Molecular Basis for the Coupling Ion Selectivity of F_1F_0 ATP Synthases: Probing the Liganding Groups for Na^+ and Li^+ in the c Subunit of the ATP Synthase from *Propionigenium modestum*

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ABSTRACT: The conserved glutamate residue at position 65 of the *Propionigenium modestum* c subunit is directly involved in binding and translocation of Na^+ across the membrane. The site-specific introduction of the cQ32I and cS66A substitutions in the putative vicinity to cE65 inhibited growth of the single-site mutants on succinate minimal agar, indicating that both amino acid residues are important for proper function of the oxidative phosphorylation system. This growth inhibition was abolished, however, if the cF84L/cL87V double mutation was additionally present in the *P. modestum* c subunit. The newly constructed *Escherichia coli* strain MPC848732I, harboring the cQ32I/cF84L/cL87V triple mutation, revealed a change in the coupling ion specificity from Na^+ to H^+. ATP hydrolysis by this enzyme was therefore not activated by NaCl, and ATP-driven H^+ transport was not affected by this alkali salt. Both activities were influenced, however, by LiCl. These data demonstrate the loss of the Na^+ binding site and retention of Li^+ and H^+ binding sites within this mutant ATPase. In the *E. coli* strain MPC848766A (cS66A/cF84L/cL87V), the specificity of the ATPase was further restricted to H^+ as the exclusive coupling ion. Therefore, neither Na^+ nor Li^+ stimulated the ATPase activity, and no ATP-driven Li^+ transport was observed. The ATPase of the *E. coli* mutant MPC32N (cQ32N) was activated by NaCl and LiCl. The mutant ATPase exhibited a 5-fold higher K_m for NaCl but no change in the K_m for LiCl in comparison to that of the parent strain. These results demonstrate that the binding of Na^+ to the c subunit of *P. modestum* requires liganding groups provided by Q32, E65, and S66. For the coordination of Li^+, two liganding partners, E65 and S66, are sufficient, and H^+ translocation was mediated by E65 alone.

The central enzyme for energy conservation in mitochondria, chloroplasts or bacteria is the ATP synthase or F_1F_0 ATPase (*1*−*4*). Two different subgroups of the F_1F_0 ATPase family have been recognized that differ with respect to the coupling ion employed. While the mitochondrial, chloroplast, and most bacterial ATPases catalyze ATP synthesis from ADP and inorganic phosphate with $\Delta\mu H^+$ [1] as the driving force, *Propionigenium modestum* is the representative for bacterial ATPases that use $\Delta\mu Na^+$ as the driving force for ATP synthesis (*5*−*7*). Except for this difference, the ATPases of the two subgroups are remarkably similar with respect to structure and function which was convincingly demonstrated by the formation of functional hybrids between *P. modestum* F_0 and *Escherichia coli* F_1 moieties (*8*). To easily discriminate between these subgroups, we propose to designate the proton- and sodium ion-translocating F_1F_0 ATPases as FP-ATPases and FS-ATPases, respectively. Both ATPase subgroups consist of the water-soluble F_1 moiety that harbors the catalytic sites for ATP synthesis/hydrolysis and has the subunit composition $\alpha_3\beta_3\gamma\delta\epsilon$ and the membrane-bound F_0 moiety that is responsible for the translocation of the coupling ions and in bacteria has the subunit composition ab_2c_{9-12} (*9*). The globular F_1 head piece is connected to the F_0 part by a narrow stalk that contains subunits provided by F_1 and F_0 (*10*, *11*).

The high-resolution structure of most of the F_1 part of the ATPase from bovine heart mitochondria has suggested a catalytic mechanism involving rotation of the central γ subunit relative to the $\alpha_3\beta_3$ hexagon, and experimental evidence in favor of this rotation was obtained recently (*12*−*14*). High-resolution structural information on the stalk region and the F_0 part of the molecule is lacking, and therefore, the molecular details of the ion translocation mechamism and of the coupling between vectorial and chemical events remain elusive.

A significant contribution to the solution of these problems has come from biochemical and molecular biological studies of the Na^+-translocating ATPase from *P. modestum*. These investigations have clearly shown that the F_0 part is exclusively responsible for recognition and transport of the coupling ions and that the direct coupling hypothesis, where the coupling protons directly participate in the chemistry of ATP synthesis at the active site(s) of F_1, is not valid (*15*). Furthermore, a carrier mechanism (not a channel mechanism) was shown to apply for the transport of the coupling ions through F_0. The transport therefore involves binding of the coupling ions from one side of the membrane, followed by a conformational switch that makes the ions accessible to the opposite side of the membrane, to which they are subsequently released (*16*). Biochemical studies further

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[1] Abbreviations: $\Delta\mu H^+$, electrochemical gradient of protons across the membrane; $\Delta\mu Na^+$, electrochemical gradient of sodium ions across the membrane; DCCD, dicyclohexylcarbodiimide; ACMA, 9-amino-6-chloro-2-methoxyacridine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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Table 1: Bacterial Strains and Plasmids Used in This Study

strains/plasmids	relevant characteristics	source/reference
strains		
E. coli DH5α	*supE44* ΔlacU169 (*ϕ*80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
E. coli DK8	bglR HfrPO1 thi-1 relA1 ilv::Tn10 (Δ uncIBEFHAGDC)	ref 25
E. coli PEF42	CM1470 with *atpIBEFHA* from *P. modestum*	ref 21
E. coli MPC32I	PEF42 with *atpE Q32I*	this study
E. coli MPC32N	PEF42 with *atpE Q32N*	this study
E. coli MPC8487	PEF42 with *atpE F84L L87V*	ref 21
E. coli MPC848732I	PEF42 with *atpE Q32I F84L L87V*	this study
E. coli MPC848766A	PEF42 with *atpE S66A F84L L87V*	this study
plasmids		
pBluescriptKS$^+$	Ap^R; cloning vector	Stratagene
pMC32I	Ap^R (pKS); containing 833 bp *atpE* fragment with *Q32I*	this study
pMC32N	Ap^R (pKS); containing 833 bp *atpE* fragment with *Q32N*	this study
pMC66A	Ap^R (pKS); containing 735 bp *atpE* fragment with *S66A*	this study
pMC203I	Ap^R (pKS); containing 180 bp *atpE* fragment with *Q32I F84L L87V*	this study
pMC237A	Ap^R (pKS); containing 82 bp *atpE* fragment with *S66A F84L L87V*	this study
pHEP100	Ap^R (pKS); containing *atpIBEFHA′* from *P. modestum* and *uncAGDC* from *E. coli*	ref 20
pMC166A	pHEP100 with *atpE S66A*	this study

indicated that the Na^+ (or H^+) binding site is at the DCCD-reactive conserved acidic residue in the C-terminal membrane domain of subunit c (cE65 in the case of the *P. modestum* ATPase) (*17*, *18*). Mutational analyses performed with subunit c of the *E. coli* ATPase are in accord with these findings (*19*). The genetic approach has recently become available to F_0 from *P. modestum*. *E. coli* clones harboring the genes for the F_0 part and the *δ* subunit from *P. modestum* and for the remaining F_1 subunits from *E. coli* expressed a functional Na^+-dependent ATPase (FS-ATPase) and conferred to these cells Na^+-dependent growth on succinate (*19*, *20*). Following mutagenesis, a mutant was isolated, for which the Na^+-dependent growth was impaired and the ATPase functioned as a proton pump (*21*). The new strain had a double mutation (cF84L/cL87V) in the extended C-terminal portion of the *P. modestum* c subunit which is lacking in the corresponding *E. coli* protein. This mutation with the specificity change for the coupling ions further corroborates the model where the c subunits harbor the binding sites for the coupling ions during transport.

