A Triple Mutation in the a Subunit of the *Escherichia coli/Propionigenium modestum* F_1F_o ATPase Hybrid Causes a Switch from Na^+ Stimulation to Na^+ Inhibition

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ABSTRACT: Previously we have shown that the Na^+-translocating *Escherichia coli* ($F_1-\delta$)/*Propionigenium modestum* ($F_o+\delta$) hybrid ATPase acquires a Na^+-independent phenotype by the c subunit double mutation F84L, L87V that is reflected by Na^+-independent growth of the mutant strain MPC8487 on succinate [Kaim, G., and Dimroth, P. (1995) *J. Mol. Biol. 253*, 726−738]. Here we describe a new class of mutants that were obtained by random mutagenesis and screening for Na^+-independent growth on succinate. All six mutants of the new class contained four mutations in the a subunit (S89P, K220R, V264E, I278N). Results from site-specific mutagenesis revealed that the substitutions K220R, V264E, and I278N were sufficient to create the new phenotype. The resulting *E. coli* mutant strain MPA762 could only grow in the absence but not in the presence of Na^+ ions on succinate minimal medium. This effect of Na^+ ions on growth correlated with a Na^+-specific inhibition of the mutant ATPase. The K_i for NaCl was 1.5 mM at pH 6.5, similar to the K_m for NaCl in activating the parent hybrid ATPase at this pH. On the other hand, activation by Li^+ ions was retained in the new mutant ATPase. In the absence of Na^+ or Li^+, the mutant enzyme had the same pH optimum at pH 6.5 and twice the specific activity as the parent hybrid ATPase. In accordance with the kinetic data, the reconstituted mutant ATPase catalyzed H^+ or Li^+ transport but no Na^+ transport. These results show for the first time that the coupling ion selectivity of F_1F_o ATPases is determined by structural elements not only of the c subunit but also of the a subunit.

The F_1F_o ATPases are responsible for ATP synthesis from ADP and inorganic phosphate in mitochondria, chloroplasts, or bacteria (*1*−*5*). These enzymes consist of a water-soluble headpiece, F_1, with the subunit composition $\alpha_3\beta_3\gamma\delta\epsilon$ and a membrane-integral sector, F_o, that in bacteria has the subunit composition ab_2c_{9-12}. The F_1 sector contains the catalytic machinery for the ATP synthesis and the F_o sector for the coupling ion movement across the membrane, and both processes are intimately coupled events.

Most F_1F_o ATPases including that from *Escherichia coli* use protons as the exclusive coupling ions and have therefore been classified as FP ATPases (*6*). F_1F_o ATPases, that use Na^+ as the physiological coupling ion, were classified as FS ATPases (*6*). The paradigm of this group is the ATPase of *Propionigenium modestum* (*7*−*9*). An important early finding made with this enzyme was the use of Na^+, Li^+, or H^+ as alternative coupling ions in a competitive manner, indicating the same translocation mechanism for either of these cations (*9*). The operation of all F_1F_o ATPases by the same principle mechanism was further corroborated by the construction of functional hybrids. First, an *E. coli* F_1/*P. modestum* F_o hybrid was obtained by in vitro reconstitution (*10*), and later molecular biology techniques led to the in vivo synthesis of an *E. coli* ($F_1-\delta$)/*P. modestum* ($F_o+\delta$) ATPase hybrid (*11, 12*). Remarkably, these hybrid ATPases exhibited the same coupling ion specificity as the *P. modestum* ATPase, showing that the F_o part is exclusively responsible for the recognition of the coupling ions. Important insights into the mode of ion translocation have been obtained with *P. modestum* F_o, reconstituted into proteoliposomes. These proteoliposomes catalyzed $\Delta\psi$-dependent[1] Na^+ transport following saturation kinetics or Na^+ counterflow (*13*). A transporter type mechanism was therefore envisaged employing a binding site that becomes occupied with the coupling ion from one side of the membrane and releases it to the other side after a conformational switch had altered the accessibility of this site.

The Na^+ binding site was subsequently defined to be at the DCCD-reactive glutamate-65 residue of subunit c of the *P. modestum* ATPase, because Na^+ ions specifically protected the enzyme from the reaction with the carbodiimide (*14, 15*). These conclusions were confirmed and extended by a mutational approach showing that cQ32 and cS66 were additional Na^+ binding ligands (*6*). Extensive mutational analysis data obtained with *E. coli* subunit c (*1*−*4*) could be well fitted into this model of ion translocation, but the conclusiveness of the model rests to a great deal on the data obtained with the *P. modestum* ATPase (*5*).

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[1] Abbreviations: $\Delta\psi$, transmembrane electrical potential; ACMA, 9-amino-6-chloro-2-methoxyacridine; DCCD, dicyclohexylcarbodiimide; PCR, polymerase chain reaction(s).

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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*Δ*M*15) *hsdR17 recA1 endA1 gyrA96 thi−1 relA1*	Bethesda Research Laboratories
E. coli DK8	bglR HfrPO1 thi−1 relA1 ilv::Tn10 (ΔuncIBEFHAGDC)	ref *17*
E. coli PEF42	CM1470 with *atpIBEFHA* from *P. modestum*	ref *11*
E. coli MPC8487	PEF42 with *atpE F84L L87V*	ref *16*
E. coli MPA762	PEF42 with *atpB K220R V264E I278N*	this study
plasmids		
pBluescriptKS$^+$	Ap^R; cloning vector	Stratagene
pMA851	Ap^R (p*KS*); containing 584 bp *atpB* fragment with *S89P K220R V264E I278N*	this study
pMA762	Ap^R (p*KS*); containing 193 bp *atpB* fragment with *K220R V264E I278N*	this study
pMA542	Ap^R (p*KS*); containing 654 bp *atpB* fragment with *V264E I278N*	this study
pMA498	Ap^R (p*KS*); containing 199 bp *atpB* fragment with *K220R I278N*	this study
pMA484	Ap^R (p*KS*); containing 161 bp *atpB* fragment with *K220R V264E*	this study
pHEP100	Ap^R (p*KS*); containing *atpIBEFHA*′ from *P. modestum* and *uncAGDC* from *E. coli*	ref *12*

Based on a random mutational approach, a double mutation in the C-terminal tail of the *P. modestum* c subunit has been identified (F84L, L87V) that caused a switch from Na^+- to H^+-coupled ATP synthesis in the *E. coli* host cells (*16*). But these mutants were not the only ones which could grow on succinate without Na^+ addition. We report here on a triple mutation in the a subunit which grows on succinate minimal medium in the absence of Na^+ ions. A characteristic of this new mutant is its growth inhibition by Na^+ ions which correlates with a specific inhibition by Na^+ of the mutant ATPase.

