ATP Synthesis by Decarboxylation Phosphorylation

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ATP Synthesis by Decarboxylation Phosphorylation

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Abstract Adenosine triphosphate (ATP) is used as a general energy source by all living cells. The free energy released by hydrolyzing its terminal phosphoric acid anhydride bond to yield ADP and phosphate is utilized to drive various energy-consuming reactions. The ubiquitous F_1F_0 ATP synthase produces the majority of ATP by converting the energy stored in a transmembrane electrochemical gradient of H^+ or Na^+ into mechanical rotation. While the mechanism of ATP synthesis by the ATP synthase itself is universal, diverse biological reactions are used by different cells to energize the membrane. Oxidative phosphorylation in mitochondria or aerobic bacteria and photophosphorylation in plants are well-known processes. Less familiar are fermentation reactions performed by anaerobic bacteria, wherein the free energy of the decarboxylation of certain metabolites is converted into an electrochemical gradient of Na^+ ions across the membrane (decarboxylation phosphorylation). This chapter will focus on the latter mechanism, presenting an updated survey on the Na^+-translocating decarboxylases from various organisms. In the second part, we provide a detailed description of the F_1F_0 ATP synthases with special emphasis on the Na^+-translocating variant of these enzymes.

1 Introduction

Adenosine triphosphate (ATP) is used as a general source of chemical energy by all living cells. The free energy released in hydrolyzing its terminal phosphoric anhydride bond to yield ADP and phosphate is utilized to drive various energy-consuming reactions, e.g., biosyntheses, membrane transport, regulatory networks, mechanical movements, nerve conduction, etc. Accordingly, the demand for ATP is impressive, amounting to a daily turnover of 50 kg in a human on average. In order to maintain a constant supply of ATP and to complete the cell energy cycle, its terminal phosphoric anhydride bond has to be continuously regenerated from ADP and phosphate. Individual cells may dispose of several distinct catabolic reaction sequences leading to ATP synthesis by substrate-level phosphorylation in the cytoplasmic compartment of the cell. The underlying mechanism involves storage of metabolic energy as an energy-rich phosphate bond and transfer of the phosphate moiety to ADP to yield ATP.

By far the greatest amount of ATP, however, is synthesized at the membrane by the ubiquitous F_1F_0 ATP synthase. This multicomponent protein complex

is able to convert the free energy stored in an electrochemical gradient of protons or Na^+ ions across the membrane into the high-energy phosphoric anhydride bond of ATP. While the mechanism of ATP synthesis by the ATP synthase itself is universal, distinct reactions are used by different cells or organelles to energize the membrane. In photosynthesis by plants, the thylakoid membrane of chloroplasts is energized by light to generate an electrochemical gradient of protons, and consequently ATP synthesis in these organelles is termed photophosphorylation. In mitochondria or aerobic bacteria, oxidation reactions drive proton flux through the respiratory chain complexes to charge the membrane, and ATP synthesis in these organelles or cells is therefore termed oxidative phosphorylation. Several anaerobic bacteria perform fermentation reactions, in which the free energy of the decarboxylation of a certain metabolite is converted into an electrochemical gradient of Na^+ ions across the membrane. The thus stored energy is used to drive ATP synthesis in a process termed decarboxylation phosphorylation (for reviews see Dimroth 1997, 2004; Buckel 2001). In this chapter we will focus on the latter mechanism. We will first give a state-of-the-art overview of the Na^+-translocating decarboxylases and succeed with a detailed description of the F_1F_0 ATP synthases. Special emphasis will be given to Na^+ translocation and torque generation by the Na^+-translocating variant of these enzymes.

2
Fermentation Pathways with Na^+-Transport Decarboxylases (NaT-DC)

2.1
Fermentation of Citrate

The fermentation of tri- or dicarboxylic acids by various bacteria includes a decarboxylation reaction which is coupled to Na^+ translocation across the membrane (Dimroth 2004). This mode of energy conversion (Bush and Saier 2002) was first recognized for oxaloacetate decarboxylase of *Klebsiella pneumoniae* (Dimroth 1980, 1982a,b). The membrane-bound enzyme complex is induced during anaerobic growth on citrate, where it catalyzes a specific step of citrate metabolism. After uptake into the cell by the Na^+-dependent citrate carrier CitS (Pos and Dimroth 1996), citrate is cleaved by citrate lyase into acetate and oxaloacetate. The latter is subsequently converted to CO_2 and pyruvate by the membrane-bound oxaloacetate decarboxylase sodium ion pump. Pyruvate is converted to acetyl-CoA and formate by pyruvate formate lyase, and part of the formate is cleaved to CO_2 and H_2 by formate hydrogen lyase. Acetyl-CoA is further metabolized to acetyl phosphate by phosphotransacetylase, and acetate kinase converts acetyl phosphate and ADP to acetate and ATP. A peculiarity of this pathway is the mode of synthesis of NAD(P)H for biosynthetic reactions. While most bacteria growing

fermentatively are faced with the problem of getting rid of reducing equivalents (mainly NADH) formed by the oxidation of the growth substrates, citrate fermentation by *K. pneumoniae* includes no oxidative steps and NADH is not generated. However, as the mean oxidation status of citrate is above average for cellular components, the cells require reducing equivalents for citrate assimilation. It has been shown that hydrogen generated in the formate hydrogen lyase reaction provides the reducing equivalents for NAD(P)H formation from $NAD(P)^+$ by a soluble or a membrane-bound hydrogenase (Steuber et al. 1999).

Overall, the citrate fermentation pathway produces 1 mol ATP per mol citrate by substrate-level phosphorylation in the acetate kinase reaction, and in addition, an electrochemical gradient of Na^+ ions is generated by the oxaloacetate decarboxylase. The chemical concentration gradient of Na^+ is used to drive citrate uptake by the electroneutral $Hcitrate^{2-}/Na^+/H^+$ symporter (CitS) (Pos and Dimroth 1996), and the electrical component of the $\Delta\mu Na^+$ is assumed to contribute driving force for ATP synthesis by the H^+-translocating F_1F_0 ATP synthase, which is constitutively expressed in this organism.

In *K. pneumoniae*, the structural genes for oxaloacetate decarboxylase are part of the gene cluster for citrate fermentation, which comprises two operons (*citS* and *citC* operons) with divergent orientation (Bott and Dimroth 1994; Bott et al. 1995; Bott 1997). The *citS* operon includes the genes for the citrate carrier (*citS*), the three structural genes for oxaloacetate decarboxylase (*oadGAB*), and a two-component regulatory system (*citAB*). The genes of the *citC* operon encode citrate lyase ligase (*citC*), the three subunits of citrate lyase (*citDEF*), and an enzyme involved in the biosynthesis of its 2′-(5″-phosphoribosyl)-3′-dephospho-CoA prosthetic group (*citG*) (Schneider et al. 2000a,b). An additional gene required for the prosthetic group biosynthesis (*citX*) has been found at a distant location, clustered with the two-component regulatory system (*citYZ*) and another citrate carrier (*citW*) (Schneider et al. 2002).

Data bank searches revealed genes for oxaloacetate decarboxylase in numerous anaerobic bacteria and in some archaea. Some of these organisms even possess several oxaloacetate decarboxylase genes. In *Salmonella typhimurium*, two copies of the *oad* genes have been identified, one being inserted into the citrate fermentation operon and expressed during anaerobic growth on citrate, and the other being associated with genes required for tartrate fermentation and expressed under anaerobic growth on tartrate (Wifling and Dimroth 1989; Woehlke et al. 1992; Woehlke and Dimroth 1994). *Vibrio cholerae* also has two copies of the *oad* genes; the *oad-2* genes are part of the citrate fermentation operon and are expressed during anaerobic growth on citrate (Dahinden et al. 2005a). The *oad-1* genes are not associated with genes for a specific fermentation pathway and conditions for their expression are unknown. The archaeon *Archaeoglobus fulgidus* contains a cluster of three *oad* genes. The gene for the oxaloacetate specific carboxyltransferase sub-

unit is not part of this cluster but found at a separate location of the genome (Dahinden and Dimroth 2004). Furthermore, the genome contains a gene for a methylmalonyl-CoA specific carboxyltransferase. It is conceivable that these different carboxyltransferases form sodium ion translocating decarboxylase complexes with different substrate specificity, and share the three proteins that are encoded by the *oad* gene cluster.

