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Control of ATP hydrolysis in chloroplasts

Heinrich Strotmann, Sigrid Kleefeld and Detlev Lohse

Institut für Biochemie der Pflanzen, Universität Düsseldorf, Universitätsstraße 1, D-4000 Düsseldorf, FRG

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Dark ATP hydrolysis catalyzed by the membrane-bound preactivated thiol-modified chloroplast ATPase was measured at constant initial ATP concentration and constant initial phosphate potential $c_{ATP}/c_{ADP} \cdot c_{P_i}$ which was adjusted by inverse variation of the concentrations of ADP and P_i. Under these conditions, the rate of ATP hydrolysis is strongly inhibited as the concentration of ADP is increased and the concentration of P_i is decreased. Inhibition is preferentially caused by ADP-dependent inactivation of ATPase molecules. At low initial ADP concentration, the transmembrane proton gradient generated by effective ATP hydrolysis protects the enzyme from deactivation in spite of progressive accumulation of ADP because energy-dependent release of ADP counteracts its binding. Deactivation due to incorporation of ADP occurs, however, when the proton gradient decreases as a consequence of exhaustion of substrate ATP.

ATP synthesis; ATPase; Chloroplast; 9-Aminoacridine fluorescence; Kinetic control; (Spinach)

1. INTRODUCTION

The direction and rate of the H^+-coupled ATPase reaction above all are determined by the magnitude of the thermodynamic parameters ΔG_{H+} and ΔG_P. A large proton-motive force at low phosphate potential enables ATP synthesis to occur, but the reverse reaction is facilitated when $n \cdot \Delta G_{H+} < \Delta G_P$ (n: H^+/ATP stoichiometry). In chloroplasts ΔG_{H+} depends on light intensity and thus may be variable under natural conditions. In the dark, ΔG_{H+} approaches zero. Hence, regulation of the chloroplast ATPase is an additional prerequisite to avoiding waste of ATP by ATP hydrolysis under energetically unfavorable conditions. Regulation of the chloroplast ATPase includes activation and deactivation caused by membrane energization and deenergization, respectively [1–4]. Moreover, the properties of the activated enzyme are altered by reduction of a disulfide to a dithiol group in the γ-subunit of CF_1 [5,6]. In vitro reduction may be achieved by DTT [7]; in vivo the reductant is thioredoxin [8,9]. Thiol modification, which is not necessary for catalysis of ATP formation in strong light, increases the efficiency of ATP synthesis at low $\Delta\tilde{\mu}H^+$ [4,10,11]. Upon relaxation of the transmembrane proton gradient, the oxidized ATPase is immediately deactivated whereas the reduced ATPase retains activity and thus is capable of hydrolyzing ATP in the dark. Dark deactivation of the reduced enzyme may be achieved by reoxidation which is, however, a relatively slow process in the intact chloroplast [12]. Extensive studies with isolated thylakoid vesicles have shown a much faster deactivation by ADP [13]. ADP-dependent deactivation correlates with tight binding of one nucleotide molecule to CF_1 [14,15] at a site which is located on the β-subunit [16] in the vicinity of the α-subunit [17]. A new membrane energization by light leads to rerelease of the tightly bound ADP related with reactivation of the ATPase [14]. In the intact chloroplast ATP and phosphate are present side by side with ADP. On transition from light to dark,

Correspondence address: H. Strotmann, Institut für Biochemie der Pflanzen, Universität Düsseldorf, Universitätsstraße 1, D-4000 Düsseldorf, FRG

Abbreviations: DTT, dithiothreitol; PMS, phenazine methosulfate

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the change in ATPase activity cannot be readily predicted because ATP and phosphate interactions also play a role in regulation. Tight binding of ATP instead of ADP is known to stabilize the enzymatic activity [18] whereas phosphate delays tight binding of ADP and thereby enzyme inactivation [14,19]. Hence, the same components which exert thermodynamic control of the ATPase reaction are also involved in kinetic control as activators and deactivators of the enzyme.

This paper reports on an experimental approach to separate the kinetic and thermodynamic effects of substrate concentrations from each other. The experiments were conducted with thylakoids preilluminated and furthermore kept in the presence of excess DTT in order to exclude any regulatory contribution of enzyme reduction/oxidation.

