Introduction of a Carboxyl Group in the Loop of the F_0 *c*-Subunit Affects the H$^+$/ATP Coupling Ratio of the ATP Synthase From *Synechocystis* 6803

Hendrika S. Van Walraven,[1,4] Marijke J. C. Scholts,[1] Holger Lill,[1] Hans C. P. Matthijs,[2] Richard A. Dilley,[3] and Ruud Kraayenhof[1]

The proton translocation stoichiometry (H$^+$/ATP ratio) was investigated in membrane vesicles from a *Synechocystis* 6803 mutant in which the serine at position 37 in the hydrophilic loop of the *c*-subunit from the wild type was replaced by a negatively charged glutamic acid residue (strain plc37). At this position the *c*-subunit of chloroplasts and the cyanobacterium *Synechococcus* 6716 already contains glutamic acid. H$^+$/ATP ratios were determined with active ATP synthase in thermodynamic equilibrium between phosphate potential (ΔG_p) and the proton gradient ($\Delta \mu_H^+$) induced by acid–base transition. The mutant displayed a significantly higher H$^+$/ATP ratio than the control strain (wild type with kanamycin resistance) at pH 8 (4.3 vs. 3.3); the higher ratio also being observed in chloroplasts and *Synechococcus* 6716. Furthermore, the pH dependence of the H$^+$/ATP of strain plc37 resembles that of *Synechococcus* 6716. When the pH was increased from 7.6 to 8.4, the H$^+$/ATP of the mutant increased from 4.2 to 4.6 whereas in the control strain the ratio decreased from 3.8 to 2.8. Differences in H$^+$/ATP between the mutant and the control strain were confirmed by measuring the light-induced phosphorylation efficiency (P/*2e*), which changed as expected, i.e., the P/*2e* ratio in the mutant was significantly less than that in the wild type. The need for more H$^+$ ions used per ATP in the mutant was also reflected by the significantly lower growth rate of the mutant strain. The results are discussed against the background of the present structural and functional models of proton translocation coupled to catalytic activity of the ATP synthase.

KEY WORDS: ATP synthase; proton translocation; H$^+$/ATP ratio; enzyme regulation; cyanobacteria; *Synechocystis* 6803 mutants; *c*-subunit; charged amino acids.

INTRODUCTION

The ATP synthase is the terminal enzyme of oxidative and photosynthetic phosphorylation whereby energy from respiratory and photosynthetic electron transfer is converted into the third phosphate bond of ATP (Mitchell, 1966). The enzyme is present in mitochondria, chloroplasts, and (photosynthetic) bacteria and its overall structure is essentially the same among various species. In the enzyme ATP synthesis and hydrolysis (catalyzed by the F_1 part) are linked to transmembrane movements of protons (catalyzed by the F_0 part), respectively dissipating or forming a proton gradient ($\Delta \mu_H^+$) across the membrane (Nicholls and Ferguson, 1992).

Key to abbreviations: Chl, chlorophyll; ΔG_p, phosphate potential; $\Delta \mu_H^+$, proton electrochemical potential difference; F_1, peripheral hydrophilic part of the ATP synthase complex; F_0, membrane-spanning hydrophobic part of the ATP synthase complex; P/*2e*, phosphorylation efficiency; PMS, phenazine methosulfate; PMSF, phenylmethylsulfonyl fluoride; S-13, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide.

[1]Department of Structural Biology, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands.
[2]Department of Microbiology, University of Amsterdam, Amsterdam, The Netherlands.
[3]Department of Biological Sciences, Purdue University, West Lafayette, Indiana.
[4]To whom correspondence should be addressed; e-mail: rieky@bio.vu.nl.

The F_1 part is composed of five different subunits, denoted α, β, γ, δ, and ε, and the F_0 part of *E. coli* harbors three different subunits: *a*, *b*, and *c*, which makes a total of eight different subunits. In ATP synthase from chloroplasts and photosynthetic bacteria an extra F_0 subunit (*b'*) is found (see Van Walraven *et al.*, 1993). Mitochondrial ATP synthase is far more complex due to the large number of additional F_0 subunits (Collinson *et al.*, 1994). A general F_1 subunit stoichiometry of $\alpha 3\beta 3\gamma \delta\varepsilon$ is found, and the stoichiometry of the F_0 subunits is *a*, *b*, *b'* (or two copies of *b* in *E. coli*), *c*9–15 with the latter number apparently variable among different sources as first observed by Stock *et al.* (1999).

