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ATP synthesis by ATPase proteoliposomes from the thermophilic cyanobacterium *Synechococcus* 6716 by ionophore-induced electric potentials and proton gradients

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ATP synthesis driven by low pre-established electric potentials and pH gradients is studied in large ATPase proteoliposomes, prepared from the ATPase complex and native lipids from the thermophilic cyanobacterium *Synechococcus* 6716. Electric potentials and pH gradients were achieved by valinomycin and nigericin, respectively, in the presence of a K^+ gradient across the membrane. External base-pulses were also applied. In this system ATP synthesis driven by valinomycin-induced K^+ influx, nigericin-induced internal acidification and by external base-pulses is demonstrated. Electric potentials and pH gradients of equivalent size lead to roughly similar ATP synthesis activities. ATP synthesis is optimal at 80–100 nM valinomycin and at 0.75–1 μM nigericin at the proper pre-set ion gradients. Uncoupler and DCCD inhibit ATP synthesis. Prior activation of the complex by thiol agents or trypsin was not required for synthesis activity. The ATP synthesis rate increases with the size of electric potential or pH gradient. The threshold value of the electrochemical gradient for significant ATP synthesis is about 30 mV. ATP production proceeds for more than 60 min. The generation of ionophore-induced electric potentials and pH gradients have been followed by oxonol VI and intraliposomal Neutral red, respectively. The extent of the absorbance changes of both probes is proportional to the size of electric potential or pH gradient. Ionophore-induced oxonol VI and Neutral red responses are stable for at least 30 min. The results are discussed in terms of membrane permeability and vesicle size.

Introduction

There are strong indications that in photophosphorylation and oxidative phosphorylation an electrochemical proton gradient (consisting of an electric potential and a pH gradient) across the membrane that contains the phosphorylating system plays an important role. Such a gradient may drive ATP synthesis and is generated by ATP hydrolysis. Therefore, the application of pre-established electric potential and/or pH gradient is a useful tool to study the kinetics of ATP synthesis (cf. Ref. 1 for review, and Refs. 2, 3).

In this type of experiment with chloroplasts, ATP mainly has been produced by acid-base transition [4], frequently combined with a valinomycin-induced potassium ion diffusion potential (inside positive) [5–8]. In mitochondria and submitochondrial particles ATP synthesis has been obtained by a diffusion potential (matrix side

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Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; F_1, the water-soluble part of the ATPase complex; F_0, the membrane-bound part of the ATPase complex; oxonol VI, bis(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol; S-13,5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide.

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negative) [9–11]. Few studies deal with ATP synthesis induced by only an electric potential with chloroplasts (e.g., Ref. 6) or ATP synthesis induced by an outside acid-pulse with mitochondria (e.g., Ref. 12).

ATP synthesis can also be demonstrated in proteoliposomes reconstituted with ATPase complexes from different origin [13–18], but with the exception of the system derived from the thermophile PS 3 [13,19], rates of ATP synthesis are very low (see Refs. 19 and 20 for reviews).

Although it has been well established that bulk-to-bulk gradients are capable of inducing the synthesis of 'sufficient' amounts of ATP, in agreement with the chemi-osmotic theory of Mitchell [21], the minimal size of the applied electrochemical potential gradient, required to initiate significant ATP synthesis, usually referred to as 'threshold value', is generally found to be rather high [4,5,7,18]. For chloroplasts [4,5,7,22] and chloroplast ATPase proteoliposomes [18] this threshold was estimated to be 150–200 mV in total (corresponding to about 2.5–3.5 pH units). The addition of activating agents dithioerythritol or dithiothreitol lowers the threshold value by about 1 pH unit [23,24]. The threshold value is thought to originate from a supposed sigmoidal relationship between the applied electrochemical gradient and the ATP synthesis rate [2].

In this paper, we report on ATP synthesis activities of ATPase proteoliposomes from the thermophilic cyanobacterium *Synechococcus* 6716 induced by either pre-established electric potentials or by pH gradients with sizes far below the threshold value for chloroplasts. These ATPase proteoliposomes, prepared by dialysis, are large (200–400 nm) and were shown to be well-coupled, with low permeability for ions [25–27]. Electric potentials and pH gradients are induced by the ionophores valinomycin and nigericin, respectively, in the presence of appropriate K^+ gradients. The data are compared with ATP synthesis activities induced by base-pulses, commonly used in studies with chloroplasts. The generation of electric potentials and pH gradients has been followed by the use of optical probes. The relation between the size of the electrochemical gradient, the extent of the probe response and the rate of ATP synthesis is also discussed concerning the nature of the threshold value.

