Mode of interaction of the single a subunit with the multimeric c subunits during the translocation of the coupling ions by F_1F_0 ATPases

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We have recently isolated a mutant (aK220R, aV264E, aI278N) of the Na^+ -translocating *Escherichia coli*/*Propionigenium modestum* ATPase hybrid with a Na^+ -inhibited growth phenotype on succinate. ATP hydrolysis by the reconstituted mutant ATPase was inhibited by external (N side) NaCl but not by internal (P side) NaCl. In contrast, LiCl activated the ATPase from the N side and inhibited it from the P side. A similar pattern of activation and inhibition was observed with NaCl and the ATPase from the parent strain PEF42. We conclude from these results that the binding sites for the coupling ions on the c subunits are freely accessible from the N side. Upon occupation of these sites, the ATPase becomes more active, provided that the ions can be further translocated to the P side through a channel of the a subunit. If by mutation of the a subunit this channel becomes impermeable for Na^+ , N side Na^+ ions specifically inhibit the ATPase activity. These conclusions were corroborated by the observation that proton transport into proteoliposomes containing the mutant ATPase was abolished by N side but not by P side Na^+ ions. In contrast, LiCl affected proton translocation from either side, similar to the sidedness effect of Na^+ ions on H^+ transport by the parent hybrid ATPase. If the ATPase carrying the mutated a subunit was incubated with $^{22}\text{NaCl}$ and ATP, 1 mol $^{22}\text{Na}^+$ /mol enzyme was occluded. With the parent hybrid ATPase, $^{22}\text{Na}^+$ occlusion was not observed. The occluded $^{22}\text{Na}^+$ could be removed from its tight binding site by 20 mM LiCl, while incubation with 20 mM NaCl was without effect. Li^+ but not Na^+ is therefore apparently able to pass through the mutated a subunit and make the entrapped Na^+ ions accessible again to the aqueous environment. These results suggest an ion translocation mechanism through F_0 that in the ATP hydrolysis mode involves binding of the coupling ions from the cytoplasm to the multiple c subunits, ATP-driven rotation to bring a Na^+ , Li^+ , or H^+ -loaded c subunit into a contact site with the a subunit and release of the coupling ions through the a subunit channel to the periplasmic surface of the membrane.

Keywords: F_1F_0 ATPases/ion translocation/*Propionigenium modestum*/rotational mechanism

Introduction

The enzyme ATP synthase or F_1F_0 ATPase plays a central role in the synthesis of ATP from ADP and inorganic phosphate in mitochondria, chloroplasts or bacteria (Futai *et al.*, 1989; Fillingame, 1990; Deckers-Hebestreit and Altendorf, 1996; Dimroth, 1997; Weber and Senior, 1997). The energy for this endergonic reaction stems from a transmembrane electrochemical gradient of protons or in some cases Na^+ ions. Accordingly, the proton or sodium ion-translocating ATPases have been classified as FP-ATPases and FS-ATPases, respectively (Kaim *et al.*, 1997). The water-soluble F_1 part of these enzymes catalyses the hydrolysis of ATP and has the subunit composition $\alpha_3\beta_3\gamma\delta\epsilon$. The membrane-embedded F_0 component is responsible for the translocation of the coupling ions across the membrane and in bacteria has the probable subunit composition ab_2c_{12} .

The crystal structure of mitochondrial F_1 (Abrahams *et al.*, 1994) in combination with the binding change mechanism (Boyer, 1993) suggested a catalytic mechanism of ATP hydrolysis that involves the rotation of the extended α -helical γ -subunit within the central cavity of the $\alpha_3\beta_3$ headpiece, and experimental evidence in favour of this rotation was obtained recently with an $\alpha_3\beta_3\gamma$ subcomplex of F_1 (Duncan *et al.*, 1995; Sabbert *et al.*, 1996; Noyi *et al.*, 1997).