Purified F_1 from bovine heart mitochondria has been crystallized to 2.8 A resolution (Abrahams *et al.*, 1994) and it appeared that the three α- and β-subunits are arranged alternately like the segments of an orange. The resolved part of the γ-subunit is located in the middle of the enzyme, running from top to bottom. This structure supports the idea that the ATP synthase acts as a molecular rotary machine, involving stepwise (120°) rotation of the γ-subunit (driven by proton translocation through F_0) within the α/β assembly, each step releasing 1 ATP. Rotation of γ has indeed been demonstrated with F_1 from chloroplasts and Bacillus PS3 (see Junge *et al.*, 1997). Recently, detailed structures of the central ATP synthase stalk subunits from mitochondria and *E. coli* were published (Gibbons *et al.*, 2000; Rodgers and Wilce, 2000). It was shown before that ε is also part of the rotor (see Wada *et al.*, 2000). The multicopy *c*-subunit appeared to be present in a ring in yeast and in chloroplasts (Seelert *et al.*, 2000; Stock *et al.*, 1999). After an earlier result was debated (see Tsunoda *et al.*, 2000) the ring of *c*-subunits was ultimately shown to rotate (for *E. coli* (Panke *et al.*, 2000), for PS3 (Tsunoda *et al.*, 2001)). In a working model, one *a*-subunit with two half-channels for protons to either side of the membrane has contact with two hairpin-structured *c*-subunits that are part of the rotating ring (Vik *et al.*, 2000). Each of the *c*-subunits is protonated by one half-channel in *a* and, after one full cycle, deprotonated by the other half-channel of *a*. Thus, γ, ε, and the *c*-ring (= rotor) rotate relative to the α, β, δ, *b*, *d'* (or b2), and *a* subunits (= stator); the rotor and stator are interchangeable (Tanabe *et al.*, 2001). In contrast to this two-channel model a one-channel model is also postulated for *Propriogenum modestum* (Dimroth *et al.*, 2000) where protons bound to a *c*-subunit can exchange freely with the cytoplasm.

The consequence of this structure-function model is that there must be a straightforward correlation between the number of *c* and catalytic β subunits and the number of protons translocated per each ATP synthesized or hydrolyzed (the H^+/ATP ratio). Although there is little

doubt about the number of catalytic subunits (three) the number of *c*-subunits was more difficult to determine and for a long time only one reliable determination was done with not much accuracy (9–12; Foster and Fillingame, 1982). Until very recently it was suggested that the exact number of *c*-subunits was 12 (Jones and Fillingame, 1998). Increasing evidence suggests that the number of *c*-subunits is probably not the same in all sources: 10 in yeast (Stock *et al.*, 1999), 14 in chloroplasts (Seelert *et al.*, 2000), 10 in *E. coli* (Jiang *et al.*, 2001), and 11 in *Propriogenum modestum* (Stahlberg *et al.*, 2001). This would imply different H^+/ATP ratios for different ATP synthases.

Also the study of H^+/ATP is prone to many pitfalls. To mention one important factor: apart from being an energetic intermediate for the catalytic reactions, a proton gradient $(\Delta\mu_H^+)$ is also required for activation of virtually all ATP synthases studied so far. In the case of chloroplasts the influence of the redox state has to be accounted for (see Junesch and Graber, 1987). When the ATP synthase is active, a number of protons are translocated across the membrane during the synthesis or hydrolysis of one molecule of ATP. This H^+/ATP ratio is a crucial parameter in the function of the ATP synthase as a rotary machine. In earlier days of the Mitchell hypothesis a ratio of 2 was postulated (Mitchell, 1966) but a ratio of 3 H^+/ATP was, until recently, generally accepted (see Nicholls and Ferguson, 1992). Only for chloroplasts and some cyanobacteria this ratio was corrected to (at least) 4 by several equilibrium (Van Walraven *et al.*, 1996) and kinetic (Berry and Rumberg, 1996) measurements. Combined with the determined number of *c*-subunits those data fit the structural model nicely (14 *c*-subunits divided by 3 β-subunits is more than 4).

However our group and others have found that the H^+/ATP ratios are about 4 in chloroplasts and in some cyanobacteria grown and studied at "standard" conditions concerning light, temperature, pH, salt concentration, etc. (Van Walraven *et al.*, 1996), but the ATP synthase from the moderately thermophilic cyanobacterium *Synechococcus* 6716 can exhibit a change in H^+/ATP depending on several factors, listed below.

1. Significant changes in the H^+/ATP take place upon variation of growth temperature (Van Walraven *et al.*, 1997). When a *Synechococcus* 6716 cell culture was grown at temperatures close to its maximal tolerance or under light limitation the H^+/ATP of membrane vesicles was increased. Light limitation and growth at high temperature led to changes in the lipid saturation and chain length in the thylakoid membrane.

2. The H$^+$/ATP ratio was also affected by the manipulations involving reconstitution of the ATP synthase into proteoliposomes (Van Walraven *et al.*, 1990). After reconstitution of the ATP synthases of either *Synechococcus* 6716 or chloroplasts with native lipids at low protein/lipid ratio the H$^+$/ATP ratio was found to be as high as 9. Coreconstitution of *Synechococcus* 6716 ATP synthase and cytochrome b-563/c-554 complex yielded an H$^+$/ATP of about 7. In membrane vesicles of *Synechococcus* 6716 the H$^+$/ATP was about 4. Obviously, the physiological significance of such large deviations from normal in artificial membrane systems is at least questionable, but organisms using high H$^+$/ATP ratios do exist in nature. In thylakoids from the alkalophilic and halophilic cyanobacterium *Spirulina platensis* a H$^+$/ATP of at least 7 is found (Bakels *et al.*, 1993).

3. The H$^+$/ATP depends on external pH during measurement and this pH dependence is different between different sources (Krenn *et al.*, 1993). The H$^+$/ATP in vesicles from *Synechococcus* 6716 increases from about 3.0 to 4.2 when the pH was increased from 7.2 to 8.2. The same increase in pH led to a decrease in H$^+$/ATP from about 4.9 to 3.0 in chromatophores from *Rhodospirillum rubrum*. Alignment of the sequences of the *a*- and *c*-subunits from the *Synechococcus* 6716 and *Rhodospirillum rubrum* ATP synthases revealed that the distribution of charged residues in the hydrophilic loops near the F$_1$ side is different between both sources. These differences might affect the H$^+$/ATP.