2. MATERIALS AND METHODS

Well-washed thylakoids were isolated from spinach leaves as in [20]. The reaction medium contained 25 mM Tricine buffer, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT and 50 μM PMS. Isolated thylakoids were added to give a final chlorophyll concentration of 25 μg/ml. Before the reactions were started by the addition of the substrates ADP and P_i or ATP the reaction mixture was illuminated for 2 min (250 $W \cdot m^{-2}$).

The formation of ATP in a subsequent light period (250 $W \cdot m^{-2}$) and its hydrolysis in the dark were followed by employing [^{14}C]ADP and unlabeled P_i. During the course of the reaction aliquots were deproteinized by $HClO_4$ (final concentration 0.6 M) and analyzed by ion-exchange TLC for labeled AMP, ADP and ATP [21]. These data were also used for the calculation of equilibrium phosphate potentials. Initial rates of ATP hydrolysis in the presence of ADP and P_i (fig.2) were measured by release of $^{32}P_i$ from [γ-^{32}P]ATP added together with ADP and P_i 15 s after preillumination. Aliquots taken 10, 20 and 30 s after the start of the reaction were deproteinized and analyzed as in [22]. Initial rates were obtained by extrapolation. The activity of ATPase at a definite time during phosphorylation or hydrolysis was determined as follows: At the desired time a mixture consisting of carrier-free [^{32}P]ATP, PEP (2 mM), pyruvate kinase (100 U/ml) and nigericin (0.5 μM) was added. At the same time light was turned off if necessary. The initial rate of release of $^{32}P_i$ was measured in samples taken after 10, 20 and 30 s (see above).

9-Aminoacridine fluorescence as a measure of transmembrane ΔpH [23] was followed using a self-constructed fluorimeter in a cylindrical cuvette with stirring device. The excitation wavelength was 400 nm, emission being detected at 494 nm. Photosynthesis was excited with red light > 630 nm (filter RG 630, Schott). The concentration of 9-aminoacridine was 5 μM.

3. RESULTS

In order to convert the oxidized ATPase into its reduced form, thylakoids were preilluminated for 2 min in the presence of 10 mM DTT. During a dark interval of 1 min 50 μM [^{14}C]ADP and 250 μM phosphate (sample a) or 250 μM [^{14}C]ADP and 50 μM P_i (sample b) were added. In the subsequent light formation of [^{14}C]ATP was observed until equilibrium was reached (fig.1A). From the measured equilibrium concentrations of [^{14}C]-ATP, [^{14}C]ADP and the calculated equilibrium concentration of phosphate the equilibrium phosphate potential was computed. The results show that quite similar concentrations of [^{14}C]ATP and similar phosphate potentials were attained in spite of the inverse starting concentrations of ADP and P_i in samples a and b.

When the light was turned off after 5 min, ATP hydrolysis was observed in sample a, but no change of the [^{14}C]ATP concentration was found in sample b. If an uncoupler was added together with turning off the light, the rate of ATP hydrolysis was increased in sample a, but no divergent result was obtained in sample b (not shown). With the same set of substrate concentrations the formation of light-induced transmembrane ΔpH and its dark decay were measured by following 9-aminoacridine fluorescence quenching (fig.1B). Using a value of 60 μl/mg chl [22] for the internal thylakoid volume the calculated equilibrium ΔpH in the light was about 3.6 in both cases. Assuming an H^+/ATP stoichiometry of 3 [24–26] and neglecting the electrical component $\Delta\psi$, the ΔpH of 3.6 yields -60.6 $kJ \cdot mol^{-1}$, i.e. the absolute values of $\Delta\tilde{\mu}H^+$ and ΔG_P correspond as expected in the equilibrium state.

Upon turning off the light ΔpH drops to a transitory level of about 3 units in sample a. The transitory dark ΔpH is obviously caused by ATP hydrolysis, which effects inward proton translocation through the ATPase complex [27]. The decline of ΔpH to zero after about 2 min matches with the point of exhaustion of the substrate ATP (see fig.1A). In sample b ΔpH immediately drops to zero after turning off the light. This corresponds to the finding that no ATP is hydrolyzed in the dark under these conditions.