2.2 Fermentation of Succinate or Lactate

Methylmalonyl-CoA decarboxylase is another member of the NaT-DC enzyme family. It has been characterized in lactate fermenting *Veillonella parvula* (Hilpert and Dimroth 1982, 1983; Huder and Dimroth 1993) and in succinate fermenting *Propionigenium modestum* (Bott et al. 1997). The decarboxylase converts (*S*)-methylmalonyl-CoA to propionyl-CoA and CO_2 and conserves energy by coupling the exergonic decarboxylation reaction to the transport of Na^+ ions across the membrane. In both bacteria, methylmalonyl-CoA decarboxylation is an essential step in the metabolism of succinate to propionate and CO_2 (Dimroth and Schink 1998; Schink and Pfennig 1982). In spite of these similarities, only *P. modestum* but not *V. parvula* is able to grow from the conversion of succinate to propionate and CO_2. In *V. parvula*, the conversion of succinate to propionate is part of the lactate fermentation pathway, which produces sufficient ATP for growth by substrate-level phosphorylation. ATP production by decarboxylation phosphorylation is therefore not mandatory in this organism, and its inability to grow on succinate may in fact be due to the lack of a suitable F_1F_0 ATP synthase (Denger and Schink 1982).

Propionigenium modestum was the first bacterium shown to gain energy for growth exclusively from a decarboxylation reaction and is thus the paradigm for the mechanism of decarboxylation phosphorylation (Fig. 1) (Hilpert and Dimroth 1982; Hilpert et al. 1984). Succinate metabolism starts with the transfer of the CoA moiety from propionyl-CoA to succinate to yield succinyl-CoA and propionate. Rearrangement of the carbon skeleton of succinyl-CoA with a B12-dependent enzyme leads to (*R*)-methylmalonyl-CoA, which is subsequently converted to (*S*)-methylmalonyl-CoA. The membrane-bound (*S*)-methylmalonyl-CoA decarboxylase conserves energy by coupling the decarboxylation reaction to the transport of Na^+ ions across the membrane. The product propionyl-CoA is used to activate a new succinate molecule to succinyl-CoA. The energy stored in the electrochemical gradient of Na^+ ions is subsequently used to drive ATP synthesis by a Na^+-translocating F_1F_0 ATP synthase. Given the bioenergetic demands for ATP synthesis in growing bacteria (~60 kJ/mol) (Thauer et al. 1977) and the free energy of the decarboxylation reaction (~ – 20 kJ/mol) (Schink and Pfennig 1982), it is clear that approximately three rounds of succinate degradation are required to support the synthesis of one molecule of ATP. The bioener-

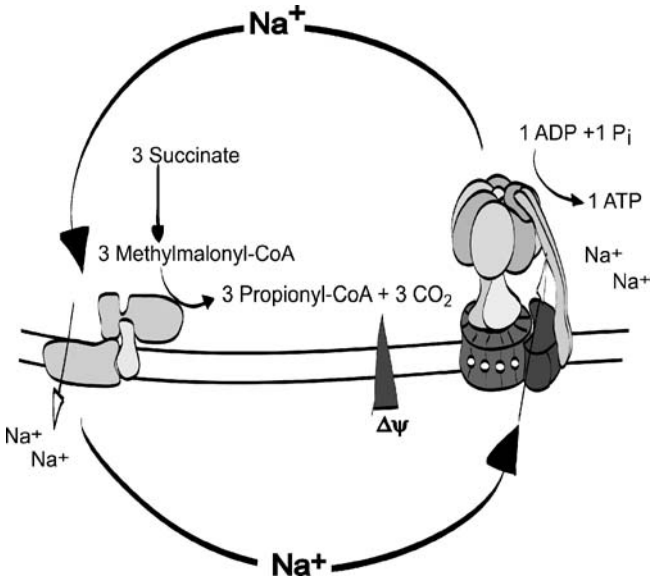

Fig. 1 ATP synthesis by decarboxylation phosphorylation in *P. modestum*

getic relationships require a proper adjustment of the ratios between chemical reaction and Na^+ translocation of the two membrane-bound complexes operating in tandem. Methylmalonyl-CoA decarboxylase has been shown to translocate one Na^+ ion electrogenically and one Na^+ ion electroneutrally (in exchange for H^+) across the membrane per decarboxylation event (Hilpert and Dimroth 1991; DiBerardino and Dimroth 1996). For the synthesis of one molecule of ATP, 3.3 Na^+ ions need to traverse the ATP synthase electrogenically (Stahlberg et al. 2001; Dimroth and Cook 2004). These ratios thus perfectly match the requirements for ATP synthesis by decarboxylation phosphorylation in *P. modestum*.

2.3 Fermentation of Malonate

Anaerobic malonate degrading bacteria are yet another example for the in vivo ATP synthesis by decarboxylation phosphorylation. A representative of this class of organisms is *Malonomonas rubra* which grows fermentatively on malonate by converting it to acetate and CO_2 (Dehning and Schink 1989). Malonate uptake into the cell has been shown to be catalyzed by a transporter consisting of two different subunits (MadL and MadM). The transported species is $Hmalonate^-$ and this is taken up in an electroneutral symport with one Na^+ ion into the cell (Schaffitzel et al. 1998). Free malonate is chemically inert, and therefore the malonate degrading enzyme machinery (Hilbi et al.

1992; Dimroth and Hilbi 1997) harbors an activation module with a built-in acetyl thioester residue on a 2′-(5″-phosphoribosyl)-3′-dephospho-CoA prosthetic group on an acyl carrier protein (acetyl-ACP) (Berg et al. 1996, 1997). This device serves to transfer the ACP moiety from acetate to malonate in the first partial reaction, thus generating the activated malonyl-ACP species and acetate. The degradation continues with the transfer of the free carboxyl group of malonate to the prosthetic biotin group of a small biotin carrier protein (Berg and Dimroth 1998). The protein with the carboxybiotin residue diffuses to a membrane-bound decarboxylase, where the carboxybiotin is decarboxylated. This reaction is coupled to the transport of two Na^+ ions out of the cell and a simultaneous uptake of one proton into the cell. As a result, one Na^+ ion is exported from the cell electrogenically (together with a positive charge), but the other is exported electroneutrally (in exchange for H^+). It can be envisaged that the latter recycles into the cell during malonate uptake by the electroneutral Hmalonate$^-$/Na^+ symporter (Schaffitzel et al. 1998). The electrogenically exported Na^+ ions are thought to energize ATP synthesis. Details of the ATP synthesizing enzyme of this bacterium have not yet been elucidated. The bioenergetic premises of ATP synthesis in *M. rubra*, however, are similar to those in *P. modestum*, requiring about three decarboxylation events to synthesize one molecule of ATP. *M. rubra* and *P. modestum* are two examples where ATP is exclusively synthesized by decarboxylation phosphorylation. These organisms are distinct with respect to the dicarboxylic acid degraded and the appropriate enzyme equipment needed to decarboxylate its specific substrate, but share the conservation of chemical energy as an electrochemical Na^+ ion gradient.

2.4 Fermentation of Glutarate

Decarboxylation phosphorylation is also the ATP-generating mechanism in *Pelospora glutarica*, a strictly anaerobic bacterium growing on glutarate as sole carbon and energy source (Matthies and Schink 1992a; Matthies et al. 2000). The metabolism of glutarate starts with its activation to glutaryl-CoA by CoA transfer from acetyl-CoA. Subsequently, the glutaryl-CoA is oxidized by a NAD^+-dependent dehydrogenase to glutaconyl-CoA. The key energy conserving reaction is the decarboxylation of glutaconyl-CoA to crotonyl-CoA by a membrane-bound biotin-containing Na^+ pump that shares many properties with other members of the NaT-DC enzyme family. Reduction of crotonyl-CoA to butyryl-CoA with NADH regenerates the NAD^+ consumed in the glutaryl-CoA dehydrogenase reaction, and CoA transfer to acetate produces butyrate and generates the acetyl-CoA consumed in the activation of glutarate. Part of the butyryl-CoA isomerizes to isobutyryl-CoA, before the CoA moiety is transferred to acetate, yielding acetyl-CoA and isobutyrate (Matthies and Schink 1992b). Glutaconyl-CoA decarboxylase is also a key en-

ergy converting reaction in the fermentation of glutamate by *Acidaminococcus fermentans*, and the enzyme from this source has been well characterized biochemically (Buckel and Semmler 1983; Buckel 2001).

3
Structure and Mechanism of the NaT-DC Enzymes

The NaT-DC enzyme family includes oxaloacetate decarboxylase, methylmalonyl-CoA decarboxylase, glutaconyl-CoA decarboxylase, and malonate decarboxylase. These are multisubunit protein complexes, composed of water-soluble and membrane-intrinsic components. The biotin prosthetic group of these enzymes accepts the CO_2 moiety from the substrate at the water-exposed carboxyltransferase site and delivers it to the membrane-bound decarboxylase site, where the decarboxylation is coupled to Na^+ ion transport across the membrane. Many fundamental investigations have been performed with the oxaloacetate decarboxylase, and we shall give a detailed account of this enzyme below.

