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Biochimica et Biophysica Acta 1229 (1995) 96–102

BBA Biochimica et Biophysica Acta

Sodium ion dependence of inhibition of the Na^+-translocating F_1F_0-ATPase from *Acetobacterium woodii*. Probing the site(s) involved in ion transport

Martin Spruth [1], Jutta Reidlinger [2], Volker Müller *

Institut für Mikrobiologie der Universität, Grisebachstr. 8, D-37077 Göttingen, Germany

Received 29 August 1994; accepted 5 December 1994

Abstract

The Na^+-translocating F_1F_0-ATPase of *Acetobacterium woodii* was stimulated not only by Na^+ but also by Li^+ and was protected by Na^+ or Li^+ from inactivation by *N,N'*-dicyclohexylcarbodiimide (DCCD), diethylstilbestrol (DES) and tributyltin (TBSn) but not *N*-ethylmaleimide (NEM) or azide. The amount of Na^+ required for half-maximal protection from DCCD inhibition corresponded to the apparent K_m for Na^+ of ATP hydrolysis. The inhibition by the amiloride derivatives hexamethylene-amiloride (HMA), ethylisopropyl-amiloride (EIPA), *N*-10-benzyl-amiloride (benzamil) and *N*-10-phenamil-amiloride (phenamil) could be relieved by Na^+ to various degrees. EIPA and HMA effectively protected the ATPase from DCCD inactivation, whereas the protection by benzamil and phenamil was only marginal indicating that the unsubstituted guanidinium group is essential for maximal protection from DCCD inactivation. These results indicate that the amiloride derivatives and Na^+ or DCCD compete for a common binding site. Chemical modification of histidine, arginine, aspartate or glutamate residues of the F_1F_0 complex resulted in an inhibition of ATP hydrolysis, indicating an essential function of these residues in the catalytic mechanism but this inhibition could not be relieved by Na^+.

Keywords: ATPase, Na^+-; DCCD; Amiloride; Inhibitor, Na^+; Ion transport; (*A. woodii*)

1. Introduction

The homoacetogenic bacterium *Acetobacterium woodii* is a strictly anaerobic bacterium able to derive energy for growth from the conversion of $H_2 + CO_2$ to acetate via the acetyl-CoA (Wood-Ljungdahl) pathway according to the equation: $4H_2 + 2CO_2 \rightarrow 1\ CH_3COOH + 2H_2O$ [1,2]. In *A. woodii*, this pathway is obligatorily coupled to the generation of a primary electrochemical sodium ion gradient across the membrane, which in turn is used to drive the synthesis of ATP [3]. The enzyme catalyzing Na^+-driven ATP synthesis has been purified and characterized, N-terminal sequences were obtained and the genes coding for three subunits have been cloned and sequenced ([4]; Forster, A., Daniel, R. and Müller, V., unpublished data). According to the biochemical and molecular data, the enzyme is of the F_1F_0-type and uses Na^+ instead of H^+ as the coupling ion. Apart from the well studied ATPase from *Propionigenium modestum* [5–7], the enzyme from *A. woodii* is the second Na^+-translocating F_1F_0-ATPase studied in a purified state.

The discovery and biochemical and molecular analysis of the Na^+-translocating F_1F_0-ATPase of *P. modestum* has already had a great impact on our understanding of ion transport through the F_1F_0 complex [5]. Using the F_0-moitey, evidence for a carrier type rather than a channel type mechanism of ion transport was obtained [8]. Very recently it was shown that the substrate Na^+ protects the Na^+-ATPase or the purified subunit *c* of *P. modestum* against DCCD inhibition [9–11]. Furthermore, the amiloride derivative EIPA, an inhibitor with a higher specificity for Na^+/H^+ antiporters than for Na^+ channels, was shown to inhibit the enzyme from *P. modestum* as well as to protect it from DCCD inhibition [10]. From these experiments it was concluded that the DCCD binding site (Asp-61 in *E. coli* or Glu-65 in *P. modestum*) con-

* Corresponding author. E-mail: wmuelle2@gwdgv1.dnet.gwdg.de. Fax: +49 551 393793.

[1] Present address: Institut für Hygiene, Leopold-Franzens-Universität, Fritz-Pregl-Str. 3, A-6010 Innsbrück, Austria.

[2] Present address: Department of Biochemistry, Adrian Building, University Road, Leicester, LE1 7RH, UK.

SSDI 0005-2728(94)00192-8

tributes to the cation binding site; since EIPA contains with the guanidinium group a structural element of an arginine residue it was speculated that an arginine residue might interact with the DCCD binding site and, therefore, be involved in ion transport through the enzyme from *P. modestum* [10].