Materials and Methods

Synechococcus 6716 was grown at 50°C as described by Lubberding et al. [28].

Lipids and ATPase complex were isolated from this strain as described before [27,29]. The 35–50% saturated ammonium sulfate fraction (P_{35-50}) was dialyzed [26] and used without further purification.

ATPase proteoliposomes from native lipids were prepared by dialysis and collected by centrifugation at 50°C according to Van Walraven et al. [25]. The reconstitution medium consisted of 10 mM Na-Tricine, 2.5 mM $MgCl_2$, and 1 mM dithioerythritol or dithiothreitol, the KCl concentration and pH are given in the figure legends.

For Neutral red experiments, reconstitutions were carried out as in Ref. 25 in the media described in the legends to Figs. 4 and 5. Lipid concentration for reconstitution was 10 $mg \cdot ml^{-1}$; ATPase complex was reconstituted in a protein-to-lipid ratio of 0.02 (w/w). Proteoliposomes were stored above 40°C and were used within 24 h. Except for Neutral red experiments (see legends to Figs, 4 and 5) as outside medium reconstitution medium was used with KCl as indicated. All experiments were carried out at 50°C. For ATP synthesis experiments final lipid concentration was 1 $mg \cdot ml^{-1}$. When ATP synthesis was driven by ionophore-induced $\Delta\psi$ or ΔpH, the proteoliposomes were preincubated for 5 min with the ionophore before starting the synthesis reaction. In all cases, ATP synthesis was started by the addition of 1 mM ADP and 2 mM potassium phosphate. ATP synthesis was followed for 60 min and the synthesis rate was calculated.

Oxonol VI and Neutral red absorbance changes were measured with an Aminco DW-2a spectrophotometer equipped with thermostatically controlled multi-purpose cuvette [30]. For probe experiments final lipid concentration was 0.25 $mg \cdot ml^{-1}$, unless otherwise indicated.

Protein concentration was determined according to Bradford [31]. ATP content in samples was determined by two methods; the luciferine-luciferase method according to Larsson and Olsson [32] in a laboratory-built luminometer, and the hexokinase/ glucose-6-phosphate dehydrogenase method according to Bergmeyer [33]. After every assay a standard amount of ATP was added.

Luciferine, luciferase and valinomycin were purchased from Boehringer (Mannheim, F.R.G.), cholic acid, DCCD, dithioerythritol, dithiothreitol and Neutral red from Sigma (St. Louis, Mo., U.S.A.), sodium cholate from ICN Pharmaceuticals (New York, U.S.A.), and nigericin and octylglucoside (*n*-octyl-β-D-glucopyranoside) from Calbiochem (La Jolla, U.S.A.). S-13 was kindly donated by Dr. P.C. Hamm (Monsanto Co., St. Louis, Mo. U.S.A.). Oxonol VI was synthesized and donated by Dr. W. Hanstein (Ruhr-Universität Bochum, F.R.G.). All other reagents were of analytical grade.

Results

In previous work [25–27] it was demonstrated that proteoliposomes consisting of the ATPase complex and natural lipids from *Synechococcus* 6716 are well-coupled when prepared by dialysis. Coupling quality was tested by uncoupler (S-13) stimulation of ATP hydrolysis activity which is usually 2–4-fold but can be increased to more than 10-fold when all procedures (including storage) are carried out above 40°C (results not shown).

Fig. 1 illustrates the different cases for the occurrence or absence of ATP synthesis induced by the ionophores valinomycin or nigericin in the presence of different K^+ gradients. The ATPase complex is expected to be mainly incorporated with the F_1 part protruding from the outside (cf. discussion in Ref. 20). Thus, ATP synthesis may only occur when an electric potential (outside negative) is generated by valinomycin-induced K^+ influx, or when an internal acidification of the liposomes is generated by nigericin-induced K^+-H^+ exchange. It is noteworthy that in these two cases the required K^+ gradients are opposite, which makes a liposome preparation very suitable for this type of experiment, as the internal K^+ concentration may easily be varied. The size of the resulting electric potential ($\Delta\psi$) or pH gradient (ΔpH) may be calculated from the initial K^+ gradient by the Nernst equation (however, see Ref. 34). At 50°C a concentration difference of a factor 10 corresponds to about 65 mV.

