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A Hybrid Adenosinetriphosphatase Composed of F_1 of *Escherichia coli* and F_o of *Propionigenium modestum* Is a Functional Sodium Ion Pump†

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ABSTRACT: Analyses on immunoblots indicated strong binding of the α- and β-subunits of the ATPase of *Propionigenium modestum* to antibodies raised against the corresponding subunits of the F_1F_o ATPase of *Escherichia coli*. Cross-reactivities of antibodies against the other ATPase subunits were not observed. The use of Na^+ or H^+ as alternate coupling ions, observed previously for the *P. modestum* ATPase [Laubinger, W., & Dimroth, P. (1989) *Biochemistry 28*, 7194-7198], is not found for the F_1F_o ATPase of *E. coli*, which is specific for protons. However, a hybrid consisting of the F_1 moiety of the *E. coli* ATPase and F_o of that from *P. modestum* performed Na^+ or H^+ transport in a reconstituted system. As with the homologous ATPase of *P. modestum*, H^+ pumping of the hybrid was abolished at Na^+ concentrations of >1 mM. The F_o sector and not F_1, therefore, determines the cation specificity of these F_1F_o ATPases.

The strict anaerobic bacterium *Propionigenium modestum* grows from the fermentation of succinate to propionate and CO_2 (Schink & Pfennig, 1982). A unique feature of the energy metabolism of this organism if the operation of a Na^+ cycle for ATP biosynthesis [Hilpert et al., 1984; for a review, see Dimroth (1987)]. The free energy of methylmalonyl-CoA decarboxylation is used to pump Na^+ ions out of the cell, and the electrochemical Na^+ ion gradient thus established drives ATP synthesis by a Na^+-translocating ATPase.

It is remarkable that in spite of this unusual cation specificity the ATPase of *P. modestum* has properties typical of an ATPase of the F_1F_o type (Laubinger & Dimroth, 1987, 1988). The structures and functions of the ATPases of *P. modestum* and *Escherichia coli* [for a review, see Schneider and Altendorf (1987)] are in fact very similar. They both consist of a water-soluble F_1 moiety, composed of five different subunits, α, β, γ, δ, and ϵ, that catalyzes hydrolysis (or synthesis) of the terminal phosphoric acid anhydride bond of ATP. The β-subunits of these two enzymes have 69% sequence homology (Amann et al., 1988a). Each F_o moiety is a complex of three different subunits, a, b, and c, that is firmly embedded within the lipid bilayer. It provides the pathway for conduction of cations (H^+ or Na^+) across the membrane.

In the ATPase complexes (F_1F_o) the chemical events of ATP hydrolysis (or synthesis) catalyzed by F_1 are coupled to the transport of cations through F_o across the membrane. Whereas the ATPase of *E. coli* translocates protons, that of *P. modestum* uses Na^+ as the coupling ion (Laubinger & Dimroth, 1988). However, at Na^+ concentrations below 1 mM, protons are also translocated by this enzyme (Laubinger & Dimroth, 1989). The Na^+ binding site was suggested to be located on the F_o portion, because the ATPase activity of F_1F_o of *P. modestum*, but not that of F_1 alone, was specifically activated by Na^+ ions (Laubinger & Dimroth, 1987).

The two ATPases also behaved similarly with respect to the inhibitors dicyclohexylcarbodiimide, venturicidin, tributyltin chloride, and azide (Laubinger & Dimroth, 1988). This homology has been extended by the immunological studies described in this paper. In addition, we report here the formation of a hybrid ATPase consisting of F_1 from *E. coli* and F_o from *P. modestum* and its specificity with respect to Na^+ and H^+ translocation.

Experimental Procedures

Materials. Fluorescein isothiocyanate conjugated goat anti-rabbit IgG was purchased from Sigma (Munich, FRG).

Bacterial Growth. Escherichia coli ML308-225 was grown in minimal medium (Davis & Mingioli, 1950) with 0.4% glucose as the energy source. *Klebsiella pneumoniae* (ATCC 138 2) was grown on citrate medium supplemented with L-(+)-glutamic acid (3 mM) and *d*-biotin (7 μg/L) as described (Dimroth, 1986). *Propionigenium modestum* was grown on succinate under strictly anaerobic conditions (Laubinger & Dimroth, 1988). *Veillonella alcalescens* was grown anaerobically on lactate as the energy source (Hilpert & Dimroth, 1986).