A possible way to investigate the molecular mechanism behind the modulation of the H$^+$/ATP ratio is mutation of charged amino acids in the *a*- and *c*-subunits from the transformable cyanobacterium *Synechocystis* 6803. In this paper, we report experiments on H$^+$/ATP determination with a mutant (strain plc37) in which the serine on position 37 in the hydrophilic loop of the *c*-subunit from the wild-type *Synechocystis* 6803 strain (Lill and Nelson, 1991) was replaced by a negatively charged glutamic acid, making it similar to that position of subunit *c* from chloroplasts and *Synechococcus* 6716 (Van Walraven *et al.*, 1993). The results indicate that in the mutant strain the H$^+$/ATP ratio is significantly increased compared to the control strain and is similar to the value observed for chloroplasts and *Synechococcus* 6716. Some possible mechanisms will be described that can explain for variation in H$^+$/ATP between different sources and under different experimental conditions.

MATERIALS AND METHODS

Chemicals

ADP, NADP$^+$, valinomycin, and all enzymes were purchased from Boehringer (Mannheim, Germany). PMS and PMSF were from Sigma (St. Louis, MO, USA) and kanamycin sulphate from Duchefa (Haarlem, The Netherlands). The Luciferine/Luciferase monitoring kit was from LKB/Pharmacia (Uppsala, Sweden). S-13 was kindly donated by Dr. P. C. Hamm (Monsanto Co., St. Louis, MO, USA). All other chemicals were of analytical grade.

Creation of Mutant plc37

We cloned a 1.5 kB *Xho*ll piece of the *atp1* operon of *Synechocystis* 6803 (Lill and Nelson, 1991) into pGEM3Z (Promega), carrying a part of coding region of the F$_0$ subunit *a* and the complete genes encoding the *c* as well as the *b'* subunits. A kanamycin resistance cartridge was inserted at a position immediately downstream of the gene encoding subunit *c* as in Lill *et al.* (1994). The serine at position 37 of subunit *c* was mutated to glutamic acid by polymerase chain reactions using overhang extension (Ho *et al.*, 1989). Plasmids with and without the S37E exchange were used to transform *Synechocystis* 6803 and mutants were segregated and verified by Western blots as before (Lill *et al.*, 1994).

Culture Conditions

The mutant plc37 and control cyanobacterium *Synechocystis* 6803 were semicontinuously cultured at 35°C in 2-l airlift fermentors in BG-11 medium (Lubberding and Bot, 1984) supplied with 25 mg/L kanamycin and bubbled with 95% N$_2$/5% CO$_2$. The cells were diluted to an optical density (at 798 nm) of ca. 0.2. The control strain contained the kanamycin resistance as the mutant, but was otherwise identical to the wild-type *Synechocystis* 6803 (see above). Growth was followed at 798 nm and cells from about a 1-week-old culture were harvested.

Determination of Growth Parameters

Relative growth of both strains was studied in light-limited continuous cultures at 35°C by optical density (at 750 nm) and cell number (Coulter counter). Cells were inoculated at low density in a 5-cm path flat vessel (Matthijs

et al., 1996) with a volume of 1.5 L, medium was supplied at a dilution rate of 0.014 and the culture was bubbled with air. Cell growth (see Fig. 4) approached equilibrium at light saturation. In control experiments in which the light flux was increased the equilibrium density proportionally followed until carbon became limiting (results not shown).

Preparation of Membrane Vesicles

Membrane vesicles from both *Synechocystis* 6803 strains were prepared according to Scholts *et al.* (1996): 400 mL of cyanobacteria at an optical density (at 798 nm) of 1.2–1.6 were harvested and incubated in 30 mL of Mannitol storage medium (500 mM Mannitol, 10 mM Tricine–KOH, 10 mM $MgCl_2$, 5 mM NaH_2PO_4, 2.5 mM K_2HPO_4, pH 7.8) supplied with 0.2% lysozyme and 100 μM PMSF for ca. 2 h at 35°C. Samples were tested for phycocyanin release upon osmotic shocking by addition of water. As soon as phycocyanin release could be observed, after centrifugation (2 min, 10,000g), the spheroplasts were resuspended in the storage Mannitol medium plus 100 μM PMSF at a chlorophyll *a* concentration of ca. 0.2 mg/mL. The spheroplasts were kept on ice and every 2 h fresh PMSF (100 μM) was added. The vesicles were prepared fresh for each day's experiments and could be used for 5–10 h.

Chlorophyll *a* concentration was measured according to Arnon *et al.* (1974).

ATP Hydrolysis Activity

ATP hydrolysis was measured by scalar proton release using the pH electrode method as described by Krab *et al.* (1993) at 35°C. Vesicles from 7-day-old cultures of the control and the plc37 strains were suspended at 20 μg Chl/mL in medium as described in Krab *et al.* (1993).

Determination of H^+/ATP Ratio

At Equilibrium Between $\Delta\mu_H^+$ and ΔG_p

There is extreme difficulty in making direct measurement of the component of total H^+ flux that is linked to ATP synthesis or hydrolysis (the background, non-energy-linked H^+ permeability of thylakoids is surprisingly large, a fact not widely appreciated, cf. Fuks and Homble, 1996). Because of that, estimating H^+/ATP ratios is done by applying chemiosomotic theory, wherein from the thermo-dynamic relation, $\Delta G = \Delta G_p + n_H^+ \Delta\mu_H^+$, where n_H^+ is the H^+/ATP ratio, at equilibrium between ATP synthesis and hydrolysis ($\Delta G = 0$) we have $\Delta G_p = n_H^+ \Delta\mu_H^+$. Therefore, a plot of ATP synthesis versus $\Delta\mu_H^+$ will give n_H^+ at the extrapolated zero rate point, when the ΔG_p is known. For this technique to be valid, the ATP hydrolysis activity must not be down-regulated as the $\Delta\mu_H^+$ approaches the threshold for the ATP synthesis direction, $\Delta G = 0$ (as it is for the oxidized higher plant chloroplast CF_1, but it does not for the DTT- or Thioredoxin-reduced CF_1 (Van Walraven *et al.*, 1996)).