Of special interest is the mechanism by which this intersubunit rotation in the F_1 moiety is connected to the movement of the coupling ions across the F_0 sector of the F_1F_0 ATPase complex. However, as a high resolution structure of F_0 is lacking, contributions to solve these problems have come mainly from biochemical and mutational studies either with F_0 from *Escherichia coli* or with that from *Propionigenium modestum*. The distinct experimental advantages of the *P.modestum* ATPase to use Na^+ , Li^+ or H^+ as alternative coupling ions have been employed to establish that the F_0 part is exclusively responsible for transport and recognition of the coupling ions (Laubinger and Dimroth, 1987, 1988, 1989). It was also shown that the transport of these ions across F_0 follows a transporter and not a channel type mechanism (Kluge and Dimroth, 1992). Various mutations in the *P.modestum* c subunit indicated the importance of this subunit in conferring a distinct coupling ion specificity to the ATPase (Kaim and Dimroth, 1995; Kaim *et al.*, 1997). Part of the Na^+ binding site is contributed by the conserved dicyclohexylcarbodiimide (DCCD)-reactive acidic amino acid in the C-terminal membrane spanning α -helix (E65 in the *P.modestum* c subunit) (Kluge and Dimroth, 1993). The coordination sphere for Na^+ is completed by the adjacent S66 and by Q32 (Kaim *et al.*, 1997), which according to structural information of the *E.coli* c subunit (Girvin and Fillingame, 1994, 1995) is on the opposite

α -helix of the helical hairpin in close proximity to E65 and S66. The mutational analyses further indicated that Li^+ binding required E65 and S66 but not Q32 as ligands, and that for H^+ translocation E65 was sufficient as binding site during transport (Kaim *et al.*, 1997). Furthermore, in using a random mutational approach, a double mutation in the C-terminal tail of the *P.modestum* c subunit (F84L, L87V) has been obtained that converts the pertinent Na^+ -translocating hybrid ATP synthase with the *P.modestum* subunits a, b, c, δ and the *E.coli* subunits α , β , γ , ϵ into a H^+ -coupled ATP synthase (Kaim and Dimroth, 1995). A possible explanation for this phenotype may be a more global structural change of the c subunit by which Q32 is displaced from the binding pocket, so that Na^+ ions can no longer be coordinated.

Another class of mutants with the phenotype of Na^+ -independent growth on succinate were obtained recently that had no mutation in the c subunit but multiple mutations in the a subunit. Specifically, the triple mutation aK220R, aV264E, aI278N proved to be responsible for the Na^+ -independent growth phenotype of the new *E.coli* strain MPA762 (G.Kaim and P.Dimroth, in preparation). Furthermore, Na^+ was a specific growth inhibitor of this strain, the enzymatic basis for which being a specific inhibition by Na^+ of the mutant ATPase. These results indicated an important contribution of the a subunit in coupling ion selectivity and therefore an active role in coupling ion translocation *per se*.

To obtain further insight into the ion translocation mechanism, we investigated the accessibility of the coupling ion binding site during transport. Evidence is presented that in the ATP hydrolysis mode, Na^+ ions are bound from the cytoplasm to the Na^+ -specific sites on the multimeric c subunits. They can only be released to the periplasmic side of the membrane through a channel in the a subunit with an appropriate specificity. Therefore, a Na^+ ion becomes occluded by the ATPase with the a subunit triple mutation in an ATP-dependent fashion. Our data point to a rotation of the multimeric c subunits versus the single a subunit during the catalysis of coupling ion transport across the membrane.

Results

Sidedness effect of Na^+ or Li^+ ions on ATPase activity of various *E.coli/P.modestum* hybrid ATPase mutants reconstituted into proteoliposomes

We have shown recently that the ATPase of strain MPA762 with a triple mutation in the a subunit (aK220R, aV264E, aI278N) is specifically inhibited by Na^+ ions with a K_i (1.5 mM) that is similar to the K_m for Na^+ activation (1.4 mM) of the parent hybrid enzyme under otherwise identical conditions (G.Kaim and P.Dimroth, in preparation). These results suggested that the Na^+ ions become bound to the c subunits of the mutant ATPase in just the same way as in the parent hybrid ATPase (Kaim *et al.*, 1997), but cannot be released from these sites due to a specificity change of the a subunit channel that does not allow Na^+ ions to pass through. It was therefore of interest to investigate the accessibility of the c subunit-specific sites from both sides of the membrane in various ATPase mutants by the effect of Na^+ or Li^+ ions on ATP hydrolysis

