The Sodium Ion Translocating Adenosinetriphosphatase of *Propionigenium modestum* Pumps Protons at Low Sodium Ion Concentrations†

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ABSTRACT: The purified ATPase (F_1F_0) of *Propionigenium modestum* has its pH optimum at pH 7.0 or at pH 6.0 in the presence or absence of 5 mM NaCl, respectively. The activation by 5 mM NaCl was 12-fold at pH 7.0, 3.5-fold at pH 6.0, and 1.5-fold at pH 5.0. In addition to its function as a primary Na^+ pump, the ATPase was capable of pumping protons. This activity was demonstrated with reconstituted proteoliposomes by the ATP-dependent quenching of the fluorescence of 9-amino-6-chloro-2-methoxyacridine. No ΔpH was formed in the presence of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone or by blocking the ATPase with dicyclohexylcarbodiimide. In the presence of valinomycin and K^+, the ΔpH increased, in accord with the operation of an electrogenic proton pump. The proton pump was only operative at low Na^+ concentrations (<1 mM), and its activity increased as the Na^+ concentration decreased. Parallel to the decrease of H^+ pumping, the velocity of the Na^+ transport increased about 6-fold from 0.1 to 4 mM NaCl, indicating a switch from H^+ to Na^+ pumping, as the Na^+ concentration increases. Due to proton leaks in the proteoliposomal membranes, fluorescence quenching was released after blocking the ATPase with dicyclohexylcarbodiimide, by trapping residual ATP with glucose and hexokinase, or by the Na^+-induced conversion of the proton pump onto a Na^+ pump. Amiloride, an inhibitor of various Na^+-coupled transport systems, was without effect on the kinetics of Na^+ transport by the *P. modestum* ATPase.

The strictly anaerobic bacterium *Propionigenium modestum* uses a Na^+ cycle for energy conservation (Hilpert et al., 1984). The organism ferments succinate to propionate and CO_2 via succinyl-CoA, (*R*)- and (*S*)-methylmalonyl-CoA, and propionyl-CoA. The only reaction of this pathway that is sufficiently exergonic for energy conservation is the decarboxylation of (*S*)-methylmalonyl-CoA to propionyl-CoA ($\Delta G^{\circ\prime} \sim -27$ kJ/mol). The decarboxylation is catalyzed by a membrane-bound biotin-containing enzyme and is coupled to the electrogenic extrusion of two Na^+ per reaction (Hilpert et al., 1984; Dimroth, 1987; Dimroth & Hilpert, 1984).

The thus established Na^+ gradient serves a Na^+-translocating ATPase as energy source for ATP synthesis (Hilpert et al., 1984; Laubinger & Dimroth, 1987, 1988). This novel type of ATP synthesis mechanism has been termed decarboxylation phosphorylation (Laubinger & Dimroth, 1988). The use of Na^+ instead of H^+ as the coupling ion by the *P. modestum* ATPase is of considerable interest. Recently, the enzyme has been purified and shown to be a typical ATPase of the F_1F_0 type (Laubinger & Dimroth, 1987, 1988). The water-soluble F_1 part consists of five polypeptides (α, β, γ, δ, ϵ), and the membrane-bound F_0 moiety is composed of three polypeptides (a, b, c). These subunits correspond to the subunits of the F_1F_0 ATPase of *Escherichia coli* with respect to molecular weights and staining intensities on SDS gels. In addition, these ATPases are similarly affected by various inhibitors including dicyclohexylcarbodiimide (DCCD),[1] which is covalently bound to subunit c of both enzymes.

