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The effect of energization and of thiol modulation on ATP hydrolysis by soluble and membrane-bound chloroplast coupling factor

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The effects of energization and of thiol modulation on ATPase activity of chloroplast coupling factor 1 are described. At 64°C the enzyme hydrolyses ATP both with Mg or Ca ions and is insensitive to thiol modulation. Cooling down to 37°C converts the enzyme to a modulation-sensitive, Ca-specific ATPase. Mg-ATPase activity of chloroplasts is only slightly stimulated by thiol modulation when measured during illumination, yet, the presence of thiol reducing agents in the light largely stimulates ATPase activity in the subsequent dark period. It is suggested that the enzyme exists in two different catalytically active states: (1) energized state, insensitive to thiol-modulation, and (2) deenergized state, stimulated by thiol-modulation.

Chloroplast coupling factor 1 (CF_1 Light activation Heat activation ATP hydrolysis Me^{2+} specificity Thiol modulation

1. INTRODUCTION

Chloroplast coupling factor 1 (CF_1) is a latent-ATPase [1]. This property is characteristic to the enzyme of photosynthetic organisms but not of mitochondria or of non-photosynthetic bacteria [2]. Activation of the enzyme, which leads to manifestation of its catalytical activity, is brought about by light-energization which induces the formation of a pH gradient across the chloroplast membrane and which is also the driving force for ATP formation [3].

Illumination of broken chloroplasts in the presence of thiol reducing agents leads to a modulation of the enzyme which is manifested by the capacity to hydrolyze ATP also in the subsequent dark period [4,5]. It has been suggested that thioredoxin is involved in the light triggering of Mg-ATPase in intact chloroplasts in vivo [6]. Since thiol reducing agents have hardly any effect on ATP synthesis in the light it has been assumed that the effect of thiol modulation is to inhibit the deactivation of the enzyme in the dark [4,5]. This hypothesis was supported also by the effect of dithiothreitol (DTT) on the exchange of tightly-bound adenine nucleotides in CF_1 which is closely correlated with the activation of the chloroplast ATPase complex (CF_0–CF_1 ATPase) [7,8]; thus, Shoshan and Selman have demonstrated that the presence of DTT during illumination decreases the rebinding of ATP to the tight-sites in the dark [9].

In [10] it has been demonstrated that a heat treatment of purified CF_1 in the presence of DTT activates a Ca-dependent ATP hydrolysis in CF_1 also indicating the involvement of thiol modulation in the activation.

There is also ample evidence for a more direct involvement of SH groups in ATP synthesis and hydrolysis from the effects of SH reagents. Illumination of chloroplasts in the presence of SH reagents such as *N*-ethylmaleimide (NEM) derivatives [11], dithionitropyridine [12] or SH oxidizing agents such as iodosobenzoate [13] or permanganate [14] inhibited photophosphorylation and

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ATP hydrolysis. Monofunctional SH reagents generally lead to energy transfer inhibition whereas bifunctional reagents lead to uncoupling of photophosphorylation [15]. These results indicated that vicinal SH groups, located in the γ subunit of CF_1, are exposed upon illumination, which are involved in the activation and in the mechanism of ATP synthesis and hydrolysis.

We have recently shown that the nonionic detergent octylglucoside activates a Mg-specific ATPase in purified CF_1 [16,17]. We have also shown that preincubation of CF_1 with OG followed by a dilution to below the critical-micellar-concentration of the detergent activates a Ca-specific ATPase in CF_1. One of the peculiar observations in these studies was that DTT hardly affects Mg-ATPase activity in the presence of OG but largely stimulates Ca-ATPase activity when present with OG during the preactivation stage.

This paper demonstrates that thiol-modulation does not affect ATPase activity of chloroplasts in the light or of CF_1 at 64°C but stimulates the activity of chloroplasts in the dark and of CF_1 after cooling to 37°C. Based on these results it is suggested that CF_1 may exist in two different catalytically active conformational states which can be distinguished by the effect of thiol-modulation on the catalytical activity and in the soluble enzyme also by differences in the Me^{2+} specificity.

2. MATERIALS AND METHODS

Chloroplast thylakoid membranes were prepared from spinach (*Spinaca oleracea*, Hybrid 424) according to [18]. Coupling factor 1 (CF_1) was prepared from spinach chloroplasts according to [19].

