ELSEVIER

Biochimica et Biophysica Acta 1505 (2001) 108–120

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Review

The Na^+ cycle in *Acetobacterium woodii*: identification and characterization of a Na^+ translocating F_1F_0-ATPase with a mixed oligomer of 8 and 16 kDa proteolipids

Volker Müller *, Sascha Aufurth, Stefan Rahlfs

Lehrstuhl für Mikrobiologie der LMU München, Maria-Ward-Strasse 1a, 80638 Munich, Germany

Received 28 July 2000; received in revised form 30 November 2000; accepted 1 December 2000

Abstract

The homoacetogenic bacterium *Acetobacterium woodii* relies on a sodium ion current across its cytoplasmic membrane for energy-dependent reactions. The sodium ion potential is established by a yet to be identified primary, electrogenic pump connected to the Wood-Ljungdahl pathway. Reactions possibly involved in Na^+ export are discussed. The electrochemical sodium ion potential generated is used to drive endergonic reactions such as flagellar rotation and ATP synthesis. Biochemical and molecular data identified the Na^+-ATPase of *A. woodii* as a typical member of the F_1F_0 class of ATPases. Its catalytic properties and the hypothetical sodium ion binding site in subunit *c* are discussed. The encoding genes were cloned and, surprisingly, the *atp* operon was shown to contain multiple copies of genes encoding subunit *c*. Two copies encode identical 8 kDa proteolipids, and a third copy arose by duplication and subsequent fusion of two genes. Furthermore, the duplicated subunit *c* does not contain the ion binding site in hair pin two. Biochemical and molecular data revealed that all three copies of subunit *c* constitute a mixed oligomer. The evolution of the structure and function of subunit *c* in ATPases from eucarya, bacteria, and archaea is discussed.

Keywords: Sodium ion cycle; ATPase; Subunit *c*; Gene duplication; Mixed oligomer; *Acetobacterium woodii*

1. Introduction

Acetobacterium woodii is a Gram-positive, strictly anaerobic bacterium which grows on a number of substrates, including hexoses and C1 compounds such as methanol or H_2+CO_2 [1]. Because 1 mol of hexose is converted to 3 mol of acetate according to Eq. 1, this fermentation is referred to as homoacetate fermentation:

$$1\ C_6H_{12}O_6 \rightarrow 3\ CH_3COOH \quad (1)$$

Hexoses are oxidized via the Embden-Meyerhof pathway to pyruvate which is then oxidized by pyruvate:ferredoxin oxidoreductase to acetyl-CoA, reduced ferredoxin and CO_2. Acetyl-CoA is converted to acetate via acetyl phosphate. The reducing equivalents gained during glycolysis and pyruvate:ferredoxin oxidoreductase are reoxidized by reducing 2 mol of CO_2 to another mol of acetate via the so-called acetyl-CoA pathway (or Wood-Ljungdahl pathway). First, CO_2 is reduced to formate by action of formate dehydrogenase, then formate is activated and bound to tetrahydrofolate (H_4F), giving rise to formyl-H_4F (Fig. 1). Water is split off, and the resulting methenyl group is reduced via methylene-H_4F

* Corresponding author. Fax: +49-89-2180-6127;
E-mail: v.mueller@lrz.uni-muenchen.de

0005-2728/01/$ – see front matter
PII: S0005-2728(00)00281-4

to methyl-H_4F. The methyl group is then transferred to a protein containing a corrinoid cofactor and iron sulfur clusters, the so-called Co/FeS protein (Co/FeS-P). From there, the methyl group is transferred to the enzyme acetyl-CoA synthase (also called carbon monoxide dehydrogenase or CO-DH) and condensed on the enzyme with carbon monoxide, derived from another mol of CO_2 oxidized by the CO-DH activity of the acetyl CoA synthase, to acetyl-CoA [2–4]. Acetate is produced by action of phosphotransacetylase and acetate kinase. During growth on fermentable sugars, the Wood-Ljungdahl pathway serves as a sink for electrons, thereby allowing the organisms to produce 4 mol of ATP/mol hexose by substrate level phosphorylation; this is one of the highest ATP yields encountered by fermentative bacteria [5].

Apart from carbohydrates, homoacetogenic bacteria can also grow on C1 compounds such as H_2+CO_2 or methanol according to:

$$4\ H_2 + 2\ CO_2 \rightarrow 1\ CH_3COOH + 2\ H_2O \quad (2)$$

$$4\ CH_3OH + 2\ CO_2 \rightarrow 3\ CH_3COOH + 2\ H_2O \quad (3)$$

During growth on H_2+CO_2 according to Eq. 2, CO_2 is reduced to acetate via the Wood-Ljungdahl pathway (Fig. 1) with electrons gained from hydrogen oxidation by action of hydrogenases. This pathway is not coupled to net ATP formation by substrate level phosphorylation: one ATP is gained in the acetate kinase reaction, but one ATP is consumed in the formyl-H_4F synthetase reaction. Therefore, the Wood-Ljungdahl pathway has to be coupled to additional ATP synthesis by ion gradient-driven phosphorylation [6]. Today, 11 genera of homoacetogens