The use of different coupling ions is the most pronounced difference between these enzymes. Sodium ion pumping by the *P. modestum* ATPase (the reversal of physiological ATP synthesis) has been demonstrated with bacterial vesicles (Hilpert et al., 1984) and with a reconstituted proteoliposomal system (Laubinger & Dimroth, 1988). Stimulation of Na^+ transport by a proton conducter has excluded the possibility that a proton gradient is an intermediate state in the coupling of Na^+ transport to ATP hydrolysis (Laubinger & Dimroth, 1988). The Na^+ transport function of this enzyme correlates with a specific activation of ATPase activity of F_1F_0 by this cation (Hilpert et al., 1984; Laubinger & Dimroth, 1987, 1988). This activation was abolished in the purified F_1 ATPase and restored upon reconstitution of F_1F_0, indicating that the Na^+ binding site is located on the F_0 sector of the enzyme complex.

In this paper, we have extended the transport measurements with the reconstituted ATPase-containing proteoliposomes. It is shown here that the ATPase functions as a proton pump at low Na^+ concentrations.

Experimental Procedures

Purification of F_1F_0 ATPase. Propionigenium modestum was grown on succinate under strictly anaerobic conditions as described (Laubinger & Dimroth, 1988). The ATPase (F_1F_0) was extracted from the isolated bacterial membranes and purified by fractionated precipitation with poly(ethylene glycol) as described (Laubinger & Dimroth, 1988).

Reconstitution of Proteoliposomes. A suspension of 60 mg of phosphatidylcholine (Sigma, type II S) in 1.8 mL of buffer A (5 mM potassium phosphate, pH 7.0, containing 5 mM $MgCl_2$, 100 mM K_2SO_4, and 1 mM dithioerythritol) was sonicated for 2 × 1 min at 80 W under a nitrogen atmosphere, while cooling with ice. The purified F_1F_0 ATPase (0.6 mg of protein in 0.1 mL of buffer A) was mixed with 0.9 mL of the suspension of the liposomes and reconstituted by the freeze–thaw–sonication procedure described by Laubinger and Dim-

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[1] Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide.

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roth (1988). The kinetics of Na^+ transport were determined with proteoliposomes prepared as above, but with 50 mM potassium phosphate buffer, pH 7.0, containing 5 mM $MgCl_2$ and 1 mM dithioerythritol, instead of buffer A and at a ratio of phospholipid to protein of 200 to 1.

Determination of Na^+ Transport. The incubation mixtures contained the following at 25 °C in 0.7 mL: 50 mM potassium phosphate buffer, pH 7.0, 5 mM $MgCl_2$, 20 units of pyruvate kinase, 6 mM phosphoenolpyruvate, 2 mM $^{22}NaCl$ (400 cpm $nmol^{-1}$), and the reconstituted proteoliposomes (obtained from 7 mg of crude lipid). The transport was initiated after 5 min by adding 2.5 mM K-ATP, and Na^+ uptake into the proteoliposomes was measured as described (Laubinger & Dimroth, 1988).

Fluorescence Assay. The standard reaction mixtures contained the following in 1.5 mL at 25 °C: 5 mM potassium phosphate buffer, pH 7.0, 5 mM $MgCl_2$, 100 mM K_2SO_4, 1.3 μM ACMA, and the reconstituted proteoliposomes (2.7–2.9 mg of lipid). After the signal had stabilized, the reaction was initiated by adding 2.5 mM K-ATP. Fluorescence was measured with an Aminco SPF 500 spectrofluorometer using an excitation wavelength of 410 nm and an emission wavelength of 480 nm. Special precautions, as described by Dimroth and Thomer (1986), were taken to reduce the endogenous Na^+ concentration as far as possible.

ATPase Assay. The ATPase activity of reconstituted proteoliposomes was measured, as described (Laubinger & Dimroth, 1988), using the same buffer as applied in the respective transport experiment.

Protein was determined according to Bradford (1976), with the modifications described (Laubinger & Dimroth, 1987).

