Studies on Na^+ and H^+ Translocation through the F_o Part of the Na^+-Translocating F_1F_o ATPase from *Propionigenium modestum*: Discovery of a Membrane Potential Dependent Step

Claudia Kluge and Peter Dimroth*

Mikrobiologisches Institut, Eidgenössische Technische Hochschule, ETH-Zentrum, Schmelzbergstrasse 7, CH-8092 Zürich, Switzerland

Received July 28, 1992; Revised Manuscript Received October 2, 1992

ABSTRACT: The purified ATPase of *Propionigenium modestum* (F_1F_o) was incorporated into liposomes, and the F_1 part was dissociated. The F_o–liposomes catalyzed proton uptake in response to a potassium diffusion potential (inside negative). Proton translocation was abolished by rebinding F_1 to the F_o–liposomes or after incubation with the c-subunit-specific inhibitor dicyclohexylcarbodiimide (DCCD). Proton uptake was also sensitive to the presence of external Na^+ or Li^+ ions and was completely abolished at 2 mM NaCl or 150 mM LiCl, respectively. However, the same concentrations of these salts in the internal volume of the F_o–liposomes were without effect, suggesting that the cation binding site is not accessible from both sides of the membrane simultaneously. An open channel-type of transport through F_o from *P. modestum* is therefore excluded. The F_o–liposomes also catalyzed Na^+ influx or efflux in response to a K^+ diffusion potential that was negative on the inside or outside, respectively. These Na^+ fluxes could not be created, however, by ΔpNa^+ of about 60–180 mV. The initial rate of Na^+ uptake depended strongly on the size of the membrane potential with no significant conductivity below –40 mV, followed by a proportional increase up to about –115 mV. In the absence of a membrane potential, the F_o–liposomes catalyzed $^{22}Na^+$ counterflow against a 28-fold concentration gradient. Uptake of $^{22}Na^+$ into F_o–liposomes against ΔpNa^+ (counterflow) was completely prevented by imposing an inside-positive potassium diffusion potential of 90 mV. The catalysis of $^{22}Na^+$ counterflow by F_o from *P. modestum* is a clear indication of a carrier (transporter)-type mechanism and excludes a channel mechanism. On the basis of our data, we propose a model of Na^+ translocation by F_o that involves a minimum of four steps: (1) binding of Na^+ from a defined surface of the membrane; (2) translocation of the binary complex to the outer surface; (3) release of Na^+; (4) return of the unloaded carrier to the original surface. The first three steps that are sufficient for Na^+ counterflow are assumed to be independent of the membrane potential, while the fourth step that is specifically required for Na^+ influx or Na^+ efflux seems to represent the membrane potential dependent reaction step.

The strictly anaerobic bacterium *Propionigenium modestum* synthesizes all of its ATP by a mechanism termed decarboxylation phosphorylation [for a review, see Dimroth (1987)]. In the course of succinate degradation to propionate and CO_2, (*S*)-methylmalonyl-CoA is decarboxylated to propionyl-CoA by a membrane-bound enzyme functioning as a Na^+ pump (Hilpert et al., 1984). The electrochemical Na^+ gradient thus established is taken advantage of by a Na^+-translocating ATPase for ATP synthesis (Laubinger & Dimroth, 1987, 1988, 1989).

Despite this peculiarity of the *P. modestum* ATPase to use Na^+ instead of H^+ as the physiological coupling ion, the enzyme is a member of the F_1F_o ATPase family. The *P. modestum* ATPase has the typical subunit structure of an eubacterial F_1F_o ATPase with five subunits (α, β, γ, δ, ϵ) forming the water-soluble F_1 moiety and three subunits (a, b, c) forming the membrane-bound F_o part of the enzyme complex (Laubinger & Dimroth, 1987, 1988). The amino acid sequence of the β subunit exhibited 69% identity with the corresponding polypeptide of *Escherichia coli* (Amann et al., 1988), while the identity between the a, b, and c subunits from these two bacteria was only 18%, 11%, and 17%, respectively (Ludwig et al., 1990; Kaim et al., 1990; Esser et al., 1990).

The function of the *P. modestum* ATPase as a primary Na^+ pump has been clearly demonstrated with the purified enzyme reconstituted into proteoliposomes (Laubinger & Dimroth, 1988). Interestingly, at low Na^+ concentrations (<1 mM) the ATPase has the capacity to act as a proton pump. Increasing the Na^+ concentration from 0 to 1 mM had an inverse effect on proton or Na^+ transport, decreasing proton pumping but increasing Na^+ pumping (Laubinger & Dimroth, 1989). Thus protons (H_3O^+) and Na^+ ions may be translocated by the same mechanism. Several lines of evidence have indicated a Na^+ binding site located on the F_o portion. The F_1F_o ATPase complex, but not F_1 alone, was specifically activated by Na^+ ions, and a hybrid constructed in vitro with F_1 from *E. coli* and F_o from *P. modestum* acted as a Na^+ pump like the *P. modestum* ATPase and unlike the *E. coli* ATPase (Laubinger & Dimroth, 1987; Laubinger et al., 1990).

More recently, a functional ATPase hybrid was also obtained by in vivo complementation of an *E. coli* deletion mutant with a recombinant plasmid containing the genes for subunits a, c, b, δ, and part of α of the *P. modestum* ATPase (Kaim et al., 1992). The functional substitution of particularly the F_o subunits of the *E. coli* ATPase by the corresponding subunits of the *P. modestum* ATPase is striking considering the low homologies in the primary structure of the respective proteins. These findings, however, provide additional strong evidence for a unifying mechanism of H^+- and Na^+-translocating F_1F_o ATPases.

* Author to whom correspondence should be addressed.

0006-2960/92/0431-12665$03.00/0

It is well established that the F_o part of several different F_1F_o ATPases conducts protons [for reviews, see Schneider and Altendorf (1987) and Fillingame (1990)]. The mechanism of proton conduction through F_o, however, is not yet understood. Particularly, it is not clear whether F_o functions as an open channel or by a carrier (transporter)-type mechanism. As some of these ambiguities might be easier to solve with F_o from the Na^+-translocating ATPase from *P. modestum*, we performed detailed studies on the ion translocating properties of this protein moiety. These results provide important new insights into the mechanism of ion translocation through F_o.

EXPERIMENTAL PROCEDURES

Purification of F_1F_o ATPase. *P. modestum* was grown on succinate under strictly anaerobic conditions as described (Laubinger & Dimroth, 1988). The ATPase (F_1F_o) was extracted from isolated bacterial membranes and purified by fractionated precipitation with poly(ethylene glycol) as described (Laubinger & Dimroth, 1988).

Reconstitution of Proteoliposomes Containing the F_1F_o ATPase. Liposomes were formed by sonicating a suspension of 60 mg of phosphatidylcholine (Sigma, Type II S) in 1.85–1.9 mL of buffer A (50 mM potassium phosphate, pH 8.0, containing 1 mM dithioerythritol) for 2 × 1 min in a waterbath sonicator. The purified F_1F_o ATPase of *P. modestum* (0.6–0.9 mg of protein) in 0.1–0.15 mL of 5 mM potassium phosphate buffer, pH 7.0, containing 1 mM dithioerythritol, 0.1 mM diisopropyl fluorophosphate, and 0.05% Triton X-100 was added to the preformed liposomes, and the suspension was incubated for 10 min at 25 °C with occasional shaking. The mixture was frozen in liquid nitrogen, kept there for 5 min, and thawed in an ice/water bath which lasted about 1 h. The proteoliposomes were sonicated in a waterbath sonicator for 2 × 5 s, collected by centrifugation (200000*g*, 50 min), and resuspended in 0.3 mL of buffer B (5 mM potassium phosphate, pH 7.0, prepared from KOH and phosphoric acid, Na^+ content <0.0005%) and used directly for translocation experiments. Alternatively, the F_1 moiety was dissociated from the proteoliposomes as described below.