3.1
Oxaloacetate Decarboxylase Na^+ Pump

As described above, oxaloacetate decarboxylase of *K. pneumoniae*, *S. typhimurium*, or *V. cholerae* is encoded by the three structural genes *oadG*, *oadA*, and *oadB*, which are part of the *citS* operon within the citrate fermentation gene cluster (Woehlke et al. 1992). *oadG* codes for the γ subunit (8.9 kDa) which consists of an N-terminal hydrophobic α helix serving as a membrane anchor and a water-soluble C-terminal domain, which is linked to the membrane domain by a flexible linker peptide consisting mainly of proline and alanine residues. The γ subunit plays an essential role in the complex formation of the decarboxylase, but appears to have no catalytic function by itself (Schmid et al. 2002a). Near the C terminus, the γ subunit contains a Zn^{2+} metal ion (Dimroth and Thomer 1983; DiBerardino and Dimroth 1995). *oadA* codes for the water-soluble α subunit (63.5 kDa) which has a three domain structure. The N-terminal domain, which harbors the carboxyltransferase catalytic site, is bound via a flexible proline/alanine-rich linker peptide to the association domain, which promotes complex formation with the γ subunit (Dahinden et al. 2005b). At its C terminus, another proline/alanine linker connects the association domain to the biotin domain with the prosthetic group bound to a lysine residue, 35 amino acid residues before the C terminus. The crystal structure of the carboxyltransferase shows a dimer of $\alpha_8\beta_8$ barrels with an active site Zn^{2+} ion at the bottom of a deep cleft that is liganded by an aspartate and two histidine residues (Studer et al. 2007). *oadB* codes for the β subunit (44.9 kDa), a very hydrophobic integral membrane protein that folds into a block of three

N-terminal membrane-spanning α helices, a hydrophobic linker peptide, inserting into the membrane but not traversing it, and a block of six C-terminal membrane-spanning α helices (Jockel et al. 1999).

A model of structure and function of the oxaloacetate decarboxylase Na^+ pump is depicted in Fig. 2. The catalytic cycle starts with the transfer of the carboxylic group from oxaloacetate to the biotin prosthetic group on the enzyme. The carboxyl transfer reaction is proposed to follow a two-step sequence. First, the Zn^{2+} ion is thought to coordinate the carboxylate in position 4 of oxaloacetate to facilitate its transfer to the ε-amino group of a conserved, essential, active site lysine residue. The biotin subsequently accepts the carboxyl group from the carbamoyl-lysine intermediate (Studer et al. 2007). Evidence for a carbamoyl-lysine was found in the crystal structure of the 5 S subunit of transcarboxylase which catalyzes exactly the same reaction as OadA (Hall et al. 2004). The carboxybiotin thus formed switches from the carboxyltransferase catalytic site to the decarboxylase site on OadB (Dimroth and Thomer 1983, 1993). It has been proposed that the Zn^{2+} on the C-terminal tail of the γ subunit coordinates the carboxyl group of carboxybiotin during the transfer to protect the chemically labile compound from spontaneous decarboxylation (Studer et al. 2007). At OadB the decarboxylation takes place, and the free biotin group is regenerated. During this Na^+-dependent reaction, a periplasmically derived proton is consumed and two sodium ions are translocated from the cytoplasm into the periplasm (DiBerardino and Dimroth 1996). Essential residues for this reaction have been identified by site-specific mutagenesis of OadB (Jockel et al. 2000a,b; Schmid et al. 2002b). Based on these studies and other biochemical investigations, a model for the reaction mechanism was proposed which is shown

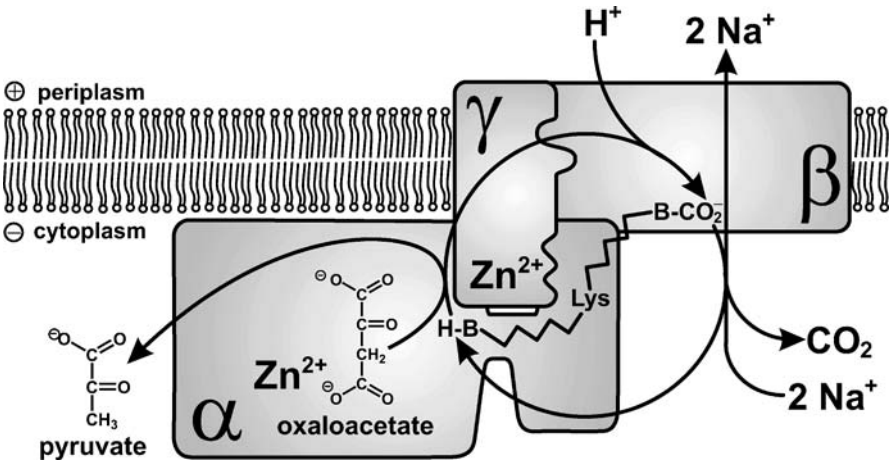

Fig. 2 Model of structure and function of the oxaloacetate decarboxylase Na^+ pump. (B-H, biotin; B-CO_2^-, carboxybiotin; Lys, biotin-binding lysine residue)

in Fig. 3. The model predicts that a number of highly conserved and functionally indispensable residues on helices IV and VIII and on region IIIa of OadB are involved in the ion translocation mechanism. At the decarboxylase site on OadB, the carboxybiotin is thought to form a stable complex, possibly with the side chain of R389 at the cytoplasmic surface of helix VIII. Site-

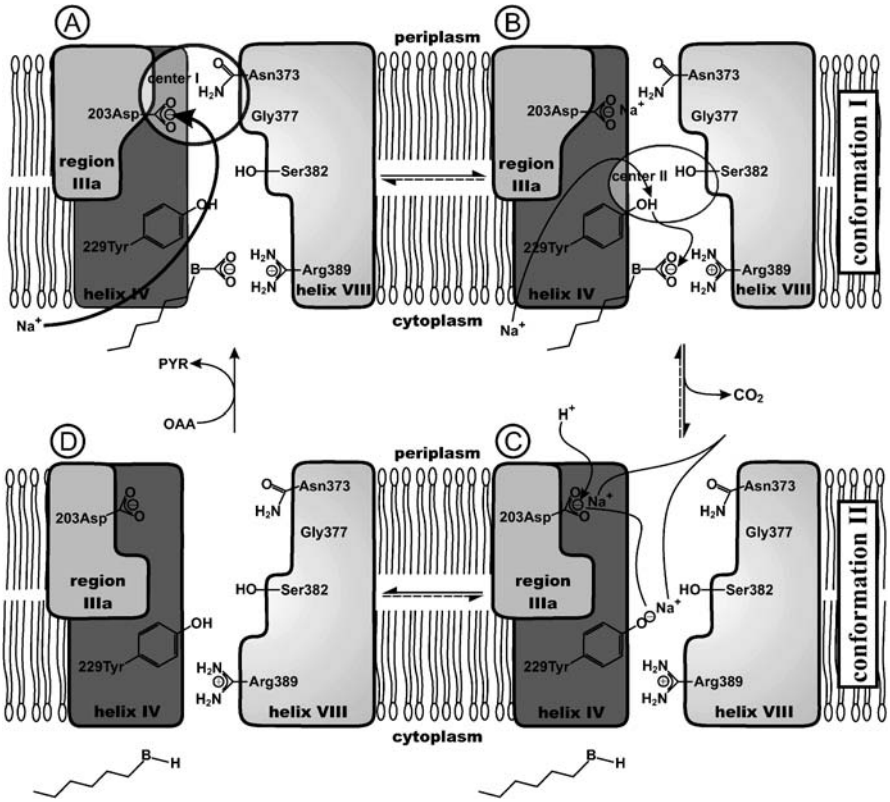

Fig. 3 Model for decarboxylation of carboxybiotin and vectorial Na^+ transport. The appropriate locations of important residues in helix IV, VIII, and in region IIIa of the β subunit and their participation in the Na^+ transport mechanism are depicted. *Panel A* shows the empty binding site region with enzyme-bound carboxybiotin (B-CO_2^-), exposing the Na^+ binding sites toward the cytoplasm. In *panel B*, the first Na^+ binding site at the D203-N373 pair has been occupied and the second Na^+ enters the Y229-S382 site, displacing the phenolic proton from Y229 with a rearrangement of the hydrogen-bonding network. The proton delivered to the carboxybiotin causes the immediate decarboxylation of this acid labile compound. This elicits the conformational change (B→C) exposing the Na^+ binding sites toward the periplasm. The ions are released into this reservoir and a proton enters the periplasmic channel and restores the hydroxyl group of Y229. In *panel D*, the Na^+ binding sites are empty and exposed toward the periplasm and the biotin is not modified (B→H). Upon carboxylation of the biotin, the protein switches back into the conformation where the Na^+ binding sites are exposed toward the cytoplasm (D→A)