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 (*16*) supplemented with thiamin (0.1 mg/L) and 35 mM succinate or 10 mM glucose. *E. coli* strain DK8 (*17*) 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 μg/mL tetracyclin was convenient. For the screening of mutants, succinate minimal agar plates without NaCl were used. To reduce the internal Na^+ concentration below 50 μM, commercially available agar was purified as described (*16*).

Cloning and DNA Sequencing. Recombinant DNA procedures were carried out according to established protocols (*18*). 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 (*19, 20*). The protocols and equipment for automated DNA sequencing were applied (Sequenator 370A and *Taq* dye-Deoxy Terminator Sequencing Kit from Applied Biosystems).

Random Mutagenesis and Screening of Random Mutants. For random mutagenesis, plasmid pHEP100 (*12*) was used, and the in vitro mutagenesis with hydroxylamine was carried out as described (*16, 21*). The mutated pHEP100 derivatives (pHM plasmid series) were constructed by replacing the nonmutated 2.8 kb *Eco*O109I/*Kpn*I DNA fragment (containing *atp*IBEFHA′ from *P. modestum*) from pHEP100 with the mutated 2.8 kb *Eco*O109I/*Kpn*I DNA fragments (*16*). After transformation into *E. coli* DK8 (*17*), the mutants were screened for growth on succinate minimal agar plates with low Na^+ content (below 40 μM) as described (*16*).

Site-Specific Mutagenesis of the P. modestum a Subunit and Introduction of the Amino Acid Substitutions into the Hybrid ATPase. In the first step, the mutations S89P, K220R, V264E, and I278N were introduced into the *P. modestum* gene for the a subunit in different combinations by polymerase chain reaction with specific oligonucleotides (see legend to Figure 1). 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) (*22*). Together with the mutations, *Eco*RI restriction sites were introduced that allowed the identification of the plasmid harboring the mutated a subunit gene. The newly constructed plasmids were designated pMA851 (584 bp insert; S89P, K220R, V264E, I278N), pMA762 (193 bp insert; K220R, V264E, I278N), pMA542 (654 bp insert; V264E, I278N), pMA498 (199 bp insert; K220R, I278N), and pMA484 (161 bp insert; K220R, V264E), respectively. In the second step, the introduction of the mutations into the hybrid ATPase was accomplished by transforming *E. coli* PEF42 expressing a functionally active Na^+-dependent hybrid ATPase with each of these plasmids. The mutants were screened for Na^+-independent growth 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 plasmids into the genome of *E. coli* PEF42 (*16*).

Biochemical Procedures. Purification and determination of the ATPase, the fluorescence quenching assays for ATP-dependent H^+ translocation, and the determination of $^{22}Na^+$ transport were performed as described (*11, 12*). The ATPase was reconstituted into proteoliposomes according to established protocols (*11, 12*). Li^+ transport into proteoliposomes was measured by flame emission analysis (*16*). Protein was determined as described (*23*) 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.

RESULTS

Random Mutagenesis, Screening, and DNA Sequence Analysis of the Mutants. We have previously shown that

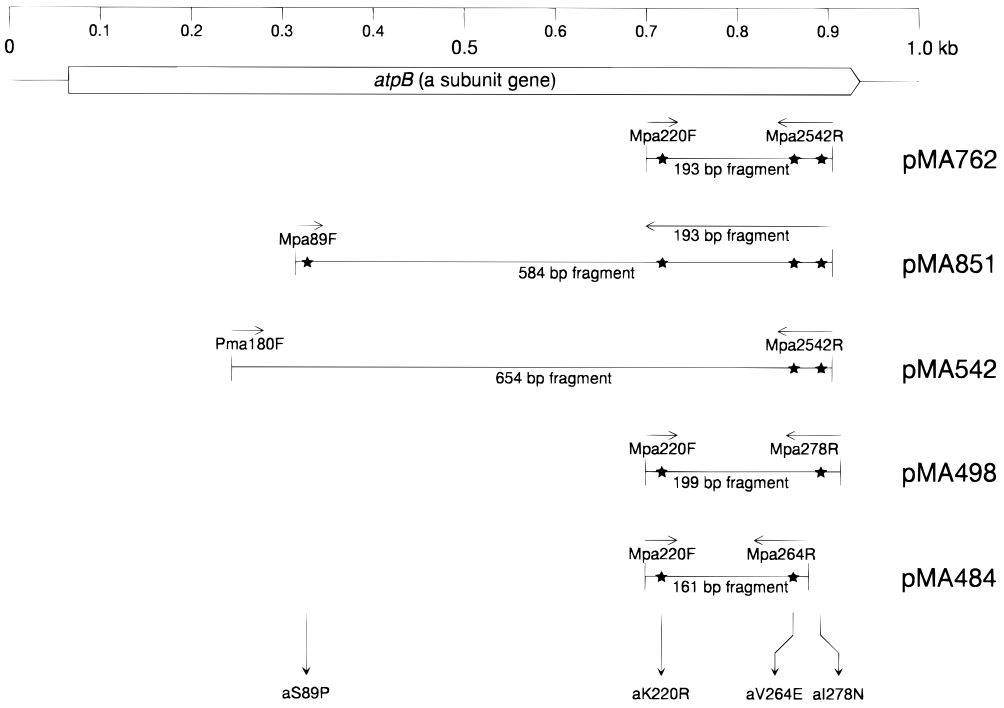

FIGURE 1: Construction of plasmids pMA762, pMA851, pMA542, pMA498, and pMA484. Chromosomal *P. modestum* DNA was amplified by PCR with the primer pairs indicated. The following oligonucleotides were used for PCR (the mutated bases are underlined; the *Eco*RI restriction site is shown in italics): Mpa220F (GA*GAATTC*GCAAGACCAACGAACATT), Mpa2542R (TTGATTATAAACCATTGT-CAGCATGATGAATACGAAACTTTGTTCCAC), Mpa89F (TTAGATGGAGTCCAAAGAATCTGAA), Pma180F (CCCCAAATGC-CTAAGGGC), Mpa278R (CAATAGATCCTTGATTATAAACCATTGT), and Mpa264R (ACGAAACTTTGTTCCACACCACTGAA). The mutated PCR products were cloned into the vector pBluescriptKS. Mutations are indicated by an asterisk.

