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# Dependence of the proton translocation stoichiometry of cyanobacterial and chloroplast $H^+$-ATP synthase on the membrane composition

H.S. van Walraven, M.J.C. Scholts, F. Koppenaal, R.H.A. Bakels and K. Krab

*Biological Laboratory, Vrije Universiteit, Amsterdam (The Netherlands)*

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**The high proton translocation stoichiometry (approx. 9 $H^+$/ATP) of ATPase proteoliposomes reconstituted from a thermophilic cyanobacterium (Van Walraven et al. (1986) FEBS Lett. 208, 138–142) has also been observed with chloroplast ATP synthase when reconstituted with cyanobacterial lipids. Both enzyme complexes in isolated and reconstituted form show highest stable trypsin-activated ATP hydrolysis activity at the same temperature (55 °C). Also, both isolated ATP synthases require the same reconstitution procedure for maximal coupling quality. The proton translocation stoichiometry has been deduced from the relation between the initial rates of ATP hydrolysis at varying sizes of the electrochemical potential gradient ($\Delta\bar{\mu}_{H^+}$). A $\Delta\bar{\mu}_{H^+}$ was imposed by valinomycin-induced $K^+$ diffusion potentials or by base-pulses which were equally efficient in inhibiting ATP hydrolysis. Kinetic experiments with the use of the pH indicator Cresol red confirm the high proton translocation stoichiometry of both types of ATPase proteoliposome. Functional co-reconstitution of both types of ATPase proteoliposome with cyanobacterial cytochrome $b_6f$ complex leads to a decrease in proton translocation stoichiometry to about 7 $H^+$/ATP. Cyanobacterial membrane vesicles take up 4.4 protons per ATP hydrolyzed. A value of 4.5 $H^+$/ATP is observed with chloroplasts in equilibrium (Gräber, P., Junesch, U. and Thulke, G. (1986) in Progress in Photosynthesis Research (Biggins, J., ed.), pp. 177–184, Martinus Nijhoff, Dordrecht). These results indicate that the proton translocation stoichiometry of the ATP synthase depends on the membrane composition. The consequence of this finding for the mechanism of proton translocation and the possible physiological relevance are discussed.**

## Introduction

According to the chemiosmotic theory of Mitchell [1] an electrochemical gradient of protons ($\Delta\bar{\mu}_{H^+}$) across a coupling membrane connects electron transfer and ATP synthesis in oxidative and photosynthetic energy transduction. In thermodynamic equilibrium, the size of $\Delta\bar{\mu}_{H^+}$ should be proportional to the phosphate potential ($\Delta G_p$) with as proportionality factor, the number of protons that has to be translocated to drive the synthesis of a single molecule of ATP (the proton translocation stoichiometry or $H^+$/ATP ratio). Under non-equilibrium conditions the rate of ATP synthesis or ATP hydrolysis depends on the total driving force of the reactions, equal to:

$$\Delta G = \Delta G_p - \left[\frac{H^+}{ATP}\right] \cdot \Delta\bar{\mu}_{H^+}$$

ATP synthesis requires that $\Delta G < 0$, and ATP hydrolysis occurs when $\Delta G > 0$. This equation serves to illustrate the importance of the value of the proton translocation stoichiometry. Values around 2–3 $H^+$/ATP have been predicted and have been observed in several membrane systems [2].

A number of experiments on electron-transfer-driven ATP synthesis does not agree with a simplistic version of the chemiosmotic hypothesis (see Ref. 3 for a review). One of the anomalies involves ATP synthesis occurring at a low size of the bulk-phase $\Delta\bar{\mu}_{H^+}$. Two ways to explain this suggest themselves directly. The first possi-

Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; $F_1$, the water-soluble part of the ATP synthase; $F_0$, the membrane-bound part of the ATP synthase; MGDG, monogalactosyldiacylglycerol; S-13, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylaniline.

Correspondence: H.S. Van Walraven, Biological Laboratory, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands.

bility is that the relevant coupling $\Delta\bar{\mu}_{H^+}$ is not the one between the bulk-phases separated by the membrane, but has a more local character. This possibility has been explored extensively in recent years [3]. A second possibility has been less prominent in literature: a high $H^+$/ATP value under some conditions may explain a number of deviations from theory. An essential test of these two possibilities is to study the extent to which it is possible to drive ATP synthesis or inhibit ATP hydrolysis by an externally applied $\Delta\bar{\mu}_{H^+}$. It is obvious that in such a system a localized $\Delta\bar{\mu}_{H^+}$ cannot be higher than the applied $\Delta\bar{\mu}_{H^+}$ (between bulk-phases by definition).

Such studies with chloroplasts tended to yield $H^+$/ATP values close to 3 [2]. However, problems with a relatively high membrane permeability, presence of adenylate kinase activity, and more generally lack of detailed knowledge about the osmotic and structural properties of the thylakoids make it extremely difficult to carry out these experiments sufficiently close to equilibrium. Recently Gräber et al. [4] have circumvented these problems by performing this type of experiment using a rapid mix-quench technique, and have reported an $H^+$/ATP of 4.5.

An alternative way to overcome some of the problems with natural membranes is to isolate the ATP synthase and reconstitute the enzyme into an artificial membrane. Chloroplast ATP synthase reconstituted with soybean phospholipids (asolectin) is able to catalyze rates of ATP synthesis and hydrolysis up to half of the physiological level [5–7]. However, the membrane permeability is very high and studies under equilibrium conditions have not been carried out. The poor coupling of these proteoliposomes follows from the rapid decrease of ATP synthesis (in less than 1 s) and also from the low uncoupler stimulation of ATP hydrolysis activity [8]. Non-equilibrium studies with chloroplast ATPase proteoliposomes indicate values of the proton translocation stoichiometry that roughly resemble those of chloroplasts [7].

We used the ATP synthase from the thermophilic cyanobacterium *Synechococcus* 6716 (optimal growth temperature 50–55 °C), reconstituted with the native lipids. These proteoliposomes have reasonable activity, large volume, low surface charge and, most importantly, low membrane permeability [9]. In early studies it appeared that much more than 3 protons per ATP synthesized or hydrolyzed must be translocated [10] and measurements with $\Delta G_p$ and $\Delta\bar{\mu}_{H^+}$ in real equilibrium yielded a proton translocation stoichiometry of 9 $H^+$/ATP [11].