Preparative Procedures. Membranes of *E. coli* wild-type ML 308-225, *K. pneumoniae*, and *V. alcalescens* were prepared as described (Vogel & Steinhart, 1976). EF_1[1] (Steffens

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et al., 1987) and EF_1F_o (Friedl et al., 1979) were isolated from everted membrane vesicles of ATPase-overproducing *E. coli* strain KY7485 (Foster et al., 1980). PF_1F_o (Laubinger & Dimroth, 1988) and PF_1 (Laubinger & Dimroth, 1987) were isolated from the membranes of *P. modestum*. In order to raise specific antibodies in rabbits, EF_1F_o was immunized in the presence of SDS. Antibodies against the individual subunits of EF_1 were kindly donated by Dr. R. D. Simoni.

Reconstitution of Proteoliposomes Containing the F_1F_o ATPase. (Method A) Freezing and Thawing. A suspension of 60 mg of phosphatidylcholine (Sigma, type II S) in 1.9 mL of buffer A (50 mM potassium phosphate, pH 8.0, containing 1 mM dithioerythritol and 2 mM NaCl) was sonicated for 2 × 1 min at 80 W under a nitrogen atmosphere, while being cooled with ice. The purified F_1F_o ATPase of *P. modestum* (0.3 mg of protein in 0.1 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 over a time period of about 1 h. The proteoliposomes were sonicated with a microtip for 2 × 5 s at 40 W, and used either directly for translocation experiments or subsequently for the dissociation of F_1.

The F_1F_o ATPase of *E. coli* was reconstituted into proteoliposomes by the same procedure, buffer A being replaced with 20 mM Tricine/KOH buffer, pH 8.0, containing 10 mM NaCl, 100 mM KCl, 2.5 mM $MgCl_2$, and 1 mM dithioerythritol or with 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM NaCl, 50 mM KCl, 2.5 mM $MgCl_2$, and 1 mM dithioerythritol.

(Method B) Removal of Detergent by Amberlite. The general conditions for the application of this method have been described (Krämer & Heberger, 1986). Amberlite XAD-2 (Sigma) was washed three times with methanol and stored under distilled water. The resin was equilibrated with the appropriate buffer, and 1.2 g of the moist beads was poured into a Pasteur pipet. Buffer B (5 mM potassium phosphate, pH 7.0, containing 5 mM $MgCl_2$, 100 mM K_2SO_4, 2 mM NaCl, and 1 mM dithioerythritol) was used for the determination of Na^+ transport, buffer C (same as buffer B, but without NaCl) was applied in the assay for H^+ transport, and buffer D (50 mM potassium phosphate, pH 8.0, containing 1 mM dithioerythritol) was used for removal of F_1 after reconstitution.

For each reconstitution experiment, 14 mg of phosphatidylcholine (Sigma, type II S) was suspended in 1.17 mL of buffer B, C, or D and sonicated under N_2 for 2 × 1 min at 80 W, while being cooled with ice. The *P. modestum* F_1F_o ATPase (0.07–0.28 mg in 0.02 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 0.21 mL of a 10% solution of the detergent $C_{12}E_8$ in the respective buffer and mixed vigorously. After 10 min at 0 °C, the protein/detergent mixture was added to the preformed liposomes (final volume 1.4 mL). The suspension was incubated for 15 min at 25 °C and recycled 15 times over the Amberlite column, prepared as described above. The proteoliposomes formed during this procedure were directly used for the transport measurements or for dissociation of F_1.

Dissociation of F_1 from Proteoliposomes Containing the F_1F_o ATPase. The proteoliposomes reconstituted by method A or B were collected by centrifugation (220000*g*, 45 min) and resuspended in 0.5 mL of buffer E [1 mM Tris, 0.5 mM K_2-EDTA, 10% (v/v) glycerol, 1 mM dithioerythritol, 2 mM NaCl (or without NaCl for proton translocation measurements), pH 9.0] per 10 mg of phospholipid. After 2 h at 25 °C, the dissociated F_1 ATPase was separated from the F_o liposomes by centrifugation (220000*g*, 45 min). The pellet was resuspended in the same volume of buffer E as before, incubated again for 50 min at 25 °C, and centrifuged. The F_1-depleted proteoliposomes were resuspended in 0.1 mL of buffer F (50 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithioerythritol, 2 mM NaCl, pH 8.0) per 10 mg of phospholipid (final volume 0.15 mL/10 mg of phospholipid) for Na^+ transport experiments or in the same volume of buffer G (20 mM Tricine/KOH, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithioerythritol, pH 8.0) for proton translocation measurements.

