The ATP synthase γ subunit provides the primary site of activation of the chloroplast enzyme: experiments with a chloroplast-like *Synechocystis* 6803 mutant

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The activation characteristics of the F_1F_o -ATP synthase (where F_1 and F_o are the hydrophilic and membrane-bound parts respectively of the enzyme) from *Synechocystis* 6803 wild-type and a *Synechocystis* 6803 mutant with a chloroplast-like insertion in the γ subunit have been studied. Activation of the ATP synthase in wild-type and mutant membrane vesicles was performed by acid–base transition-induced generation of a proton motive force ($\Delta\bar{\mu}_{H^+}$). Since the mutant containing the regulatory segment of the chloroplast γ subunit showed thiol-

modulation (typical of the chloroplast enzyme), this segment is indeed involved in the regulation of enzyme activation. It is shown that the ATP synthase from *Synechocystis* 6803 wild type corresponds functionally to the reduced form of the chloroplast ATP synthase, in view of the low $\Delta\bar{\mu}_{H^+}$ required for activation of the enzyme and the high stability of the active state. Both the cyanobacterial wild-type and mutant ATP synthases can be activated by methanol, which apparently does not require the presence of the γ subunit regulatory segment.

INTRODUCTION

The F_1F_o -ATP synthases (where F_1 and F_o are the hydrophilic and membrane-bound parts respectively of the ATP synthase) from chloroplasts and cyanobacteria are highly related [1]. However, differences have been found between chloroplasts and different cyanobacterial strains in one important regulatory aspect, namely the redox-linked modulation of the enzyme. All F_1F_o -ATP synthases from photosynthetic membranes require a proton gradient (proton motive force; $\Delta\bar{\mu}_{H^+}$) to become active, but the enzyme from the cyanobacterium *Synechococcus* 6716 [2,3] shows no effect of the redox state on $\Delta\bar{\mu}_{H^+}$ -dependent activation, in contrast with F_1F_o -ATP synthases from chloroplasts [4] and from another cyanobacterial strain, *Spirulina platensis* [5]. In chloroplast F_1F_o -ATP synthase, a stretch of nine amino acid residues, including two cysteines in the γ subunit of chloroplast F_1 , is likely to be involved in this redox effect [6]; this stretch is absent from the cyanobacterial enzymes [7,8]. The cyanobacterium *Spirulina platensis*, however, contains two other cysteine residues in the γ subunit that might be involved in its redox regulation [9].

A mutant F_1F_o -ATP synthase has been prepared from *Synechocystis* 6803 with a chloroplast-like segment containing two cysteine residues inserted into the γ subunit [10]. Currently, mutants are being constructed containing the two non-conserved cysteine residues present in *Spirulina platensis*. Since a method is now available for the isolation of energetically well-coupled thylakoid vesicles from *Synechocystis* 6803 [11,12], the effects of these mutations on the functional properties of cyanobacterial F_1F_o -ATP synthases can be studied. In the present paper we describe the ΔpH requirements for the physiological activation of the ATP synthases in *Synechocystis* 6803 wild type and mutant

strains under oxidized and reduced conditions. Artificial activation of the organisms by methanol was also studied.

MATERIALS AND METHODS

Organisms and preparation of membrane vesicles

The cyanobacterium *Synechocystis* sp. PCC 6803 wild type, as well as the mutants of this strain (Cys 1-10 and Cys 3-3 [10]), were grown as described in [11].

Spheroplasts were prepared from cyanobacterial cells by lysozyme treatment [11,12], and kept at 0 °C in the presence of 100 μM PMSF. Immediately prior to the measurements, an osmotic shock was applied to the reaction medium to obtain thylakoid vesicles.

