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ATP synthesis by F-type ATP synthase is obligatorily dependent on the transmembrane voltage

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ATP synthase is the universal enzyme that manufactures cellular ATP using the energy stored in a transmembrane ion gradient. This energy gradient has two components: the concentration difference (ΔpH or ΔpNa^+) and the electrical potential difference ΔΨ, which are thermodynamically equivalent. However, they are not kinetically equivalent, as the mitochondrial and bacterial ATP synthases require a transmembrane potential, ΔΨ, but the chloroplast enzyme has appeared to operate on ΔpH alone. Here we show that, contrary to the accepted wisdom, the 'acid bath' procedure used to study the chloroplast enzyme develops not only a ΔpH but also a membrane potential, and that this potential is essential for ATP synthesis. Thus, for the chloroplast and other ATP synthases, the membrane potential is the fundamental driving force for their normal operation. We discuss the biochemical reasons for this phenomenon and a model that is consistent with these new experimental facts.

Keywords: acid bath procedure/ATP synthase/chloroplasts/membrane potential

Introduction

The ultimate goal of energy metabolism in living cells is the production of ATP from ADP and inorganic phosphate. The majority of the ATP is produced by the enzyme ATP synthase, also called F_1F_0 ATP synthase, F-ATP synthase or F_1F_0 ATPase. This enzyme transforms energy from a transmembrane electrochemical gradient of protons, or in some cases Na^+ ions, into the phosphoric acid anhydride bond of ATP. Closely related ATP synthases are found in the bacterial cytoplasmic membrane, the inner membrane of mitochondria and the thylakoid membrane of chloroplasts. These enzymes consist of a membrane-embedded portion, F_0, which contains the ion translocation machinery, and the soluble F_1 moiety that harbours the nucleotide-binding sites in which ATP is synthesized or hydrolysed (for reviews, see Deckers-Hebestreit and Altendorf, 1996; Dimroth, 1997; Weber and Senior, 1997).

A great deal of experimental work on the structure and function of the ATP synthase is summarized schematically in Figure 1. The extramembraneous F_1 module has the subunit composition $\alpha_3\beta_3\gamma\delta\varepsilon$. The crystal structure of the $\alpha_3\beta_3\gamma$ subcomplex of F_1 (Abrahams *et al*., 1994) shows three α and three β subunits alternating at the periphery of a spherical structure with a centrally located γ subunit interacting asymmetrically with the three catalytic β subunits. This inherent asymmetry of F_1 correlates with Boyer's binding change mechanism, supporting the view that the binding changes in the β subunits are elicited by the rotation of the γ subunit (Boyer, 1993). Several recent studies have verified the ATP-driven rotation of the γ subunit within the $\alpha_3\beta_3$ headpiece (Duncan *et al*., 1995; Sabbert *et al*., 1996; Noyi *et al*. 1997), and it was also shown that subunit ε rotates with γ as a unit (Capaldi *et al*., 1996; Bulygin *et al*., 1998; Kato-Yamada *et al*., 1998). In the ATP synthesis mode, the rotation should be reversed, and it has to be driven by the downhill movement of the coupling ions through the F_0 sector.

The composition of F_0 from bacteria is $ab_2c_{9–12}$, and evidence from electron and atomic force microscopy indicates that the c subunits form a ring that is flanked at the periphery by the a and the two b subunits (Birkenhäger *et al*., 1995; Singh *et al*., 1996; Takeyasu *et al*., 1996). The a subunit is thought to fold through the membrane with five or six α-helical domains (Yamada *et al*., 1996; Jäger *et al*., 1998; Long *et al*., 1998; Valiyaveetil and Fillingame, 1998). The b subunits have an N-terminal membrane anchor and attach with their C-terminal domain via subunit δ to one α subunit of the $\alpha_3\beta_3$ hexagon (Lill *et al*., 1996; Ogilvie *et al*., 1997). Subunit c of *Escherichia coli* in chloroform/methanol/water packs into two helices that are supposed to be membrane-integral, connected by a short hydrophilic loop (Girvin *et al*., 1998). In contrast, the c subunit of *Propionigenium modestum* in dodecylsulfate micelles folds into four clearly defined α helices, suggesting that the N- and the C-terminal helices span the membrane, whereas the two additional helices and the interhelical connections are located cytoplasmatically (Matthey *et al*., 1999). This folding of the c subunits locates the Na^+-binding site at the membrane boundary, between the interhelical segments I/II and III/IV. Biochemical evidence showing free access of most of the c subunit-binding sites to Na^+ from the aqueous phase are perfectly in accord with this structure (Kaim *et al*., 1998).

In the model shown in Figure 1, the ATP synthase has the appearance of a counter-rotating rotor and stator comprising, respectively, $c_{9–12}\gamma\varepsilon$ and $ab_2\delta\alpha_3\beta_3$. Indeed, several lines of evidence with the ATP synthase of *P.modestum* strongly suggest a counter-rotation of the c and a subunit assemblies during translocation of the coupling Na^+ ions. (i) The c subunits carry a Na^+-binding site, with the side chains of Q32, E65 and S66 serving as ligands (Kaim *et al*., 1997). (ii) With an a subunit triple mutation, the coupling ion specificity of the ATP synthase changed from Na^+, Li^+ or H^+ (wild-type) to only Li^+ or H^+ (Kaim and Dimroth, 1998a). (iii) This enzyme occluded one Na^+ per mutant ATP synthase in an ATP-dependent fashion (Kaim *et al*., 1998). (iv) In the mutant enzyme with partially dicyclohexylcarbodiimide-modified