activity. The hybrid ATPases of *E.coli* strains MPA762 (aK220R, aV264E, aI278N) (G.Kaim and P.Dimroth, in preparation), MPC8487 (cF84L, L87V) (Kaim and Dimroth, 1995) and of the parent strain PEF42 (Kaim and Dimroth, 1993) were therefore reconstituted into proteoliposomes and the effect of internal (P side) or external (N side) Na^+ or Li^+ ions on ATPase activities were determined. In order to avoid confusion it should be noted that in accordance with P.Mitchell we have defined the side of a membrane to which the coupling ions are pumped as the P (positive) side and the side from which they are pumped as the N (negative) side. The results of Figure 1 show that the ATPase with the triple mutation in the a subunit (strain MPA762) was specifically inhibited by Na^+ ions, but only if these were present at the N side, whereas P side Na^+ ions were without effect. Na^+ ions can therefore apparently bind to the c subunits from the N side, but not from the P side of the proteoliposomes and when bound cause inhibition, probably because they cannot be released through the mutated a subunit to the P side of the membrane. In contrast, the MPA762 ATPase was activated by LiCl added from the N side, but inhibited if the same LiCl concentration was present at the P side. The activation can be explained by binding of Li^+ ions to the c subunits leading to an increased ATPase activity compared with the enzyme with H^+ -occupied c subunits. Importantly, there is apparently no restriction to Li^+ for release through the a subunit to the P side of the membrane during ATP-driven transport.

The observed inhibition of ATPase activity by P side (internal) Li^+ ions is more difficult to interpret. One should, however, consider the large Li^+ concentration gradient against which H^+ pumping has to occur and which may favour the reverse reaction, i.e. Li^+ entering through the a subunit to the binding site on the c subunit(s) and perhaps further to the N side. This certainly should slow down the H^+ -coupled ATP hydrolysis. The validity of this interpretation was tested by varying the concentration ratio across the membrane. The ATPase activity of proteoliposomes containing 50 mM LiCl at the P side was measured after the addition of 10, 25 or 50 mM LiCl at the N side of the membranes. The results shown in Figure 1 clearly indicate activation of the ATPase by increasing the N side LiCl concentrations to reach the same ATPase activity (0.5 U/mg protein) at equimolar LiCl concentrations on both sides of the membranes as observed without LiCl present on either side. If the membranes were permeabilized with Triton X-100 increasing P side LiCl concentrations (up to 50 mM) were without effect on the ATPase activity, which remained constant at 0.5 U/mg protein (data not shown).

The parent hybrid ATPase isolated from *E.coli* PEF42 was stimulated if Na^+ ions were added from the N side and inhibited by P side NaCl. Here again Na^+ activates, because these cations have direct access to the c subunits from the N side and because the Na^+ -bound enzyme is more active than its H^+ -bound counterpart. The inhibition by Na^+ from the P side is on the other hand compatible with partial reversed operation of the ATPase, elicited by the large Na^+ gradient. This was shown by the activation of the parent ATPase if 5 mM NaCl was present at the P side and the NaCl concentration at the N side was increased (Figure 1). Furthermore, after the Na^+ ion

Fig. 1. Effect of Na^+ or Li^+ on the ATP hydrolysis activity from *E. coli* MPA762 (aK220R, aV264E, aI278N), the parent strain PEF42 and *E. coli* MPC8487 (cF84L, cL87V) after reconstitution into proteoliposomes. Open triangles indicate the presence of NaCl or LiCl within the liposomes (P side) and closed triangles indicate the presence of NaCl or LiCl outside the liposomes (N side). The black squares indicate a constant NaCl concentration of 5 mM or a constant LiCl concentration of 50 mM at the P side of the liposomes and increasing NaCl or LiCl concentrations at the N side. ATPase activities were measured in 50 mM potassium phosphate buffer containing 100 mM K_2SO_4 adjusted to pH 6.5 with KOH.

gradient was abolished by the addition of Triton X-100 or the ionophore monensin to the proteoliposomes, no inhibition of ATPase activity by N side NaCl could be observed (data not shown). It may be interesting to note in this context, that for the soluble ATPase, the activation profiles by Na^+ or Li^+ were the same as for the proteoliposomal enzymes with the alkali ions added from the N side (Kluge and Dimroth, 1993; Kaim and Dimroth, 1995; Kaim *et al.*, 1997). The reconstituted ATPase with an impaired Na^+ binding site on its c subunit (strain MPC8487) (Kaim and Dimroth, 1995) was not activated by Na^+ ions from the N side, as expected. Na^+ ions were also without effect when added to the P side, which is in accord with the above notion that inhibition by the appropriate alkali ions from the P side is due to an acceleration of the back reaction, elicited by the large alkali ion gradient. Without a Na^+ binding site on the c subunit, this back reaction is obviously not possible, and simple access of the a subunit channel by Na^+ is therefore apparently not inhibitory.