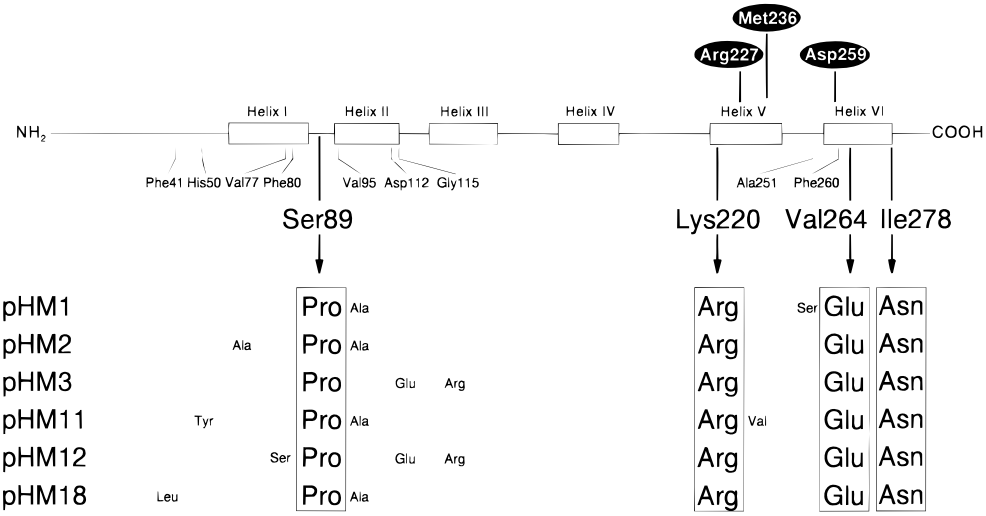

FIGURE 2: Random mutations in the gene of the a subunit of the pHM plasmids containing the mutated hybrid *atp* operon. The upper lines show the putative helices of the *P. modestum* a subunit and the distribution of the random mutations. The amino acid residues Arg227, Met236, and Asp259 in *P. modestum* (black underlayed ovals) correspond to residues Arg210, Glu219, and His245 in the *E. coli* a subunit, respectively. The lower section summarizes the amino acid replacements in the *P. modestum* a subunit.

after transforming plasmid pHEP100 into the *E. coli atp* deletion strain DK8, Na^+-dependent growth on succinate was acquired (*12*). Plasmid pHEP100 harbors the ATPase genes for subunits a, b, c, and δ from *P. modestum* and those for α, β, γ, and ϵ from *E. coli*. Random mutagenesis of the hybrid *atp* operon with hydroxylamine and screening for Na^+-independent growth on succinate allowed the isolation of 18 transformants (*16*). Based on DNA sequence analysis of these mutants, two families could be distinguished. The 12 mutants of the first family contained the F84L, L87V double mutation in the c subunit besides various other mutations in the a and c subunits. It was shown previously that the double mutation in subunit c is sufficient to confer the Na^+-independent phenotype to the *E. coli* host cells (*16*).

The F84L, L87V double mutation was absent in the remaining six mutants with the phenotype of Na^+-independent growth on succinate. DNA sequence analyses of the gene for the a subunit of these mutants revealed a total of 38 mutations (Figure 2). Of these mutations, 18 were found between residues 41 and 115 in putative helices 1 and 2, and 20 were located between residues 220 and 278 in putative helices 5 and 6. Importantly, all mutants contained the four

amino acid substitutions S89P, K220R, V264E, and I278N. These were therefore the key candidates for generating the new phenotype.

Site-Specific Mutagenesis of aS89P, aK220R, aV264E, and aI278N. To investigate whether the four mutations in the a subunit are sufficient for the Na^+-independent growth phenotype, these were introduced into the plasmid pMA851 (Figure 1) by PCR-based site-specific mutagenesis (*22*). The plasmid was then transformed into *E. coli* PEF42 that harbors the genes for the Na^+-dependent hybrid ATPase on the chromosome. The transformants were screened for Na^+-independent growth on succinate minimal agar plates to identify clones in which the a subunit mutations had been introduced into the genome by homologous recombination. To verify the mutations, chromosomal DNA fragments comprising the gene of the a subunit were amplified by PCR and sequenced on both strands. As a result, only the three mutations aK220R, aV264E, and aI278N could be identified, whereas the aS89P mutation was not found on the genome of the transformants analyzed.

The aS89P substitution is therefore obviously not required to determine the Na^+-independent growth phenotype. Consequently, the crossovers leading to homologous recombination (*11*) could have occurred downstream of aS89. Transformants with the Na^+-independent growth phenotype were also obtained with plasmid pMA762 that contains only the three mutations aK220R, aV2654E, and aI278N (Figure 1). The introduction of all three mutations into the chromosome of these transformants was confirmed by DNA sequencing as described above. We conclude from these results that the triple mutation in the a subunit of the hybrid ATPase (K220R, V264E, I278N) is exclusively responsible for the phenotype of Na^+-independent growth on succinate and that no further mutations are required. This conclusion was confirmed with plasmids pMA542, pMA498, and pMA484, which contained the three possible combinations of double mutations (aK220R/aV264E; aK220R/aI278N; aV264E/aI278N), respectively (Figure 1). After the transformation of these plasmids into *E. coli* PEF42, no transformants were obtained after screening for Na^+-independent growth on succinate minimal medium. The new mutant strain with the aK220R, aV264E, aI278N triple mutation was designated *E. coli* MPA762 and was used for all further characterizations.