Activation of Mg-ATPase in chloroplast thylakoids was performed by a 3 min preillumination of chloroplasts (100 μg chlorophyll) at 23°C with saturating white light (7×10^6 ergs/cm^2/s) in 1 ml containing 30 mM Na-tricine (pH 8), 30 mM KCl, 2 mM $MgCl_2$, 5 mM Na-phosphate, 30 μM PMS and 5 mM DTT. After 3 min the light was either turned off or left on and after 2 additional minutes the reaction was started by addition of 2 mM [γ-^{32}P]ATP (35 μCi/mmol), 5 mM phosphoenolpyruvate and 20 units pyruvate kinase. The reaction was stopped with 5% trichloroacetic acid and $^{32}P_i$ was extracted as previously described [16,17]. ATPase activity of soluble CF_1 was measured by incubating CF_1 (0.5 mg/ml) in a solution containing: 40 mM Na-tricine (pH 8), 10 mM ATP containing [γ-^{32}P]ATP (20 μCi/mmol), 10 mM DTT and either 4 mM $MgCl_2$ or 15 mM $CaCl_2$ [17]. 30 mM octylglucoside was included where indicated. CF_1 was added to the solution after 1–2 min of preincubation at the desired temperature. The incubation was terminated by the addition of a 10-fold access of ice-cold 5% trichloroacetic acid, and $^{32}P_i$ was extracted [17]. Pre-activation of CF_1–ATPase was performed under identical conditions but in the absence of [γ-^{32}P]ATP. The incubation was terminated after 2 min at 64°C (in the absence of OG) or at 45°C (in the presence of 30 mM OG) by cooling the enzyme suspensions to 23°C, transferring 100 μl samples through Sephadex G-50 columns pre-equilibrated with 40 mM Na-tricine (pH 8) plus 0.1 mM EDTA by the Sephadex-centrifugation procedure [20], followed by a 5-fold dilution with tricine–EDTA buffer. ATP hydrolysis was measured by incubating 4 μg pre-activated CF_1 for 10 min at 37°C in 0.5 ml containing: 40 mM Na-tricine (pH 8), 4 mM ATP containing [γ-^{32}P]ATP (20 μCi/mmol) and either 2 mM $MgCl_2$ or 8 mM $CaCl_2$. [γ-^{32}P]ATP was prepared by illumination of chloroplasts in the presence of ADP and $^{32}P_i$ as previously described [16,17].

Octylglucoside was obtained from CalBiochem and all other reagents were obtained from Sigma Chemical Co.

3. RESULTS

3.1. *The effects of temperature and of octylglucoside on the Me^{2+} specificity of ATPase activity of soluble CF_1 and on its sensitivity to thiol modulation*

The effect of temperature on Ca-ATPase and on Mg-ATPase activities of latent CF_1 in the presence or absence of OG is demonstrated in fig.1.

Increasing the temperature from 37°C to 64°C (in the absence of OG) stimulates both Ca-ATP and Mg-ATPase activities of CF_1. Yet, the ratio Mg-ATPase/Ca-ATPase increases with increasing the temperature from 0.3 at 45°C to 0.8 at 64°C which is the temperature that is routinely used for heat-activation of CF_1–Ca-ATPase [10]. In the presence of 30 mM OG, CF_1 is a Mg-specific ATPase at all the temperatures tested, as previ-

ously described [16,17]. At above 45°C and in the presence of OG, the enzyme is inactivated within a few minutes [17].

A comparison of the initial rates of ATP hydrolysis at 64°C in the presence of either Ca or Mg shows an initial lag of about 12 s in the onset of ATP hydrolysis in the presence of Mg but not with Ca while the steady-state rates of hydrolysis in the presence of Ca or Mg are quite similar (29 and 25 μmol ATP hydrolyzed.mg protein^{-1}.min^{-1} respectively, not shown). This may be due to a slower activation of Mg-ATPase as compared to Ca-ATPase, and suggests that a higher energy barrier has to be overcome for Mg-ATPase activation. This is consistent with the observation that a higher temperature is required for Mg-ATPase activation in the absence of OG (fig.1). The situation seems to be reversed in the presence of OG, since Mg-ATPase is activated at much lower temperatures than Ca-ATPase (fig.1).

The specificity of CF_1-ATPase for Mg and Ca is remarkably altered after cooling or diluting the CF_1–OG mixture as is demonstrated from the comparison of tables 1 and 2. Under these conditions CF_1 is converted to a Ca-specific ATPase and the maximal rates of ATP hydrolysis in the presence of Mg under optimal conditions (with respect to Mg and ATP concentrations) are 10–15% of the Ca-ATPase activity, as was also reported previously [17,21].