Sidedness effect of Na^+ or Li^+ ions on ATP-driven H^+ -transport by various *E. coli*/*P. modestum* hybrid ATPase mutants

The results of proton translocation into proteoliposomes, elicited by the ACMA fluorescence quenching approach, are shown in Figure 2. As expected, the ATPase with the triple mutation in subunit a isolated from *E. coli* MPA762 catalysed proton accumulation on the P side, which was gradually diminished in the presence of 2 mM or 5 mM NaCl on the N side and completely abolished by 10 mM NaCl (Figure 2A). Thus, inhibition of proton transport and ATP hydrolysis activity by N side Na^+ ions correlate with each other. With up to 10 mM NaCl on the P side of the proteoliposomes, proton transport by this mutant ATPase was not affected, again correlating with the missing effect of P side Na^+ ions on ATPase activity (Figure 2B). If LiCl was added to the N side of the proteoliposomes containing the mutant ATPase, H^+ transport gradually declined to non-detectable levels on increasing the LiCl concentration from 10 to 50 mM. This result

was to be expected by the competition of Li^+ and H^+ for the same binding sites on the c subunits (Laubinger and Dimroth, 1989; Kluge and Dimroth, 1992, 1993; Kaim and Dimroth, 1995) that are accessible from the N side. Surprisingly, however, a similar inhibition pattern of proton transport was observed, if the same LiCl concentrations that had been added to the N side were present on the P side of the proteoliposomes (Figure 2B). As considerable ATPase activity persists, even at a P side LiCl concentration of 50 mM, where proton transport is completely abolished, the inhibition of proton transport by P side Li^+ ions is only partially due to the inhibition of the ATPase. Similar inhibition patterns with the same concentrations of N side and P side Na^+ ions were also observed with proteoliposomes containing the parent hybrid ATPase (Figure 2A and B). The addition of NaCl to either the N side or the P side of proteoliposomes containing the ATPase with an impaired Na^+ binding site on its c subunits (strain MPC8487) was, on the other hand, without any effect on ATP-driven proton transport. These results clearly indicate that the target site for inhibition of H^+ translocation by Na^+ (enzyme from strain PEF42) or Li^+ (enzyme from strain MPA762) is the coupling ion binding site on subunit c. Occupation of this site on the multiple c subunits occurs either directly from the N side or indirectly via subunit a from the P side.

Occlusion of $^{22}\text{Na}^+$ by the ATPase with the a subunit triple mutation from strain MPA762

We have concluded from results presented above that the ATPase from strain MPA762 contains intact Na^+ binding sites on its c subunits which are directly accessible in solution or from the N side (facing F_1) after reconstitution into proteoliposomes. These sites are not accessible, however, through the mutated a subunit, neither in solution nor from the P side of reconstituted proteoliposomes. It was possible, therefore, that ATP hydrolysis would force a c subunit containing a Na^+ ion into the contact site with the a subunit so that the Na^+ ion becomes occluded. The mutant ATPase was therefore incubated with 0.1 or 1 mM $^{22}\text{NaCl}$ and ATP for 30 s and the enzyme was subsequently

Fig. 2. Effect of NaCl and LiCl concentrations on ATP-driven fluorescence quenching of ACMA by reconstituted proteoliposomes at pH 7.5. ACMA quenching was initiated by adding 2.5 mM K-ATP ($\downarrow$) to reaction mixtures containing the ATPases of the triple-mutant MPA762, the parent strain PEF42 and the double-mutant MPC8487 reconstituted into proteoliposomes. The indicated NaCl and LiCl concentrations were either applied at the N side of the liposomes (A) or were present at the P side of the liposomes (B). Quenching was released by the addition of 2 μ M CCCP ($\downarrow$).

