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# Activation of the $H^+$-ATP synthases of a thermophilic cyanobacterium and chloroplasts – a comparative study

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**The activation requirements of the ATP synthases of the thermophilic cyanobacterium *Synechococcus* 6716, studied in coupled membrane vesicles and in the isolated or reconstituted complex, and of the ATP synthase of spinach chloroplasts were compared. It was found that methanol, heat treatment or dithiothreitol did not activate ATP hydrolysis in *Synechococcus* 6716. In contrast to the chloroplast enzyme, activation could only be accomplished with sulfite, octyl glucoside, a proton electrochemical potential difference and trypsin. The lack of activation by dithiothreitol, heat and methanol in the cyanobacterial ATP synthase can be explained by the absence of three cysteine residues in the regulatory $\gamma$ subunit of the $F_1$ part. The threshold value of the proton electrochemical potential difference at which ATP synthesis occurs at low $\Delta G_p$ was about 9.5 kJ mol$^{-1}$ for the cyanobacterial ATP synthase. This is similar to the threshold value of the reduced form of the enzyme in chloroplasts. With cyanobacterial membrane vesicles, an $H^+$/ATP stoichiometry slightly exceeding 4 was obtained in ATP hydrolysis as well as in ATP synthesis, measured as a function of an artificially applied proton electrochemical potential difference. These findings are discussed in terms of a single structural difference between the cyanobacterial and the chloroplast enzyme. When comparing the enzyme of *Synechococcus* 6716 with that of chloroplasts, our results indicate that the difference in activation requirements of both ATP synthases resides in a different arrangement of the $\gamma$ and $\epsilon$ subunits.**

## Introduction

The ATP synthase from chloroplasts and cyanobacteria is an enzyme that has to be activated in order to express its activity in synthesizing or hydrolyzing ATP.

Hydrolysis activity of chloroplast ATP synthase can be induced by trypsin [1], sulfhydryl agents such as dithiothreitol and dithioerythritol [2], heat [3], methanol [4], detergents such as octyl glucoside [5], and sulfite [6]. Most of the research dealing with activation has been done on the water-soluble $F_1$ portion isolated from chloroplasts. Apart from these treatments, membrane-bound $CF_1F_0$ is dependent on activation by a $\Delta\tilde{\mu}_{H^+}$ for both synthesis and hydrolysis activity. In chloroplasts, $\Delta\tilde{\mu}_{H^+}$ has been induced in vitro by light [7], by acid-base transition [8] or an additional valinomycin-induced $K^+$-diffusion potential [9,10].

It has been shown that the $\gamma$ subunit in $CF_1$ plays a prominent role in the mechanism of activation [11]. The $\gamma$ subunit of chloroplast ATP synthase contains four cysteine residues and three trypsin cleavage sites. Activation of the ATP synthase by sulfhydryl agents results from reduction of a disulfide bond between two cysteines in the $\gamma$ subunit [12]. The cysteines most

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Abbreviations: $F_1$, the hydrophilic part of the ATP synthase (prefix C refers to chloroplasts, prefix S to *Synechococcus*), $F_0$, the membrane-bound part of the ATP synthase, DCCD, *N,N'*-dicyclohexylcarbodiimide; octyl glucoside, *n*-octyl $\beta$-D-glucopyranoside; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; $\Delta\tilde{\mu}_{H^+}$, proton electrochemical potential difference; PMS, phenazinemethosulfate; S-13, 5-chloro-3-*t*-butyl-2'-chloro-4'-nitrosalicylanilide.

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probably involved are Cys-199 and Cys-205 in the sequence alignment of Werner et al. [13]. Several sites in the polypeptide can be cleft by trypsin which results in an active state, more or less similar to that accomplished by reduction by a sulfhydryl agent [14,15].

The region containing the disulfide bond presumably is also involved in heat activation, not by reduction but by a conformational change [14], and an additional displacement of the $\epsilon$ subunit has also been suggested [16].

Reduction of the $\gamma$ subunit in membrane-bound $CF_1F_0$ by dithiothreitol leads to a decrease in the value of $\Delta\bar{\mu}_{H^+}$ required for activation. This was demonstrated by Jünesch and Gräber [17], who presented a reaction scheme in which the relations between the different active states of the enzyme are depicted. A modified version of this scheme is presented in Fig. 1. When chloroplasts are illuminated in the presence of dithiothreitol, the inactive, oxidized state of the ATP synthase (state $E_i^{ox}$) becomes activated and reduced (state $E_a^{red}$). In state $E_a^{red}$ the ATP synthase is able to catalyze high rates of ATP hydrolysis and ATP synthesis. When the ATP synthase is in its $E_i^{ox}$ form, membrane energization ($\Delta\bar{\mu}_{H^+}$) leads to an active state, $E_a^{ox}$. The activation of the oxidized form requires a higher $\Delta\bar{\mu}_{H^+}$ then ATP synthesis itself. Hence, ATP hydrolysis rarely occurs: at low levels of $\Delta\bar{\mu}_{H^+}$ the ATP synthase is inactive, whereas at high levels of $\Delta\bar{\mu}_{H^+}$ ATP synthesis is likely to occur (depending on the value of $\Delta G_p$). Recently, it was found that the part of the $\gamma$ subunit presumably involved in sulfhydryl agent and heat-dependent activation is missing in a number of cyanobacterial strains [13,18,19,20], while two trypsin cleavage sites are still present [13,20]. In this respect, these strains provide comparative material to study the role of the disulfide involved cysteines in the $\gamma$ subunit. Consequently, it may be expected that the ATP synthase of these organisms can be activated only by a proton electrochemical potential difference and trypsin, and not by dithiothreitol or heat treatment. Indeed, in earlier studies it was already shown that ATP hydrolysis catalyzed by $F_1$ isolated from *Mastigocladus laminosus* or *Synechococcus* 6716 was not stimulated by dithiothreitol or heat pretreatment [21,22].