directed sulfhydryl labeling with methanethiosulfonate reagents has identified helix VIII to align the channel for Na^+ and H^+ conductance across the membrane (Wild et al. 2003). Evidently, the proton which moves from the periplasmic reservoir through this channel must reach the carboxybiotin near the cytoplasmic surface to account for the consumption of a proton in the decarboxylation reaction. According to the model (Schmid et al. 2002b), the Na^+ channel is initially open to the cytoplasm. In this conformation, the two different Na^+ binding sites are of high affinity (K_s ~1 mM). The first Na^+ is thought to bind at a site near the periplasmic surface (center I), which includes the side chains of D203 and probably also of N373. Subsequently, the second Na^+ binds to the Y229 and S382 including site (center II). At this integral membrane location (center II) the Na^+ ion will only be tolerated after charge balancing, i.e., after dissociation and removal of a proton from the site. The proton from the phenolic hydroxyl group of Y229 is therefore assumed to dissociate as the Na^+ ion is approaching and to move to the carboxybiotin, where it is consumed during decarboxylation of this acid-labile compound. Concomitantly, the biotin prosthetic group leaves the site and OadB changes its conformation. This exposes the Na^+ binding sites toward the periplasm and simultaneously decreases their Na^+ binding affinities. The Na^+ ions dissociate into the periplasmic reservoir, while a proton enters the periplasmic channel and restores the hydroxyl group of Y229. Hence, each decarboxylation event is coupled to the transport of two Na^+ ions from the cytoplasm to the periplasm and the consumption of a periplasmically derived proton.

The amino acid sequences of the membrane-embedded β subunits of the NaT-DC family are very similar, and therefore these partial enzymes are thought to act by a common reaction mechanism. Distinct primary structures are found, however, for the carboxyltransferase subunits/domains, suggesting that these partial enzymes operate by different mechanisms. The crystal structures of the carboxyltransferase of glutaconyl-CoA decarboxylase (Wendt et al. 2003) and oxaloacetate decarboxylase (Studer et al. 2007) are indeed not related to each other and indicate a different reaction mechanism for each of them.

4
ATP Synthesis Energized by an Electrochemical Na^+ Ion Gradient

4.1
H^+- and Na^+-Translocating ATP Synthases

The mechanism of decarboxylation phosphorylation is completed by the synthesis of ATP, utilizing the electrochemical gradient of Na^+ ions as energy source. The enzyme responsible for ATP synthesis is a Na^+-translocating F_1F_0 ATP synthase. F_1F_0 ATP synthases are ubiquitous from bacteria to plants and

animals, but usually use H^+ rather than Na^+ as the coupling ion (Boyer 1993; Capaldi and Aggeler 2002; Dimroth et al. 2006). This is reasonable, since the oxidation of nutrients in animals or bacteria or photosynthesis in plants leads to the formation of an electrochemical proton gradient across the membrane. Some bacteria with a fermentative metabolism, e.g., *P. modestum*, *M. rubra*, or *P. glutarica*, synthesize ATP exclusively by decarboxylation phosphorylation, while others can synthesize ATP also by substrate-level phosphorylation. Detailed studies have been performed with the closely related ATP synthases from *P. modestum* and *Ilyobacter tartaricus*. The *P. modestum* enzyme was the first F_1F_0 ATP synthase found to act with Na^+ and not with H^+ as the physiological coupling ion (Laubinger and Dimroth 1987, 1988, 1989). The advantage of this coupling ion specificity is to provide a direct link to the Na^+-translocating decarboxylase, completing the Na^+ cycle without an intermediate interconversion of a Na^+ into a H^+ gradient with a Na^+/H^+ antiporter. The ATP synthases of *P. modestum, I. tartaricus,* or *Escherichia coli* are encoded by eight structural genes that are organized in an operon (Krumholz et al. 1992; Kaim et al. 1992). The first gene of the operon, which is termed i gene, encodes an integral membrane protein that is not part of the ATP synthase complex. In an i gene deletion clone of *E. coli*, the cells were still able to synthesize ATP by the F_1F_0 ATP synthase, albeit at a reduced level (Gay 1984). This suggests that the i gene product is not essential for the production of a functional ATP synthase complex, but that it might help to make the assembly more efficient. Downstream of the i gene are the structural genes for the ATP synthase, starting with the gene for subunit a. This is followed by the genes for the other membrane-bound subunits c and b and by the genes for the water-soluble subunits δ, α, γ, β, and ε. The similarity between the *atp* operons from *P. modestum* and *E. coli* allowed the construction of hybrid ATP synthases harboring parts from either bacterium (Kaim and Dimroth 1993, 1994). The hybrids were constructed in the *E. coli* strain CM1470 that carries a deletion in the genes for the ATP synthase subunits a, c, b, δ, and part of α. As this strain does not form a functional ATP synthase, it is unable to grow by oxidative phosphorylation with succinate as carbon source. This defect could be complemented by transformation with plasmid pAP42 which harbors those ATP synthase genes from *P. modestum* that were lacking on the chromosome of *E. coli* CM1470. As a result of homologous recombination, the *P. modestum* genes were integrated into the chromosome of *E. coli* CM1470, yielding the new strain *E. coli* PEF42, which synthesizes a functional ATP synthase hybrid and is thus able to grow on succinate minimal medium. These results impressively buttress the common origin of H^+- and Na^+-translocating F_1F_0 ATP synthases. The Na^+-translocating ATP synthases occur exclusively in anaerobic bacteria. Other representatives of this class were found in *Acetobacterium woodii* (Müller et al. 2001) and in *Clostridium paradoxum* (Ferguson et al. 2006).

The F_1F_0 ATP synthases are nanosize rotary engines, organized as an assembly of two entities, F_1 and F_0, which are connected by a central and a peripheral

stalk (Fig. 4). Each of these protein complexes functions as a reversible rotary motor and exchanges energy with the opposite motor through mechanical rotation of the central stalk. During ATP synthesis, the electrochemical ion gradient fuels the membrane-embedded F_0 motor to rotate the central stalk in its intrinsic direction. Conversely, ATP hydrolysis by the F_1 motor causes reverse rotation of the shaft, which converts the F_0 motor into an ion pump. Under normal circumstances, the F_0 motor generates the larger torque and drives the F_1 motor in the ATP synthesis direction. However, in fermenting bacteria, when the respiratory enzymes are not active, the F_1 motor hydrolyzes ATP to use the F_0 motor as the generator of the indispensable membrane potential. An interesting example is the anaerobic thermoalkaliphilic bacterium *C. paradoxum*

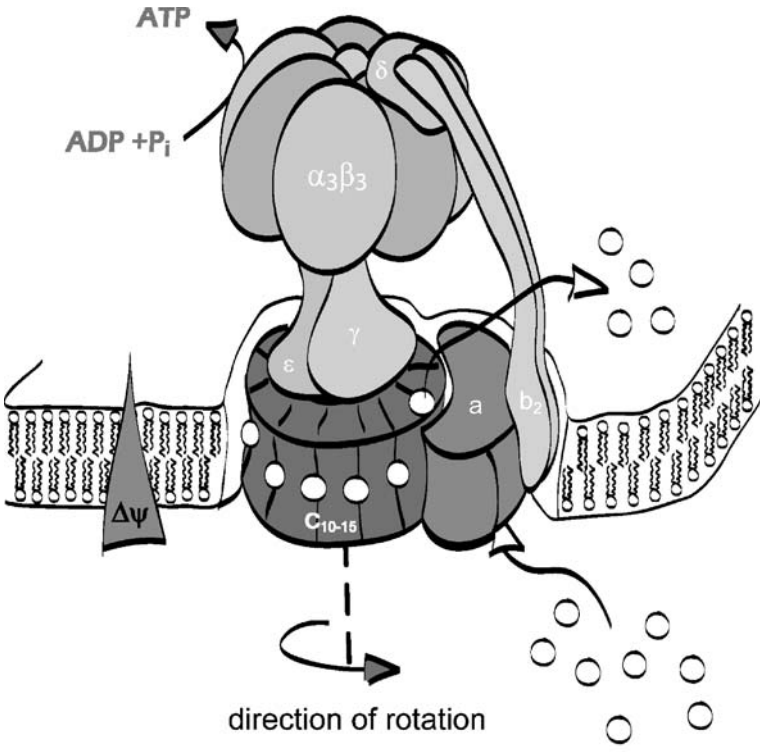

Fig. 4 Cartoon of structure and function of a bacterial ATP synthase. F_1 (subunits $\alpha_3\beta 3\gamma\delta\varepsilon$) and F_0 (subunits ab_2c_{10-15}) are two motors that exchange energy by rotational coupling. The rotary subunits are $\gamma\varepsilon c_{10-15}$ and the membrane anchored and cytoplasmic stator subunits are $ab_2\alpha_3\beta_3\delta$. During ATP synthesis, coupling ions (*shown as white circles*) pass through the F_0 motor from the periplasm to the cytoplasm, inducing rotation and enabling the F_1 motor to synthesize ATP

(Ferguson et al. 2006), which generates ATP in the fermentation of glucose to acetate and CO_2, and uses these acids to keep the cytoplasmic pH near neutral in spite of the alkaline environment. The physiological function of the F_1F_0 ATPase of this organism is to generate a membrane potential by hydrolyzing some of the ATP and in order to not compromise pH homeostasis, the ATPase uses Na^+ and not H^+ as the coupling ion.