rapidly passed over a cation exchange column to remove all exchangeable $^{22}\text{Na}^+$ from the enzyme. The results of Table 1A indicate that up to 1 mol Na^+ per mol enzyme could not be removed by the ion exchanger and must therefore have been occluded within the ATPase. Interestingly enough, Na^+ occlusion was ATP-dependent: ATP could not be substituted by ADP and inorganic phosphate. An occlusion of Na^+ ions was also not observed if the mutated ATPase was replaced by the parent hybrid enzyme of strain PEF42. Hence, Na^+ ions apparently bind to the multiple c subunits, from where they can be readily removed by a cation exchanger. Upon ATP hydrolysis, however, one Na^+ -loaded c subunit is moved into the contact site with the a subunit. In this configuration, the Na^+ binding site of the c subunit is no longer directly accessible, but requires a channel provided by the a subunit to be released and to be trapped by the ion exchanger. As Na^+ ions cannot penetrate through the channel of the mutated a subunit, the one Na^+ ion that is bound to the c subunit at the contact site with the Na^+ -impermeable a subunit remains trapped in the enzyme.

Further experiments were performed to explore whether the occluded $^{22}\text{Na}^+$ ions could be removed from the occluded site by adding large concentrations of NaCl or LiCl (20 mM each), followed by another 30 s incubation, before adsorption of exchangeable Na^+ ions by the cation exchanger. As shown in Table 1B, NaCl was unable to remove the occluded $^{22}\text{Na}^+$ ions from the enzyme, but LiCl caused almost complete liberation of the entrapped $^{22}\text{Na}^+$. As the main difference of the mutant ATPase with respect to these alkali ions is its accessibility for Li^+ but not for Na^+ through the a subunit channel, one has to conclude that the occluded Na^+ ions are removed from their tight binding site by Li^+ ions entering through the a subunit channel.

Discussion

The results of this communication provide compelling evidence for an intimate cooperation of the single a subunit with the multiple c subunits in coupling ion translocation across the F_0 sector of F_1F_0 ATPases and confirm the

Table I. ATP-dependent occlusion of $^{22}\text{Na}^+$ by the (EF₁- δ) (PF₀+ δ) hybrid ATPase (control, strain PEF42) and that with the triple mutation in subunit a (K220R, V264E, I278N; strain MPA762)

A	ATPase (strain)	1 mM $^{22}\text{NaCl}$	2.5 mM ATP 0.1 mM $^{22}\text{NaCl}$	2.5 mM ATP 1 mM $^{22}\text{NaCl}$	2.5 mM ADP 1 mM $^{22}\text{NaCl}$
			$^{22}\text{Na}^+$ occluded (mol Na^+ /mol ATPase)		
	PEF42	<0.01	<0.01	<0.01	<0.01
	MPA762	<0.01	0.6	0.9	<0.01
B		2.5 mM ATP	2.5 mM ATP		
		1 mM $^{22}\text{NaCl}$	1 mM $^{22}\text{NaCl}$		
		20 mM NaCl (chase)	20 mM LiCl (chase)		
			$^{22}\text{Na}^+$ occluded (mol Na^+ /mol ATPase)		
	MPA762	0.9	0.1		

Enzymes are incubated with $^{22}\text{NaCl}$ (0.1 or 1 mM) and either 2.5 mM ATP or 2.5 mM ADP as indicated. After 30 s the occluded $^{22}\text{Na}^+$ was separated from free $^{22}\text{Na}^+$ by ion exchange chromatography on Dowex 50, K^+ , and measured by γ -counting. (A) The mean results from three different experiments are depicted. (B) For chasing, 20 mM NaCl or 20 mM LiCl was added to the enzyme samples after incubation with 1 mM $^{22}\text{NaCl}$ and 2.5 mM ATP and the incubation was continued for another 30 s prior to separation of the entrapped $^{22}\text{Na}^+$ by chromatography on Dowex 50, K^+ .

earlier suggestion of a specific interaction between the two subunits (Steffens *et al.*, 1988). We have described elsewhere that by a triple mutation in the *P.modestum* a subunit, the coupling ion specificity of the corresponding *E.coli/P.modestum* hybrid ATPase was altered (G.Kaim and P.Dimroth, in preparation). While Li^+ or H^+ could still be employed as the coupling ion, the capacity for Na^+ translocation was abolished and this alkali ion was inhibitory to ATPase functions. Mutants of the *P.modestum* c subunit with altered coupling ion specificities have also been described (Kaim and Dimroth, 1995; Kaim *et al.*, 1997). Hence, there is hardly any doubt that for the translocation of the coupling ions, a sophisticated interplay between the a subunit and the c subunits is required. This notion is in accord with concepts developed for the *E.coli* ATPase based on extensive mutational studies of the a and c subunits (Futai *et al.*, 1989; Fillingame, 1990; Deckers-Hebestreit and Altendorf, 1996; Weber and Senior, 1997). A number of amino acid residues have thus been identified on these subunits that appear to be important for H^+ translocation.