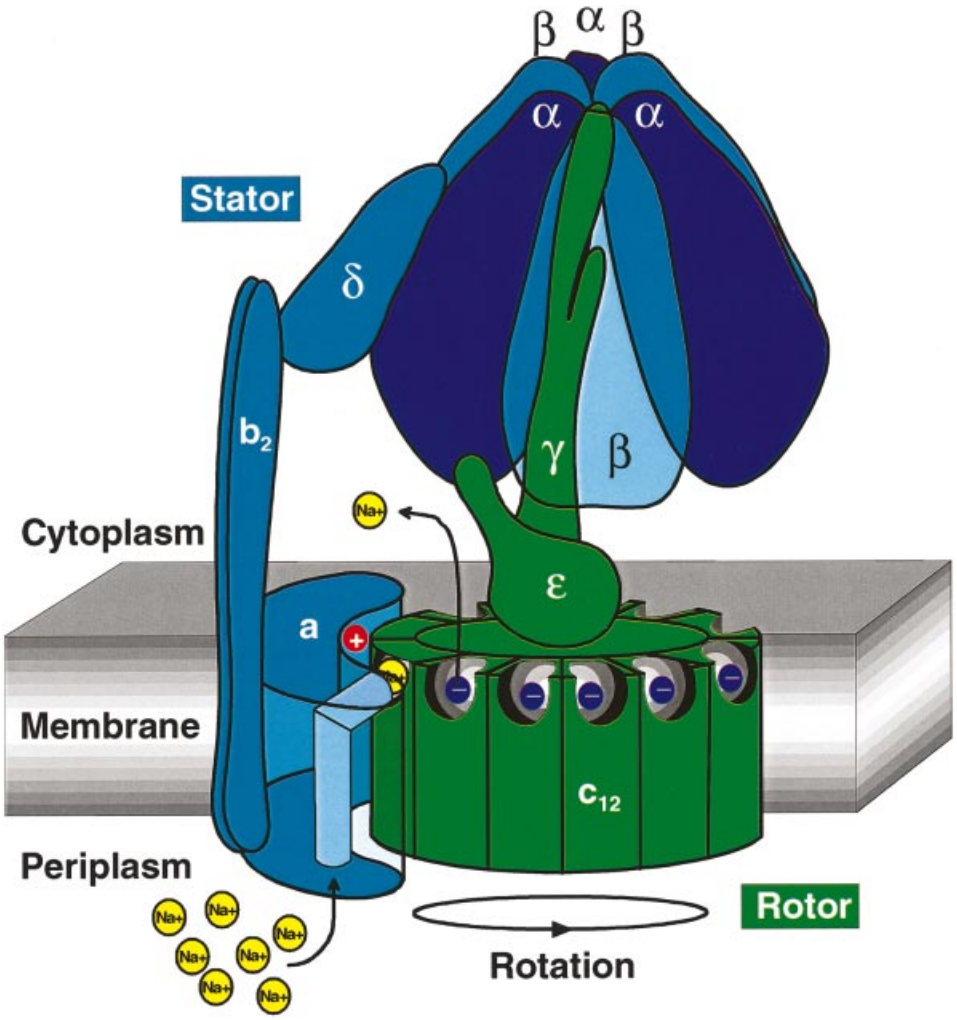

Fig. 1. Cartoon of the structure and function of the *P.modestum* ATP synthase. Schematic view of the enzyme consisting of the F_1 headpiece with the catalytic binding sites on the three β subunits, the F_0 motor module with the Na^+-binding sites on the c subunits and an Na^+-conducting channel within the a subunit. The rotor (green) comprises subunits $c_{9–12}\gamma\varepsilon$ and the stator (blue) comprises the $ab_2\alpha_3\beta_3\delta$ assembly. The pathway of Na^+ during ATP synthesis is from the periplasm through the a subunit stator channel onto an empty c subunit rotor site at the rotor–stator interface. The Na^+ ion dissociates from this site into the cytoplasm after the rotor has turned. The positive stator charge (aR227) that contributes in the torque-generating mechanism through electrostatic attraction of negatively charged empty rotor sites is indicated in red.

c subunits, Na^+ occlusion was impaired if ATP was added first and Na^+ second, but not if this order was reversed (Kaim and Dimroth, 1998b). (v) With the wild-type ATP synthase reconstituted into proteoliposomes, Na^+ ions were exchanged rapidly between the two compartments of the membrane (Kaim and Dimroth, 1998b). The exchange was abolished if the system switched from an idling into a torque-generating operation mode, either by the hydrolysis of ATP or with the driving force provided by an electric potential. A Na^+ concentration gradient, on the other hand, was unable to induce the switch. This intriguing result strongly suggests that the transmembrane voltage is indispensable for generation of the rotational torque required for ATP synthesis and contradicts the general opinion that $\Delta\Psi$ and ΔpH serve as kinetically equivalent driving forces for ATP production.

In a classical study, Jagendorf and Uribe (1966) reported ATP synthesis by chloroplasts in the dark after incubation with succinate buffer, pH 4.0, and dilution into Tris buffer, pH 8.0, and ascribed this to be driven entirely by the ΔpH. However, it has never been considered that this frequently cited 'succinate bath' procedure or 'acid bath procedure' could also establish a $\Delta\Psi$. We show here that this is the case and that phosphatidylcholine membranes are highly permeable for the succinate monoanion. As illustrated in Figure 2, this electrogenic charge movement develops a membrane potential in accordance with the concentration difference of the succinate anion that arises from the pH difference on the two sides of the membrane. We further demonstrate that the electric potential is essential for ATP formation by the chloroplast enzyme. The obligatory requirement for $\Delta\Psi$ for ATP production is therefore a common property of the ATP synthases from chloroplasts, mitochondria (Thayer and Hinkle, 1975) and bacteria (Kaim and Dimroth, 1998b,c).

Results

Generation of a membrane potential by acid–base transition depending on the nature of the (di) carboxylic acid

More than 30 years ago, Jagendorf and Uribe (1966) reported ATP synthesis by the chloroplast ATP synthase after applying the 'acid bath procedure', where a ΔpH of

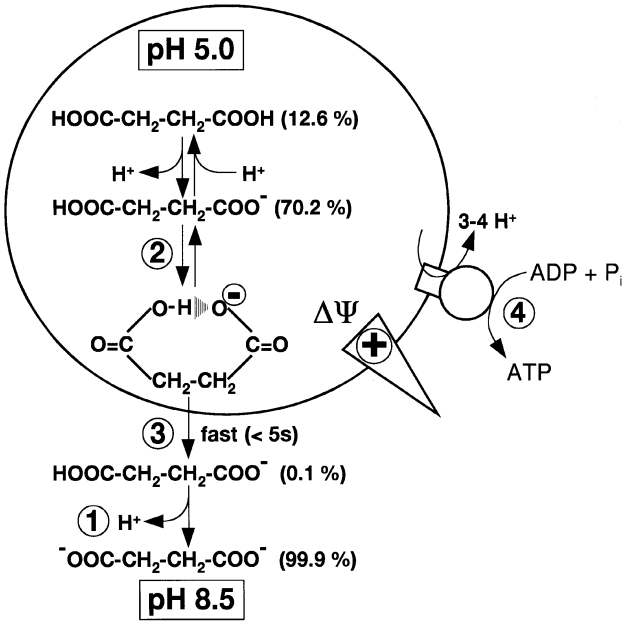

Fig. 2. Generation of a membrane potential by the 'acid bath procedure'. The procedure involves two consecutive steps, the acidic and the basic stage. During the acidic stage, the proteoliposomes containing 5 mM K^+-phosphate buffer, pH 7.2, are incubated with 100 mM succinate buffer, pH 5.0 (not shown). At this pH, 70.2% of the succinate is present as monoanion and 12.6% is in the undissociated form. As both succinate species are membrane permeable (see below), they will diffuse into the liposome interior in response to the concentration gradient. The membrane potential transiently generated by the uptake of the succinate monoanion collapses rapidly due to the proton permeability of the liposome membrane (see Figure 4). After 2 min at 25°C, the succinate has equilibrated between the outside and the inside, and the pH is 5.0 on both sides. The cartoon depicts the events occurring during the shift to the basic stage by 1:1 dilution into 100 mM glycylglycine buffer, pH 8.5. The pH jump shifts the external equilibrium towards the succinate dianion (99.9%), resulting in a large concentration gradient of the succinate monoanion across the membrane (1). Part of the succinate monoanion folds into a ring, where the negative charge is delocalized between the two carboxylic groups, and in this form it becomes membrane permeable (2). Consequently, it diffuses through the membrane from the inside to the outside, following its concentration gradient until the developing electric potential becomes equal to the concentration difference of the succinate monoanion on both sides (3). The membrane potential thus formed is essential for the synthesis of ATP by the chloroplast ATP synthase (4). Note that the membrane potential rapidly collapses by proton movements from the inside to the outside. The membrane permeability for protons is increased by both $\Delta\Psi$ and ΔpH.