Dissociation of F_1 from Proteoliposomes Containing the F_1F_o ATPase. For the dissociation of F_1, the centrifuged proteoliposomes (10 mg of phospholipid) were resuspended in 0.5 mL of buffer C [1 mM Tris, 0.5 mM K_2-EDTA, 10% (v/v) glycerol, 1 mM dithioerythritol, adjusted to pH 9.25 with 100 mM KOH] (Laubinger & Dimroth, 1990). After 2 h at 25 °C, the dissociated F_1 ATPase was separated from the F_o–liposomes by centrifugation (200000*g*, 60 min). The pellet was resuspended in the same volume of buffer C as before, incubated for 50 min at 25 °C, and centrifuged. F_1-depleted proteoliposomes were resuspended in 0.3 mL of buffer B.

Rebinding of F_1 from P. modestum (PF_1) to F_1-Depleted Proteoliposomes. PF_1 was purified from membrane vesicles as described (Laubinger & Dimroth, 1987), and buffer was changed to buffer D (5 mM potassium phosphate, pH 7.0, containing 1 mM dithioerythritol and 5 mM $MgCl_2$). F_1-depleted proteoliposomes prepared from 5 mg of crude lipids were incubated with 0–0.2 mg of purified F_1 ATPase for 45 min at 4 °C. The membrane-bound enzyme was subsequently separated from excess F_1 ATPase by centrifugation (200000*g*, 50 min) and resuspended in 0.3 mL of buffer B. For ATP-dependent Na^+ transport experiments, the proteoliposomes were suspended in buffer D containing 100 mM K_2SO_4.

Imposition of an Artificial Membrane Potential. Proteoliposomes in buffer B were loaded with 200 mM KCl by incubation overnight at 4 °C. Immediately prior to the transport experiments, the proteoliposomes were diluted into the appropriate incubation mixture (5–94-fold in different experiments). A K^+ diffusion potential was imposed by addition of valinomycin (about 50 pmol/mg of lipid).

Determination of H^+ Uptake. Proton uptake was determined by the quenching of ACMA[1] fluorescence. The standard reaction mixtures contained in 1.5 mL at 25 °C the following: 2 mM Tricine/KOH buffer, pH 7.2, 5 mM $MgCl_2$, 200 mM choline chloride, 1.3 μM ACMA, and the F_1-depleted proteoliposomes loaded with 200 mM KCl (15–20 μL; 0.8–1.1 mg of lipid). The pH of the mixture was adjusted to pH 7.1 by the addition of 1.5 μL of 0.1 M KOH. After the signal had stabilized, the reaction was initiated by adding 67 nM valinomycin. Fluorescence was measured with a Shimadzu RF-5001PC spectrofluorophotometer using an excitation wavelength of 410 nm and an emission wavelength of 480 nm.

Determination of Na^+ Transport into Proteoliposomes (Influx). The standard incubation mixtures contained in 0.7 mL at 25 °C the following: 2 mM Tricine/KOH buffer, pH 7.2, 5 mM $MgCl_2$, 200 mM choline chloride, 2 mM $^{22}NaCl$ (360–380 dpm/nmol), and F_1-depleted proteoliposomes loaded with 200 mM KCl (15–20 μL; 0.8–1.1 mg of lipid). The pH of the mixture was adjusted to pH 7.1 by the addition of 1 μL of 0.1 M KOH. After 5 min, a membrane potential was applied by adding 96 nM valinomycin. Samples (90 μL) were taken at various times and passed over small columns of Dowex 50, K^+ to adsorb the external $^{22}Na^+$ (Dimroth, 1982). The resin was washed twice with 0.3 mL each of 2 mM Tricine/KOH buffer, pH 7.2, containing 5 mM $MgCl_2$ and 200 mM sucrose. The radioactivity of $^{22}Na^+$ eluted from the columns which reflects $^{22}Na^+$ entrapped in the proteoliposomes was determined by γ-scintillation counting.

Determination of ATP-Dependent Na^+ Transport into F_1F_o–Liposomes. Na^+ transport into F_1F_o–liposomes was determined with incubation mixtures containing in 0.7 mL at 25 °C the following: 50 mM potassium phosphate buffer, pH 7.0, 2 mM $^{22}NaCl$ (390 dpm/nmol), 5 mM $MgCl_2$, 6 mM phosphoenolpyruvate, 20 units of pyruvate kinase, and reconstituted F_1F_o–liposomes (1 mg of lipid). After 5 min, the transport was initiated by adding 1.25 mM ATP (potassium salt), and the concentration of $^{22}Na^+$ inside the proteoliposomes was determined as described above.

Determination of Na^+ Transport out of Proteoliposomes (Efflux). F_1-depleted proteoliposomes in buffer B were incubation overnight with 20 mM $^{22}NaCl$ (180–200 dpm/nmol) and 180 mM choline chloride. It was assured by control experiments that complete equilibration of internal and external Na^+ was obtained after 10 h. The proteoliposomes (20 μL, about 1 mg of lipid) were added to 0.7 mL of 2 mM Tricine/KOH buffer, pH 7.1, containing 5 mM $MgCl_2$, and 200 mM KCl at 25 °C. If not indicated otherwise, efflux was initiated after 5 min by adding 96 nM valinomycin, and the concentration of $^{22}Na^+$ retained inside the liposomes was measured as described above.

Counterflow. F_1-depleted proteoliposomes in buffer B were incubated overnight with 100 mM NaCl. Twenty microliters of the Na^+-loaded F_o–liposomes (about 1.4 mg of lipid) was added to 0.7 mL of 2 mM Tricine/KOH buffer, pH 7.3,

[1] Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; the term carrier is used here to define a transporter type membrane protein like, e.g., the lactose carrier.

containing 5 mM $MgCl_2$, 100 mM choline chloride and about 1 μCi of $^{22}NaCl$ (carrier-free) at 25 °C. $^{22}Na^+$ uptake was determined after separation of external and internal $^{22}Na^+$ on Dowex 50, K^+ (see above). The columns were washed with 2 × 0.3 mL of 2 mM Tricine/KOH buffer, pH 7.2, containing 5 mM $MgCl_2$ and 100 mM sucrose.

Assays. ATPase activity of reconstituted proteoliposomes and orientation of the ATPase in the proteoliposomes was determined by a coupled spectrophotometric assay as described elsewhere (Laubinger & Dimroth, 1988). Protein was determined according to Bradford (1976) with the modifications described (Laubinger & Dimroth, 1987). The concentration of Na^+ was determined by atomic absorption spectroscopy at 588.9 nm with a Shimadzu AA646 atomic absorption/flame emission spectrophotometer.