The operation of the *P. modestum* ATPase with Na^+, Li^+, or H^+ as alternative coupling ions offered unique possibilities for discerning the molecular basis for the selection between these cations. Clearly, cE65 is an appropriate H^+ binding site, but for Na^+ or Li^+ binding, additional liganding groups in an appropriate spacial vicinity are required. Remarkable differences in the primary structures of the c subunits are a serine residue at position 66 and a glutamine residue at position 32 of the *P. modestum* enzyme versus a conserved alanine and an isoleucine residue at the corresponding positions of the *E. coli* ATPase. As these residues are in close vicinity to the conserved acidic residue in the *E. coli* c subunit, for which part of the structure was determined in chloroform/methanol/H_2O (4:4:1) (*22*−*24*), S66 and Q32 of the *P. modestum* c subunit are key candidates for Na^+ or Li^+ binding ligands. We show here that the Na^+- or Li^+-dependent ATPase functions were completely abolished in all mutants with a cS66A substitution. The cQ32I mutation abolished Na^+-dependent but not Li^+-dependent ATPase functions, and in the ATPase with a cQ32N mutation, its Na^+- and Li^+-dependent functions were retained at a reduced affinity for Na^+ and no change in the affinity for Li^+. All mutants in addition retained the H^+-dependent ATPase functions, and these could be improved by additionally introducing the F84L/L87V double mutation into the c subunit.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media. The bacterial strains, cloning vectors, and recombinant plasmids used in this work are listed in Table 1. For routine cloning procedures, *E. coli* DH5α was applied. All *E. coli* strains were cultivated in M13 minimal media (*21*) supplemented with thiamine (0.1 mg/L) and 35 mM succinate or 10 mM glucose. *E. coli* strain DK8 (*25*) harboring *ilv::Tn10* was additionally supplemented with leucine, isoleucine, and valine at concentrations of 0.2 mM each. The same strain showed the Tet^R phenotype, and selection with 30 *μ*g/mL tetracyclin was convenient. For the screening of mutants, succinate minimal agar plates without NaCl were used. In order to reduce the internal Na^+ concentration to below 50 *μ*M, commercially available agar was purified as described (*21*).

Cloning and DNA Sequencing. Recombinant DNA procedures were carried out according to established protocols (*26*). DNA fragments were isolated from agarose gels with QIAEX (Diagen). DNA sequences were determined by the chain-termination method in combination with site-specific primers (*27*, *28*).

Site-Specific Mutagenesis of the P. modestum c Subunit. The mutations Q32I, S66A, F84L, and L87V were introduced into the *P. modestum* gene for the c subunit in different combinations by polymerase chain reaction (PCR) with specific primers (Table 2). Genomic DNA of *P. modestum* was amplified as depicted in Figure 1, and the mutated PCR products were cloned into the vector pBluescriptKS (*Eco*RV). Together with the mutations, different restriction sites that allowed the identification of the plasmid harboring the mutated subunit c gene were introduced (Table 2). The newly constructed plasmids were designated pMC32I (Q32I), pMC32N (Q32N), pMC66A (S66A), pMC203I (Q32I/F84L/L87V) and pMC237A (S66A/F84L/L87V).

Introduction of the Site-Specific Amino Acid Substitutions into the Hybrid ATPase. The introduction of the mutations into the hybrid ATPase was accomplished as previously described (*21*). In detail, *E. coli* PEF42 expressing a

Table 2: Oligonucleotides Used for PCR[a]

primer	sequence (5′−3′)	position	mutation
Pma180V	CCCCAAATGCCTAAGGGC	*uncB*; bp 180−197	none
Mpc32VI	GGA**AT**AGGGTATGCA***GCCGGC***AAA (*Nae*I)	*uncE*; bp 94−117	Q32I
Mpc32VN	GGA**A**A**C**GGGTATGCA***GCCGGC***AAA (*Nae*I)	*uncE*; bp 94−117	Q32N
Mpc66VA	***CGATCG***CGGAA**G**C**T**ACTGGTATCT (*Pvu*I)	*uncE*; bp 184−207	S66A
Mpc8487R	CCCA***AGTACT***CCAACAA**G**TGCCTT (*Sca*I)	*uncE*; bp 243−267	F84L L87V
Pmb507R	TAGCTTCTGCATATCTTCTAC	spacer *uncF-H*; bp 13−33	none

[a] All oligonucleotides bind to the *atp* operon of *P. modestum* as target DNA. The mutated bases are underlined, and the corresponding restriction sites are shown in italics.

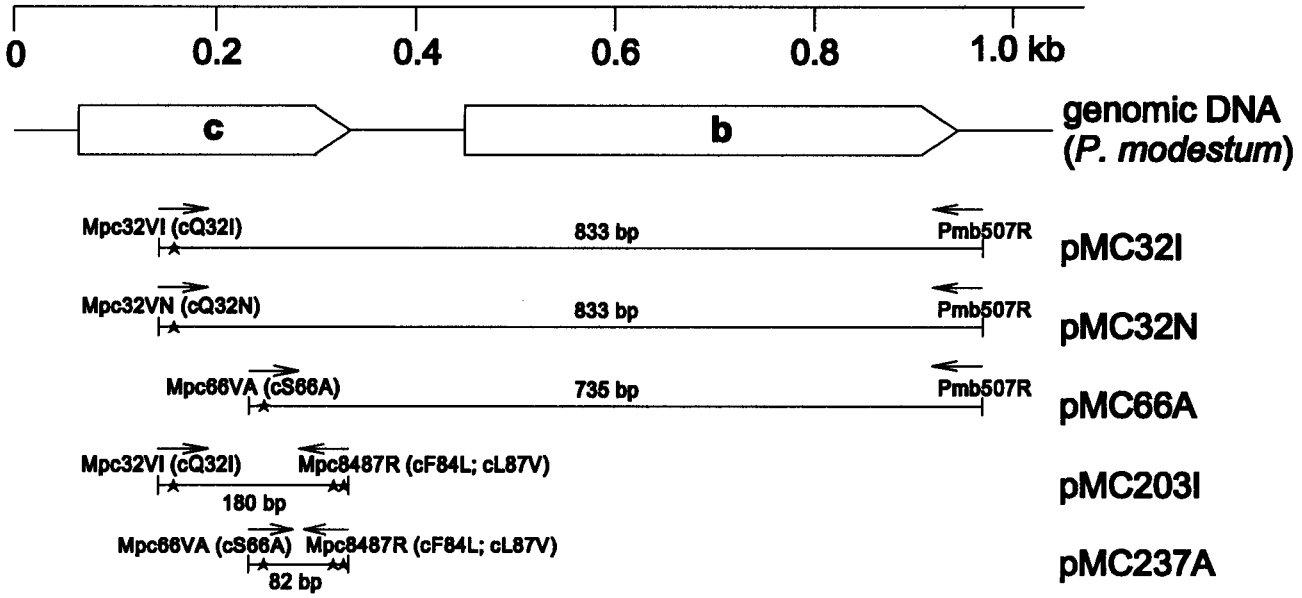

FIGURE 1: Construction of plasmids pMC32I, pMC32N, pMC66A, pMC203I, and pMC237A. The mutated PCR products were cloned into the vector pBluescriptKS. Mutations are indicated by an asterisk. ATPase genes are designated as the corresponding subunits.

functionally active Na^+-dependent hybrid ATPase was transformed with plasmids pMC32I, pMC32N, pMC66A, pMC203I, and pMC237A. The mutants were screened on succinate agar plates containing minimal amounts of Na^+ salts (insufficient for the growth of *E. coli* PEF42) and no ampicillin to select for homologous recombination of the mutated plasmid inserts into the genome of *E. coli* PEF42. After incubation for 48 h at 37 °C, colonies were obtained with plasmids pMC203I and pMC237A. The resulting mutant strains were termed MPC848732I and MPC848766A, respectively.