~250 mV was thought to serve as the only driving force. We became intrigued by this procedure through recent experiments with the Na^+-translocating ATP synthase of *P.modestum*. Results indicated that the enzyme switched from an idling into a torque-generating operation mode only by applying $\Delta\Psi$ but not with ΔpNa^+ (Kaim and Dimroth, 1998b) (see Introduction). Surprisingly, when proteoliposomes containing this enzyme were subjected to the 'acid bath procedure', ATP formation was observed (Kaim and Dimroth, 1998b). The synthesis was strictly dependent on the presence of Na^+ (10 mM on either side) and was therefore certainly not energized by ΔpH. Hence, diffusion of a charged succinate species was expected to create an electric potential which provided the driving force for ATP production.

To validate this concept, we measured the membrane potential generated by the 'acid bath procedure' with different organic acids, using the lipophilic anion [^{14}C]thiocyanate as the membrane-permeable probe. The results in Table I show that potentials of ~124 mV were obtained with the dicarboxylic acids succinate, malonate, malate, oxaloacetate or maleinate. With glutarate, the potential dropped to 51 mV and, with oxalate, fumarate or the monocarboxylic acids acetate or propionate, the calculated potentials were in the range of only 10 mV. This value resembles those of controls in the absence of an acidic buffer or of liposomes whose internal lumen was acidified with MES buffer. Hence, the monoanions of certain dicarboxylic acids appear to be membrane-permeable, developing a Nernst potential in accordance with the concentration difference of the monoanionic species on both sides (see Figure 2 and Discussion). Upon correlating the chemical nature of the organic acids with their capacity to develop a membrane potential, the most striking difference exists between maleinate with a *cis* and fumarate with a *trans* double bond. As only maleinate creates the potential, an obvious interpretation is folding of appropriate dicarboxylates into a ring while traversing the membrane. The negative charge is thus spread to both carboxylic groups and the dielectric barrier for crossing the membrane decreases. In agreement with this idea is the formation of similar potentials with all dicarboxylic acids that can adopt a stable ring structure within the membrane (Table I), whereas no potential is induced by oxalate or by monocarboxylates, which cannot fold into an intramolecular ring. The inferior ability of glutarate to generate a membrane potential is likely to correlate with the decreased stability of its ring structure, apparently overcompensating the increase in hydrophobicity by adding an additional methylene group.

The membrane permeability of several ^{14}C-labelled dicarboxylic acids was also investigated by the uptake of radioactivity into liposomes. The results of Figure 3 indicate that malonate, succinate, maleinate or fumarate do not diffuse into the liposomes at pH 8.5, where both carboxylic groups are dissociated. Applying a K^+/valinomycin diffusion potential of 60 mV does not change the situation, obviously because the membrane is not permeable for the species with two negative charges. As expected, the electric potential of 60 mV elicited a 10-fold increase in the internal concentration of the probe [^{14}C]thiocyanate which was independent of the pH applied. Neither at pH 5.0 nor at pH 4.0 with or without an electric potential was fumarate uptake into the lumen of the liposomes observed, indicating that this compound in its mono- or dianionic form is membrane impermeable. At these two pH values, the other dicarboxylic acids tested traversed the membrane yielding internal concentrations that remained constant between 10 and 60 s. Upon applying a K^+/valinomycin diffusion potential of 60 mV, the internal concentration of malonate or maleinate increased ~10-fold at pH 5.0 and pH 4.0, which is expected since 83–98% of these dicarboxylic acids exist as the membrane-permeable monoanionic species. For succinate, however, this increase was considerably less, reflecting that at pH 4.0 only 38% of this compound is in the single protonated state (see Table I for pK_a values).

Transmembrane voltage as an obligatory driving force for ATP synthesis

Numerous reports on the energetics of ATP synthesis have appeared, particularly with the ATP synthase from chloro-

Table I. Membrane potentials generated by the 'acid bath procedure' with various dicarboxylic acids

Buffer	Formula	pK_{a1}	pK_{a2}	Membrane potential (mV)
Malonate	HOOC-CH_2-COOH	2.85	5.69	124 ± 3
Succinate	HOOC-CH_2-CH_2-COOH	4.16	5.61	124 ± 4
Malate	HOOC-HCOH-CH_2-COOH	3.40	5.11	123 ± 2
Oxaloacetate	HOOC-CO-CH_2-COOH	2.35	4.21	125 ± 4
Maleinate	HOOC-CH=CH-COOH (*cis*)	1.83	6.07	124 ± 5
Fumarate	HOOC-CH=CH-COOH (*trans*)	3.03	4.44	11 ± 3
Glutarate	HOOC-CH_2-CH_2-CH_2-COOH	4.31	5.41	51 ± 3
Oxalate	HOOC-COOH	1.23	4.19	9 ± 5
Acetate	HOOC-CH_3	4.75		11 ± 2
Propionate	HOOC-CH_2-CH_3	4.87		13 ± 5
Morpholinoethane sulfonic acid (MES)				8 ± 2
None				8 ± 2

The liposomes were incubated for 2 min with acidic buffer containing 100 mM of the (di) carboxylic acid, pH 5.0, diluted into 100 mM glycylglycine, pH 8.5, containing 34 μM [^{14}C]NaSCN (0.5 μCi), and the potential was calculated from the distribution of [^{14}C]SCN^- between both sides of the membrane as described in Materials and methods. The values obtained are the mean of four independent experiments, and the upper and lower ranges are indicated.

Fig. 3. ΔΨ-dependent uptake of several dicarboxylic acids or [^{14}C]SCN^- into liposomes. The buffer kept at pH 8.5 (**A**), pH 5.0 (**B**) or pH 4.0 (**C**) contained 2 mM of ^{14}C-labelled NaSCN (▲), 2 mM [^{14}C]maleinate (●), 2 mM [^{14}C]malonate (▼), 2 mM [^{14}C]succinate (■) or 2 mM [^{14}C]fumarate (◆). A K^+/valinomycin diffusion potential of 60 mV was induced by the addition of KCl (↓) and the uptake of the radioactivity was determined. For details, see Materials and methods.