Growth Characteristics of E. coli MPA762. Growth curves in succinate minimal medium with and without NaCl addition were recorded with the mutant strain *E. coli* MPA762 and the parent strain *E. coli* PEF42 (Figure 3A,B). In the absence of Na^+, strain MPA762 grew on succinate minimal media, as expected. The growth curves were the same as that of strain PEF42 in the presence of 10 mM NaCl. Both strains reached the stationary phase after about 35 h at an optical density at 600 nm of 1.2. Quite surprisingly, the growth of strain MPA762 was gradually diminished in a NaCl-dependent manner, and virtually no growth could be observed in the presence of 10 mM NaCl. Please recall that Na^+ ions affect the growth of strain PEF42 on succinate exactly opposite to strain MPA762; i.e., growth of PEF42 is Na^+-dependent, and no growth occurs in its absence [Figure 3 and (*11*)]. Hence, the new mutant strain MPA762 has gained the ability to grow on succinate in the absence of Na^+ ions and simultaneously has acquired a specific growth inhibition by this alkali ion.

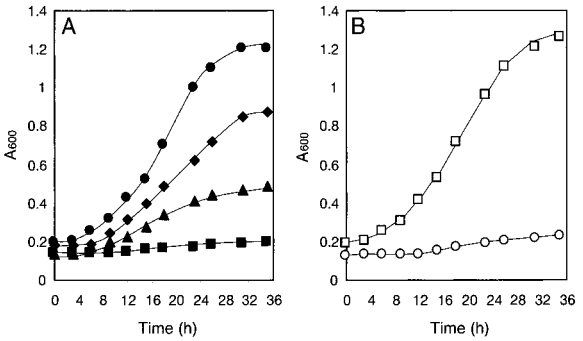

FIGURE 3: Effect of Na^+ on growth of *E. coli* strains MPA762 (A) and PEF42 (B). The growth curves were recorded in 1% succinate minimal medium with cultures containing (○,●) 23 to 35 μM NaCl, (◆) 1 mM NaCl, (▲) 5 mM NaCl, or (□,■) 10 mM NaCl, respectively.

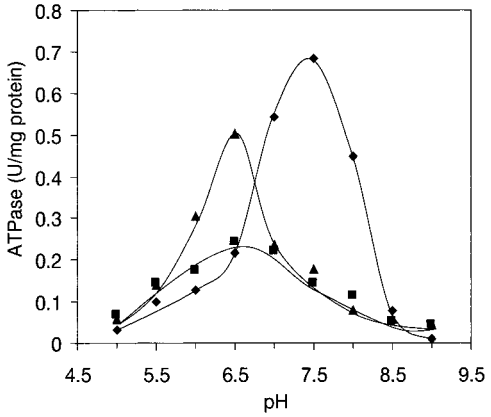

FIGURE 4: Dependence of ATPase activity from various *E. coli* strains on pH: PEF42 (■), MPC8487 (◆), and MPA762 (▲). The ATPase activities were determined with purified enzyme in a buffer consisting of 20 mM each of K-glycine, K-MOPS, and K-tricine, 100 mM K_2SO_4, and 0.05% Triton X-100 at the pH indicated. The estimated endogenous Na^+ concentration was below 50 μM.

Effect of pH and Alkali Ions on the New Mutant ATPase. Biochemical properties of the mutant ATPase were investigated to assess the phenotypic alterations on the enzyme itself. The pH/rate profile of the ATPase from strain MPA762, measured in the absence of Na^+ or Li^+ ions, shows a rather sharp optimum at pH 6.5 (Figure 4). With the purifed enzyme, the specific ATPase activity at this pH was about 0.5 unit/mg of protein and decreased about 3−4-fold at 1 pH unit difference to either side. The same specific activities as for this mutant ATPase were found for that from the parent strain PEF42 at pH values <5.5 and >7.0, again without Na^+ or Li^+ addition, but the sharp rise in activity at pH 6.5 was not observed. The optimum activity of this ATPase at pH 6.5 was therefore with 0.2 unit/mg of protein less than half that of the new mutant ATPase. It is interesting that the shift in the pH optimum to 7.5 that is observed for the ATPase of the c subunit mutant strain MPC8487 is not observed in the ATPase with the triple mutation in the a subunit. This shift of the pH optimum is therefore not absolutely essential for an efficient proton-coupled ATP synthesis that can support growth of the *E. coli* cells by virtue of a *P. modestum/E. coli* hybrid ATPase.

The effect of LiCl on the ATPase activity from the mutant strain MPA762 and the parent strain PEF42 at three different