Since no colonies could be detected for plasmids pMC32I, pMC32N, and pMC66A, the screening was performed on succinate plates containing 10 mM NaCl. The transformants were incubated at 37 °C and scored for growth after 48 h, yielding small colonies ($\phi \leq 1$ mm) and large colonies ($\phi > 2$ mm) for PEF42/pMC32N. Fourteen of the small colonies were analyzed. The *E. coli* strain with the Q32N mutation was named MPC32N. As no colonies were obtained for plasmids pMC32I and pMC66A, we screened for recombination events on minimal agar plates with glucose as substrate. After incubation for 24 h at 37 °C, large colonies ($\phi > 2$ mm) and small colonies ($\phi \leq 1$ mm) could be distinguished for PEF42/pMC32I, but not for PEF42/pMC66A (uniform colonies; $\phi > 2$ mm). Six of the small colonies were isolated and shown to harbor the Q32I mutation. The new strain was named *E. coli* MPC32I. In all mutants described above, the presence of the intended substitutions and the absence of additional mutations were confirmed by DNA sequencing.

Construction of Plasmid pMC166A. Since attempts to construct the S66A amino acid substitution in the *P. modestum* c subunit of *E. coli* PEF42 via homologous recombination failed (see above), we used the previously established pHEP100 plasmid system for the construction of this mutant (*20*). For this purpose, the "megaprimer" method, based on site-directed mutagenesis by PCR, was used (*29*). The strategy for the mutant construction is shown in Figure 2. The first PCR was performed using the mutant primer (Mpc66VA) and the flanking primer (Pmb507R). The resulting 735 bp product was purified and used as one of the primers ("megaprimer") in the second PCR with the other flanking primer (Pma180V). Genomic DNA of *P. modestum* was used as the template in both polymerase chain reactions. The final 1673 bp product was isolated and digested with the restriction enzyme *Bfr*I. The restriction yielded a 1152 bp DNA fragment containing the S66A mutation on both strands. The *Bfr*I restriction of plasmid pHEP100 produced an 11 889 bp and a 1152 bp DNA fragment. The latter was replaced with the mutated 1152 bp (cS66A) PCR product in the subsequent ligation. The resulting plasmid was named pMC166A. The exclusive S66A mutation in the *P. modes-*

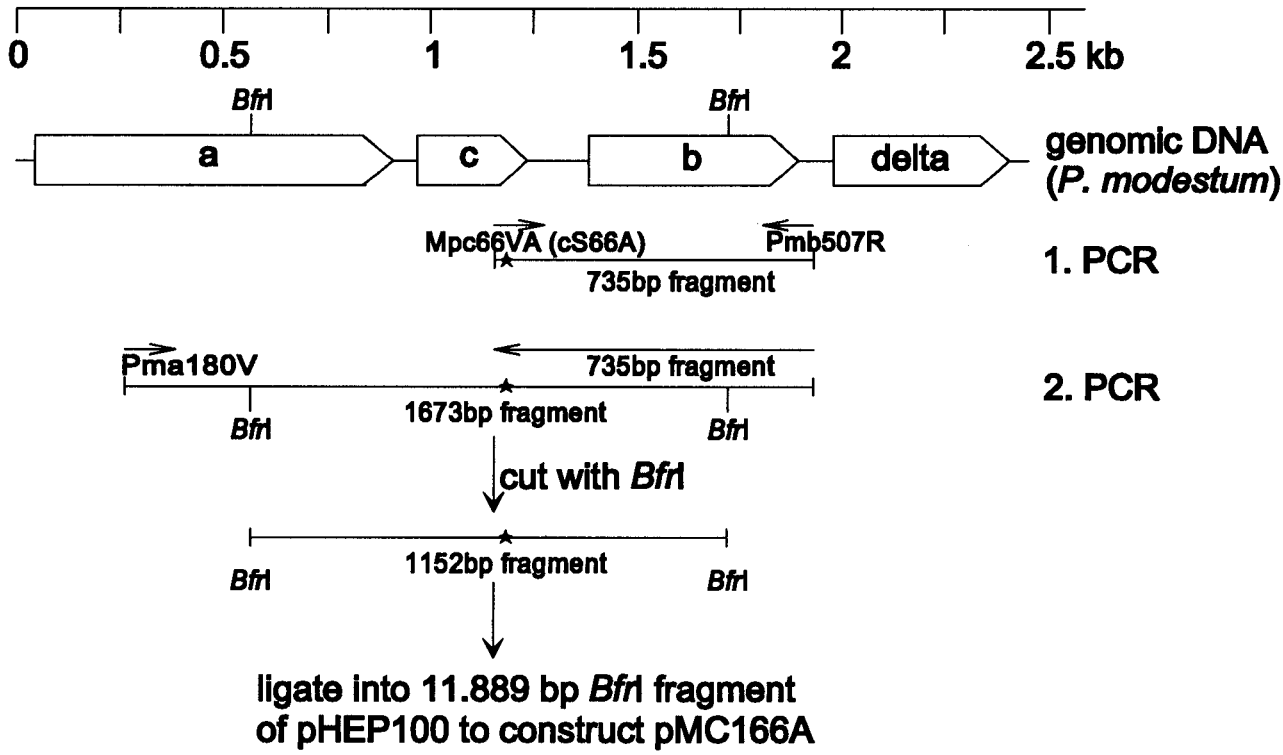

FIGURE 2: Construction of plasmid pMC166A. PCR products were amplified and cloned as described in the text. Mutations are indicated by an asterisk. ATPase genes are designated as the corresponding subunits.

tum c subunit gene of the hybrid ATPase was demonstrated by DNA sequencing.

Fluorescence Assay for NADH-Dependent H^+ Translocation. Membranes were prepared with a French press in P-buffer [50 mM potassium phosphate (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, and 10% (v/v) glycerol]. After fractional centrifugation, the F_1 moiety of the ATPase was stripped from the membranes by washing with E-buffer [1 mM Tris-HCl (pH 8.0), 0.5 mM K_2-EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol]. The stripped membranes were directly assayed by NADH-driven ACMA fluorescence quenching. The standard reaction mixture contained in 1.5 mL at 25 °C 50 mM potassium phosphate buffer (pH 7.5), 5 mM $MgCl_2$, 1.3 μM ACMA, and 200 μg of protein. After the signal had stabilized, the reaction was initiated by adding 25 μM potassium-NADH (Sigma). Fluorescence was measured with a Shimadzu RF-5001 PC spectrofluorometer using 410 nm as excitation and 480 nm as emission wavelengths.

Biochemical Procedures. Purification and determination of the ATPase and the fluorescence quenching assays for ATP-dependent H^+ translocation were performed as described (*19*). Reconstitution of ATPase into proteoliposomes was performed according to established protocols (*19*, *20*). Li^+ transport into proteoliposomes was measured by flame emission analysis (*21*). Protein was determined as described (*30*) with bovine serum albumin as the standard. Samples containing Triton X-100 were determined by comparison with protein standards containing the same amount of Triton X-100.