In this paper we compare the activation requirements of the ATP synthase of the thermophilic cyanobacterium *Synechococcus* 6716 with that of the spinach chloroplast enzyme.

We will show that apart from activation by trypsin treatment and $\Delta\bar{\mu}_{H^+}$, the *Synechococcus* ATP synthase may be activated by treatment with sulfite or octyl glucoside, agents that have not been shown to act directly on the structure of the $\gamma$ subunit [4–6].

## Materials and Methods

*Organism and culture conditions.* Thermophilic cyanobacterium *Synechococcus* PCC 6716 was batch-cultured in BG11 medium [23], at 50°C in a 2 liter airlift fermenter as described in Ref. 22.

*Preparation of cyanobacterial membrane vesicles.* Cells were harvested in the linear growth phase and treated with 0.1% lysozyme at 37°C as described [22] in a medium without $K^+$. Osmotic shock was carried out in 5 mM $MgCl_2$, 10 mM Tricine-NaOH (pH 7.8). KCl concentration in this medium was adapted to the appropriate $[K^+]_{in}$ of the vesicles (see legends to tables and figures). After osmotic shock, the vesicles were centrifuged and resuspended in the supernatant to a chlorophyll *a* concentration of about 1 mg $ml^{-1}$. All steps were carried out at room temperature.

*Preparation of broken chloroplasts.* Isolation of chloroplasts was carried out as in Schuurmans et al. [24], except that osmotic shock was done in 8 ml 5 mM $MgCl_2$ for 1.5 min, after which 32 ml of isolation medium containing 300 mM sucrose, 10 mM KCl, 3 mM $MgCl_2$ and 30 mM Tricine-NaOH (pH 7.8) was

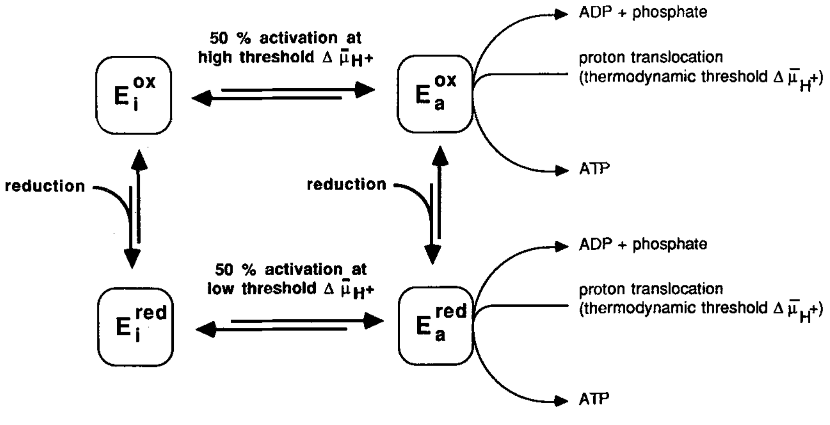

Fig. 1. The relations between the different states of the chloroplast ATP synthase. ox, oxidized; red, reduced; i, inactive; a, active. The low and high $\Delta\bar{\mu}_{H^+}$ values for 50% activation are approx. 12 and 19 kJ $mol^{-1}$, respectively. The thermodynamic threshold value depends on the current value of $\Delta G_p$. (modified from Ref. 17).

added. After centrifugation at 3500 × *g* for 3 min the chloroplasts were resuspended in isolation medium to a chlorophyll concentration of about 2.5 mg ml$^{-1}$. All steps were carried out at 4°C.

*Isolation and reconstitution of Synechococcus and chloroplast ATP synthase.* The ATP synthases from *Synechococcus* 6716 and spinach chloroplasts were isolated according to Lubberding et al. [25] and Pick and Racker [26], respectively. The fractions precipitated between 35% and 50% saturated ammonium sulfate ($P_{35\text{-}50}$) were used without further purification. They were stored at 4 °C in a medium containing 5 mM $MgCl_2$ and 100 mM Tricine-NaOH (pH 7.8) at a protein concentration of about 10 mg ml$^{-1}$ (*Synechococcus* ATP synthase) or in liquid nitrogen in a medium containing 5 mM $MgCl_2$, 200 mM sucrose and 20 mM Tricine-NaOH (pH 8) at a protein concentration of 30 mg ml$^{-1}$ (chloroplast ATP synthase). ATP synthase proteoliposomes were prepared by overnight detergent dialysis at 50°C [27] with native lipids extracted from *Synechococcus* 6716 [28] in a concentration of 10 mg ml$^{-1}$ and a protein-to-lipid ratio of 0 02 (w/w). Proteoliposomes were stored at 4 °C.

*Chlorophyll concentrations.* Chlorophyll concentrations in cyanobacterial vesicles and broken chloroplasts were determined according to Arnon et al. [29] and Bruinsma [30], respectively.

*Protein concentrations.* Protein concentrations were determined by the Pierce method [31] using bovine serum albumin as standard.

*ATP hydrolysis activity.* Activity was determined continuously in the presence of an ATP-regenerating system coupled to NADH oxidation, the principle of which is described by Bergmeyer [32]. The reaction was started by addition of 5 mM ATP. NADH oxidation was monitored spectrometrically at 340–400 nm in an Aminco DW-2 dual wavelength spectrophotometer supplied with a thermostated multipurpose cuvette [33]. The assay medium used was as described by Stutterheim [34], omitting $K_2CO_3$. Unless stated otherwise, the assay temperature was 50°C.

In some cases (see text) ATP hydrolysis was measured as inorganic phosphate release using the assay method developed by LeBel [35], as modified by Jagendorf [36]. The concentration of ATP in the reaction mixture was 5 mM.

*Phosphorylation reactions.* Phosphorylation reactions were performed at 50°C with cyanobacterial vesicles employing a modified method originally described by Jagendorf and Uribe [8], with the following two-stage protocol:

(i) Cyanobacterial vesicles prepared in the presence of 10 mM KCl were incubated for 1 min in 2 ml acidifying medium (A, see below) at a chlorophyll concentration of about 60 $\mu$g ml$^{-1}$.