4.2 The F_1 Motor

For the F_1 motor, no differences between H^+- and Na^+-translocating ATP synthases have been reported so far. The motor consists of a hexameric assembly of alternating α and β subunits around a central coiled-coil γ subunit. The F_1 complex is intrinsically asymmetric, owing to different interactions of the central γ subunit with each of the catalytic β subunits, and provides them with different conformations and nucleotide affinities at their catalytic sites. On rotation of the γ subunit, the conformations of the β subunits change sequentially such that each β subunit adopts the same conformations of varying affinity during one rotational cycle. As a result, three molecules of ATP are synthesized. This model, known as the binding change mechanism (Boyer 1993), explains a wealth of biochemical and kinetic data. Important details of this mechanism, however, are still under active investigation. The rotational model is consistent with the crystal structure of F_1, which shows a marked asymmetry in the conformations and nucleotide occupancy of the catalytic β subunits (Abrahams et al. 1994). Based on the structure, the rotational catalysis was experimentally proven by a variety of biochemical and spectroscopic techniques. Most convincingly, the rotation of a micrometer-sized fluorescent actin filament attached to the central shaft γ subunit has been directly visualized by video microscopy of single F_1 molecules (Noji et al. 1997). Rotation was also observed when the actin filament was attached to subunit ε or to the c_{10-15} oligomer (Kato-Yamada et al. 1998; Pänke et al. 2000; Sambongi et al. 1999; Tsonuda et al. 2001). These studies confirmed several cross-linking studies, which have been important to define subunits γ, ε, and c_{10-15} as the rotor subunits and subunits α_3, $\beta_3\delta$, a, and b_2 as the stator subunits (Capaldi et al. 2000). It was found that cross-links between subunits γ, ε, and c did not block ATP-driven proton translocation, whereas cross-links between subunits γ or ε and α or β led to an inhibition of the enzyme. Consistent with these data is a medium-resolution electron density map of an F_1 complex from yeast with an attached c_{10} ring, which shows a physical connection between the γ and ε subunits and the c ring at its cytoplasmic loops (Stock et al. 1999).

Single-molecule experiments with small magnetic or gold beads or with fluorescent dyes instead of the large actin filament provided insights into mechanistic details of the enzyme. Rotation in the fully coupled Na^+-ATP synthase was observed both in synthesis and hydrolysis mode (Kaim et al. 2002).

Rotation of the rotor was counterclockwise in the ATP hydrolysis direction and clockwise in the ATP synthesis direction, when viewed from the F_0 domain (Diez et al. 2004; Itoh et al. 2004), and performed up to 700 revolutions per second (Nakanishi-Matsui et al. 2006). In F_1, the γ subunit rotates in steps of 120° for each ATP molecule hydrolyzed. Each 120° step can be further divided into four stages. In the ATP binding dwell, an ATP molecule binds to the empty β_1 site and elicits a rapid 80° substep rotation of the γ subunit. In the following catalytic dwell, ATP is thought to be cleaved at the β_2 site and ADP and/or phosphate is released from the β_3 site. This initiates a 40° substep rotation of the γ subunit completing its 120° rotation (Yasuda et al. 2001; Nishizaka et al. 2004; Shimabukuru et al. 2003). The rotational behavior of F_1F_0 resembled that of F_1, indicating that friction in the F_0 motor is negligible during ATP-driven rotation. Tributyltin chloride, a specific inhibitor of the ion access route in subunit a (von Ballmoos et al. 2004), inhibited rotation by 96% (Ueno et al. 2005), in accordance with strict coupling between mechanical and ion translocation events. When the performance of the F_1 motor was probed by sophisticated single-molecule experiments in femtoliter-sized chambers, the hydrolysis of three ATP molecules per revolution was directly observed and showed very high mechanochemical coupling (Rondelez et al. 2005). If the γ subunit of F_1 was forced to rotate in the ATP synthesis direction, ATP synthesis from ADP and phosphate was observed. Interestingly, the mechanochemical coupling efficiency was low for an F_1 subcomplex lacking the ε subunit, but reached more than 70% after the reconstitution with this protein (Rondelez et al. 2005). The ε subunit thus has an important role in the synthesis of ATP, but the mechanism for this function has not yet been elucidated.

4.3 The F_0 Motor

The F_0 motor is a membrane-bound protein complex consisting of an oligomeric ring of c subunits, a single a subunit, and a dimer of b subunits, which flank the c ring laterally (Mellwig and Böttcher 2003; Rubinstein et al. 2003). Early on, the c subunit has attracted much interest, because of its very hydrophobic character and its small size, which facilitated its purification by extraction into organic solvent mixtures. Based on amino acid sequencing and specific labeling studies with a hydrophobic diazirine derivative, subunit c was predicted to fold as a helical hairpin, consisting of two membrane spanning α helices and a cytoplasmic connecting loop (Hoppe et al. 1984). Importantly, the C-terminal α helix contains a conserved acidic residue, approximately in the middle of the membrane, which plays a profound role in proton translocation. Accordingly, mutagenesis of the respective aspartic acidic residue in *E. coli* (D61) to asparagine, or its chemical substitution with the specific F-ATPase inhibitor dicyclohexylcarbodiimide (DCCD), results in the impairment of proton translocation (Hoppe et al. 1982; Sebald et al. 1980). Hence, the D61 residues

are thought to extract protons from one and deliver them to the other side of the membrane, as the c ring rotates. Accordingly, the F_0 motor of *P. modestum*, which conducts Na^+, harbors specific binding sites for Na^+ on its c ring (Kluge and Dimroth 1992; Kaim et al. 1997). Biochemical evidence for these sites was obtained by DCCD labeling experiments (Kluge and Dimroth 1993a,b; 1994). The ATP synthase of *P. modestum* was specifically labeled at cE65, which is equivalent to cD61 of *E. coli*. The chemical modification with DCCD consumes a proton, and consequently the reaction rate increased from alkaline to acidic pH values, following a titration curve with an apparent pK of 6.5, which reflects the pK of cE65 (Kluge and Dimroth 1993a). Importantly, Na^+ ions protected from this modification and shifted the pK of cE65 into the acidic range, indicating a competition of Na^+ and H^+ binding to the same site.

The Na^+ binding sites on the c ring were confirmed by mutational studies. First, random mutagenesis of the c ring and selection for Na^+-independent growth on succinate minimal medium led to the identification of a double mutant in subunit c, which abolished Na^+ binding and made the holoenzyme to a more efficient H^+-translocating ATP synthase (Kaim and Dimroth 1995). As these c-subunit mutations are near the C terminus, in considerable distance to the ion binding glutamate 65, they must exert their effect via a long-distance conformational change at the binding site. Second, using site-specific mutagenesis, the Na^+ binding site was identified to be contributed by the triad cQ32, cE65, and cS66 (Kaim et al. 1997). These three residues are conserved in all Na^+-translocating F_1F_0 ATP synthases. In the high-resolution structure of the c ring from *I. tartaricus*, the side chains of these three amino acids were indeed seen to be Na^+ binding ligands (Meier et al. 2005). The topography of the binding site within the middle of the membrane was identified by photocrosslinking experiments using a photoactivatable DCCD derivative attached to cE65 (von Ballmoos et al. 2002a). This became specifically bound to the fatty acid side chains of the phospholipids. Quenching experiments with a fluorescent DCCD derivative attached to cE65 and phospholipids carrying a spin label at different positions of the fatty acid side chains were consistent with this membrane location (von Ballmoos et al. 2002b).