RESULTS

Growth Characteristics of E. coli Strains with Mutations in the c Subunit of a P. modestum/E. coli ATPase Hybrid. As described in the introductory section, cE65 of the *P. modestum* ATPase comprises part of the Na^+-binding active site for the transport of this coupling ion. Prominent candidates for the additional Na^+-liganding amino acids were S66 and Q32 because these are probably in an appropriate spatial vicinity with respect to E65 and because most proton-translocating ATPases contain apolar residues at the equivalent positions. To investigate the role of these amino acids in Na^+ binding, we changed cS66 to A and cQ32 to I and N as described in Experimental Procedures.

Neither strain MPC32I nor DK8/pMC166A was able to grow on succinate minimal medium with or without NaCl addition (Table 3). As the two parent strains grow well in succinate minimal medium supplemented with NaCl but not on succinate without NaCl addition, the reason for the growth impairment of the mutants could be the loss of the Na^+ binding site in the mutant ATPases. In contrast to these mutants, *E. coli* MPC32N could grow on succinate minimal medium, but only at 10 mM NaCl, whereas 2 mM NaCl was sufficient to support growth of the parent strain PEF42 (*19*). The most obvious explanation for these results is a decrease in the Na^+ binding affinity of the ATPase elicited by the cQ32N mutation.

The *P. modestum*/*E. coli* ATPase hybrid is optimized for protons as coupling ions by introducing the F84L/L87V double mutation into the c subunit (strain MPC8487) (*21*). Therefore, of interest was the study of the cQ32I and cS66A substitution in the MPC8487 background. Resulting strains MPC848732I and MPC848766A harboring the triple mutations grew on succinate which was independent of NaCl addition. Hence, oxidative phosphorylation in these strains obviously operates with H^+ as the coupling ion. The ATPase became optimized for this function by the cF84L/cL87V double mutation, irrespective of whether cS66 was additionally mutated to A or whether cQ32 was additionally mutated to I. In summary, these results demonstrate that cS66 and cQ32 play an important role in the Na^+-coupled ATP synthesis mechanism but can be replaced by other residues in a H^+-coupled ATP synthase.

Table 3: Phenotypic Properties of Chromosomal and Plasmid-Harboring Strains with Mutations in the c Subunit of the *E. coli*/*P. modestum* F_1F_0 ATPase Hybrid

strain/plasmid	mutations	growth on succinate (no NaCl)[a] (%)	growth on succinate (10 mM NaCl)[a] (%)	ATPase activity (membranes)[b] (mU/mg)	activation by Na^+	activation by Li^+
PEF42	none	24	100	400	+	+
MPC8487	F84L/L87V	95	96	390	−	+
MPC32I	Q32I	20	22	100	−	+
MPC32N	Q32N	21	79	320	+	+
DK8/pMC166A	S66A	14	15	80	−	−
MPC848732I	Q32I/F84L/L87V	61	63	240	−	+
MPC848766A	S66A/F84L/L87V	92	94	380	−	−

[a] Growth yield in 1% succinate minimal medium, calculated relative to *E. coli* PEF42. [b] The ATPase activities in membranes were corrected for residual activities obtained from *E. coli* strains with a Δ*unc* genotype. For maximum ATPase activities, cells were grown in succinate minimal medium. The ATPase activities of all membranes were recorded in the presence of 10 mM NaCl.

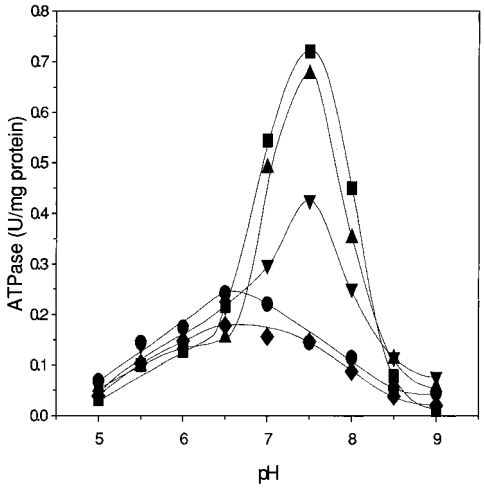

FIGURE 3: Dependence of ATPase activity from variuos *E. coli* strains on pH: PEF42 (●), MPC8487 (■), MPC848766A (▲), MPC848732I (▼), and MPC32N (◆). The ATPase was assayed after extraction from the membrane at the pH indicated. The estimated endogenous Na^+ concentration was below 50 μM.

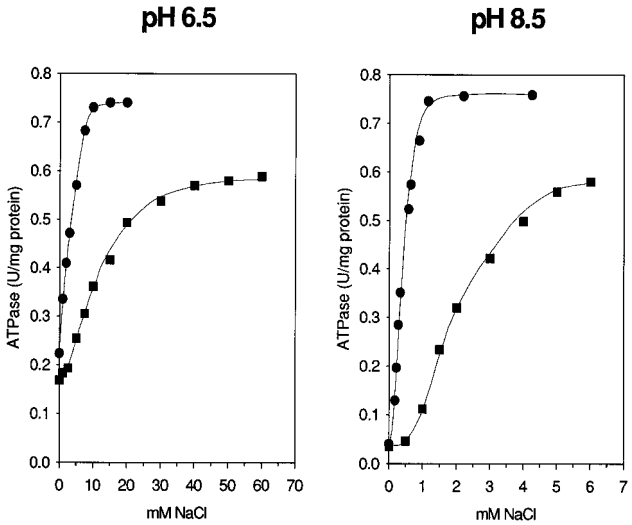

FIGURE 4: Activation profiles of the parent ATPase from *E. coli* PEF42 (●) and the mutated ATPase from *E. coli* MPC32N (■) by NaCl at pH 6.5 and 8.5. The ATPase activities were determined with purified enzymes and the buffer consisted of potassium-glycine, potassium-MOPS, and potassium-tricine (each at 20 mM), 100 mM K_2SO_4, and 0.05% Triton X-100 adjusted to the respective pH with KOH.

Biochemical Characterization of Mutant ATPases. The ATPase activities in the membranes of various mutant strains varied between 80−100 mu/(mg of protein) for cells harboring the cQ32I or cS66A single mutations and 400 mu/(mg of protein) for the parent strain PEF42 (Table 3). Activities between 240 and 390 mu/(mg of protein) were found, if these c subunit mutations were added to the cF84L/cL87V double mutation or for strain MPC32N with the cQ32N single mutation. It is also apparent that the succinate (−)- or (+)-phenotype correlates with ATPase activities of <100 or 240 mu/(mg of protein), respectively.

We have previously shown that introduction of the F84L/L87V double mutation into the c subunit shifts the pH optimum of the ATPase from 6.5 to 7.5 concomitant with a significant (Na^+-independent) activity increase (*21*). These properties reflect an improvement of H^+-coupled ATP synthesis so that the double-mutant strains acquire the succinate (+)-phenotype. The results of Figure 3 show similar pH profiles for ATPases from the single-mutant strains and from the parent strain PEF42 (in the absence of Na^+). Likewise, the pH profiles from the ATPases with triple mutations resemble that from the ATPases with the double mutation in the C-terminal tail of its c subunit. These data thus indicate the inevitability of the cF84L/cL87V double mutation for the improvement of the H^+-coupled ATP synthase activity of the *P. modestum* ATPase that is required for Na^+-independent growth on succinate.