(ii) Then 1 ml of this incubation mixture was mixed with an equal volume of alkaline medium (B, see below). After 0.5–1 min of incubation, a 0.5 ml aliquot was drawn from this reaction mixture, and ATP synthesis was immediately quenched by addition of 50 $\mu$l 6 M perchloric acid, followed by neutralization with 50 $\mu$l 6 M KOH in 1 M Tris and storage on ice. The pH of the acid stage and the alkaline stage mixture were measured at 50°C directly after these acid-base transitions in order to determine the actual $\Delta$pH value.

The A medium contained 10 mM succinic acid, 2 mM $MgCl_2$, 10 mM KCl, 2 mM $NaH_2PO_4$, 2 $\mu$M valinomycin, 10 $\mu$M DCMU and 10 mM Tricine. The pH was varied with NaOH over a range of pH 5–6.5, depending on the desired $\Delta$pH. The B medium contained 10 mM KCl, 2 mM $MgCl_2$, 1 mM $NaH_2PO_4$, 1 mM ADP, 10 $\mu$M DCMU and 200 mM Na-Tricine (pH 8.6).

The yield of ATP in 0.1 ml of the above mentioned aliquots was measured by the hexokinase/glucose-6-phosphate dehydrogenase assay method [32] in 1.8 ml buffer containing 1 mM glucose, 0.8 mM NADP, 7 mM $MgCl_2$, and 80 mM Tris-HCl (pH 7.6). NADPH formation was monitored at 340 nm.

Treatment of the enzyme and membrane preparations with the various ATP synthase activating agents is described in the legends to the tables and figures.

NADH, NADP, ATP, ADP, phospho*enol*pyruvate, valinomycin, and all enzymes were purchased from Boehringer (Mannheim, F.R.G.). Dithiothreitol, trypsin type XI, soybean trypsin inhibitor and DCCD were purchased from Sigma (St. Louis, U.S.A.). DCMU was from ICN K&K Laboratories. (Cleveland, U.S.A.). Elon was from J.T. Baker (Deventer, The Netherlands). S-13 was a gift from Dr. P.C. Hamm (Monsanto, St. Louis, U.S.A.).

## Results

### *Stimulation of ATP hydrolysis activity of the isolated ATP synthases from Synechococcus 6716 and spinach chloroplasts*

Table I summarizes the outcome of experiments in which the effect of various treatments on the activity of both types of isolated ATP synthase was studied. As reported earlier [37], the rate of ATP hydrolysis catalyzed by either of the two enzymes is up to 10-times faster at 50°C than at 25°C. In general, the effects of various pretreatments are much better expressed at the higher temperature. Also, more trypsin is needed at 25°C in order to obtain stable increased activity within the experimental time. The results from the distinct experiments are obtained with different preparations of ATP synthase which vary in basal activity.

Trypsin, heat and dithiothreitol are effective in stimulating the hydrolysis activity of chloroplast ATP synthase. This corresponds to previous data obtained with

TABLE I

*The effects of various activation treatments on the ATP hydrolysis activity of isolated ATP synthase complex from Synechococcus ($SF_1F_0$) and chloroplasts ($CF_1F_0$)*

For preparations see Materials and Methods. ATP hydrolysis was assayed by the method of [34]. The concentration of ATP synthase during the assay was 10–20 $\mu$g ml$^{-1}$ and the reaction was started with 5 mM ATP. Unless otherwise indicated, the storage media (see Materials and Methods) were used for incubations. In Expt. 1, trypsin was preincubated at the measurement temperature and in the assay medium for 10 min and stopped by a 10-fold excess of trypsin-inhibitor. In Expts. 2 and 3 trypsin (250 and 25 $\mu$g ml$^{-1}$ at 25 and 50 ° C, resp.) was added about 10 min after the start of the reaction and the maximal rate was then reached in about 5–10 min. Heat was applied for 2 min at a protein concentration of 0.5 mg ml$^{-1}$ in the presence of 40 mM ATP. The samples were diluted 10-times before the assay. Preincubation with dithiothreitol was 5 min at room temperature at a protein concentration of 1–2 mg ml$^{-1}$. Assay temperatures are indicated in the column headings. The standard deviation of the measurements varies from 2% (120 mM DTT+ trypsin treatment of $CF_1F_0$) up to 17% (heat treatment of $CF_1F_0$ at 50–60 ° C), as derived from mainly triplicate determinations on a single preparation.

| Activation method | ATP hydrolysis rate (nmol/min per mg protein) | | | |
|---|---|---|---|---|
| | $SF_1F_0$ | | $CF_1F_0$ | |
| | 25 ° C | 50 ° C | 25 ° C | 50 ° C |
| 1. Trypsin treatment | | | | |
| None | 64 | 120 | 28 | 573 |
| 10–100 $\mu$g ml$^{-1}$ | n.d. | 670 | 29 | 1075 |
| 0.1–1 mg ml$^{-1}$ | 137 | 787 | 38 | n.d. |
| 2a. Heat activation | | | | |
| 20 ° C | 38 | 230 | 76 | 450 |
| 50–60 ° C | 25 | 136 | 103 | 2800–3800 |
| 75 ° C | n.d. | 55 | 0 | 150 |
| 2b. Heat activation + trypsin treatment | | | | |
| 20 ° C | 88 | 615 | 96 | 1150 |
| 50–60 ° C | 98 | 550 | 114 | 3400–4550 |
| 75 ° C | n.d. | 85 | 35 | 150 |
| 3a. Dithiothreitol | | | | |
| None | 13 | 131 | 34 | 550 |
| 5–80 mM | 13 | 134 | 41 [a] | 820 [a] |
| 120 mM | 2 | 73 | 41 [a] | 700 [a] |
| 3b. Dithiothreitol + trypsin treatment | | | | |
| None | 48 | 205 | 65 | 850 |
| 5–80 mM | 47 | 177 | 75 | 830 |
| 120 mM | 2 | 124 | 62 | 700 |

[a] Increase in ATP hydrolysis activity only occurred under turnover conditions 2–3 min after start of the assay.