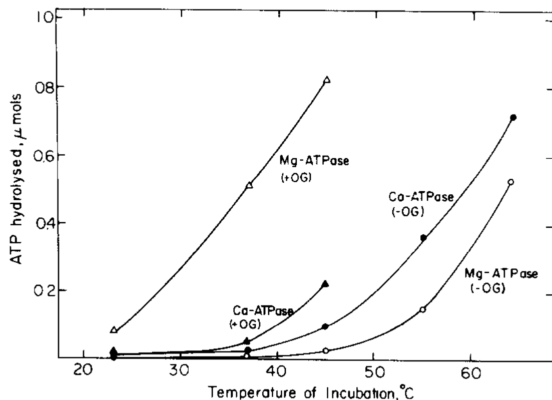

Fig.1. The effect of temperature on Ca-ATPase and on Mg-ATPase activities of CF_1 in the presence and in the absence of OG. ATP hydrolysis was measured by incubating CF_1 for 1 min at the indicated temperatures in the presence or absence of 30 mM octylglucoside as described in section 2.

Table 1

The effect of DTT on ATP hydrolysis at 64°C and in the presence of OG

Addition to assay medium	ATP hydrolyzed μmol hydrolyzed.mg protein^{-1}.min^{-1}			
	At 64°C		At 45°C + OG	
	Ca-ATPase	Mg-ATPase	Ca-ATPase	Mg-ATPase
–	15.0	10.4	5.8	18.6
+ 20 mM DTT	16.2	10.6	6.2	19.6

ATP hydrolysis was measured as in fig.1 by incubation of CF_1 (0.5 mg/ml) for 2 min at 64°C or at 45°C (+ 30 mM OG) in the presence or absence of 20 mM DTT

Table 2

The effects of DTT, on heat-activation and on OG-activation of CF_1-ATPase

Additions to preincubation medium	Rate of ATP hydrolysis μmol hydrolyzed.mg protein^{-1}.min^{-1}			
	Heat-activated at 64°C		OG-preactivated at 45°C	
	Ca-ATPase	Mg-ATPase	Ca-ATPase	Mg-ATPase
–	0.4	0.05	0.4	0.05
ATP	6.3	1.05	4.2	0.8
ATP + DTT	11.6	1.95	9.8	1.7
DTT	0.2	0		

CF_1 (0.5 mg/ml) was preactivated for 2 min at 64°C or at 45°C (plus 30 mM OG) in the presence of the indicated additions. The concentrations of ATP and DTT in the preincubation medium were 10 mM, respectively. The incubation was terminated by cooling the samples to 23°C followed by Sephadex-centrifugation. For additional details see section 2

DTT has only a minor effect on Ca-ATPase or Mg-ATPase in the presence of OG or at 64°C (2–8% stimulation, table 1), however, after cooling or OG dilution, the effect of DTT is clearly manifested on both Ca-ATPase and Mg-ATPase (2-fold stimulation, table 2). This ATP-dependent DTT activation requires its presence during the pretreatment (preincubation with OG or at 64°C)

rather than in the assay medium since separation of the DTT before the assay by Sephadex-centrifugation does not inhibit ATPase activity. In this state the enzyme remains fully active for several days. Similarly, addition of 5 mM DTT to the assay medium of enzyme preparations which were preactivated in the absence of DTT only slightly stimulate ATP hydrolysis (not shown). The effect of DTT is, therefore, due to thiol-modulation during the heat (or OG) preactivations. Yet, it is not manifested in the catalytical properties of the enzyme at 64°C or in the presence of OG.

It appears, therefore, that the enzyme possesses different catalytical properties during and following activation. During activation (in the presence of OG micelles or at 64°C) the activity is characterized by a relatively high Mg-ATPase/Ca-ATPase ratio (> 0.8) and by the minor effect of thiol modulation on ATP hydrolysis. Following activation the enzyme is converted to Ca-specific ATPase (Mg-ATPase/Ca-ATPase < 0.2) and the rate of ATP hydrolysis is markedly stimulated by thiol modulation.