The recently described Na^+-translocating ATPase from *A. woodii* clearly is of the F_1F_0-type but differs from typical eubacterial F_1F_0-ATPases, including the enzyme of *P. modestum*, in some aspects: first, it apparently contains just 6 subunits in contrast to 8 for the enzymes from *P. modestum* or *E. coli*. Second, all attempts including standard techniques to detach the F_1 moiety from membranes or the F_1F_0 complex were unsuccessful, indicating a different type of F_1-F_0 interaction (which might be the result of the different subunit composition?). Third, it is inhibited by nitrate, which does not inhibit other F_1F_0-type enzymes but archaebacterial and V_1V_0-ATPases.

Preliminary data obtained with the ATPase from *A. woodii* indicated that, despite the differences from the enzyme from *P. modestum*, it is also protected from DCCD inhibition by Na^+ [4,12]. However, the influence of other ions on DCCD inhibition and concentration dependencies were not determined. We have now analyzed the protection by alkali cations from DCCD inhibition in more detail and, in addition, have determined the inactivation of the ATPase by DCCD in the presence of different amiloride derivatives. Taken together, these results indicate that the ATPases from *A. woodii* and *P. modestum* use similar groups in the enzyme for binding and/or transport of the ion.

Substrate protection from inhibitor action is a usefull tool to determine ion (= substrate) binding sites in ion translocating proteins. It was another aim of this study to identify additional Na^+ binding sites using this approach. Therefore, the dependence on Na^+ of inhibition of the ATPase by different compounds was examined. These studies showed that Na^+ also protects from TBSn and DES inactivation and revealed crucial histidine, arginine, aspartate and glutamate residues within the enzyme complex.

2. Materials and methods

2.1. Growth of organisms and purification of ATPase

A. woodii (DSM 1030) was grown to mid-exponential growth phase as previously described [13], with the addition of 4 g/l glycine to the medium. Preparation of membranes and F_1F_0-ATPase from *A. woodii* was carried out as described [4].

2.2. Reaction with inhibitors and chemical modifiers

Stock solutions of DCCD, diethylpyrocarbonate (DEPC), DES, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl), TBSn, tetranitromethane (TNM) and venturicidin were prepared fresh for each experiment in ethanol, *N*-ethylmaleimide (NEM) in *n*-pentane, amiloride, benzamil, EIPA, HMA and phenamil in dimethylsulfoxide, phenyglyoxal in 50 mM KH_2PO_4 (pH 8.0), 5 mM $MgCl_2$ and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) in H_2O. Membranes or the purified enzyme were preincubated with the inhibitors for a time period indicated for each experiment at 30° C in the absence (the contaminating Na^+ concentration in the buffer systems was 0.1 mM) or presence of Na^+ (5 mM) before ATPase activities were determined. The preincubation was carried out directly in the cuvette or with a concentrated enzyme solution; in the latter case, aliquots were diluted into reaction buffer to stop the reaction with the inhibitor. For incubation with DEPC, EDAC and phenylglyoxal 50 mM KH_2PO_4 (pH 6.5) (DEPC and EDAC) or 7.9 (phenylglyoxal), 5 mM $MgCl_2$ was used. All other incubations were carried out in 100 mM Tris (pH 7.5) (adjusted with maleic acid), 5 mM $MgCl_2$. Controls received the solvents only.

2.3. Protection from DCCD inhibition by amilorides

The enzyme was preincubated first with DCCD and the amiloride derivative for 30 min. By diluting the sample 80-fold the non-covalently bound amiloride derivative was removed, whereas the covalently bound DCCD remained bound to the enzyme, thereby inhibiting ATP hydrolysis.

2.4. Labeling with [^{14}C]DCCD

ATPase from *A. woodii* (120 μg) was incubated with 50 μM [^{14}C]DCCD (specific activity: 54 mCi/mmol) in the absence or presence of 10 mM Na^+ for 1.5 h at 30° C (total volume, 300 μl). The sample was diluted 5-fold into equilibration buffer (50 mM Tris (pH 7.5), 0.5 mM $MgCl_2$, 0.2% Triton X-100) and applied to a Q-Sepharose column (2 ml bed volume). After washing with 25 ml equilibration buffer, ATPase was eluted with equilibration buffer which contained in addition 2 M KCl. Aliquots of the fractions were counted for radioactivity with a liquid scintillation counter. An ethanolic stock of [^{14}C]DCCD was prepared by evaporating the toluene under a gentle stream of air and dissolving the [^{14}C]DCCD in ethanol. SDS gel electrophoresis and autoradiography was performed as described [14,15].