RESULTS

Preparation of F_o–Liposomes. Studies of the properties of ion conduction through the F_o part of the *P. modestum* ATPase required incorporation of the F_o moiety into membranes with low intrinsic ion conductivity. Initial attempts to dissociate F_1 from the detergent-solubilized F_1F_o ATPase failed. Instead, we reconstituted the whole F_1F_o complex into proteoliposomes, then dissociated the F_1 part and finally collected the F_o–liposomes by ultracentifugation. Proteoliposomes containing F_1F_o were obtained by a freeze–thaw–sonication procedure. About 50% of F_1 of these proteoliposomes was oriented to the outside as shown by comparing ATPase activities with and without Triton X-100 (Laubinger & Dimroth, 1988). The major part of F_1 that was oriented to the outside was dissociated on incubation with EDTA at pH 9.0 and low ionic strength because only 4% of the initial ATPase activity remained in the isolated F_o–liposomes. Incubation of the F_o–liposomes with F_1 restored the original ATPase activity of the F_1F_o–liposomes. In addition, the reconstituted F_1F_o–liposomes had the same activity of ATP-driven Na^+ or H^+ transport as the original proteoliposomes prior to dissociation and reassociation of F_1. Dissociation of F_1 did therefore not lead to any significant denaturation of F_o. In the isolated proteoliposomes that were obtained after dissociation and reconstitution with F_1, the ATPase activity in presence of Triton X-100 was 85% of its initial value prior to dissociation of F_1. These data indicate that in our F_o–liposome preparation most F_1 was removed from the outside while most internal F_1 remained bound to the internally oriented F_o subcomplexes.

Proton Translocation through the F_o Part of the P. modestum ATPase. A unique property of the *P. modestum* ATPase is to pump Na^+ ions (Laubinger & Dimroth, 1988). At low Na^+ concentrations (<1 mM), however, the enzyme switches to pump protons (Laubinger & Dimroth, 1989). The isolated F_o part of the *P. modestum* ATPase was therefore expected to conduct protons at these low Na^+ concentrations. Proton uptake into the F_o–liposomes was determined by the quenching of ACMA fluorescence. The results of Figure 1 indicate proton uptake into the F_o–liposomes in response to a K^+ diffusion potential (inside negative) applied by adding valinomycin to the KCl-loaded F_o–liposomes. Proton uptake into control liposomes not containing F_o occurred at a much slower rate. The fluorescence signal rapidly decreased by adding the proton conductor CCCP, as expected. After incubation of the F_o–liposomes with F_1, the proton conduction through F_o was blocked. Proton translocation was also abolished after incubation with DCCD which specifically modifies an acidic amino acid residue of the c-subunit (Ludwig et al., 1990). These properties of F_o from *P. modestum* are analogous to those of F_o from other F_1F_o ATPases [for reviews, see Schneider and Altendorf (1987) and Fillingame (1990)].

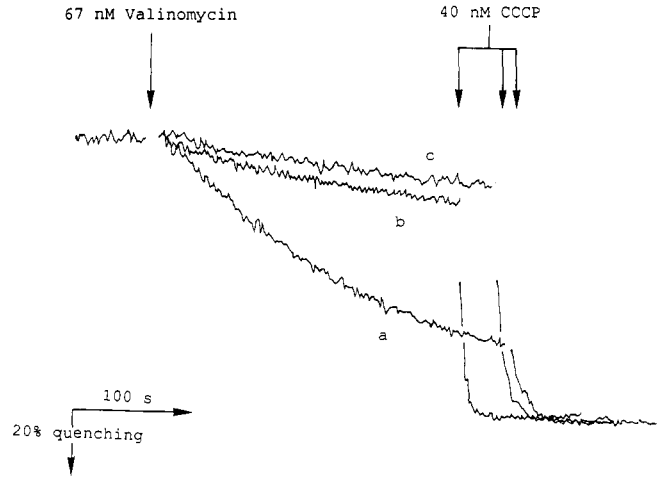


FIGURE 1: $\Delta\psi$-induced quenching of ACMA fluorescence by liposomes containing the F_o part of the *P. modestum* ATPase. (a) F_o–liposomes were prepared, loaded with 200 mM KCl, and diluted 100-fold into buffer of low K^+ content (<2 mM) to induce $\Delta\psi$ by valinomycin addition as described under Experimental Procedures. (b) Same as panel a, but including incubation of the KCl-loaded F_o–liposomes with F_1. (c) Same as panel a, but including incubation of the KCl-loaded F_o–liposomes with 20 μM DCCD (30 min, 25 °C). The kinetics obtained with liposomes (without protein) were the same as those shown in panel c. Additions of valinomycin and CCCP were made as indicated. For details, see Experimental Procedures.

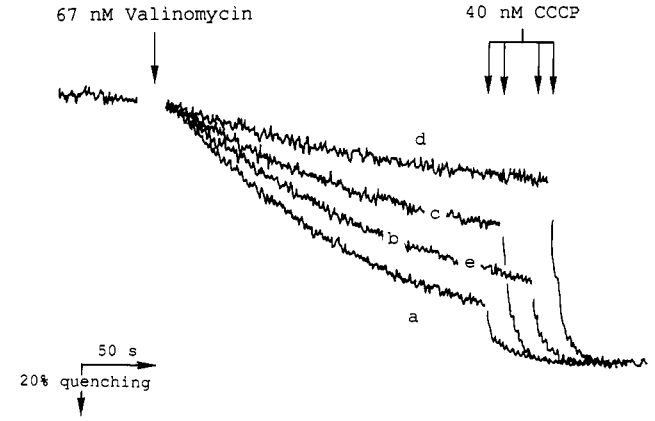


FIGURE 2: Effect of Na^+ ions on the $\Delta\psi$-induced quenching of ACMA fluorescence by F_o–liposomes. Application of $\Delta\psi$ to F_o–liposomes was performed as described in the legend of Figure 1. The incubation mixtures had an endogenous Na^+ concentration of about 50 μM. (a) Without addition of NaCl; (b) with 0.02 mM NaCl; (c) with 0.2 mM NaCl; (d) with 2 mM NaCl; (e) F_o–liposomes loaded with 2 mM NaCl in addition to the usual 200 mM KCl. Additions of valinomycin and CCCP were made as indicated. Details of the fluorescence quenching assay are described under Experimental Procedures.

The effect of Na^+ addition on H^+ uptake into F_o–liposomes is shown in Figure 2. Rate and extent of the quenching decreased as the Na^+ concentration increased and with 2 mM NaCl proton conduction through F_o was completely abolished. If 2 mM NaCl was added to F_o–liposomes taking up protons, this led to an immediate cessation of the proton translocation (not shown). Proton uptake into F_o–liposomes was also inhibited by LiCl, albeit at about 100 times the concentrations required by NaCl for the same effect.