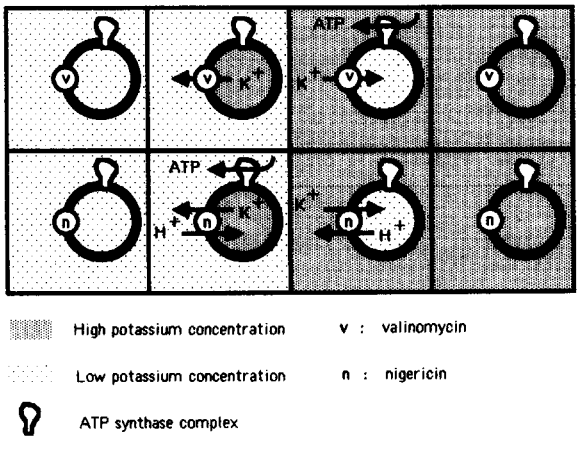

Fig. 1. The use of a K^+ gradient to drive ATP synthesis by ATPase proteoliposomes. Utilization of a K^+ gradient is induced by addition of valinomycin (upper row) or nigericin (lower row). Indicated are the situations in which ATP synthesis is expected.

Fig. 2 shows two typical examples of ATP synthesis experiments carried out under the conditions mentioned above. In the experiment of Fig. 2A, ATP synthesis is observed driven by a valinomycin-induced $\Delta\psi$ of 65 or 85 mV. However, also with a 'reversed' gradient ($\Delta\psi = -45$ mV) ATP production is observed. The experiments in Fig. 2B show that the latter ATP synthesis activity can be removed by centrifugation. Moreover, such an activity does not require the presence of phosphate (not shown), and hence is due to adenylate kinase activity. Also shown in Fig. 2B is nigericin-induced ATP synthesis driven by a reversed KCl gradient corresponding to a ΔpH of 1.

The ATP synthesis rates from a number of this type of experiment are listed in Table 1. Indeed, ATP synthesis is only observed as predicted in Fig. 1. The ATP synthesis activites induced by different components of the electrochemical gradient of equivalent size are quite similar. At higher $\Delta\psi$ or ΔpH the synthesis rates increase. Significant ATP synthesis is induced even at a $\Delta\psi$ of 45 mV or an equivalent ΔpH. In Table I, the results of three different series of experiments are given. Due to the long duration of each experiment not all controls can be done in the same series. Adenylate kinase activity is very variable; therefore it is advisable to remove this activity by collecting the proteoliposomes by centrifugation (as in Fig. 2B and in Expts. 2 and 3 given in Table I). In Expt. 1,

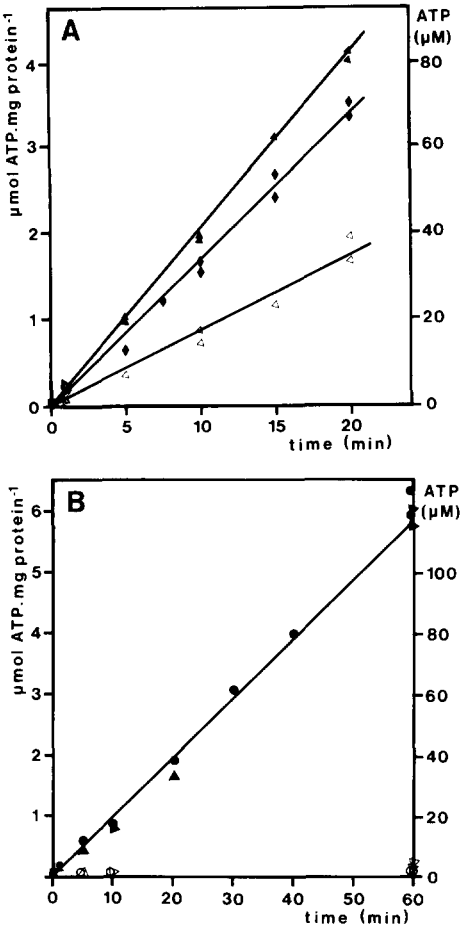

Fig. 2. Time-dependence of ionophore-induced ATP synthesis by ATPase proteoliposomes in the presence of a K^+ gradient. The experimental conditions are given in Materials and Methods and in the legend of Table I. (A) Not centrifuged proteoliposomes; valinomycin-induced $\Delta\psi$ of -45 mV (△), 65 mV (◆) and 85 mV (▲). (B) Centrifuged proteoliposomes; valinomycin-induced $\Delta\psi$ of -65 mV (△) and 65 mV (▲); nigericin-induced ΔpH of -1 (○) and 1 (●). Initial ATP concentration was less than 1 μM.