2.5. Assays

ATPase activity was routinely determined by a coupled spectrophotometric assay with pyruvate kinase and lactate dehydrogenase as described [4]. The buffer was 100 mM Tris (pH 7.5) (adjusted with maleic acid), 5 mM $MgCl_2$. In case of interference of inhibitors with the enzymatic assay, the ATP-dependent formation of orthophosphate

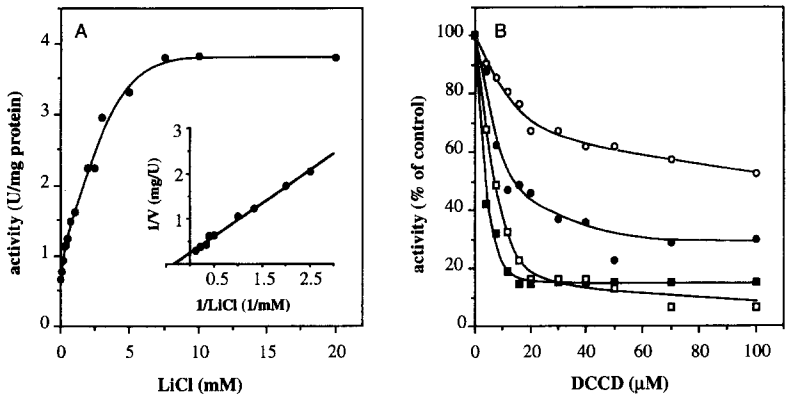


Fig. 1. Stimulation of ATP hydrolysis (A) and protection from DCCD inhibition (B) by Li^+ of the ATPase from *A. woodii*. Panel A, ATP hydrolysis by the purified ATPase was determined at the indicated Li^+ concentrations. For the double-reciprocal plot (inset) the activity at 0 mM LiCl was subtracted. In panel B, the enzyme was incubated with DCCD for 30 min at 30° C in the presence of 0 (■), 5 (□) 15 (●) and 50 mM (○) LiCl. Activities are given as percent of controls determined in the absence of DCCD (cf. panel A).

was monitored [16]. The enzymatic assay was started by adding a preincubated mixture of ATP, NADH, phospho-*enol*pyruvate, pyruvate kinase and lactate dehydrogenase whereas the discontinous assay was started by addition of ATP. Because of the broad absorption peak of the amiloride derivatives around 350 nm orthophosphate formation was monitored at 440 nm when the amiloride concentration exceeded 200 μM. In all cases, it was verified that the inhibitors did not affect the assay system. To determine the effect of Na^+ or Li^+, Tris-ATP was prepared as described [4] and used as substrate. Protein concentrations were determined by a modified Lowry procedure using bovine serum albumin as standard [16,17]. Triton X-100 was removed prior to the test by extraction with diethyl ether/ethanol (3:1, v/v).

2.6. Materials

DEPC, DES, EDAC, NBD-Cl, NEM, Venturicidin, Ba_2-ATP and phenylglyoxal were obtained from Sigma (München, Germany). Pyruvate kinase and lactate dehydrogenase were from Boehringer Mannheim. Poly(ethylene glycol) 6000 was obtained from Serva (Heidelberg, Germany). DCCD and TNM were purchased from Aldrich (Steinheim, Germany). [^{14}C]DCCD was from Amersham Buchler (Braunschweig, Germany), Na_2-ATP and NADH from Gerbu (Gaiberg, Germany) and TBSn from Fluka (Neu-Ulm, Germany). Tris (p.a.) and KH_2PO_4 (suprapur) used for Na^+-free buffers were obtained from Merck (Darmstadt, Germany). Amiloride derivatives were from Dr. E.J. Cragoe, Jr., Lansdale, PA, USA.

3. Results

3.1. Effect of alkali cations on ATPase activity and DCCD inhibition

Li^+ is known to substitute for Na^+ in a number of Na^+ translocating proteins. ATP hydrolysis as carried out by