The ATPase with the cQ32N mutation was the only one which retained the activation by Na^+ ions (Table 3). In contrast, the cQ32I or the cS66A mutation completely abolished this characteristic property of the *P. modestum* ATPase. These results therefore suggest that the hydroxyl group of S66 and the amide groups of Q32 or N32 are Na^+-binding ligands. Further support of this supposition was obtained by studying the effect of Na^+ on the ATPase with the Q32N substitution in more detail. The Na^+ activation profiles at pH 6.5 and 8.5 are shown in Figure 4. At both pH values, the mutant ATPase required Na^+ concentrations about 5 times higher for half-maximal or maximal activity, respectively, than the parent ATPase of strain PEF42. On the other hand, Hill plot analyses revealed that the cooperativity of both ATPases with respect to Na^+ activation was the same ($n_H = 1.0$ at pH 6.5; $n_H = 2.2$ at pH 8.5). These results indicate that reduction of the side chain by one CH_2 group in going from Q32 to N partially deteriorates the Na^+-binding site, requiring increased Na^+ concentrations for binding, while the substitution by I knocks out Na^+ binding completely. These findings are completely in accord with the growth studies described above.

Activation by Li^+ was observed with all mutant ATPases except those with a cS66A substitution (Table 3). The hydroxyl group of S66 is therefore clearly required as a

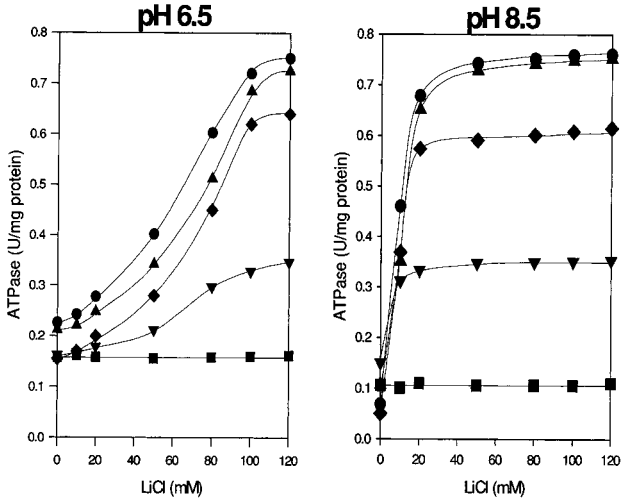

FIGURE 5: Activation profiles of ATPase from various *E. coli* strains by LiCl at pH 6.5 and 8.5: PEF42 (●), MPC848732I (▲), MPC32N (◆), MPC32I (▼), and MPC848766A (■). The ATPase activities were determined with enzymes purified from different strains. The buffer consisted of potassium-glycine, potassium-MOPS, and potassium-tricine (each at 20 mM), 100 mM K_2SO_4, and 0.05% Triton X-100 adjusted to the respective pH with KOH.

ligand not only for Na^+ but also for Li^+ binding. Activation profiles for several mutant ATPases by LiCl at pH 6.5 and 8.5 are shown in Figure 5. As expected from the results of Na^+ activation, the ATPase with the Q32N substitution was also well activated by Li^+ ions. In contrast to the Na^+ activation profiles, however, the Li^+ concentrations required for half-maximal activation were not significantly different for this mutant ATPase with respect to that from the parent strain PEF42. This result could indicate that the amide group of Q or N at position 32 of the c subunit is not required as ligand for Li^+ binding. This view is corroborated by a distinct Li^+ activation of the ATPases with a Q32I substitution. Interestingly, the maximal activity of the Li^+-stimulated ATPase harboring the F84L, L87V double mutation in addition to the Q32I substitution was higher by a factor of more than 2 than that of the ATPase with the Q32I mutation only. We have previously shown that the F84L/L87V double mutation does not alter the Li^+ activation profile of the *P. modestum* ATPase (*21*). Hence, introduction of the Q32I substitution in the vicinity of the Li^+ binding site severely affects the maximal velocity of the Li^+-stimulated ATPase, and this effect can be cured by the additional introduction of the double mutation.

H^+ and Li^+ Transport Experiments. To determine the coupling ion specificity of the mutant ATPases, H^+ and Li^+ transport experiments were performed. Proton transport by a selection of ATPases reconstituted into proteoliposomes, monitored by ACMA fluorescence quenching, is shown in Figure 6. The highest proton pumping activities were observed with the ATPase with the cQ32N mutation (MPC32N) and those with the triple mutations (MPC848732I and MPC848766A), while those with the corresponding single mutations (cQ32I in MPC32I or cS66A in pMC166A) were poor proton pumps only (not shown in part). Proton pumping by the ATPases with a cS66A mutation (pMC166A or MPC848766A) was not affected by Na^+ or Li^+ ions, indicating the loss of the binding site for either of these alkali ions. In ATPases with a cQ32I substitution (MPC32I or MPC848732I), NaCl still did not affect proton pumping but LiCl clearly reduced this activity. Hence, cS66 appears to be required for Na^+ or Li^+ binding, and cQ32 appears to be additionally required for Na^+ binding but not for Li^+ binding.

This conclusion is in accord with the effect of NaCl or LiCl on proton pumping by the ATPase with the cQ32N mutation (Figure 6). This activity was clearly inhibited by either of these alkali salts. Mutant and parent ATPases corresponded similarly to 20 mM LiCl, but NaCl concentrations required for comparable inhibition of proton pumping were about 5 times higher for the mutant than for the parent enzyme. These results support our conclusion that cQ32 is not a Li^+ binding ligand and further indicate that the amide group of this residue is involved in the liganding of Na^+ ions. The decreased Na^+ binding affinity in the ATPase with the cQ32N mutation probably results from a less perfect Na^+ binding geometry due to the smaller chain length of the N compared to the Q side chain.

To determine whether the poor proton pumping activities of the ATPases with the c subunit mutations Q32I or S66A were due to an inhibition of proton translocation through F_0, we investigated the passive proton permeability of F_1-stripped bacterial membranes. The kinetics and the extent of ACMA fluorescence quenching elicited by NADH addition are a measure of the tightness of these membranes for protons. If the quenching data with and without DCCD (an F_0-specific inhibitor) are compared, it is evident that passive proton flux occurs through all three F_0 moieties investigated, albeit with different activities (Figure 7). Clearly, the passive proton permeability increased from wild-type F_0 over that with the Q32I mutation to that with the S66A mutation. In accord with previous results, the proton permeability through *P. modestum* F_0 was disrupted in the presence of Na^+ or Li^+ ions (*16*). Interestingly, NaCl addition had no effect on the proton fluxes through F_0 containing the c subunit mutations Q32I (strain MPC32I) or S66A (strain DK8/pMC166A). LiCl blocked the passive proton permeability through F_0 with the cQ32I mutation and did not affect proton flux through F_0 with the cS66A substitution. These results are completely compatible with the effect of Na^+ or Li^+ on ATP-driven H^+ movement by the various mutant ATPases described above. Hence, Na^+, Li^+, or H^+ competes for binding and transport in wild-type F_0; Na^+ binding is abolished by mutating cQ32 to I, and Na^+ and Li^+ binding are abolished by mutating cS66 to A. At present, the mechanism for the increased proton permeability by the F_0 moieties with the Q32I or S66A mutation is unknown. Please note that the increased passive proton conduction through F_0 is in contrast to the low proton pumping activity of F_1F_0, indicating perhaps that these mutations affect the coupling between proton movement and F_1 functions or lead to an unphysiological path for the protons through the isolated F_0 moieties.

