 that is displayed by the purified Fd-Th reductase. This sensitivity has been noted before [20], and is not displayed by the unpurified enzyme ([21] and this paper). Sensitivity to O_2 is a property of the reductase that appears during its purification, though the reason for this interesting phenomenon is unknown.

4. Discussion

The mechanism by which dithiols modulate the ATPase activity of CF_0–CF_1 is not well understood. This paper does not attempt to elucidate the details of the mechanism, but does point out that the process probably occurs in vivo and suggests that the thioredoxin system is the physiological dithiol reductant

Table 2
Effect of Fd-Th reductase and Th on light-triggered CF_0–CF_1 ATPase activity

Omission from activation medium	ATPase activity [μmol P_i released/(mg chlorophyll . h)]	
	Dark	Light
Complete[a]	28	129
– Reductase	25	33
– Fd	26	53
– Th_f	26	121
– DCMU/ascorbate/DCPIP	25	33
Complete (gas phase air)	25	28

[a] Conditions were as in section 2 except chlorophyll was 0.6 mg/ml, and other additions were 1.4 μM Fd-Th reductase, 2.2 μM Th_f, 3.3 μM DCMU, 5 mM ascorbate, 0.1 mM DCPIP. The activation tube was flushed with N_2 (5 min) in the dark, then samples (50 μl) taken for assay of dark ATPase activity immediately prior to illumination (7.5 min)

involved. Experiments using both a crude stromal fraction and purified Fd-Th reductase show that these stromal proteins cause the ATPase activity of CF_0-CF_1 to be expressed following a period of illumination of broken chloroplasts. Illumination probably has two functions under these conditions:

(i) To photoreduce the Fd-Th reductase (via Fd);

(ii) To generate a transthylakoid ΔpH and thus bring CF_0-CF_1 into its active state at the dual pH optimum.

In view of the relatively small effects of Th_f, it is likely that Fd-Th reductase interacts directly with CF_0-CF_1 in its active state. This would be consistent with the observations in [12] that the δ subunit of CF_1 has Th-like activity.

It should be noted that the highest rates of ATP hydrolysis were observed after illuminating intact chloroplasts in the absence of added dithiols. Since the percentage of intact chloroplasts present was only 50–70% as judged by their permeability to ferricyanide, it seems likely that the ATPase activity of those chloroplasts that were permeable to ferricyanide was also induced by illumination. One explanation for this result is that Fd-Th reductase is normally loosely bound to the thylakoid and is not dislodged during chloroplast preparation until the organelles are subjected to an osmotic shock. At this stage, we do not rule out the possibility that other stromal dithiols also participate in the dithiol modification of CF_0-CF_1 in the intact chloroplast.

Finally, the results emphasise how CF_0-CF_1 can exist in several states of differing activity depending on the experimental conditions used. It seems likely however that the state capable of catalysing net hydrolysis of ATP at high rates is the physiological state of the enzyme in vivo during illumination. This conclusion is supported by experiments of Dr R. H. Marchant (personal communication) which show that preillumination of spinach leaves increases the ATPase activity detected in subsequently isolated chloroplasts. At this stage it is not clear what advantage is gained by the dithiol modification since the unmodified coupling factor is competent as an ATP synthetase [1]. Moreover, it is undesirable that the ATPase activity be expressed in vivo during darkness. In fact, evidence suggests that the CF_0-CF_1 undergoes inactivation in the intact chloroplast after a light to dark transition [17,18]. The mechanism by which this occurs is not known and is currently under investigation.

Acknowledgements

We thank Dr Jennifer Moyle for general discussion and Robert Harper, Alan Jeal and Stephanie Key for expert technical assistance and help in preparing the manuscript. Financial support for this work was provided by a grant from the Swiss National Science Foundation to P.S. and by Glynn Research Ltd.

References

[1] Jagendorf, A. T. (1977) in: Encyclopaedia of Plant Physiol. (Trebst, A. and Avron, M. eds) new ser., vol. 5, pp. 307–337, Springer-Verlag, Berlin.
[2] Kaplan, J. H. and Jagendorf, A. T. (1967) J. Biol. Chem. 243, 972–979.
[3] Smith, D. J., Stokes, B. O. and Boyer, P. D. (1976) J. Biol. Chem. 251, 4165–4171.
[4] Harris, D. A. and Crofts, A. R. (1978) Biochim. Biophys. Acta 502, 87–102.
[5] Gräber, P., Schlodder, E. and Witt, H. T. (1979) Biochim. Biophys. Acta 461, 426–440.
[6] Mitchell, P. (1968) Chemiosmotic Coupling and Energy Transduction, Glynn Research Ltd., Bodmin, Cornwall.
[7] Heldt, H. W., Werden, K., Milovancev, M. and Geller, G. (1973) Biochim. Biophys. Acta 314, 224–241.
[8] Wimmer, M. J. and Rose, I. A. (1977) J. Biol. Chem. 252, 6769–6775.
[9] Petrack, B., Craston, A., Sheppy, F. and Farron, F. (1965) J. Biol. Chem. 240, 906–914.
[10] Bakker-Grunwald, T. and Van Dam, K. (1974) Biochim. Biophys. Acta 347, 290–298.
[11] Buchanan, B. B., Wolosiuk, R. A. and Schürmann, P. (1979) Trends Biochem. Sci. 4, 93–96.
[12] McKinney, D. W., Buchanan, B. B. and Wolosiuk, R. A. (1979) Biochem. Biophys. Res. Commun. 86, 1178–1184.
[13] Heber, U. and Santarius, K. A. (1970) Z. Naturforsch. 256, 718–728.
[14] Hall, D. O. (1972) Nature New Biol. 235, 125–126.
[15] Mitchell, P. (1954) J. Gen. Microbiol. 11, 73–82.
[16] Schürmann, P. (1979) Experientia 35, 943.
[17] Inoue, Y., Kobayashi, Y., Shibata, K. and Heber, U. (1979) Biochim. Biophys. Acta 504, 142–152.
[18] Mills, J. D. and Hind, G. (1979) Biochim. Biophys. Acta 547, 455–462.
[19] Carmeli, C. (1969) Biochim. Biophys. Acta 189, 256–266.
[20] Schürmann, P. and Jacquot, J. P. (1979) Biochim. Biophys. Acta 569, 309–312.
[21] Buchanan, B. B., Schürmann, P. and Kalberer, P. P. (1971) J. Biol. Chem. 246, 5952–5959.