$CF_1$ (see Ref. 38 for review). Especially, pretreatment with heat (with a temperature optimum of 50–60°C) leads to very high activities which can still be increased by subsequent addition of trypsin.

In contrast to studies on $CF_1$, where both reduction and activation start immediately upon dithiothreitol addition [14] , the activation of $CF_1F_0$ becomes manifest during the measurement of ATP hydrolysis only some minutes after the start (not shown). This lagging activation might be related to proton translocation. A similar phenomenon, self-activation (but observed in the absence of dithiothreitol) has been reported to occur in chromatophores and reconstituted ATP synthase proteoliposomes from *Rhodospirillum rubrum* [39]. Interestingly, the effects of dithiothreitol and trypsin treatment are not additive, in contrast to the effects of heat treatment and trypsin.

Table I also shows that heat treatment or dithiothreitol do not increase the ATP hydrolysis activity of isolated *Synechococcus* ATP synthase. At high temperature or at high dithiothreitol concentration even inhibition is observed. Trypsin, on the other hand, increases the activity 5- to 10-times (see also Ref. 37). In testing the stimulating effect of octyl glucoside, ATP synthase proteoliposomes were used and octyl glucoside was added during the assay. Because the isolated ATP synthase preparation contains substantial amounts of octyl glucoside [25], detergent-depleted proteoliposomes have been used to study the activating effect of this detergent. Octyl glucoside slightly increases the ATP hydrolysis activity of the cyanobacterial ATP synthase proteoliposomes (135%) at 30 mM but inhibits (58% residual activity) when the concentration is increased to 50 mM, as assayed at 50°C.

### *Effects of methanol, sulfite, and octyl glucoside on ATP hydrolysis in cyanobacterial membrane vesicles*

We tested a number of compounds that stimulate ATP hydrolysis catalyzed by chloroplasts. Table II shows the effects of these compounds on ATP hydrolysis catalyzed by well-coupled membrane vesicles of *Synechococcus* 6716.

To observe any stimulating effect of methanol, it has to be present during ATP hydrolysis [4]. Because

TABLE II

*Effects of various stimulating agents on ATP hydrolysis in membrane vesicles of Synechococcus 6716*

The effect of methanol was measured as inorganic phosphate release [36]. The effect of octyl glucoside was measured in assay at a chlorophyll concentration of 5 $\mu$g ml$^{-1}$ after 10 min preincubation at a chlorophyll concentration of 250 $\mu$g ml$^{-1}$ in the presence of 5 mM ATP and 30 mM octyl glucoside. The effect of sulfite was measured after 5 min preincubation at room temperature and a chlorophyll concentration of 250 $\mu$g ml$^{-1}$. Preincubations were carried out in a medium containing 5 mM $MgCl_2$ and 10 mM Tricine-NaOH (pH 7.8). Rates are given relative to the rate in the absence of stimulating agent.

| Agent | Concentration | Relative rate of hydrolysis (%) |
|---|---|---|
| $CH_3OH$ | 5–40 vol.% | 57 |
| Octyl glucoside | 17.5 mM | 154 |
| $Na_2SO_3$ (dark) | 50 mM | 120 |
| $Na_2SO_3$ (light) | 50 mM | 150 |
| NaCl (dark and light) | 100 mM | 100 |

methanol interferes with the pyruvate kinase/lactate dehydrogenase system used in the assay, ATP hydrolysis in this case is followed by measuring phosphate release. In *Synechococcus* 6716 membrane vesicles the only effect of methanol after 120 s of incubation is a slight inhibition of ATP hydrolysis. Stimulation by octyl glucoside and sulfite, on the other hand, could be demonstrated (maximum values are given in Table II). As with the isolated complex, octyl glucoside increases the ATP hydrolysis activity. However, a decrease in activity to 70% of the original level was observed when the concentration of octyl glucoside was increased above 17.5 mM (not shown). This is more or less analogous to the inhibition observed at higher octyl glucoside concentrations in ATP synthase proteoliposomes (see Table I) and contrasts with the earlier findings that $CF_1$ remains activated at octyl glucoside concentrations up to 60 mM [5].

Finally, Table II shows that also sulfite enhances ATP hydrolysis and that this enhancement is larger under illumination. The addition of an uncoupler inactivated ATP hydrolysis in the presence of sulfite, also after light activation (not shown). A control experiment with NaCl at a comparable concentration revealed that the stimulating effect is not caused by sodium ions.

### *Effect of heat pretreatment on ATP hydrolysis activity in cyanobacterial membrane vesicles and broken chloroplasts*

Chloroplast $F_1$ ATP synthase can be activated by heat [3]. Fig. 2A shows the effect of heat pretreatment at different temperatures. Activities were measured at two different assay temperatures (25 and 50°C). It can be seen that (as is the case with isolated ATP synthase from *Synechococcus*, cf. Table I), heat treatment of cyanobacterial membrane vesicles only has an inhibiting effect on ATP hydrolysis at both assay temperatures. Broken chloroplasts were subjected to the same treatment and ATP hydrolysis was assayed at 25°C and 50°C (Fig. 2B). The absence of a stimulating effect of heat treatment is rather surprising and in contrast with the effect of this treatment on isolated $CF_1F_0$ (see Table I). It can be deduced that the effect of assay temperature is not as prominent in membrane preparations (Fig. 2A and Fig. 2B) as in the isolated enzyme (Table I). Both in cyanobacterial membranes and in chloroplasts increase of the assay temperature from 25°C to 50°C results in an increase in activity by only a factor of 2.