adenylate kinase activity of 80 (valinomycin experiments) or 11 (nigericin experiments) nmol ATP · min^{-1} · mg $protein^{-1}$ was not removed; the data in Table I have been corrected for this. Isolated and not-reconstituted ATPase complex shows no ionophore-induced ATP synthesis under any circumstances (data not shown). 24 hour old proteoliposomes show 30% loss of activity. As expected, ATP synthesis is affected by the uncoupler S-13 and by DCCD. Both ΔpH- and $\Delta\psi$-induced ATP synthesis are inhibited 50–60% by 1 μM S-13. ATP synthesis is inhibited almost completely at higher S-13 concentrations (1.5–2 μM) (not shown). The DCCD inhibition is 90–100% at high concentrations (50 μM) and is reached only after preincubation of the proteoliposomes with the inhibitor for about 20 min. The optimal valinomycin concentration for ATP synthesis (80–100 nM) is relatively low, compared to concentrations used by others. Effects of different valinomycin concentrations have also been shown in a previous paper [26] where the generation of a pH gradient by ATP hydrolysis was stimulated by low concentrations of valinomycin (10–50 nM), while at high concentrations (above 1 μM) the pH gradient became transient and rapidly collapsed again. For ΔpH-driven ATP synthesis nigericin must be added at 0.75–1 μM. The rate of nigericin-induced ATP synthesis is only slightly less than the rate of ATP synthesis driven by a base-pulse. Ionophore-induced ATP synthesis continues over an extremely long period, at least 60 min. Activation by trypsin treatment, which is absolutely necessary for induction of ATP hydrolysis and $^{32}P_i$-ATP exchange [25,26,29], was not necessary for ATP synthesis.

The generation of valinomycin-induced $\Delta\psi$ and nigericin-induced ΔpH was studied with the use of added oxonol VI and intraliposomal Neutral red, respectively. These two probes were succesfully used to monitor the components of the electrochemical gradient induced by ATP hydrolysis [25,26].

In Fig. 3 the extent of the absorbance change of oxonol VI after ionophore addition is plotted as a function of the initial ion gradient. Both valinomycin-induced K^+ diffusion potentials (closed symbols) and proton diffusion potentials (open symbols) are shown. In the latter case an initial pH difference between internal and external medium is relaxed by the uncoupler S-13, here used as a proton-ionophore. The oxonol VI responses are linearly related to the extent of the initial ion gradient ($\Delta\log[K^+]$ or ΔpH).

We have demonstrated recently [25,26] that Neutral red can be used within a distinct pH range for intraliposomal pH measurements. Internal pH changes induced by the addition of nigericin in the presence of a K^+ gradient have been followed by

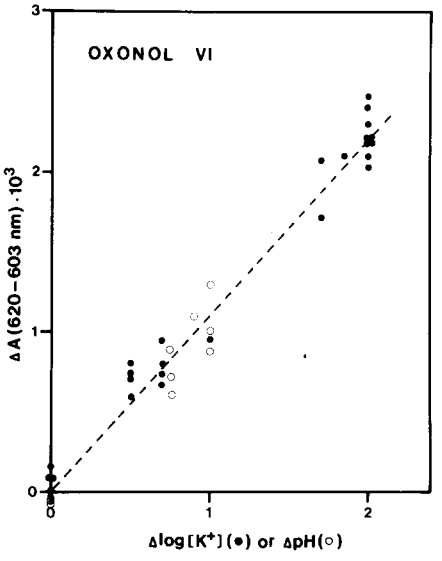

Fig. 3. Dependence of ionophore-induced oxonol VI absorbance change on the size of the initial ion gradient. K^+ diffusion potentials (●) were induced by addition of 100 nM valinomycin to a suspension of proteoliposomes with 10 mM internal K^+ at pH 7.5. Osmolarity was kept constant with LiCl. Proton diffusion potentials (○) were induced by addition of 250 nM S-13 to proteoliposomes with an internal pH of 6.5, and 10 mM internal and external KCl. Oxonol VI concentration was 0.5 μM.

