Evidence for a high proton translocation stoichiometry of the H^+-ATPase complex in well coupled proteoliposomes reconstituted from a thermophilic cyanobacterium

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Evidence is presented for a high proton translocation stoichiometry (H^+/ATP) of approx. 9 in ATPase proteoliposomes with extremely low permeability for ions, reconstituted from a thermophilic cyanobacterium. A proportional relation between the phosphate potential (ΔG_p) and the proton-motive force (Δp) was observed in thermodynamic equilibrium. A bulk-to-bulk Δp was imposed by valinomycin-induced K^+ diffusion potentials of different size while the initial ΔG_p was varied. In all cases equilibrium was reached in about 1.5 h. A high H^+/ATP ratio was also deduced from the relation between the initial rates of ATP synthesis or hydrolysis at varying ΔG_p and Δp. The implications of these results for the mechanism of energy transduction in energy-conserving membranes are discussed.

ATPase proteoliposome *Energy transduction* *Protonmotive force* *Diffusion potential* *Phosphate potential* *Proton translocation stoichiometry*

1. INTRODUCTION

There is abundant experimental evidence that in oxidative and photosynthetic ATP synthesis an electrochemical gradient of protons (Δp) across (part of) the membrane is an essential intermediate in energy transduction. However, whether a bulk-to-bulk Δp exclusively represents the energized state according to the chemiosmotic theory of Mitchell [1], or whether there are other (merely localized) pathways for protons is still a matter of debate. Data giving evidence for the occurrence of the latter possibilities have been reviewed recently [2–4].

If any chemiosmotic type of mechanism applies, there should be a unique relationship between the proton-motive force (Δp) relevant for that mechanism and the rate of the energy-linked reactions. Moreover, at equilibrium, the size of this Δp should be proportional to the phosphate potential (ΔG_p) by a factor equal to the number of protons translocated per ATP synthesized or hydrolysed. There have been indications that the relevant Δp does not operate between the bulk phases ([2], but see [5,6]), which has led to a number of 'localized' variants of the chemiosmotic hypothesis [3,4]. In cases where it was attempted to establish the stoichiometry of proton translocation by ATPases of different origin from the relative sizes of bulk-phase Δp and the ΔG_p at or close to equilibrium, or from kinetic measurements of proton translocation driven by ATP hydrolysis (e.g. [5–12]), values of about 2–3 H^+/ATP have been obtained.

Unfortunately, bioenergetic membrane studies are often severely hampered by the complexity of the natural membrane system, leading to conflicting results from the same type of experiments (e.g. [5] vs [13]). The difficulties comprise: side effects of added compounds (e.g. uncouplers) for Δp

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variation, adenylate kinase activity, variable membrane permeability for ions and lack of knowledge about and variability of the internal volume, surface charge, ionic strength, buffering capacity, etc. As a consequence, these systems are often not in true thermodynamic equilibrium and the estimates of Δp may be different.

In principle, reconstituted proteoliposomes offer very promising systems for studying elementary energy-transducing events. A Δp can be imposed by various methods. However, until now most ATPase proteoliposomes appeared to have low enzyme activity and/or poor coupling quality [14]. Therefore, it has only been possible to study initial events on the millisecond or seconds time scale, far from equilibrium, which is a serious drawback for a quantitative approach. Recently, we reported on relatively high ATP synthesis activities, maintained for long periods of time, by large and well coupled ATPase proteoliposomes from the thermophilic cyanobacterium *Synechococcus* 6716, induced by pre-established electric potentials or pH gradients of low magnitudes [15]. Here we report on the relation between the size of bulk-to-bulk Δp imposed by valinomycin-induced K^+ diffusion potentials and both the level of ΔG_p and the rate of ATP synthesis and ATP hydrolysis at varying ΔG_p. The proteoliposomes are comparatively ion-tight and are therefore able to approach thermodynamic equilibrium between Δp and ΔG_p which leads to better estimates of the true proton translocation stoichiometry. Under the experimental conditions these ATPase proteoliposomes function according to a chemiosmotic type of energy transduction. However, they demonstrate a strongly deviating H^+/ATP stoichiometry.