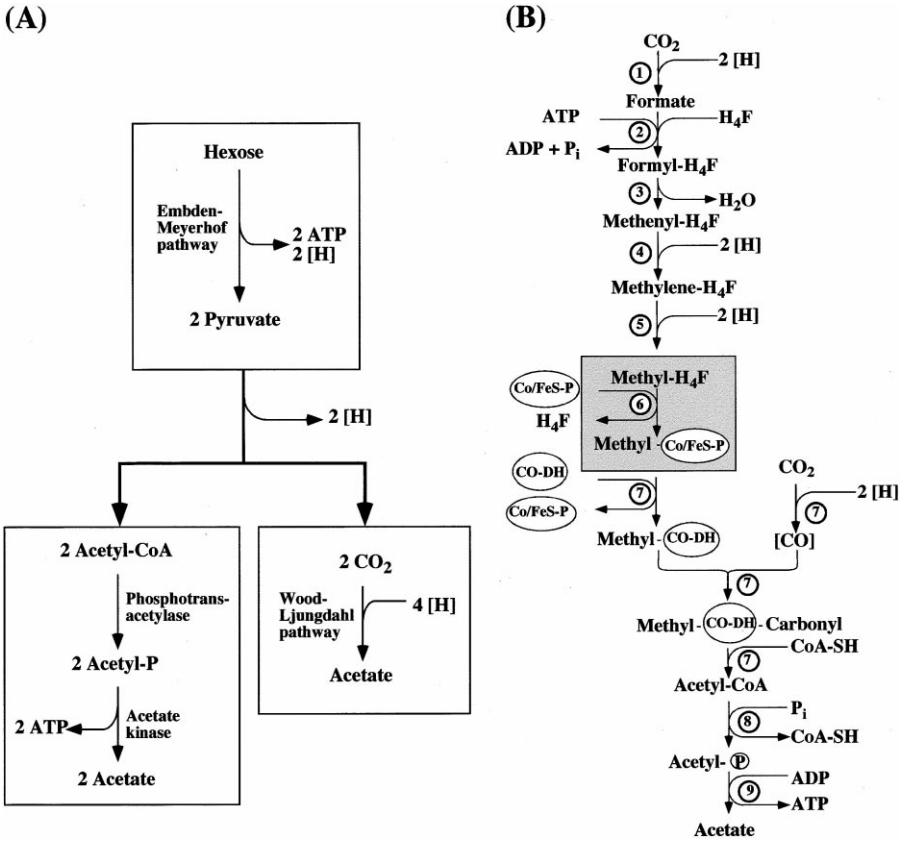

Fig. 1. Pathway of acetate formation from fructose (A) and H_2+CO_2 (B) in *A. woodii*. The $\Delta\tilde{\mu}_{Na^+}$ generating reaction is not known but it is assumed that a methyl transfer reaction (grey box) is involved in Na^+ export. H_4F, tetrahydrofolate; Co/FeS-P, corrinoid/iron sulfur protein; CO-DH, carbon monoxide dehydrogenase; CoA-SH, coenzyme A; 1, formate dehydrogenase; 2, formyl-H_4F synthetase; 3, methenyl-H_4F cyclohydrolase; 4, methenyl-H_4F dehydrogenase; 5, methylene-H_4F reductase; 6, methyltransferase; 7, carbon monoxide dehydrogenase; 8, phosphotransacetylase; 9, acetate kinase. [H] denotes reducing equivalents (two electrons). Panel A was adapted from Drake [70].

are distinguished [7], but they fall into only two classes with respect to their energy metabolism: the proton organisms and the sodium ion organisms. The former have cytochromes, and a proton motive electron transport chain is postulated [8]. The latter group, for which *A. woodii* is a model organism, is devoid of cytochromes, but contains membrane-bound corrinoids [9]. The Wood-Ljungdahl pathway is strictly sodium ion dependent in these organisms [10–12]. In recent years it turned out that a sodium ion cycle is operative in *A. woodii*. A sodium motive force is generated by a yet to be identified primary pump and the $\Delta\tilde{\mu}_{Na^+}$ established is then used to drive flagellar rotation, but more important, ATP synthesis [13,14].

2. Generation of a sodium motive force in *A. woodii*

From studies with cell suspensions of *A. woodii* it is apparent that the Wood-Ljungdahl pathway is sodium ion dependent and accompanied by the generation of a sodium motive force across the cytoplasmic membrane. Inhibitor studies clearly revealed that $\Delta\tilde{\mu}_{Na^+}$ is generated by a primary, electrogenic pump connected to the Wood-Ljungdahl pathway. Further studies using substrates which are fed into the Wood-Ljungdahl pathway at different levels identified the reaction sequence leading from methylene-H_4F to a methylated intermediate as Na^+ dependent [10]. This reaction sequence contains the methylene-H_4F reductase, and one (or two) methyltransferase reactions according to:

$$\text{Methylene} - H_4F + 2[H] \rightarrow \text{Methyl} - H_4F \quad (4)$$

$$\text{Methyl} - H_4F + \text{Co/FeS} - P \rightarrow \text{Methyl}-\text{Co/FeS} - P + H_4F \quad (5)$$

$$\text{Methyl} - \text{Co/FeS} - P + \text{CO} - \text{DH} \rightarrow \text{Methyl}-\text{CO} - \text{DH} + \text{Co/FeS} - P \quad (6)$$

Eq. 4 is catalyzed by the methylene-H_4F reductase. Because the enzyme was found to be localized to 100% in the cytoplasm (R. Heise and V. Müller, unpublished data), we do not consider this reaction sequence to be sodium motive. Furthermore, studies with the sodium ion-dependent homoacetogen *Peptostreptococcus productus* also made an involvement of the methylene-H_4F reductase in Na^+ transport unlikely [15,16]. This leaves reactions 5 and 6 as most likely candidates for Na^+ transport. This reaction sequence has never been studied in sodium ion-dependent homoacetogens; all we know are results from studies with proton organisms. Since these do not have a primary Na^+ pump, it might turn out that sodium ion-dependent homoacetogens use a modification of the pathway. However, in proton organisms such as *Moorella thermoacetica* the methyl group is first transferred to the Co/FeS-P by action of a soluble methyltransferase (Eq. 5) [17]. Subsequently, the methyl group is transferred to the final acceptor, a subunit of the CO-DH (Eq. 6) [18]. In the sodium ion-dependent methanogenic archaea, an analogous reaction takes place [19–21]. There, the methyl group from methyltetrahydromethanopterin is also transferred to a corrinoid, but in contrast to *M. thermoacetica*, the corrinoid is not part of a second protein but a cofactor of the multisubunit, membrane-bound methyltetrahydromethanopterin:coenzyme M methyltransferase [22–24]. This enzyme also catalyzes the second partial reaction, the demethylation of the corrinoid. The acceptor of the methyl group is a small coenzyme (coenzyme M; 2-methylthioethane sulfonate) which can be chemically synthesized and used as natural methyl group acceptor in enzyme assays. In contrast, the methyl group acceptor in homoacetogens is a protein, a major drawback in the setup of an enzyme assay. This is one of the reasons why this reaction has not been studied in sodium ion-dependent homoacetogens. However, in methanogens the demethylation (analogous to Eq. 6) of the methyl-corrinoid intermediate was shown to be Na^+ dependent and therefore suggested to drive Na^+ transport [21]. The analogy of the pathways in homoacetogens and methanogens as well as the finding of membrane-bound corrinoids in sodium ion-dependent homoacetogens led to the proposal that the methyl transfer from methyl-H_4F to CO-DH is the site of Na^+ extrusion in homoacetogens. However, this still has to be verified by experimental analyses.

3. *A. woodii* synthesizes ATP by means of a reversible, Na^+ translocating ATP synthase

Upon addition of H_2+CO_2, *A. woodii* produced acetate and Na^+ was extruded from the cells giving rise to a chemical Na^+ potential (ΔpNa^+) of −90 mV. Experiments with cell suspensions revealed that the Na^+ gradient drives phosphorylation of ADP [10]. ATP synthesis was not inhibited by protonophores but by sodium ionophores, indicating a direct coupling of ATP synthesis to the Wood-Ljungdahl pathway by $\Delta\tilde{\mu}_{Na^+}$ [25]. Resting cells of *A. woodii* synthesized ATP in response to an artificial ΔpNa; the rate and extent increased by addition of tetraphenyl borate which eliminates the electrical charge generated by the influx of Na^+ [14]. When proton diffusion potentials were applied, ATP synthesis was also observed. Proton diffusion potential-driven ATP synthesis was strictly dependent on Na^+ and inhibited by sodium ionophores. Furthermore, ATP synthesis was inhibited by *N*,*N*′-dicyclohexylcarbodiimide (DCCD) or venturicidin, but not by vanadate, which is in accordance with the action of a F_1F_0-ATP synthase. The reverse reaction, Na^+ movement coupled to ATP hydrolysis was studied in inverted membrane vesicles. The ATPase activity at low Na^+ (100 μM Na^+) and high proton concentration (pH 4.9) was 70 mU/mg protein, indicating that the enzyme also translocates H^+ at low Na^+ concentrations. However, ATPase activity was stimulated 1.4–1.6-fold by Na^+. Upon hydrolysis of ATP, Na^+ was actively transported into the lumen of the inverted vesicles. In the presence of 0.2 mM NaCl, a 24-fold accumulation of Na^+ was achieved. The ATP-dependent accumulation of Na^+ was inhibited by sodium ionophores but not by protonophores, indicating a primary event. Furthermore, Na^+ transport was stimulated after dissipation of the membrane potential, indicating that the primary Na^+ transport was electrogenic. ATP hydrolysis as well as Na^+ transport was inhibited by DCCD, venturicidin, and azide, but not by vanadate [26]. Altogether, this is conclusive evidence for the presence of an ATP synthase in *A. woodii* which uses Na^+ as coupling ion.