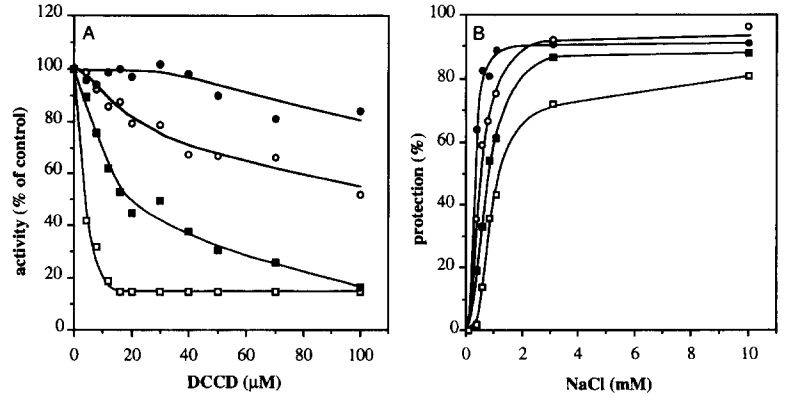


Fig. 2. Protection of the ATPase from *A. woodii* from DCCD inhibition by Na^+. Panel A, purified ATPase (4–14 μg in a volume of 0.8 ml) was incubated with DCCD for 30 min at 30° C in the presence of 0.1 (□), 0.4 (■), 1.1 (○) or 10 mM (●) NaCl. Activities are given as percent of controls determined in the absence of DCCD. Panel B, replot of the data of panel A, activities are given as protected activities (0% protection = residual activity at 0.1 mM NaCl) at 8 (●), 20 (○), 50 (■) and 100 μM (□) DCCD.

the Na^+-ATPase from *A. woodii* was also stimulated by Li^+ (Fig. 1A) but not by K^+ (data not shown); however, the apparent K_m for Li^+ was 2.5 mM compared to 0.4 mM for Na^+ [4] and the maximal activity was only 74% of the one obtained with saturating concentrations of Na^+. In addition, Li^+ but not K^+ protected the enzyme from DCCD inhibition but the amount required for half-maximal protection was increased compared to Na^+ (Fig. 1B).

3.2. Dependence of protection from DCCD inhibition on the Na^+ concentration

Upon preincubation with DCCD (15 μM) for 30 min in the absence of Na^+ (the contaminating Na^+ concentration in the buffer system was 0.1 mM), ATP hydrolysis was almost completely inhibited (Fig. 2A). Increasing Na^+ concentrations led to a relief of inhibition (Fig. 2A): at 100 μM DCCD and 0.4, 1 or 10 mM Na^+ the enzyme was inhibited by 83, 48 and 16%, respectively. From a replot of the percentage of protection caused by Na^+ at different DCCD concentrations (Fig. 2B), the apparent concentration of Na^+ required for half-maximal protection from DCCD inhibition was estimated to be 0.2, 0.4, 0.7 or 1.0 mM Na^+ at 8, 20, 50 and 100 μM DCCD. These values are in the same range as the apparent K_m for Na^+ of ATP hydrolysis [4].

3.3. Dependence of Na^+ protection from DCCD inhibition on the pH

The rate of reaction of DCCD with the enzyme was accelerated by lowering the pH (data not shown), which is in accordance with a reaction mechanism involving the protonated carboxylate. Correspondingly, the protective effect of Na^+ was pH-dependent: at more acidic pH values, higher concentrations of Na^+ were required for protection from DCCD inactivation: 78% of the maximal activity was obtained at pH 8.5, 0.6 mM Na^+ and 100 μM DCCD, whereas at pH 6.5 there was no protection against 100 μM DCCD, even in the presence of 5 mM Na^+.

3.4. Labeling of the ATPase with [^{14}C]DCCD and effect of Na^+

DCCD is known to react with the 'active carboxylate' (Asp-61 in *E. coli* or Glu-65 in *P. modestum*) in subunit *c* of the ATPase giving rise to a dicyclohexylurea bound via an N-acyl bond to the protein. To demonstrate directly that the presence of Na^+ abolished the reaction of DCCD with the ATPase radiolabeling studies with [^{14}C]DCCD in the absence or presence of Na^+ were performed. After incubation of the enzyme with [^{14}C]DCCD in the absence of Na^+, peptides with molecular masses of 44 kDa and 4.8 kDa were labeled (Fig. 3). These polypeptides represent the oligomer and monomer of subunit *c*, respectively [4]. When the Na^+ concentration was increased to 10 mM the enzyme was no longer labeled by [^{14}C]DCCD (Fig. 3), indicating that the presence of Na^+ abolished the reaction of DCCD with subunit *c*.