Binding of F_1 from E. coli (EF_1) to Proteoliposomes Containing PF_o. For Na^+ transport experiments, proteoliposomes, reconstituted with PF_1F_o according to method A and depleted of PF_1, were divided into two portions of 0.4 mL each. EF_1 (0.01 mL, 0.26 mg of protein) was added to one sample; the other served as a control. Both samples were incubated for 16 h at 25 °C and then centrifuged (220000*g*, 45 min). The proteoliposomes were each suspended in 0.4 mL of 50 mM Tris-HCl buffer, pH 8.0, containing 50 mM KCl, 2.5 mM $MgCl_2$, 2 mM NaCl, and 1 mM dithioerythritol.

For H^+ transport experiments, the proteoliposomes reconstituted with PF_1F_o by method B and depleted of PF_1 were used. The reconstituted EF_1PF_o hybrid was formed by incubation of 0.18 mL of the PF_o-containing proteoliposomes with 26 μg of EF_1 ATPase for 16 h at 25 °C.

Determination of Na^+ Transport. Measurements of Na^+ transport into reconstituted proteoliposomes were performed as described (Laubinger & Dimroth, 1988). For the experiments with the EF_1PF_o ATPase hybrid, the composition of the assay buffer was optimized for the requirements of the *E. coli* ATPase. The incubation mixtures contained the following: 50 mM Tris-HCl buffer, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 20 units of pyruvate kinase, 6 mM phosphoenolpyruvate, 2 mM $^{22}NaCl$ (330 cpm/nmol), the proteoliposomes with the ATPase hybrid, and valinomycin (3 μM), if indicated. The transport was initiated by the addition of 5 mM K-ATP.

Determination of H^+ Translocation. Proton translocation was determined by the quenching of ACMA fluorescence, as described (Laubinger & Dimroth, 1989). With the ATPase hybrid, the reaction mixture contained the following: 20 mM Tricine/KOH, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 1.3 μM ACMA, and the proteoliposomes with the ATPase hybrid. The reaction was initiated by the addition of 5 mM K-ATP.

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

Protein was determined according to Bradford (1976) with the modifications described (Laubinger & Dimroth, 1987). The protein content of the membranes used for Western blot analyses was determined as described (Dulley & Grieve, 1975).

SDS gel electrophoresis (gradient slab gels: 7.5–17.5% acrylamide) was performed according to Douglas et al. (1979). Immunoblotting was performed as described by Deckers-

[1] Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; EF_1F_o, ATPase complex of *Escherichia coli*; EF_1, extrinsic ATPase component of *E. coli*; EF_o, intrinsic membrane component of *E. coli* ATPase; PF_1F_o, ATPase complex of *Propionigenium modestum*; PF_1, extrinsic ATPase component of *P. modestum*; PF_o, intrinsic membrane component of *P. modestum* ATPase; EF_1PF_o, ATPase hybrid composed of F_1 from *E. coli* and F_o from *P. modestum*; $C_{12}E_8$, dodecyl octaoxyethylene ether; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

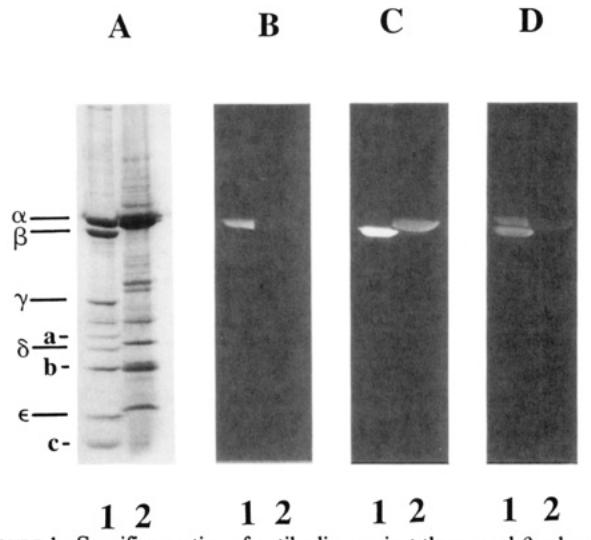

FIGURE 1: Specific reaction of antibodies against the α- and β-subunits of the EF_1 complex with their corresponding antigens in EF_1F_o and PF_1F_o. (A) SDS gel electrophoresis of purified EF_1F_o (lane 1) and PF_1F_o (lane 2) complexes (about 10 μg of protein each) visualized with Serva blue G-250. The subunits of the EF_1F_o complex are marked. (B–D) Immunofluorescence of the blotted F_1F_o complexes on nitrocellulose after incubation with antibodies raised against the α-subunit (B), the β-subunit (C), or EF_1 (D), respectively. All antisera were diluted 250-fold.