From the data of Krab *et al.* (1993) it is clear that vesicles of *Synechoccus* 6716 from 7-day-old cultures have the F_1 fully active after isolation and do not even need light ($\Delta\mu_H^+$) for observing ATP hydrolysis. Other cyanobacterial thylakoids, including *Synechocystis* 6803, share this same property (Bakels *et al.*, 1991; Krenn *et al.*, 1997). Because the point about hydrolysis being active without reducing agents and $\Delta\mu_H^+$ is so important for the thermodynamic arguments used for determining the H^+/ATP ratio, we again measured ATPase activity in the control and the mutant strains of *Synechocystis* 6803 (see Results), and reaffirmed that reducing agents and $\Delta\mu_H^+$ are not necessary for sustained ATP hydrolysis, and that ATP hydrolysis activity remained active through the vesicle preparation steps.

The H^+/ATP ratio was determined at the threshold $\Delta\mu_H^+$ for ATP synthesis/hydrolysis driven by acid–base transition as carried out according to Krenn *et al.* (1993) at 35°C (cf. Bakels *et al.*, 1991, for additional details). All media were supplied with 100 μM PMSF. First, 0.1 mL of spheroplasts in the storage Mannitol medium with a concentration of 0.1–0.3 mg Chl/mL were osmotically shocked for 10 min in 0.4 mL of "acid stage" medium "A" at pH 7.5. Medium "A" contained 10 mM succinic acid, 2 mM $MgCl_2$, 10 mM KCl, 2 mM NaH_2PO_4, 2 μM valinomycin, 10 μM DCMU, and 10 mM Tricine. Then 0.2 mL of these vesicles were added to 1 mL of "acid stage" medium "A" with variable pH for 1 min and finally acid–base transition was carried out by addition of 0.5 mL of this suspension to 0.5 mL of "base stage" medium "B." Medium "B" contained 10 mM KCl, 2 mM $MgCl_2$, 1 mM NaH_2PO_4, 1 mM ADP, 10 μM DCMU, and 200 mM Na–Tricine at the selected alkaline pH. After 1 min, 5 μL samples were tested for ATP content. ATP production was measured with the luciferin/luciferase assay according to the manufacturer's instructions. The pH of the acid- and base-stages was measured afterwards to determine the exact value of ΔpH (and $\Delta\mu_H^+$). All ATP production was corrected for myokinase activity and ATP already present by uncoupling with 5 μM S-13. ΔG_p values were calculated according to Krab and Van Wezel (1992).

From P/2e

Light-induced linear electron transport and ATP synthesis rates were measured respectively by the reduction of $K_3Fe(CN)_6$ and the reduction of $NADP^+$ in a coupled enzymatic reaction. First, 1 mL of spheroplasts from the storage Mannitol medium with a concentration of ca. 0.2 mg Chl/mL were osmotically shocked in the dark at 35°C for 10 min in 27 mL of reaction medium (15 mM Tricine–NaOH (pH 8.0), 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 2.5 mM K_2HPO4, 1 mM glucose, and 100 μM PMSF). Then the suspension was placed on ice and 1.2 mM $K_3Fe(CN)_6$, 1 mM ADP (final concentrations) and the above reaction medium were added to bring the total volume to 32 mL. Three milliliter aliquots of that suspension at 35°C were given 5 min of saturating white-light intensity. Then the reaction was stopped by cooling on ice and addition of 0.3 mL $HCLO_4$ (6 M), and the suspension was neutralized by addition of 6 M KOH–1 M Tris until the final pH was 7.5 ± 0.5. The $KCLO_4$ precipitate was removed by centrifugation and remaining $K_3Fe(CN)_6$ of the supernatant was measured at 420 nm. Subsequently the ATP formation was measured in a coupled enzymaic reaction (Bergmeyer, 1970) by the reduction of $NADP^+$. Samples kept dark by wrapping in aluminum foil provided a control for dark ATP formation. Samples given the uncoupler S-13 (1 μM final concentration) and illuminated provided an indication of the coupling quality of the spheroplasts. Each data point was the result of triplicate determinations and P/*2e* determinations on vesicles from both strains have been carried out 16 times over a period of 8 months.

RESULTS

Mutation of the *c*-Subunit of the ATP Synthase From *Synechocystis* 6803

In Fig. 1 the amino acid sequences of part of the F_0 *c*-subunit from thylakoid ATP synthases from spinach chloroplasts and *Synechococcus* 6716 (Van Walraven *et al.*, 1993) are compared with those from the transformable cyanobacterium *Synechocystis* 6803 (Lill and Nelson, 1991). This part includes the hydrophilic loop at the site where F_1 binds (Girvin *et al.*, 1998). The loop region (residues 35–50) is completely conserved between *Synechocystis* 6803, *Synechococcus* 6716, and spinach with the exception of residue 37 that is serine (S) in *Synechocystis* 6803, and glutamic acid (E) in the two other *c*-subunits. By site-directed mutagenesis the S was replaced by E in the mutant strain plc37. Since E at position 37 is already present in the other thylakoid ATP synthases and in view of the high sequence conservation of thylakoid ATP synthase, in particular the *c*-subunit (88–89% identity) (Van Walraven *et al.*, 1993), it is unlikely that this mutation has any other side effect on the structure or function of the enzyme.