Results

Proton Transport into Reconstituted Proteoliposomes. It has previously been demonstrated with reconstituted proteoliposomes that the ATPase of *P. modestum*, an enzyme of the F_1F_0 type, acts as a primary Na^+ pump (Laubinger & Dimroth, 1988). This Na^+ transport function can be correlated with a specific activation of the ATPase activity of F_1F_0 by Na^+ ions. The residual activity in the absence of Na^+ ions (about 10% of V_{max}), however, was puzzling. As it could indicate an activation by protons, we measured the effect of pH on the ATPase activity with or without Na^+. The results shown in Figure 1 indicate different pH profiles in the absence or presence of Na^+ ions. In the absence of Na^+, the ATPase has its pH optimum at pH 6.0, and the activity at pH 5.0 and 7.5 decreases by 40% and 80%, respectively. In contrast, the pH optimum in the presence of 5 mM NaCl is at pH 7.0, and the activity decreases at pH 5.0 or 7.5 by 86% or 6%, respectively. A 12-fold activation of the ATPase activity by 5 mM NaCl at pH 7.0 is thus opposed to only a 1.5-fold activation at pH 5.0. These data may suggest that the ATPase is activated not only by Na^+ ions but also by protons, albeit less efficiently.

We have therefore investigated whether protons were also transported by this ATPase. Proton transport into the liposomes was monitored by the quenching of ACMA fluorescence. A decrease in fluorescence is thought to result from accumulation of the dye inside the liposomes in response to the ΔpH generated by proton pumping of the ATPase complex. The results of Figure 2 indicate a significant quenching of ACMA fluorescence following ATP addition and the reversion of this reaction by adding the uncoupler CCCP. The extent of the ATP-dependent fluorescence quenching increased in the presence of valinomycin in accordance with its origination from an electrogenic H^+-translocating ATPase.

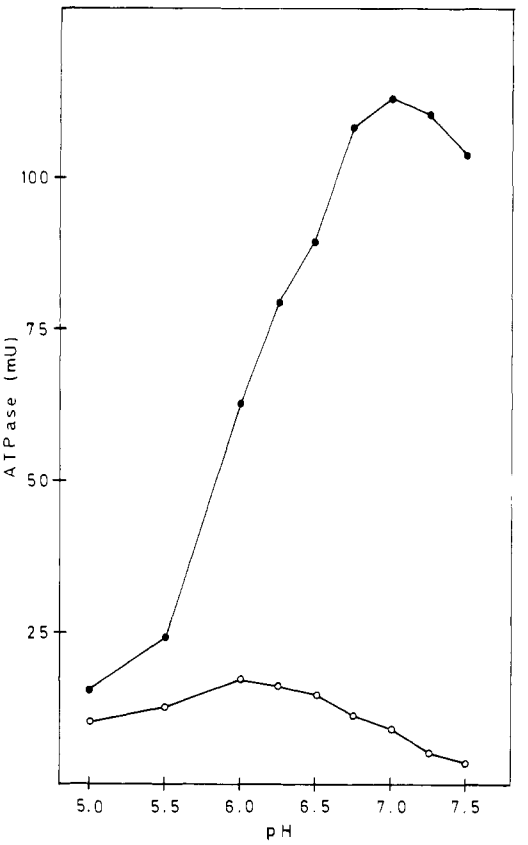

FIGURE 1: pH profiles of the ATPase (F_1F_0) of *P. modestum* in the absence (○) or presence (●) of 5 mM NaCl. The ATPase activities were determined spectrophotometrically (Laubinger & Dimroth, 1987) in incubation mixtures containing the following in 1.0 mL at 25 °C: 50 mM potassium phosphate buffer of the pH indicated, 5 mM $MgCl_2$, 0.3 mM NADH, 3 mM phosphoenolpyruvate, 15 units of lactate dehydrogenase, 10 units of pyruvate kinase, 2.5 mM K-ATP, and 0.11 unit of the ATPase.