The effect of the Na^+ or Li^+ concentration within the F_o–liposomes on proton translocation was studied by loading these vesicles with NaCl or LiCl together with the usual 200 mM KCl. The results of Figure 2 show that an internal Na^+

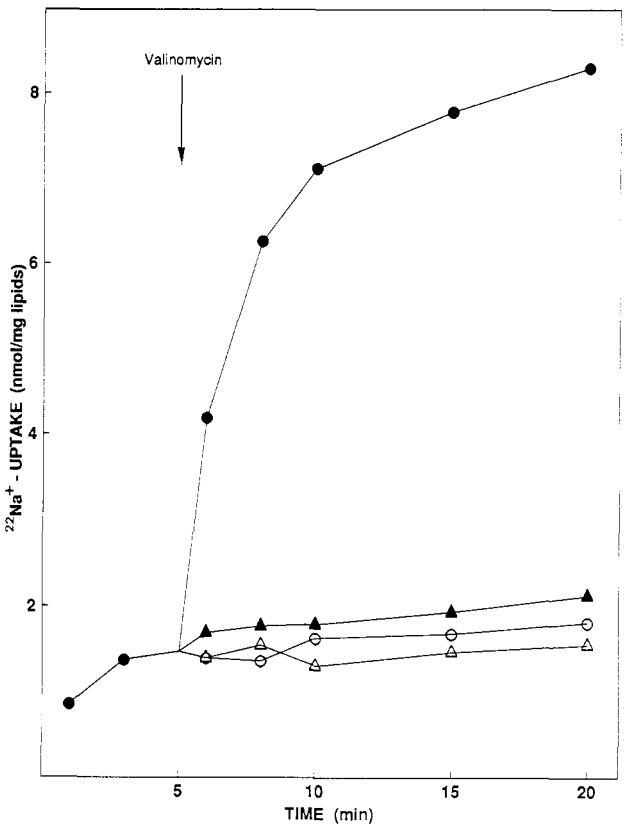


FIGURE 3: $\Delta\psi$-induced flux of $^{22}Na^+$ into F_o–liposomes. $\Delta\psi$ was created as a K^+-diffusion potential by adding valinomycin to KCl-loaded F_o–liposomes prepared from 1.1 mg of crude lipids. Uptake of $^{22}Na^+$ into these F_o–liposomes was subsequently determined at an internal Na^+ concentration of 0.046 mM (●). Control experiments were performed after incubation of F_o–liposomes with F_1 (▲) or after DCCD treatment (○). Also shown is a control with liposomes without F_o (△).

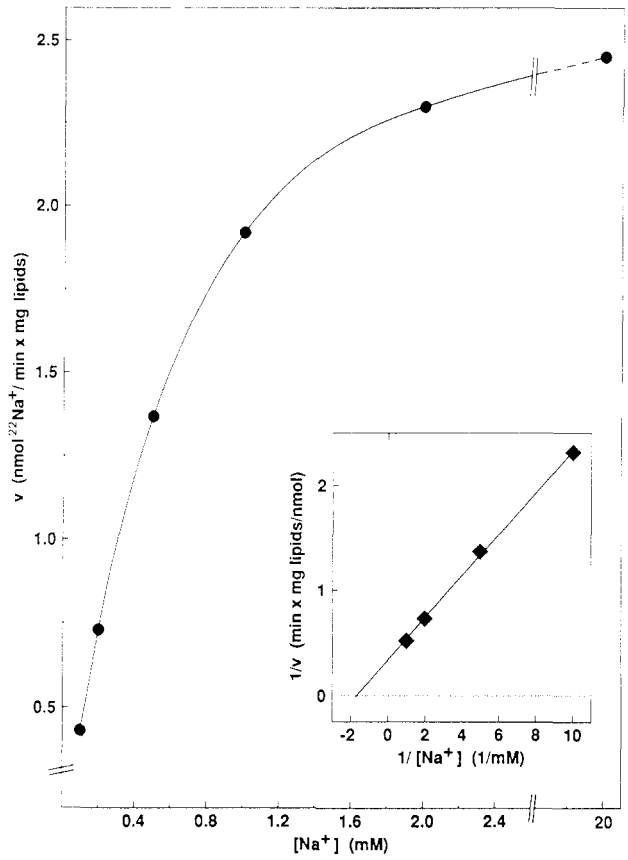


FIGURE 4: Effect of Na^+ concentration on initial velocity of Na^+ uptake into F_o–liposomes. The initial velocity of $^{22}Na^+$ flux into KCl-loaded F_o–liposomes prepared from 1.3 mg of crude lipids after creating $\Delta\psi$ with valinomycin of 100 mV (1:47 dilution) was determined. (Inset) Double-reciprocal plot of initial velocity against Na^+ concentration. Experimental details are described under Experimental Procedures.

concentration of 2 mM was without effect on H^+ uptake. The quenching kinetics were the same as with 20 μM NaCl added to the outside. This external Na^+ concentration results from the 1:100 dilution of the F_o liposomes loaded with 2 mM NaCl into assay mixture. Similarly, a 1:100 dilution of F_o liposomes loaded with 150 mM LiCl yielded quenching kinetics comparable to those obtained by adding 1.5 mM LiCl to the outside. These results show that concentrations of Na^+ or Li^+ which completely block H^+ uptake into F_o–liposomes when present on the outside have no effect if present within the proteoliposomes. The external cation binding site of F_o is therefore not freely accessible from within the liposomes.

Sodium Uptake into F_o–Liposomes. Inhibition of proton translocation into F_o–liposomes by Na^+ or Li^+ indicates competition of these cations for a common binding site on the protein. Sodium translocation into the F_o–liposomes was determined by incubation of these proteoliposomes with $^{22}Na^+$ and subsequent separation by ion-exchange chromatography of external Na^+ from that entrapped in the internal volume. Any uptake of $^{22}Na^+$ into the proteoliposomes determined with this technique will from the beginning strongly argue against a channel-type mechanism for F_o of *P. modestum*, because the ion exchanger will bind all $^{22}Na^+$ ions that are freely accessible to the outside via an open channel.

Figure 3 shows the results of $^{22}Na^+$ uptake into proteoliposomes containing F_o of the *P. modestum* ATPase. If the only driving force for Na^+ uptake consisted of ΔpNa^+ of about −100 mV, the rate of $^{22}Na^+$ uptake was very low. This rate was not significantly increased by increasing ΔpNa^+ to −180 mV (data not shown). However, if valinomycin was added to K^+-loaded F_o–liposomes, creating $\Delta\psi$ of about −100 mV in response to the K^+ gradient, $^{22}Na^+$ was rapidly translocated to the inside of these F_o–liposomes. In control liposomes without F_o, no $^{22}Na^+$ uptake was observed, indicating that F_o and not valinomycin is responsible for $^{22}Na^+$ translocation. This conclusion was confirmed by inhibition of the $\Delta\psi$-induced $^{22}Na^+$ uptake after incubation of the F_o–liposomes with the c-subunit-specific probe DCCD. The results were further confirmed by the complete inhibition of $^{22}Na^+$ uptake after incubation with F_1, also indicating that $^{22}Na^+$ translocation is catalyzed by F_o having its F_1-binding site exposed to the outside.

The results of Figure 4 show the dependence of the initial velocity of Na^+ uptake by F_o–liposomes from the external Na^+ concentration. The curve shows typical saturation kinetics yielding K_T for Na^+ of 0.6 mM. This value is very similar to K_m for Na^+ in activating ATP hydrolysis (Laubinger & Dimroth, 1987) or to K_T for Na^+ in ATP-driven Na^+ transport by F_1F_o (Laubinger & Dimroth, 1989). These results clearly indicate that the translocation of Na^+ through F_o requires binding of the alkali ion to specific sites of the protein and additionally suggests that the Na^+ binding site exposed to the outside of F_o–liposomes does not significantly change its affinity for Na^+ ions upon F_1 binding.

