Modulation of the proton-translocation stoichiometry of H⁺-ATP synthases in two phototrophic prokaryotes by external pH

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The stoichiometry between proton translocation and ATP synthesis/hydrolysis was studied in two different photosynthetic prokaryotes, the thermophilic cyanobacterium *Synechococcus* 6716 and the purple bacterium *Rhodospirillum rubrum*. The H⁺/ATP ratio was determined by acid–base transitions as a function of the external pH. The H⁺/ATP ratio of the *Synechococcus* 6716 ATP synthase was found to increase with increasing pH. In contrast, in *R. rubrum* this ratio decreased with increasing pH. These results were qualitatively supported by experiments using the fluorescence probe 9-aminoacridine. The

degree of coupling between the H⁺ flux and the ATP synthesis/hydrolysis reaction is apparently modulated by the conditions under which the proton pump has to work. Such modulation of the H⁺/ATP ratio may be of physiological significance for an organism, for example when ATP synthesis is necessary at low proton-electrochemical potential difference ($\Delta\bar{\mu}_{\text{H}^+}$ levels). The different pH dependencies of the H⁺/ATP ratios in these organisms are considered in relation to the differences in the charged amino acids that are present in the F₀ subunits a and c.

INTRODUCTION

The inner membrane of mitochondria, the thylakoid membrane of chloroplasts and of cyanobacteria, as well as the plasma membrane of bacteria contain ATP synthases that catalyse the formation of ATP in response to a transmembrane proton gradient [1]. By acting in reverse, these enzymes can also generate a proton gradient by the hydrolysis of ATP. The overall structure of the ATP synthases in these systems is very similar. They are composed of two main parts: an integral membrane-protein complex, the F₀ part, that mediates proton translocation, and a membrane-associated multisubunit protein, the F₁ part, that contains the catalytic and nucleotide-binding sites (for a review see [2]). The hydrophilic F₁ part is generally found to consist of five different subunits, α , β , γ , δ and ϵ , in a stoichiometry of 3:3:1:1:1. The subunit composition of the hydrophobic F₀ part is found to be distinct in ATP synthases from various species. The *Escherichia coli* F₀ part consists of three different subunits: a, b and c (in a ratio of 1:2:10–12; see [3]). In cyanobacteria and purple bacteria, two distinct b subunits are found (b and b') [4]. The F₀ part of the chloroplast enzyme has essentially the same structure as the cyanobacterial F₀, whereas eukaryotic enzymes have more complicated structures [2]. The genes coding for various ATP synthases have been cloned and the nucleotide sequences and gene organization studied for ATP synthases from bacteria, cyanobacteria, yeasts and plants, as well as mammals [2]. The different α and β subunits show the highest homology, whereas the a and c subunits of ATP synthases only contain some residues that are strictly conserved [2,5].

Earlier estimations of the number of protons translocated per molecule of ATP synthesized or hydrolysed (the H⁺/ATP ratio) point to a value of 3 for ATP synthases from different origins, such as bacterial vesicles, chloroplasts and mitochondria [6]. However, for the isolated and reconstituted ATP synthase (in native lipids) from the cyanobacterium *Synechococcus* 6716, a

stoichiometry as high as 9 was observed [7]. However, after the co-reconstitution of this cyanobacterial ATP synthase with the *Qbc* (plastoquinone-containing cytochrome *b*-563/*c*-554) redox complex isolated from the same organism, the proton-translocation stoichiometry was lowered to about 7 [8,9]. An H⁺/ATP ratio of 4 was found in cyanobacterial thylakoid vesicles, both under conditions of ATP hydrolysis and of ATP synthesis, measured as a function of an artificially applied proton-electrochemical potential difference, $\Delta\bar{\mu}_{\text{H}^+}$ [10]. These results suggest that the H⁺/ATP ratio can be modulated by the alteration of the membrane composition [11]. A higher stoichiometry than 3 has also been described for the spinach-chloroplast ATP synthase [12]; two independent experimental approaches indicated a stoichiometry of 4 for this chloroplast enzyme [13]. It appears, therefore, that the different values that have been found for the H⁺/ATP ratio of the *Synechococcus* 6716 ATP synthase may not be completely explained by trivial experimental differences. There may be a significant physiological function for an H⁺/ATP ratio that is higher than expected on purely thermodynamic considerations, in organisms that have to cope with relatively low $\Delta\bar{\mu}_{\text{H}^+}$ levels, such as alkalophilic bacteria and uncoupler-resistant mutants [14]. This is particularly important when the ATP synthase is the sole or the major mechanism for ATP production. In addition, it would be advantageous if the H⁺/ATP ratio could be tuned to variable physiological conditions.