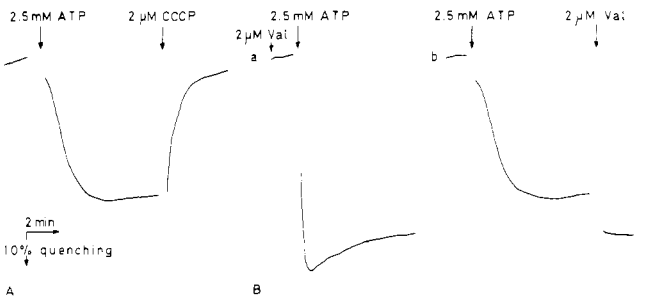

FIGURE 2: Quenching of ACMA fluorescence by reconstituted proteoliposomes. (A) The quenching of fluorescence was initiated by adding 2.5 mM K-ATP and abolished with 2 μM CCCP, as indicated. (B) Same as (A) but with valinomycin (Val) (2 μM) applied either before (a) or after ATP addition (b). See Experimental Procedures for details of the fluorescence quenching assay.

The effect of the ATPase inhibitor DCCD on the generation of a ΔpH is shown in Figure 3. With 50 μM inhibitor, which completely abolished the ATPase activity, the ATP-dependent decrease of ACMA fluorescence was also abolished, indicating that the catalytically active ATPase is in fact required for ΔpH generation. If a ΔpH was first generated and the ATPase subsequently inhibited by DCCD, the proton gradient was rapidly abolished, as indicated by the release of the fluorescence quench. The proteoliposomes are therefore apparently leaky for protons, so that maintenance of a ΔpH requires the continuous operation of the ATP-driven proton pump. This conclusion was confirmed by the release of fluorescence quenching after turning off H^+ pumping by trapping the re-

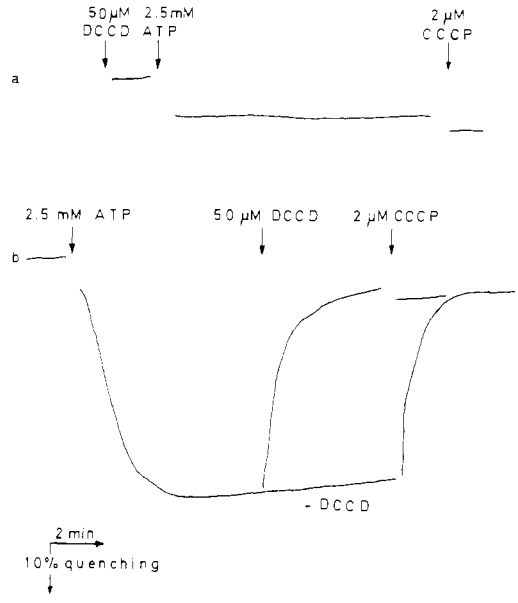

FIGURE 3: Inhibition of ACMA fluorescence quenching by DCCD. Additions of DCCD and K-ATP were made as indicated in the figure. For details of the fluorescence quenching assay, see Experimental Procedures.