To investigate the cause of the different results obtained with reconstituted ATP synthase from chloroplasts and from *Synechococcus* 6716, we have reconstituted both enzymes in the cyanobacterial lipids. The present paper describes the determination of the proton translocation stoichiometry of reconstituted ATP synthase from the thermophilic cyanobacterium and from spinach chloroplasts in two different types of experiment. In one type of experiment the rate of ATP hydrolysis was measured as a function of $\Delta\bar{\mu}_{H^+}$ applied. The stoichiometry then was determined from the threshold level at which ATP hydrolysis was fully inhibited. In the other type of experiment proton uptake coupled to ATP hydrolysis was directly measured with the use of a pH-indicator. Finally, the effect of the membrane composition on the proton translocation stoichiometry was studied.

## Materials and Methods

ATP synthases from either *Synechococcus* 6716 or spinach chloroplasts were isolated according to Lubberding et al. [12] and Pick and Racker [13], respectively. The fractions precipitating between 35% and 50% saturated ammonium sulfate ($P_{35-50}$) were used without further purification and were stored at 4 °C (cyanobacterial ATP synthase) or in liquid nitrogen (chloroplast ATP synthase). Cytochrome $b_6f$ complex from *Synechococcus* 6716 was isolated by a procedure described for *Anabaena variabilis* [14] as modified by F. Koppenaal [48]. Cyanobacterial lipids were extracted as in Ref. 15.

All types of ATPase proteoliposomes were prepared with lipids from the thermophilic cyanobacterium *Synechococcus* 6716 by overnight detergent dialysis [16]. Unless otherwise indicated the reconstitution temperature was 50 °C. Lipid concentration for reconstitution was 10 mg · ml$^{-1}$. The protein-to-lipid ratio of the ATPase proteoliposomes was about 0.02 (w/w) unless otherwise indicated. Cytochrome $b_6f$ was used for coreconstitution in a concentration of 2.2 μM cytochrome *b*-563. Proteoliposomes were stored at 4 °C and could be used for up to 1 week.

Membrane vesicles from *Synechococcus* 6716 were prepared by lysozyme treatment of the cells, osmotic shock and washing steps [17] and were resuspended at a chlorophyll concentration of 0.3 mg · ml$^{-1}$.

Unless otherwise indicated, the medium used for reconstitution, shock/wash and reactions contained 10 mM Tricine-NaOH (pH 7.8 at 50 °C), 10 mM KCl, 2.5 mM $MgCl_2$ and 1 mM dithiothreitol.

All experiments were carried out at 50 °C unless otherwise indicated. Proteoliposomes were used at a concentration of 0.5 mg · ml$^{-1}$. Membrane vesicles were used at a concentration corresponding to 3 μg chlorophyll · ml$^{-1}$. Unless otherwise indicated, the ATP synthases in the different systems were activated by trypsin treatment (25 μg · ml$^{-1}$). The reactions were started by the addition of ATP (pH approx. 7.8) to a final concentration of 5 mM.

$\Delta\bar{\mu}_{H^+}$ was applied as a $\Delta\psi$ (valinomycin-induced $K^+$

diffusion potential), a $\Delta$pH (calibrated NaOH pulse in the presence of valinomycin) or a combination of both. Valinomycin was used at a concentration of 100 nM (0.15 $\mu \cdot$ mg lipid$^{-1}$). $\Delta\psi$ is defined as inside minus outside and $\Delta$pH as outside minus inside. The size of $\Delta\psi$ was calculated according to the Nernst equation ($\Delta\psi = 6.5 \log([K^+]_{out}/[K^+]_{in})$ (kJ $\cdot$ mol$^{-1}$) at 50 °C).

All ATP hydrolysis activities were determined continuously in an enzymatic ATP regenerating system of which the principle is described by Bergmeyer [18]. The reactions involved are described in the text. The reaction medium was supplied with 0.2 mM phospho*enol*-pyruvate and 0.2 mM NADH. The NADH content was followed spectrophotometrically at 340–400 nm. Some determinations of ATP hydrolysis activity were carried out in the standard reaction medium as described by Stutterheim et al. [19]. Either reaction medium yielded similar results. pH changes were determined by measuring the absorbance (573–700 nm) of the pH indicator Cresol red in a weakly buffered external medium. Absorbance changes were calibrated by the addition of standard amounts of oxalic acid. All absorbances were measured with an Aminco DW-2a spectrophotometer, equipped with a thermostatically controlled multi-purpose cuvette [20].

$\Delta G_p$ was calculated according to Rosing and Slater [21] with $\Delta G_0'$ estimated to be 31.2 kJ $\cdot$ mol$^{-1}$.

Protein concentration was measured according to Bradford [22].

Lysozyme, phospho*enol*pyruvate, NADH, lactate dehydrogenase, pyruvate kinase, ATP and valinomycin were purchased from Boehringer (Mannheim, F.R.G.); cholic acid, sodium cholate, dithiothreitol and Cresol red from Sigma (St. Louis, MO, U.S.A.); and octylglucoside from Calbiochem (La Jolla, U.S.A.) or from Sigma. S-13 was donated by Dr. P.C. Hamm (Monsanto Co., St. Louis, MO). All other chemicals were of analytical grade.

## Results

*Characterization of chloroplast ATP synthase reconstituted with lipids from the thermophilic cyanobacterium Synechococcus 6716*

First we have tested the temperature dependence of the hydrolysis activity of the isolated and reconstituted chloroplast ATP synthase. Fig. 1 shows that this ATP synthase reconstituted with cyanobacterial lipids is maximally active at 55 °C. This is not due to the presence of cyanobacterial lipids, since isolated chloroplast ATP synthase has a stable ATP hydrolysis activity at 50 °C which is much higher than that at 30 and 40 °C (results not shown). At temperatures higher than 55 °C the hydrolysis activity of both isolated and reconstituted complex decreases during measurement. This temperature dependence is similar to that observed with