Hebestreit and Altendorf (1986) with a fluorescein isothiocyanate conjugated goat anti-rabbit IgG for visualization.

Results

Homologies between ATPases of Different Microorganisms. In a Western blot analysis of membranes from *E. coli*, *K. pneumoniae*, *P. modestum*, and *V. alcalescens* the following cross-reactivities of antibodies raised against subunits of the EF_1F_o complex were observed: strong homology exists between *E. coli* and *K. pneumoniae*. Every antiserum directed against the eight individual subunits of the EF_1F_o complex recognized a corresponding protein in membranes of *K. pneumoniae*. This has also been verified with the purified F_1F_o ATPase from *K. pneumoniae* (Kauffer et al., 1987). With membranes of *P. modestum*, the β-subunit of the ATPase was strongly recognized, while the α-subunit gave only a faint signal, if analyzed with antibodies against the corresponding subunit from the *E. coli* ATPase. With membranes of *V. alcalescens* no cross-reactivities could be detected with antibodies against subunits of EF_1F_o (data not shown).

The results of Figure 1 show the reaction of antibodies against the α- and β-subunits of the EF_1 complex with their corresponding antigens in purified EF_1F_o and PF_1F_o. The results are similar to those obtained with bacterial membranes (see above), showing strong homology between the β-subunits and a much weaker homology between the α-subunits. No cross-reactivity could be observed with antibodies against the γ-, δ-, or ε-subunits of EF_1 or with those raised against the individual subunits of the EF_o complex (data not shown).

E. coli F_1F_o ATPase Is Unable To Pump Na^+ Ions. The discovery that the ATPase of *P. modestum* could pump Na^+ or H^+ under appropriate conditions (Laubinger & Dimroth, 1989) prompted us to investigate whether the ATPase of *E. coli* could pump Na^+ in addition to its well-established function as a proton pump. We were, however, unable to detect such a function using proteoliposomes that were shown to retain the usual proton pumping activity. Even under conditions that should favor Na^+ over H^+ pumping, if these were competitive coupling ions (pH 8.0, 10 mM NaCl), Na^+ was not accumulated by the proteoliposomes above the background that is observed in the absence of ATP. The ATPase of *E. coli* is therefore a specific proton pump.

Table I: Reconstitution of the ATPase of *P. modestum*[a]

reconstitution method	orientation with F_1 to the outside (%)	nmol min^{-1} (mg of lipid)$^{-1}$ ATPase activity −val	ATPase activity +val	Na^+ transport −val	Na^+ transport +val	Na^+/ATP ratio
A	25	4.4	4.4	0.7	6.1	1.4
B	7	1.4	1.6	2.5	4.1	2.6

[a] The phospholipid to protein ratio was 200:1. For details, see Experimental Procedures. val = valinomycin. The orientation of the ATPase in the proteoliposomes was determined by activity measurements in the absence and presence of Triton X-100, as described (Laubinger & Dimroth, 1988).

Properties of Proteoliposomes Reconstituted with the P. modestum ATPase by either Freezing and Thawing or Detergent Removal with Amberlite. During the course of our studies on the formation of ATPase hybrids, two different reconstitution methods have been used. Method A (freezing and thawing) was superior for Na^+ transport measurements, and method B (detergent removal) was advantageous for measuring proton transport. In most transport experiments described in this paper, for each cation the superior reconstitution method was used. The properties of the reconstituted proteoliposomes are summarized in Table I. The percentage of the ATPase with the substrate binding site oriented to the outside was 25% in proteoliposomes prepared by method A and only 7% if the reconstitution was performed by method B. On dissipation of the membrane potential by valinomycin, the ATPase activity of proteoliposomes prepared by method A was not affected, but the initial rate of Na^+ transport increased about 7-fold, yielding a Na^+ to ATP coupling ratio of 1.4. With proteoliposomes prepared by method B, valinomycin stimulated the ATPase activity 1.1-fold and Na^+ uptake 1.6-fold, yielding a Na^+ to ATP stoichiometry of 2.6. The percentage of ATPase molecules oriented with the F_1 moiety to the outside that are competent to function in Na^+ transport is thus higher in proteoliposomes prepared by method B. These proteoliposomes, however, contain 93% of the ATPase oriented with the substrate binding site to the inside and have, therefore, low ATPase and Na^+ transport activities. If the protein to phospholipid ratio used in the reconstitution experiments with either method was raised from 1:200 to 1:50, the Na^+ to ATP coupling ratio dropped, indicating that less ATPase molecules were properly reconstituted to function in Na^+ transport (data not shown).