Activation and Inactivation of Cyanobacterial ATP Synthase

When the CF_1 enzyme is active the H^+/ATP ratio can be determined at the value of $\Delta\mu_H^+$ where no net ATP synthesis or hydrolysis takes place (the "threshold" value; equilibrium between $\Delta\mu_H^+$ and ΔG_p). When the $\Delta\mu_H^+$ is applied by acid–base transition in the presence of valinomycin and similar concentrations of K^+ on both sides of the membrane to abolish $\Delta\psi$, the $\Delta\mu_H^+$ then reflects a delocalized proton gradient mainly composed of ΔpH. As discussed in the Materials and Methods section, the *Synechocystis* 6803 CF_1 is already active in the stock vesicle suspension without the need for reducing agents or light ($\Delta\mu_H^+$). This point is shown to be true for the vesicles used in this work by the data of Table I, for the control and plc37 strains, both prepared from 7-day-old cultures. Just as was shown in other studies (Krab *et al.*, 1993; Krenn *et al.*, 1997, for both *Synechocystis* 6803 and *Synechococcus* 6716) there was an active ATP hydrolysis in the dark after 3.75 mM ATP was added and before giving any il-

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                              27           37           47
Spinach chloroplasts      GQGTAAGQAV   EGIARQPEAE   GKIRGTLLLS
Synechococcus 6716        GQGNASGQAV   EGIARQPEAE   GKIRGTLLLT
Synechocystis 6803        GQGNASGQAV   SGIARQPEAE   GKIRGTLLLT


Synechocystis 6803 mutant GQGNASGQAV   EGIARQPEAE   GKIRGTLLLT
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Fig. 1. Alignment of part of *c*-subunits of chloroplast and cyanobacterial thylakoid ATP synthase including the mutant *Synechocystis* 6803 strain plc37. For Refs. see Van Walraven *et al.* (1993).

Table I. The Effect of $\Delta\mu_H^+$ and ADP on ATP Hydrolysis Activity in Membrane Vesicles of the *Synechocystyis* 6803 Strain plc37 and the Control Strain

	Control		plc37	
	Without ADP	With ADP[a]	Without ADP	With ADP[a]
ATP hydrolysis rates (μmol/min·mg Chl)[b]				
Before illumination	3.13	2.18	2.26	1.70
Immediately after illumination	3.75	4.06	2.13	2.96
Immediately after illumination (uncoupled)	0.96	0.28	0.15	0.00

[a] ADP (0.25 mM, when added) was present during osmotic shock in the reaction vessel before addition of ATP.
[b] $\Delta\mu_H^+$ was induced by illumination in the presence of phenazine methosulfate (PMS, 10 μM). ATP (3.75 mM) was added after the spheroplasts were osmotically shocked for 5 min in the reaction vessel. Uncoupling was achieved by addition of 0.5 μM S-13.

lumination. However, Table I also shows that when ADP was added before measurement, ATP hydrolysis activity was lower, probably owing to ADP binding at the CF_1 catalytic site, as has been shown for *Synechococcus* 6716 and reduced higher plant thylakoids (cf. Krab *et al.*, 1993, and references therein). By illumination ADP is released from the catalytic site and ATP hydrolysis proceeds at a higher rate. These effects indicate that the ATP hydrolysis is due to CF_1 activity and it is therefore logical to conclude that the CF_1 enzyme will go to an equilibrium state ($\Delta G = 0$) between ATP synthesis and hydrolysis when the $\Delta\mu_H^+$ approaches the threshold $\Delta\mu_H^+$ value, as is necessary to use the zero ATP yield point for applying the $\Delta G_p = n_H^+ \Delta\mu_H^+$ relationship to determine n_H^+.

Determination of the H⁺/ATP Ratio From Equilibrium Studies

From Fig. 2(A) it is clear that at similar external pH (8.40 and 8.34, respectively) the ATP synthase of the mutant strain plc37 translocates significantly more protons per ATP than the control strain (wild type with kanamycin resistance). Equilibrium is reached at a $\Delta\mu_H^+$ value of about 8 kJ/mol in the plc37 strain and in the control strain at about 13 kJ/mol, which leads to estimates of the H⁺/ATP of 4.6 and 2.8, respectively, in this particular experiment. At a lower external pH the difference between the control and mutant strain is much less (Fig. 2(B)). In Fig. 2 and in many other such experiments (not shown), ATP hydrolysis activity below the equilibrium $\Delta\mu_H^+$ was seen, indicating that ATP synthesis activity came to equilibrium with ATP hydrolysis activity, and therefore we can apply the $\Delta G_p = n_H \Delta\mu_H^+$ equilibrium assumption and estimate n_H^+ from the zero ATP yield point.