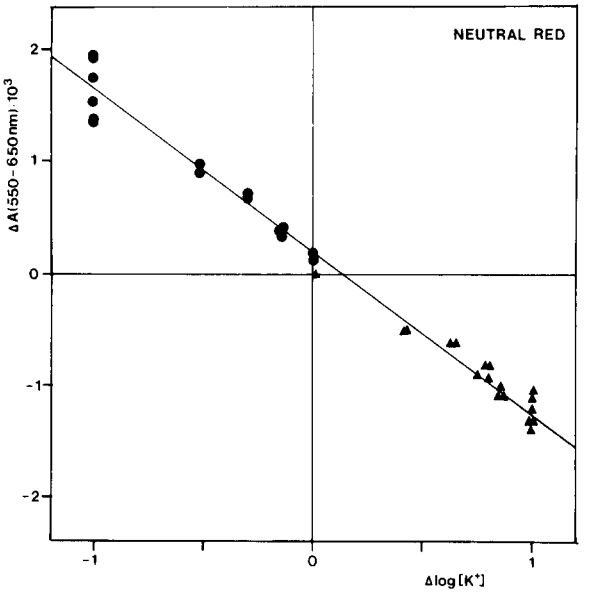

Fig. 4. Dependence of nigericin-induced absorbance change of Neutral red on initial K^+ gradient. Internal pH changes were induced by addition of 500 nM nigericin to proteoliposomes with inside medium consisting of 1 mM Na-Tricine, 2.5 mM $MgCl_2$, 10 μM Neutral red and either 100 mM KCl (●) or 10 mM KCl (▲). Osmolarity was kept constant with choline chloride, KCl plus choline chloride was 100 mM. The outside medium was free of Neutral red and was buffered with 10 mM Hepes. Apart from the difference in KCl and choline chloride concentration, the further composition of the outside medium was equal to the composition of the inside medium. Starting pH was 6.5.

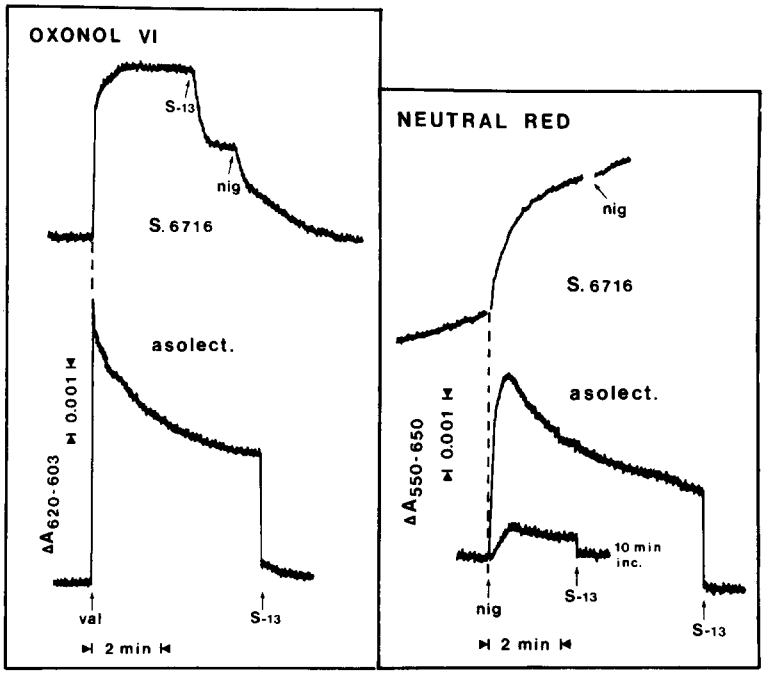

Fig. 5. Time-courses of valinomycin-induced oxonol VI response and nigericin-induced intraliposomal Neutral red response in proteoliposomes prepared with *Synechococcus* 6716 lipids or asolectin. Conditions were as described in the legends to Figs. 2 and 3 except that the asolectin liposomes were present at 0.5 $mg \cdot ml^{-1}$ and were measured at 20°C. In 'oxonol VI' the external KCl concentration was 1 M, corresponding to a $\Delta\psi$ of 130 mV. In 'Neutral red' the external KCl concentration was 10 mM (corresponding to a ΔpH of 1) in the experiment with the *Synechococcus* 6716 proteoliposomes, and 1 mM (corresponding to a ΔpH of 2) in the experiment with the asolectin proteoliposomes. S-13 concentration was 1 μM. Val, valinomycin; nig, nigericin.