Determination of ATP hydrolysis activity

ATP hydrolysis activity was determined in three different ways. First, light-triggered ATP hydrolysis was measured by monitoring the release of scalar protons with a sensitive pH electrode [13] in a set-up as described by Krab et al. [3] in 2 mM Tricine/NaOH (pH 8.0) with 5 mM $MgCl_2$, 50 mM KCl, 50 mM NaCl, 2.5 mM K_2HPO_4 , 50 μM phenazine methosulphate (PMS), 100 μM PMSF and 5 mM ATP. The activities were measured at 35 °C and corrections were made for the background acidification due to the presence of PMSF.

In acid–base transition experiments, ATP hydrolysis activity was assessed (in the presence of uncoupler [3]) by measuring the oxidation of NADH in an ATP-regenerating system [14]. Spheroplasts at a concentration of 70 μg of chlorophyll/ml were first

Abbreviations used: $\Delta\bar{\mu}_{H^+}$, proton electrochemical potential difference or proton motive force; DTT, dithiothreitol; F_1 , hydrophilic part of the ATP synthase; F_o , membrane-bound part of the ATP synthase; PMS, phenazine methosulphate; S-13, 5-chloro-3-t-butyl-2'-chloro-4'-nitrosalicylanilide.

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shocked by a 7 min incubation in medium A (10 mM succinic acid, 2 mM MgCl_2 , 10 mM KCl, 2 mM NaH_2PO_4 , 2 μM valinomycin and 10 mM sodium/Tricine at pH 7.6) [2]. Vesicles with a chlorophyll concentration of 9 μg of chlorophyll/ml were then incubated at 35 °C in acidic medium (medium A, with the pH varied using NaOH over the range pH 5.7–8.6, depending on the desired ΔpH) for 1 min, and subsequently diluted 1:1 (v/v) in alkaline medium B (2 mM MgCl_2 , 10 mM KCl, 1 mM NaH_2PO_4 and 200 mM sodium/Tricine at pH 8.6). Immediately after performing the acid–base transition (within 5–10 s), ATP hydrolysis was measured [15] enzymically by diluting samples 1:10 (v/v) in assay medium containing 5 mM ATP and 0.5 μM of the uncoupler S-13 (5-chloro-3-t-butyl-2'-chloro-4'-nitrosalicylanilide) [3]. Activities were measured at 35 °C in the presence of 100 μM PMSF.

In methanol activation experiments, ATP hydrolysis was measured as the release of P_i according to the method of LeBel et al. [16]. The reactions were performed in 20 mM Tricine (pH 7.8) in the presence of 2.5 mM Mg^{2+} and 5 mM ATP (a molar ratio of 0.5). The reactions were started by the addition of spheroplasts and proceeded for 4 min at 35 °C. The first 2 min at 35 °C were necessary to obtain membrane vesicles, as was confirmed by phycocyanin release (A_{620}). A blank sample was treated in the same way as the experimental samples, except that spheroplasts were incubated for 2 min before addition of colouring reagent.

In some experiments the actual ATP concentration was verified with the luciferin/luciferase assay [17].

Other methods

Activation of ATP hydrolysis by methanol was carried out as described by Anthon and Jagendorf [18]. Chlorophyll *a* concentrations were determined by the method of Arnon et al. [19].

Chemicals

ATP, ADP, NADH, phosphoenolpyruvate, valinomycin and all enzymes were purchased from Boehringer (Mannheim, Germany). PMS and PMSF were from Sigma (St. Louis, MO, U.S.A.). The luciferin/luciferase kit was obtained from Pharmacia-LKB Biotechnology (Uppsala, Sweden). S-13 was a gift from Dr. P. C. Hamm (Monsanto Co., St. Louis, MO, U.S.A.). All other chemicals were of analytical grade.