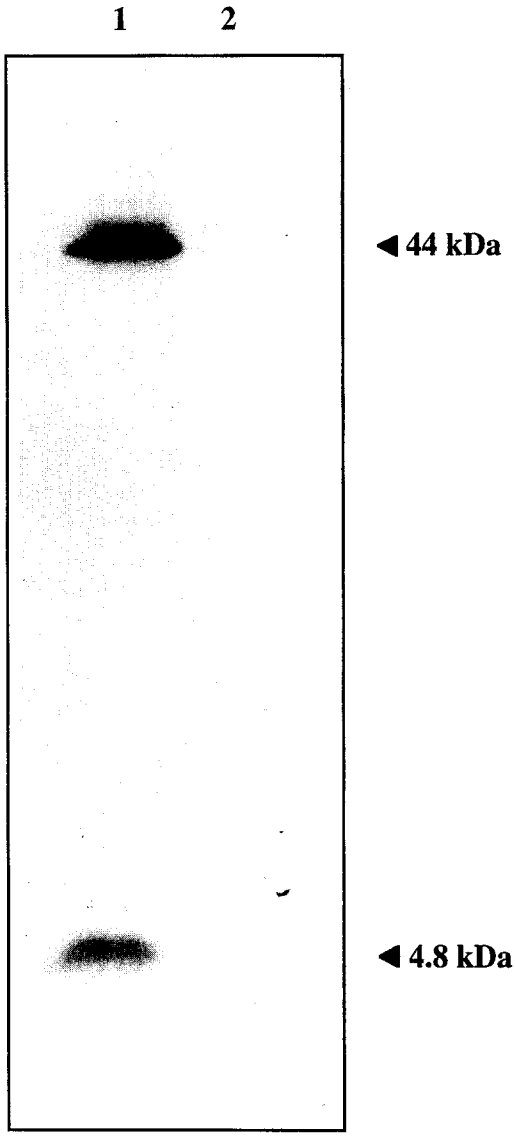


Fig. 3. Autoradiography of [^{14}C]DCCD-labeled ATPase from *A. woodii*. Purified ATPase was labeled with [^{14}C]DCCD (50 μM) in the absence (lane 1) or presence (lane 2) of 10 mM Na^+ as described in Materials and methods.

3.5. Inhibition of the ATPase by amiloride derivatives and the effect of Na^+

In a previous study we have shown that in the presence of Na^+ the ATPase from *A. woodii* is not only inhibited by EIPA but also by HMA and benzamil but, interestingly, not by phenamil [4]; the two former compounds are more specific inhibitors for Na^+/H^+ antiporters than for Na^+ channels whereas the two latter are more specific for Na^+ channels than for antiporters [18]. This inhibition pattern was unexpected and, since the amilorides are known com-

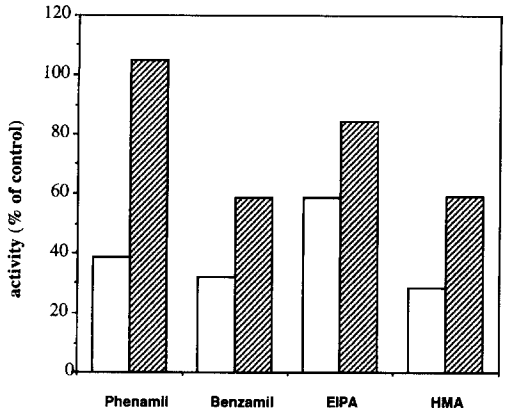


Fig. 4. Protection of the ATPase from *A. woodii* from amiloride inhibition by Na^+. Purified ATPase (5–20 μg) was incubated with the amilorid derivatives (100 μM each) for 30 min at 30° C in the absence (non-shaded columns) or presence (shaded columns) of 5 mM NaCl. ATP hydrolysis was monitored by orthophosphate formation.

petitive inhibitors of Na^+-translocating proteins, the effect of Na^+ on the inhibition was determined. Phenamil, benzamil, EIPA and HMA (each at a concentration of 100 μM) inhibited ATP hydrolysis by 61.5, 68, 41.5 and 71.5%, respectively, in the absence of Na^+. Of interest was now the effect of Na^+: in the presence of 5 mM Na^+ during the preincubation period, the inhibition caused by phenamil was completely relieved, whereas Na^+ protection from benzamil, EIPA and HMA inhibition was significant but less pronounced (Fig. 4).

When the enzyme was preincubated with phenamil in the absence of Na^+, subsequent addition of Na^+ after 30 min also led to a relief of inhibition. Furthermore, inhibition could be relieved by diluting the amiloride derivative containing enzyme solution, indicating that the amiloride derivatives do not bind covalently.