TABLE I

Rates of $\Delta\psi$- and ΔpH-driven ATP synthesis in ATPase proteoliposomes. $\Delta\psi$ is defined as inside minus outside; ΔpH as outside minus inside. Internal K^+ concentration was 10 mM in valinomycin and base-pulse experiments and 100 mM in nigericin experiments. In base-pulse experiments initial pH was 7.2, in all other cases 7.5. See Materials and Methods for further details (–, not determined).

Pre-established Electrochemical gradient		Perturbation	ATP synthesis rate (nmol/min per mg protein)		
$\Delta\psi$ (mV)	ΔpH		Expt. 1	Expt. 2	Expt. 3
		Valinomycin (nM)			
−65		100	0	0	2
0		100	5	0	2
45		100	20	–	–
65		100	80	99	99
85		100	120	–	–
65		40	–	–	34
65		800	–	–	80
65		100 + S-13 (1 μM)	–	59	–
65		100 + DCCD (7 μM)	–	–	70
65		100 + DCCD (50 μM)	0	0	0
		Nigericin (μM)			
	−1.00	1	0	0	0
	0.00	1	0	0	0
	0.65	1	59	–	–
	1.00	1	103	100	108
	1.00	1 + 24 hour old	–	–	70
	1.00	1 + S-13 (1 μM)	–	–	40
	1.00	1 + DCCD (50 μM)	6	0	0
		Base pulse			
	0.00	–	–	0	0
	1.00	+	–	134	131
	1.50	+	–	266	214

Neutral red, trapped inside the liposomes during reconstitution. The results are given in Fig. 4. The observed absorbance change of Neutral red is proportional to the initial K^+ gradient, and thus to the calculated ΔpH at both positive and negative pH changes.

The time-dependences of the oxonol VI and Neutral red responses are given in Fig. 5. The responses in proteoliposomes prepared with native lipids are compared with the responses in proteoliposomes prepared according to the same procedure with asolectin. Asolectin (a mixture of soybean phospholipids) is used by others in most studies on ATP synthesis by reconstituted ATPase proteoliposomes. In proteoliposomes prepared from native lipids, both the oxonol VI response after valinomycin addition and the Neutral red response after nigericin addition, develop in 2–5 min, and remain stable for at least 30 min (not shown). In asolectin proteoliposomes, the signals develop quickly and decrease to a certain level immediately thereafter. In this latter type of liposome, preincubation with the applied gradient for 10 min before ionophore addition results in a drastically decreased probe response, which shows that dissipation of the initial gradient is much faster than in the native lipids. Both probe responses are sensitive to S-13. The effect of S-13 on the oxonol VI response in *Synechococcus* 6716 proteoliposomes is small, excess valinomycin also decreases the oxonol VI response (not shown). A second nigericin addition causes no additional Neutral red response.

The stability of the probe responses in the proteoliposomes prepared from lipids from *Synechococcus* 6716 indicates that these proteolipo-

somes are extremely impermeable for ions, in contrast to proteoliposomes prepared from asolectin. Similar results have been obtained for liposomes without incorporated protein (cf. accompanying paper Ref. 34).

Discussion

The results presented show that ATPase proteoliposomes from the thermophilic cyanobacterium *Synechococcus* 6716 can produce ATP at 50°C driven by either $\Delta\psi$ or ΔpH. This ATP synthesis activity is not due to adenylate kinase activity. The electric potential ($\Delta\psi$) is established by addition of valinomycin in the presence of a K^+ gradient, and ΔpH may be applied either by a base-pulse to the outside medium, or by addition of nigericin in the presence of a K^+ gradient. In spite of various attempts with chloroplasts, (cf. Refs. 1 and 2 for review) nigericin-induced ATP synthesis has not to our knowledge been demonstrated before. Nigericin is a common tool for dissipation of proton gradients rather than for its generation and subsequent ATP synthesis. Proteoliposomes can easily be loaded with KCl, which offers a great advantage over intact organelle systems. In the latter, the internal K^+ concentration can only be roughly estimated.