In fig.1C, the actual enzyme activities of ATPase during the course of the reaction were determined using the following procedure: The reactions were run as in fig.1A, but with unlabeled ADP and P_i. At the indicated times a pulse of carrier-free [γ-^{32}P]ATP together with PEP, pyruvate kinase and nigericin was employed together with turning off the light. The initial rate of ATP hydrolysis was followed by analyzing the released $^{32}P_i$. By the action of pyruvate kinase/PEP all of the ADP present in the medium is converted to ATP within less than 1 s and mixed with [^{32}P]ATP. Hence, the concentration of ^{32}P-labeled ATP is constant and identical in every sample. Through the action of nigericin ΔpH is immediately brought to zero which facilitates attainment of the maximal rate of ATP hydrolysis. In order to establish the same assay conditions in the two series, the pulse mixture was supplemented with extra ATP in sample a.

No significant differences in enzyme activity could be detected in the light. When the light was turned off, however, a transitory high activity was maintained in sample a while in sample b the ATPase activity immediately decreased to zero within less than 30 s. In sample a activity decreased to zero after about 2 min.

In the experiment shown in fig.2, ATP hydrolysis by pre-illuminated thylakoids was measured at a constant initial concentration of ATP (50 μM) and constant initial phosphate potential (60 kJ·mol^{-1}) but inversely varied concentrations of ADP and P_i. The initial rate of ATP hydrolysis as a function of ADP concentration is plotted in fig.2. In order to keep ΔpH = 0, nigericin was added together with the substrates in a parallel series. The results show a dramatic inhibition of ATP hydrolysis with increasing ADP and decreasing P_i concentration. 50% inhibition is

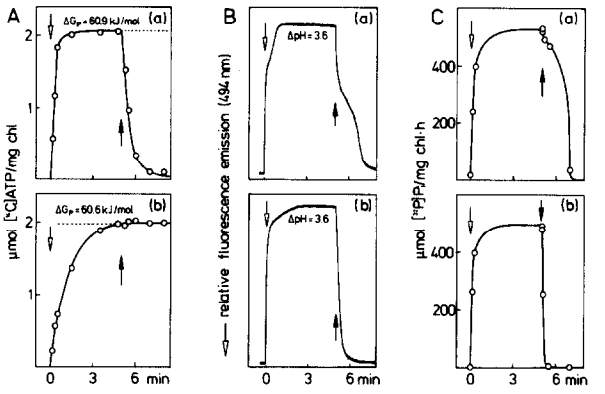

Fig.1. Formation and hydrolysis of ATP (A), change of 9-aminoacridine fluorescence (B), and change in ATPase activity (C) in a light and subsequent dark period. After preillumination of the thylakoids for 2 min (250 W·m^{-2}), either 50 μM ADP and 250 μM P_i or 250 μM ADP and 50 μM P_i (b) were added during a 1 min dark period. At t = 0 (open arrows) the samples were re-illuminated for 5 min before the light was turned off (closed arrows). In A ADP was ^{14}C-labeled. The equilibrium phosphate potential (in kJ/mol) was computed from the measured equilibrium concentrations of [^{14}C]ATP and [^{14}C]ADP and the calculated equilibrium phosphate concentration according to

$$\Delta G_P = \Delta G_P^0 + RT\ln\frac{[ATP]}{[ADP][P_i]}$$

with ΔG_P^0 = 32.5 kJ/mol [29] and T = 293 K. In B, the equilibrium ΔpH was computed using

$$\Delta pH = \log\left(\frac{F_0 - F}{F}\cdot\frac{V_t}{V_c}\right) \quad [23],$$

(F_0, total fluorescence; F, actual fluorescence; V_t, total volume; V_c, internal thylakoid volume). The procedure for determination of ATPase activity (C) is described in the text.

observed at 20 μM (coupled system) or 15 μM ADP (presence of uncoupler), respectively. At equal concentrations of ADP and ATP (50 μM), the rate of ATP hydrolysis was almost zero in both the absence and presence of uncoupler.