RESULTS

Figure 1 presents a qualitative indication of the energetic thresholds for ATP hydrolysis by wild-type *Synechocystis* 6803 membrane vesicles. In Figure 2 of [11], it was shown that ATP is synthesized at increasing rates with increasing light intensity above a threshold lamp voltage of approx. 10 V (corresponding to an irradiance of 48 $\text{W}\cdot\text{m}^{-2}$). ATP hydrolysis activity in the dark induced by pre-illumination requires a much lower light intensity (about 1 $\text{W}\cdot\text{m}^{-2}$; see Figure 1). When an acid–base transition is applied either to drive ATP synthesis (cf. [12]) or to induce ATP hydrolysis (see below), a threshold $\Delta\tilde{\mu}_{\text{H}^+}$ value of approx. 10 kJ/mol is found for both processes, suggesting that phosphorylation is limited by the activity of F_1F_0 -ATP synthase. This apparent contradiction can be explained by the fact that phosphorylation decreases the actual proton gradient. Therefore the same light intensity does not result in equal $\Delta\tilde{\mu}_{\text{H}^+}$ values in the two sets of experiments.

Activation of ATP hydrolysis induced by an acid–base transition was measured enzymically in *Synechocystis* 6803 wild-type

Figure 1 Light-triggered ATP hydrolysis activity of thylakoid membrane vesicles of wild-type *Synechocystis* 6803

Activity is shown as function of light intensity, presented as irradiance ($\text{W}\cdot\text{m}^{-2}$), at the entrance of the reaction vessel. Activities were measured by the release of scalar protons (see the Materials and methods section). ATP hydrolysis was started by addition of ATP and determined after pre-illumination for 30 s. The chlorophyll *a* concentration was 8 $\mu\text{g}/\text{ml}$. Chl, chlorophyll.

and mutant membrane vesicles (see Figures 2 and 3). A minimal ΔpH of 1.7 or a minimal $\Delta\tilde{\mu}_{\text{H}^+}$ of approx. 10 kJ/mol was necessary for activation of the wild-type ATP synthase (Figure 2A). When these wild-type vesicles were illuminated in the presence of dithiothreitol (DTT) and PMS prior to the acid–base transition, the minimal $\Delta\tilde{\mu}_{\text{H}^+}$ required (Figure 2B) was also approx. 10 kJ/mol, which implies that in wild-type *Synechocystis* 6803 there is no effect of the redox state on the $\Delta\tilde{\mu}_{\text{H}^+}$ threshold for activation. From the amino acid sequence (see Figure 2 in [10]) it is clear that there is no reducible disulphide bridge present in the wild-type γ subunit.

However, in the *Synechocystis* 6803 mutant with the chloroplast-like insertion in its γ subunit, a significant difference was observed in the threshold $\Delta\tilde{\mu}_{\text{H}^+}$ value required for activation of ATP hydrolysis. With membrane vesicles illuminated in the presence of DTT prior to the acid–base transition, a shift to a lower $\Delta\tilde{\mu}_{\text{H}^+}$ values from approx. 10 kJ/mol (Figure 3A) to approx. 6 kJ/mol (Figure 3B) was observed. Lowering of the energetic threshold by treatment with DTT is also found for chloroplast ATP synthase [4]. Since cyanobacterial vesicles are rather sensitive to low pH values in the acidic region, no data at higher $\Delta\tilde{\mu}_{\text{H}^+}$ values than the ones indicated could be obtained. Because *Synechocystis* 6803 membrane vesicles are not stable for prolonged periods, they were produced by osmotic shock of spheroplasts in the cuvette immediately before the measurements. Since the internal K^+ concentration of spheroplasts is high, a change in membrane potential could not be applied to increase the $\Delta\tilde{\mu}_{\text{H}^+}$.