4.4 Subunit C Structures

Two different structures of the *E. coli* c subunit were solved by NMR at pH 5 and 8, respectively, in an organic solvent mixture (Girvin et al. 1998; Rastogi and Girvin 1999). In the pH 5 structure, which is thought to present the conformation during exposure to the lipids, D61 has an inward facing orientation. In the pH 8 structure, however, which is thought to represent the conformation in the interface with subunit a, D61 is exposed to the surface. The latter conformation matches cross-linking data between subunits c and a (Jiang and Fillingame 1998). To account for these results, a c ring model

was proposed, in which the C-terminal helix performs a large 140° swiveling versus the N-terminal helix (Fillingame et al. 2003) in the interface with subunit a. This relocates the proton binding D61 to the surface to permit loading or unloading of the site from and to subunit a. NMR investigations of the *P. modestum* c subunit revealed different secondary structures in SDS and in the organic solvent mixture, and neither was similar to one of the *E. coli* c subunit structures (Matthey et al. 1999, 2002). Therefore, an artifactual folding of the protein due to the unphysiological environment of a c monomer in an organic solvent mixture or a harsh detergent could not be excluded.

In the native ATP synthase, the c subunits assemble into an oligomeric ring, the stoichiometry of which varies depending on the species. Rings with ten monomers exist in yeast mitochondria (Stock et al. 1999), the thermophilic bacterium PS3 (Mitome et al. 2004), and possibly in *E. coli* (Jiang et al. 2001), whereas rings found in the bacterium *I. tartaricus* (Stahlberg et al. 2001) and *P. modestum* (Meier et al. 2003) have 11 monomers. The archaeon *Methanopyrus kandleri* harbors a gene in which 13 c subunits are fused (Lolkema et al. 2003), suggesting that this organism produces a corresponding ring with 13 fused c subunits. Furthermore, ring stoichiometries of 14 and 15 have been found in chloroplasts (Seelert et al. 2000) and the cyanobacterium *Spirulina platensis* (Pogoryelov et al. 2005), respectively. An even larger ring with 20 hairpins resulting from ten double-size subunits and harboring ten ion binding sites exists in the V-type ATPase from *Enterococcus hirae* (Murata et al. 2005). The number of subunits in each ring also indicates the number of ions transported across the membrane during each cycle of the ATP synthase. Consequently, as the F_1 motor contains three catalytic sites and synthesizes three molecules of ATP per cycle, a variation in the number of c subunits and ion binding sites automatically leads to different H^+ (Na^+) to ATP ratios. ATP synthases with large c rings have a high H^+ (Na^+) to ATP ratio, which would be advantageous for ATP synthesis at low ion motive force. Conversely, ATP synthases with small c rings might prevail in organisms with constantly high ion motive force: the low H^+ (Na^+) to ATP ratio of these enzymes results in a more efficient use of energy. A mismatch to the threefold symmetry of the F_1 motor created by F_0 motors with 10, 11, 13, or 14 c subunits has been regarded as functionally important (Stock et al. 1999; Murata et al. 2004) in order to prevent the enzyme from becoming trapped in deep energy minima. However, recent data from the c_{15} ring of the *S. platensis* ATP synthase show that symmetry mismatch is not mandatory for function (Pogoryelov et al. 2005).

4.5
Structure of the C Ring From *I. tartaricus*

The Na^+-translocating ATP synthases of *P. modestum* or *I. tartaricus* have the distinct advantage of containing an oligomeric c ring of excessive stabil-

ity (Laubinger and Dimroth 1988; Neumann et al. 1998; Meier and Dimroth 2002). This facilitated the purification of the c ring from the *I. tartaricus* ATP synthase in high yield and purity (Meier et al. 2003). The isolated c ring could be reconstituted with subunits a and b to form a functional F_0 moiety, indicating that it retained its native conformation (Wehrle et al. 2002a). The initial investigations on the c ring structure were performed by atomic force microscopy and electron microscopy after reconstitution into lipid vesicles and crystallization in two dimensions (Stahlberg et al. 2001; Meier et al. 2003). The structure was solved at medium resolution (4 Å) by electron crystallography (Vonck et al. 2002) and at high resolution (2.4 Å) by X-ray crystallography (Meier et al. 2004) (Fig. 5). In the structure of the oligomer, each c ring is composed of 11 subunits. Of these, each monomer is folded as a helical hairpin with the loop at the cytoplasmic side and the termini at the periplasmic side, as was predicted earlier by independent methods (Girvin et al. 1998; Hoppe et al. 1984). The structure (Fig. 5) shows a cylindrical, hourglass-shaped protein complex which has a height of ~70 Å and protrudes from the membrane on either side. Its outer diameter is ~40 Å in the middle and ~50 Å at the top

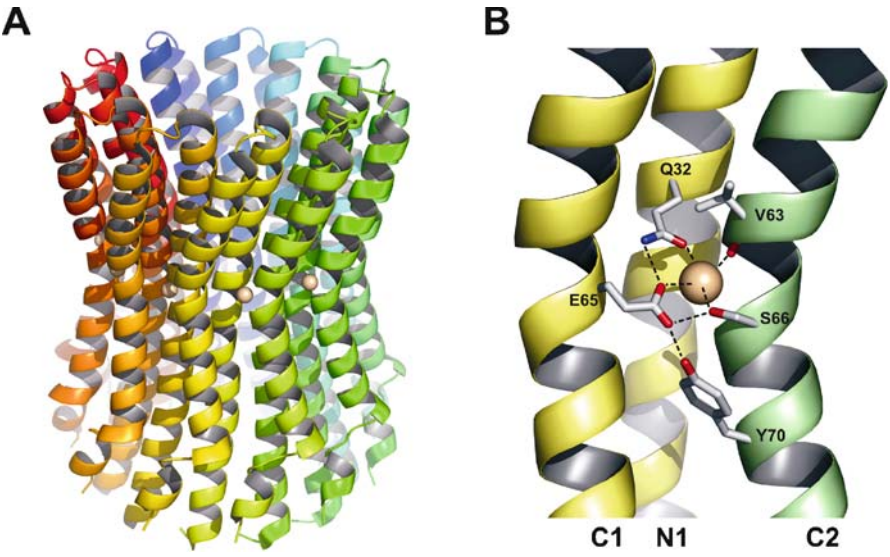

Fig. 5 Structure of the *I. tartaricus* c_{11} ring in ribbon form. **A** Individual subunits are shown in different colors. The *spheres* indicate the bound Na^+ ions. **B** Close-up of the Na^+ binding site formed by the inner (N1) and outer helix (C1) of one c subunit and the outer helix (C2) of the neighboring c subunit. Na^+ coordination and selected hydrogen bonds are indicated with *dashed lines*. The structure shows the locked conformation. During opening, the side chain of Y70 might relocate into a cavity underneath the binding site, thus destabilizing the hydrogen-bonding network and allowing unloading and loading of the binding site to and from subunit a

and bottom. The 11 N-terminal helices are closely spaced within an inner ring surrounding a cavity of ∼17 Å. The tight helix packing leaves no space for side chains and is accounted for by a highly conserved motif of four glycine residues in the inner, N-terminal helix (Vonck et al. 2002). Each N-terminal helix is connected to a C-terminal helix by a loop formed by the highly conserved peptide R45, N46, P47 which is exposed to the cytoplasmic surface (Watts et al. 1995; Hermolin et al. 1999). The C-terminal helices pack into the grooves formed between N-terminal helices, producing the outer ring. All helices show a bend of about 20° in the middle of the membrane (at P28 and E65 in the N-terminal and C-terminal helices, respectively) causing the narrow part of the hourglass shape. Moreover, the bend tilts the helices in the cytoplasmic half out of the plane by ∼10°, yielding a right-handed twisted packing. When the c ring is viewed from the cytoplasm, it rotates counterclockwise during ATP synthesis against the drag imposed by the F_1 motor components. Thus, the resulting torque might decrease the bend and increase the interhelical distance in the cytoplasmic part of the c ring, depending on the energies involved. Such a conformational change under load might serve to store elastic energy in the c ring, adding to that described for the central and peripheral stalk subunits (Junge et al. 2001). The internal surface of the c ring is very hydrophobic and was shown by photocross-linking experiments to be filled with phospholipids in the natural environment of the membrane (Oberfeld et al. 2006).