The initial velocity of ATP-driven Na^+ transport into F_1F_o–liposomes was 14.8 nmol/(min·mg of lipid). After dissociation of F_1 the resulting F_o–liposomes catalyzed Na^+ uptake with a rate of 4.5 nmol/(min·mg of lipid) at a membrane potential of −90 mV. The maximum rate of Na^+ influx into F_o–

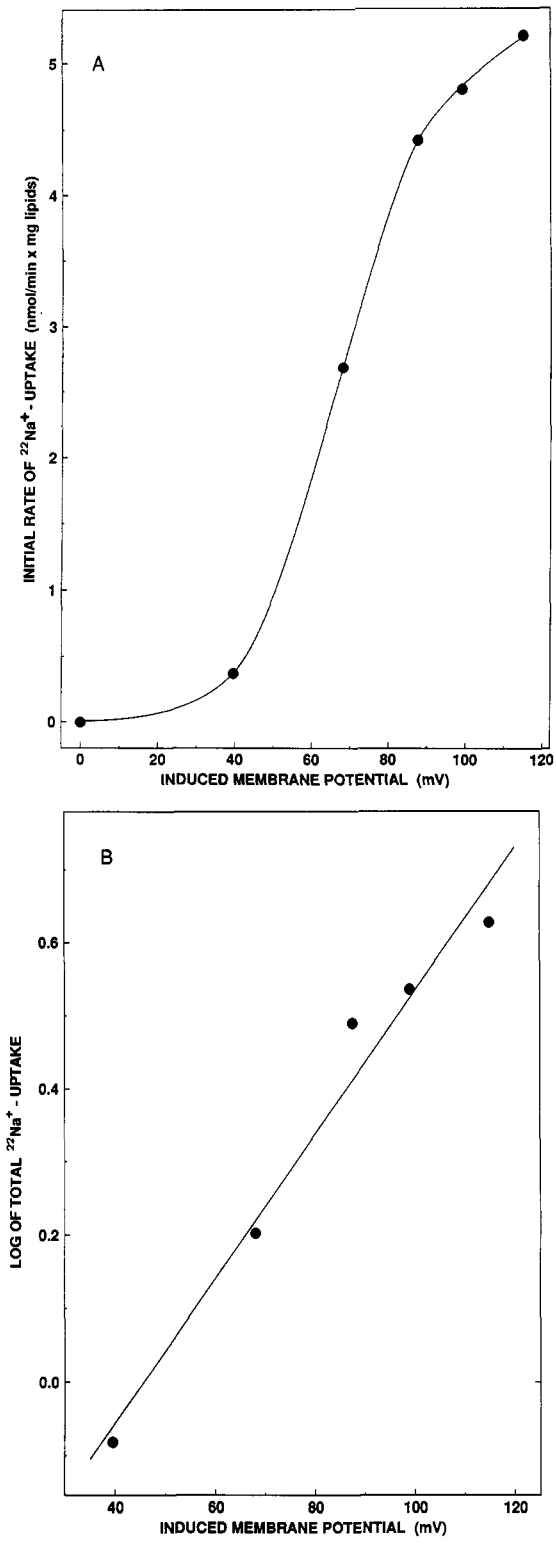


FIGURE 5: Effect of $\Delta\psi$ on $^{22}Na^+$ uptake into liposomes containing the F_o part of the *P. modestum* F_1F_o ATPase. K^+-loaded vesicles (8, 15, 23, 50, and 150 μL) were diluted into 0.7-mL standard incubation mixtures, and $^{22}Na^+$ uptake was determined after addition of valinomycin as described under Experimental Procedures. The rate of $^{22}Na^+$ uptake in the absence of $\Delta\psi$ was determined with 8 μL of vesicles in the absence of valinomycin. The K^+ diffusion potential was calculated by the Nernst equation. Initial velocity of Na^+ uptake (A) and total amount of Na^+ uptake, determined from steady-state values after 15 min incubation (B) are shown.

liposomes, calculated from the data shown in Figure 5A, was 13 $Na^+/(F_o \cdot s)$, taking into account that about 50% of the F_o molecules did not contribute to Na^+ uptake since their F_1 binding site was oriented to the inside of the liposomes and probably contained F_1 bound. This rate is in the typical range of a carrier (transporter) but orders of magnitude too low for a typical channel.

Effect of Membrane Potential. As the $\Delta\psi$ was the main determinant for Na^+ conduction into the F_o–liposomes, we measured the dependency of Na^+ conduction on the membrane potential. The results of Figure 5A show the dependence of Na^+ conductivity, reflected by the initial velocity of Na^+ uptake, to the imposed diffusion potential. Na^+ conductivity at and below –40 mV was very low and increased proportionally between about –40 and –115 mV. It should be noted that the membrane potentials were calculated from the potassium gradients. Especially at high $\Delta\psi$ values the K^+ buffer capacity is very low, and the calculated potentials may be overestimated. Thus it is not clear whether the increase of Na^+ conductivity between –40 to –115 mV is in fact linear or levels off at potentials above –90 mV as it appears in the figure. The results of Figure 5B show that the logarithm of $^{22}Na^+$ uptake in the steady state was in linear proportion to the membrane potential, suggesting an electrogenic Na^+ transport where the Na^+ concentration gradient equilibrates with the electrical charge difference across the membrane.

$^{22}Na^+$ Counterflow. As shown above, $^{22}Na^+$ uptake into F_o–liposomes in response to ΔpNa^+ of –100 to –180 mV was very slow. Importantly, however, some increase of internal $^{22}Na^+$ occurred, if carrier-free $^{22}Na^+$ was added to F_o–liposomes that contained 2 mM NaCl on both sides of the membrane (equilibrium exchange). Increasing the Na^+ concentration on the inside at a constant $^{22}Na^+$ concentration on the outside led to an increased uptake of radioactivity. The F_o–liposomes thus catalyzed $^{22}Na^+$ counterflow. To study the counterflow kinetics, the F_o–liposomes were loaded with 100 mM NaCl and diluted 28-fold into Na^+-free buffer containing 1 μCi of the carrier-free isotope $^{22}Na^+$. The dilution thus established ΔpNa^+ of 86 mV from the inside to the outside. As shown in Figure 6, $^{22}Na^+$ was rapidly taken up into the proteoliposomes under these conditions. These data indicate that the translocation of Na^+ through the membrane is reversible. The F_o moiety apparently carries unlabeled Na^+ ions from the inside to the outside following the direction of the Na^+ concentration gradient. In the reverse reaction, $^{22}Na^+$ is taken up from the outside and moves to the inside. This leads to an accumulation of radioactivity in the internal vesicular space, because $^{22}Na^+$ is trapped there by dilution with the large excess of unlabeled Na^+ ions. No $^{22}Na^+$ translocation to the inside of F_o–liposomes was observed, however, if these were not loaded with NaCl or if a $\Delta\psi$ of 90 mV (inside positive) was applied.

Sodium Efflux from F_o–Liposomes. For a comparison with the Na^+ influx studies described above, $^{22}Na^+$ efflux from F_o–liposomes that had been loaded with the radioactive tracer was determined. The results listed in Table I show that the efflux of $^{22}Na^+$ was very slow if the only driving force was a ΔpNa^+ between 60 and 90 mV. Even slower $^{22}Na^+$ efflux rates were observed with control liposomes or with proteoliposomes containing the F_1F_o ATPase complex. On the addition of monensin, however, the F_o–liposome preparation was almost instantaneously depleted of internal $^{22}Na^+$, because the ionophore makes internal $^{22}Na^+$ accessible to the ion exchanger present on the outside, where the alkali ion is trapped. These results indicate that ΔpNa^+ of about 90 mV is not sufficient to significantly drive Na^+ flux out of F_o–liposomes, consistent with the data on Na^+ influx described above.