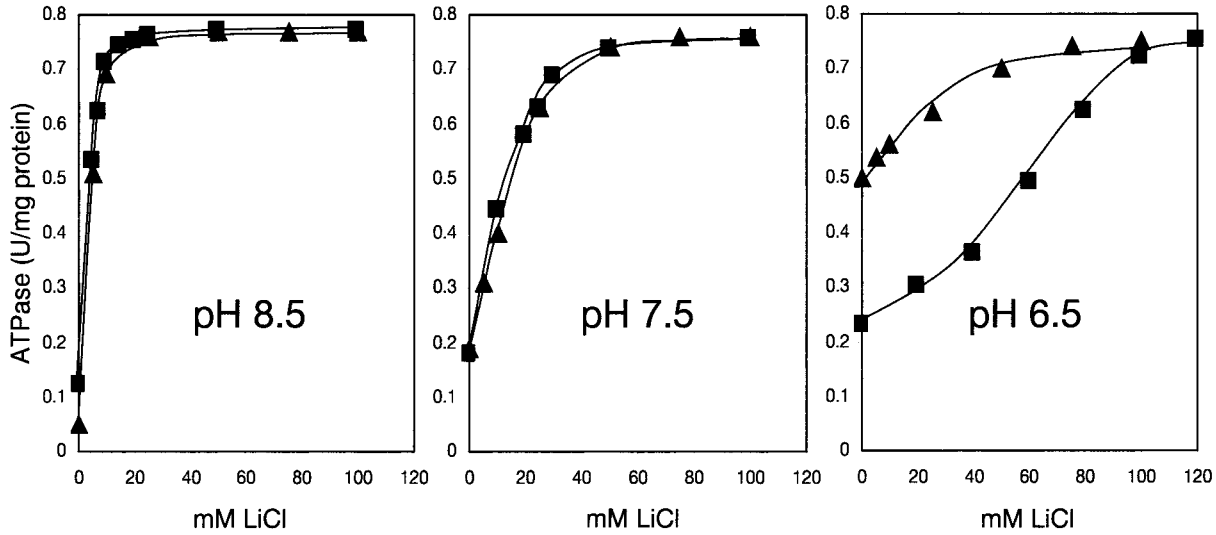

FIGURE 5: Activation profiles of the ATPase from *E. coli* PEF42 (■) and *E. coli* MPA762 (▲) by LiCl at pH 8.5, pH 7.5, and pH 6.5. The ATPase activities were determined with purified enzyme in a buffer consisting of 20 mM each of K-glycine, K-MOPS, and K-Tricine, 100 mM K_2SO_4, and 0.05% Triton X-100 adjusted to the respective pH with KOH.

pH values is shown in Figure 5. At pH 8.5, the activity of the mutant ATPase increased from 0.05 unit/mg in the absence of LiCl to 0.75 unit/mg at 20 mM LiCl and above. At this pH, the activation profile was very similar to that of the parent hybrid ATPase from strain PEF42. At pH 7.5, the Li^+-independent ATPase activity was 0.2 unit/mg of protein, and saturation with the alkali ion was reached above 50 mM LiCl. At pH 6.5, the ATPase was already quite active in the absence of Li^+ ions (0.5 unit/mg) and maximal activation to 0.75 unit/mg of protein required LiCl concentrations of about 100 mM. For saturation at this pH, similar LiCl concentrations are required for the parent hybrid ATPase. However, the Li^+-independent ATPase activities from the PEF42 ATPase were about 2.5-fold less than that with the a subunit triple mutation, as already reflected by the pH profiles (Figure 4).

The effect of NaCl on the activity of the ATPase with the triple mutation in subunit a and the parent ATPase is shown in Figure 6. At pH 6.5, the MPA762 ATPase activity observed in the absence of alkali ions of 0.5 unit/mg of protein decreased with increasing NaCl concentrations to about 10% of its initial value at 10 mM NaCl. These results clearly show that Na^+ ions act as specific inhibitor for this mutant ATPase, while the parent ATPase was specifically activated by this alkali ion (Figure 6). Analysis of the data of Figure 6 revealed a K_i for NaCl of 1.5 mM for the mutant ATPase which is similar to the K_m of 1.4 mM that has been observed for the activation by NaCl of the parent hybrid ATPase of strain PEF42 under similar conditions (*11*). Sodium ions are therefore bound with similar affinity to the Na^+ binding site(s) of the c subunits of the parent hybrid ATPase or of the ATPase with the a subunit triple mutation. This is reasonable because the Na^+ binding site(s) on the c subunits should not be affected by the mutations in the a subunit. However, the binding of Na^+ to the c subunits causes the most possible adverse effect, i.e., activation of the parent hybrid ATPase vs inhibition of the new mutant ATPase.

H^+, Li^+, and Na^+ Transport Experiments. The coupling ion specificity of the mutant ATPase was further investigated

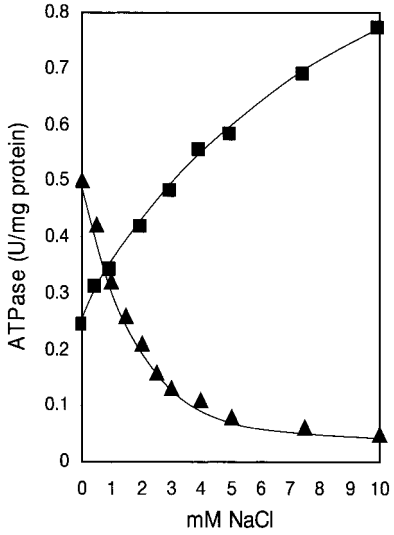

FIGURE 6: Effect of NaCl on the ATPase from *E. coli* MPA762 (▲) and *E. coli* PEF42 (■). The ATPase activities were determined with purified enzyme in 50 mM potassium phosphate buffer containing 100 mM K_2SO_4, 0.05% Triton X-100 adjusted to pH 6.5 with KOH, and the NaCl concentrations indicated.

by H^+, Li^+, and Na^+ transport experiments. Proton transport was studied at pH 7.5 by ACMA fluorescence quenching with proteoliposomes containing the mutant ATPase from strain MPA762 and the parent hybrid ATPase from *E. coli* PEF42. The results, shown in Figure 7, indicate ATP-driven proton uptake into the proteoliposomes which was inhibited in both cases to about the same extent with the same concentrations of LiCl and NaCl, respectively. The results are in accord with a competition of H^+, Li^+, and Na^+ to bind to the coupling ion binding site on subunit c (*15*). The validity of these conclusions was corroborated by Li^+ transport experiments with reconstituted proteoliposomes containing the mutant or the parent ATPases at pH 7.5. The results of Figure 8A show a rapid accumulation of Li^+ ions inside the proteoliposomes containing either the mutant or the parent ATPase following ATP addition. Again, the presence of 2 mM NaCl drastically decreases the Li^+ transport, and with 10 mM NaCl, the Li^+ transport was reduced to the level of the control reaction, i.e., Li^+ transport