As the maximal hydrolysis activity could not be attained, it was not possible to determine the $\Delta\tilde{\mu}_{\text{H}^+}$ at which the activation is half-maximal with the present vesicle preparations. However, a minimal $\Delta\tilde{\mu}_{\text{H}^+}$ for activation of approx. 10 kJ/mol corresponds well to the $\Delta\tilde{\mu}_{\text{H}^+}$ required to activate the reduced form of chloroplast ATP synthase [4]. The oxidized form of the chloroplast enzyme requires more than 19 kJ/mol for activation [4]. Therefore the F_1F_0 -ATP synthase from *Synechocystis* 6803, similar to the enzyme from *Synechococcus* 6716 but not that from *Spirulina platensis* [5], corresponds functionally to the reduced

Figure 2 Activation of ATP hydrolysis by an acid–base transition of *Synechocystis* 6803 wild-type vesicles

To obtain the data in (B), the vesicles were incubated in medium A [2] at pH 7.6 supplemented with 80 μ M PMS and 20 mM DTT. Illumination (at maximal light intensity) of these preparations for 3 min at 25 °C was followed by a 4 min incubation at 0 °C. Chl, chlorophyll.

form of the chloroplast enzyme. Actually, the threshold values for both the oxidized and reduced forms of the *Synechocystis* 6803 mutant enzyme are lower than those of the corresponding forms of the chloroplast enzyme.

Activated reduced chloroplast F_1F_0 -ATP synthase remains stable in the absence of a $\Delta\bar{\mu}_{H^+}$ providing that ADP is absent [20]. A similar stability was observed for the ATP synthase from wild-type and mutant *Synechocystis* 6803 under reduced and oxidized conditions. ATP hydrolysis activity, as measured with a pH electrode, was stable for only a few minutes, and decreased slowly due to inactivation by tight binding of the product ADP [21]. However, ATP hydrolysis activity measured in an ATP-regenerating system was stable for at least 15 min.

The activity of *Synechocystis* 6803 ATP synthase (expressed on the basis of chlorophyll content, as in Figures 1–4) shows a variability which can be explained by the variable chlorophyll content of the different vesicle preparations, as is also observed in other cyanobacteria [5] and in chloroplasts [4].

As is clear from Figures 1–4, our membrane vesicles show a variable background ATP hydrolysis activity. A similar background activity is also observed for *Synechococcus* 6716 [3], which appeared to be the consequence of ATP synthases that are maintained in an active form (possibly due to respiration) and a small percentage of other (plasma membrane) types of ATPase. This background activity hampers an accurate estimation of the

Figure 3 Activation of ATP hydrolysis by an acid–base transition in vesicles of the *Synechocystis* γ subunit mutant strain (Cys 1-10)

The experimental procedures were as for Figure 2. Chl, chlorophyll.

Figure 4 Stimulation by methanol of ATP synthase activity in *Synechocystis* 6803 wild-type (●) and γ subunit mutant (○) vesicles

ATP hydrolysis was measured as P_i release (see the Materials and methods section) at a concentration of 9 μ g of chlorophyll (Chl)/ml. Corrections were made for P_i contamination of ATP preparations.

threshold $\Delta\bar{\mu}_{H^+}$ for activation. However, the raw data (not corrected for background activity) show that the reduction-induced shifts of the threshold $\Delta\bar{\mu}_{H^+}$ are significant.

As described previously, the ATP hydrolysis activity of *Synechocystis* 6803 wild-type thylakoids can be enhanced by trypsin treatment, but not by thiol reduction or short heat treatment [11]. By contrast, thiol reduction, heat and trypsin treatments all increased the hydrolysis activity of the mutant enzyme. Similarly, the ATP hydrolysis activity of isolated *Synechocystis* 6803 wild-type F_1 is unmasked by trypsin, whereas the mutant F_1 is activated by incubation with trypsin as well as by 50 mM DTT [10].

Another activator of ATP synthase is methanol [18,22]. The *Synechocystis* 6803 wild-type and mutant enzymes could both be activated by methanol, with similar titration profiles (Figure 4). The 3-fold higher ATP hydrolysis activity in 30% methanol found for *Synechocystis* 6803 (Figure 4) is in accordance with the reported stimulation of mitochondrial F_1 [22]. However, methanol is able to enhance the Mg-ATPase activity of F_1 from *Chlamydomonas*, spinach [18] and *Spirulina platensis* [5] by a factor of about 40. Under the conditions tested, the optimal Mg/ATP ratio for the methanol-activated *Synechocystis* enzyme was found to be 0.5, similar to results obtained with other ATP synthases [5,18].