The *P.modestum* a and c subunit mutants with altered coupling ion specificity have a distinct advantage in deciphering the sidedness of coupling ion access to these subunits. In reconstituted proteoliposomes only the ATPase molecules with the F₁ sector facing outwards must be considered, because ATP does not penetrate the membrane and can only be hydrolysed if F₁ is accessible from the outside. With proteoliposomes containing the *E.coli/P.modestum* hybrid ATPase with the triple mutation in the a subunit (G.Kaim and P.Dimroth, in preparation), ATPase as well as proton transport activities were inhibited by external (N side) but not by internal (P side) Na^+ ions (Figures 1 and 2). These results thus indicate that the Na^+ binding sites on the multiple c subunits of the mutant ATPase are accessible from the N side but not from the P side of the proteoliposomes. We have shown in this study that the mutant ATPase acts as a H^+ pump in the absence of N side Na^+ ions. Binding of Na^+ to the coupling ion binding sites on the c subunits inhibited H^+ pumping, as expected from an ATPase that can use H^+ or Na^+ as alternative coupling ions. The simultaneous

inhibition of the ATPase activity by N side Na^+ ions, however, indicates that the Na^+ ions cannot be transported completely across the membrane by the mutant enzyme. The most reasonable explanation for this behaviour is an impaired Na^+ permeability of the a subunit ion channel as a result of the a subunit triple mutation. If a Na^+ -loaded c subunit is moved by ATP hydrolysis into the contact side with the Na^+ -impermeable a subunit, the bound Na^+ ions cannot be released to the other side of the membrane and coupled ATPase activity is therefore abolished.

In contrast to Na^+ , Li^+ activated the mutant ATPase from the N side, thus indicating that the Li^+ transport activity was not impaired by the a subunit triple mutation. Further in accordance with the Li^+ permeability of this a subunit is the inhibition of ATPase or H^+ transport activity by P side Li^+ ions, which can be attributed to the large ion gradient against which pumping of the coupling ions has to occur (see Results). Furthermore, the data indicate accessibility of the coupling ion binding site(s) on the c subunits for Li^+ but not for Na^+ from the P side via the mutated a subunit. Hence the a subunit apparently forms a cation-selective channel that connects the interior of the proteoliposomes with the coupling ion binding site of that c subunit that is in proper contact with the a subunit. These conclusions were corroborated by the inhibition of proton transport into proteoliposomes containing the parent hybrid ATPase by Na^+ ions present on either side of the membrane, thus indicating that the Na^+ ions have access to the c subunit binding sites from both membrane sides. Also in accordance with this model of ion translocation through the F₀ moiety of the ATPase is the lack of any effect of Na^+ ions on proton translocation into proteoliposomes containing the ATPase with an impaired Na^+ binding site on its c subunits (with the mutation F84L, L87V) (Kaim and Dimroth, 1995). Previously, a Li^+ binding site has been introduced by site-directed mutagenesis into the c subunits of the *E.coli* ATPase (Zhang and Fillingame, 1995). The Li^+ -inhibited ATPase functions of this enzyme can now readily be explained on the basis of an ion selective channel of the

Fig. 3. Rotational model for coupling ion movement via the a and c subunits of the F_1F_0 ATPase across the membrane. The model shows Na^+ ion movement from the cytoplasm to the periplasm (ATP hydrolysis mode of the *P. modestum* ATPase; view from the periplasm). The c subunits are accessible from the cytoplasm and are able to bind/release the Na^+ ions in a reversible manner. After occupation of the Na^+ ion binding sites on the multiple c subunits from the cytoplasm, ATP hydrolysis moves a Na^+ loaded c subunit into close contact with the release channel on the a subunit. Simultaneously, an empty c subunit is displaced from this position. The Na^+ ion of the c subunit making the new contact with subunit a is subsequently removed from its binding site and released to the periplasm via the ion-selective a subunit channel. In the a subunit triple-mutant MPA762, the Na^+ ion indicated with an asterisk (*) cannot be released to the periplasm and remains trapped within the ATPase complex. The critical charged residues on the a subunit (aR227 and aD259, *P. modestum* numbering, indicated with a plus and a minus) and cE65 on the c subunit may be essential to drive the rotation of the c subunits by the attraction and repulsion of electrical charges. The Na^+ (or Li^+ or H^+) ion translocation that is connected to this rotation probably contributes to the electrical charge forces, e.g. by transiently neutralizing the negative charge of cE65 at the coupling ion binding site.