4. The ATPase from *A. woodii* is a F_1F_0-ATPase

The ATPase was purified to apparent homogeneity after solubilization of membranes with Triton X-100 by (poly)ethyleneglycol precipitation and gel filtration. This preparation contained (only) six subunits with apparent molecular masses of 57 (α), 52 (β), 35 (γ), 19 (δ), 16 (ε), and 4.8 ($c_{2/3}$) kDa [27]. It should be noted that subunits c_2 and c_3 are absolutely identical but encoded by two genes (see below). Their molecular mass was underestimated in previous studies (4.8 kDa); the apparent molecular mass in 10% polyacrylamide gels according to Schägger and von Jagow [28] is 7 kDa. The 52 kDa subunit cross-reacted with antibodies against the β subunit of the F_1F_0-ATPase of *Escherichia coli*, and the 7 kDa subunit was labeled by [^{14}C]DCCD [27]. Later studies using separation of membrane protein complexes by blue native-PAGE and subsequent SDS-PAGE in the second dimension revealed the presence of three additional subunits of 19 (*b*), 18 (*a*), and 16 (c_1) kDa [29]. These experiments gave biochemical and immunological evidence that the ATPase is of the F_1F_0 class. This was corroborated by analyzing the structure of the six-subunit enzyme by electron microscopy. Electron micrographs of negatively stained specimens revealed the typical structure of two domains connected by a stalk. The dimensions of the two domains and the stalk are comparable to F_1F_0-ATPases from other organisms, and the F_1 part of the isolated enzyme showed a hexagonal symmetry suggesting an $\alpha_3\beta_3$ structure [30].

The final proof that the ATPase of *A. woodii* is a

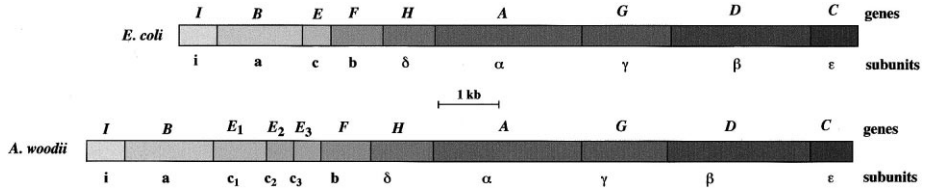

Fig. 2. Comparison of the *atp* operons from *A. woodii* and *E. coli*. *atp* genes and the corresponding subunits are indicated.

F_1F_0-ATPase was obtained after cloning and sequencing the encoding genes which revealed an *atp* operon in *A. woodii* very similar to other organisms (see below) [31–34]. The *atp* operon contains homologues of the nine genes present in the *E. coli atp* operon (Fig. 2). The order of the genes is *atpIB-E*$_1$*E*$_2$*E*$_3$*FHAGDC*. Northern blot analyses revealed that the genes form one polycistronic message [33]. In contrast to any other known F_1F_0-ATPase operon, the *atp* operon from *A. woodii* contains three tandemly organized genes (*atpE*$_1$, *atpE*$_2$, *atpE*$_3$) encoding proteolipids (subunit *c*, see below).

5. The F_1F_0-ATPase from *A. woodii* is a primary Na^+ pump

The following kinetic data were obtained with the six-subunit complex which apparently lacked (or had substoichiometric amounts of) subunits *a*, *b*, and c_1 [27]. ATP hydrolysis was strictly dependent on the presence of Na^+. At pH 7.5, ATP hydrolysis in the absence of Na^+ was virtually zero, but was stimulated 6-fold by addition of Na^+. Maximal hydrolysis was obtained at 1.5 mM Na^+, the K_m for Na^+ was 0.4 mM [27]. Li^+ is known to substitute for Na^+ in a number of Na^+ translocating proteins; this was also the case for the ATPase from *A. woodii*. However, the apparent K_m for Li^+ was 2.5 mM and the maximal activity was only 74% of the one obtained at saturating Na^+ concentrations [35]. KCl did not stimulate ATP hydrolysis. Of interest was the effect of the pH on the sodium ion dependence. At pH 5.3, the enzyme had a significant activity even in the absence of Na^+. This indicates that the enzyme couples to protons at low Na^+ and high H^+ concentrations. This hypothesis was confirmed by the observation that the enzyme had two different pH optima depending on the Na^+ concentration (pH 5.5–6.0 at 0.1 mM Na^+; pH 7–9 at 5 mM Na^+) [27].

To unequivocally prove that the F_1F_0-ATPase from *A. woodii* is a primary electrogenic Na^+ pump, the purified ATPase was incorporated into liposomes. Upon addition of ATP, $^{22}Na^+$ was rapidly transported into the lumen of the proteoliposomes. The rates of ATP hydrolysis and Na^+ transport were 1 μmol and 0.27 μmol min^{-1} (mg protein)$^{-1}$, respectively, indicating a suboptimal coupling of ATP hydrolysis and Na^+ transport. The low coupling efficiency might be due to the fact that the ATPase preparation lacked (or had only substoichiometric amounts) of subunits *a*, *b*, and c_1. However, inhibitor studies clearly demonstrated that the Na^+ transport was a primary, electrogenic event directly coupled to ATP hydrolysis (Fig. 3) [27].

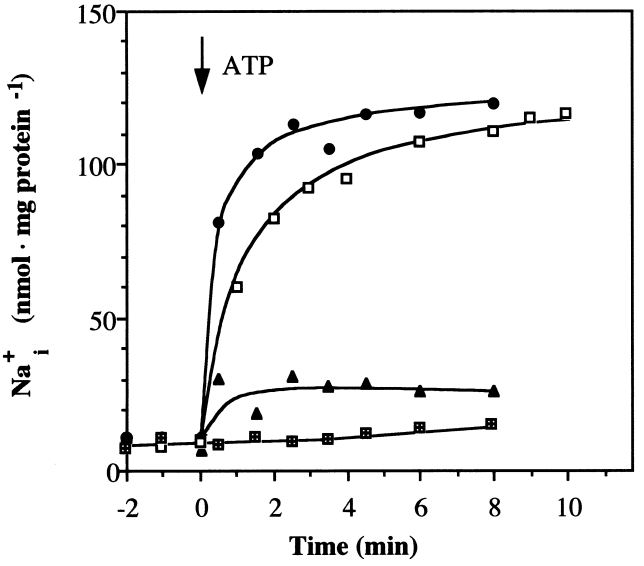

Fig. 3. Na^+ transport catalyzed by proteoliposomes containing the purified ATPase of *A. woodii*. The six-subunit enzyme [27] was incorporated into liposomes, incubated for 30 min in the presence of 2 mM Na^+, and the reaction was started by addition of K_2-ATP. The assay contained protonophore SF6847 (●), Na^+ ionophore ETH 2120 (▲), DCCD (■), or no addition (□).