plasts. From these studies, it was concluded that ΔΨ and ΔpH contribute kinetically equally to the synthesis of ATP so that each driving force is readily substitutable by the other when of the same size. For ΔpH generation in these experiments, the 'acid bath procedure' with succinate has been used almost exclusively. As outlined above, this method not only generates the desired ΔpH but also a ΔΨ of appreciable size. To avoid any interference with a membrane potential, a variety of earlier ATP synthesis experiments were conducted in the presence of K^+/valinomycin (Sone *et al*., 1977; Maloney and Schattschneider, 1980; Bokranz *et al*., 1985; Fischer *et al*., 1994; Possmayer and Gräber, 1994). Unfortunately, however, it was not quantitated whether these additions effectively clamped the potential to zero. Therefore, we measured the membrane potentials elicited during the acid–base transition in the presence of 200 mM KCl on both sides of the membrane and with various lipid:valinomycin ratios by the uptake of [^{14}C]thiocyanate into the liposomes. The results shown in Figure 4 indicate that in the absence of valinomycin a potential of ~140 mV develops instantaneously during the acid–base transition and then gradually declines. The size of the potential is in reasonable agreement with the Nernst potential calculated from the distribution of the succinate monoanion between the two sides of the membrane (see Discussion). The observed decline in the potential can be attributed to the efflux of protons which is driven by ΔpH and by ΔΨ. The permeability coefficient of protons through phosphatidylcholine membranes is 10^{-4}–10^{-8} cm/s (Perkins and Cafiso, 1987). Interestingly, a lipid:valinomycin ratio of 920:1 affected the Nernst potential only marginally and, even at a lipid:valinomycin ratio of 230:1, an electric potential of ~95 mV was developed. To clamp the potential to zero effectively, a lipid:valinomycin ratio of 92:1 was required. Hence, the membrane potential elicited by the acid–base transition was essentially unaffected by the lipid:valinomycin ratio of 850:1 used by Possmayer and Gräber (1994) and indisputably participated as a driving force for ATP synthesis. Thus, the membrane is highly permeable for the succinate monoanion, leading to an electric potential that can only be balanced by compensating K^+ fluxes in the presence of significant amounts of valinomycin.

The observations described above prompted us to measure the rate of ATP synthesis by the reconstituted ATP

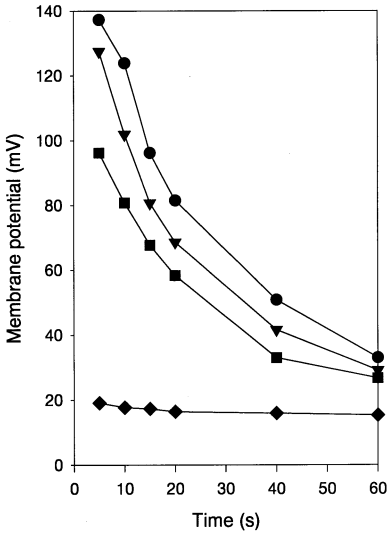

Fig. 4. Effect of various phospholipid:valinomycin ratios on the electric potential elicited by succinate diffusion. The uptake of [^{14}C]thiocyanate into liposomes was measured after applying the 'acid bath procedure'. Following incubation in succinate buffer, pH 5.0, the suspension was diluted 1:1 into Tricine buffer, pH 8.5, containing 34 μM [^{14}C]NaSCN with no valinomycin (●) or lipid:valinomycin ratios of 920:1 (▼), 230:1 (■) or 92:1 (◆). For details, see Materials and methods.

synthase from chloroplasts at $\Delta pH = 0$ and at $\Delta pH = 3.5$ in response to K^+/valinomycin diffusion potentials that were calculated by the Nernst equation to range from 0 to 60 mV. The results of Figure 5A show that the velocities follow titration curves with inflection points at ~35 mV. Importantly, there is no ATP synthesis activity of the chloroplast enzyme at $\Delta pH = 200$ mV in the absence of $\Delta\Psi$, and the contribution of $\Delta\Psi$ to the rate of ATP synthesis is far more important than that of the ΔpH. At potentials of 30 mV and below, the rate of ATP synthesis is only marginally increased by superimposing a ΔpH of 200 mV. The ΔpH effect increases with increasing potentials but, even at $\Delta\Psi =$ 60 mV, a superimposed ΔpH of 200 mV increases the velocity of ATP synthesis only by a factor of 1.6, far less than expected if ΔpH and $\Delta\Psi$ were kinetically equivalent driving forces as was concluded from previous studies (Fischer *et al*., 1994; Possmayer and Gräber, 1994).

Figure 5B shows the effect of K^+/valinomycin diffusion potentials ranging from 0 to 120 mV on the rate of ATP synthesis by the reconstituted *E.coli* ATP synthase. The ATP synthesis rates observed with this enzyme are ~60% lower than those obtained with the chloroplast ATP synthase. Up to $\Delta\Psi = 40$ mV, the velocity of ATP synthesis is very low and not significantly different, whether a ΔpH is additionally present or not. The midpoint potential is at ~80 mV and thus markedly higher than that of the chloroplast ATP synthase. Also similar to the results with this enzyme is an increasing effect of the superimposed ΔpH on the ATP synthesis rate as the potential is increased. At $\Delta\Psi = 120$ mV, the rate increase by superimposing a ΔpH of 200 mV is 1.5-fold and thus again far too low for a kinetic equivalence of these forces.

For further comparison, dependencies of ATP synthesis rates on $\Delta\Psi$ with or without a ΔpNa^+ of 77 mV by the *P.modestum* ATP synthase are shown in Figure 5C. With only $\Delta pNa^+ = 77$ mV, the enzyme is unable to synthesize ATP, and no significant activities are observed up to a $\Delta\Psi$ of 40 mV, irrespective of whether ΔpNa^+ was additionally present or not. The curves have inflection points at ~80 mV, and the effect of a superimposed ΔpNa^+ on the ATP synthesis rate is more pronounced at higher potentials. For instance, the rate increase by $\Delta pNa^+ = 77$ mV is 1.3-fold at $\Delta\Psi$ between 60 and 120 mV, indicating no kinetic equivalence of the two driving forces in promoting ATP synthesis.

In summary, these results show that the three ATP synthases investigated respond primarily to the electric potential. Without $\Delta\Psi$ or at low $\Delta\Psi$ values, the rates of ATP synthesis are either zero or very low and, with increasing $\Delta\Psi$, the increments of the ATP synthesis rates due to superimposed ΔpH (or ΔpNa^+) increase, but far less than expected if ΔpH was kinetically equivalent to $\Delta\Psi$ as the driving force for ATP synthesis.

In order to confirm the results obtained for the reconstituted chloroplast ATP synthase, acid–base transition experiments were performed with broken chloroplasts in the dark. After acidification with 20 mM succinate, pH 5.0, and 1:1 dilution of the thylakoid membrane suspension into 100 mM Tris buffer, pH 8.5, ATP synthesis immediately commenced and reached a maximum of 100 nmol of ATP per mg chlorophyll after 20 s (Figure 6A). Interestingly, if the basic buffer contained 2 mM of the membrane-permeable compound sodium tetraphenylboron, ATP synthesis was completely abolished. This could be explained if the membrane potential generated by the efflux of the monoanionic succinate species was destroyed by the counteracting influx of the tetraphenylboron anion. This conclusion was corroborated by monitoring in parallel experiments the size of the membrane potential at various times after the acid–base transition (Figure 6B). With succinate as the acidic buffer, a membrane potential of 110 mV was found 5 s after the acid–base transition that decreased to 10 mV after a further 15 s. In contrast, no membrane potential was generated if the tetraphenylboron anion was additionally present on the external side of the thylakoid membranes. With fumarate as the acidic buffer, the rate of ATP synthesis was approximately five times lower than with succinate and, at maximum, 30 nmol of ATP per mg chlorophyll were synthesized (Figure 6A). ATP synthesis was abolished in the presence of 2 mM sodium tetraphenylboron. As shown in Figure 6B, an initial membrane potential of 60 mV is generated in the 'acid bath procedure' with fumarate but not in the additional presence of the membrane-permeable anion tetraphenylboron. Hence, there is a clear correlation also in the thylakoids between ATP synthesis and the presence of a membrane potential. However, the capacity of thylakoids to develop a membrane potential and to synthesize ATP with fumarate as acidic buffer in the 'acid bath procedure' is not shared by liposomes (Table I) or proteoliposomes reconstituted with purified ATP synthases (Kaim and Dimroth, 1998b,c). The difference may be due to the presence of an electrogenic fumarate transporter in the thylakoid membrane which generates the potential during fumarate efflux.