The validity of these conclusions was further investigated by Li^+ transport experiments. The results of Figure 8 show ATP-dependent Li^+ ion transport into proteoliposomes containing the ATPase from the parent strain PEF42 with an initial rate of about 5 nmol min^{-1} mg of $(lipids)^{-1}$. The Li^+ transport decreased to 60 and 30%, respectively, of this value if cQ32 was mutated to I in the cF84L/L87V or in the wild-type background, respectively. Only residual Li^+ transport into liposomes containing the poorly coupled ATPase of strain DK8/pMC166A occurred, and no Li^+ influx could be determined for the enzyme of strain MPC848766A.

A comparison of Li^+ transport into proteoliposomes containing the parent ATPase or that with the cQ32N

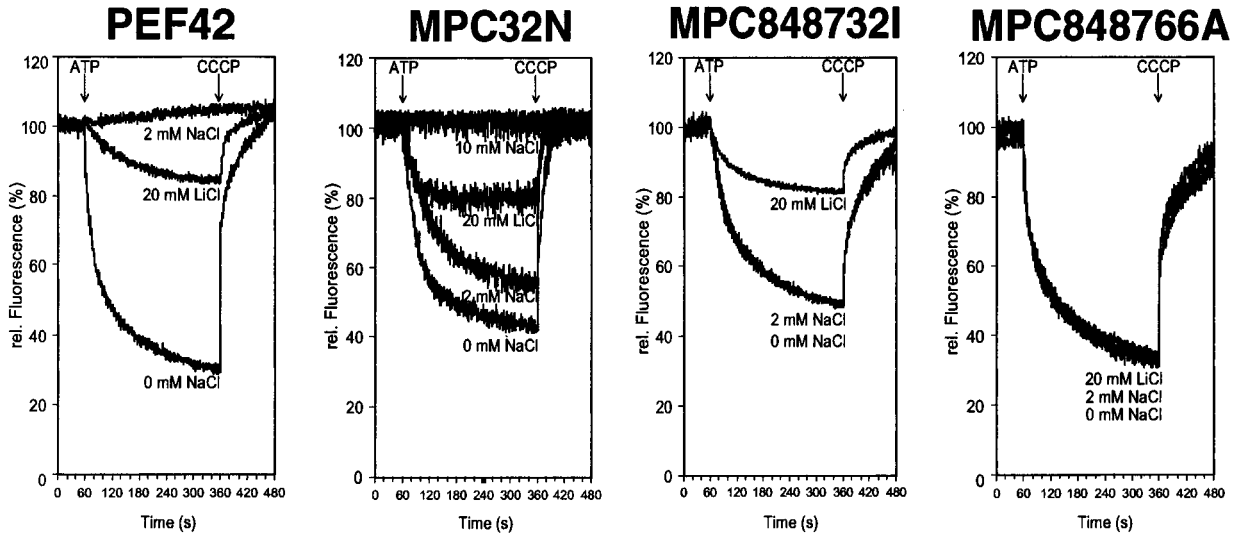

FIGURE 6: Effect of Na^+ and Li^+ on ATP-driven fluorescence quenching of ACMA by reconstituted proteoliposomes at pH 7.5. ACMA quenching was initiated by adding 2.5 mM K-ATP (↓) to reaction mixtures containing the purified hybrid and mutant ATPase reconstituted into proteoliposomes and the concentrations of NaCl and LiCl indicated. Quenching was released by the addition of 2 μM CCCP (↓).

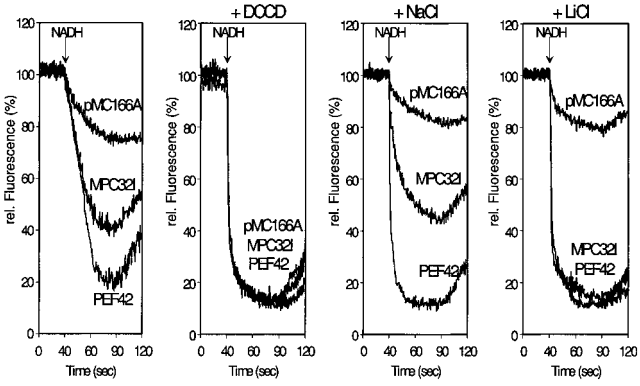

FIGURE 7: Comparison of proton permeabilities of parent and mutant membranes by NADH-driven ACMA quenching. The reaction mixture contained in a total of 1.5 mL 50 mM potassium phosphate buffer (pH 7.5), 0.2 mg of F_1-depleted membranes of the strains indicated, and 50 μM NADH, added at the arrow (Na^+ content of the reaction mixtures was below 50 μM). Increased proton permeability through the F_0 part of the ATPase is indicated by a diminished fluorescence quenching reaction. The reversal of the quenching response is due to exhaustion of the substrate NADH.

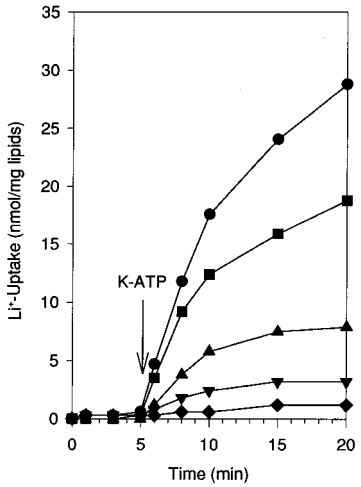

FIGURE 8: Comparison of Li^+ transport into reconstituted proteoliposomes of parent and mutant ATPases at pH 7.5. The same amounts of purified enzyme were reconstituted according to the freeze−thawing method. The assay contained 20 mM LiCl and 30 μM valinomycin: (●), *E. coli* PEF42 (■), *E. coli* MPC848732I (▲), *E. coli* MPC32I, (▼) *E. coli* DK8/pMC166A, and (◆) *E. coli* MPC848766A. Controls without ATP or with 20 μM DCCD did not catalyze any Li^+ transport (not shown).

substitution is shown in Figure 9. In the absence of Na^+, the mutant enzyme transported Li^+ ions almost as effectively as the parent enzyme. In the presence of 2 mM NaCl, Li^+ transport by the mutant enzyme was hardly affected, but that catalyzed by the parent ATPase was significantly inhibited. For a similar inhibition of Li^+ transport by the mutant enzyme, the NaCl concentration had to be increased to 10 mM. Hence, the Na^+ concentrations required for inhibition of Li^+ transport by the two ATPase specimens match those causing similar effects on H^+ transport (see above, Figure 6). These results add to the evidence for competition between Na^+, Li^+, and H^+ for the same binding site, which is at cE65 and involves cS66 as a ligand for Na^+ or Li^+ ions and cQ32 as an additional ligand for Na^+ ions.

DISCUSSION

There is now compelling evidence which shows that the two types of F_1F_0 ATPases (S and P type, see the introductory section), which can be distinguished by their coupling ion specificity, perform the same principle mechanism of ion translocation and coupling between vectorial and chemical events (for reviews, see 6 and 7). The Na^+-, Li^+-, or H^+-translocating *P. modestum* ATPase can therefore be employed as a model system, with which the ion translocation mechanism can be probed. This system has obvious experimental advantages. Na^+ or Li^+ movements can be measured more accurately than proton movements, and the putative binding site for alkali ions can be more conclusively explored using a mutational approach. While the essential feature of a proton binding site is a group that can be protonated or deprotonated under physiological conditions, an alkali ion binding site requires a set of liganding groups with an appropriate spatial geometry to trap the ion within this binding pocket. The knowledge of the liganding amino acids therefore indicates that these are in close spatial vicinity providing distinct distances between the liganding groups and the alkali ion. Furthermore, as Na^+, Li^+, and H^+ have been shown to compete for the same binding site in *P. modestum* F_0 (*17*, *31*), the proton binding site must contribute part of the liganding groups for the alkali ions and can therefore be identified, too.