6. Kinetic properties of the Na^+-F_1F_0-ATPase from *A. woodii*

The following kinetic data were obtained with the six-subunit complex in which subunits *a*, *b*, and c_1 were not detected [27]. The ATPase had a high specificity for ATP; other nucleotides were hydrolyzed with lower rates and compounds such as pyrophosphate, ADP or AMP were not hydrolyzed (Table 1). With respect to divalent cations, the enzyme had a lower substrate specificity; however, the absence of Mg^{2+} abolished ATP hydrolysis completely. For MgATP, an apparent K_m of 0.4 mM was determined. The pH optimum was 7–9 at physiological sodium ion concentrations. The enzyme could not be stimu-

Table 1
Kinetic properties of the Na^+-F_1F_0-ATPase from *A. woodii*

Property	Values
Molecular mass (kDa)	590
Subunits and their molecular masses (kDa)[a]	α (57), β (52), γ (35), δ (19), *b* (19), *a* (18), ε (16), c_1 (16), c_2 (7), c_3 (7)
Substrate specificity (relative specific activity (%))	ATP (100) > ITP (39) > GTP (31) > CTP (9) > UTP (2)
Apparent K_m (Mg-ATP) (mM)	0.4
Activating cations (apparent K_m in mM)	Na^+ (0.4), Li^+ (2.5)
Activating anions[b]	SO_3^{2-}
Coupling ion	Na^+ at physiological conditions; H^+ at low Na^+ and high H^+
pH optimum	7–9 (5 mM Na^+), 5–6 (0.1 mM Na^+)

[a]The masses of δ and *b* and ε and c_1 are not absolutely identical and depend on the gel system used [27,29]. Rounded values are given. Subunits c_2 and c_3 are identical.
[b]Not stimulated by HCO_3^-, SO_4^{2-}, PO_4^-, and CH_3COO^-.

lated by addition of different alcohols, and of the anions tested, only sulfite stimulated ATP hydrolysis (Table 1) [27]. ATP hydrolysis was inhibited by typical inhibitors of F_1F_0-ATPases such as DCCD, diethylstilbestrol (DES), tributyltin (TBSn), *N*-ethylmaleimide (NEM) or azide (Table 2). Surprisingly, the enzyme was also inhibited by nitrate (Table 2), which was believed to be specific for V_1V_0- and A_1A_0-ATPases. In addition to the classical inhibitors, *N*-10-benzylamiloride (benzamil), a compound known to inhibit Na^+ channels in eucaryotes [36] and the sodium ion-driven, bacterial flagellar motor [37] also inhibited ATPase activity. Of its analogues tested, 5-(*N*,*N*-hexamethylene)amiloride (HMA), 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), and *N*-10-phenylamiloride (phenamil) but not amiloride inhibited the enzyme. Surprisingly, the ATPase was protected by Na^+ or Li^+ from inactivation by DCCD, DES, TBSn and the amiloride derivatives, while Na^+ or Li^+ had no effect on inhibition by NEM or azide [35]. This observation led to experiments in which the Na^+ binding site was probed (see below).

Table 2
Inhibitor sensitivity of the Na^+-F_1F_0-ATPase of *A. woodii*

Inhibitor	Concentration (μM)	Activity after preincubation without NaCl (%)[a,b]	Activity after preincubation with 5 mM NaCl (%)[a]
DCCD	5	52	97
	20	18	95
	100	0	80
Azide	10	48	48
	50	17	17
Amiloride	100	100	100
Benzamil	100	32	60
Phenamil	100	38.5	100
EIPA	100	58.5	85
HMA	100	28.5	60
Vanadate	500	97	97
Nitrate	10^4	49	49
DES	12.5	70	100
	100	35	100
TBSn	10	15	35
Venturicidin	10	50	50

[a]The ATPase was preincubated with the inhibitor and in the presence or absence of NaCl for 30 min. The reaction was then started by addition of 5 mM Na_2-ATP. 100% activity corresponds to the activity that is reached when the enzyme is preincubated for 30 min without inhibitor under otherwise identical conditions.
[b]The contaminating Na^+ concentration in the preincubation was 0.1 mM.

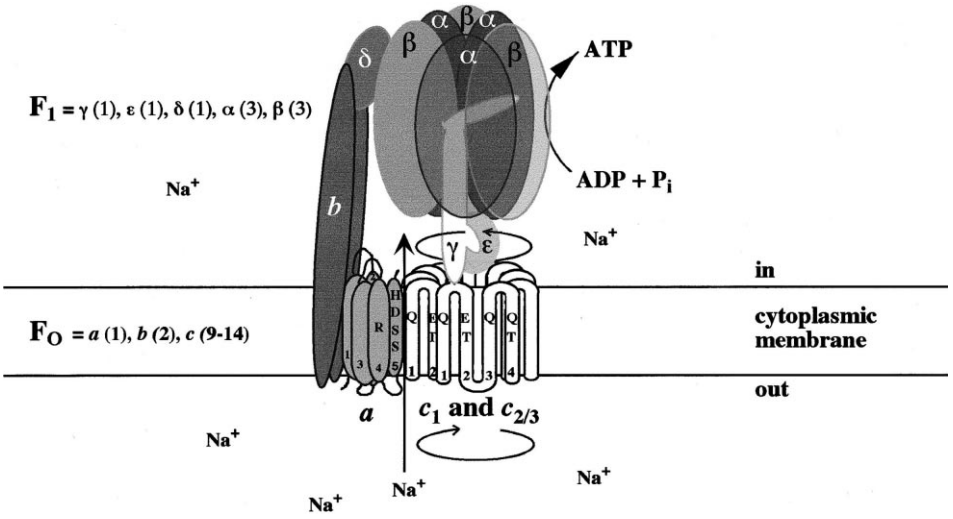

Fig. 4. Hypothetical structure of the Na^+-F_1F_0-ATPase from *A. woodii*. The model is based on biochemical and molecular data and comparisons to known high resolution structures of F_1F_0-ATPases. Transmembrane helices of subunits *a* and *c* are numbered and residues hypothetically involved in Na^+ transport are indicated. Arrows denote directions of rotation. The number of subunits in the enzyme as determined in various studies is given in parentheses.

7. Subunit composition and function of the F_0 domain

From the biochemical and molecular data it is obvious that the ATPase from *A. woodii* is a member of the F_1F_0 class. The overall structure as revealed by primary sequence comparisons [31–34], subunit composition analyses [27,29] and electron microscopic studies [30] is similar to other F_1F_0-ATPases. Therefore, it is reasonable to assume that the molecular architecture and function of the enzyme from *A. woodii* is similar to those from other organisms. A hypothetical model is depicted in Fig. 4. Three copies of each subunit α and β alternate around a central stalk built by subunit γ. Subunit ε is part of the central stalk and connects it with the membrane domain via subunit *c*. A ring of subunit *c* (9–14 copies), subunit *a* (one copy), and subunit *b* (two copies) are localized in the membrane. It has been clearly shown that the F_1F_0-ATPase is a rotary device [38–41]. Ion flow through the membrane along the *a*-*c*-interface is most likely coupled to a rotation of the ring of proteolipids, and this rotation concomitantly leads to a rotation of the central stalk (subunit γ) [41–43]. Rotation of subunit γ within the $\alpha_3\beta_3$ headpiece results in the liberation of ATP from the β subunits [44]. Such a mechanism requires a stator, which is most likely built by subunits *b* and δ (Fig. 4).