EF_1PF_o ATPase Hybrid Reconstituted into Proteoliposomes Pumps Na^+ or H^+ Ions. To investigate the formation of a hybrid between PF_o and EF_1 and its possible function in ion translocation, we attempted to dissociate PF_1 from proteoliposomes that were reconstituted with the *P. modestum* ATPase complex (PF_1F_o). During two successive incubations with low ionic strength Tris/EDTA buffer at pH 9.0, more than 96% of the ATPase activity was released into the supernatant. The Na^+ transport activity simultaneously dropped to 3% of its original value. The proteoliposomes retained the F_o moiety, because the addition of an excess of PF_1 restored 77% of the original ATPase and 62% of the Na^+ transport activity (Table II).

Incubation of the PF_o-containing proteoliposomes with an excess of EF_1 resulted in a huge increase of membrane-bound ATPase activity (Table II). Please note that the specific activity of the EF_1 used in these experiments was 70 times higher than that of the PF_1. If this ratio remained the same after binding to PF_o, the ATPase activity of the original

Table II: Dissociation of PF_1 from Proteoliposomes Containing PF_1F_o and Reconstitution of the Enzyme Complex[a]

step	ATPase activity		Na^+ transport activity		Na^+ transport to ATP hydrolysis (mol/mol)
	units	%	units	%	
proteoliposomes	0.22	100	0.33	100	1.5
first Tris/EDTA treatment	0.04	18			
second Tris/EDTA treatment	0.009	4	0.009	3	1.0
incubation with PF_1	0.17	77	0.204	62	1.2
incubation with EF_1	18		0.126	38	0.007

[a] The ATPase was reconstituted by freezing and thawing (method A) with 0.3 mg (1.0 unit) of PF_1F_o and 60 mg of phospholipids. The F_1 moiety was subsequently dissociated with Tris/EDTA buffer, pH 9.0. Reconstitution of the enzyme complex was achieved by incubation of the proteoliposomes containing PF_o with PF_1 (0.3 mg, 0.16 unit) or EF_1 (0.6 mg, 23 units). The proteoliposomes were collected after each treatment by centrifugation. For details, see Experimental Procedures.

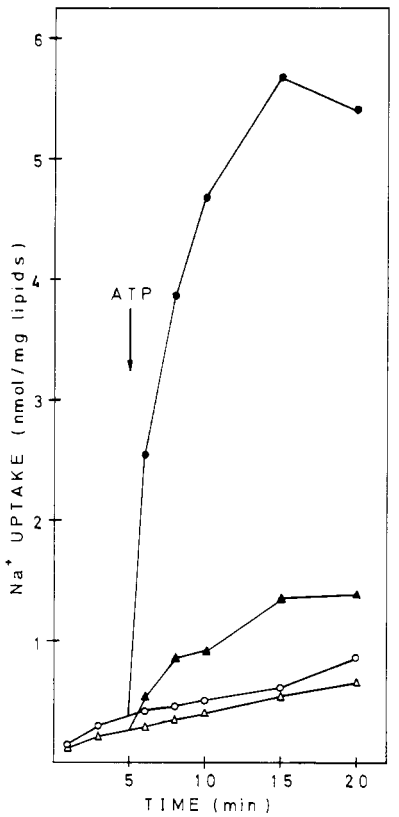

FIGURE 2: Kinetics of Na^+ transport into proteoliposomes containing the EF_1PF_o ATPase hybrid. The PF_1F_o ATPase was reconstituted according to method A, followed by dissociation of PF_1 and substitution by EF_1, as described under Experimental Procedures. The transport of Na^+ ions was determined in the presence of 3 μM valinomycin; it was initiated by ATP addition, as indicated (●). Control without ATP (○). Na^+ transport of the PF_1-depleted proteoliposomes with (▲) and without (△) ATP.