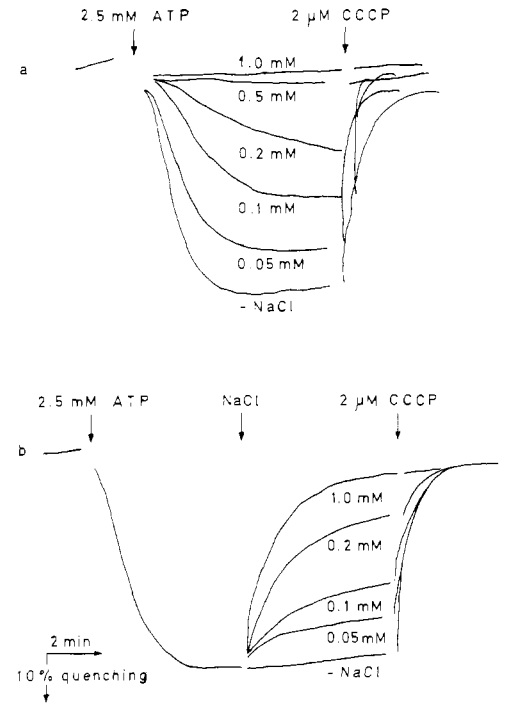

FIGURE 4: Effect of Na^+ ions on the quenching of ACMA fluorescence by reconstituted proteoliposomes. (a) Fluorescence quenching was initiated by adding 2.5 mM K-ATP (arrow) to reaction mixtures containing the concentrations of NaCl indicated. The quenching was released by adding 2 μM CCCP. (b) Fluorescence quenching was initiated by adding 2.5 mM K-ATP to Na^+-free reaction mixtures. Subsequently, NaCl (different concentrations) and CCCP (2 μM) were added, as indicated. Details of the fluorescence quenching assay are described under Experimental Procedures.

sidual ATP with glucose and hexokinase (not shown). In summary, these results demonstrate that the ATPase of *P. modestum* can act as a proton pump.

Effect of Na^+ Concentration on H^+ and Na^+ Pumping by the P. modestum ATPase. In the experiments shown in Figure 4, we studied the effect of Na^+ concentration on the proton pump activity of the *P. modestum* ATPase. The results indicate that Na^+ ions had a marked effect on the ATP-dependent quenching of ACMA fluorescence. The strongest

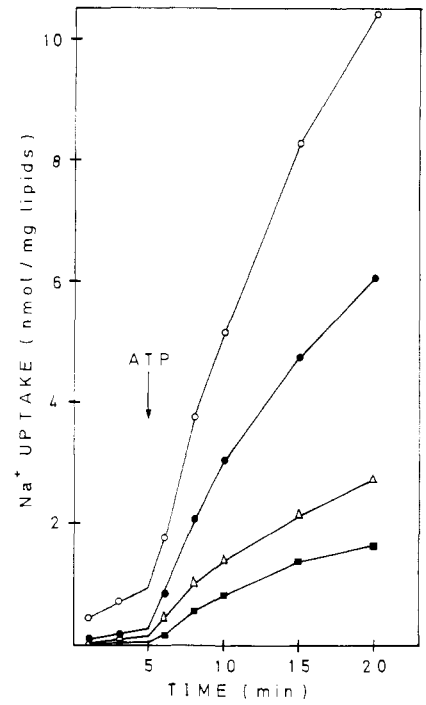

FIGURE 5: Effect of Na^+ concentration on the kinetics of Na^+ uptake into reconstituted ATPase containing proteoliposomes. The uptake of Na^+ ions was determined as described under Experimental Procedures with proteoliposomes prepared from 7 mg of crude lipids. The $^{22}NaCl$ concentrations in the incubation mixtures (total volume 0.7 mL) were (○) 4, (●) 1, (△) 0.2, and (■) 0.1 mM.

quenching was observed in the absence of Na^+ ions, and the rate and extent of the quenching decreased, as the Na^+ concentrations increased. Half-maximal activity was observed at about 0.1 mM NaCl, and as little as 1 mM NaCl completely prevented the fluorescence quenching, indicating that the pumping of protons has been switched off. If the proton gradient was first established by the ATPase in the absence of Na^+ ions, the fluorescence quenching was released by the subsequent addition of NaCl. The rate and extent of the rise of fluorescence increased with increasing Na^+ concentrations. At NaCl concentrations of about 0.1–0.2 mM, the ΔpH was diminished by half, and 1 mM NaCl completely abolished the proton gradient, because the fluorescence quenching was not further diminished by the subsequent addition of CCCP. These results resemble those obtained with the ATPase inhibitor DCCD or with the addition of an ATP trap (see above) and confirm the conclusion that the proton pump activity is severely inhibited in the presence of NaCl. The rate of H^+ pumping decreases in relation to the Na^+ concentration applied and is apparently completely turned off at about 1 mM NaCl.