Discussion

According to the chemiosmotic theory, the driving force for ATP synthesis is contributed by the transmembrane

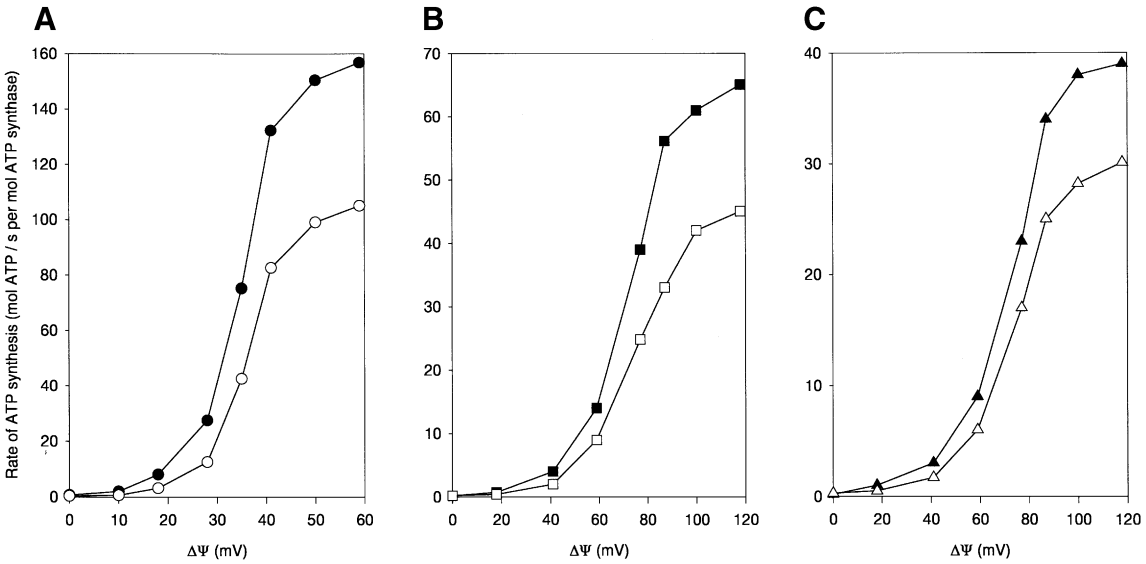

Fig. 5. Relative contribution of the electric potential ($\Delta\Psi$) and the H^+ or Na^+ concentration gradient (ΔpH; ΔpNa^+) to the rate of ATP formation by three different ATP synthases. (**A**) Reconstituted chloroplast ATP synthase. K^+/valinomycin diffusion potentials were applied in the absence (○) or presence (●) of ΔpH = 206 mV. (**B**) Reconstituted *E.coli* ATP synthase. K^+/valinomycin diffusion potentials were applied in the absence (□) or presence (■) of ΔpH = 206 mV. (**C**) Reconstituted *P.modestum* ATP synthase. K^+/valinomycin diffusion potentials were applied in the absence of ΔpNa^+ (5 mM NaCl present on either side of the membrane) (△) or in the presence of ΔpNa^+ = 77 mV (outside 5 mM NaCl, inside 100 mM NaCl) (▲). ATP was determined as described in Materials and methods. Please note that ATP synthesis at $\Delta\Psi$ = 0 was measured in the presence of valinomycin and 1 mM KCl on either side of the membrane in order to avoid interference with the generation of an electric potential through the movement of ions during ATP synthesis.

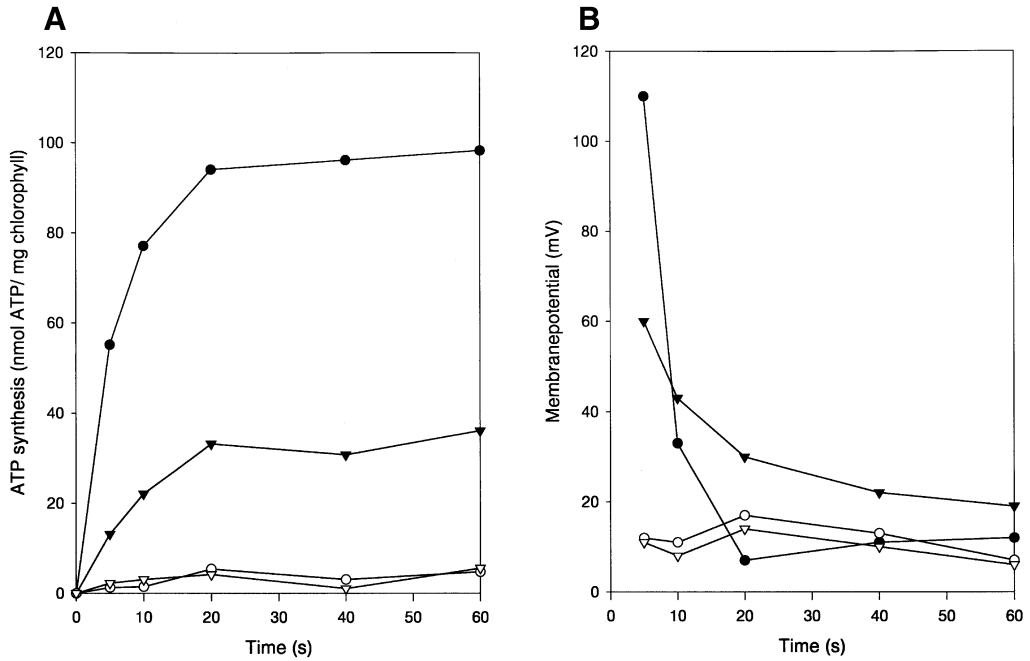

Fig. 6. ATP synthesis and electric potential ($\Delta\Psi$) generated by the 'acid bath procedure' in thylakoid membranes. (**A**) ATP synthesis by isolated thylakoid membranes. Thylakoid membranes were subjected to an acid–base transition as described in Materials and methods using 20 mM succinate (●) or 20 mM fumarate (▼), pH 5.0, as the acidic, and 100 mM Tris–HCl, pH 8.5, as the basic buffer. In control experiments, thylakoids equilibrated in the acid stage with succinate (○) or fumarate (▽) were subjected to the basic stage buffer containing, additionally, 2 mM sodium tetraphenylboron. (**B**) Electric potentials generated across thylakoid membranes by the 'acid bath procedure'. During the acid stage, thylakoids were loaded with 20 mM succinate (●, ○) or 20 mM fumarate (▼, ▽), pH 5.0. The suspensions subsequently were diluted 1:1 into 100 mM Tris–HCl buffer, pH 8.5, containing 23 μM [^{14}C]NaSCN (0.5 μCi) without (●, ▼) or with 2 mM sodium tetraphenylboron (○, ▽). The membrane potential was calculated from the uptake of [^{14}C]thiocyanate as described in Materials and methods.

electric potential and the pH gradient. Thermodynamically, this is indisputable, given that ATP synthesis is coupled to the (electrogenic) translocation of protons across the membrane. However, it has often been accepted that the two parameters of the proton motive force ($\Delta\Psi$ and ΔpH) are also kinetically equivalent, so that either one of them is equally capable of energizing ATP synthesis. Evidence for an entirely ΔpH-driven ATP synthesis mechanism

reported for the chloroplast ATP synthase (Jagendorf and Uribe, 1966) has indeed been a landmark for the general acceptance of the chemiosmotic theory. Subsequently, numerous studies were performed to determine quantitatively the effect of $\Delta\Psi$ and ΔpH on the rate of ATP synthesis, and it was concluded that the two forces are not only thermodynamically but also kinetically equivalent.