In Fig. 3 several H⁺/ATP determinations at different values of external pH are summarized for both mutant

and control strain. It appears that the mutation has a dramatic effect both on the value of H⁺/ATP ratio and on its dependence on external pH. At the pH at which most previous experiments have been performed (pH 8; see Van Walraven *et al.*, 1996) the mutant strain has an H⁺/ATP of 4.3 compared to 3.3 in the control strain. Furthermore, the dependence of H⁺/ATP of strain plc37 on external pH resembles that of *Synechococcus* 6716; an increase in the H⁺/ATP from 4.2 to 4.6 upon increasing pH from 7.6 to 8.4. In the control strain a decrease from 3.8 to 2.8 H⁺/ATP was found upon increasing pH from 7.6 to 8.3, which is more in line with *Rhodospirillum rubrum* chromatophores (see Krenn *et al.*, 1993, for further information on the difference in H⁺/ATP and charged amino acids in the *c*-subunit loop).

Determination of the H⁺/ATP Ratio From Kinetic Studies (P/*2e*)

The finding that the H⁺/ATP in membranes from the mutant strain was higher than compared to the control strain was corroborated by flux experiments where P/*2e* was determined. At pH close to 8 the P/*2e* was found to be 0.903 ± 0.087 for the mutant and 1.247 ± 0.087 for the control strain at high light intensity. At all pH values tested (7.8–8.6) vesicles from the mutant strain produced significantly less ATP compared to control vesicles under the same conditions, which points to an increased H⁺/ATP ratio. The assumption was made that the difference between the two *Synechocystis* 6803 strains solely lies in the mutation at residue 37 of the ATP synthase *c*-subunit, so that other factors such as proton flux not coupled to ATP synthesis are taken as equal in membranes from both strains. It is difficult to calculate an actual H⁺/ATP from these kinetic determinations but studies of Berry and Rumberg (1999) showed that in spinach chloroplasts under
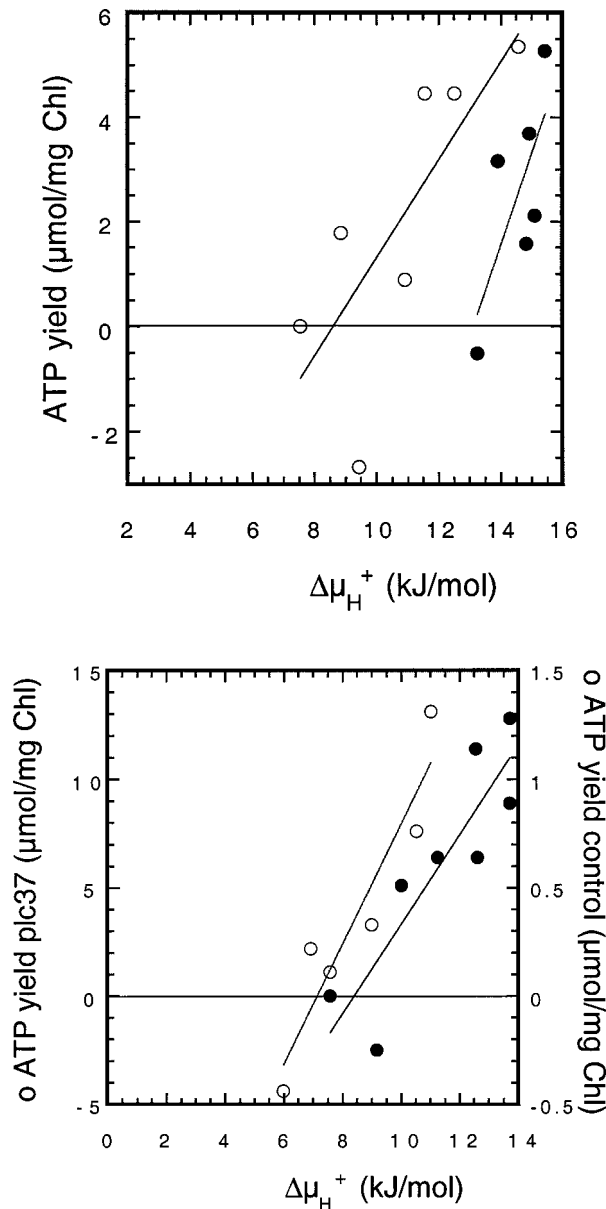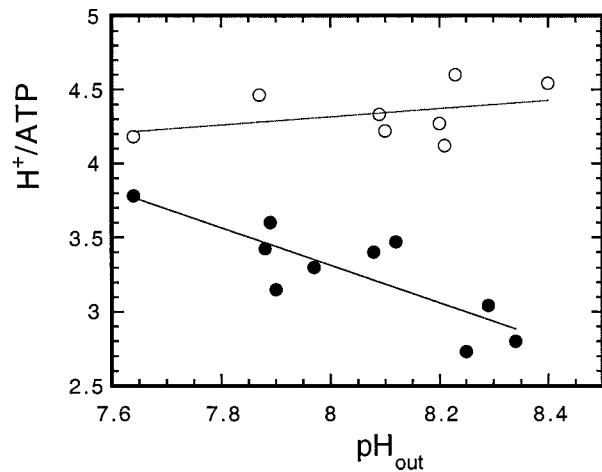
Fig. 3. H$^+$/ATP as a function of external pH of the thylakoid ATP synthases from membrane vesicles of *Synechocystis* 6803 strain plc37 (O) and the control strain (●). The H$^+$/ATP ratios were determined from the x-axis intercepts of plots similar to those shown in Fig. 2.

conditions of strong light intensity and low membrane permeability, H$^+$/$2e$ is close to 4. If we extrapolate those findings to the closely related cyanobacterium the P/$2e$ values would be consistent with H$^+$/ATP ratios of 4.4 for the mutant and 3.2 for the control strain corresponding well with the data from Fig. 3.