DISCUSSION

The activation experiments clearly indicate that the ATP synthase from the *Synechocystis* 6803 mutant, containing the regulatory segment of the chloroplast γ subunit, shows thiol-modulation typical of the chloroplast system. The results strongly support the view that this part of the γ subunit indeed regulates the activation of the ATP synthase. The reduced form of the *Synechocystis* 6803 mutant enzyme, however, is not equivalent to any known chloroplast state. The low threshold value of this reduced mutant enzyme implies that thiol-modulation of the enzyme enables the organism to synthesize ATP at very low $\Delta\bar{\mu}_{H^+}$ values, as is found for *Spirulina platensis* [5]. Therefore another region of the γ subunit (besides the cysteine bridge sequence) might also be involved in activation.

The role of the cysteine bridge in the regulation of ATP synthase was also investigated by Ross et al. [23]. These investigators deleted the cysteine bridge from the *Chlamydomonas reinhardtii* F_1 γ subunit. Their mutant enzymes were not modulated by DTT, but stimulation of activity by DTT was observed for the *Chlamydomonas* wild-type enzyme. Recently, these investigators [24] observed that the spacer region of five amino acids between the disulphide bond is also involved in the redox regulation of chloroplast ATP synthase. Other recent experiments [25], however, indicated that cleavage of the γ subunit between residues 215 and 232 is sufficient to fully activate the enzyme without reduction of the γ subunit disulphide bond. In addition, cleavage within this region is found to be responsible for loss of affinity for the inhibitory ϵ subunit [25].

Like heat and detergents, alcohols are believed to activate F_1F_0 -ATP synthase by causing dissociation of the ϵ subunit, and the effect of removal of this subunit from F_1 seems to mimic the changes in the γ subunit upon reduction or trypsin cleavage [6]. A close physical proximity has been reported between the γ subunit disulphide bridge, the γ subunit dark-accessible thiol group and a thiol group in the ϵ subunit, indicating that, in chloroplasts, the γ and ϵ subunits are physically and functionally closely linked [26]. Anthon and Jagendorf [27] showed that activation of F_1F_0 -ATP synthase by methanol involves several distinct effects. Methanol not only overcomes the inhibition by the ϵ subunit, but it also increases the affinity of F_1 for anions (such as SO_3^{2-}) that reverse the inhibitory effect of Mg^{2+} and, furthermore, it increases the V_{max} of Mg-ATP hydrolysis [27].

Soteropoulos et al. [28] found that reduction of the γ subunit disulphide bond in chloroplast F_1 decreased the affinity for the ϵ subunit, and therefore methanol might indeed affect the γ - ϵ interaction. Since *Synechocystis* 6803 wild-type and mutant ATP synthases are equally activated by methanol, the γ subunit regulatory segment is unlikely to be involved in methanol activation. The absence of the cysteine residue from the ϵ subunit of *Synechocystis* 6803, as in other cyanobacterial ϵ subunits (*Synechococcus* 6716, *Synechococcus* 6301 and *Anabaena* 7120), could imply altered γ - ϵ arrangements. Recently, a regulatory role in conformational coupling has been suggested for the ϵ subunit [29], and the region between residues 202 and 212 of the γ subunit of *Escherichia coli* F_1 -ATPase was found to be involved in ϵ subunit binding [30]. On the other hand, the interaction between the catalytic sites on F_1 seems to be affected by bivalent cations as well as by the light-accessible cysteine residue present in the chloroplast and cyanobacterial γ subunits [31].

Unfortunately, more concrete mechanisms for activation of F_1F_0 -ATP synthase involving the γ subunit cannot be suggested at this stage. Crystal structures of photosynthetic ATP synthases are not yet available, and high-resolution details of the relevant part of the γ subunit have not been resolved for bovine heart mitochondrial F_1 [32].

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