2. MATERIALS AND METHODS

ATPase proteoliposomes were prepared from the native lipid mixture and ATPase of the thermophilic cyanobacterium *Synechococcus* 6716 by overnight dialysis at 50°C according to Van Walraven et al. ([16] see also references in [15]) with a protein to lipid ratio of about 0.01 (w/w). The reconstitution medium contained 100 mM Na-Tricine (pH 7.5 at 50°C), 10 mM KCl, 2.5 mM $MgCl_2$ and 1 mM dithioerythritol. Lipid concentration for reconstitution was 10 mg/ml. The ATPase proteoliposomes were collected by centrifugation for 1.5 h at 150000 × *g* at 50°C and resuspended. Proteoliposomes were stored above 40°C and were used within 12 h.

All experiments were carried out at 50°C. As external (reaction) medium the reconstitution medium was used with variable KCl concentrations. K^+ diffusion potentials ($\Delta\psi$) were induced by valinomycin addition (0.1 μg/mg lipid, Boehringer Mannheim) to proteoliposomes in the presence of a K^+ gradient and the diffusion potentials were allowed to stabilize for 1 min. $\Delta\psi$ is defined as inside minus outside. The reactions were started by the addition of the ATP, ADP and potassium phosphate mixture of choice (see figure legends).

In the case of the ΔG_p determinations in equilibrium studies (figs 1 and 2) the lipid concentration was 50 mg/ml (0.5 mg protein). After the reaction time the ATP and ADP contents were determined immediately. ΔG_p was calculated according to [17], with $\Delta G_{o)}$ estimated to be 31.2 kJ/mol.

The ATP content in samples was determined by the luciferine-luciferase method following the instructions for use supplied with the LKB ATP monitoring kit (Turku, Finland) (see also [18]) in a laboratory-built luminometer (obtained from the Arrhenius Lab., Stockholm). The ATP and ADP contents in samples and during simultaneous measurements (see below) were determined enzymatically as described by Bergmeyer [18]. NADH absorbance changes were measured at 340 nm with an Aminco DW-2a spectrophotometer, equipped with a thermostatically controlled multipurpose cuvette [19]. After every assay standard amounts of either ATP or ADP were added.

During the initial activity measurements (fig.3) the lipid concentration was 1 mg/ml (0.01 mg protein). The reaction medium contained either 0.8 mM $NADP^+$ and 1 mM glucose for ATP determinations or 0.2 mM phospho*enol*pyruvate and 0.2 mM NADH for ADP determinations. After a reaction time of 2 min the enzymes hexokinase/glucose-6-phosphate dehydrogenase (for ATP) or pyruvate kinase/lactate dehydrogenase (for ADP) were added. In studies carried out in the presence of an enzymatic ATP- or ADP-regenerating system the enzymes were already present at the start. The enzymes were dialyzed before use in order to remove ammonium sulfate.

Protein concentration was determined according to Bradford [20].

3. RESULTS AND DISCUSSION

The reported experiments were carried out with proteoliposomes that were not activated beforehand, did not contain significant adenylate kinase activity and that showed an uncoupler stimulation of ATP hydrolysis activity by a factor exceeding 10 (see also [15]).

Fig.1 is a compilation of several experiments showing the dependence of the ΔG_p on the initial size of Δp $(=\Delta\psi)$ in actual thermodynamic equilibrium. Independently of the initial ΔG_p (30, 52 or 75 kJ/mol) a linear relationship was observed which can be extrapolated through zero. The proportionally factor between Δp and ΔG_p is approx. 9. Only ΔG_p values between 30 and 80 kJ/mol can be determined with precision. Out of this region small deviations in ATP and ADP determinations give rise to large errors in ΔG_p.

The time-dependent changes of ATP concentration of the three representative experiments from fig.1, started at different initial ΔG_p, are given in fig.2. In all cases equilibrium was reached in about

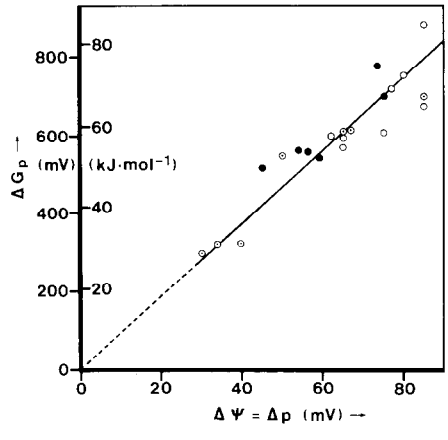

Fig.1. Dependence of ΔG_p on the size of the pre-established Δp in ATPase proteoliposomes under conditions of thermodynamic equilibrium. Experimental conditions are given in section 2. The initial ΔG_p of data indicated with (●) was 75 kJ/mol (1 mM commercially available ATP contaminated with 1.3% ADP and phosphate), with (○) 52 kJ/mol (0.5 mM ATP, ADP and phosphate) and with (⊙) 29–32 kJ/mol (1 mM commercially available ADP contaminated with 0.1–0.25% ATP, plus 2 mM phosphate). The line was calculated by least-squares fit.