a subunit that in the case of *E. coli* is impermeable for Li^+ ions.

Hence, our data let us postulate two channels for the coupling ions, each channel running about half way across the membrane. The first half channel is provided by the c subunits and connects its coupling ion binding site to the outer surface of the proteoliposomes or to the cytoplasm of a bacterial cell. This channel is accessible for all c subunits except those that are in close physical contact to the a subunit. The second half channel is provided by the a subunit and connects the coupling ion binding site of the c subunit, that is in contact with a, to the inner volume of the proteoliposomes or the periplasm of a bacterial cell.

The observed occlusion of Na^+ ions by the ATPase with the a subunit triple mutation is fully in accord with the model of an ion-selective a subunit channel connecting the coupling ion binding site of a particular c subunit with the internal volume of the proteoliposomes. Important further insights into the ion translocation mechanism derived from the fact that one Na^+ ion is occluded per ATPase and that the occlusion is ATP-dependent. These results strongly favour a rotational model for the catalysis of ion translocation across the F_0 moiety. Such a model is drawn in Figure 3. As a high resolution structure of F_0 is not available, the model assumes an arrangement of the multiple c subunits (probably 12) as a ring, with the a subunit located on one side outside this ring, as deduced by electron spectroscopic imaging (Birkenhäger *et al.*,

1995) and atomic force microscopy of *E. coli* F_0 (Singh *et al.*, 1996; Takeyasu *et al.*, 1996). ATP-driven coupling ion movement starts with the occupation of the coupling ion sites on the c subunits from the cytoplasm (Figure 3). ATP hydrolysis by an $\alpha_3\beta_3\gamma$ subcomplex of F_1 has been shown to lead to a rotation of the central γ subunit within the $\alpha_3\beta_3$ hexagon (Duncan *et al.*, 1995; Sabbert *et al.*, 1996; Noyi *et al.*, 1997). This rotation could be connected to the ring of c subunits and could thus move a Na^+ , Li^+ or H^+ -loaded c subunit into the contact site with the a subunit, from where the coupling ions can be released through the a subunit channel into the periplasm. This rotation simultaneously displaces another c subunit with an empty coupling ion binding site from the contact site with the a subunit channel. This c subunit can subsequently be loaded again with an appropriate coupling ion from the cytoplasm. In summary, the rotational model provides the most reasonable mechanism for the interaction of a single a subunit with the multimeric c subunits during the translocation of the coupling ions. The model is in accordance with all experimental data reported here and elsewhere and is most significantly supported by the $^{22}\text{Na}^+$ occlusion experiments. Rotational driving force is apparently transmitted via ATP-driven rotation of the γ -subunit on the ring of c subunits. The resulting movement of a Na^+ -loaded c subunit into the contact with the Na^+ -impermeable a subunit creates an immobile complex in which the Na^+ ion is occluded. In the wild-type ATPase, however, the Na^+ ions are released from this contact site

through the a subunit, resulting in continued rotation of the c subunits without Na⁺ occlusion. Na⁺ is not occluded by the mutant ATPase in the absence of ATP, probably because the Na⁺-loaded c subunit will not be kept within the contact site with the mutated a subunit without the rotational driving force elicited by ATP hydrolysis.

Our results further indicate that the entrapped radioactive ²²Na⁺ could not be released from its occluded position by an excess of unlabelled Na⁺ ions, but an excess of Li⁺ ions made the occluded ²²Na⁺ accessible to an ion exchange column. As the main difference of the ATPase with respect to these alkali ions is the permeability of the mutated a subunit for Li⁺ but not for Na⁺, it is obvious that Li⁺ ions can displace the ²²Na⁺ from its occluded position via the a subunit channel. Possibly, the Li⁺ ions became bound to the empty c subunit that had been displaced before from the contact site with the a subunit channel. The occupation of this site with Li⁺ may trigger a reverse rotation of the c subunits so that the Na⁺-loaded c subunit moves back into a position in which the Na⁺ ions will be accessible from outside and are therefore readily removed by the ion exchanger.