7.1. The c-oligomer is a heterooligomer of 8 and 16 kDa proteolipids

The F_1F_0-ATPase operon from *A. woodii* differs from all other F_1F_0-ATPase operons known by the presence of multiple genes encoding proteolipids (Fig. 2). $AtpE_2$ (subunit c_2) and $AtpE_3$ (subunit c_3) are 100% identical on the amino acid level and only 18 substitutions occurred on the DNA level. This is strong evidence for a duplication of an ancestral gene. The deduced molecular mass of the polypeptides is 8.18 kDa. As their bacterial homologues, they are supposed to be organized in the membrane like a hair pin with two transmembrane helices connected by a polar loop. For the sake of simplicity, these two identical subunits and their genes are abbreviated as $c_{2/3}$ or $AtpE_{2/3}$ and $atpE_{2/3}$, respectively. Most interestingly, $atpE_1$ with 546 base pairs is more than double the size of $atpE_{2/3}$. The first and second halves are 66% identical on the DNA level, indicating a duplication of a precursor gene and subsequent fusion of the two gene copies. The deduced molecular mass of $AtpE_1$ (subunit c_1) is 18.37 kDa with four predicted transmembrane helices arranged in two hair pins. Only 60% of the residues of hair pins one and two are identical. 70 and 72% of the residues of hair pins one and two, respectively, are

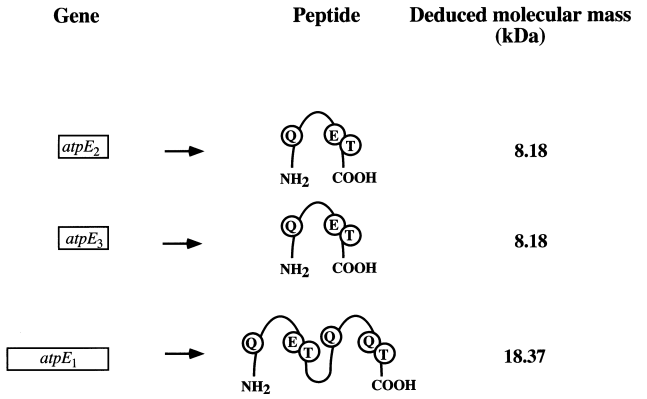

Fig. 5. Gene-polypeptide correspondence of subunits c_1, c_2, and c_3 from the Na^+-F_1F_0-ATPase of *A. woodii*. The hypothetical Na^+ binding motif is indicated.

identical in $AtpE_{2/3}$. However, the membrane-buried Na^+ binding residue (Glu62 in $AtpE_{2/3}$; Glu79 in hair pin one of $AtpE_1$), which is also conserved and involved in H^+ transport in H^+-F_1F_0-ATPases, is substituted by a glutamine residue in hair pin two (Fig. 5). $AtpE_1$ contains an enlarged N-terminus of 17 residues which is not present in $AtpE_{2/3}$ [34].

Since the finding of a duplicated proteolipid encoding gene in a F_1F_0-ATPase operon has important consequences for our understanding of the evolution of structure and function of ATPases, it had to be verified that *atpE*$_1$ indeed encodes a duplicated proteolipid. Therefore, *atpE*$_1$ was cloned into an expression vector. When *atpE*$_1$ was expressed in an ATPase-deficient mutant of *E. coli*, a polypeptide with an apparent molecular mass of 16 kDa was synthesized. However, this experiment did not rule out a possible, specific splicing of the primary 16 kDa product into two 8 kDa polypeptides in *A. woodii*. To test this, a specific polyclonal antiserum was generated against subunit c_1. Western blot analyses of membranes from cells grown on fructose revealed that *atpE*$_1$ encodes a polypeptide with an apparent molecular mass of 16 kDa in *A. woodii* [33].

The proteolipid oligomer of *A. woodii* is very stable and withstands usual denaturation conditions such as heating at 100°C in SDS buffer. N-Terminal sequence analyses revealed the presence of subunit $c_{2/3}$ in the oligomer, but c_1 could not be detected this way. However, Western blot analyses with an antiserum against c_1 revealed its presence in the oligomer. Upon autoclaving the sample for 3 min the proteolipid oligomer was disrupted, the monomers were released, and subunits c_1 and $c_{2/3}$ were unequivocally detected by Western blot analyses [29]. That proves that c_1 is a genuine subunit of the ATPase from *A. woodii*. The proteolipid oligomer of the F_1F_0-ATPase from *A. woodii* is the first known to contain a mixture of 8 and 16 kDa polypeptides. The stoichiometry of the different polypeptides in the *c*-oligomer has not yet been determined.

Subunit c_1 is not only duplicated but also does contain a glutamine residue (Gln162) instead of the membrane-buried glutamate in hair pin two which is essential for ion transport since it is part of the proposed sodium ion binding site (Gln-Glu-Thr) in subunit *c* (see below). Although the free electron pair of the amino group of Gln162 could in principle ligand the sodium ion (as does Gln46 in helix one and Gln129 in helix three), the substitution might have consequences for the coupling efficiency of the rotary motor of the ATPase. Current views on the function of the motor assume an electrostatic attraction of Na^+ (H^+) by a highly conserved Glu (Asp) in subunit *c* [45,46]. Due to the neutralization of the charge of Glu (Glu62 in $c_{2/3}$ and Glu79 in c_1 of *A. woodii*) by Na^+ (H^+), the *c*-ring may cross the electric barrier and rotate into the hydrophobic zone, driven by the electrostatic interaction of an Arg of subunit *a* with another free Glu on the next monomer of the *c*-ring. This leads to a rotation of the *c*-ring relative to subunits *a* and *b*. For the F_1F_0-ATPase of *E. coli* it was demonstrated that the ATPase can tolerate the exchange of one Asp61 in the *c*-oligomer with an Asn residue and is still able to translocate H^+ [47]. The ATPase from *Methanococcus jannaschii* contains a triplicated proteolipid with only two proton translocating groups, but still this enzyme functions as an ATP synthase [48]. In view of this discussion the determination of the exact stoichiometry of the proteolipid monomers in the *c*-oligomer of *A. woodii* is of great interest; this remains a challenging task for the future.

7.2. The sodium ion binding site in F_0

Substrate protection from inhibitor action is a useful tool to determine substrate-protein interactions and to determine ion (=substrate) binding sites. Na^+ protected the ATPase against inactivation by the F_0-directed inhibitors DCCD, DES, TBSn, and

the amiloride derivatives phenamil, benzamil, EIPA, and HMA (Table 2) [35]. On the other hand, inactivation by azide or NBD-Cl was not prevented by Na^+, indicating that sodium ions protect the Na^+-ATPase from inhibition by reagents acting on F_0. The DCCD-Na^+ interaction was analyzed in more detail. DCCD is known to react with the proton translocating (Na^+ binding) residue (Glu62 in *A. woodii* subunit $c_{2/3}$) giving rise to a dicyclohexylurea bound via an *N*-acyl bond to the protein. Upon incubation of membranes from *A. woodii* with [^{14}C]DCCD in the absence of Na^+, the *c*-oligomer and subunit $c_{2/3}$ were labeled. When the Na^+ concentration was increased to 10 mM, the enzyme was no longer labeled by [^{14}C]DCCD, indicating that the presence of Na^+ abolished the reaction of DCCD with subunit *c* [35]. The rate of reaction of DCCD with the enzyme was pH dependent; correspondingly, the protective effect of Na^+ was pH dependent. At pH 7.5, 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. On the other hand, there was no protection against 100 μM DCCD at pH 6.5 [35]. The same Na^+-DCCD interaction was observed with the ATPase from *Propionigenium modestum* [49].