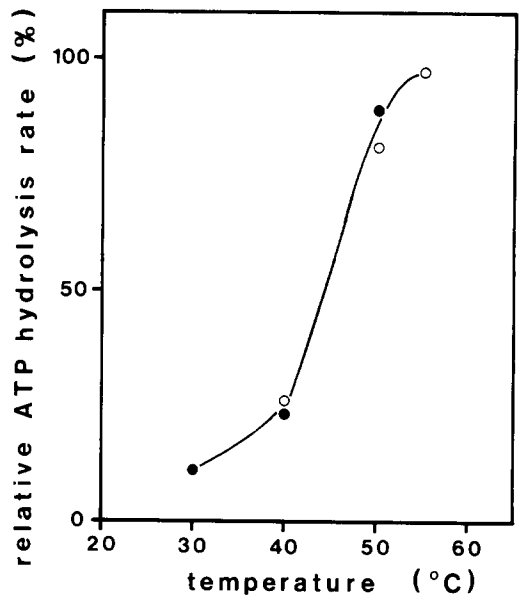


Fig. 1. Temperature dependence of ATP hydrolysis activity of chloroplast ATP synthase reconstituted with cyanobacterial lipids. The protein-to-lipid ratio in this experiment was 0.1 (w/w) and the proteoliposomes were prepared at 30 °C (●) plus an additional night at 40 °C (○). 100% activity corresponds to 400 nmol/min per mg protein. Further details are given in Material and Methods.

isolated and reconstituted *Synechococcus* 6716 ATP synthase [15] and apparently is an intrinsic property of the enzyme itself, accidentally coinciding with the optimal growth temperature of the cyanobacterium (50–55 °C).

Table I shows that the ATP hydrolysis activities at 50 °C of both types of isolated ATP synthase can be stimulated by trypsin treatment. The activity without trypsin activation is an order of magnitude higher for the chloroplast enzyme. This is probably due to a higher sensitivity of the chloroplast complex to activation by the heat treatment implicit in the activity measurement at high temperature [23].

From Table II it can be concluded that a reconstitution temperature of 50 °C is essential for optimal coupling quality (stimulation of ATP hydrolysis by S-13) of proteoliposomes prepared from chloroplast ATP synthase and cyanobacterial lipids. This could be expected because of the high phase-transition temperatures of

TABLE I

*The ATP hydrolysis activities of isolated cyanobacterial (S) and chloroplast (C) ATP synthase and the effect of trypsin treatment*

For experimental details see Materials and Methods.

| Source | ATP hydrolysis activity ($\mu$mol/min per mg protein) | |
|---|---|---|
| | no trypsin | trypsin |
| S | 0.1–0.2 | 1–2 |
| C | 2.5 | 5 |

TABLE II

*Reconstitution conditions for chloroplast ATP synthase with cyanobacterial lipids*

The effects of reconstitution temperature and protein to lipid ratio on the rate of ATP hydrolysis and uncoupler stimulation. All activities were determined at 50 °C. See Materials and Methods for further details.

| Reconstitution temperature (°C) | Protein/lipid ratio (w/w) | ATP hydrolysis activity (nmol/min per mg protein) | |
|---|---|---|---|
| | | no addition | +100 nM S-13 |
| 30 | 0.1 | 356 | 388 |
| 30–40 [a] | 0.1 | 324 | 340 |
| 50 | 0.2 | 133 | 186 |
| 50 | 0.02 | 291 | 466–582 |
| 50 | 0.01 | 252 | 277 |
| 50 | 0.005 | 417 | 500 |

[a] An additional night at 40 °C.

some of the lipids [9]. Table II also shows that raising the reconstitution temperature leads to an apparent loss of activity of more than 50%. This is not due to damage to the complex during reconstitution but to a lower incorporation of protein in proteoliposomes prepared at 50 °C as compared to 30 °C. This is probably because dialysis at high temperature is much faster. The non-incorporated ATP synthase denatures upon detergent depletion. That only a small portion of the added ATP synthase is incorporated at 50 °C is illustrated in Table II, where it is shown that activity increases with lower protein to lipid ratio. From the table we conclude that maximal S-13 stimulation (and thus coupling quality) is obtained at a protein to lipid ratio of 0.02 (w/w). This again is in harmony with the results obtained by reconstitution studies with the *Synechococcus* 6716 ATP synthase [15].

Thus, the optimal conditions concerning catalytical activity and coupling quality for both types of ATP synthase and cyanobacterial lipids are a reconstitution and measuring temperature of 50 °C and a lipid to protein ratio of 1 : 50. These conditions have been used in the following studies. Even so, the coupling quality is less than that observed with *Synechococcus* 6716 ATPase proteoliposomes [10,11].

*Determination of the $H^+$/ATP ratio of reconstituted cyanobacterial and chloroplast ATP synthase*

In an earlier study [11], the actual value of the $H^+$/ATP of *Synechococcus* 6716 ATPase proteoliposomes has been determined from the proportional relation between the phosphate potential ($\Delta G_p$) and the size of the $\Delta\bar{\mu}_{H^+}$ in thermodynamic equilibrium. This type of experiment is very laborious, since it requires vast amounts of proteoliposomes to reach equilibrium within reasonable time. Also, a very good coupling quality (uncoupler stimulation of ATP hydrolysis activity exceeding 10) is necessary for a pre-established $\Delta\bar{\mu}_{H^+}$ to remain constant on the required time-scale [11]. Since chloroplast ATP synthase reconstituted with cyanobacterial lipids does not yield such well coupled systems this experiment cannot be carried out by this