### *Stimulation of ATP hydrolysis in cyanobacterial membrane vesicles by trypsin*

We determined the relation between the trypsin concentration and the rate of ATP hydrolysis in cyanobacterial membrane vesicles. Trypsin was supplied in the assay medium. After 5 min incubation the action of trypsin was stopped with a 10-fold excess of soybean trypsin inhibitor. The rate of ATP hydrolysis was followed continuously at a chlorophyll *a* concentration of 1.8 μg $ml^{-1}$ and a protein concentration (without trypsin and trypsin inhibitor) of 44 μg $ml^{-1}$. Trypsin activates ATP hydrolysis maximally up to 186% (100% activity corresponds to 2.2 μmol ATP/min per mg Chl *a*) at a concentration of 0.1 mg $ml^{-1}$. This concentration corresponds well with that found for the isolated enzyme of *Synechococcus* and chloroplasts (Table I).

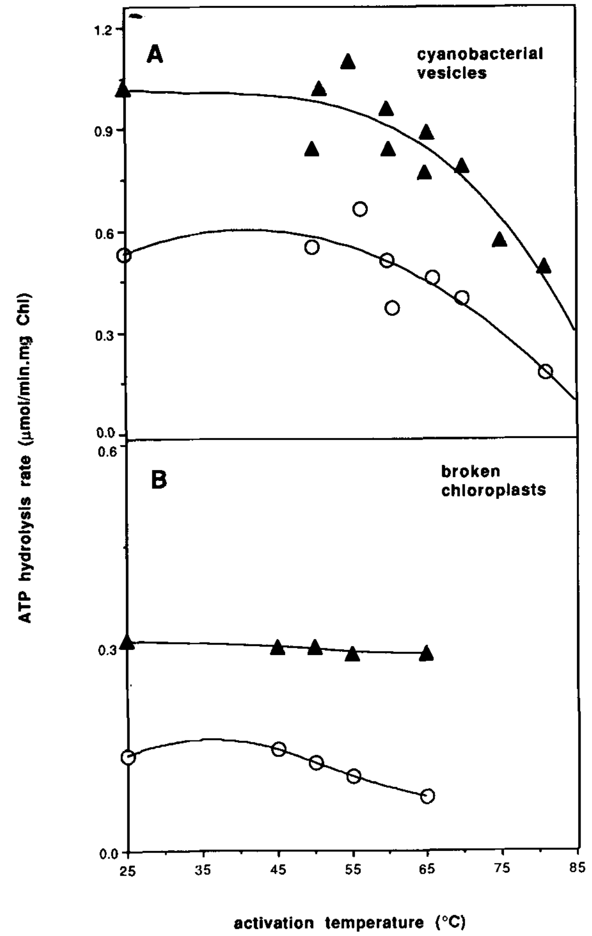

Fig. 2. Response of ATP hydrolysis upon heat pretreatment in membrane vesicles of *Synechococcus* 6716 (A) and broken chloroplasts (B). The incubation method was modified after [3]. Vesicles were diluted to a chlorophyll *a* concentration of 160 μg $ml^{-1}$ in incubation buffer containing 40 mM ATP, 10 mM $MgCl_2$ and 100 mM Tricine-NaOH (pH 7.8) in a total volume of 1 ml at the given incubation temperatures. Broken chloroplasts were diluted to a chlorophyll concentration of 500 μg $ml^{-1}$ in incubation buffer containing 40 mM ATP, 3 mM $MgCl_2$, 300 mM sucrose, and 30 mM Tricine-NaOH (pH 7.8) in a total volume of 1 ml. After 2 min temperature treatment 40 μl of the incubation mixtures were assayed in a volume of 1.9 ml at 50°C (closed triangles) and at 25°C (open circles).

### *Stimulation of ATP hydrolysis by light and artificially applied $\Delta\bar{\mu}_{H^+}$*

As has been mentioned, the chloroplast ATP synthase can be activated by a $\Delta\bar{\mu}_{H^+}$, both in case of ATP