4. DISCUSSION

Our results show that ATP hydrolysis catalyzed by chloroplast ATPase may proceed at high rates

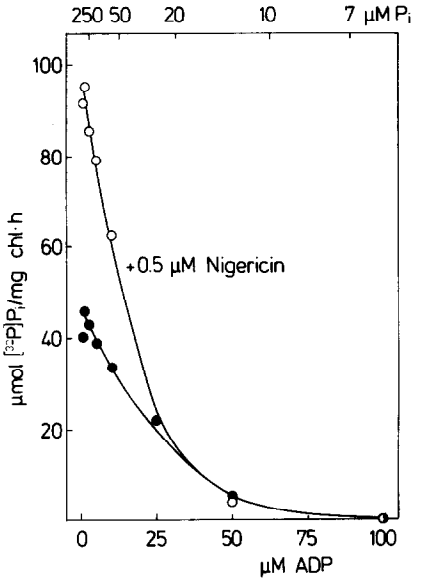

Fig.2. The initial rate of dark ATP hydrolysis at constant initial ATP concentration (50 μM) and constant initial phosphate potential (60 kJ/mol) adjusted by the addition of ADP and P_i at varying concentrations with constant $c_{ADP}c_{P_i} = 6.25 \times 10^{-10}$ M^2. [γ-^{32}P]ATP, ADP and P_i ± 0.5 μM nigericin were added together after 2 min preillumination (250 $W \cdot m^{-2}$) following 15 s dark treatment.

or may be completely inhibited even when the concentrations of ATP and thermodynamic conditions are identical. The reason for this is an efficient kinetic control of enzyme activity. If regulation of the ATPase by reversible thiol modification is excluded – as reported here – the rate of ATP hydrolysis may be affected by ADP and P_i in a different manner because of their different affinities to the catalytic site. At $\Delta\tilde{\mu}H^+ = 0$ the affinity of the active site for ADP is lower by a factor of 2 than that for ATP [28]. Accordingly, 50% competitive inhibition may be expected at an ADP/ATP ratio of 2. On the other hand, 50% inhibition of ATP hydrolysis is obtained with phosphate at concentrations as high as 20 mM [29]. When the concentrations of ADP and P_i are varied inversely to adjust the same phosphate potential, part of the observed variance in the extent of inhibition may therefore be ascribed to competition of ADP and lack of competition of P_i with substrate ATP at the catalytic site. Another obvious factor is enzyme deactivation by tight binding of ADP. At low proton-motive force tight binding of ADP to CF_1 causes deactivation, whereas tight binding of ATP leads to stabilization of ATPase activity (see section 1). The rate constant for ADP binding is one order of magnitude higher than that for ATP binding [18]. Hence the deactivating effect of ADP apparently exceeds the competitive effect of ADP. This is confirmed by the finding that 50% inhibition of the rate of ATP hydrolysis is observed at an ADP/ATP ratio as low as 0.3 (fig.2).

The situation is complex when ATP hydrolysis is allowed to form a transmembrane proton gradient (absence of uncoupler). On the one hand, $\Delta\tilde{\mu}H^+$ causes inhibition of ATP hydrolysis because of energetic control. On the other, $\Delta\tilde{\mu}H^+$ effects release of tightly bound nucleotides which altogether leads to partial compensation of enzyme deactivation. The opposing effects of $\Delta\tilde{\mu}H^+$ explain why the rate of ATP hydrolysis is increased by uncoupling at low concentrations of ADP and why the concentration of ADP required for half-maximal inhibition is somewhat higher in coupled than in uncoupled chloroplasts (fig.2).

The effects of nucleotides and $\Delta\tilde{\mu}H^+$ discussed are sufficient to explain the observed reactions on light-dark transition in fig.1. When the reaction was started with excess ADP over P_i (sample b) the ADP/ATP ratio was 3.8 before the light was turned off. This ADP/ATP ratio is larger than that necessary for rapid and complete enzyme deactivation. Measurement of enzyme activity by the technique employed, which excludes any substrate competition at the active site, indicates that the lack of dark ATP hydrolysis is indeed due to deactivation of the ATPase. In sample a the ADP/ATP ratio at the beginning of the dark period was 0.04. Under these conditions, the majority of active enzyme molecules is stabilized by ATP rather than inactivated by ADP. As ATP hydrolysis proceeds, the ADP/ATP ratio increases dramatically. Nevertheless, high ATPase activity is maintained because $\Delta\tilde{\mu}H^+$ coupled with ATP hydrolysis counteracts tight nucleotide binding. Only when ATP hydrolysis and coupled inward proton translocation become slower due to exhaustion of ATP is breakdown of activity effected by progressive incorporation of ADP.

The mechanism of self-regulation of the ATPase

by its own substrates discussed appears a reasonable and economic way to balance the energy charge of the adenylate system inside the chloroplast on changing environmental conditions.

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