3.6. Inhibition of the ATPase by DCCD and effect of amiloride derivatives

When used at 100 μM in the absence of Na^+, the amiloride derivatives inhibited ATP hydrolysis (cf. Fig. 4) but they did not protect the enzyme from DCCD inactivation (data not shown). Higher concentrations (600 μM) of phenamil, benzamil, EIPA and HMA led to a strong inhibition of the ATPase with residual activities of 9.5, 12, 12 and 8.5%, respectively, and the inhibition could at least partially be relieved by addition of Na^+ (data not shown). Of interest was now the effect of the amilorides on DCCD inactivation of the ATPase. Therefore, the enzyme was first preincubated in the absence of Na^+ for 30 min with DCCD (20 μM) alone or with a combination of DCCD and the amiloride (600 μM) and then subsequently diluted to remove the non-covalently bound amiloride before ATP hydrolysis was determined in the presence of Na^+. In the absence of amilorides the enzyme was inhibited by 91% by DCCD; in contrast, in the presence of phenamil, benzamil, EIPA or HMA the enzyme was only inhibited by 76, 78, 60 and 39%, respectively, by DCCD. These results indicate that HMA and, to a gradually lesser extent, also EIPA protected the enzyme from DCCD inactivation, whereas the protection from DCCD inhibition by phenamil and benzamil was only marginal.

3.7. Inhibition of the ATPase by F_0-directed inhibitors other than DCCD and effect of Na^+

Na^+ did not protect the Na^+-ATPase against inactivation by the F_1-directed inhibitors azide and NBD-Cl, indicating that Na^+ protects only from inhibiton by reagents acting on the F_0 domain. Therefore, other F_0-directed inhibitors were tested. DES at a concentration of 12.5 and 100 μM led to an inhibition of ATP hydrolysis by 30 and 65%, respectively, when the Na^+ concentration was 0.1 mM. Raising the Na^+ concentration to 5 mM led to a complete protection from DES inhibition. Interestingly, addition of Na^+ to the enzyme preincubated with DES led to a reversal of inhibition, indicating that DES does not bind covalently to the enzyme. TBSn at a concentration of 10 μM inhibited ATPase activity by 85% in the absence and 65% in the presence of 5 mM Na^+. Venturicidin inhibited the ATPase to 50% at most. This value was already obtained at a concentration of 10 μM irrespective of the absence or presence of Na^+. From these data it can be assumed that the binding of Na^+ to the F_0-moiety induces a conformational change which results in a reduced accessibility of DES and TBSn.

3.8. Inhibition of the ATPase by chemical modification of histidine, arginine or glutamate residues and effect of Na^+

There is a large body of evidence for the involvement of arginine, glutamate and histidine residues in ion transport through the F_0-domain of the ATPase (for a review see [19]). Therefore, we tested whether a chemical modification of such groups might inhibit the Na^+-ATPase of *A. woodii* and, if so, whether inhibition could be prevented by the presence of Na^+. The histidine-directed reagent DEPC decreased the rate of ATP hydrolysis in a concentration dependent manner with maximal inhibition (60%) at 0.5 mM. The inhibition was relieved by hydroxylamin but the same degree of inhibition was observed in the presence or absence of Na^+. The water soluble, carboxyl group-directed EDAC also inhibited the rate of ATP hydrolysis in a concentration dependent manner but inhibition was independent of Na^+. At 1 mM EDAC, 70–75% inhibition was observed. Phenylglyoxal which modifies arginine residues inhibited the enzyme: at 1 and 10 mM phenylglyoxal the activity was inhibited by 56 and 90%, respectively, but Na^+ did not prevent from inhibition. TNM (1 mM) which

modifies tyrosine residues led to an inhibition of 78% in the absence as well as in the presence of Na^+. There was no significant inhibition by the sulfhydryl-directed agent, NEM, either in the presence or in the absence of Na^+.

4. Discussion

The F_1F_0-ATPase is one of the most fundamental ion pumps in nature which uses the energy stored in transmembrane ion gradients to synthesize ATP. In *E. coli* the enzyme is composed of eight subunits assembled into two domains, the F_1 (α-, β-, γ-, δ- and ϵ-subunits) and the F_0 (*a*-, *b*- and *c*-subunits) with an apparent stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon ab_2c_{9-12}$. The peripheral F_1-domain catalyzes ATP synthesis from ADP + P_i and the membrane-integral F_0-domain catalyzes the ion transport across the membrane. For each ATP synthesized, 2–4 ions have to be transported across the membrane (for reviews see [19–22]).