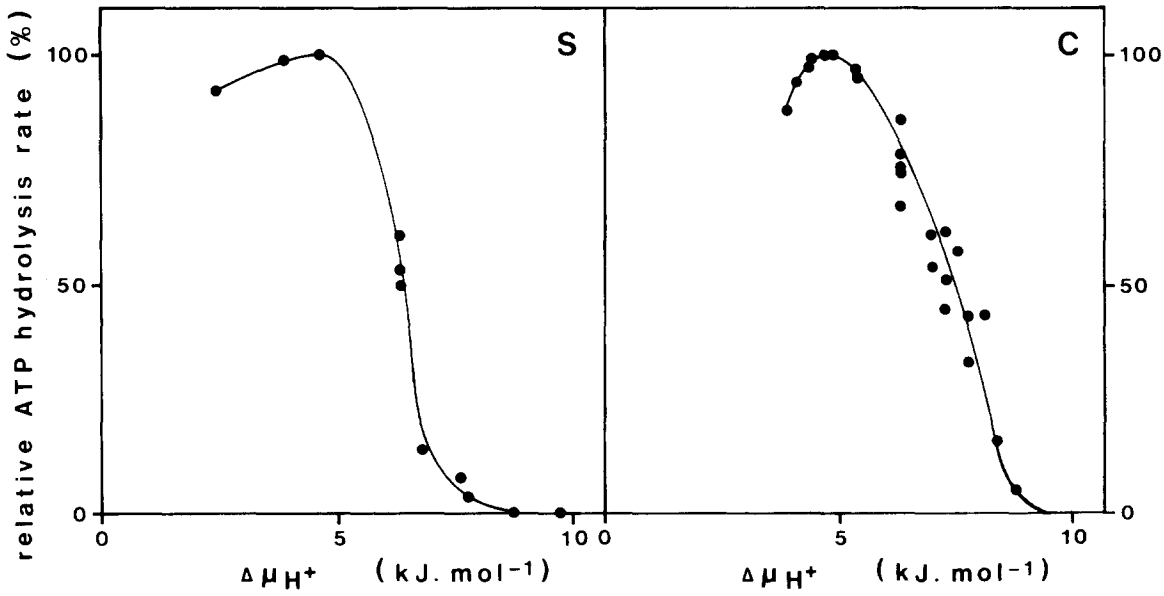


Fig. 2. Dependence of the initial rates of ATP hydrolysis on the size of a pre-established $\Delta\bar{\mu}_{H^+}$ in proteoliposomes containing either cyanobacterial (S) or chloroplast (C) ATP synthase reconstituted with cyanobacterial lipids. $\Delta G_p$ was approx. 85 kJ·mol$^{-1}$ (medium containing 5 mM ATP and an enzymatic ATP regenerating system). See Materials and Methods for further experimental details ($[K^+]_{in} = 10$ mM). The data shown in each panel have been obtained from eight experiments performed with two different preparations. The ATP hydrolysis activity varied between preparations and 100% activity corresponds to approx. 150 (S) and 300 (C) nmol/min per mg protein, respectively.

method. However, a good indication of the order of magnitude of the proton translocation stoichiometry is given by the relation between the initial rates of ATP hydrolysis and varying pre-established $\Delta\bar{\mu}_{H^+}$. We have demonstrated [11] that the result from this experiment agrees with the equilibrium studies mentioned above.

In Fig. 2 the $\Delta\bar{\mu}_{H^+}$-dependent inhibition of the ATP hydrolysis activities of both *Synechococcus* 6716 and chloroplast ATP synthase reconstituted with cyanobacterial lipids is demonstrated. For *Synechococcus* 6716 proteoliposomes the ATP hydrolysis activity reaches zero at a $\Delta\bar{\mu}_{H^+}$ of 8.7 kJ · mol$^{-1}$. At this point 'equilibrium' has been reached and the $\Delta G_p$ to $\Delta\bar{\mu}_{H^+}$ ratio is 9.8. The ATP hydrolysis activity of chloroplast ATP synthase reconstituted with cyanobacterial lipids is completely inhibited at a $\Delta\bar{\mu}_{H^+}$ of 9.7 kJ · mol$^{-1}$. There the $\Delta G_p$ to $\Delta\bar{\mu}_{H^+}$ ratio is 8.8.

In contrast to the previously reported experiments [11] the reconstituted ATP synthases in this study were activated by trypsin treatment. The maximal ATP hydrolysis activity and the dependence of this activity on the size of $\Delta\bar{\mu}_{H^+}$ of reconstituted *Synechococcus* 6716 ATP synthase are similar irrespective of trypsin treatment. However, trypsin pre-activated proteoliposomes show less additional $\Delta\bar{\mu}_{H^+}$-dependent activation (cf. Fig. 3 in Ref. 11). Fig. 2 shows that ATP hydrolysis activity of both types of proteoliposome is maximal at a $\Delta\bar{\mu}_{H^+}$ of 4.3–4.8 kJ · mol$^{-1}$.

In most of the experiments given in Fig. 2 the $\Delta\bar{\mu}_{H^+}$ consisted of a $\Delta\psi$. A $\Delta$pH or a combination of $\Delta\psi$ and $\Delta$pH is equally efficient in inhibiting ATP hydrolysis. For driving ATP synthesis this equal efficiency has been observed before [10]. A $\Delta\psi$ up to 100 mV ($\Delta\bar{\mu}_{H^+} = 9.7$ kJ · mol$^{-1}$) is stable for at least 15 min in both types of proteoliposome. A higher $\Delta\psi$ slowly collapses within minutes, as was seen by a steady increase in ATP hydrolysis activity and also by direct measurements (Koppenaal, F., unpublished results). $\Delta\bar{\mu}_{H^+}$-dependent inhibition of ATP hydrolysis activity is sensitive to uncouplers. Addition of the protonophore S-13 at a $\Delta\psi$ of 100 mV ($\Delta\bar{\mu}_{H^+}$ of 9.7 kJ · mol$^{-1}$) restores the ATP hydrolysis activity of 80–85%. A full recovery or more, depending on the coupling quality of the proteoliposomes, cannot be expected at the used buffer concentration (10 mM), since the $K^+$ gradient cannot be compensated by sufficient numbers of protons. In earlier work [10] it was shown that under these conditions S-13 was able to uncouple only about half of a valinomycin-induced $K^+$ diffusion potential. Increasing the external KCl concentration does not cause artefactual inhibition of ATP hydrolysis activity. Up to more than 300 mM KCl in the absence of valinomycin (which would lead to a $\Delta\bar{\mu}_{H^+}$ of 9.7 kJ · mol$^{-1}$ in the presence of the ionophore) there is no effect on the ATP hydrolysis activity. 500 mM KCl causes a 10% inhibition and at 1 M KCl 60% of the activity still remains.