3.2. *The effect of thiol modulation on Mg-ATPase activity of chloroplasts during illumination and after light triggering*

Figure 2 demonstrates the effect of DTT when present during the illumination of chloroplasts, on Mg-ATPase activity in the light and in the subsequent dark period. Chloroplasts have been preilluminated for 3 min in the presence or absence of DTT and [γ-^{32}P]ATP hydrolysis was measured 2 min later in the dark or in the light. In order to avoid rephosphorylation of the released $^{32}P_i$ the reaction was carried out in the presence of a large pool of inorganic phosphate (5 mM) and an ATP regenerating system (phosphoenolpyruvate and pyruvate kinase). The experiment shows that in the dark the rate of ATP hydrolysis is much faster than in the light as could be expected for a proton-translocating ATPase in the presence of a large opposing proton gradient. The experiment also demonstrates that in the light DTT only slightly stimulates ATP hydrolysis (by about 20%) while it largely stimulates ATP hydrolysis in the subsequent dark period (by about 150%). DTT had hardly any effect on ATP hydrolysis when added 2 min after illumination, just before the assay, in agreement with previous results. It appears there-

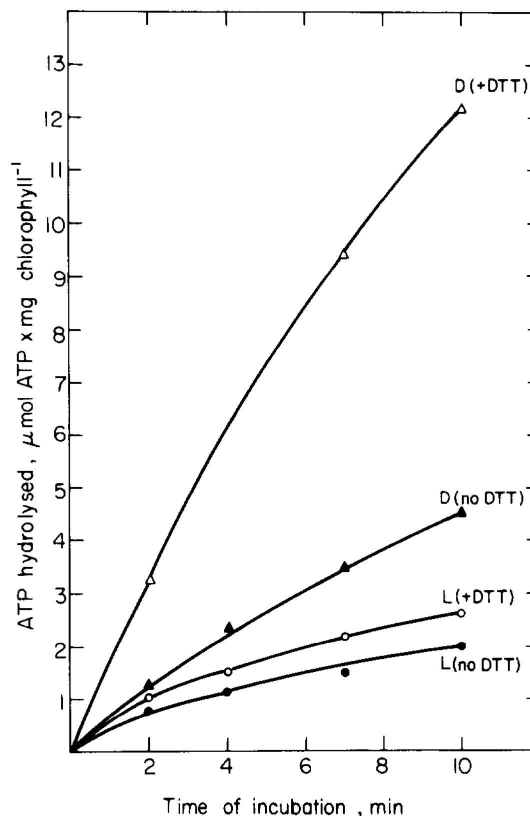

Fig.2. The effect of DTT on Mg-ATPase activity of chloroplasts in the light and in the dark. Chloroplasts (100 μg) have been preilluminated 3 min at 23°C in the presence or absence of 5 mM DTT and the hydrolysis of [γ-^{32}P]ATP was measured 2 min later in the light or in the dark as described in section 2.

fore that thiol modulation takes place during illumination in agreement with Bakker-Grunwald and Van Dam [5]. The DTT stimulation of dark ATPase activity is not due to a slower decay of the ΔpH since it is completely dissipated during the 2 min in the dark in either the presence or absence of DTT (not shown). This differential effect of DTT on ATP hydrolysis in the light and in the dark may be due either to: (a) the fact that in the light ATP hydrolysis takes place against a large transmembrane ΔpH and the rate limiting step in the reaction may be a partial reaction which is coupled to proton uptake and therefore the effect of thiol modulation is not manifested, or (b) the catalytical mechanisms of ATP hydrolysis in the light and in the dark are different, namely, thiol modu-

lation stimulates ATP hydrolysis in the deenergized chloroplasts, but not in energized chloroplasts.

4. DISCUSSION

The results described here indicate that there is a close analogy between the effect of illumination on membrane-bound CF_1 and the effect of heat or OG micelles on the soluble enzyme with respect to thiol modulation: (a) Both illumination of chloroplasts and heating or the presence of OG in CF_1 expose thiol groups to DTT in agreement with previous reports (reviewed in [22]). (b) The effect of thiol modulation on the catalytical activity (ATP hydrolysis) is not manifested in illuminated chloroplasts or in CF_1 at either 64°C or with OG but becomes apparent in the dark (chloroplasts) and after cooling or dilution (CF_1).

It appears, therefore, that CF_1 undergoes 'energization' by ΔpH (chloroplasts) or by either heat or by the contact with OG micelles (CF_1) which induces the exposure of an s–s bond to reduction by DTT and is manifested also by differences in the mechanism of ATP hydrolysis with regard to the involvement of thiol groups in the catalytical mechanism and in soluble CF_1 also with regard to the divalent cation specificity.