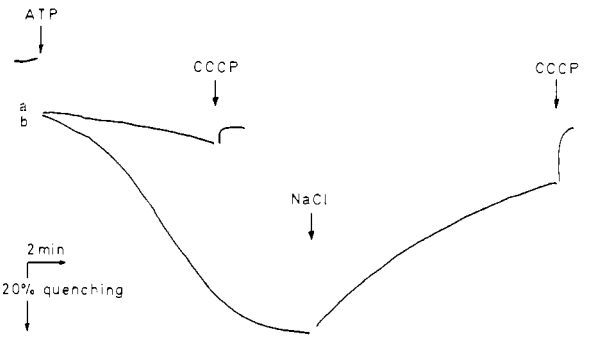

FIGURE 3: Quenching of ACMA fluorescence by reconstituted EF_1PF_o ATPase containing proteoliposomes. The PF_1F_o ATPase was reconstituted according to method B at a protein to phospholipid ratio of 1:50, followed by dissociation of PF_1 and substitution by EF_1, as described under Experimental Procedures. Fluorescence quenching by PF_o- (curve a) or EF_1PF_o- (curve b) containing proteoliposomes was determined in the absence of Na^+ ions. Additions of CCCP (2 μM) and NaCl (2 mM) were made as indicated.

proteoliposomes (0.22 unit) would rise to 15.4 units upon replacement of all PF_1 by EF_1. The observed ATPase activity after incubation of the PF_1-depleted proteoliposomes with EF_1 (18 units) is therefore in the range expected, if an EF_1PF_o hybrid would form. Compelling evidence for the formation of such a hybrid was obtained by the results of Na^+ and proton transport experiments, described below.

The results of Table II indicate that, on incubation of the PF_o-containing proteoliposomes with EF_1, 38% of the Na^+ transport activity of the proteoliposomes containing undissociated PF_1F_o was recovered. Thus, relative to the transport activity reconstituted with PF_1, that reconstituted with EF_1 is 62%. The hybrid ATPase shows therefore a significant Na^+ transport activity. The Na^+/ATP coupling ratio of the original proteoliposomes was 1.5, and that after dissociation and reconstitution with PF_1 was 1.2. In the EF_1PF_o hybrid, however, the Na^+ to ATP stoichiometry was only 0.007, mainly due to the large increase of the ATP hydrolyzing activity. This ATPase activity could result from unspecifically adsorbed EF_1 and/or poor coupling of the EF_1PF_o hybrid. Unlike PF_1, EF_1 may not associate properly to PF_o, or PF_o may be limiting the rate of Na^+ translocation but may not significantly affect the rate of ATP hydrolysis.

The kinetics of Na^+ transport into the reconstituted proteoliposomes are shown in Figure 2. Whereas little Na^+ uptake was observed in the absence of ATP, its addition to EF_1PF_o-containing proteoliposomes initiated a rapid accumulation of the alkali ion. The internal Na^+ concentration increased during the first 10 min and then slowly declined due to exhaustion of phosphoenolpyruvate, the source of ATP regeneration. The PF_o-containing proteoliposomes prior to reconstitution with EF_1 catalyzed only slow Na^+ transport due to its residual ATPase activity. With these vesicles, a Na^+ to ATP coupling ratio of 1 was observed, which is in the same range as found prior to the dissociation of F_1 (1.5, Table II). The proteoliposomes, therefore, do not become significantly more leaky for Na^+ ions by dissociation of the F_1 moiety from the ATPase complex. The Na^+ transport increase after binding of EF_1 is thus certainly the result of a functional EF_1PF_o Na^+ pump and not caused by the closing of Na^+ leaks.

Proton transport into the reconstituted proteoliposomes was determined by the quenching of ACMA fluorescence. The results of Figure 3 show ATP-dependent fluorescence quenching of proteoliposomes containing the EF_1PF_o ATPase hybrid and only little quenching by PF_o-containing proteoliposomes, probably due to the residual ATPase activity (about 4%, cf. Table II). The quenching was released by CCCP as expected. Proton transport by the EF_1PF_o hybrid was only observed in the absence of Na^+ ions (not shown), and if NaCl was added after a proton gradient was established, the

fluorescence quenching was partially released. These results are very similar to observations made previously with the homologous PF_1F_o ATPase (Laubinger & Dimroth, 1989) and indicate that the F_o sector and not F_1 is responsible for the competition between Na^+ and H^+ as coupling cations of the *P. modestum* ATPase.