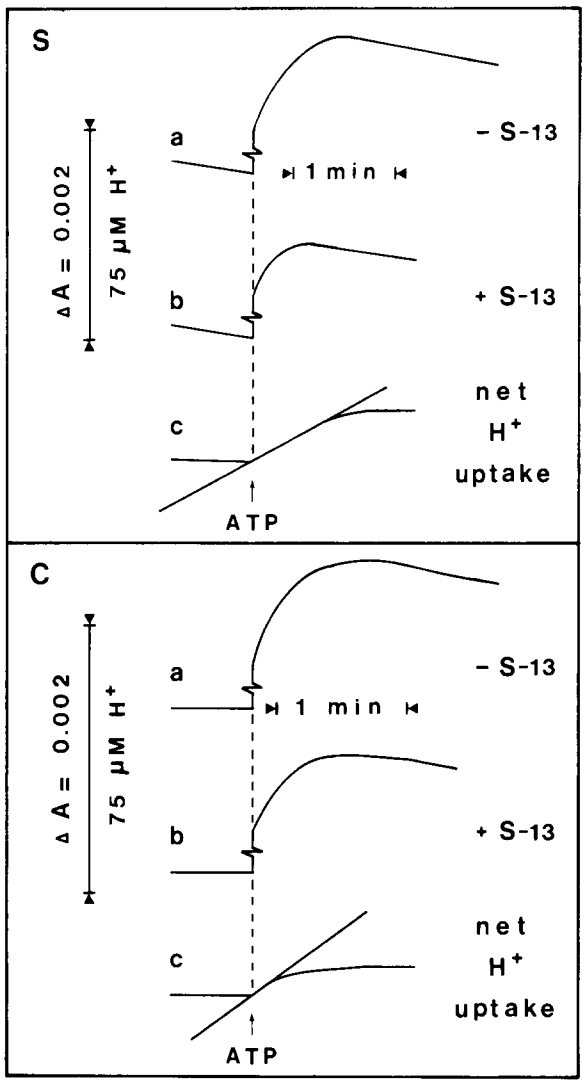


Fig. 3. Kinetic determination of the initial $H^+$ uptake upon ATP hydrolysis by proteoliposomes from either cyanobacterial (S) or chloroplast (C) ATP synthase reconstituted with cyanobacterial lipids. 10 μM Cresol red was present to measure pH changes. Experiments were carried out in the presence of 100 nM valinomycin to eliminate $\Delta\psi$. The medium contained an ATP-regenerating system and was buffered with 2 mM Tricine-NaOH (pH 7.8). Further details are explained in the text. (a) Complete system; (b) control in the presence of 100 nM S-13; (c) difference a – b.

Under the conditions used we assume that neither $\Delta G_p$ nor $\Delta\bar{\mu}_{H^+}$ changes during the experiments in Fig. 2. The $\Delta G_p$ remains constantly high due to the presence of an ATP-regenerating system. The initial rates of ATP hydrolysis are determined in the first minute after ATP addition. In the case of a pre-established $\Delta\psi$ the ion and buffer concentrations are sufficiently high to prevent any changes in $\Delta\psi$ or $\Delta$pH ( = 0) during measurement. In the case of a base-pulse the presence of valinomycin is required to prevent the otherwise rapid generation of a $\Delta\psi$ within seconds upon ATP addition [24]. Indeed, in the absence of valinomycin the $x$-axis

intercept for ATP hydrolysis activity shifts with about 2.9 kJ · mol$^{-1}$ to lower $\Delta\bar{\mu}_{H^+}$ for both types of proteoliposome (results not shown).

Possible sources of error that may influence the size of the proton translocation stoichiometry have been discussed in the previous paper [11], but none of them will lead to much lower values.

Essentially, the different methods used above and in the previous paper [11] are based on the same (thermodynamic) principle. Therefore, the proton translocation stoichiometry of both types of proteoliposome has also been determined by a different (kinetic) approach. With the use of direct pH measurements the actual number of protons taken up per ATP hydrolyzed has been determined. Due to the high temperature and low KCl concentration during measurements the use of a sensitive pH electrode causes artefactual problems. Cresol red has successfully been used before to follow bulk pH changes [24] and can be applied to monitor external alkalinization upon ATP addition. Also, in another study with light-induced proton fluxes catalyzed by *Synechococcus* 6716 Photosystem I incorporated in asolectin, Cresol red yields similar results compared to a sensitive pH electrode (Hotting, E.J., personal communication).

Upon ATP addition to ATPase proteoliposomes the following reactions lead to changes in proton concentration:

ATP hydrolysis

$$ATP^{4-} + H_2O \rightarrow ADP^{3-} + HPO_4^{2-} + t_1H^+ \quad (1)$$

($t_1$ is a pH-dependent factor approx. equal to 1 at pH 8)

proton uptake coupled to ATP hydrolysis

$$nH^+_{out} \rightarrow nH^+_{in} \quad (2)$$

in which $n$ is the value of the $H^+$/ATP ratio. In principle, when the amount of ATP added is large enough, the rate of ATP hydrolysis can be calculated from the steady-state rate of acidification (from reaction 1), and then $n$ follows from the initial rate of alkalinisation (reactions 1 and 2).

However, due to contamination of ATP by ADP and the inhibiting effect of the resulting $\Delta$pH on the rate of ATP hydrolysis, a steady state is not obtained. This problem has also been encountered by Sholtz et al. [25] in the kinetic determination of the proton translocation stoichiometry of the $H^+$-ATP synthase of rat liver mitochondria. Following the example of Sholtz et al. [25] we used our 'standard' ATP regenerating system (see Materials and Methods) which maintains the $\Delta G_p$ at approx. 85 kJ · mol$^{-1}$ to overcome this problem. Additional reactions of the regenerating system are:

the pyruvate kinase reation

$$ADP^{3-} + t_3H^+ + \text{phospho}\textit{enol}\text{pyruvate}^{3-} \rightarrow ATP^{4-} + \text{pyruvate}^- \quad (3)$$

the lactate dehydrogenase reaction

$$NADH + t_4H^+ + \text{pyruvate}^- \rightarrow NAD^+ + \text{lactate}^- \quad (4)$$

The possibility of following the steady-state rate of ATP hydrolysis by measuring pH changes depends on the value of $t_1 - t_3 - t_4$.

For the two types of proteoliposome a typical example of such a pH measurement during ATP hydrolysis is shown in the two panels of Fig. 3. A possible ATP-induced reaction will be due to several additional factors and needs to be corrected. Therefore, in the traces b ATP is added to proteoliposomes uncoupled with S-13. The traces show an initial alkalinization but no steady state change in pH. The absence of the steady-state change means that at pH 7.8 $t_1 - t_3 - t_4$ is zero, so that the rate of ATP hydrolysis has to be determined independently. The initial alkalinization is due to: the difference in pH between the reaction medium and the ATP solution; the removal of the ADP contamination by the ATP regenerating system. In the absence of active proteoliposomes the same result is obtained. Calibration of the ATP regenerating system with standard additions of ADP shows that 0.78 proton per NADH is taken up in reactions 3 and 4 (results not shown). In traces a, the experiment has been carried out in the absence of uncoupler. The initial alkalinization now includes the actual proton uptake by the ATP synthase. After correcting traces a for the side reactions by subtraction of the traces b, the actual proton uptake by the proteoliposomes is shown in traces c.