Table I: Na^+ Efflux from Liposomes, F_o–Liposomes, and F_1F_o–Liposomes Loaded with $^{22}NaCl$ following Dilution into Na^+-free Buffer[a]

sample	dilution	% $^{22}Na^+$ remaining in liposomes or F_o–liposomes after				
		1 min	5 min	15 min	30 min	90 min
F_o–liposomes	1:10[b]		102.3	98.8	91.7	77.0
liposomes	1:10[b]		98.1	95.7	95.8	91.3
F_o–liposomes	1:14		97.9	88.1	85.1	70.5
F_o–liposomes + 2μM monensin	1:14	1.5	0.4	0.9	0.6	0.9
liposomes	1:14		101.0	98.1	96.8	92.6
F_o–liposomes	1:35		93.6	82.6	77.3	65.7
liposomes	1:35		95.1	91.0	93.4	89.7
F_o–lipsomes ($\Delta\psi$ 91 mV)[c]	1:35	67.0	64.0	56.4		
liposomes ($\Delta\psi$ 91 mV)[c]	1:35	93.2	91.1	75.3		
F_1F_o–liposomes	1:10[b]		93.3	94.4	90.7	89.2
liposomes	1:10[b]		93.9	91.8	89.1	94.0

[a] F_1F_o–liposomes were prepared and depleted of F_1 as described under Experimental Procedures. For each proteoliposome preparation, control liposomes (without protein) were treated the same way. After equilibration overnight with 20 mM $^{22}NaCl$ (180 dpm/nmol), proteoliposomes or liposomes were added to 0.7 mL of 2 mM Tricine/KOH, pH 7.1, containing 5 mM $MgCl_2$ and 20 mM choline chloride (25 °C), yielding the dilutions indicated. [b] In 50 mM Tris/HCl, pH 8.0, containing 2.5 mM $MgCl_2$ and 20 mM choline chloride. [c] Equilibration overnight with 100 mM KCl and 20 mM $^{22}NaCl$; the assay contained 100 mM choline chloride.

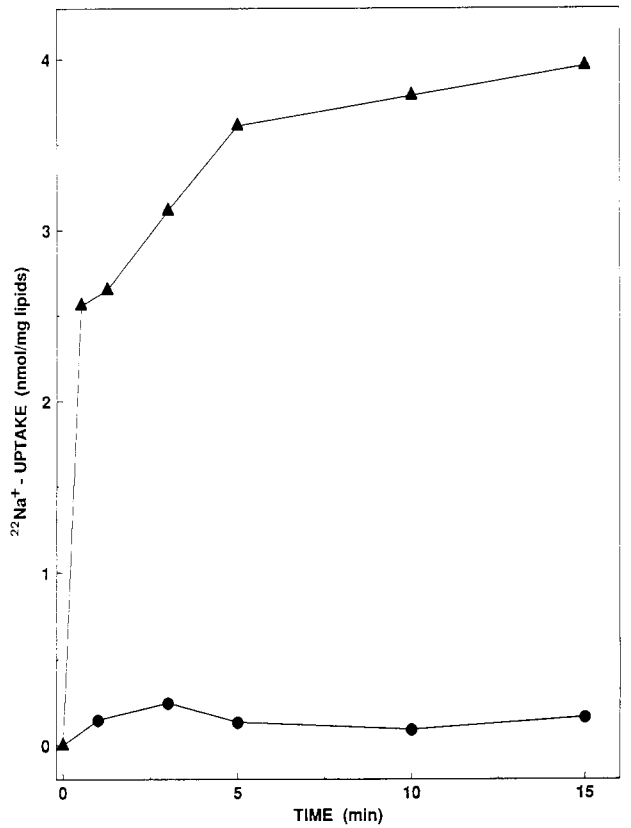


FIGURE 6: $^{22}Na^+$ counterflow into F_o–liposomes loaded with 100 mM NaCl. Counterflow was initiated by diluting 20 μL of these proteoliposomes into 0.7 mL of Na^+-free buffer that contained 1 μCi $^{22}NaCl$ (carrier-free) to generate ΔpNa^+ of about 86 mM from the inside to the outside. $^{22}Na^+$ uptake was subsequently determined with samples taken after different incubation periods (▲). In another experiment, $\Delta\psi$ of 90 mV (inside positive) was applied by adding 170 mM KCl and valinomycin to the incubation mixture (●). For details, see Experimental Procedures.

Application of a calculated potassium diffusion potential of 90 mV (outside negative) greatly increased the rate of $^{22}Na^+$ efflux from the F_o–liposomes to 4.7 nmol/(min·mg of lipid). An almost identical value [4.5 nmol/(min·mg of lipid)] was observed for the initial rate of Na^+ influx into the same F_o–liposomes by applying $\Delta\psi$ of −90 mV (inside negative). After the rapid efflux of about 30% of internal $^{22}Na^+$ within 1 min, further $^{22}Na^+$ efflux was slow, indicating that only a minority of the Na^+-loaded vesicles contained an F_o moiety within their membrane.

In order to compare better the kinetics of Na^+ influx and efflux into and from F_o–liposomes, these were loaded with $^{22}Na^+$ by $\Delta\psi$-driven influx through F_o rather than by overnight equilibration. Efflux was then initiated by reversing the potential upon addition of excess KCl to the outside. The results shown in Figure 7 indicate that under these conditions about 90% of the $^{22}Na^+$ taken up into F_o–liposomes in response to $\Delta\psi$ (inside negative) are released again by reversion of $\Delta\psi$ (outside negative). The residual 10% of the accumulated $^{22}Na^+$ which was released after adding monensin probably passed into free liposomes during the incubation with the radioactive tracer. It could not be released by adding more KCl to the outside, thus inducing a higher $\Delta\psi$ than that applied in the opposite direction to drive $^{22}Na^+$ uptake. Furthermore, the amount of $^{22}Na^+$ that was not released by efflux through F_o increased by lowering the protein to phospholipid ratio of the proteoliposomes. Except for this small percentage of nonexchangeable Na^+ ions, the kinetics of $^{22}Na^+$ influx and efflux were very similar. These results therefore suggest that inward and outward fluxes of Na^+ are catalyzed by the same F_o moieties. The flux direction is clearly determined by the sign of the $\Delta\psi$, whereas the orientation of F_o within the membrane appears to be at least less significant.

DISCUSSION

Proton Conduction by F_o from the P. modestum ATPase. We show here that the F_o part of the *P. modestum* ATPase conducts protons in the direction of a negative membrane potential like the F_o part of other F_1F_o ATPases [for reviews, see Schneider and Altendorf (1987) and Fillingame (1990)]. In further agreement with the properties of these enzymes is the inhibition of proton flux with DCCD which reacts with a specific aspartate or glutamate residue in the middle of the C-terminal membrane-spanning α-helix of subunit c (Sebald & Hoppe, 1981). Also in common is the blockade of proton movement after reconstitution of the holoenzyme by adding F_1. An important difference to the F_o parts from the proton-translocating ATPases, however, is the inhibition of proton translocation through *P. modestum* F_o by Na^+ or Li^+ ions at concentrations of about 1 and 100 mM, respectively. These results reflect the physiological function of F_1F_o from *P. modestum* as a Na^+-translocating ATP synthase and are reminiscent of the inhibition of ATP-driven active proton pumping by F_1F_o with similar concentrations of Na^+ ions (Laubinger & Dimroth, 1989). Our results are in agreement with the hypothesis that Na^+ and H^+ (H_3O^+) ions may compete for a common binding site within the F_o sector of the *P.*