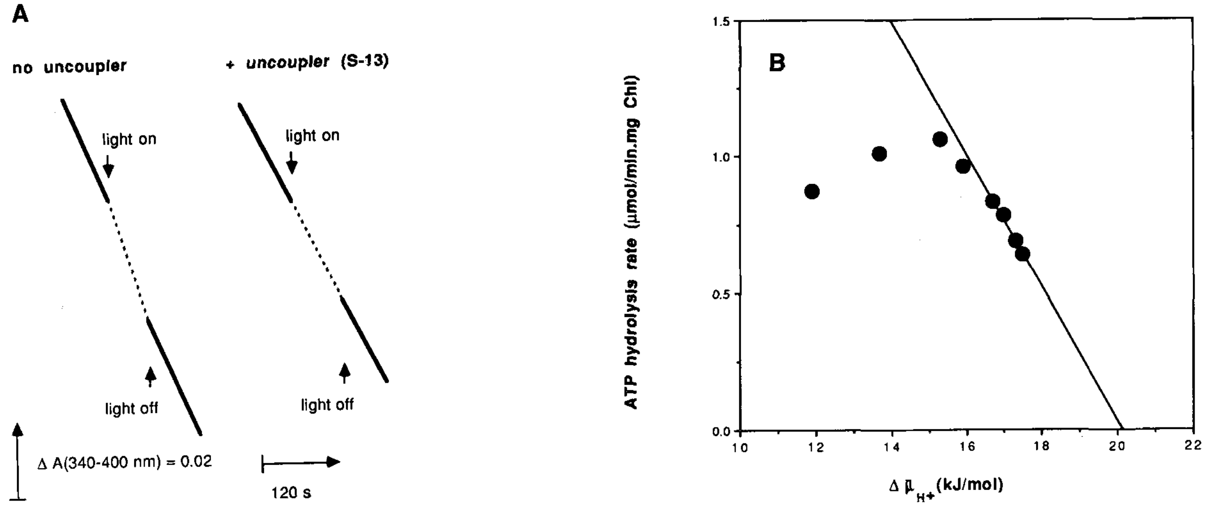

Fig. 3. Stimulation of ATP hydrolysis in membrane vesicles of *Synechococcus* 6716 by light (A) and an applied artificial proton electrochemical gradient (B). (A) Membrane vesicles at a chlorophyll *a* concentration of 5 $\mu g\ ml^{-1}$ were illuminated ($\lambda \geq 665$ nm) during hydrolysis in the absence or presence of 1 $\mu M$ S-13. (B) Initial rate of ATP hydrolysis as a function of a valinomycin-induced $K^+$-diffusion potential applied during assay. This potential is calculated using the Nernst equation. The chlorophyll *a* concentration was 3.5 $\mu g\ ml^{-1}$, the concentration of valinomycin was 0.1 $\mu M$. The line shown is a linear extrapolation to 0.0 $\mu$mol ATP/ min per mg Chl *a* using the four points at highest $\Delta\tilde{\mu}_{H^+}$ in the plot. Further details, see text.

synthesis or ATP hydrolysis. This is demonstrated in two types of experiment we have carried out with membrane vesicles of *Synechococcus* 6716. Fig. 3A shows an experiment in which cyanobacterial vesicles hydrolyzing ATP were illuminated for 1 min with saturating actinic light. ATP hydrolysis was followed continuously in the dark before and after illumination (during illumination the photomultiplier was switched off). The dashed line thus connects the start and the end of the 'blind' part of the trace. From the deflection of the dashed line it can be calculated that the rate of ATP hydrolysis increases by 30% in the light. When the uncoupler S-13 was added no light stimulation of ATP hydrolysis occurred (Fig. 3A), suggesting that stimulation was due to generation of $\Delta\tilde{\mu}_{H^+}$.

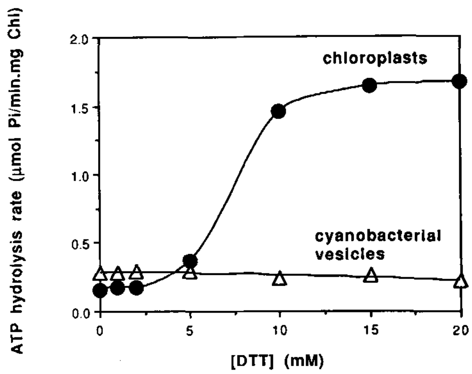

Fig. 4. Hydrolysis of ATP in broken chloroplasts and membrane vesicles of *Synechococcus* 6716 treated with dithiothreitol (DTT), in the light. Broken chloroplasts (●) and cyanobacterial membrane vesicles (△) were incubated in white light during 10 min at room temperature. The chlorophyll concentration was 200 $\mu g\ ml^{-1}$ for both preparations. PMS was present at 100 $\mu M$, total volume was 1 ml. The incubations were carried out in chloroplast isolation medium or in 5 mM $MgCl_2$/10 mM Tricine-NaOH (pH 7.8). Aliquots of 100 $\mu l$ were assayed for inorganic phosphate release according to Ref. 36 at 37 °C.

A $\Delta\tilde{\mu}_{H^+}$ can also be generated by a valinomycin-induced $K^+$-diffusion potential. In Fig. 3B it is demonstrated how a diffusion potential influences the initial rate of ATP hydrolysis in cyanobacterial membrane vesicles. With an inner $K^+$ concentration of 1 mM, this diffusion potential was imposed by increasing the outer $K^+$ concentration from 10 to 720 mM stepwise in the presence of valinomycin. The ATP-regenerating system (see Materials and Methods) maintains the $\Delta G_p$ at the high value of about 85 kJ $mol^{-1}$ (see Ref. 37). Fig. 3B also shows that ATP hydrolysis is inhibited at high values of $\Delta\tilde{\mu}_{H^+}$. Prior to this inhibition, however, ATP hydrolysis is stimulated slightly by $\Delta\tilde{\mu}_{H^+}$, reaching a maximum between 13.4 and 15.8 kJ $mol^{-1}$. The $H^+$/ATP stoichiometry can be estimated by extrapolating the curve in Fig. 4B to the right to zero hydrolysis rate. When extrapolation is done by a straight line, the rate is zero at 20.1 kJ $mol^{-1}$. At a phosphate potential of 85 kJ $mol^{-1}$ the $H^+$/ATP stoichiometry would then be approx. 4.2.

### *Influence of light and dithiothreitol on ATP hydrolysis in broken chloroplasts and cyanobacterial membrane vesicles*

In a parallel experiment broken chloroplasts and cyanobacterial membrane vesicles were illuminated in the presence of the cyclic electron transfer mediator PMS [40] in order to generate a $\Delta\tilde{\mu}_{H^+}$ by light-driven proton uptake. The dithiothreitol concentration in the incubation medium was varied from 0 to 20 mM to study the effect of the reducing agent on light-activated ATP hydrolysis by thylakoids. Hydrolysis was measured by phosphate release rather than by the enzymatic method (see Materials and Methods) because PMS strongly interferes with this latter assay. The results shown in Fig. 4 clearly demonstrate that only the broken chloroplasts are strongly stimulated by an increasing dithiothreitol concentration, while cyanobacterial membrane vesicles, already performing their maximum activity, remain indifferent to this treatment. In dithiothreitol-treated chloroplasts, addition of 0.1 mM of DCCD inhibited ATP hydrolysis by 70–90%. In the cyanobacterial vesicles DCCD inhibited by only about 20% (not shown).