The effect of Na^+ concentration on the kinetics of Na^+ transport into the proteoliposomes is shown in Figure 5. The initial rate of Na^+ uptake increased 6-fold on increasing the Na^+ concentration from 0.1 to 4 mM. A reciprocal plot of initial velocity versus Na^+ concentration yielded a straight line with an apparent K_M of 0.4 mM (not shown) which is similar to that found for the activation of ATP hydrolysis by Na^+ (0.8 mM) (Laubinger & Dimroth, 1988). Proton pumping by the ATPase is 50% inhibited by Na^+ concentrations that are about 2–4 times lower than the apparent K_M for this ion in its own transport. In summary, the results show that within a certain range of Na^+ concentrations the rate of proton translocation is decreased, as Na^+ transport is increased. The low apparent $K_{1/2}$ for Na^+ as an inhibitor for proton translocation is consistent with an alternative coupling of the *P. modestum* ATPase with either Na^+ or H^+ ions.

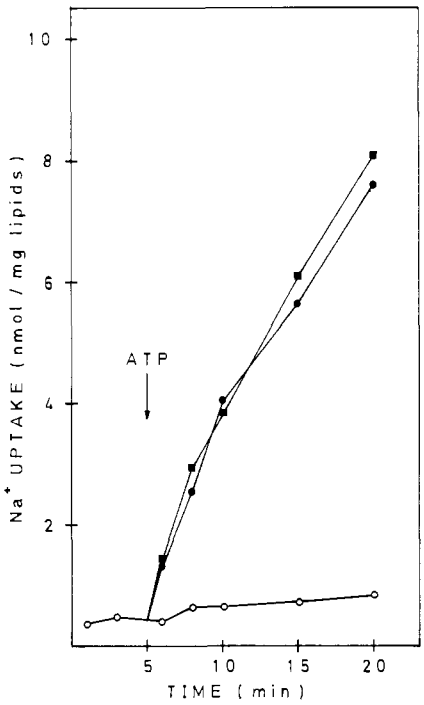

FIGURE 6: Effect of amiloride on the kinetics of Na^+ uptake into reconstituted ATPase containing proteoliposomes. The uptake of Na^+ ions was determined as described under Experimental Procedures with proteoliposomes prepared from 7 mg of crude lipids. Amiloride (0.5 mM final concentration) was added as a solution in dimethyl sulfoxide (■). The control (●) contained the same concentration of dimethyl sulfoxide (0.5%). Control without ATP (○).

The rate of Na^+ uptake increased about 6-fold in the presence of 3 μM valinomycin, without affecting the ATP hydrolysis rate (not shown). The ratio of Na^+ transport/ATP hydrolysis was thus 1 and 0.15 in the presence or absence of valinomycin, respectively. Valinomycin similarly increased the rate and extent of proton translocation (Figure 2) and was without effect on the rate of ATP hydrolysis (not shown). These data indicate that only a fraction of the ATPase present in the proteoliposomes is correctly incorporated to perform ATP hydrolysis coupled to Na^+ (H^+) translocation; the major fraction of the ATPase molecules, however, hydrolyzes ATP uncoupled from Na^+ (H^+) transport. The effect of monensin is also in accord with this notion: the rate of ATP hydrolysis was not stimulated although the transport of Na^+ was completely abolished. In the presence of 5 mM NaCl, the ATPase of the proteoliposomes was activated 1.8-fold. This activation is considerably less than observed for the soluble enzyme but is of the same degree as in the presence of Triton X-100 (Laubinger & Dimroth, 1987). A conformation favorable for ATP hydrolysis may thus be stabilized by either phospholipid, Triton X-100, or Na^+ ions.