Despite the large body of biochemical and molecular data on the structure and function of F_1F_0-ATPases, the mechanism of ion translocation through the F_0 domain is not yet known. DCCD is a potent inhibitor of F_1F_0-ATPases and it is known to react with the carboxyl group of an aspartate (Asp-61 in *E. coli*) or glutamate (Glu-65 in *P. modestum*) residue of subunit *c* (the 'active carboxylate') [19]. This acidic residue is well conserved in both Na^+- and H^+-translocating ATPases and a variety of biochemical and molecular data, mostly obtained for the H^+-translocating enzyme from *E. coli*, suggest an involvement of this residue in ion translocation (for a review see [19]). Interestingly, the purified enzyme from *P. modestum* was protected from DCCD inhibition by Na^+ and this was interpreted in terms of a competitive binding for Na^+ and DCCD at the 'active carboxylate,' indicating a function of the 'active carboxylate' as one ligand involved in complexing the Na^+ ion. Kluge and Dimroth developed a model in which DCCD reacts with the protonated carboxyl group, whereas Na^+ binds to the dissociated, negatively charged species by an association/dissociation mechanism. Increasing the Na^+ concentration shifts the equilibrium towards the unprotonated species of the carboxyl group thus decreasing the rate of reaction of the 'active carboxylate' (i.e., carboxylic acid) with DCCD [10]. Although the enzymes from *P. modestum* and *A. woodii* are apparently different in their subunit composition and inhibitor sensitivity, the results obtained in this study, i.e., the protective effect of the substrates Na^+ or Li^+ on DCCD inhibition of the purified Na^+-ATPase of *A. woodii*, the correlation in the amount of Na^+ or Li^+ required for stimulation and for protection, and the observation that the Na^+ concentration required for half-maximal protection was increased when the pH was lowered, are in accordance with this model and further strenghten the importance of this group in ion complexation in F_1F_0-ATPases.

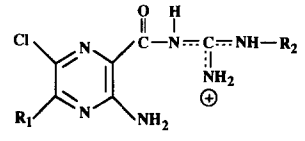
Apart from the similarities, the Na^+-ATPases from *P. modestum* and *A. woodii* differ significantly in their sensitivity towards DCCD. In the presence of 5 mM Na^+ the enzyme from *A. woodii* is apparently completely resistant towards DCCD, even at high concentrations (100 μM), whereas the enzyme from *P. modestum* is inhibited to 82% under the same conditions (Reidlinger, J. and Müller, V., unpublished data). In the presence of 200 μM DCCD and 50 mM Na^+ the enzyme from *A. woodii* is also completely protected, whereas Na^+ does not significantly protect the enzyme from *P. modestum* under these conditions [9,12]. It will be interesting to determine the structural difference of the two enzymes from *P. modestum* and *A. woodii* responsible for this kinetic difference but this has to await the cloning and sequencing of the genes coding for the F_0-domain of the enzyme from *A. woodii*.

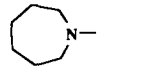
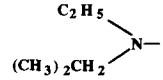
An interesting observation is the inhibition of the Na^+-ATPases from *A. woodii* and *P. modestum* by amiloride derivatives and the protection from DCCD inactivation by these compounds. They are known inhibitors of Na^+-translocating proteins in eukaryotes [18] as well as the Na^+/H^+ antiporter of methanogenic Archaea [23], the Na^+-ATP synthase of *Methanosarcina mazei* [24] and the Na^+-driven flagellar motor of alkaliphilic bacteria [25] as well as of *A. woodii* (Müller, V., unpublished). The amilorides can be divided into two groups (Table 1). Amiloride itself did not inhibit the ATPase from *A. woodii* [4] but substitution of the amino group in position 1 by an hydrophobic cycloalkyl residue as in HMA or by a hydrophobic branched

Table 1
Structures of amiloride derivatives

TABLE I

Structures of amiloride derivatives



	R_1	R_2
HMA		—H
EIPA		—H
Benzamil	NH_2—	—CH_2— 
Phenamil	NH_2—	

alkyl chain such as in EIPA and a substitution of one of the terminal amino hydrogen atoms by a benzyl or phenyl group created potent inhibitors. Apparently, the hydrophobicity has to be increased by substitutions mentioned above in order for the amiloride derivatives to act as ATPase inhibitors. Phenamil inhibition could be completely relieved by Na^+ and both benzamil and phenamil were poor in protecting the enzyme from DCCD inhibition. Therefore, a substitution at the terminal hydrogen of the guanidinium group leads to weaker affinities of the amilorides towards the enzyme compared to Na^+ or DCCD. The relief of phenamil inhibition by Na^+ explains why the enzyme from *A. woodii* was not inhibited by this compound in a previous study [4] in which Na_2-ATP was used as a substrate.