The comparison between our experimental system with ATPase proteoliposomes prepared from asolectin shows that the use of the native lipids of the thermophilic cyanobacterium (for composition, see Ref. 27) leads to an extremely impermeable membrane. This follows from the stability of the ionophore-induced $\Delta\psi$ and internal acidification. In addition to this, it is essential to keep preparations above 40°C. This leads to a spectacular uncoupler stimulation of ATP hydrolysis activity (a factor of 10 or more, compare Ref. 20) and is required for ATP production with the reported properties.

An important difference between this type of proteoliposome and other systems (see Refs. 1–3 for review) is the extreme stability in time of ATP synthesis (at least 60 min) apparently also related to the low permeability of the proteoliposome membrane. Asolectin proteoliposomes reconstituted from chloroplast ATPase behave like chloroplasts with regard to ATP synthesis [17] and produce ATP for less than 1 s upon establishment of an electrochemical gradient. With the ATPase complex from the thermophilic bacterium PS III reconstituted with natural lipids, Sone et al. [13] observed ATP production for about 30 s. The longest ATP synthesis duration reported so far has been obtained with mitochondria [10], which synthesize ATP for some minutes in the presence of a valinomycin-induced ion diffusion potential.

A second striking difference between ATP synthesis in our system and in other systems is the small electrochemical potential gradient which is sufficient here to observe ATP synthesis (e.g., a 'threshold value' for $\Delta\psi$ of about 30 mV). A very low threshold value will result in prolonged ATP synthesis, since the electrochemical potential gradient can be consumed to the hilt. In chloroplasts and in proteoliposomes reconstituted from chloroplast ATPase complex and asolectin, there is a threshold value for ATP synthesis of 2.5–3.5 pH units, corresponding to 150–200 mV [4,5,7,18,22]. As already mentioned in the introduction, this value of the electrochemical potential difference is usually reached by a combination of an acid-base transition and valinomycin-induced K^+ ion diffusion potential. There are several factors that may determine the size of the threshold. In the following we will discuss some of these, and their possible significance for the results of our experiments, and the results obtained in chloroplast ATPase systems by others.

Firstly, it has been proposed that activation of the ATPase complex requires energy [35,36]. When chloroplast ATPase has been activated with a thiol agent such as dithioerythritol or dithiothreitol, the threshold is diminished to about 100 mV (1.5 pH unit) [23,24]. We consider it unlikely that a similar explanation may be given for the (small) threshold observed in ATPase proteoliposomes prepared from ATPase complex and native lipids from *Synechococcus* 6716, since we showed here that artificial activation of the complex is not required for ATP synthesis in this system. This does not mean that the ATPase activity does not depend on conformational triggering. Such a triggering is believed to be required not only for ATP hydrolysis but also for ATP synthesis; it has been suggested [37] that such triggering of ATP synthesis results from a certain electric potential value and flash-in-

duced ATP synthesis does not require added thiol agents.

A second factor contributing to the threshold level may be the internal volume, or rather, the internal amount of the relevant ion species [34]. We have shown that a small internal volume or a small internal K^+ concentration of liposomes leads to a threshold value in the valinomycin-induced oxonol VI response, and have discussed the possibility that the same phenomenon may apply to ATP synthesis [34]. It is possible that this phenomenon also contributes to the large remaining threshold (about 100 mV) in chloroplasts, but this is not the case in our system. In our system the oxonol VI calibration curve (Fig. 3) goes through the origin, which is to be expected on the basis of the size of the proteoliposomes (diameter 200–400 nm, [25]) and the internal K^+ concentration (10 mM). There is supporting evidence that increasing the chloroplast internal volume leads to a lower threshold for ATP synthesis ([6], see also discussion in Ref. 34). For the internal volume effect, a basal electric potential difference (positive outside) in the chloroplasts is required [34]. Such a basal potential difference may be caused by a difference in surface charge between the two sides of the thylakoid membrane.

Thirdly, thermodynamics may put a limit to the amount of ATP that may be synthesized at any given level of $\Delta\psi$ or ΔpH. The size of this limit is determined by the number of ions that have to move across the membrane to synthesize one molecule of ATP, and by the maximal phosphate potential that can be reached. We have the impression that the low $\Delta\psi$ or ΔpH that can drive ATP synthesis in our system requires the former to be rather high or the latter to be rather low. At present, this subject is being further investigated.

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