It was already mentioned above that the amiloride derivatives benzamil, HMA, EIPA, and phenamil are potent inhibitors of the ATPase from *A. woodii*. Dilution experiments revealed that they do not bind covalently to the enzyme [35]. 5 mM Na^+ completely protected against inactivation by phenamil, whereas Na^+ protection from benzamil, HMA, and EIPA was significant but less pronounced [35]. The functional group in the amilorides is the guanidinium group which is also present in the side chain of arginine. Therefore, it is assumed that an arginine residue of the enzyme and Na^+ compete for a common binding site. That this common binding site may be Glu62 of subunit *c* is an attractive idea, since the amiloride derivatives also protect the enzyme from inactivation by DCCD. This is in accordance with the model of ion transport, in which Glu62 of subunit *c* and Arg158 (*A. woodii* numbering) of subunit *a* play a crucial role [45].

The Na^+ binding site in subunit *c* of F_1F_0-ATPases was elucidated by various techniques. First, the group of Fillingame created a mutant version of subunit *c* of H^+-F_1F_0-ATPase in *E. coli* in which Ala62 was changed to Ser. This enzyme was able to bind but not to translocate lithium ions. Although Na^+ was not bound, this was the first evidence for the involvement of Ser62 (corresponding to Thr63 in *A. woodii*) in Li^+/Na^+ binding [50]. The group of Dimroth defined, in addition, Gln32 (corresponding to Gln29 in *A. woodii* subunit $c_{2/3}$, and Gln46 and Gln129 in subunit c_1) as part of the binding site by site-directed mutagenesis [51]. The residues Glu62, Thr63, and Gln29 are conserved in *A. woodii*. Furthermore, Pro25 (*A. woodii* numbering) of subunit *c* is only conserved in the two Na^+-F_1F_0-ATPases [32]. This might indicate the involvement of Pro25 in Na^+ binding, but this has to be verified by site-directed mutagenesis.

Apart from subunit *c*, a second subunit, subunit *a*, is required for ion transport; both are envisaged to make the ion channel of the ATPase. Three residues of subunit *a* from H^+-F_1F_0-ATPases, Arg210, Glu219, and His245 (*E. coli* numbering), were indicated by mutant studies to be directly involved in H^+ transport but recent studies demonstrated that Glu219 and His245 are not essential for H^+ transport [52–54]. The residues involved in Na^+ binding of subunit *a* from Na^+-F_1F_0-ATPases are unknown but a comparison of the sequences of subunit *a* from the Na^+-F_1F_0-ATPases from *P. modestum* [55,56] and *A. woodii* to those from H^+-F_1F_0-ATPases revealed determinants likely to be important for Na^+ binding [34]. Whereas subunit *c* of the Na^+-F_1F_0-ATPases of *P. modestum* [57] and *A. woodii* are more closely related to each other than to subunit *c* from H^+-F_1F_0-ATPases (60% identity versus 23% to *E. coli*), this is not the case with subunit *a*: 30–45% of the residues from *A. woodii* are identical in subunit *a* from other enzymes, irrespective of whether they transport Na^+ or H^+. However, the similarity increases if only single transmembrane helices are compared, and the degree of similarity increases towards the C-terminus. A striking 71% of the residues of helix 5 of subunit *a* are identical in Na^+-F_1F_0-ATPases whereas only 30–50% of the residues are identical when Na^+- and H^+-ATPases are compared. This high degree of structural conservation of helix 5 of Na^+-F_1F_0-ATPases is indicative for a conservation of function and might indicate a

participation of helix 5 in Na^+ transport. The high degree of conservation of helix 5 of subunit *a* in Na^+-F_1F_0- compared to H^+-F_1F_0-ATPases might be due to the fact that the liganding of Na^+ requires more amino acids in a critical spatial organization than the binding of H^+. Helix 4 of subunit *a* harbors the residue Arg210 (*E. coli* numbering) which was shown to be absolutely essential for H^+ transport in *E. coli* [52]. This residue is strictly conserved among species (Arg158 in *A. woodii*). A mutation of three residues of subunit *a* of the Na^+-F_1F_0-ATPase of *P. modestum* (Lys219, Val263, Ile277) led to altered Na^+ binding. With the exception of Ile277 these are not conserved in *A. woodii*. However, even in *P. modestum* they were not envisaged to be involved directly in Na^+ liganding but their mutation was seen to result in a more global structural change of subunit *a* leading to a perturbation of the Na^+ binding pocket [58]. From sequence comparisons the group of candidates involved in Na^+ transport could be limited to Arg158, Thr174, Ser175, His192, Tyr194, Asp196, Ser199 and Ser204 (*A. woodii* numbering) [34]. However, their role in Na^+ transport still has to be determined by experimental analyses.

8. Why multiplication and duplication of the proteolipid encoding gene?

One of the most striking and unique features of the *atp* operon of *A. woodii* is the presence of multiple copies of proteolipid encoding genes. Multiplication of proteolipid encoding genes has been found before only in V_1V_0-ATPases from eucarya [59–61] and A_1A_0-ATPases from archaea [62]. What could be the selective pressure for multiplication of proteolipid encoding genes? One has to keep in mind that the subunits of the ATPase are present in different stoichiometries ($a_1b_2c_{9-14}\delta\alpha_3\gamma\beta_3\varepsilon$), and the proteolipid (subunit *c*) has by far the highest copy number in the complex. Most of our knowledge concerning the regulation of the synthesis of the proteolipid derived from the paradigm *E. coli*. There, the proteolipid encoding gene is part of a polycistronic message, and enhanced synthesis of the proteolipid is achieved by enhancement of translation. In addition, but to a lesser extent, regulation by differential mRNA stability contributes to differential gene expression [63,64]. Apparently, multiplication of the *atpE* gene and embedding the copies into the operon is another way to increase the concentration of subunit *c*. This strategy is apparently realized by *A. woodii*, but this does not exclude additional mechanisms.