Stoichiometries were calculated from initial rates of proton uptake as shown in Fig. 3, and from the steady-state rate of ATP hydrolysis. The results of these experiments are given in Table III. In one set of experiments (indicated by [a] and [b] in Table III), the steady-state rate of ATP hydrolysis under uncoupled conditions was used. The rationale for this is that the true initial rate of ATP hydrolysis cannot be determined under the exact conditions of Fig. 3 because of the simultaneous removal of contaminating ADP by the regenerating system. We have chosen the steady-state rate under uncoupled conditions because initial ATP hydrolysis occurs at $\Delta\bar{\mu}_{H^+} = 0$, so that there is no back-pressure to inhibit ATP hydrolysis, and no $\Delta\bar{\mu}_{H^+}$-dependent activation. In a second set of experiments (indicated by [c] in Table III), proteoliposomes were used in which the ATP synthase was not activated by trypsin treatment. Under these conditions ATP hydrolysis is so slow that it is possible to distinguish between this process and the

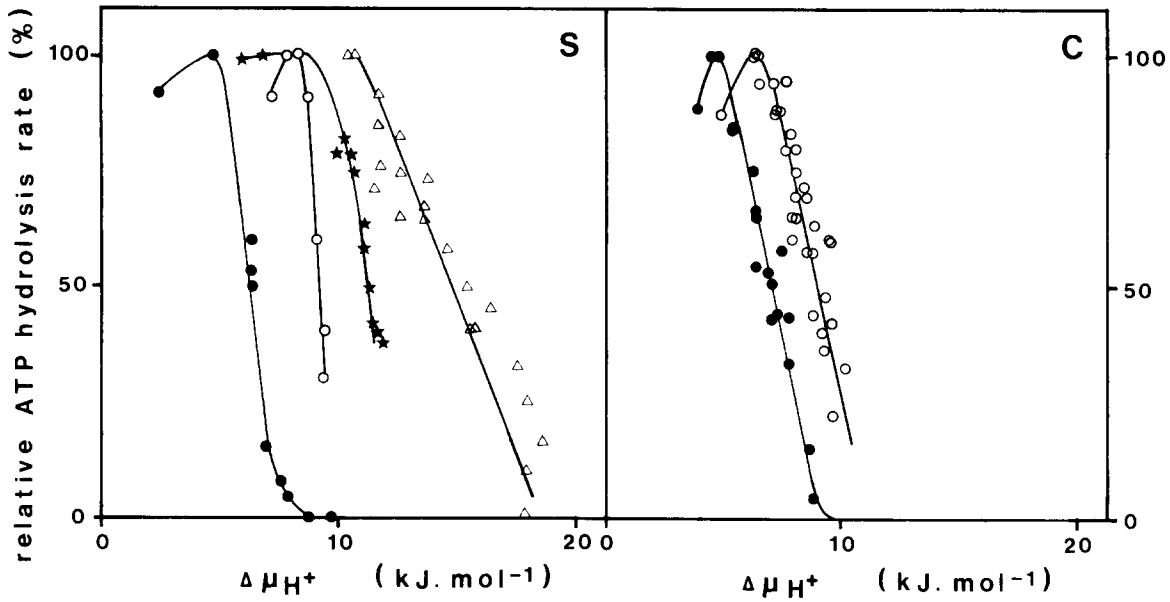


Fig. 4. Dependence of the initial rates of ATP hydrolysis on the size of the pre-established $\Delta\bar{\mu}_{H^+}$ in different membrane systems. For experimental conditions see legend to Fig. 2. The data of at least ten experiments with three different preparations of each type of proteoliposome and of eight experiments with four different preparations of membrane vesicles have been pooled. The ATP hydrolysis activity of the membrane vesicles varied between different preparations and 100% activity corresponds to approx. 600 nmol/min per mg Chl (1 μmol/min per mg ATP synthase). The activities of the ATPase proteoliposomes varied around the same value as has been given in the legend of Fig. 2. ●, ATPase proteoliposomes; ○, ATPase proteoliposomes co-reconstituted with cytochrome $b_6f$ complex; △, membrane vesicles, $[K^+]_{in} = 1$ mM; ★, unactivated membrane vesicles, $[K^+]_{in} = 10$ mM. In the case of membrane vesicles the $\Delta\bar{\mu}_{H^+}$ consisted only of a $\Delta\psi$ and the media did not contain dithiothreitol.

removal of contaminating ATP in both the pH traces and in ATP hydrolysis determinations.

Table III shows that under all conditions tested, high $H^+$/ATP ratios are obtained with both types of proteoliposome. This result is independent of differences in activity and/or coupling quality. It is difficult to give an exact number of $H^+$/ATP by this method because the results critically depend on the determination of the initial rate of proton uptake and ATP hydrolysis. However, important is that the thermodynamically determined high proton translocation stoichiometry of about 9 $H^+$/ATP observed here and before [11] is confirmed by a different type of experiment.

TABLE III

*Kinetic determination of the $H^+$/ATP ratio with cyanobacterial (S) and chloroplast (C) ATP synthase reconstituted in proteoliposomes*

Rates and extents of $H^+$ uptake were determined as in Fig. 3. ATP hydrolysis was measured as described in Materials and Methods. For further details see text.

| Source | Expt. | $H^+$ uptake (μmol/min per mg protein) | | ATP hydrolysis (nmol/min per mg protein) | | $H^+$/ATP |
|---|---|---|---|---|---|---|
| | | initial | total | basal | +100 nM S-13 | |
| S | 1 [a] | 1.9 | 1.75 | 110 | 200 | 9.5 |
| | 2 [b] | 1.2 | 0.8 | 108 | 174 | 7 |
| | 3 [c] | 0.63 | 2.5 | 80 | – | 8.9 |
| C | 1 [a] | 2.7 | 0.8 | 200 | 300 | 9 |
| | 2 [c] | 0.33 | 1.14 | 38 | – | 8.7 |

[a] Kinetic trace of $H^+$ uptake shown in Fig. 3.
[b] Same, but with proteoliposomes aged for 7 days at 4°C.
[c] ATP synthase not pre-activated by trypsin treatment.