Since the pH of the medium is an obvious possible regulating factor, we describe in this paper the effect of the external pH on the H⁺/ATP ratio of the ATP synthases from the thermophilic cyanobacterium *Synechococcus* 6716 and from the purple bacterium *Rhodospirillum rubrum*. The comparison of these organisms was considered to be useful since their growth conditions imply that they have different degrees of stress on their energy transduction. We give a possible explanation for the difference in pH dependence, based on differences in the charged amino acids that are present in the F₀ subunits.

Abbreviations used: $\Delta\bar{\mu}_{\text{H}^+}$, proton-electrochemical potential difference; ΔG_p , phosphate potential; 9-AA, 9-aminoacridine; *n*, number of protons translocated per molecule of ATP synthesized/hydrolysed.

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MATERIALS AND METHODS

Organisms and culture conditions

The thermophilic cyanobacterium *Synechococcus* PCC 6716 (kindly donated by Dr. R. Rippka, Pasteur Institute, Paris) was cultured in BG11 medium at 50 °C in a 2 litre airlift fermenter using the method described by Lubberding and Bot [15].

The photosynthetic purple non-sulphur bacterium *R. rubrum* was grown anaerobically at 30 °C in the light (two tube lights of 40 W at 30 cm) in a medium containing 1 % (w/v) yeast extract, 1 % (w/v) bactopecton and 0.15 % NaCl, pH 7.2.

Preparation of membrane vesicles and chromatophores

Synechococcus 6716 cells were harvested in the linear growth phase and incubated with 0.1 % lysozyme at 37 °C [15] to obtain spheroplasts. Subsequently, membrane vesicles were obtained after an osmotic shock in 10 mM MgCl₂, 10 mM KCl and 10 mM Tricine/NaOH (pH 7.8). Vesicles were resuspended in the supernatant to a chlorophyll *a* concentration of 1.8 mg·ml⁻¹. Vesicles were either stored frozen at -80 °C (after the addition of 0.3 M sucrose) or used within 36 h after isolation.

R. rubrum cells were collected by centrifugation after 2 days of growth, washed and resuspended in 30 mM Tricine/NaOH (pH 8.0) containing 10 mM KCl and 5 mM MgCl₂. Chromatophores were prepared by sonication for 10 × 30 s at an amplitude of 20 µm in the presence of 0.02 % lysozyme, followed by differential centrifugation [16]. After removal of whole cells by low-speed centrifugation (10 min, 4000 *g*) a high-speed centrifugation step (60 min, 50 000 *g*) gave a preparation that was enriched in chromatophores. For further purification a discontinuous sucrose gradient (5 %, 40 % and 45 % sucrose, w/v), was used; after centrifugation for 16 h in a swing-out rotor at 220 000 *g*, the chromatophores could be collected from the 40 % (w/v) sucrose fraction. The chromatophores were stored at -80 °C in the presence of 0.3 M sucrose.

The chlorophyll *a* concentration of cyanobacterial vesicle preparations was determined by the method of Arnon et al. [17]. The concentration of bacteriochlorophyll in the chromatophores was measured after extraction with an acetone/methanol (7:2, v/v) mixture (ϵ_{770} 75 mM⁻¹·cm⁻¹) [18].

Determinations of the H⁺/ATP ratio

Acid-base transition experiments

ATP-synthesis activities were studied at 50 °C for *Synechococcus* 6716 membrane vesicles and at 30 °C for *R. rubrum* chromatophores. Phosphorylation was induced by acid-base transitions as described by Bakels et al. [10] with some minor modifications. Cyanobacterial vesicles (at a chlorophyll *a* concentration of 85 µg·ml⁻¹) or chromatophores (at a bacteriochlorophyll concentration of 40 µg·ml⁻¹) were incubated for 1 min in an acidic medium containing the permeant buffer succinic acid [10]. Then the actual acid-base transition was performed by mixing 1 ml of the acidified sample with 1 ml of the alkaline medium [10] and incubating for 1 min. An aliquot of 0.25 ml was drawn from this mixture, and ATP synthesis was stopped with 50 µl of 6 M HClO₄, which was then neutralized with 50 µl of 6 M KOH in 1 M Tris. The amount of ATP present in these samples was measured immediately using the luciferin-luciferase assay method [19]; the luminescence signal was calibrated by standard ATP additions. The pH of the acidic medium with *Synechococcus* 6716 vesicles or *R. rubrum* chromatophores and of the acid-alkaline mixture, at 50 °C and

30 °C respectively, was monitored immediately (with a Hamilton mini-electrode) to establish the extent of the pH rise. Valinomycin (1 µM) and equal internal and external concentrations of potassium ions (10 mM) were present to nullify any membrane potential component of the $\Delta\bar{\mu}_{H^+}$, and 5 µM 3-(3,4-dichlorophenyl)-1,1-dimethylurea was added to prevent possible photophosphorylation. The lowest pH values of the acidic stage were 4.8 and 5.3 for cyanobacterial vesicles and chromatophores respectively, since aggregation and precipitation occurred at more acidic pH values.