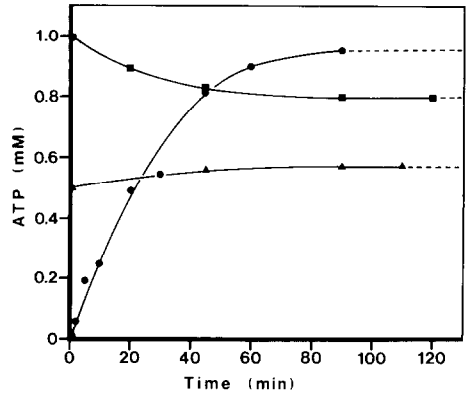

Fig.2. Time dependence of ATP synthesis or hydrolysis by ATPase proteoliposomes reaching equilibrium at different initial ΔG_p and Δp. Experimental conditions are given in section 2. (●) Δp, 65 mV; initial ADP concentration, 1 mM; phosphate, 2 mM; (■) Δp, 65 mV; initial ATP concentration, 1 mM; (▲) Δp, 62 mV; initial concentrations of ATP, ADP and phosphate, 0.5 mM.

1.5 h. Note that small differences in ΔG_p as the result of either a slightly different Δp or a small deviation from the line drawn in fig.1 correspond to relatively large variations in ATP, ADP or phosphate concentrations. The long duration of such experiments is caused by both low enzyme activities at low values of Δp and the limited protein concentration due to the low maximal ATPase incorporation in the proteoliposomes. Since the reactions are very slow it is not necessary to terminate them in a quench mixture before determining the ATP and ADP contents.

In fig.3. the initial rates of ATP synthesis or ATP hydrolysis at different pre-established Δp and ΔG_p are given. The activities show a clear correlation with the size of Δp and at increasing pre-established ΔG_p the intercepts shift to higher values of Δp. With the same system a similar dependence of ATP synthesis and hydrolysis activities on the size of Δp, only consisting of a pH gradient, and ΔG_p was reported in [16]. At the lowest value of ΔG_p (32 kJ/mol) hydrolysis of ATP cannot be detected due to the low ATP concentration (2.5 μM). Likewise, ATP synthesis cannot be measured at the highest ΔG_p (about 85 kJ/mol) in the presence of an ATP-regenerating system. The bending of those two curves could be caused by small immediate deviations of ΔG_p after the onset of ATP synthesis or ATP hydrolysis, respectively. The proportion of these changes may

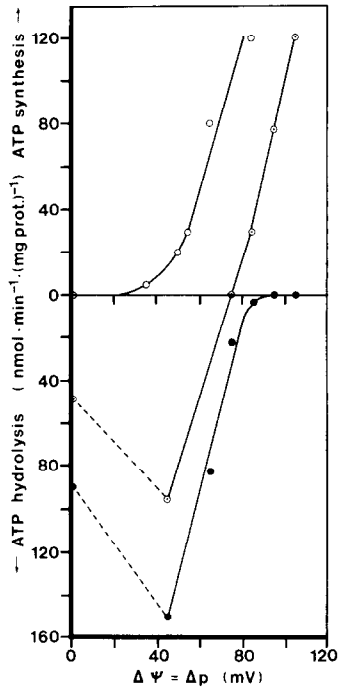

Fig.3. Dependence of initial rates of ATP synthesis and ATP hydrolysis on the size of the pre-established Δp and the effect of ΔG_p. ΔG_p was (○) 32 kJ/mol (1 mM ADP contaminated with 0.25% ATP, plus 2 mM phosphate), (⊙) 75 kJ/mol (1 mM ATP with 1.3% ADP contamination) and (●) approx. 85 kJ/mol (with medium containing 1 mM ATP and an enzymatic ATP-regenerating system, section 2).

differ between low and high reaction rates and is difficult to determine. Extrapolation through the X-axis of the three curves for initial ΔG_p of 32, 75 and about 85 kJ/mol (corresponding to 331, 777 and 880 mV) shows intercepts for Δp of 45, 75 and 85 mV, respectively, where the ATP synthesis and hydrolysis activities are zero. In these cases the ΔG_p to Δp ratios are 7.4, 10.4 and 10.4, respectively.