### *The energy threshold for ATP synthesis in cyanobacterial membrane vesicles*

We investigated the energy threshold for ATP synthesis in *Synechococcus* 6716 to see whether its ATP synthase is closer to the oxidized or to the reduced state of the chloroplast ATP synthase. In these experiments the initial $\Delta G_p$ was 40.4 kJ mol$^{-1}$. ATP synthesis was accomplished by acid-base transition of membrane vesicles, in the presence of valinomycin in order to prevent generation of any membrane potential. Furthermore, DCMU was added to inhibit photophosphorylation (see Materials and Methods). Proton gradient-driven ATP production was corrected for secondary ATP-producing processes (like myokinase activity) by subtracting ATP formation monitored in the presence of 1.5 $\mu$M of the uncoupler S-13.

The amount of ATP formed at a wide range of $\Delta\tilde{\mu}_{H^+}$ values (11.7, 14.4 and 17.3 kJ mol$^{-1}$) was maximal within 15 s. Therefore, in Fig. 5 the $\Delta\tilde{\mu}_{H^+}$ dependence of the yield of ATP is shown. Extrapolation in Fig. 5 shows that ATP synthesis occurs when $\Delta\tilde{\mu}_{H^+}$ exceeds a threshold value of 9.5 kJ mol$^{-1}$ (corresponding to a ΔpH of 1.5). At $\Delta\tilde{\mu}_{H^+}$ values higher than 15 kJ mol$^{-1}$ the data points are more scattered. This may be due to the 60 s incubation of the vesicles, which at the low pH values required for the larger ΔpH values can lead to destabilization of the high energy state [8]. From the threshold value of 9.5 kJ mol$^{-1}$ and the initial phosphate potential of 40.4 kJ mol$^{-1}$ a H$^+$/ATP stoichiometry of 4.3 can be calculated. This value is in good agreement with that derived from Fig. 3B and with other observations with cyanobacteria [37] and chloroplasts [41,42].

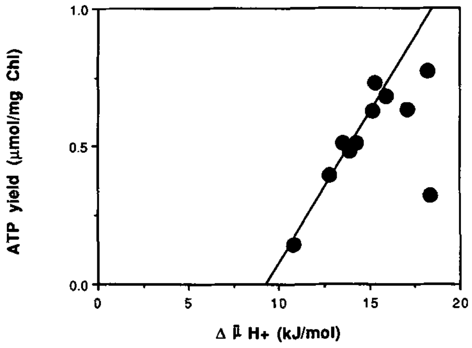

Fig. 5. ATP yield as a function of the applied proton electrochemical potential gradient in membrane vesicles of *Synechococcus* 6716. The proton gradient was applied by performing an acid-base transition as described in Materials and Methods. The chlorophyll concentration was 34 $\mu$g ml$^{-1}$. $\Delta G_p$ is 40.4 kJ mol$^{-1}$, calculated from $\Delta G_0' = 34.3$ kJ mol$^{-1}$ [56], $[\mathrm{ADP}] = 9.8 \cdot 10^{-4}$ M, $[\mathrm{P_i}] = 1.48 \cdot 10^{-3}$ M, and [ATP] $= 1.4 \cdot 10^{-5}$ M (as a measured contamination of the ADP used). Reaction time was 30 s. The ATP yield was corrected for myokinase activity (see Results); $\mathrm{pH_{out}}$ was $8.01 \pm 0.02$. The data shown have been obtained from two experiments performed with two different membrane preparations. The line shown is a linear extrapolation to a yield of 0.0 $\mu$mol ATP/mg Chl *a* using the five points at lowest $\Delta\tilde{\mu}_{H^+}$ in the plot. For further reaction conditions see Materials and Methods.

A 5 min pre-incubation of the vesicles with 2 mg trypsin per mg chlorophyll *a* resulted in complete inhibition of ATP synthesis (data not shown). This inhibition was observed for ΔpH values ranging from 2 to 3, which is the range where (in these vesicles) significant ATP synthesis does occur. Such inhibitory effect of trypsin treatment has been described for chloroplasts and may indicate an uncoupling effect [43]. However, shorter preincubation at lower trypsin-to-chlorophyll ratios might actually stimulate phosphorylation [45].

## Discussion

### *Activation by methanol, sulfite, octyl glucoside, trypsin and heat pretreatment*

We have shown here that sulfite and octyl glucoside stimulate ATP hydrolysis activity catalyzed by the *Synechococcus* 6716 ATP synthase. Also, the cyanobacterial ATP synthase is activated by treatment with trypsin. In contrast to the situation with chloroplast ATP synthase, however, methanol treatment, reduction with dithiothreitol and heat treatment have no stimulating effect on ATP hydrolysis activity of *Synechococcus* ATP synthase. The slight inhibitory effect of methanol on *Synechococcus* 6716 ATP synthase is not characteristic for cyanobacteria in general. In *Spirulina platensis* methanol stimulates ATP hydrolysis activity [40].

In contrast to chloroplast ATP synthase, the *Synechococcus* enzyme is activated by octyl glucoside only up to a critical concentration (17.5 and 30 mM for membrane-bound and isolated enzyme, respectively). In this respect the effect of octyl glucoside on the cyanobacterial enzyme more resembles that of sodium salts of short chain fatty acids on $\mathrm{CF_1}$ [5]. In contrast to chloroplasts [6], sulfite does not replace $\Delta\tilde{\mu}_{H^+}$ in stimulating ATP hydrolysis activity in the cyanobacterium.

It is difficult to conclude whether the differences in response of the two enzymes to the activator depends on the presence or absence of a disulfide group in the $\gamma$ subunit. Recently, evidence was obtained that sulfite does act on the $\gamma$ subunit of the chloroplast enzyme, but not on the disulfide group [44]. Therefore, cyanobacteria are promising objects to study the structural basis of sulfite stimulation.

Heat activation of chloroplast ATP synthase has been studied in isolated $CF_1$ [3,14,16]. We have shown heat activation of the isolated ATP synthase from chloroplasts ($CF_1F_0$), but we failed to demonstrate the same phenomenon when the chloroplast ATP synthase was still associated with the thylakoid membrane. This rather puzzling observation will be a matter for forthcoming studies.