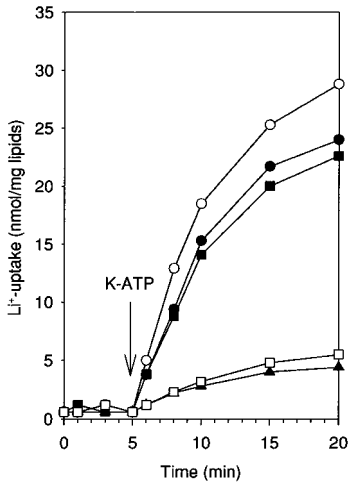

FIGURE 9: Comparison of Li^+ transport into reconstituted proteoliposomes of the parent ATPase from *E. coli* PEF42 and of the ATPase from *E. coli* MPC32N at pH 7.5. The same amounts of purified enzyme were reconstituted according to the freeze−thawing method. The assay contained 20 mM LiCl and 30 μM valinomycin: (○) *E. coli* PEF42 with 38 μM and (□) 2 mM NaCl and (●) *E. coli* MPC32N with 43 μM, (■) with 2 mM, and (▲) with 10 mM NaCl. Controls without ATP or with 20 μM DCCD did not catalyze any Li^+ transport (not shown).

Two sets of mutants have been employed to determine the binding site for the different coupling ions. In the first series of single mutants, we observed impairment or alteration of various Na^+- or Li^+-dependent activities of the *P. modestum* ATPase, such as growth on succinate, activation of the ATPase activity by alkali ions, interference of alkali ions with proton transport, Li^+ transport, and interference of Na^+ ions with Li^+ transport (see Results). The congruent results of these studies were an impairment of all Na^+- or Li^+-dependent activities upon substituting S66 with A. The Na^+-dependent activities of the ATPase were also abolished by mutating Q32 to I. Importantly, however, in the ATPase with the Q32N substitution, the Na^+-dependent activities were retained albeit at a decreased Na^+ binding affinity. This is exactly what one would expect, if the amide group of Q32 contributes ligands for the coordination of the Na^+ ions. The larger distance between the Na^+ ion and the amide group, resulting from the Q32N mutation, obviously affects the binding affinity to this alkali ion, while a residue without the amide ligand (Q32I substitution) completely abolishes Na^+ binding.

It is also interesting that the Li^+-dependent properties of the ATPase were retained in both Q32N or Q32I mutations. The smaller Li^+ ion is therefore evidently liganded without the participation of the amide group of Q32. From these results, one would predict that the Q32N and Q32I single mutations do not significantly affect the global structure of the molecule but rather are of local significance. This prediction can likely be extended also to the S66A single mutation with arguments discussed below for the double and triple mutations. The most likely interpretation of these results is that S66 of the *P. modestum* c subunit contributes an essential liganding group for Na^+ and Li^+ binding and that the amide group of Q32 contributes an additional ligand for Na^+ but not fot Li^+ binding. Together with our previous discovery that glutamate 65 is the essential residue promoting Na^+ or H^+ binding during the translocation of these cations (*17*, *18*), these results provide important insights into the mechanism for selecting and translocating the coupling ions across the F_0 sector of the ATPase. Clearly, in the three-dimensional structure of the c subunit, the carboxylate of E65 and the hydroxyl group of the adjacent S66 must be oriented to give appropriate distances for the binding of Na^+ or Li^+ ions. In addition, the amide group of glutamine 32 must complete the coordination sphere for the Na^+ ions and must therefore also be an appropriate distance from this ion. An overview of the Na^+ or Li^+ binding ligands and of the mutational alterations of the liganding groups is shown in Figure 10.

In a second series of mutations, potential alkali ion-liganding residues were altered in the background of the cF84L/cL87V double mutation. This double mutation has previously been demonstrated to impair the Na^+-dependent properties of the *P. modestum* ATPase with retention of the Li^+-dependent properties. In addition, the mutation led to an improvement of the proton-coupled ATP synthase function as shown by Na^+-independent growth of the mutant strain on succinate. This is reflected *in vitro* by an about 3-fold increase of the Na^+-independent specific ATPase activity in combination with a shift of the pH optimum from pH 6.5 to 7.5 in going from the wild-type ATPase to that with the double mutation. The molecular basis for these properties could be a more global structural change of the c subunit, possibly initiated by a different interaction of the altered amino acids in the C-terminal part of the molecule with amino acids on the opposite strand of the helical hairpin, thereby affecting the geometry of the Na^+ binding sphere around E65. Specifically, one could imagine that Q32 is sufficiently displaced in the mutant from its original location so that it is no longer capable of acting as a Na^+ binding ligand (Figure 10). E65 and S66 are on the other hand adjacent residues of a putative α-helical structure. An alteration of the geometry of the liganding groups provided by these residues is therefore less likely. Our results on the retention of Li^+ binding and impairment of Na^+ binding by the F84L/L87V double mutation are completely in accord with this model. The model is further corroborated by triple mutations. The Li^+-dependent activities were thus retained by changing Q32 to I and were abolished by changing S66 to A, both in the background of the F84L/L87V double mutation. Please note that the parent ATPase and those with single amino acid exchanges around E65 have the same pH activation profile with an optimum at pH 6.5, whereas for all ATPases harboring the F84L/L87V double mutation, the activation profile was shifted to yield an optimum at pH 7.5. The shift of the pH profile in ATPases with the double mutation probably reflects a global structural change of the c subunit affecting the environment of E65 (see above). Upon displacement of Q32, the environment around E65 may become more hydrophobic which should lead to an increase of the p*K* of the E65 carboxylate, in accordance with the observed shift of the pH optimum by 1 unit to alkaline. The identical pH profile of the parent ATPase and that with the Q32N single mutation on the other hand argue against a global structural change elicited by this mutant. For the S66A single mutant, the pH profile was difficult to determine, because of the low ATPase activity of this mutant, but the data indicate a profile similar to that of the parent ATPase and certainly not shifted to that of the ATPases harboring the double mutation.

These results provide compelling evidence of how the specificity for the coupling ions by F_1F_0 ATPases is determined. Essential for Na^+ translocation is a coordination