The Effect of Different H$^+$/ATP on Growth Rates

An increase in H$^+$/ATP due to a single mutation in the ATP synthase c-subunit should have an impact on growth rate of the cyanobacterium. From Fig. 4 it is clear that the mutant strain plc37 grows slightly slower than the control strain, in keeping with its herein-demonstrated requirement for more protons used per ATP synthesized. The same difference in growth between the mutant and control strain was observed with the semicontinuously cultured cells used for vesicle preparation (not shown).

DISCUSSION

In these experiments the data on ATP yield after the acid–base jump versus $\Delta\mu_H^+$ (used to estimate the H$^+$/ATP ratio) scatter rather badly, but that is unavoidable in such experiments using cyanobacterial vesicles owing to (1) the large background ATP hydrolysis activity; (2) the difficult-to-control proteolysis activity. Cyanobacterial vesicles have to be prepared fresh (cannot be frozen) and can be used for up to ca. 5 h. Therefore, the number of data
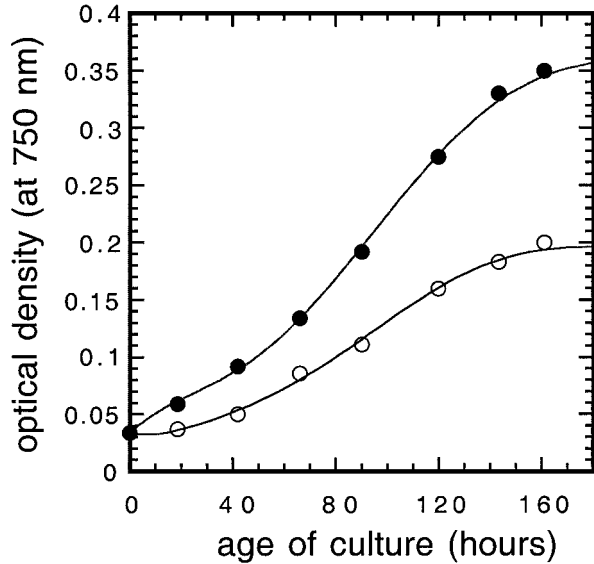
Fig. 2. Representative experiments of ATP synthesis (within 1 min) as a function of $\Delta\mu_H^+$ applied by acid–base transition by vesicles from *Synechocystis* 6803 strain plc37 (O) and the control strain (●). The $\Delta\mu_H^+$ mainly consists of a ΔpH due to equal concentrations of K$^+$ (10 mM) on both sides of the membrane and the presence of 1 μM valinomycin. Each experiment has been carried out at least three times. The experiments were carried out at 35°C at a chlorophyll concentration of 2 μg/mL. For all data points the ATP productions were corrected for myokinase activity and ATP already present in the sample under uncoupled conditions (5 μM S-13). For vesicles from *Synechocystis* 6803 strain plc37 (O) pH$_{out}$ was 8.40 and initial ΔG_p was 36.31 kJ/mol (A) or pH$_{out}$ was 7.87 and initial ΔG_p was 33.46 kJ/mol (B). For vesicles from the control strain (wild type with kanamycin resistance) (●) pH$_{out}$ was 8.34 and initial ΔG_p was 35.97 kJ/mol (A) or pH$_{out}$ was 7.64 and initial ΔG_p was 32.12 kJ/mol (B). See Materials and Methods section for further experimental details.

Fig. 4. Relative growth determined by optical density (at 750 nm) for *Synechocystis* 6803 strain plc37 (O) and the control strain (●). One representative experiment out of four experiments is shown. Growth determined by cell number correlated well with the optical density determinations.

points from one vesicle preparation is limited. However, the high background ATP hydrolysis activity is useful in that it maintains active CF_1 enzymes and the threshold $\Delta\mu_{H^+}$ for ATP synthesis is not complicated by the issue of CF_1 activation. However, the disadvantages in using the cyanobacteria are offset by the advantage that mutagenisis of the CF_0 subunits is possible in this system but currently unattainable with the higher plant chloroplast genetic systems.

The results presented in this work, despite the relatively bad scatter in the data, show quite clearly that, in the cyanobacterial strain *Synechocystis* 6803, replacement of serine 37 in the hydrophilic loop of the ATP synthase *c* subunit with a glutamic acid increases the H^+/ATP ratio. The result seems quite logical in that the mutant subunit *c* being made identical in position 37 to that of higher plant thylakoid and *Synechococcus* 6716 subunit *c* (Fig. 1), results in the H^+/ATP ratio becoming more similar to that of the latter two systems.

The increased H^+/ATP ratio in the plc37 mutant was corroborated by the P/2*e* ratio results and by the effects on growth. Thus, by three separate criteria, we show evidence consistent with a higher H^+/ATP coupling ratio in the serine 37 to glutamate replacement mutant.