*Dependence of the proton translocation stoichiometry on membrane protein composition*

To study the effect of membrane protein composition, we have compared the effect of an applied $\Delta\bar{\mu}_{H^+}$ on ATP hydrolysis catalysed by either ATPase proteoliposomes or by a preparation of cyanobacterial thylakoid membrane vesicles. Also, we have studied the effect of the presence of a single other membrane protein by coreconstitution of the cyanobacterial $b_6f$ complex with either of the two ATP synthases. The results are shown in Fig. 4 (the data referring to ATPase proteoliposomes are taken from Fig. 2). Upon extrapolation of the data for proteoliposomes containing the cytochrome $b_6f$ complex ATP hydrolysis will vanish at a $\Delta\bar{\mu}_{H^+}$ of 11.6 and 12.5 kJ · mol$^{-1}$, respectively. This corresponds to a lowering of the $H^+$/ATP ratio to approx. 7. The data referring to the cyanobacterial thylakoid membrane vesicles with an internal KCl concentration of 1 mM extrapolate to a $\Delta\bar{\mu}_{H^+}$ of 19.3 kJ · mol$^{-1}$, corresponding to a $H^+$/ATP ratio of 4.4. Membrane vesicles with an internal KCl concentration of 10 mM show a steeper dependence between ATP hydrolysis rate and $\Delta\bar{\mu}_{H^+}$ with an intercept at approx.

14.5 kJ · mol$^{-1}$. This is probably due to artefactual inhibition of ATP hydrolysis caused by high KCl concentrations (1 M KCl is required to induce a $\Delta\bar{\mu}_{H^+}$ of 12.5 kJ · mol$^{-1}$). After correction for this inhibition the $H^+$/ATP ratio approaches the value found for membrane vesicles with 1 mM internal KCl (not shown). In the experiments with membrane vesicles, $\Delta\bar{\mu}_{H^+}$ was applied as $\Delta\psi$ only, because the vesicles are not able to maintain a $\Delta$pH well enough for this type of measurement.

The coupling quality of the membrane systems used in the experiments of Fig. 4 declines with increasing complexity. The uncoupler stimulation of ATP hydrolysis activity of the proteoliposomes containing ATP synthase only is more than a factor 3; for proteoliposomes with co-reconstituted cytochrome $b_6f$ this decreases to a factor 1.6 and membrane vesicles show an uncoupler stimulation of only 1.1–1.2.

## Discussion

*Comparison between the ATP synthases from Synechococcus 6716 and chloroplasts*

The results reported here show a great similarity in behaviour of the ATP synthase from chloroplasts and from *Synechococcus* 6716. This is the case both for the isolated enzymes with respect to temperature, and for the enzymes reconstituted into proteoliposomes provided the same (cyanobacterial) lipids are used. This similarity is also found for other properties such as $K_m$ for ATP, $Mg^{2+}$ dependence, inhibition by DCCD and pH optimum (see Refs. 9,13). The fact that both types of ATP synthase behave so similarly is also reflected by their high degree in sequence homology. The actual DNA sequences of the *Synechococcus* 6716 ATP synthase genes will be published elsewhere (Van Walraven, H.S. and Walker, J.E.) but the $\alpha$, $\beta$, a and c subunits are particularly well conserved [26] between chloroplasts and two cyanobacterial species *Synechococcus* 6716 (preliminary report [27]) and *Synechococcus* 6301 [28].

The main difference between *Synechococcus* 6716 ATP synthase and that from chloroplasts is that, in contrast to the former [9], the latter can be activated by heat treatment [23] or by reducing agents like dithiothreitol or dithioerythritol [29]. A part of the $\gamma$ subunit from chloroplasts containing two cystein residues that can be reduced [30,31] is lacking in cyanobacteria (van Walraven, H.S. and Walker, J.E., unpublished data, and Ref. 28). This part may also be involved in heat activation [30]. The site in the $\gamma$ subunit probably involved in trypsin activation [31] is present both in chloroplast and cyanobacterial ATP synthase. Trypsin appeared to have a similar effect as reducing agents on ATP synthesis and hydrolysis activity in chloroplasts [32].

The specific activity of chloroplast ATP synthase in thylakoid membranes can be as high as approx. 4 $\mu$mol/min per mg ATP synthase [33] at room temperature. Table I shows that for the isolated chloroplast enzyme this is 5 $\mu$mol/min per mg ATP synthase at 50 °C. Incorporation in cyanobacterial lipids leads to a decrease in specific activity of about 80% (uncoupled rate). The specific activity of the *Synechococcus* 6716 ATP synthase in membrane vesicles is about 1 $\mu$mol/min per mg ATP synthase at 50 °C. This hardly changes for the isolated cyanobacterial enzyme (Table I) and incorporation in cyanobacterial lipids here leads to a decrease in specific activity of about 50% (uncoupled rate). The relatively high rates observed in chloroplast thylakoid membranes and with the isolated chloroplast enzyme compared to systems containing *Synechococcus* 6716 lipids may be due to the degree of saturation of the associated monogalactosyldiacylglycerol (MGDG). ATP synthase from chloroplasts, and perhaps also from cyanobacteria, is highly activated by the presence of polyunsaturated but not by saturated MGDG [34] and *Synechococcus* 6716 only contains the latter [15]. Reconstitution of the chloroplast enzyme with cyanobacterial lipids results in 'dilution' of unsaturated MGDG by saturated MGDG. Consequently, the uncoupled specific activities of the two types of ATP synthase proteoliposome have similar (low) values. Also, chloroplast ATP synthase reconstituted with *Synechococcus* 6716 lipids has a low rate of ATP synthesis driven by an imposed $\Delta\bar{\mu}_{H^+}$ (Gräber, P., personal communication).

*Implications of a variable proton translocation stoichiometry of ATP synthases*

Initially the expectation was that the different values of $H^+$/ATP obtained with chloroplast [4,7] and *Synechococcus* 6716 [10,11] ATP synthases, respectively, reflected an intrinsic difference between these enzymes. Our results clearly show that this is not the case. When the two ATP synthases are incorporated into the same lipid membrane, determinations of the $H^+$/ATP ratio yielded the same result, viz values of around 9. This shows that the membrane environment of the enzyme is very important for the outcome of this type of experiment. This conclusion is strengthened by the results with cyanobacterial membrane vesicles and co-reconstituted proteoliposomes (Fig. 4). In our experiments the proton translocation stoichiometry decreases with increasing complexity of the membrane. However, even the lowest value observed with membrane vesicles is a $H^+$/ATP as high as 4.4. It is interesting to note that 4.5 rather than 3 $H^+$/ATP have also been observed with chloroplasts in equilibrium [4].