To determine the ATP contamination of the ADP preparation (present in the alkaline medium), the acid-base transition was performed without vesicles. To correct for the ATP already present in the membrane vesicles, a control was performed in which 1 ml of acidified sample was added to the alkaline medium together with perchloric acid. A correction for the ATP synthesized by adenylate kinase present in the membrane vesicles was also found to be necessary. Since the adenylate-kinase activity is also pH-dependent, uncoupled control series were performed in the presence of 0.5 µM 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide.

The phosphate potentials (ΔG_p) were calculated using the method of Krab and van Wezel [20].

9-Aminoacridine (9-AA) fluorescence measurements

An alternative method to determine the H⁺/ATP ratio was also performed, using the fluorescence changes of the probe 9-AA, as described by Strotmann and Lohse [21] with some minor modifications. The measurements were performed using an Oriel 3090 fibre-optic fluorimeter and a thermostated multipurpose 1.8 ml cuvette [22]. Actinic light was provided at a wavelength of > 645 nm via a 10 mm-diameter light guide (Schott). The measurement temperature was 35 °C with *Synechococcus* 6716 vesicles and 30 °C for *R. rubrum* chromatophores. Vesicles (at a chlorophyll *a* concentration of 20 µg·ml⁻¹) or chromatophores (final bacteriochlorophyll concentration of 10 µg·ml⁻¹) were added to 2.5 mM Tricine buffer (of adjusted pH) containing 5 mM MgCl₂, 10 mM KCl, 2.5 µM 9-AA and 50 µM *N*-methylphenazonium methosulphate. Valinomycin (50 nM for vesicles and 2 µM for chromatophores [23]) was added to prevent the generation of a membrane potential. Since the ATP synthases both from *Synechococcus* 6716 and from *R. rubrum* lack the disulphide bond in the γ subunit [10], dithiothreitol was omitted during the pre-illumination step.

Chemicals

NADH, NADP, ATP, ADP, valinomycin and all enzymes were purchased from Boehringer (Mannheim, Germany). 3-(3,4-Dichlorophenyl)-1,1-dimethylurea was from ICN K&K Laboratories (Cleveland, OH, U.S.A.). The luciferin-luciferase mixture was obtained from Pharmacia-LKB Biotechnology (Uppsala, Sweden). *N*-Methylphenazonium methosulphate was from Sigma (St. Louis, MO, U.S.A.) and 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide was a gift from Dr. P. C. Hamm (Monsanto Co., St. Louis, MO, U.S.A.). All other chemicals were of analytical grade.

RESULTS

The effect of pH on the proton-translocation stoichiometry was studied using the classical acid-base transition method [24] adapted for this particular purpose. In acid-base transition experiments, a pH jump (and/or a K⁺-diffusion potential) is used

Figure 1 ATP yield as a function of the applied proton gradient in *R. rubrum* chromatophores at different external pH values

The $\Delta\mu_{H^+}$ was generated by performing acid–base transitions as described in the Materials and methods section. The ATP yields shown were corrected for the amount of ATP that was not due to ATP-synthase activity. Abbreviation used: Bchl, bacteriochlorophyll.

Figure 2 Effect of the external pH on the H^+ /ATP ratio of the ATP synthase from *Synechococcus* 6716 (at 50 °C; ●—●) and the ATP synthase from *R. rubrum* (at 30 °C; ▲—▲), measured by acid–base transitions

as the driving force for ATP synthesis. The measurements were performed with coupled membrane vesicles from the cyanobacterium *Synechococcus* 6716 as well as with *R. rubrum* chromatophores. The energy-threshold values for ATP synthesis were determined from the curves of ATP yield against $\Delta\mu_{H^+}$ (see Figure 5 in [10]), and the initial ΔG_p values at certain pH, temperature, ionic strength and substrate concentration values were calculated accurately using the method of Krab and van Wezel [20]. The data from an acid–base transition experiment with chromatophores at varying external pH are shown in Figure 1. From these threshold values (ranging from 6.6 kJ/mol at pH 7.2 to 12 kJ/mol at pH 8.2) and the ΔG_p values (ranging here from 33.4 kJ/mol at pH 7.2 to 38.4 kJ/mol at pH 8.2), the H^+ /ATP ratios at different pH values could be determined. For *Synechococcus* 6716 the threshold values ranged from 11.9 kJ/mol at pH 7.2 to 10.5 kJ/mol at pH 8.2, while the ΔG_p values varied from 36.1 kJ/mol at pH 7.2 to 42.8 kJ/mol at pH 8.2.