According to the results presented here and elsewhere (Kaim and Dimroth, 1998b,c), this conclusion is no longer tenable. We provide compelling evidence that $\Delta\Psi$ is indispensable for ATP synthesis and cannot be replaced by large ΔpH values for the ATP synthases of chloroplasts or *E.coli* or by large ΔpNa^+ values for the ATP synthase of *P.modestum*. For investigations of the response of ATP synthases to ΔpH, the 'acid bath procedure' has been used almost exclusively. Thylakoids or proteoliposomes acidified with succinate to pH 4–5 were diluted rapidly into Tris buffer, pH 8–8.5, and ATP formation was monitored. The driving force for the ATP synthesis was attributed to the ΔpH applied. Remarkably, it had already been noticed in the original publication (Jagendorf and Uribe, 1966) that the nature of the buffer used to acidify the chloroplast interior played an important role in ATP synthesis. Upon acidification with HCl or glutamate, appreciable amounts of ATP were not formed but, by including 10 mM succinate, the basal level of ATP synthesis increased 15-fold. We have now clearly demonstrated that the 'acid bath procedure' with succinate as the acidic buffer not only establishes the desired ΔpH but also a $\Delta\Psi$ of significant size. As outlined in Figure 2, this is based on the unexpectedly high permeability of the membrane for the succinate monoanion. The pK_{a2} of succinate is 5.2 and, therefore, the concentration of the succinate monoanion decreases by three orders of magnitude from the inside to the outside if the internal pH is 5.0 and the external pH is 8.0. According to the Nernst equation, a potential of ~180 mV could therefore be generated, which is in reasonable agreement with the experimentally determined values of ~140 mV (Figure 4). Based on the chemical nature of the dicarboxylic acids suitable for generating a membrane potential, we postulate that membranes are permeable only for those monoanionic species in which the charge is delocalized upon folding into a ring (Figure 2, Table I). Consistent with this supposition is a K^+/valinomycin diffusion potential-induced 80-fold increase in the membrane permeability for succinate, malonate or maleinate at pH values at which the monoanionic species predominate (Figure 3). The permeability of fumarate which is unable to fold into a ring is, however, not increased under these conditions. Our conclusion is also supported by ATP synthesis experiments, with reconstituted proteoliposomes being about equally effective with succinate, malonate or maleinate as acidic buffer in the 'acid bath procedure' but not with fumarate (Kaim and Dimroth, 1998b,c).

For bacteria, $\Delta\Psi$ is a more useful driving force for ATP synthesis than ΔpH, because they are faced with the problem of strongly varying pH values in the environment. At high external pH values, exemplified in extreme cases by the alkaliphiles, the pH gradient has to be inverted to maintain the viability of the cells, and hence $\Delta\Psi$ is not merely the exclusive driving force for ATP synthesis, but in addition has to compensate for the opposing ΔpH. In mitochondria, the contribution to the proton motive force is mainly by $\Delta\Psi$ and to a lesser extent by ΔpH, and, therefore, an obligatory contribution of $\Delta\Psi$ to the ATP synthesis mechanism is compatible with the physiology of these organelles (Thayer and Hinkle, 1975). Chloroplasts, however, convert the $\Delta\Psi$ generated during photosynthesis rapidly into ΔpH, and in the steady state the ΔpH with ~180 mV significantly exceeds the residual $\Delta\Psi$ ($\leqslant$50 mV) (Gräber and Witt, 1976). ATP synthesis in these organelles therefore utilizes the ΔpH as the main thermodynamic driving force, but for kinetic reasons the process is obligatorily dependent on $\Delta\Psi$ (Figure 5A). Interestingly, however, the midpoint potential for ATP synthesis with 30 mV is only about half that found for the bacterial ATP synthases, obviously reflecting an adaptation of the chloroplast enzyme to the low electric potential present across the thylakoid membranes. It is important that our results with the reconstituted chloroplast ATP synthase also apply to the enzyme under *in vivo* conditions, i.e. in the chloroplast under saturating light in the steady state. Although there is no explicit mention in the literature that the $\Delta\Psi$ component is kinetically indispensable for ATP synthesis under these conditions, some of the reported data can easily be reconciled with this proposition. Portis and McCarty (1976) mentioned that gramicidin failed to collapse the pH gradient below 2.4 units, but fully abolished phosphorylation by thylakoids in the steady state. Most probably, the gramicidin destroyed the $\Delta\Psi$, and the significant ΔpH by itself is unable to drive ATP synthesis. McCarty further noticed significant inhibition of ATP synthesis by chloroplast and subchloroplast particles by valinomycin, which increased continuously with the valinomycin concentration applied (McCarty, 1969). At the highest valinomycin concentration tested (2 μg/ml; corresponding to a lipid:valinomycin ratio of ~750:1), the inhibition was 50–60%. However, no saturation was achieved with this amount of valinomycin, and phosphorylation might have been destroyed completely if higher concentrations were applied. Please note that the data of Figure 4 show that lipid:valinomycin ratios of ~100:1 are required to clamp the potential generated during the acid bath procedure completely to zero. Finally, the curious notion that ATP synthesis elicited by the 'acid bath procedure' is heavily dependent on the nature of the acid used to bring the internal pH of thylakoids down to 4–5 (Jagendorf and Uribe, 1966) is explained by the different capacities of these acids to create a $\Delta\Psi$, as shown in this communication (Table I). The extension of our experiments from proteoliposomes containing the reconstituted chloroplast ATP synthase to isolated thylakoid membranes gives comparable results and thus strongly supports this conclusion. The measured ATP synthesis correlates perfectly with the generation of an electric potential in these organelles. The addition of the membrane-permeable tetraphenylboron anion, however, completely dissipates the electric potential and abolishes ATP synthesis. Hence, a congruent explanation of the observations with thylakoids and reconstituted proteoliposomes is an obligatory dependency of ATP synthesis by the chloroplast enzyme on $\Delta\Psi$.