The duplication of the proteolipid encoding gene per se is not of great consequence for the function of the ATPase. It was shown recently, that genetically engineered duplicated proteolipids from *E. coli* are functional in H^+ transport and ATP synthesis [65,66]. The striking feature, however, of subunit c_1 is not its size but rather the fact that the ion translocating residue is not conserved in helix two. A loss of one ion translocating residue is also encountered in the '16 kDa proteolipids' from eucarya and this loss has the dramatic consequence that the V_1V_0-ATPases are not able to synthesize ATP in vivo. The capability to synthesize ATP is directly dependent on the number of ions translocated per ATP synthesized. According to $\Delta G_p = -n \times F \times \Delta p$, a phosphorylation potential (ΔG_p) of approx. 50–70 kJ/mol is sustained by the use of $n = 3$–4 ions/ATP at a physiological electrochemical ion potential of −180 mV (Δp). However, if the number of protons is lower, then ATP can no longer be synthesized. Although it was demonstrated that the number of monomers in the *c*-ring may vary from nine to 14 [43,65,67,68], for the following calculation it is assumed that like in the case of the bacterial and archaeal '8 kDa proteolipids' with two transmembrane helices, 12 monomers and 12 proton translocating carboxyl groups are present per oligomer. Taking into account three ATP synthesizing or hydrolyzing centers, this gives a stoichiometry of four ions/ATP. In contrast, six copies of the '16 kDa proteolipid' with four transmembrane helices constitute the proteolipid oligomer of V_1V_0-ATPases [69]. Since the ion translocating group is lost in the first pair of transmembrane helices, the stoichiometry is only two ions/ATP, which is too low to allow ATP synthesis. On the other hand, if the number of ions is low, the same ΔG_p can account for a much higher Δp, making the enzyme a better proton pump, a function required by the physiology of the eucaryotic cell. In general, the smaller the number of carboxylates per ring, the worse is the coupling efficiency. Taking this into account it is now reasonable to assume that a cell could, depending on the cellular needs, alter the function of the ATPase

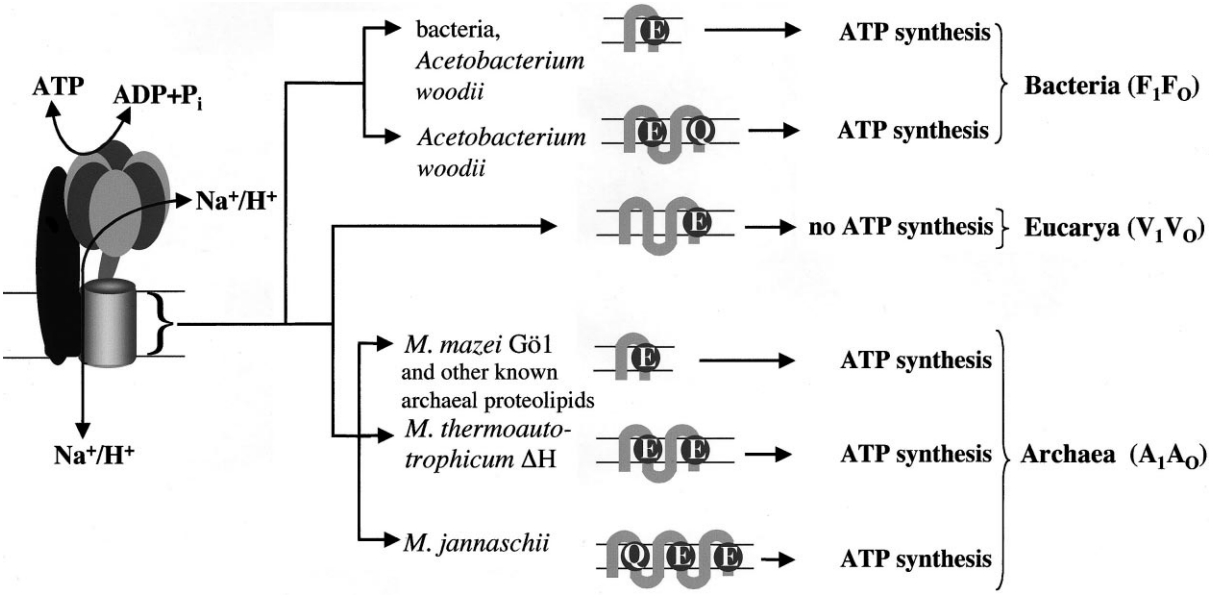

Fig. 6. Evolution of structure and function of subunit *c* of ATPases. *M. mazei*, *Methanosarcina mazei*.

between ATP synthesis and ATP hydrolysis by varying the number of proton (ion) translocating residues. This is a very attractive idea for *A. woodii*, which can grow by fermentation or by anaerobic respiration or by using pathways which do neither involve substrate level phosphorylation nor respiration but methyl transfer-driven Na^+ extrusion (during the operation of the Wood-Ljungdahl pathway). During fermentation the enzyme has to work as an ion pump generating the membrane potential whereas during autotrophic growth on H_2+CO_2 it has to work as a synthase. The switch from pump to synthase could be performed by changing the ratio of c_1/$c_{2/3}$. This hypothesis is a challenging task for future experiments.

9. Evolutionary consideration

The finding of a duplicated proteolipid in a F_1F_0-ATPase is without precedence in bacteria. Duplicated proteolipids were, for a long time, regarded as an exclusive feature of eucaryal V_1V_0-ATPases [69,70]. Duplication and triplication of proteolipid encoding genes with subsequent fusion of the genes was described very recently for the archaea *Methanobacterium thermoautotrophicum* and *M. jannaschii* ([48]; C. Ruppert and V. Müller, unpublished data). Therefore, it is now evident that multiplied and fused proteolipid encoding genes are not exclusively present in members of the domain *Eucarya*, but also in the other domains of life. This does not necessarily argue against the commonly favored view of evolution of ATPases, but could result from horizontal gene transfer which is very often underestimated in natural systems. A view of the evolution of structure and function of subunit *c* of ATPases is depicted in Fig. 6.

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Graduiertenkolleg 'Chemische Aktivitäten von Mikroorganismen'. We are indebted to Prof. G. Gottschalk for generous support and for his contribution during the early stages of this project.

References

[1] W.E. Balch, S. Schoberth, R.S. Tanner, R.S. Wolfe, J. Syst. Bacteriol. 27 (1977) 355–361.
[2] L.G. Ljungdahl, Annu. Rev. Microbiol. 40 (1986) 415–450.
[3] H.G. Wood, FASEB J. 5 (1991) 156–163.