Figure 2 shows the results of the acid–base transition experiments (performed in triplicate) for both organisms. With increasing pH, the proton-translocation stoichiometry of the

ATP synthase from the cyanobacterium *Synechococcus* 6716 was found to increase. In *R. rubrum* chromatophores, the H^+ /ATP ratio was found to decrease at more alkaline pHs.

To investigate the effect of pH on the H^+ /ATP ratio, the fluorescence responses of 9-AA at different light intensities and phosphate potentials were also determined. In this method [21], it is assumed that the 9-AA signal at a certain ΔG_p reflects the actual ΔpH . The number of protons translocated per molecule of ATP synthesized or hydrolysed (n) can be determined, because when the ATP synthesis rate equals the ATP hydrolysis rate, the driving force is proportional to the Gibbs free energy, $\Delta G_p = n \cdot \Delta\mu_{H^+}$. This phosphate potential is the sum of a standard term, ΔG_p° , and a variable term including the actual concentrations of the substrates, ATP, ADP and inorganic phosphate. In these experiments (performed in quadruplicate) the effect of the attenuation of light intensity and the addition of substrate mix on the 9-AA fluorescence signal was determined. The 9-AA fluorescence signal change after the addition of a given substrate mixture was recorded as a function of the proportion of light-induced 9-AA fluorescence quenching (in the absence of substrates). For cyanobacterial membrane vesicles, it was clear that at a certain proportion of quenching (supposed to correspond qualitatively with a certain ΔpH), the fluorescence signals that were obtained after the addition of ATP, ADP and P_i mixtures were always higher at the higher medium pH, corrected for the pH dependence of 9-AA fluorescence itself (results not shown). Since ΔG_p as well as ΔG_p° (or $\Delta G_0'$) increase with increasing pH [20], these results indicate that n is not constant, but increases with pH. In contrast, *R. rubrum* chromatophores showed the opposite behaviour in these experiments (results not shown). However, since the use of 9-AA for quantitative ΔpH measurements is open to criticism [25,26], we consider these results to be indicative of general trends only, and, qualitatively, the results agree with the more accurate acid–base transition experiments.

DISCUSSION

Both methods to determine the H^+ /ATP ratio at varying ΔG_p and pH clearly indicate that this stoichiometry is variable. The two photosynthetic micro-organisms tested show opposite responses to the medium pH change, and in each case an H^+ /ATP ratio significantly higher than 3 could be observed. The acid–base transition method provides the most reliable approach. The results of the fluorescence probe method [21] were qualitatively in agreement with the acid–base transition results; however, this fluorescence method is only applicable to a limited range of substrate concentrations and has some intrinsic problems.

ATP synthesis by chromatophores on energization by either an artificial K^+ -diffusion potential alone or such a potential together with a ΔpH has been described before, while only a low level of ATP formation was found with a ΔpH alone [23]. After reconstitution, however, the ATP synthase from *R. rubrum* was found to catalyse ΔpH -driven ATP synthesis [27], and both membrane-potential gradient ($\Delta\psi$) and ΔpH appeared to be equivalent as the driving force for ATP synthesis [28]. Recently, ATP synthesis driven by artificially induced ion gradients has been described by Turina et al. [29] for chromatophores from another purple bacterium, *Rhodobacter capsulatus*. The acid–base transition experiments described here show that ATP synthesis by chromatophores from *R. rubrum* can be driven by an artificially generated pH gradient. Our experiments with chromatophores as well as with cyanobacterial vesicles clearly indicate that the degree of coupling between ATP synthesis (or

hydrolysis) and H^+ translocation depends on the conditions under which the proton pump has to work, as has been already observed by Pietrobon et al. [30]. However, in this case a real mechanistic change in H^+/ATP was observed. The possibility of ATP synthesis under low-energy conditions is already discussed in [8]. As reported recently [31], the H^+/e^- ratio of cytochrome *o* from *E. coli* has also been found to be pH-dependent. This H^+/e^- ratio decreased at more alkaline pH values. Although the membrane exhibited a higher proton permeability at high pH, it was probable that there was a change in the proton-pumping stoichiometry.