In general, results on the contribution of bioenergetic parameters to ATP synthesis are more conclusive if obtained with reconstituted proteoliposomes than with natural membrane vesicles. In the earlier times of bioenerg-

etics, membrane vesicles from, for example, mitochondria or chloroplasts have been the preferred study objects simply because purified ATP synthase preparations were not available. Since the natural membranes contain, in addition to the ATP synthase, many other proteins that might interfere in a very unpredictable way with the results on ATP synthase energetics, the conclusions cannot be as rigorous as with the reconstituted systems. For example, the 'acid bath procedure' with succinate as the acidic buffer is equally feasible in order to energize ATP synthesis in thylakoids or in reconstituted proteoliposomes containing the purified chloroplast ATP synthase. The same procedure with fumarate as the acidic buffer, however, generates a membrane potential that supports ATP synthesis in thylakoids but not in reconstituted proteoliposomes (Kaim and Dimroth, 1998b,c, and Figure 6). Hence, it is likely that thylakoids contain an electrogenic fumarate transporter that builds up the potential which in turn drives ATP synthesis. Another example refers to ATP synthesis in submitochondrial particles. We show here that malonate is a suitable acidic buffer to generate a membrane potential in the 'acid bath procedure'. With submitochondrial particles, however, ATP was not synthesized by following the 'acid bath procedure' with malonate but, by superimposing a K^+/valinomycin diffusion potential, ATP synthesis was found (Thayer and Hinkle, 1975). It is conceivable that the electroneutral malonate–phosphate exchanger (Saint-Macary and Foucher, 1983) rapidly dissipates the malonate gradient so that $\Delta\Psi$ cannot be generated under these conditions by diffusion of the malonate monoanion and, hence, ATP synthesis, which is $\Delta\Psi$ dependent, is not observed.

The most important facet of our findings probably relates to the ATP synthesis mechanism. The obligatory requirement for a transmembrane voltage for the synthesis of ATP is a key element in the coupling mechanism and must be taken into account in any of its model descriptions. Different models for the ion translocation mechanism have been proposed, and the more recent ones are based on a rotation of the c subunit ring against the a subunit (Vik and Antonio, 1994; Duncan *et al*., 1995; Junge *et al*., 1997; Elston *et al*., 1998; Kaim and Dimroth, 1998b; Kaim *et al*., 1998; Dimroth *et al*., 1999). In one model, the rotor sites on the c subunit ring (rotor) have been placed in the middle of the membrane (Vik and Antonio, 1994; Duncan *et al*., 1995; Junge *et al*., 1997; Elston *et al*., 1998). They communicate with the two different sides of the membrane through two non-colinear access channels within the stator (a subunit). The rotor performs Brownian motion against the stator within a narrow angle. Electrostatic constraints inhibit the movement of an empty, negatively charged rotor site into the area that is in contact with lipids. Importantly, however, the rotor progresses in one direction if a pH gradient is applied. This ensures occupation of rotor sites from the side of low pH and deprotonation of these sites on the side of high pH, and consequently determines the direction of rotation. According to this model, ΔpH acts as the principle driving force for rotation and, hence, for ATP synthesis, an assumption which is not compatible with the observed obligatory role of the membrane potential.

We have recently proposed an alternative model that is based on several lines of evidence obtained mainly with the Na^+-translocating ATP synthase of *P.modestum* (Kaim and Dimroth, 1998b; Kaim *et al*., 1998). Only one periplasmatically oriented access channel is present in the stator through which empty rotor sites can be occupied by Na^+ (or H^+). The coupling ions are subsequently released into the cytoplasm after the rotor has turned. The concept for the motor function of the ATP synthase involves electrostatic interactions between the positive stator charge (aR227) and the negatively charged rotor sites (cE65) that are modulated periodically by charge balancing as the coupling ions move across.

Further elaboration and extension of this concept has inspired Oster and colleages to present a new model (Dimroth *et al*., 1999). This is the only model that considers the obligatory requirement for the membrane potential for ATP synthesis (Kaim and Dimroth, 1998b,c) and gives a quantitative account of how the transmembrane voltage is transduced into rotary torque. Importantly, the motor works by a combination of rotor diffusion and electrostatic effects, including the interaction between charged rotor and stator sites mentioned above. The model is reinforced by our present extension of the mandatory role of the membrane potential to the chloroplast ATP synthase, which makes it likely that this is of general significance for the entire family of these enzymes.

Materials and methods

Materials

The ATP synthases of *E.coli* and *P.modestum* were purified and determined by their ATP hydrolysis activity as described (Laubinger and Dimroth, 1988). The chloroplast ATP synthase was a gift from Dr N.Dencher.

Isolation of thylakoid membranes

Chloroplasts were prepared by two 5 s homogenizations of 25 g spinach leaves in 180 ml of buffer A at 0°C in a Waring blendor. Buffer A contained 25 mM HEPES, pH 7.6, 330 mM sorbitol, 30 mM KCl, 5 mM NaCl, 5 mM ascorbate, 4 mM cysteine, 2 mM Na^+-EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.5 mM K_2HPO_4. After filtration through cheesecloth, the chloroplast suspension was centrifuged for 5 min at 1500 *g*. The pelleted chloroplasts were resuspended carefully with a pipette in 5 ml of buffer A containing, in addition, 5% dimethylsulfoxide, then frozen and stored under liquid nitrogen. The chloroplasts were thawed before use, diluted to a concentration of 0.25 mg of chlorophyll per ml with buffer A and centrifuged as above. The pellet was resuspended in buffer B containing 25 mM HEPES, pH 7.6, 30 mM KCl, 5 mM NaCl, 2 mM Na^+-EDTA, 1 mM $MgCl_2$ and 1 mM $MnCl_2$ to the same concentration as in the previous step and incubated for 5 min at 0°C for breakage. The thylakoid membranes were collected by centrifugation for 5 min at 2000 *g* and resuspended in buffer B to a concentration of 0.5 mg of chlorophyll per ml.

Determination of the inner liposome volume

Three liposome suspensions were prepared by sonicating 20.9, 41.3 or 62.5 mg of soybean phosphatidylcholine (Sigma, Type II S) in 1.8 ml of 50 mM potassium phosphate buffer, pH 7.0. The suspensions were frozen in liquid nitrogen for 15 min, and thawed in an ice–water bath for 2 h. To separate the external phosphate, 200 µl of each suspension were loaded onto a Sephadex-G75 column (130×6 mm) equilibrated with 5 mM MOPS, pH 7.0. From the total and inorganic phosphate content determined in 22 samples (Ames and Dubin, 1960), a mean inner volume of 5.5 µl/mg of lipid was calculated.

Determination of membrane potentials induced by various mono- or dicarboxylic acids

The membrane potential was determined from the distribution of the lipophilic anion [^{14}C]thiocyanate between the external medium and

intravesicular fluid and calculated by the Nernst equation (Rottenberg, 1979):

$$\Delta\Psi = \frac{2.3\ R\ T}{F} \log \frac{[SCN^-]_{in}}{[SCN^-]_{out}}$$

Liposomes were formed as described above containing 44 mg of lipids per ml of 5 mM potassium phosphate buffer, pH 7.2. Acidification of the interior was achieved by adding 10 µl of the liposome suspension to 590 µl of acidic medium, pH 5.0, containing 100 mM of the respective acid (see Table I). After incubation for 2 min at 25°C, the external pH was increased to 8.1–8.6 with 9–13 µl of 5 M NaOH. The suspension was diluted with 600 µl of 100 mM glycylglycine buffer, pH 8.5 containing 34 µM [^{14}C]NaSCN (0.5 µCi). After 20, 40 and 60 s, aliquots of 150 µl were subjected to rapid filtration, and the radioactivity inside the liposomes was measured by liquid scintillation counting (Kaim and Dimroth, 1998c).