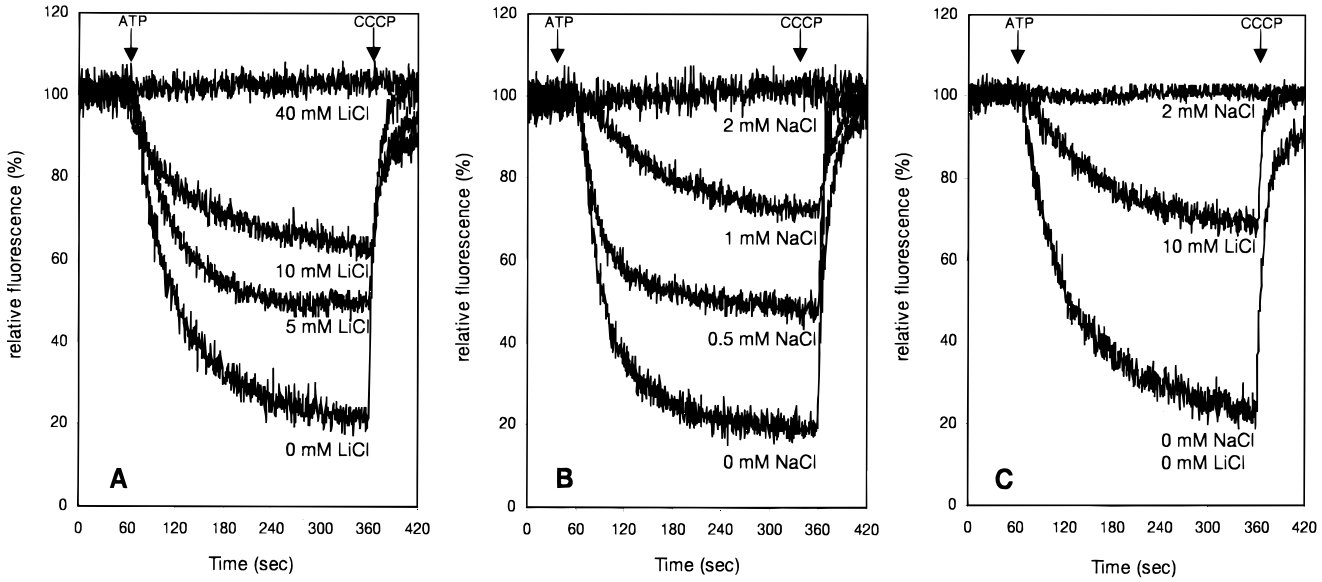

FIGURE 7: Effect of Na^+ or Li^+ on ATP-driven fluorescence quenching of ACMA by reconstituted proteoliposomes at pH 7.5. ACMA quenching was initiated by adding 2.5 mM K-ATP (↓) to reaction mixtures containing the mutant ATPase of *E. coli* MPA762 (A, B) or the purified parent ATPase of *E. coli* PEF42 (C) reconstituted into proteoliposomes and the concentrations of NaCl or LiCl indicated. Quenching was released by the addition of 2 μM CCCP (↓).

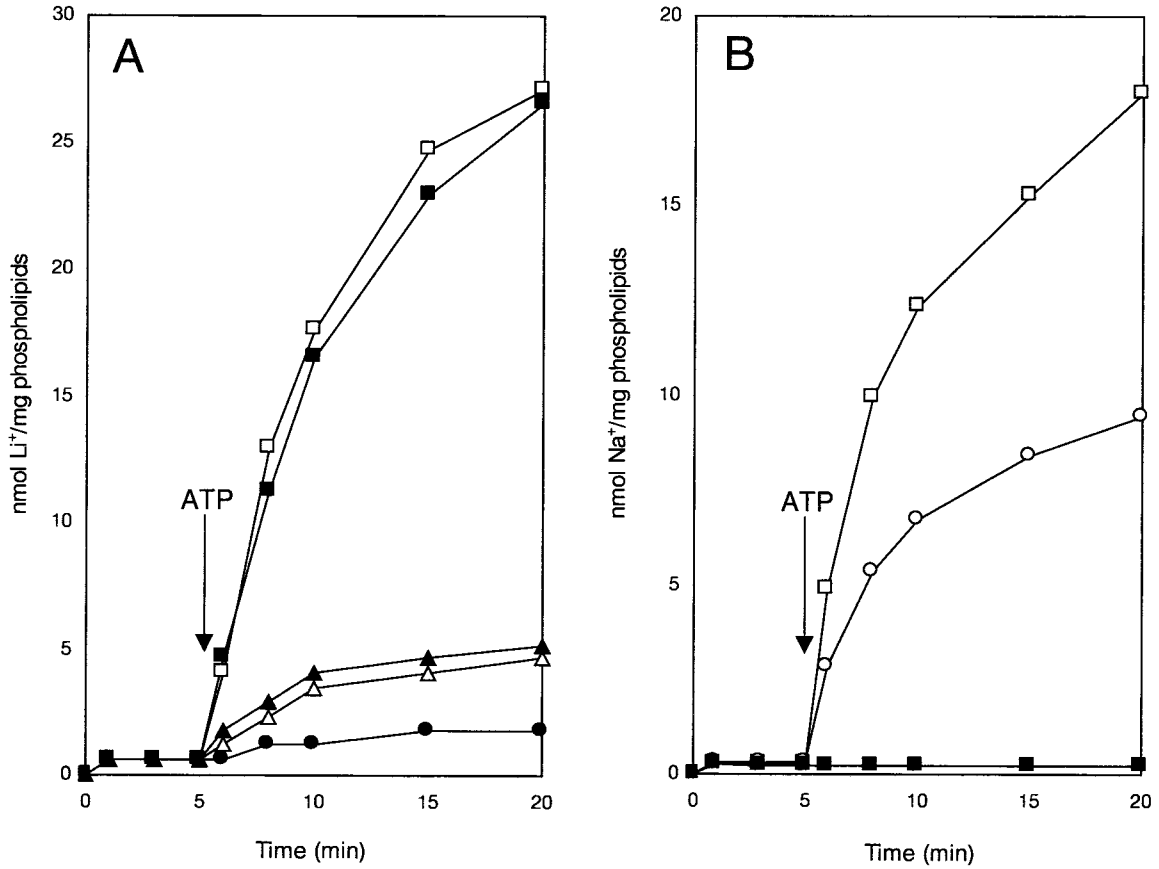

FIGURE 8: Comparison of Li^+ (A) and Na^+ (B) transport into reconstituted proteoliposomes containing the parent or the mutant ATPases at pH 7.5. The same amounts of purified enzymes were reconstituted by the freeze−thaw−sonication method. The assay contained (A) 20 mM LiCl or (B) 2 mM ^{22}NaCl and 30 μM valinomycin: (open symbols) *E. coli* PEF42 and (closed symbols) *E. coli* MPA762. In (A), Li^+ transport is shown: (□) PEF42, (△) PEF42 with 2 mM NaCl, (■) MPA762, (▲) MPA762 with 2 mM NaCl, and (●) MPA762 with 10 mM NaCl. In (B), Na^+ transport is shown: (□) PEF42, (○) PEF42 with 20 mM LiCl, and (■) MPA762. Controls without ATP or with 20 μM DCCD did not catalyze any Li^+ or Na^+ transport (not shown).