Amiloride is a potent inhibitor for many Na^+-coupled transport systems (Benos, 1982), including Na^+ channels, Na^+/H^+ antiporter, and the Na^+-driven flagellar motor of alkalophilic *Bacillus* (Sugiyama et al., 1988). The inhibitor might therefore interact with Na^+-coupled transport systems through a common binding site. The effect of 0.5 mM amiloride on the transport of Na^+ by the *P. modestum* ATPase is shown in Figure 6. There is no difference to the control in the absence of the drug, indicating that the ATPase of *P. modestum* is devoid of an amiloride binding site that affects the transport of Na^+ ions. In addition, these results confirm our previous conclusion (Laubinger & Dimroth, 1988) that the Na^+ transport by the *P. modestum* ATPase is a primary event and not catalyzed by a proton-translocating ATPase in combination with a Na^+/H^+ antiporter, which would be expected to be amiloride sensitive.

Discussion

The two membrane-bound enzymes methylmalonyl-CoA decarboxylase and ATPase effect a Na^+ circuit in *P. modestum*, that couples the exergonic decarboxylation of methylmalonyl-CoA to ATP synthesis (Hilpert et al., 1984; Laubinger & Dimroth, 1988). The decarboxylation of methylmalonyl-CoA ($\Delta G^{\circ\prime} \sim -27$ kJ/mol) is coupled to an extrusion of two Na^+ ions per reaction (Dimroth & Hilpert, 1984). In order to account for the energetic requirements of ATP synthesis under physiological conditions ($\Delta G' \sim +50$ kJ/mol), the Na^+/ATP stoichiometry is expected to be about 4. As the highest ratio of Na^+ transport to ATP hydrolysis found with the reconstituted proteoliposomal system was 1, these considerations are in accord with the notion that only a fraction of the ATPase molecules has been properly reconstituted under the conditions employed here. In spite of using Na^+ as a coupling ion, the ATPase of *P. modestum* has a remarkable structural and functional relationship to the H^+-translocating ATPase of *E. coli* and other enzymes of this type (Laubinger & Dimroth, 1988). The use of different coupling ions appeared therefore to be the most pronounced difference between these enzymes.

Our present investigations show that partial correspondence among these ATPases exists even with respect to the coupling ion, since they both have the capacity to catalyze H^+ translocation. Whereas proton transport is the principal activity of the *E. coli* ATPase, the enzyme of *P. modestum* pumps protons only at low Na^+ concentrations. The rate of proton pumping decreased gradually from 0 to 1 mM NaCl, where it was turned off completely. Simultaneously with this deceleration of proton pumping, the rate of Na^+ pumping increased, thus indicating a switch of the ATPase from a H^+ to a Na^+ pump at increasing Na^+ concentrations. The best explanation for this property would be a competition of Na^+ and H^+ ions for a common binding site on the enzyme. At pH 7 and Na^+ concentrations of about 10^{-4} M, this site may be occupied by either Na^+ or H^+, and both cations are translocated, whereas at Na^+ concentrations of 10^{-3} M and above only the alkali ion is transported. It should be noted in this context that the affinity of the ATPase for protons exceeds its affinity for Na^+ by about 3 orders of magnitude. On the basis of ATP hydrolysis rates in the presence or absence of Na^+ ions (Figure 1), however, one may expect that at pH 7 the V_{max} of H^+ pumping will not be higher than one-tenth the V_{max} of Na^+ transport. It is clear, therefore, that the efficiency of the *P. modestum* ATPase to function as a Na^+ pump is based primarily on a high V_{max} and not on a low K_M for this alkali ion. However, since *P. modestum* requires 0.35 M NaCl for optimum growth (Schink & Pfennig, 1982) Na^+ will certainly be the only coupling ion under physiological conditions.