[4] S.W. Ragsdale, Crit. Rev. Biochem. Mol. Biol. 26 (1991) 261–300.
[5] V. Müller, in: Encyclopedia of Life Sciences, Macmillan, London, 2001, in press.
[6] G. Fuchs, FEMS Microbiol. Rev. 39 (1986) 181–213.
[7] G. Diekert, in: A. Balows, H.G. Trüper, M. Dworkin, W. Harder, K.-H. Schleifer (Eds.), The Prokaryotes, Springer-Verlag, New York, 1992, pp. 517–533.
[8] L.G. Ljungdahl, in: H. Drake (Ed.), Acetogenesis, Chapman and Hall, New York, 1994, pp. 63–88.
[9] W. Dangel, H. Schulz, G. Diekert, H. König, G. Fuchs, Arch. Microbiol. 148 (1987) 52–56.
[10] R. Heise, V. Müller, G. Gottschalk, J. Bacteriol. 171 (1989) 5473–5478.
[11] G. Geerligs, P. Schönheit, G. Diekert, FEMS Microbiol. Lett. 57 (1989) 253–258.
[12] H. Yang, H.L. Drake, Appl. Environ. Microbiol. 56 (1990) 81–86.
[13] V. Müller, S. Bowien, Arch. Microbiol. 164 (1995) 363–369.
[14] R. Heise, J. Reidlinger, V. Müller, G. Gottschalk, FEBS Lett. 295 (1991) 119–122.
[15] G. Wohlfahrt, G. Geerligs, G. Diekert, Eur. J. Biochem. 192 (1990) 411–417.
[16] G. Wohlfahrt, G. Diekert, Arch. Microbiol. 155 (1991) 378–381.
[17] J. Seravalli, S. Zhao, S.W. Ragsdale, Biochemistry 38 (1999) 5728–5735.
[18] S. Menon, S.W. Ragsdale, J. Biol. Chem. 274 (1999) 11513–11518.
[19] R. Fischer, P. Gärtner, A. Yeliseev, R.K. Thauer, Arch. Microbiol. 158 (1992) 208–217.
[20] B. Becher, V. Müller, G. Gottschalk, FEMS Microbiol. Lett. 91 (1992) 239–244.
[21] D.S. Weiss, P. Gärtner, R.K. Thauer, Eur. J. Biochem. 226 (1994) 799–809.
[22] T. Lienard, B. Becher, M. Marschall, S. Bowien, G. Gottschalk, Eur. J. Biochem. 239 (1996) 857–864.
[23] P. Schulz, S.P. Albracht, J.M. Coremans, G. Fuchs, Eur. J. Biochem. 171 (1988) 589–597.
[24] P. Gärtner, A. Ecker, R. Fischer, D. Linder, G. Fuchs, R.K. Thauer, Eur. J. Biochem. 213 (1993) 537–545.
[25] R. Heise, V. Müller, G. Gottschalk, FEMS Microbiol. Lett. 112 (1993) 261–268.
[26] R. Heise, V. Müller, G. Gottschalk, Eur. J. Biochem. 206 (1992) 553–557.
[27] J. Reidlinger, V. Müller, Eur. J. Biochem. 223 (1994) 275–283.
[28] H. Schägger, G. von Jagow, Anal. Biochem. 166 (1987) 369–379.
[29] S. Aufurth, H. Schägger, V. Müller, J. Biol. Chem. 275 (2000) 33297–33301.
[30] J. Reidlinger, F. Mayer, V. Müller, FEBS Lett. 356 (1994) 17–20.
[31] A. Forster, R. Daniel, V. Müller, Biochim. Biophys. Acta 1229 (1994) 393–397.
[32] S. Rahlfs, V. Müller, FEBS Lett. 404 (1997) 269–271.
[33] S. Rahlfs, S. Aufurth, V. Müller, J. Biol. Chem. 274 (1999) 33999–34004.
[34] S. Rahlfs, V. Müller, FEBS Lett. 453 (1999) 35–40.
[35] M. Spruth, J. Reidlinger, V. Müller, Biochim. Biophys. Acta 1229 (1995) 96–102.
[36] T.R. Kleyman, E.J. Cragoe Jr., J. Membr. Biol. 105 (1988) 1–21.
[37] S. Sugiyama, E.J. Cragoe Jr., Y. Imae, J. Biol. Chem. 263 (1988) 8215–8219.
[38] R.L. Cross, T.M. Duncan, J. Bioenerg. Biomembr. 28 (1996) 403–408.
[39] D. Sabbert, S. Engelbrecht, W. Junge, Nature 381 (1996) 623–625.
[40] H. Noji, R. Yasuda, M. Yoshida, K. Kinosita, Nature 386 (1997) 299–302.
[41] Y. Sambongi, Y. Iko, M. Tanabe, H. Omote, A. Iwamoto-Kihara, I. Ueda, T. Yanagida, Y. Wada, M. Futai, Science 286 (1999) 1722–1724.
[42] H. Noji, K. Hasler, W. Junge, K.J. Kinosita, M. Yoshida, S. Engelbrecht, Biochem. Biophys. Res. Commun. 260 (1999) 597–599.
[43] D. Stock, A.G.W. Leslie, J.E. Walker, Science 286 (1999) 1700–1705.
[44] P.D. Boyer, Biochim. Biophys. Acta 1140 (1993) 215–250.
[45] T. Elston, H. Wang, G. Oster, Nature 391 (1998) 510–513.
[46] P. Dimroth, H. Wang, M. Grabe, G. Oster, Proc. Natl. Acad. Sci. USA 96 (1999) 4924–4929.
[47] O.Y. Dmitriev, K. Altendorf, R.H. Fillingame, Eur. J. Biochem. 233 (1995) 478–483.
[48] C. Ruppert, H. Kavermann, S. Wimmers, R. Schmid, J. Kellermann, F. Lottspeich, H. Huber, K.O. Stetter, V. Müller, J. Biol. Chem. 274 (1999) 25281–25284.
[49] C. Kluge, P. Dimroth, Biochemistry 32 (1993) 10378–10386.
[50] Y. Zhang, R.H. Fillingame, J. Biol. Chem. 270 (1995) 87–93.
[51] G. Kaim, F. Wehrle, U. Gerike, P. Dimroth, Biochemistry 36 (1997) 9185–9194.
[52] G. Deckers-Hebestreit, K. Altendorf, Annu. Rev. Microbiol. 50 (1996) 791–824.
[53] P.E. Hartzog, B.D. Cain, J. Biol. Chem. 269 (1994) 32313–32317.
[54] F.I. Valiyaveetil, R.H. Fillingame, J. Biol. Chem. 272 (1997) 32635–32641.
[55] G. Kaim, W. Ludwig, P. Dimroth, K.H. Schleifer, Nucleic Acids Res. 18 (1990) 6697.
[56] G. Kaim, W. Ludwig, P. Dimroth, K.H. Schleifer, Eur. J. Biochem. 207 (1992) 463–470.
[57] W. Ludwig, G. Kaim, W. Laubinger, P. Dimroth, J. Hoppe, K.H. Schleifer, Eur. J. Biochem. 193 (1990) 395–399.
[58] G. Kaim, P. Dimroth, Biochemistry 37 (1998) 4626–4634.
[59] R. Hirata, L.A. Graham, A. Takatsuki, T.H. Stevens, Y. Anraku, J. Biol. Chem. 272 (1997) 4795–4803.
[60] T. Oka, R. Yamamoto, M. Futai, J. Biol. Chem. 272 (1997) 24387–24392.
[61] T. Oka, R. Yamamoto, M. Futai, J. Biol. Chem. 273 (1998) 22570–22576.

[62] V. Müller, C. Ruppert, T. Lemker, J. Bioenerg. Biomembr. 31 (1999) 15–27.
[63] J.E. McCarthy, H.U. Schairer, W. Sebald, EMBO J. 4 (1985) 519–526.
[64] J.E. McCarthy, Mol. Microbiol. 4 (1990) 1233–1240.
[65] P.C. Jones, R.H. Fillingame, J. Biol. Chem. 273 (1998) 29701–29705.
[66] P.C. Jones, J. Hermolin, R.H. Fillingame, J. Biol. Chem. 275 (2000) 11355–11360.
[67] H. Seelert, A. Poetsch, N.A. Dencher, A. Engel, H. Stahlberg, D.J. Müller, Nature 405 (2000) 418–419.
[68] D.L. Foster, R.H. Fillingame, J. Biol. Chem. 257 (1982) 2009–2015.
[69] N. Nelson, L. Taiz, Trends Biochem. Sci. 14 (1989) 113–116.
[70] H.L. Drake, in: H.L. Drake (Ed.), Acetogenesis, Chapman and Hall, New York, 1994, pp. 3–62.