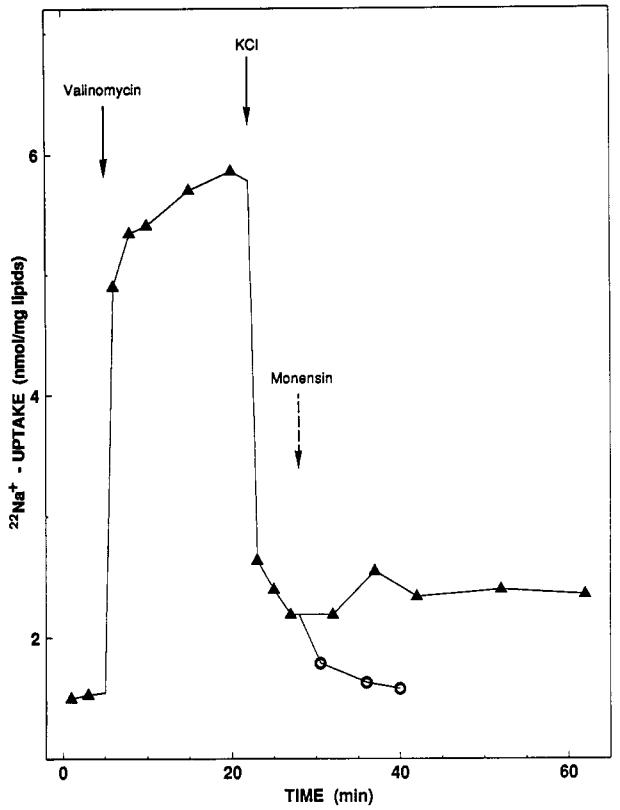


FIGURE 7: Uptake of $^{22}Na^+$ into F_o–liposomes induced by a K^+-diffusion potential (inside negative) and inversion of the flux direction at a reversed potential (outside negative). F_o–liposomes were loaded with 23 mM KCl and 50 mM choline chloride. Forty microliters of these F_o–liposomes (2 mg of crude lipids) was diluted into 1.4 mL of the standard reaction mixture used for $^{22}Na^+$ uptake determination. $^{22}Na^+$ influx was initiated by adding valinomycin (arrow) in order to create $\Delta\psi$ (inside negative). After 22 min, KCl was added to a final concentration of 320 mM (marked by arrow) to create $\Delta\psi$ (outside negative), and $^{22}Na^+$ retained inside the F_o–liposomes was determined for additional 40 min (▲). As a control, 6 nM monensin was added after 28 min (dashed arrow) (○).

modestum ATPase and that the mechanism for the translocation of these different ions may be the same (Laubinger & Dimroth, 1989). This hypothesis is of course not compatible with a proton translocation mechanism through a network of a hydrogen-bonded chain within the membrane ("proton wire") (Nagle & Morowitz, 1978) but rather favors the translocation of a distinct molecule (H_3O^+). Incidently, crown ethers can form very similar coordination complexes with H_3O^+ or with Na^+ (Boyer, 1988).

Importantly, inhibition of $\Delta\psi$-driven proton flux into F_o–liposomes by Na^+ or Li^+ depends on the side to which these ions are added. While proton uptake was completely abolished with 2 mM NaCl or 150 mM LiCl added to the outside of the proteoliposomes, the same concentrations of these ions within the liposomes were without effect. Externally exposed cation binding sites of F_o are therefore apparently not readily accessible from the inner surface of the membranes. F_o from *P. modestum* thus evidently does not represent a simple pore.

Na^+ Influx. A pore-type mechanism was also excluded from $^{22}Na^+$ uptake experiments into F_o–liposomes. $^{22}Na^+$ accumulated inside the proteoliposomes in response to $\Delta\psi$ (inside negative) was not released by passage through Dowex 50, K^+. The internal $^{22}Na^+$ was therefore not freely accessible from the outside through F_o, whereas in the presence of monensin the proteoliposomes were completely depleted of $^{22}Na^+$ by the same procedure. Interestingly, $^{22}Na^+$ uptake into F_o–liposomes required $\Delta\psi$ (inside negative) and could not be accomplished by ΔpNa^+ of the same size. The flux of Na^+, therefore, appears to be directed by the potential but not by the concentration gradient. Like proton uptake, Na^+ uptake into F_o–liposomes was abolished with the c-subunit-specific inhibitor DCCD or after rebinding of F_1. The influx of Na^+ is therefore catalyzed by F_o with its F_1 binding site exposed to the outside.

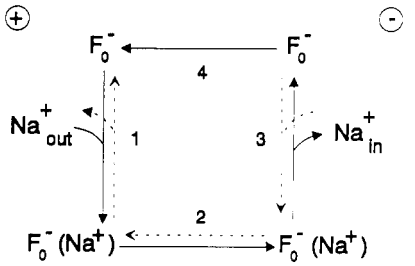
The maximum rate of Na^+ influx into F_o–liposomes [13 $Na^+/(F_o \cdot s)$] is similar to that of ATP-driven Na^+ transport by F_1F_0 [6 $Na^+/(F_1F_o \cdot s)$], suggesting that the same basic Na^+ translocation mechanism applies for the isolated F_o moiety or for F_o bound to F_1. The rate of Na^+ translocation through F_o from *P. modestum* is comparable to that of H^+ translocation through F_o from most other F_1F_o ATPases [for a review, see Schneider and Altendorf (1987)]. These rates are typical for carriers which at a time expose the substrate binding site to one side of the membrane only and move the substrate across it by a conformational change that exposes the binding site to the opposite surface [for a review, see Stein (1986)]. The observed rates are at least 5 orders of magnitude lower than those of typical channels or pores, which when open are accessible from both membrane surfaces simultaneously. Exceptionally high rates of proton conduction through F_o from spinach chloroplasts were reported [6×10^5 $H^+/CF_o \cdot s$) at 100 mV], indicating a channel-type mechanism for this protein (Lill et al., 1987). Such a mechanism, however, can be clearly excluded for F_o from *P. modestum* not only from the observed rates but also from many other data described in this paper.

Effect of Membrane Potential. A very important observation is the profound effect of the membrane potential on Na^+ translocation through F_o. The initial rate of Na^+ uptake into F_o–liposomes was very low below –40 mV and increased sharply between about –40 and –115 mV. This dependency of the Na^+ conductivity on $\Delta\psi$ could indicate a potential-dependent step in the catalytic cycle which would be rate-limiting at lower potentials where the rate of Na^+ uptake was $< V_{max}$. Also important is the linear relationship between the logarithm of Na^+ uptake and the membrane potential in the steady state, indicating that the size of the ΔpNa^+ established across the membrane is directly related to the $\Delta\psi$ applied and thus that the transport of Na^+ through F_o is an electrogenic event, as one would expect.