With the isolated enzyme from chloroplasts we have shown (Expt. 2b in Table I) that the effects of heat and trypsin treatment are additive. Also, treatment of $CF_1F_0$ with heat and dithiothreitol is additive [14]. On the other hand, simultaneous treatment of isolated $CF_1F_0$ with dithiothreitol and trypsin at high activator concentration (Expt. 3b in Table I) does not result in a higher activity. This suggests that either reduction of the $\gamma$ subunit disulfide bond or the cleavage of the $\gamma$ subunit by trypsin leads to a more-or-less similar conformational change [15]. This idea is supported by the observation that the result of the trypsin action is functionally fully comparable with that of dithiothreitol [45]. The additive character of the effect of heat treatment to both dithiothreitol and trypsin treatments indicates that this third type of activation may be due to a mechanism not involving the disulfide bond in the $\gamma$ subunit [14,16].

### *Activation by a proton electrochemical potential gradient and dithiothreitol*

Like in chloroplasts, the ATP synthase from *Synechococcus* 6716 can be activated by a $\Delta\tilde{\mu}_{H^+}$ (Fig. 3). Unlike the chloroplast enzyme, the *Synechococcus* enzyme cannot be activated by treatment with dithiothreitol (Fig. 4). Obviously, this latter finding is a direct consequence of the absence of the relevant part of the $\gamma$ subunit in the *Synechococcus* enzyme. In the absence of a protonmotive force, micromolar concentrations of ADP lead to deactivation of the cyanobacterial enzyme within 1 min (unpublished results). The trapping of the ADP released during ATP hydrolysis prevents this inhibition. The type of inhibition is comparable to that described for the reduced chloroplast ATP synthase [46,47].

It is interesting to notice that activation by dithiothreitol has been reported for the ATP synthase of the cyanobacterium *Spirulina platensis* [40]. Unfortunately, the $\gamma$ sequence of this species has not yet been elucidated.

### *Possible relation between dithiothreitol, methanol and heat activation*

It is tempting to assume that a single structural factor is responsible for the absence of chloroplast-like activation by methanol, heat treatment and dithiothreitol of the *Synechococcus* 6716 ATP synthase. The inclusion of the dithiothreitol effect in this list would imply that the missing part in the $\gamma$ subunit sequence is involved. Alcohols, heat and some detergents are known to dissociate the $\epsilon$ subunit in $CF_1$ [16,48,49]. In chloroplasts a close physical proximity between a sulfhydryl residue in the $\epsilon$ subunit and the $\gamma$ subunit disulfide and the $\gamma$ subunit 'dark site'-SH was reported [50]. This may indicate that these two subunits are closely linked (both physically and functionally [50]) so that disturbance of this $\epsilon$-$\gamma$ arrangement by tryptic cleavage [51], or removal of $\epsilon$, results in partial activation of the entire $CF_1$ and a better accessibility for dithiothreitol or further trypsin action (cf. Refs. 14, 51).

It is plausible that this $\epsilon$-$\gamma$ interaction may be the single structural factor suggested above. The absence of the sulfhydryl-containing sequence in the $\gamma$ subunit and the cysteine in the $\epsilon$ subunit of *Synechococcus* [20] would prevent or alter the $\epsilon$-$\gamma$ arrangement, typical for inactive chloroplast ATP synthase, due to a different conformation of both the cyanobacterial $\gamma$ and $\epsilon$ subunit. This could result in that the *Synechococcus* ATP synthase's being always in an 'activated' state.

This idea is strengthened by the fact that the substrate specificity with respect to Mg-ATP and Ca-ATP of the *Synechococcus* enzyme (Ref. 22 and Bakels, unpublished data) resembles that of the methanol-activated chloroplast enzyme, but differs from that of the inactive chloroplast enzyme [4].

### *Energy threshold for ATP synthesis: physiological implications*

The low threshold value of $\Delta\tilde{\mu}_{H^+}$ for ATP synthesis of the *Synechococcus* ATP synthase (9.5 kJ $mol^{-1}$) is even lower than that of the reduced form (12.5 kJ $mol^{-1}$, cf. Ref. 17) of the chloroplast enzyme. This means that the cyanobacterial enzyme does not require an extremely high $\Delta G_p$ to catalyse ATP hydrolysis. It may be conjectured that the enzyme in *Synechococcus* 6716 is structurally similar to the ATP synthase in chloroplasts in its reduced form.

The same low threshold value of 9.5 kJ $mol^{-1}$ has been found for *Rhodospirillum rubrum* [52], a photosynthetic bacterium which also lacks a disulfide group in its ATP synthase $\gamma$ subunit [53].

The high threshold level for ATP synthase activation observed in chloroplasts is thought to prevent ATP hydrolysis in the dark [17,54]. This would then imply that in *Synechococcus* 6716 protection against dark ATP hydrolysis is not guaranteed by oxidation of the $\gamma$ subunit.

During light–dark transition under aerobic conditions *Synechococcus* 6716 is able to maintain its cellular ATP level almost immediately at 75% of its light-level [55]. This may indicate that a $\Delta\bar{\mu}_{H^+}$ generated by oxidative phosphorylation in the dark would help the ATP synthase involved in photophosphorylation to refrain from ATP hydrolysis. The simplest way to achieve this is if the same ATP synthase is involved in both oxidative and photophosphorylation. An additional mechanism against excessive dark hydrolysis may be the tight binding of ADP in the absence of $\Delta\bar{\mu}_{H^+}$ [46,47]. It has been shown that the genes of the ATP synthase subunits are present as single copies in the genomic DNA of *Synechococcus* [18,20]. The use of a single ATP synthase in photophosphorylation and oxidative phosphorylation, with the correspondingly different structure of its $\gamma$-subunit, is probably not common to all cyanobacterial species. Especially cyanobacteria which do have a redox-regulated ATP synthase (e.g., *Spirulina* [40]) are interesting in this respect. Physiological and biochemical studies on this type of organism will be subject of our future studies.

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