The functional group in the amilorides is the guanidinium moiety which in its protonated form is assumed to interact with the Na^+ binding site by building a salt bridge [10]. According to the proposal of Kluge and Dimroth [10], in a first step Na^+ binds to the unprotonated carboxylate of Glu-65 (or Asp-61) of subunit *c*. Second, Na^+ is displaced by an arginine, giving rise to a carboxylate-arginine salt bridge. One of these steps is accompanied by conformational changes which are transmitted to the F_1 moiety leading to the synthesis of ATP. Our results are in full accord with this hypothesis. It should be mentioned that derivatives of benzamil and phenamil can be synthesized which can be used as a photolabel to identify subunits involved in ion transport.

The fact that Na^+ also protects from inhibition by TBSn and DES might be taken as a further indication that binding or transport of the ion through the F_0 domain induces a conformational change which then reduces the accessibility of the F_0 domain towards the inhibitors. Neither inhibitor binds covalently to the enzyme. However, a specific competition between the inhibitors and Na^+ for a common binding site can not be ruled out. On the other hand, the substrate Na^+ did not prevent the ATPase of *A. woodii* from inhibition by DEPC, phenylglyoxal and EDAC, indicating that the enzyme contains essential histidine, arginine and glutamate residues which in addition to a possible function in ion transport are important for Na^+-independent reactions of the catalytic cycle.

Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft. The skilful technical assistance of S. Bowien is gratefully acknowledged. We are indebted to Prof. G. Gottschalk for generous support and encouragement.

References

[1] Balch, W.E., Schobert, S., Tanner, R.S. and Wolfe, R.S. (1977) Int. J. Syst. Bacteriol. 27, 355–361.
[2] Wood, H.G. and Ljungdahl, L.G. (1991) in Variations in Autotrophic Life (Shively, J. and Barton, L.L., eds.), pp. 201–250, Academic Press, San Diego.
[3] Müller, V. and Gottschalk, G. (1994) in Acetogenesis (Drake, H.L., ed.), pp 127–156, Chapman & Hall, New York.
[4] Reidlinger, J. and Müller, V. (1994) Eur. J. Biochem. 223, 275–283.
[5] Dimroth, P., Laubinger, W., Kluge, C., Kaim, G., Ludwig, W. and Schleifer, H.K. (1992) Ann. N.Y. Acad. Sci. 671, 310–322.
[6] Laubinger, W. and Dimroth, P. (1987) Eur. J. Biochem. 168, 475–480.
[7] Laubinger, W. and Dimroth, P. (1988) Biochemistry 27, 7531–7537.
[8] Kluge, C. and Dimroth, P. (1992) Biochemistry 31, 12665–12672.
[9] Kluge, C. and Dimroth, P. (1993) J. Biol. Chem. 268, 14557–14560.
[10] Kluge, C. and Dimroth, P. (1993) Biochemistry 32, 10378–10386.
[11] Kluge, C. and Dimroth, P. (1994) FEBS Lett. 340, 245–248.
[12] Heise, R., Müller, V. and Gottschalk, G. (1991) Eur. J. Biochem. 206, 553–557.
[13] Heise, R., Reidlinger, J., Müller, V. and Gottschalk, G. (1991) FEBS Lett. 295, 119–122.
[14] Schägger, H. and von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[15] Chamberlain, J.P. (1979) Anal. Biochem. 98, 132–135.
[16] Heinonen, J.E. and Lahti, R.J. (1981) Anal. Biochem. 113, 313–317.
[17] Kresze, G.-B. (1983) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), pp. 84–99, Verlag Chemie, Weinheim.
[18] Kleyman, T.R. and Cragoe Jr., E.J. (1988) J. Membr. Biol. 105, 1–21.
[19] Fillingame, R.H. (1990) in The Bacteria (Krulwich, T.A., ed.), pp. 345–391, Academic Press, San Diego.
[20] Schneider, E. and Altendorf, K. (1987) Microbiol. Rev. 51, 477–497.
[21] Senior, A.E. (1990) Annu. Rev. Biophys. Biophys. Chem. 19, 7–41.
[22] Futai, M., Noumi, T. and Maeda, M. (1989) Annu. Rev. Biochem. 58, 111–136.
[23] Schönheit, P. (1993) in Alkali Cation Transport Systems in Bacteria (Bakker, E.P., ed.), pp. 179–202, CRC Press, Boca Raton.
[24] Becher, B. and Müller, V. (1994) J. Bacteriol. 176, 2543–2550.
[25] Imae, Y. and Atsumi, T. (1989) J. Bioenerg. Biomembr. 1, 705–716.