There is no indication for the existence of distinct Na^+- and H^+-translocating ATPases in *P. modestum*. The subunit pattern of the purified enzyme indicates a single enzyme species and not a mixture of two different ATPases (Laubinger & Dimroth, 1988). In addition, a single sequence was obtained by protein sequencing of the N-terminal region of subunit c (unpublished results). The low $K_{1/2}$ of about 0.1 mM for Na^+ as an inhibitor of H^+ transport also argues against distinct catalytic entities for the transport of Na^+ and H^+. The existence of Na^+- and H^+-translocating ATPases in one organism is also unlikely from functional considerations. If ATP is synthesized under consumption of a Na^+ gradient as antici-

pated for *P. modestum*, this ATP would be immediately cleaved by a H^+-translocating ATPase pumping H^+ out of the cell. The result would be the conversion of a Na^+ into a H^+ gradient but not the net synthesis of ATP.

The capacity of the *P. modestum* ATPase to act as a proton pump may have implications from an evolutionary point of view. It is conceivable that the ATPases of *E. coli* and *P. modestum* derived from a common ancestor gene. Whereas in *E. coli* the proton coupling survived, in *P. modestum* a switch from H^+ to Na^+ as coupling ion has occurred during evolution in accordance to the specific demands of this organism. The silent H^+-translocating activity could thus be a remnant of the evolutionary development of the Na^+ ATPase from a H^+ ATPase. Recently, direct evidence for the relationship between the *P. modestum* and H^+-translocating ATPases was obtained by immunological and sequencing studies. Antiserum raised against the β-subunit of the ATPase of *E. coli* or *Sulfolobus acidocaldarius* reacted with the β-subunit of the *P. modestum* ATPase in immunoblots (unpublished results). The amino acid sequences of the β-subunits of the ATPases of *P. modestum* and *E. coli* were 69% homologous (Amann et al., 1988), and the N-terminal sequences (41 residues) of the membrane-bound subunits c from these two enzymes were 21% homologous (unpublished results).

A specific proton conduction mechanism, suggested for ATPases, is that of a proton wire, in which a hydrogen-bonded chain of protonatable groups adds a H^+ on one side of the membrane and delivers an H^+ on the other side (Nagle & Morowitz, 1978). The use of Na^+ or H^+ as coupling cations by the *P. modestum* ATPase suggests a common conduction mechanism for these cations and argues therefore against proton wires. Recognizing recent discoveries of Na^+-coupled energy transductions, Boyer (1988) suggested an interaction of the protein with the cations by the formation of coordination complexes with appropriately positioned oxygen and/or nitrogen atoms. From analogy to coordination complexes formed by crown ethers, either Na^+ or H_3O^+ could be complexed by the protein. The translocation would be completed by a conformational change which exposes the cation binding region to solute on the other side of the membrane. Our present findings are much in favor of this cation conduction mechanism.

An alternate coupling with Na^+ or H^+ is not unique for the *P. modestum* ATPase. Protons may also substitute for Na^+ ions in the Na^+/K^+ ATPases from human erythrocytes (Blostein, 1985; Polvani & Blostein, 1988) or from pig kidney cells (Hara & Nakao, 1986). Other examples for membrane proteins that may have a common conduction mechanism for protons and metal ions (Na^+ or Li^+) are the melibiose carrier of *E. coli* (Suchiya & Wilson, 1978) and the alanine carrier of the thermophilic bacterium PS3 (Hirata et al., 1984). The melibiose carrier uses Na^+, H^+, or Li^+ as the symported cation. Numerous mutants have been isolated in which the sugar and/or cation specificity was affected (Kawakami et al., 1988; Botfield & Wilson, 1988). All these mutations consisted of substitutions of single amino acid residues clustered mainly in three areas of the porter, which is supposed to reflect the substrate binding region (Botfield & Wilson, 1988). In a number of such mutants, the ability to couple melibiose transport to H^+ was lost, and either Na^+ or Li^+ could be used as a coupling ion, or an absolute requirement for Na^+ was developed (Kawakami et al., 1988). For the ATPase, these observations may suggest that only minor changes of the protein sequence could change the cation specificity from H^+ to Na^+ coupling.

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