Perhaps the most instructive feature of the c ring structure is the arrangement of the Na^+ binding sites. Eleven bound Na^+ ions are seen in the c ring structure near the middle of the membrane facing toward the outer surface of the c ring (Fig. 5) (Meier et al. 2004), which confirms previous cross-linking data (von Ballmoos et al. 2002a). Each of the 11 Na^+ ions is bound at the interface of an N-terminal and two C-terminal helices. The coordination sphere is formed by side chain oxygen atoms of Q32 and E65 of one subunit and the side chain oxygen atom of S66 and the backbone carbonyl oxygen atom of V63 of the neighboring subunit (Fig. 5). An intriguing observation is that E65 acts not only as one of the Na^+ binding ligands but also as the recipient of hydrogen bonds from the side chains of Q32, S66, and Y70. This arrangement generates a stable, locked conformation of the binding site, from which the horizontal transfer of Na^+ to subunit a is prevented. This implies that the present locked conformation of the binding site converts to an open one at the subunit a/c interface in order to allow the horizontal ion transfer to and from subunit a.

The a subunit abuts the c ring laterally and is implicated in providing access to the ion binding site at the c subunit in the subunit a/c interface from either one or both sides of the membrane, depending on the model. Subunit a is an extremely hydrophobic protein containing five to six transmembrane helices (Jäger et al. 1998; Long et al. 1998; Valiyaveetil and Fillingame 1998). Of particular interest is the interface of the a subunit with the c ring, which

has to guarantee the stability of the a/c complex while allowing an almost frictionless rotation between these protein components, as revealed by single-molecule experiments (Ueno et al. 2005). These peculiarities have impeded structural determinations of subunit a. So far, biochemical and mutational studies suggest that the universally conserved aR226 residue (*P. modestum* numbering), which is localized approximately at the same level in the membrane as the cE65, is important in ion translocation.

5
Mechanism of the F_0 Motor

5.1
The Proton Motor

The F_0 motor converts the energy stored in an electrochemical ion gradient into mechanical rotation. Hence, the torque generating mechanism is an integral part of the transport of the coupling ions across the membrane. Theoretical considerations and a large body of experimental data have led to the proposal of several models for the F_0 motor function, which are considerably distinct in detail but share common features as well (Aksimentiev et al. 2004; Feniouk et al. 2004; Xing et al. 2004). The initial model of the H^+-driven motor of the *E. coli* ATP synthase (Junge et al. 1997; Vik and Antonio 1994) predicts that only a protonated, uncharged cD61 site can move from the subunit a/c interface into the lipid phase. To account for sufficient torque generation and to prevent proton leakage in the subunit a/c interface, the initial model was extended by positioning the essential positive stator arginine between the access and the exit pathway of the ion (Elston et al. 1998). The model predicts that inlet and outlet channels are located in a noncoaxial manner in subunit a (Fig. 6). The ion is proposed to enter the site through the inlet channel from the low-pH reservoir and to exit through the outlet channel into the high-pH reservoir, after performing an almost complete rotation. The unprotonated site is located between the two channels, and without an external driving force the motor is in an idling mode, and the site shuttles between the channels with equal probability to either side. In the presence of a ΔpH, the proton concentration in each of the separate aqueous access channels is unequal, and the site will be protonated more frequently at the position of the channel with higher proton concentration. The protonated site is now able to move out of the a/c subunit interface into the lipid phase. Simultaneously, a new site enters the interface, where its ion is displaced by the stator arginine. The negatively charged empty site acts as a ratchet, due to the large energy penalty of its backward movement into the lipid phase. In this model, the pH gradient determines the direction of rotation and simultaneously acts as the main driving force for the generation of torque.

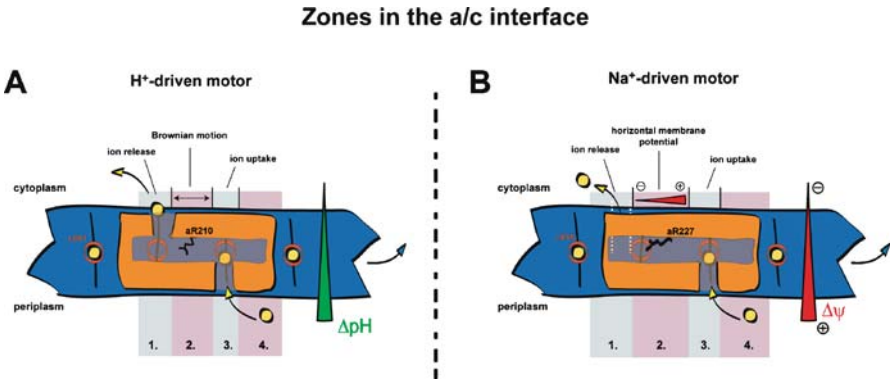

Fig. 6 Model for torque generation in the H^+- and Na^+-translocating F_0 motor. **A** Two-channel model with a ratchet-type mechanism for H^+-dependent enzymes. The crucial events during ion translocation in ATP synthesis direction in the a/c interface can be divided into four zones. (1) The occupied rotor site enters the interface and releases its coupling ion through the outlet channel into the cytoplasm with high pH. Deprotonation prevents the backward rotation into the lipid phase acting as a molecular ratchet. (2) The negative charge of the binding site is compensated by the stator arginine. In this functionally symmetric state, Brownian back and forth motions toward either channel are possible. (3) As the inlet channel, which is in contact with the periplasm where the pH is low, contains more protons than the outlet channel, which is in contact with the cytoplasm, the binding site is more frequently protonated from the periplasm. Therefore, the ΔpH determines the direction of rotation. (4) The loaded binding site can now move out of the interface into the lipid bilayer, whereby the next binding site enters the interface and experiences the events described in (1). **B** Push-and-pull model for Na^+-dependent enzymes. (1) In ATP synthesis direction, an occupied rotor site enters the interface from the left and releases its bound Na^+ ion toward the cytoplasm. This process is aided by the stator arginine. (2) The arginine compensates for the now negatively charged empty site. The horizontal component of the membrane potential, however, pulls the arginine to the left and pushes the glutamate to the right. Therefore, the electrical component of the ion motive force determines the direction of rotation from left to right. (3) The hydration of the binding site within the inlet channel stabilizes this conformation and allows loading of the binding site from the periplasm. Movement of the binding site from zone 2 to 3 pulls the next rotor site into the a/c interface as described in (1). (4) The binding site that has been occupied from the periplasm is allowed to rotate out of the interface into the lipid phase. The event is aided by a push mechanism during the events described in (2)

Experiments with engineered cysteines within helices II, IV, and V show good accessibility of these residues from the periplasmic side to approximately the middle of the membrane, which would be in accord with a periplasmic inlet channel. From the cytoplasmic surface, cysteines were only accessible within helix IV, and those cysteines were very close to the membrane surface (Angevine and Fillingame 2003; Angevine et al. 2003). Conclusive evidence for a cytoplasmic access channel within subunit a is therefore not available so far.

5.2 The Sodium Motor

In contrast to the proton-translocating ATP synthases, where the path of the ion across the membrane is intrinsically difficult to establish, the Na^+-translocating counterparts provide unique experimental options for these investigations. For example, (1) Na^+ concentrations on either side of the membrane can be manipulated at will without compromising the stability of the enzyme, (2) Na^+ translocation can be conveniently measured with the radioactive isotope $^{22}Na^+$ (Dimroth 1982b) or with a Na^+-binding fluorophore (von Ballmoos and Dimroth 2004), and (3) screening methods can be used to select for mutants with impaired or modified Na^+ binding characteristics. Many details of the ion translocation through the F_0 motor and of the mechanical rotation connected to it have therefore been explored with the Na^+-translocating ATP synthase of *P. modestum*. These experiments support a model where the ion binding sites on the c ring are accessible from the periplasmic surface through a corridor in subunit a, and from the cytoplasmic surface through a route that is either c ring-intrinsic or within the interface between the c ring and subunit a (Dimroth et al. 2006).