To establish the role of protons during the catalytic process at the molecular level, a combination of results from biochemical and molecular biological studies is appropriate. Molecular biological studies with *E. coli* [3] indicated the involvement of the *a* and *c* subunits in proton-pumping activity, and the proton pathway is presumably formed by the fourth membrane-spanning helix of subunit *a* and the C-terminal part of subunit *c* [32]. Since the nucleotide sequences coding for the subunits of the ATP synthase from *Synechococcus* 6716 have been determined [33], the primary structure of these subunits could be derived and some amino acid sequences have also been partly determined [33]. The DNA sequence of a gene cluster coding for subunits of the F_0 part of ATP synthase from *R. rubrum* has been described [4] and the corresponding protein sequences have also been derived from the DNA sequence and partly determined directly. After compiling the sequences for 15 different *a* subunits from the SwissProt database, an alignment was performed with the CLUSTAL V program [34]. For the *a* subunits from *Synechococcus* 6716 and *R. rubrum*, only 23% sequence similarity was found. Although the comparison of primary structures can show large variations in subunits from different species, the secondary and tertiary structure predictions and the distribution of hydrophobic and hydrophilic amino acids can still be quite similar, which indicates a similar function for such subunits (see [35]). When the correct protein sequences are known (as for subunit *c*), the topology of membrane proteins can be predicted quite accurately [36]. Consensus exists about the alignment of subunit *c* sequences, where two transmembrane helices are linked by a conserved hydrophilic sequence containing proline [3]. The number and location of the membrane-spanning segments in subunit *a* are more difficult to determine, and different topographies have been proposed, containing five, six, seven or eight membrane spans [37]. In agreement with [38], we consider that it is most probable that only five hydrophobic stretches will form transmembrane helices. Figure 3 represents alternative alignments of subunits *a* and *c* that have been refined manually. According to these proposed alignments, the ATP synthase from *R. rubrum* seems to have less (or oppositely) charged amino acid residues in the transition region between the membrane part and the peripheral part of the complex. The difference in the number of charged amino acid residues in this part of the enzyme between the two prokaryotes might affect the proton-translocation stoichiometry due to protonation and/or salt-bridge formation. Furthermore, the number of glycine residues in the sequences coding for the *a* and *c* subunits of *Synechococcus* 6716 and *R. rubrum* were found to be similar. However, only a few glycine residues appear to be equivalently located. Noumi et al. [39] found for the yeast vacuolar $H^+/ATPase$ that amino acids with exchangeable protons can be replaced by glycine residues, and these investigators have previously suggested that the small and relatively hydrophilic glycine residues participate in proton conductance.

The differences in amino acid sequence do not appear to be related to the difference in physiological growth temperatures of

Figure 3 Proposed alignment of the amino acid sequences of the *a* (a) and *c* (b) subunits of the ATP synthases from *Synechococcus* 6716 (*S. 6716*) and from *R. rubrum*.

Charged amino acid residues are given in bold. Extra or oppositely charged residues of the *Synechococcus* 6716 ATP synthase are indicated by an asterisk (*). The broken lines between the sequences indicate the putative transmembrane segments.

the two organisms. Since *Synechococcus* 6716 can also grow at 35 °C (instead of 50 °C) it is not actually an obligate thermophilic organism. Furthermore, the temperature dependence of isolated and reconstituted chloroplast ATP synthase was found to be the same as that of isolated and reconstituted *Synechococcus* 6716 ATP synthase; both enzymes showed optimal activity at 50 °C [8]. Therefore, the differences in the amino acid sequence of the F_0 subunits of *Synechococcus* 6716 and of *R. rubrum* may serve to establish different pH-regulated sites in the protein complex in the vicinity of the membrane-medium interface.

Ultimately, we wish to obtain information on the residues that are specifically involved in the regulation of H^+ transport, since structure-function relations can be clarified by the mutagenesis of essential amino acids. However, site-directed mutagenesis of the H^+ -ATP synthase turns out to be a more difficult approach than was first thought [40], for example, since the *ATP* (or *UNC*) genes are essential, it has not yet been possible to grow a cyanobacterial ATP-synthase mutant that is modified in an essential amino acid for more than a few divisions.

This work is supported in part by the Foundation for Biophysics, with financial aid from the Netherlands Organization for Scientific Research (NWO). The authors thank Dr. K. Krab for fruitful discussions, Mr. A. P. N. de Boer for his help in compiling and aligning the sequences and Mr. H. A. van der Stroom for his assistance in cultivating *R. rubrum*.

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