The membrane potential developed across thylakoid membranes was determined by the analogous method using a value of 10 µl of interior thylakoid volume per mg of chlorophyll for calculation (H.Strothmann, personal communication). Thylakoid membranes (100 µl) containing 0.12 mg of chlorophyll (1.2 µl interior volume) were added to 800 µl of acidic buffer, pH 5.0. The subsequent steps were performed identically to those described in the 'acid bath procedure' (see below) with the modification that the basic buffer additionally contained 23 µM [^{14}C]NaSCN (0.5 µCi).

Uptake of dicarboxylic acids into liposomes

Liposomes were formed as described above with 20 mg of lipids per ml of buffer A, B or C (A: 50 mM Tricine, pH 8.5; B: 50 mM MES, pH 5.0; C: 50 mM MES/phosphoric acid, pH 4.0) containing 1 mM KCl. A 100 µl aliquot of the liposomes was diluted with 600 µl of identical buffer containing 36 µM valinomycin and 2 mM of a ^{14}C-labelled dicarboxylic acid (0.5 µCi; 790–800 c.p.m./nmol) as described in the legend to Figure 2. The suspension was incubated at 25°C and samples of 70 µl were taken after 10, 40 and 60 s to determine the uptake rate in the absence of $\Delta\Psi$. The electrophoretic uptake of the dicarboxylates was initiated by applying a diffusion potential ($\Delta\Psi$ = 59 mV) by adding 9 mM KCl, and samples of 70 µl were taken after 5, 10 and 15 s. The samples were filtered and the radioactivity retained was quantitated by liquid scintillation counting (Kaim and Dimroth, 1998c).

Determination of membrane potentials in the presence of different valinomycin:lipid ratios

Liposomes were prepared by sonicating 22 mg of phospholipids in 1 ml of buffer containing 20 mM Tricine, 20 mM succinate, pH 8.0 and 200 mM KCl. After the freeze–thaw procedure (see above), 10 µl of the liposomes were diluted into 590 µl of acidic medium and incubated for 2 min on ice. The acidic medium, pH 5.0, contained 20 mM succinate, 5 mM NaH_2PO_4 and 200 mM KCl. Valinomycin was added to yield concentrations of 0.5, 2 and 5 µM, respectively. After the acidic stage, the pH was shifted to 8.1–8.3 by the addition of 8–11 µl of 5 M NaOH, and the liposomes were diluted on ice with 600 µl of basic medium, pH 8.5, containing 200 mM Tricine, 5 mM NaH_2PO_4, 200 mM KCl and 34 µM [^{14}C]NaSCN (0.5 µCi). After 5, 10, 15, 20, 40 and 60 s, 150 µl aliquots of the incubation mixture were subjected to rapid filtration and the entrapped [^{14}C]SCN^- was determined (Kaim and Dimroth, 1998c). The membrane potentials were calculated as described above.

ATP synthesis experiments

Liposomes were prepared by sonicating 56 mg of phospholipids in 1.8 ml of buffer twice for 1 min in a water bath sonicator. The buffer contained 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 1 mM KCl and either 100 mM Tricine, pH 8.5 (*E.coli* ATP synthase), 100 mM Tricine, pH 7.2 (*P.modestum* ATP synthase), or 100 mM Tricine, pH 8.5, plus 50 mM dithiothreitol (DTT) (chloroplast ATP synthase). A 93.5 µg aliquot of purified ATP synthase (155–170 pmol based on molar masses of 530–550 kDa) from chloroplasts, *E.coli* or *P.modestum* was added to obtain a lipid:protein ratio of 600:1. Since ~60% of the F_1 parts are facing the exterior of our proteoliposomes (Laubinger and Dimroth, 1988), 100 pmol of the ATP synthase is able to synthesize ATP. Proteoliposomes with the *E.coli* or *P.modestum* ATP synthase were incubated for 10 min at 25°C, and those with the chloroplast ATP synthase were incubated with 50 mM DTT for 2 h at 25°C to reduce the disulfide bridge on the γ subunit (Junesch and Gräber, 1987). After freezing for 5 min in liquid nitrogen and thawing for 2 h in an ice–water bath, the proteoliposomes were collected by centrifugation at 250 000 *g* for 45 min, resuspended in 1 ml of the identical buffer and sonicated twice for 5 s in a bath-type sonicator. After 1000-fold dilution of the proteoliposomes with the respective buffer, samples (100 µl) were diluted into 350 µl of 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 2.5 mM ADP and 100 mM Tricine, pH 8.5 (*E.coli* ATP synthase), 100 mM Tricine, pH 7.2 (*P.modestum* ATP synthase), or 100 mM Tricine, pH 8.5, plus 50 mM DTT (chloroplast ATP synthase). After incubation with 0.16 µM valinomycin for 2 min, different membrane potentials were applied to the proteoliposomes by variation of the external KCl concentration (see legend to Figure 4). Samples (90 µl) were added 5, 10 and 15 s after energetization to 10 µl of 10% trichloroacetic acid (TCA), and ATP was determined by the luciferin luciferase assay (Kaim and Dimroth, 1994).

To generate a ΔpH of 206 mV, proteoliposomes were prepared with 100 mM MES, pH 5.0, 5 mM $MgCl_2$, 5 mM NaH_2PO_4 and 1 mM KCl (50 mM DTT added additionally in the case of the chloroplast enzyme). After shifting the external pH to 8.2–8.4 by the addition of 5–9 µl of 5 M NaOH, K^+/valinomycin diffusion potentials were applied and the rate of ATP synthesis was determined with the samples (see above). In the case of the *P.modestum* ATP synthase, a ΔpNa^+ of 77 mV was generated by reconstituting the enzyme in the presence of 95 mM NaCl under otherwise identical conditions. External Na^+ ions were removed by passing 100 µl of the proteoliposomes over a Dowex 50 column (H^+ form). The proteoliposomes were collected by centrifugation and resuspended in 70–80 µl of 100 mM Tricine buffer, pH 7.2. K^+/valinomycin diffusion potentials were applied in samples as described above, and the ATP synthesis rates were determined.

ATP synthesis with thylakoid membranes was performed by the 'acid bath experiment' as described (Jagendorf and Uribe, 1966), with the following modifications. In order to exclude light-driven electron transport processes, the thylakoid membranes were pre-incubated for 1 h in complete darkness before acidification. The experiments were performed in a dark room and the acidic stage was initiated by adding 500 µl of the thylakoid membranes (25 mg of chlorophyll) to 400 µl of acidic buffer containing 20 mM succinate or 20 mM fumarate, pH 5.0. After 60 s, the pH was adjusted to 8.1–8.3 by the addition of 4–7 µl of 5 M NaOH and the suspension was diluted 1:1 with 900 µl of basic buffer containing 100 mM Tris–HCl, pH 8.5, 5 mM $MgCl_2$, 5 mM NaH_2PO_4 and 2.5 mM ADP. Samples of 90 µl were taken after different incubation periods, the reaction was terminated with 10 µl of 20% TCA and ATP was determined as described (Kaim and Dimroth, 1994). The final pH was measured directly after each experiment (pH 8.2–8.6).

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