Discussion

We show here that the β-subunit of the Na^+-translocating ATPase of *P. modestum* is a strong antigen for antibodies raised against the corresponding protein of the H^+-translocating ATPase of *E. coli*. This relationship is consistent with 69% sequence homology among these two β-subunits (Amann et al., 1988a). Immunological homology of the β-subunit of PF_1F_o has also been demonstrated with an antiserum directed against the β-subunit of the F_1-analogous ATPase of *Sulfolobus acidocaldarius* (Lübben et al., 1988). The α-subunit of the *P. modestum* ATPase was also an antigen for antibodies against the corresponding protein of *E. coli*, but cross-reactivities for other ATPase subunits were not observed. In contrast, all eight subunits of the ATPase of *K. pneumoniae* were recognized by an antibody mixture against F_1F_o of *E. coli*. These results are in accord with phylogenetic relationships among these bacteria. Whereas *E. coli* and *K. pneumoniae* are closely related enterobacteria, *P. modestum* is a strict anaerobe that is distinctly more distantly related (Schink & Pfennig, 1982). In membranes of *V. alcalescens*, no cross-reactivities could be detected with antibodies against subunits of EF_1F_o, although especially the antibodies against the highly conserved β-subunit (Amann et al., 1988b) showed binding to the corresponding protein in many other organisms (Lübben et al., 1987). These data are consistent with the lack of ATPase activity in the membranes of *V. alcalescens* (unpublished observation), indicating that an F_1F_o ATPase does not exist within these strictly anaerobic bacteria. This is probably the reason why *V. alcalescens*, which has the same enzyme equipment for the conversion of succinate to propionate and CO_2 as *P. modestum* (Dimroth, 1987), cannot grow like this organism on succinate (Schink & Pfennig, 1982).

The homology between EF_1F_o and PF_1F_o partially extends even with respect to the coupling cation, because the *P. modestum* ATPase switches from Na^+ to H^+ translocation at Na^+ concentrations of <1 mM (Laubinger & Dimroth, 1989). Although Na^+ is the only physiological coupling ion of the *P. modestum* ATPase (internal Na^+ concentration of >10 mM), the affinity of the enzyme for protons exceeds that for Na^+ by about 4 orders of magnitude. We were unable to detect Na^+ translocation by the *E. coli* ATPase, even at pH 8.0 and 10 mM NaCl. This enzyme is therefore either completely specific for protons, or its affinity for protons is at least 6 orders of magnitude higher than that for Na^+ ions.

On treatment of proteoliposomes containing PF_1F_o with Tris/EDTA buffer, pH 9.0, about 96% of the F_1 ATPase was dissociated. The residual ATPase transported Na^+ at about the same Na^+/ATP stoichiometry as that of the untreated proteoliposomes, indicating that Na^+ leaks were not created within the same particles that contained the residual ATPase complexes. By use of 4 μL/mg of phospholipid as the internal volume of proteoliposomes prepared by freezing, thawing, and sonication (Laubinger, 1987), it can be calculated that under our conditions for reconstitution approximately one ATPase Na^+ pump (oriented with the substrate binding site to the outside) was present in every 2.7 proteoliposomes. One can thus predict that the number of particles containing F_o in addition to an undissociated F_1F_o is very low. Therefore, the Na^+/ATP stoichiometry should not be altered significantly by partial dissociation of the F_1 moiety. The results are in accord with these expectations; they do not indicate, however, whether or not F_o functions as an open Na^+ channel.

The Na^+ transport activity of these proteoliposomes increased about 14-fold after reconstitution with EF_1. By the same arguments, as described above, this increase of Na^+ pumping cannot be accounted for by the blocking of Na^+ leaks of proteoliposomes containing in addition a functional PF_1F_o complex. The additional Na^+ pump activity must be attributed, therefore, to the function of reconstituted EF_1PF_o as a Na^+ pump. This reconstituted ATPase hybrid could pump not only Na^+ ions but also protons at Na^+ concentrations of <1 mM. The properties of the hybrid with respect to cation specificity are thus very similar to those of the homologous PF_1F_o ATPase (Laubinger & Dimroth, 1989). The formation of a functional hybrid provides further evidence for the relationship between the *E. coli* and *P. modestum* ATPases. Especially, those areas of the protein structure that are involved in the binding of F_1 and F_o must have been highly conserved.

Attempts to construct the reciprocal PF_1EF_o hybrid have so far not been successful. This is mainly due to the fact that the specific activity of PF_1 is more than 20 times lower than that of EF_1 (Laubinger & Dimroth, 1987), which makes the recording of H^+ translocation difficult.