without ATP. The specific inhibition of the mutant ATPase by Na^+ ions (Figure 6) indicated that this enzyme is unable to pump Na^+ ions. This was confirmed by the Na^+ transport experiments shown in Figure 8B. No ATP-driven $^{22}Na^+$ uptake was observed with proteoliposomes containing the mutant ATPase, while in control liposomes containing the wild-type enzyme $^{22}Na^+$ was accumulated. $^{22}Na^+$ transport by this enzyme was inhibited by LiCl as expected because

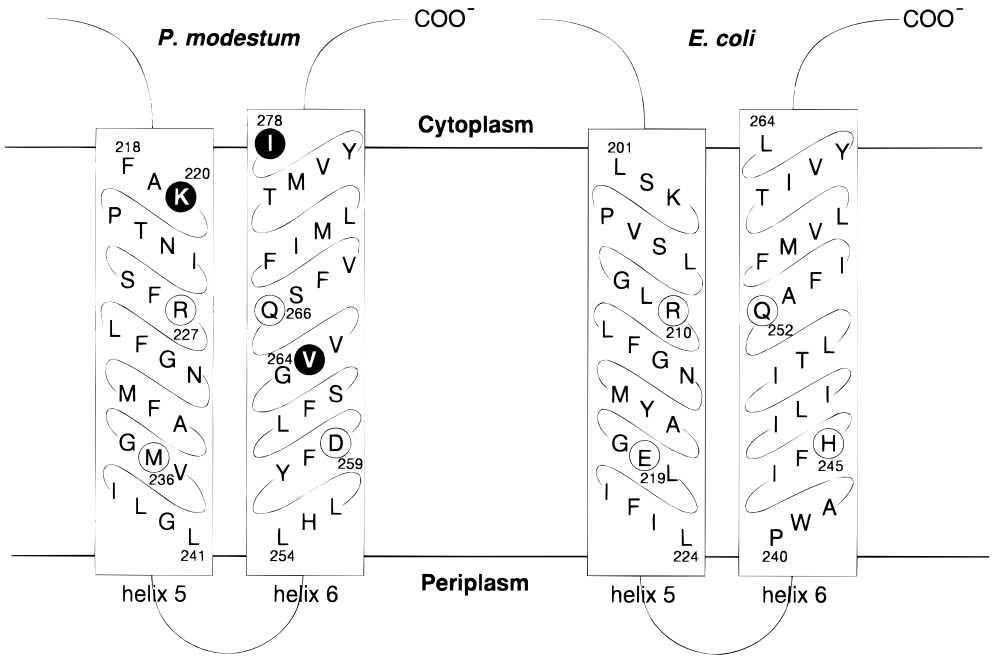

FIGURE 9: Comparison of amino acid sequences of the predicted transmembrane helices 5 and 6 of the a subunits from the F_1F_o ATPase of *P. modestum* and *E. coli*. Critical residues for coupling ion translocation are circled; the three residues of the *P. modestum* a subunit that were mutated in the enzyme with the Na^+-inhibited phenotype are underlayed in black.

Li^+ competes with Na^+ for binding to the coupling ion binding site on subunit c.

DISCUSSION

Structural analysis of most of the F_1 part of the ATP synthase from bovine heart mitochondria together with biochemical data suggested an ATP synthesis mechanism via rotation of the central γ subunit inside the $\alpha_3\beta_3$ hexagon (*24*, *25*). Evidence for such a rotation was obtained by biochemical and biophysical techniques as well as by direct visualization (*26*−*28*). As the driving force for ATP synthesis is the electrochemical gradient of protons or sodium ions across the membrane, the rotation has to be driven by the downhill movement of these coupling ions through the F_o sector of the enzyme. How this is accomplished is essentially unresolved.

All three types of subunits of *E. coli* F_o (ab_2c_{9-12}) are required to form a functional proton-translocating particle (*29*). Most researchers believe that the b subunits are part of the stator and that the a and c subunits take an active part in the ion translocation itself. The c subunits are present in 9−12 copies (*30*) and form strong aggregates in some ATPases, even resisting dissociation by boiling in SDS (*8*). A ring-like structure of c subunits in the membrane has therefore been postulated, and images of F_o by electron and atomic force spectroscopy support this view (*31*, *32*). Evidence is also available that the a subunit is located outside the ring of c subunits (*33*).

Subunit a is highly hydrophobic, and according to recent topological studies, it may consist of six membrane-spanning α-helices (*34*). Extensive mutational analyses of the *E. coli* a subunit identified the C-terminal portion comprising the last two membrane-spanning helices as critical for H^+ translocation (*35*−*39*). The amino acids forming these two helices are schematically drawn for both the *P. modestum* and the *E. coli* a subunit in Figure 9. The amino acids that are most conspicuous to play a critical role in H^+ translocation (R210, E219, H245, Q252, *E. coli* numbering) (*40*, *41*) are circled, and those within the *P. modestum* a subunit giving rise to the Na^+-inhibited growth phenotype are underlayed in black. It should be noted that the acidic residue is not conserved at position 219, but moved in some ATPases to position 245. Accordingly, ATP-driven H^+ translocation is significantly diminished by E219 or H245 single mutations but not by the E219H, H245E double mutation (*42*). According to amino acid alignments, the *E. coli* residues aR210, aE219, and aH245 correspond to the *P. modestum* residues aR227, aM236, and aD259, respectively. The critical positively and negatively charged amino acids are therefore conserved in the *P. modestum* a subunit, although the acidic amino acid that can be moved in the *E. coli* a subunit from helix 5 to helix 6 is already in the latter position in the *P. modestum* enzyme.