Fig.3 also demonstrates that a Δp-dependent activation is necessary to induce full ATP hydrolysis activity in non-activated proteoliposomes since the activity at a Δp of 0 mV is lower than at 45 mV. At low Δp other methods of activation must be applied, like trypsin treatment [16]. The ATPase complex from chloroplasts can be activated in the same way but requires much higher values of Δp [21].

In thermodynamic equilibrium the ΔG_p to Δp ratio should be equal to the proton translocation stoichiometry. From our results values between 7.5 and 10.5 H^+/ATP can be deduced. A high stoichiometry could already be foreseen from earlier work [15,16] where unusually low threshold values of pre-established Δp for ATP synthesis were found. The most reliable and constant value of 9 H^+/ATP is derived from fig.1.

There are several possible sources of error that may lead to wrong estimates of the proton translocation stoichiometry. Firstly, one has to be certain that the system has reached thermodynamic equilibrium. The proteoliposomes used here have hardly any membrane leakage and no disturbing adenylate kinase activity [15]. As in all experiments the ATP and ADP contents did not change after 1.5 h (see fig.2), it can be fairly stated that at least the results given in fig.1 were determined under equilibrium conditions.

Secondly, there may occur errors in the estimates of ΔG_p and Δp. As to ΔG_p this is not very likely since both ATP and ADP contents are determined by various methods and compared with standard amounts of adenine nucleotides, so that possible quenching due to the presence of proteoliposomes did not interfere (cf. section 2). Concerning Δp, erroneous estimates are not very likely either. Δp was generated by a valinomycin-induced K^+ diffusion potential ($\Delta\psi$), and thus exists between the bulk phases by nature. Due to the low permeability for ions, large internal volume and high buffering (100 mM) the ion capacity of the proteoliposomes should be sufficient to prevent significant changes of $\Delta\psi$ and ΔpH during the long-term experiments, under phosphorylating conditions. We did not attempt to monitor the intravesicular pH simultaneously under these conditions, using a trapped pH indicator. Under non-phosphorylating conditions Δp was shown earlier to be stable for extremely long periods ([15] and references therein). If, however, Δp were to change somewhat during ATP synthesis in the present experiments, due to instability or shortage of internal K^+, it would decrease and hence lead to even higher H^+/ATP ratios. Therefore, in the worst case our H^+/ATP ratios are underestimates, rather than overestimates.

Thirdly, it might be possible that ions other than protons are involved in the synthesis of ATP, e.g. K^+. This possibility can be ruled out by the fact

that in these proteoliposomes ATP synthesis can also be driven by an external base-pulse and by a nigericin-induced pH gradient [15]. The latter requires an opposite K^+ gradient as compared to a valinomycin-induced $\Delta\psi$.

Also alkalophilic bacteria maintain a high ΔG_p [22] at a very low value of Δp and the isolated membrane vesicles are still capable of ATP synthesis [23] under those conditions. To explain these phenomena two explanations have been put forward [23]. The first is that the bulk-phase Δp that has been measured is not the one relevant for driving ATP synthesis. The second is the possibility that higher stoichiometries of proton translocation coupled to ATP synthesis may apply. While in our case the bulk phase Δp is not an intermediate between energy-yielding and energy-consuming reactions, but is the actual energy source for ATP synthesis. Only the second explanation can be correct. This, to quote Guffanti et al. [23], '...would raise interesting questions with respect to proton pumping...', especially with respect to the mechanism of ATP synthesis by this thermophilic cyanobacterium.

Although under these conditions the ATPase proteoliposomes act by a chemiosmotic type of mechanism concerning the role of the bulk-phase Δp the high H^+/ATP ratio is not in agreement with Mitchell's hypothesis [1]. To explain this phenomenon and possibly also high ΔG_p to Δp ratios observed in other systems the theory needs adjustment. It is still uncertain whether the high H^+/ATP stoichiometry, which has been demonstrated unequivocally here and also for alkalophilic bacteria [22,23], is only a specific feature of these special types of bacteria, or whether it also applies to other energy-conserving systems. In the latter systems these high stoichiometries may not have become apparent due to methodological difficulties, as discussed above.

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