5.2.1 The Ion Path

In the following we will describe some of the experiments that have led to the model of the sodium F_0 motor and we will then give a detailed description of this model. *E. coli* deletion mutants in the *atp* operon are unable to grow on a nonfermentable carbon source such as succinate. This defect could be complemented by expressing the *E. coli* F_1 ($-\delta$) together with the *P. modestum* F_0 ($+\delta$) genes on a plasmid and thus allowed the construction and investigation of mutants in the F_0 genes of the Na^+-dependent enzyme (Kaim and Dimroth 1993, 1994). The parent *E. coli*/*P. modestum* hybrid ATP synthase showed the same Na^+-dependent growth characteristics as the F_1F_0 ATP synthase of *P. modestum*. In a random mutagenesis approach, mutants with a Na^+-independent growth phenotype could be isolated, which could be grouped into two categories. Mutants of the first category contained a double mutation in the C-terminal tail of subunit c (Kaim and Dimroth 1995). The mutation affected the geometry of the binding site in such a way that Na^+ ions were no longer able to bind, whereas the ability for Li^+ or H^+ binding was retained. The second group of mutants contained a triple mutation in subunit a, which affected the ion specificity of the periplasmic access route to the binding site, so that Na^+ access was abolished whereas Li^+ or H^+ access was retained (Kaim and Dimroth 1998a). The mutant cells could grow on succinate only in the absence, but not in the presence, of Na^+ ions, suggesting that sodium ions were unable to get access to the binding

site through the periplasmic entrance channel, and simultaneously prevented protons from reaching the binding sites via this route. Furthermore, Na^+ ions inhibited the ATP hydrolysis activity of the mutant ATP synthase, and one Na^+ per F_1F_0 became firmly occluded in the subunit a/c interface after ATP addition (Kaim et al. 1998; Kaim and Dimroth 1998a). Hence, after ATP-driven rotation of the binding site into the interface, any further rotation is prevented if the Na^+ ion cannot be released from the binding site into the periplasm due to blockage of this route by the subunit a triple mutation. Besides by mutation, the periplasmic access route for Na^+ was also blocked by the inhibitor tributyltin chloride (von Ballmoos et al. 2004). A photoactivatable derivative of this compound was found to specifically label subunit a. Sodium ions were protected from this labeling, indicating a competition between tributyltin chloride and Na^+ for the binding at the same site. The most obvious candidate for this site is the Na^+ access pathway. This supposition is in accord with biochemical data, showing that tributyltin chloride not only blocks the hydrolysis of ATP, but also transport of sodium ions across the membrane.

So far, neither inhibitors nor mutants have been found for the cytoplasmic access route to the binding site, and it is not resolved as to whether this route is localized entirely in the c ring or in the interface between the c ring and subunit a. Although several lines of evidence compellingly demonstrate that cytoplasmic access to the binding site can occur outside the subunit a/c interface in a time scale of seconds (von Ballmoos et al. 2002; Meier et al. 2003), it is not certain whether this ion path can account for physiological rates in the millisecond timescale. Among these evidences are (1) the demonstration of Na^+ or H^+ binding to the c subunit sites within the isolated c ring; (2) ATP-driven occlusion of $^{22}Na^+$ by the a subunit triple mutant, even after partial modification of the c ring with DCCD (Kaim and Dimroth 1998b)—this observation compulsorily requires that the radioactive $^{22}Na^+$ enters a site between the DCCD-modified c subunit and the a subunit; and (3) the exchange of Na^+ between both sides of the membrane with partially DCCD-modified c subunits (Kaim and Dimroth 1998b). This result requires shuttling of the rotor between the periplasmic access channel in the subunit a/c interface and a position outside the a/c interface, where the ions must exchange between the cytoplasm and the binding site.

5.2.2 The Driving Force

Experiments have also been performed to determine the driving forces required for torque generation. According to Mitchell's chemiosmotic hypothesis, the chemical concentration gradient of the coupling ion and the membrane potential contribute equally to the driving force in a system at thermodynamic equilibrium. However, under physiological conditions, far from

thermodynamic equilibrium, each individual component can contribute kinetically unequally to the driving force. It was found that in the absence of external energy, the F_0 motor, either isolated or connected to the F_1 motor, performs an idling motion of the rotor versus the stator, which causes Na^+ ions to exchange between the exterior and interior compartments separated by the membrane (Kluge and Dimroth 1992; Kaim and Dimroth 1998b). Exchange by the isolated F_0 motor is not converted into unidirectional rotation coupled to unidirectional ion flux by applying large Na^+ concentration gradients, but only with a threshold membrane potential of about 40 mV (Kluge and Dimroth 1992; Dimroth et al. 2003). Hence, the membrane potential is the kinetically indispensable driving force for torque generation and cannot be compensated by a Na^+ concentration gradient. These results were corroborated by ATP synthesis experiments with reconstituted ATP synthases from *P. modestum*, *E. coli*, or chloroplasts. With each of these enzymes, ATP synthesis was only observed after applying a certain membrane potential and not with large Na^+ or H^+ concentration gradients (Kaim and Dimroth 1999).

It is clear that the principal torque generating and ion translocation events occur in the subunit a/c interface, and it would therefore be of great interest to learn more about the critical parts of this protein assembly. As mentioned above, probably the most important residue for function in subunit a is the universally conserved arginine 226 (*P. modestum* numbering) near the middle of the penultimate transmembrane helix. Mutants of this residue in the *P. modestum* a subunit have provided important insights into its role for the operation of the F_0 motor (Wehrle et al. 2002b). All the evidence is consistent with an attribution of the positively charged residue to repel the Na^+ ions from approaching binding sites into the appropriately positioned release route to either the periplasmic or the cytoplasmic surface, depending on the direction of rotation. The most conservative R226K mutant was completely inactive in ATP-driven ion transport at pH 7.0, similar to the corresponding *E. coli* mutant (Valiyaveetil and Fillingame 1997), but interestingly attained catalytic power between pH 8 and 9. In the R226H mutation, the activity profile was shifted into the acidic range, with maximal activities between pH 6.5 and 7.5. ATP synthase with an R226A substitution catalyzed Na^+-dependent ATP hydrolysis, which was completely inhibited by DCCD, but not coupled to Na^+ transport. Taken together, these results suggest that a positive charge at position 226 is necessary to accomplish the dissociation of the Na^+ ions from approaching rotor sites. Furthermore, an arginine with its delocalized positive charge appears to be better suited for an unrestricted diffusion of the negatively charged empty rotor site than a lysine with a positive point charge. Hence, at neutral pH, when the lysine is completely protonated, it electrostatically attracts a negatively charged rotor site so powerfully that the charge has to be attenuated by its partial deprotonation at higher pH values, in order for the rotor site to escape.

5.2.3 The Model for Torque Generation

On the basis of these experimental data, a model for torque generation by the Na^+-translocating F_0 motor was proposed which is in accordance with mathematical calculations (Dimroth et al. 1999, 2006; Xing et al. 2004). In this model, the binding sites in the rotor/stator interface have to interact in a coordinated manner with the coupling ion, the positive stator charge (R226), and the membrane potential, as shown schematically in Fig. 6 for the ATP synthesis direction. During its journey through the interface with the stator, a rotor site moves along a sequence of states as follows (Xing et al. 2004). As the occupied site (site 1) enters the a/c interface, it is in the vicinity of the positive stator charge, which forces the bound sodium ion to dissociate. The negative charge of site 1 is now compensated by the positive stator charge. In the idling mode, when no external energy source is applied, the rotor can shuttle back and forth against the stator up to a certain angle. However, in the presence of a membrane potential (cytoplasmic negative), the movement becomes directional (see below), bringing the empty site 1 in juxtaposition with the periplasmic access route of subunit a. Upon hydration of the site, the free energy drops to trap it at its present position, until a Na^+ ion has been bound. So far the movement of site 1 has simultaneously pulled the next occupied rotor site (site 2) into the a/c interface. Site 2 now follows the same events as site 1 before, to release its Na^+ ion and to be pulled into juxtaposition with the stator charge, and this movement pushes the occupied site 1 out of the interface into the lipid phase. Accordingly, two rotor sites operate together in a push and pull fashion to achieve rotation of the rotor versus the stator.

To account for the essence of the membrane potential for torque generation (see above), it can be assumed that only part of it drops vertically between the periplasmic channel entrance and its terminus in the middle of the membrane and that between the latter and the terminus of the cytoplasmic access route, the membrane potential drops in a horizontal direction. This horizontal potential causes the stator charge to orient toward the incoming rotor sites and the charged rotor site to become attracted to the periplasmic entrance channel. Hence, the membrane potential acts as the main kinetic driving force for unidirectional rotation and ATP synthesis.

The Na^+ and the H^+ motors are distinct in their use of the ion release pathway, which is thought to be located in subunit a in the proton motor, whereas evidence now suggests the presence of an ion release pathway in the c ring of the sodium motor. The other main difference is in the energy source that drives each F_0 motor out of the relaxed, idling mode into unidirectional rotation to produce torque. Apart from these differences, both models are similar. Each motor operating in the direction of ATP synthesis performs a molecular cycle, in which a specific binding site on the c ring captures a coupling ion from the side with high electrochemical potential and releases it to the side

with low electrochemical potential. As loading and unloading of the binding site can only be accomplished at defined positions at the subunit a/c interface, ion translocation is closely connected to the rotation of the c ring. Hence, the mechanical rotation is linked to the ion translocation events in the F_0 motor components, and is transmitted through the camshaft-like rotating central stalk into the ATP synthesizing F_1 motor.

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