This idea is summarized schematically in fig.3. It is assumed that energization induces a conformational change in CF_1 from a deactivated state (latent ATPase) to a catalytically-active state. The activation involves exposure of an s–s bond in the γ subunit to reduction by thiol reducing agents. Yet, the modulation does not affect the catalytical properties of the enzyme at this energized state. Upon deenergization the modulated enzyme relaxes to a different catalytically active state which is characterized by a higher activity than the deactivated-demodulated state.

This idea is consistent with several previous indications for conformational changes in thylakoid-bound CF_1 in response to illumination such as the release of bound nucleotides [7,8], alterations in the affinities for ATP and ADP [23,24], exposure of SH [22,25] and of lysine groups [26] to chemical modifications, light-dependent fluorescence signals from probes covalently attached to the enzyme [26,27] and light-dependent water exchange into CF_1 [28]. Similarities between the effect of light (chloroplasts) and of heat (CF_1) on the exposure of

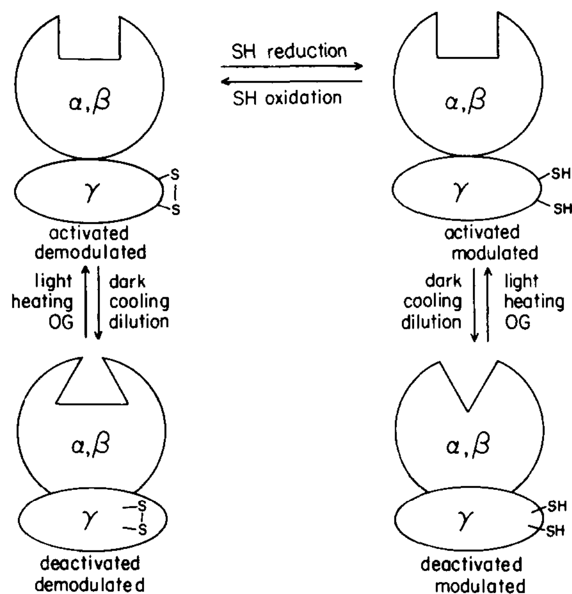

Fig.3. Summarizing scheme of the effects of energization and of thiol modulation CF_1-ATPase.

SH groups in CF_1 have also been reported [22]. The analogies between the effect of energization on the membrane-bound and on the soluble enzyme are also evident from the similarities in the properties of CF_1 in the presence of OG as compared to the light-activated membrane-bound enzyme. In both cases the enzyme is characterized by a Mg-specific ATPase activity [16,17], by the stability to high salt concentrations [17], and by ADP exchange at the tight-binding sites (Pick and Bassilian, unpublished).

There are two major differences between the membrane-bound and the soluble enzyme which are inconsistent with the generalized scheme in fig.3: (1) Deenergization of demodulated chloroplasts induces a complete reversal to the latent-ATPase state [5]. In contrast deenergization of CF_1 induces relaxation to a stable Ca-ATPase state. This difference may suggest that complete reversal to the latent ATPase state is strictly dependent on the interactions with CF_0 and is impaired by the detachment of CF_1 from the membrane. (2) Deenergization of thiol-modulated CF_1 induces Ca-specific ATPase activity rather than Mg-ATPase activity as in chloroplasts. These changes in the Me^{2+} specificity may be also a reflection of the conformational changes induced in CF_1 upon detachment from the membrane [17]. A possible,

alternative explanation for this difference is that in chloroplasts, which hydrolyze ATP in the dark, deenergization is not complete since ATP hydrolysis is coupled to proton uptake and generation of a ΔpH [29]. In this respect it is interesting to mention the work of Webster et al. [30] with *R. rubrum* chromatophores which demonstrated that the presence of high uncoupler concentration alters the Me^{2+} specificity of ATP hydrolysis from a Mg-specific to a Ca-specific ATPase. These results are consistent with the hypothesis suggested here. The demonstrations of the higher uncoupler sensitivity of Mg-ATPase as compared to Ca-ATPase in chloroplast thylakoids [1] and in purified CF_0–CF_1-ATPase proteoliposomes [31] is also consistent with this hypothesis.

Finally, one of the implications of these results is that energization results in a modification of the interactions between the α-β (catalytical subunits) and between the γ subunit which is the site of SH modulation. These results are consistent with the suggestion of McCarty [32] that the subunit may be the proton gate which is activated upon energization and is coupled to ATP hydrolysis.

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