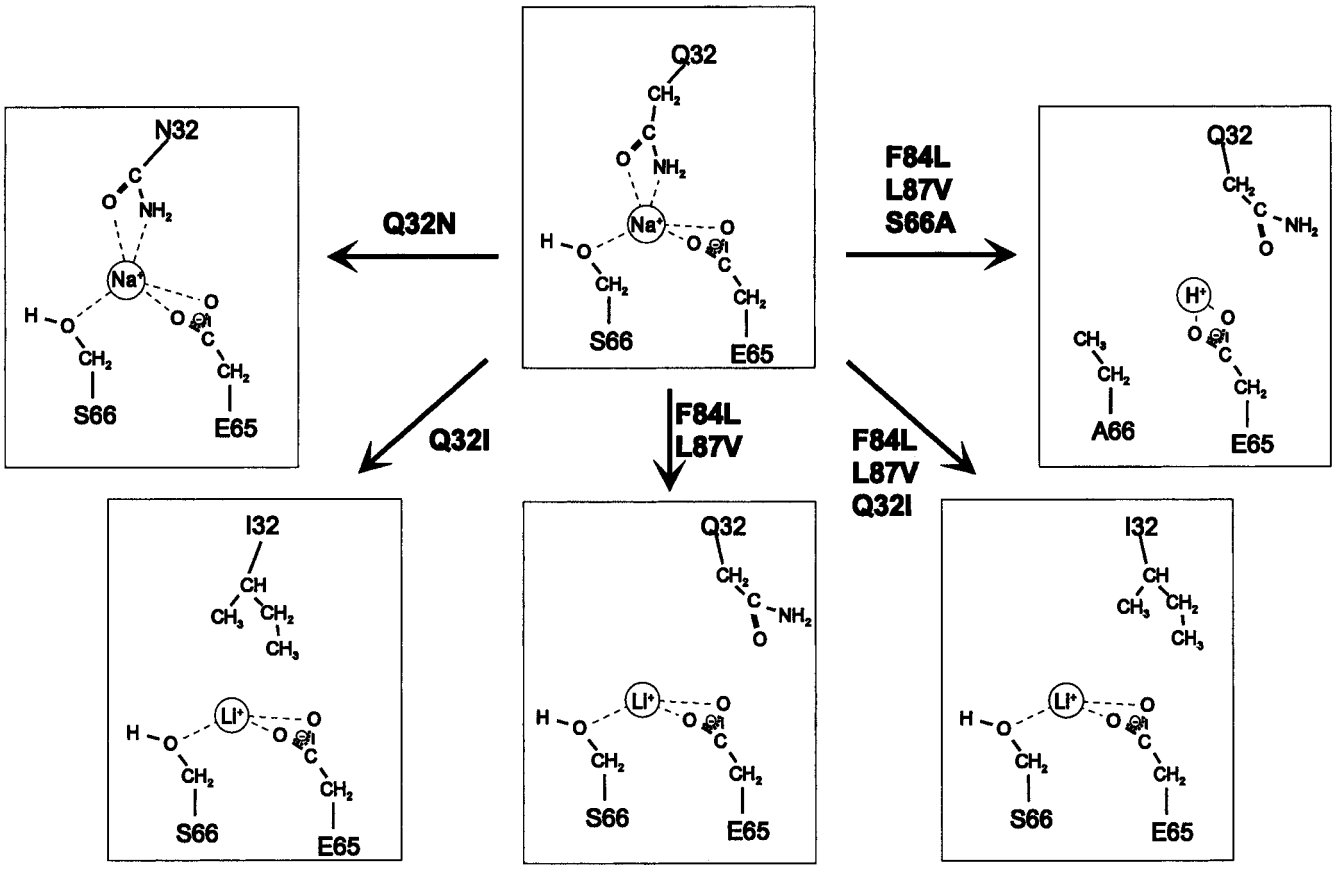

FIGURE 10: Model of the cation binding site of the *P. modestum* c subunit. The center panel illustrates the coordination of the Na^+ ion within the wild-type c subunit involving residues Q32, E65, and S66. The introduced amino acid substitutions are indicated by an arrow, and the effects on cation binding are summarized in the neighboring panels. Please note that Na^+ binding likewise includes Li^+ and H^+ binding and that Li^+ binding also includes H^+ binding.

sphere for this cation including cE65 and additional amino acids cS66 and cQ32. For the smaller Li^+ ions, the coordination sphere consists of cE65 and cS66, and for proton translocation, cE65 is sufficient as a binding site. To accomplish the translocation of these coupling ions, they must reach their binding site from one side of the membrane and must eventually be released to the other surface, which obviously requires a switch in the accessibility of the binding site. The most simple way for the three different ions to reach their binding site is via a water-filled access channel. The alkali ions hydrated by (partial) stripping off their water shell will be coordinated by the liganding groups described above. Likewise, the carboxylate of E65 might take over a proton from a water molecule or from H_3O^+. As the hydrolysis of ATP is activated at high pH by Na^+ or Li^+ ions in a cooperative manner ($n_H = 2.6$) (*18*), we conclude that at least three of these binding sites at different c subunits must be occupied simultaneously to reach maximal activity. These results indicate that the binding sites at these c subunits are probably accessible without the participation of another subunit. Subunit a which is believed to participate in the ion transport besides subunit c could have a specific role in releasing the bound coupling ions from the binding site on the c subunit and in guiding them to the other side of the membrane (in the ATP hydrolysis mode of the enzyme). This process may include ion exchange with a specific arginine residue (*18*) and may involve rotation of the ring of c subunits relative to the a subunit. Ion translocation through the F_0 part may thus lead to a rotation that could be connected to the rotational movement of the γ subunit relative to the $\alpha_3\beta_3$ hexagon that has been noticed for the F_1 part of the ATPase (*12*−*14*).

Some observations reported for the *E. coli* ATPase are congruent with the model for the c subunit function described here. Determination of part of the *E. coli* c subunit structure by NMR indicated two antiparallel membrane-spanning α-helices that are connected by a hydrophilic loop (*22*−*24*). The conserved DCCD-sensitive acidic residue in the C-terminal membrane-spanning helix (D61), which is at a position equivalent to *P. modestum* cE65, is essential for H^+ translocation (for a review, see ref 3). The *E. coli* ATPase, which is specific for protons, lacks the Na^+ binding ligands of the *P. modestum* c subunit (S66 is replaced by A and Q32 is replaced by I). According to the structure, the isoleucine residue of the *E. coli* c subunit is located in the vicinity to D61 on the opposite helix. Upon mutation of residues neighboring D61 of the *E. coli* c subunit to the corresponding ones of the *P. modestum* c subunit, a Li^+ binding site was created (*32*). Unlike that of the *P. modestum* enzyme, however, Li^+ binding produced a dead end complex that was unable to translocate this alkali ion across the membrane. A Na^+ binding site was not acquired by the mutated *E. coli* c subunit. In light of our present study, this can be readily explained by the absence of a Na^+ binding ligand on the opposite helix (Q32 in the *P. modestum* c subunit).

If all oxygen and nitrogen atoms of the amino acid side chains identified to participate in Na^+ binding would provide ligands for this alkali ion, it would be coordinated by five different ligands. This number is in the range expected for this alkali ion (*33*), especially within a protein, where the

number of liganding groups should be at the low end of the range covered by the same alkali ion in small molecule structures such as crown ethers, because of sterical restrictions. We cannot exclude, however, the fact that additional ligands are derived from oxygen atoms of the backbone or from bound water molecules. As in the case of Li^+ binding, the amide group of Q32 is not involved; this is left with only three oxygen atoms from the side chains of E65 and S66 as potential ligands. Therefore, for proper coordination of this alkali ion, water molecules may be included. An example of replacing a ligand provided by the protein for coordinating a large alkali ion (K^+) by a water molecule for coordinating a smaller alkali ion (Na^+ or Li^+) has been described for the alkali binding dialkylglycine decarboxylase for which the structure was determined with different alkali ions bound (*34*). For this enzyme, it was shown that the basis for activation by the larger K^+ and inhibition by the smaller Na^+ ion is a different conformation of the protein assumed upon binding of either of these alkali ions.

In summary, these results demonstrate that the molecular basis for the coupling ion specificity of F_1F_0 ATPases is a cation binding site in subunit c that includes the conserved acidic residue in the C-terminal α helix (E65 in the case of *P. modestum*) and in case of alkali ion binding additional liganding groups in the appropriate spatial vicinity. After binding of the coupling ion to this site, the next step of the transport must be a conformational switch by which the binding site becomes accessible to the opposite surface of the membrane. After releasing the coupling ions to this side, the cycle is completed by a second conformational switch that restores the original accessibility of the binding site. The molecular bases of these switches and for the coupling mechanism between ion movement and ATP synthesis/hydrolysis at the F_1 moiety remain challenges for the future.

ACKNOWLEDGMENT

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