Reversibility of Na^+ Flux. A sensitive method to assess reversibility is by $^{22}Na^+$ counterflow. Therefore, $^{22}Na^+$ was added to the outside of F_o–liposomes to which ΔpNa^+ of about 86 mV was applied from the inside to the outside. Under these conditions, radioactive sodium ions rapidly accumulated on the inside while no $^{22}Na^+$ counterflow was observed, if $\Delta\psi$ of 90 mV (outside negative) was applied. $^{22}Na^+$ uptake into these F_o–liposomes clearly occurs by the counterflow mechanism, since no uptake of radioactivity was observed if the F_o–liposomes contained no unlabeled Na^+ ions on the inside. The counterflow of $^{22}Na^+$ against its chemical gradient again is a clear rejection criterion for a pore-type mechanism for F_o from *P. modestum* [see Stein (1986) for a review]. The data show reversibility of Na^+ flux across the membrane through F_o in the absence of a membrane potential. No unidirectional Na^+ movement was found under these conditions, while in presence of $\Delta\psi$ of about 100 mV only unidirectional Na^+ movement but no exchange of internal and external Na^+ ions was observed.

The reversibility of Na^+ flux through F_o was further shown by loading the proteoliposomes with $^{22}Na^+$ through F_o by applying $\Delta\psi$ (inside negative). As more than 90% of these

Scheme I: Schematic Representation of Reactions Involved in Na^+ Influx and Counterflow As Catalyzed by the F_o Moiety of the *P. modestum* ATPase[a]



[a] Na^+ counterflow proceeds by the reversible reactions 1, 2, and 3 (solid and dashed lines) in the absence of a membrane potential. Na^+ uptake (solid lines) in addition requires the membrane potential-dependent reorientation of the unloaded carrier (step 4). At membrane potentials of about −100 mV, the velocity of step 4 must be so high that counterflow does not occur. F_o catalyzes Na^+ efflux, if the membrane potential is reversed (outside negative).

internal $^{22}Na^+$ ions were released upon reverting the membrane potential (outside negative) with similar kinetics as $^{22}Na^+$ uptake, it appears appropriate to assume that the translocation of $^{22}Na^+$ through F_o may occur independent of the orientation of F_o within the membrane but strictly following the direction of the applied membrane potential. Also in accord with this notion is the abolition of $\Delta\psi$-driven Na^+ uptake by rebinding F_1 to the outside. F_o oriented with the F_1 binding site to the inside of the proteoliposomes does not seem to significantly take part in Na^+ translocation under our conditions, probably because F_1 released to the inside during dissociation rebinds to internally oriented F_o in subsequent handling of the proteoliposomes.

A model summarizing the results of Na^+ translocation through F_o from *P. modestum* is shown in Scheme I. Na^+ influx (or H^+ influx in the absence of Na^+) is assumed to involve at least four steps: (1) binding of Na^+ to F_o from the outer surface of the membrane; (2) translocation of the binary complex to the inner surface; (3) release of Na^+; (4) return of the unloaded F_o to the outer surface.

Alternately, counterflow of Na^+ involves steps 1–3 only. As counterflow occurs in the absence of a membrane potential, whereas Na^+ influx or efflux are absolutely dependent on $\Delta\psi$, we predict that reorientation of unloaded F_o (step 4) represents a membrane potential-dependent step. The Na^+ binding site of unloaded F_o predicted to carry a negative charge is forced to move to the positively charged surface, thereby directing the movement of Na^+ ions from the positive to the negative surface of the membrane.

The initial velocity of Na^+ counterflow (in the absence of $\Delta\psi$) and V_{max} of Na^+ uptake (at −115 mV) were about the same [1.9–2.7 μmol/(min·mg of F_o)], suggesting that step 4 is not rate-limiting for Na^+ uptake under these conditions. Fast reorientation of the unloaded F_o at high $\Delta\psi$ (step 4) is also in accord with the lack of Na^+ counterflow under these conditions. Step 4 should become rate-limiting for Na^+ uptake, however, at low membrane potentials, where the rate of Na^+ uptake significantly decreases to reach zero in the absence of $\Delta\psi$. As one would predict from this model, these conditions are favorable for the catalysis of Na^+ counterflow.

These and previous results on the ATPase of *P. modestum* seem to indicate that the basic events of cation translocation through isolated F_o are the same as through F_o of the F_1F_o complex. F_o and F_1F_o share similar V_{max} and K_T (Na^+) values for Na^+ translocation or pumping. K_T (Na^+) is also similar to K_m (Na^+) in activating ATP hydrolysis by F_1F_o. Free F_o and F_1F_o perform proton translocation or pumping, respectively, which are both prevented by the same Na^+ concentrations or by incubation with DCCD. However, while Na^+ translocation through isolated F_o is induced by $\Delta\psi$, with F_1F_o this process requires ATP hydrolysis. Reorientation of the Na^+ binding site accomplished by $\Delta\psi$ in free F_o may therefore be mediated in the complex by conformational changes of F_1 that are elicited by ATP hydrolysis. One may speculate that the $\Delta\psi$-induced conformational change in free F_o may be induced in F_1F_o by a distinct motion of charged amino acid residues. Thus, ATP-coupled Na^+ translocation is accomplished. Conversely, ATP hydrolysis by F_1F_o but not by F_1 alone is significantly enhanced by Na^+ ions, suggesting again that the complex is only functional in performing a complete cycle of conformational changes involving F_1 and F_o that includes binding of Na^+ ions to the F_o moiety.

ACKNOWLEDGMENT

We thank the radiochemical group of the ETH Zürich for providing the opportunity to perform $^{22}Na^+$ measurements.

REFERENCES

Amann, R., Ludwig, W., Laubinger, W., Dimroth, P., & Schleifer, K. H. (1988) *FEMS Microbiol. Lett. 56*, 253–260.
Boyer, P. D. (1988) *Trends Biochem. Sci. 13*, 5–7.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Dimroth, P. (1982) *Eur. J. Biochem. 121*, 443–449.
Dimroth, P. (1987) *Microbiol. Rev. 51*, 320–340.
Esser, W., Krumholz, L. R., & Simoni, R. D. (1990) *Nucleic Acids Res. 18*, 5887.
Fillingame, R. H. (1990) *The Bacteria 12*, 345–391.
Hilpert, W., Schink, B., & Dimroth, P. (1984) *EMBO J. 3*, 1665–1670.
Kaim, G., Ludwig, W., Dimroth, P., & Schleifer, K. H. (1990) *Nucleic Acids Res. 18*, 6697.
Kaim, G., Ludwig, W., Dimroth, P., & Schleifer, K. H. (1992) *Eur. J. Biochem. 207*, 463–470.
Laubinger, W., & Dimroth, P. (1987) *Eur. J. Biochem. 168*, 475–480.
Laubinger, W., & Dimroth, P. (1988) *Biochemistry 27*, 7531–7537.
Laubinger, W., & Dimroth, P. (1989) *Biochemistry 28*, 7194–7198.
Laubinger, W., Deckers-Hebestreit, G., Altendorf, K., & Dimroth, P. (1990) *Biochemistry 29*, 5458–5463.
Lill, H., Althoff, G., & Junge, W. (1987) *J. Membr. Biol. 98*, 69–78.
Ludwig, W., Kaim, G., Laubinger, W., Dimroth, P., Hoppe, J., & Schleifer, K. H. (1990) *Eur. J. Biochem. 193*, 395–399.
Nagle, J. F., & Morowitz, H. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 298–302.
Schneider, E., & Altendorf, K. (1987) *Microbiol. Rev. 51*, 477–497.
Sebald, W., & Hoppe, J. (1981) *Curr. Top. Bioenerg. 12*, 1–64.
Stein, W. D. (1986) *Transport and Diffusion Across Cell Membranes*, Academic Press, Orlando, FL.

Registry No. ATPase, 9000-83-3; Na^+, 7440-23-5; H^+, 12408-02-5; Li^+, 7439-93-2.