Dependence of $H^+$/ATP on the membrane composition has also been shown by Van der Bend et al. [35] in experiments with light driven ATP synthesis by co-re-

constituted yeast mitochondrial ATP synthase and bacteriorhodopsin. The stoichiometry was 2–3 $H^+$/ATP when reconstitution was performed with asolectin and 8 $H^+$/ATP with egg-yolk phosphatidylcholine. Like the lipids from *Synechococcus* 6716, egg phosphatidylcholine is rather saturated and uncharged and forms well-coupled proteoliposomes with an uncoupler stimulation of ATP hydrolysis activity up to 4 (vs. 1.1 with asolectin).

Thus, it seems that an extremely high proton translocation stoichiometry ($> 4.5$ $H^+$/ATP) is an effect of reconstitution conditions of ATP synthase. At first sight the high $H^+$/ATP in the ATPase proteoliposomes observed here and before [11] is, to quote Kell [36]: "a very unusual and probably unimportant exception". However, the present findings show that the ATP synthase intrinsically has the possibility to couple ATP synthesis or hydrolysis to the transfer of at least 9 protons! This suggests that there is a mechanistic way for the enzyme to increase the $H^+$/ATP from the value found in intact membranes (2–4) to higher values, depending on the environment. This possibility may turn into actuality when ATP synthesis under conditions of low $\Delta\bar{\mu}_{H^+}$ is required.

Two most striking examples for this are alkalophilic bacteria and uncoupler-resistant *Bacillus* mutants, both extensively studied by the group of Krulwich (see Refs. 37 and 3 for reviews on their bioenergetics). These bacteria and derived membrane vesicles maintain a high $\Delta G_p$ at a $\Delta\bar{\mu}_{H^+}$ (generated by electron transfer) of as low as 2.9 $kJ \cdot mol^{-1}$. In these bacteria $\Delta\bar{\mu}_{H^+}$ is so low, due either to a large reversed $\Delta pH$ or to the presence of an uncoupler during growth. Pleading against a high $H^+$/ATP for their ATP synthases as an explanation for ATP synthesis at low $\Delta\bar{\mu}_{H^+}$ is the finding that a pre-established $\Delta\bar{\mu}_{H^+}$ ($\Delta pH$ or $\Delta\psi$) of equivalent size is not able to maintain a high $\Delta G_p$. This, as pointed out in the introduction, would favor the involvement of a higher local $\Delta\bar{\mu}_{H^+}$. However, with alkalophilic bacteria a pre-established $\Delta pH$ of 1 (equivalent to a $\Delta\bar{\mu}_{H^+}$ of 5.8 $kJ \cdot mol^{-1}$) can sustain ATP synthesis for considerable time [38] which can only be explained with a $H^+$/ATP $> 6$. Unfortunately, no quantitative thermodynamic or kinetic studies have yet been undertaken with these types of bacterium. Investigation of the membrane composition of alkalophilic bacteria and uncoupler-resistant *Bacillus* mutants reveals the following differences:
obligate alkalophilic bacteria have a lower ratio of saturated to unsaturated fatty acids and a lower protein-to-lipid ratio in the membrane compared to facultative alkalophilic strains [39];
two strains of uncoupler-resistant mutants have, compared with the wild type, a higher ratio of saturated to unsaturated fatty acids and protein to lipid ratio [40.41].
A single mutation is responsible for uncoupler-resistance in both bacteria [40] and several studies indicate that this mutation is probably located in the gene for lipid desaturation [41]. The authors suggest [40] that the occurrence of localized proton translocation between the components of the electron transfer chain and the ATP synthase (resulting in a high $\Delta G_p$ to $\Delta\bar{\mu}_{H^+}$ ratio) may be manipulated by the membrane composition. Since the alterations are so different between the two types of bacterium this possibility does not seem very likely. A direct effect of membrane environment on the ATP synthase resulting in a change of $H^+$/ATP is the other option also given in Ref. 40.

The lowering of observed $H^+$/ATP with increasing complexity of membrane composition in our experiments, may be related to an increasing surface charge density of the membranes. This would be in line with the results of Van der Bend et al. [35], because in liposomes from egg-yolk phosphatidylcholine a lower surface charge is expected than in liposomes prepared from asolectin. However, determinations of surface charge density of liposomes containing ATP synthase and/or cytochrome $b_6f$ from *Synechococcus* 6716 did not reveal significant differences (Koppenaal, F., unpublished results). This could be expected, since the amounts of protein that can be incorporated are very small.

Another possibility is that the lowering of observed $H^+$/ATP with increasing complexity of membrane composition is related to a decreasing coupling quality (as judged from uncoupler stimulation of ATP hydrolysis) in our preparations. The results of Van der Bend et al. [35] also show this correlation. Well-coupled vesicles from chloroplasts enriched in Photosystem I (with an uncoupler stimulation of ATP hydrolysis activity exceeding a factor 1.5) (see Ref. 42) translocate 6 $H^+$/ATP (unpublished results) and the coupling membranes of alkalophilic bacteria are very impermeable and can maintain a pre-established $\Delta pH$ for more than 20 min [38]. In contrast to this, however, rat liver mitochondria show high uncoupler stimulation (exceeding a factor 10 by 2,4-dinitrophenol) of ATP hydrolysis activity [43] but translocate only 3 $H^+$/ATP [44].

This shows that for now there is no general correlation between the overall membrane properties and the $H^+$/ATP values obtained. Possibly only the direct environment of the ATP synthase causes structural changes of the enzyme, leading to changes in proton translocation stoichiometry.

More information about the three-dimensional structure of the ATP synthase is needed to find out how many protons per ATP can be translocated mechanistically. The a and c subunits are supposed to play a crucial role in proton translocation through $F_0$. For *E. coli* a pathway for protons has been proposed [45] of which the homologous residues are almost identical between the three types of thylakoid ATP synthase discussed above (see sequence homologies in Ref. 46).

Up to now even the number of c subunits of chloroplast ATP synthase is still a matter of debate (Ref. 47 and references therein).

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