The results presented here and in previous publications (Laubinger & Dimroth, 1987, 1988, and 1989) have far-reaching consequences on our knowledge of the ATPase reaction mechanism in general. We can now exclude all models that are based on a direct interaction of the "vectorial" protons in the chemical events of ATP synthesis and hydrolysis (Mitchell, 1974) on the basis of the following arguments: (i) protons are not the only coupling ions of F_1F_o ATPases, because the enzyme from *P. modestum* uses Na^+ or H^+ as alternate coupling ions under certain conditions; (ii) the ATPase of *P. modestum* is not a completely different type of ATPase but rather a member of the F_1F_o family; (iii) the structural relationship of this enzyme to other members of the family is complemented by the formation of a functional EF_1PF_o hybrid; (iv) this hybrid has the same cation specificity as the homologous PF_1F_o ATPase but is clearly distinct from EF_1F_o; (v) these specificities provide strong arguments for the location of the cation binding site on F_o and not on F_1.

We suggest therefore that, according to the model of Boyer (1975), ATP synthesis is initiated by the binding of the cations to specific sites on F_o from the side of high electrochemical potential. The mechanism of alternate coupling by the *P. modestum* ATPase indicates that these sites must accommodate binding of either Na^+ ions or protons (Laubinger & Dimroth, 1989). The protons could be transported as H_3O^+, because hydronium ions and Na^+ ions effect a very similar type of binding by crown ethers (Boyer, 1988). This cation binding could trigger a conformational change that exposes the cations to the other side of the membrane and brings about ATP synthesis on the F_1 moiety of the enzyme complex. After dissociation of the cations, the enzyme is expected to return to its original conformation.

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Substitution of Arginine for Histidine-47 in the Coenzyme Binding Site of Yeast Alcohol Dehydrogenase I†

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ABSTRACT: Molecular modeling of alcohol dehydrogenases suggests that His-47 in the yeast enzyme (His-44 in the protein sequence, corresponding to Arg-47 in the horse liver enzyme) binds the pyrophosphate of the NAD coenzyme. His-47 in the *Saccharomyces cerevisiae* isoenzyme I was substituted with an arginine by a directed mutation. Steady-state kinetic results at pH 7.3 and 30 °C of the mutant and wild-type enzymes were consistent with an ordered Bi-Bi mechanism. The substitution decreased dissociation constants by 4-fold for NAD^+ and 2-fold for NADH while turnover numbers were decreased by 4-fold for ethanol oxidation and 6-fold for acetaldehyde reduction. The magnitudes of these effects are smaller than those found for the same mutation in the human liver β enzyme, suggesting that other amino acid residues in the active site modulate the effects of the substitution. The pH dependencies of dissociation constants and other kinetic constants were similar in the two yeast enzymes. Thus, it appears that His-47 is not solely responsible for a pK value near 7 that controls activity and coenzyme binding rates in the wild-type enzyme. The small substrate deuterium isotope effect above pH 7 and the single exponential phase of NADH production during the transient oxidation of ethanol by the Arg-47 enzyme suggest that the mutation makes an isomerization of the enzyme–NAD^+ complex limiting for turnover with ethanol.

Alignment of the amino acid sequences of the homologous horse liver and yeast alcohol dehydrogenases (EC 1.1.1.1) suggests that His-47 in the yeast enzyme (Arg-47 in the liver enzyme) binds the phosphate of the AMP moiety of coenzyme (Jörnvall et al., 1978; Eklund et al., 1976, 1981). Molecular modeling shows that Nδ of the imidazole ring can form a hydrogen bond with a phosphate oxygen (Eklund et al., 1987; Plapp et al., 1987). Histidine might interact more weakly than arginine and allow faster release of coenzymes, increasing turnover numbers and altering pH dependencies.

Inactivation studies using diethyl pyrocarbonate implicated one histidine residue in the mechanism of *Saccharomyces cerevisiae* alcohol dehydrogenase I, although two histidines (His-47 and His-51) are in the active site. The free enzyme showed a pK value of 7.1 for inactivation (Dickenson & Dickinson, 1975a). The reacting residue could not be identified due to the lability of ethoxyformylated histidine.

A mutant yeast alcohol dehydrogenase with Arg-47 was generated previously by selecting for mutants that could grow in the presence of allyl alcohol (Wills, 1976; Wills et al., 1981). Wills proposed that the mutants survived because the redox

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