An alternative rotational model has previously been proposed for proton translocation by the *E.coli* ATPase via subunits a and c (Vik and Antonio, 1994; Duncan *et al.*, 1995; Junge *et al.*, 1997). This model assumes loading of the c subunits via an a subunit half channel that is open to one side of the membrane and discharging of the loaded c subunits via an a subunit half channel that is open to the other side of the membrane. Several lines of evidence, e.g. the complete protection by Na⁺ from the labelling of multiple c subunits by DCCD or the cooperative effect of Na⁺ on the activation of the *P.modestum* ATPase (Kluge and Dimroth, 1993) are, however, not compatible with this model.

Attraction and repulsion by electrical charges could very well be the principal driving forces for the rotation. A well-known property of the *P.modestum* ATPase is an absolute requirement at pH >8.0 of Na⁺ ions (or Li⁺ ions) for ATPase activity. The negative charge contributed by E65 of subunit c must therefore apparently be compensated by Na⁺ ions (or another coupling ion) to enable the ATPase to move this particular c subunit into the contact site with the a subunit. A negatively charged amino acid on the a subunit might repel the c subunit with an unbalanced negative charge of E65 and prevent rotation and therefore ATP hydrolysis. It is interesting therefore, that a conserved glutamate residue on either helix 5 or 6 appears to be essential for proton translocation by the *E.coli* ATPase (Cain and Simoni, 1986, 1988). The conserved and absolutely essential arginine residue in the fifth transmembrane helix of subunit a may be required to release the bound coupling ion by ion exchange and/or for further movement of the c subunits against the a subunit by electrostatic forces.

Materials and methods

Bacterial strains and media

The bacterial strains *E.coli* PEF42 (Kaim and Dimroth, 1993), *E.coli* MPC8487 (Kaim and Dimroth, 1995) and *E.coli* MPA762 (G.Kaim and P.Dimroth, in preparation) were used in this work. All *E.coli* strains were cultivated in M13 minimal media supplemented with thiamine (0.1

mg/l) and 35 mM succinate or 10 mM glucose (Kaim and Dimroth, 1993). For the screening of the mutants, succinate minimal agar plates with an internal Na⁺ concentration below 50 µM were used as described (Kaim and Dimroth, 1995).

Biochemical procedures

Purification and determination of the ATPase and the fluorescence quenching assays for ATP-dependent H⁺-translocation were performed as described (Kaim and Dimroth, 1995). Protein concentration was determined as described (Bradford, 1976) with bovine serum albumin as standard. Samples containing Triton X-100 were determined by comparison with protein standards containing the same amount of Triton X-100.

Occlusion of ²²Na⁺ by the mutant ATPase

The ATPases of strain *E.coli* PEF42 and *E.coli* MPA762 were purified to a specific activity of 2 U/mg protein as described (Kaim and Dimroth, 1993, 1994). The incubation mixtures contained the following at 25°C in 0.1 ml: 5 mM potassium phosphate buffer, adjusted with KOH to pH 7.2, 5 mM MgCl₂, 0.1% Triton X-100, 1 mM ²²NaCl (1.5×10⁴–3.5×10⁴ c.p.m./nmol) and 4 mg (~3 nmol) of each purified ATPase. Thirty seconds after the addition of 2.5 mM potassium ATP, the external ²²Na⁺ was separated from that occluded by the ATPase by passage over 1 ml Dowex 50, K⁺, and purging the column with 0.6 ml wash buffer (5 mM Tricine, adjusted with KOH to pH 7.2, 5 mM MgCl₂, 100 mM saccharose and 0.1% Triton X-100). Controls were run in the absence of ATP or in the presence of ADP. Chasing experiments were performed by adding 20 mM NaCl or 20 mM LiCl to the occluded samples 30 s after ATP addition. After incubation for another 30 s, the samples were applied to Dowex 50, K⁺, as described above. The radioactivity of occluded ²²Na⁺ passing through the column was determined by γ counting.

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