Earlier (Scholts *et al.*, 1996; Van Walraven *et al.*, 1996) we published H^+/ATP ratios around 4 for thiol-modulated chloroplasts, *Synechococcus* 6716, and a mutant of *Synechocystis* 6803. The acid–base transitions in

those studies were done with an external pH of about 8 and the actual H^+/ATP values for chloroplasts and *Synechococcus* 6716 were at least 4 but often more (4.2–4.3; see Van Walraven *et al.*, 1996) whereas from Fig. 1 of Scholts *et al.* (1996) one could calculate a H^+/ATP of less than 4. At that time (late nineties) when we believed in a H^+/ATP of 4 for "green photosynthetic membranes" (later adopted for other sources, see, e.g., Jones and Fillingame, 1998) at pH 8 we explained such small differences as experimental variation. A further complication of older work is that the temperature at which the experiments were performed differs between different systems (50°C for *Synechococcus* 6716, 37°C for *Synechocystis* 6803, and room temperature for chloroplsts). Higher temperature and pH have a considerable increasing effect on the value of ΔG_p (and thus H^+/ATP) and especially very high temperatures are difficult to account for (Krab and Van Wezel, 1992). In the present study much more data have been carefully obtained using two independent methods (equilibrium and kinetic) at identical experimental conditions for the mutant and control strain of *Synechocystis* 6803.

There is accumulating evidence from several studies that the *c*-ring, ε, and γ rotates as an ensemble relative to the stator subunits α, β, δ, *b* (and *b'*), and subunit *a* (Tanabe *et al.*, 2001). However that still leaves several possibilities for a change in gear of the ATP synthase (H^+/ATP ratio).

1. Since the number of *c*-subunits varies between different sources and H^+/ATP $= c/\beta$, H^+/ATP is variable as well (see Arechaga and Jones, 2001; Tomashek and Brusilow, 2000). This is the case for CF_0CF_1 where the number of *c*-subunits is 14 (Seelert *et al.*, 2000) and the H^+/ATP between 4 and 4.5 (Berry and Rumberg, 1996; Van Walraven *et al.*, 1996). It also has been described that the preferable number of *c*-subunits in *E. coli* is 10 (Jiang *et al.*, 2001) and determinations of the H^+/ATP gives values of about 3 (Kashket, 1982) although the ratio appeared to be pH dependent. Also from studies with *E. coli* mutants where the number of *c*-subunits can be manipulated, evidence for a change in H^+/ATP was obtained although the actual value was not measured (Schemidt *et al.*, 1998; Tomashek and Brusilow, 2000). Yeast mitochodrial ATP synthase contains 10 *c*-subunits (Stock *et al.*, 1999) and the H^+/ATP in mammalian mitochondria is close to 3 (4 including the translocation of substrates; Hinkle *et al.*, 1991). However, H^+/ATP determinations in *E. coli* (Kashket, 1982) and mammalian mitochondria

(Hinkle *et al.*, 1991) are rather old and need to be reevaluated. Furthermore, it cannot be excluded that *c*-subunits are lost or gained in the isolation procedure (Seelert *et al.*, 2000; Stock *et al.*, 1999). In the studies on cyanobacterial ATP synthase a different growth condition may have an effect on the number of *c*-subunits. Also there is a possibility that a change in charge in the loop of the *c*-subunit, as in the mutant in this study, has an effect on packing of the *c*-ring, recently suggested by Arechaga and Jones (2001). In the forthcoming studies we will investigate the number of *c*-subunits in ATP synthase from cyanobacterial mutants under different growth conditions. An interesting phenomenon occurs in plants where growth conditions affect the size of the *c*-ring of the vacuolar ATPase (Ratajczak, 2000) that is evolutionarily related to the ATP synthase.

2. In the case of pH dependence of the H$^+$/ATP ratio during the actual assay described here and before (Krenn *et al.*, 1993) it is difficult to conceive how a pH change lasting only 1 min or less (in the alkaline phase of an acid–base transition experiment) can have such a rapid effect on the structure of the ATP synthase as described above. As a working hypothesis we developed a model in which extra proton binding sites (charged aminoacids) in the hydrophilic loop of the *c*-subunit affect the H$^+$/ATP ratio. Also their degree of protonation varies depending on obvious factors such as pH but maybe also on protein and lipid composition in their vicinity. It would then be expected that the H$^+$/ATP depends on pH during measurement and that this dependence differs between different ATP synthases due to the difference in proton binding sites and a different degree of protonation at a given pH. Indeed the H$^+$/ATP in vesicles from *Synechococcus* 6716 increases when the pH was increased whereas the same increase in pH led to a decrease in H$^+$/ATP in chromatophores from *Rhodospirillum rubrum* (Krenn *et al.*, 1993). A similar type of "gear change" by tight proton binding to some of the c-subunits was recently postulated by Grabe and Oster (2001) for vacuolar ATPases involved in hyperacidification of vacuoles. By lowering the H$^+$/ATP in this way it is possible to reach higher values of ΔpH. Also, a recent paper (Jones, 2001) describes the effect of ATP-induced proton pumping and pH regulation of an introduction of a carboxyl group in the first transmembrane helix (position 28) of the *E. coli* subunit *c*.

3. Another possibility given in the literature (Birkenhager *et al.*, 1999) is, although there are a number of *c*-subunits present in the ATP synthase, not all of them are involved proton transport during rotation at all times.

Modulation of the H$^+$/ATP might be an important tool for long-term adaptation (number of *c*-subunits) and short-time tuning (tight binding of protons or *c*-subunits placed outside the ring) of the efficiency of energy transduction. Cyanobacteria and many other (phototropic) bacteria are normally exposed to harsh and fluctuating environmental conditions involving temperature, light, pH, and salinity. A large increase in H$^+$/ATP as observed in *Spirulina platensis* and possibly other extremophiles, but also smaller changes, provide the organisms with the opportunity to continue ATP synthesis in "lower gear" to survive conditions of low $\Delta\mu_H$.

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