The mutational studies of this work strongly support and extend the functional role of subunit a in the process of ion translocation across the membrane. The three mutations (K220R, V264E, I278Q) that cause the Na^+-independent growth phenotype on succinate are within the critical C-terminal region of the a subunit. It is interesting that neither of the mutations found by the random approach affected the putatively functionally critical residues R227 or D259. Therefore, these residues are probably equally important for the translocation of Na^+ ions or protons. The triple mutation in subunit a causes a change in the coupling ion specificity of the ATPase from Na^+, Li^+, or H^+ to only Li^+ or H^+. These results show for the first time that the ion selectivity of F_1F_o ATPases is determined by specific structural elements not only of subunit c but also of subunit a.

We have convincingly demonstrated in our previous work that subunit c of the *P. modestum* ATPase carries a binding site for the coupling ions Na^+, Li^+, or H^+ (*6*). This site

comprises the conserved acidic residue in the C-terminal membrane-spanning helix of the hairpin (E65). The adjacent S66 residue serves as an additional ligand for Na^+ or Li^+, and Q32 on the opposite helix is specifically required for Na^+ binding. A cQ32I mutation, therefore, abolished all Na^+-dependent activities of the ATPase with full retention of Li^+-dependent or H^+-translocating activities (*6*). A similar phenotype was also observed with a F84L, L87V double mutation in the C-terminal tail of the protein (*16*). A reasonable explanation for the properties of this mutant is the alteration of interhelix interactions within subunit c by which Q32 becomes displaced from the binding sphere and is therefore no longer capable to serve as a Na^+ binding ligand.

With these results in mind, we would like to propose an additional binding site for the coupling ions on subunit a. In our model, this binding site would consist of a carboxylate, perhaps the conserved acidic amino acid in helix 5 of the *E. coli* a subunit (E219) or in helix 6, respectively, of the *P. modestum* a subunit (D259). The acidic residue would be sufficient to accommodate proton binding, whereas for metal ion binding by the *P. modestum* a subunit additional liganding amino acids must be present. The triple mutation in the *P. modestum* a subunit might perturb the coordination sphere for Na^+ liganding, especially if liganding groups are distributed to two different helices, while Li^+ binding which requires less ligands is not changed, very similar to the situation already worked out for subunit c (*6*).

The characteristics of the a subunit triple mutation described here are completely compatible with this model: (i) the mutant ATPase is fully functional with Li^+ or H^+ as coupling ion, but has lost the capacity for Na^+ translocation; (ii) furthermore and importantly, the mutant ATPase is activated by Li^+ ions such as the wild-type enzyme, but is inhibited by Na^+ ions; (iii) the K_i for Na^+ inhibition of the mutant ATPase is the same as the K_m for Na^+ activation of the wild-type enzyme under otherwise identical conditions. Please note that at pH 6.5 where these experiments had to be conducted for technical reasons, there is no apparent cooperativity neither for Na^+ activation nor for Na^+ inhibition by the appropriate enzyme. We conclude from these results that the Na^+ binding sites on the multimeric c subunits are not affected by the triple mutation in the a subunit.

If there is a binding site for the coupling ions not only on subunit c but also on subunit a, this has a significant impact on the ion translocation mechanism. In the following, we propose a model that takes present and previous results into account: the Na^+ (Li^+ or H^+) binding sites of most c subunits of the ring are freely accessible from the n(negative)-side (cytoplasmic side in bacteria), whereas the corresponding binding site on subunit a is accessible from the p(positive)-side of the membrane. The active-site carboxylates of the c subunits have a p*K* of 7.0 in the absence of Na^+ ions that is shifted to p*K* 6.0 in the presence of 1 mM Na^+ (*14*). At pH >8.0, ATP hydrolysis is Na^+-dependent in a highly cooperative manner ($n_H = 2.6$), indicating occupancy of at least three sites simultaneously for maximal activation (*14*). This would be difficult to explain by models in which the binding sites on subunits c are loaded and unloaded via two different channels in subunit a that communicate with the two different sides of the membrane (*26*, *43*, *44*). In these models, the c subunit ring would have to rotate in order to complete the ion translocation, and during this rotation the coupling ions would be occluded by the c subunits. Such a model is also not compatible with DCCD-labeling studies: subunits c can be labeled with this compound, and Na^+ ions provide complete protection (*15*).

As ATP hydrolysis turns the γ subunit, it is attractive to hypothesize that this motion is mechanically coupled to the rotation of the c subunit ring. In this way, all c subunits would make phase-shifted the same contact with the fixed a subunit. Once in contact with a, the coupling ion could no longer dissociate to the n-side but would be released through the a subunit to the p-side of the membrane. Please recall that at pH >8.0 the binding sites on subunits c have to be occupied with Na^+ for ATP hydrolysis to occur (*14*) and according to our model for the ring of c subunits to rotate. In the a subunit triple mutation, ATP is hydrolyzed, if the c subunit binding sites are occupied with Li^+ or H^+ but not with bound Na^+ ions. In our view, the triple mutation destroys the Na^+ binding site on subunit a which prevents release of the Na^+ ion from the c subunit at the contact site to the a subunit to the p-side of the membrane. It is quite interesting that an *E. coli* ATPase mutant in which four c subunit residues were replaced by the corresponding ones from *P. modestum* (V60A, D61E, A62S, I63T) was functional as a proton pump but was specifically inhibited by Li^+ ions (*45*). As the a subunit from the *E. coli* enzyme probably does not accommodate Li^+ binding and translocation, these observations are completely compatible with ours on the a subunit triple mutation and therefore in accord with the model.

In summary, we propose that the ATPase functions as a perfectly coupled molecular machine in which ATP hydrolysis moves the ring of c subunits versus the a subunit, but only if the following conditions are met: the binding sites on the c subunits that are accessible from the n-side have to be occupied with the coupling ions and the coupling ions have to be released via the a subunit to the p-side of the membrane. Fully in accord with this model are recent $^{22}Na^+$ occlusion experiments. We discovered an ATP-dependent occlusion of one $^{22}Na^+$ per ATPase harboring the a subunit triple mutation, while no $^{22}Na^+$ was occluded by the wild-type enzyme or in the absence of ATP (*46*).

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