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ATP Synthases With Novel Rotor Subunits: New Insights into Structure, Function and Evolution of ATPases

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ATPases with unusual membrane-embedded rotor subunits were found in both F_1F_0 and A_1A_0 ATP synthases. The rotor subunit *c* of A_1A_0 ATPases is, in most cases, similar to subunit *c* from F_0. Surprisingly, multiplied *c* subunits with four, six, or even 26 transmembrane spans have been found in some archaea and these multiplication events were sometimes accompanied by loss of the ion-translocating group. Nevertheless, these enzymes are still active as ATP synthases. A duplicated *c* subunit with only one ion-translocating group was found along with "normal" F_0 *c* subunits in the Na^+ F_1F_0 ATP synthase of the bacterium *Acetobacterium woodii*. These extraordinary features and exceptional structural and functional variability in the rotor of ATP synthases may have arisen as an adaptation to different cellular needs and the extreme physicochemical conditions in the early history of life.

KEY WORDS: Archaea; A_1A_0 ATPase; *Acetobacterium*; Na^+ F_1F_0 ATPase; rotor diversity; ion specificity.

STRUCTURE OF A_1A_0 ATPases

Ever since archaea have been studied, their ability to thrive in extremely hot, acidic, alkaline or saline environments has attracted much attention. These harsh conditions require special cellular adaptation mechanisms that confer stability on proteins at temperatures at or above 100°C, pHs at around 1 or 12 and salt concentrations up to 5 M salt. In addition, archaea have unique metabolic pathways that are not found in bacteria and often use low energy substrates for living. The principle mechanisms of energy conservation, substrate level phosphorylation and chemiosmosis, also apply to archaea but it is hypothesized that ancient forms of energy conserving mechanisms not found in bacteria or eukarya are present in members of this domain of life due to their phylogenetic position close to the root of the tree of life (Schäfer *et al*., 1999).

The A_1A_0 ATP synthases from archaea are evolutionarily closely related to eukaryal V_1V_0 ATPases but only distantly to F_1F_0 ATPases. However, like F_1F_0 but unlike V_1V_0 ATPases they are reversible enzymes and their physiological function is to synthesize ATP. The enzymes from all three classes of ATPases are rotary machines that are composed of two motors connected by a central stalk. The difference in function between V_1V_0 ATPases and A_1A_0 ATP synthases is based on variations in the membrane-embedded motor, as discussed later.

The enzymes from the strictly anaerobic methanogenic archaea, the methanogens, are hitherto the best-investigated specimens of this unique class of ATPases. The A_1A_0 ATPase has at least nine subunits (A_3:B_3:C:D:E:F:H:a:c_x), but the actual subunit stoichiometry and the cellular localization of most subunits are unknown (Müller *et al*., 1999; Müller and Grüber, 2003). A A_1 subcomplex heterologously produced in *E. coli* made up of the five different subunits A, B, C, D, and F is asymmetric, with a headpiece that is approximately 94 Å long and 92 Å wide and a stalk with a length of approximately 84 Å and 60 Å in diameter (Grüber *et al*., 2001a,b). The complex possesses a pseudo-hexagonal arrangement of six peripheral globular masses, reflecting the major subunits A and B. Subunits C and F are exposed in the complex, whereas subunit D is well protected from trypsin degradation (Grüber *et al*., 2001). The shielding of subunit D from trypsin is an important finding

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since this subunit has been proposed as the structural and functional homolog of the γ subunit of F_1F_0 ATPases (Coskun *et al*., 2002; Grüber *et al*., 2001a,b; Müller *et al*., 1999).

The A_0 domain represents the membrane-embedded motor and contains only two membrane-intrinsic subunits, *a* (stator) and *c* (rotor). Subunit *a* is very similar to subunit *a* of V_1V_0 ATPases with a hydrophilic *N*-terminal and a hydrophobic *C*-terminal domain of apparent molecular masses of ~39 and ~33 kDa, respectively, in *M. mazei* Gö1 (Müller *et al*., 1999). The hydrophilic domain is predicted to be highly α helical and assumed to be the functional homolog of the soluble domain of subunit *b* of F_1F_0 ATPases. The hydrophobic *C*-terminus of subunit *a* is predicted to have seven transmembrane helices and is assumed to be functionally similar to subunit *a* of V_1V_0/F_1F_0 ATPases; however, similarity on amino acid sequence level is below 20%. The arginine essential for ion translocation (*a*Arg-735 in *S. cerevisiae*, (Kawasaki-Nishi *et al*., 2001)) is conserved in subunit *a* (equivalent to Arg-557 of *M. mazei* Gö1); this residue is most likely the essential positive charge on the stator in A_0 (see below). The second subunit of the A_0 domain is subunit *c*, which builds the rotor. Subunit *c* of A_1A_0 ATPases has an extraordinary variability that is discussed in detail in the following section.

Despite several attempts over the years, so far only one A_1A_0 ATPase, from the hyperthermophile *M. jannaschii* (optimal growth temperature 85°C) could be purified without loss of subunits (Lingl *et al*., 2003). The first projected structure of an intact A_1A_0 ATP synthase was determined by electron microscopy of single particles at a resolution of 1.8 nm (Coskun *et al*., 2004). The enzyme has an overall length of 25.9 nm and is organized in an A_1 headpiece (9.4 × 11.5 nm), and a membrane domain A_0 (6.4 × 10.6 nm), which are linked by a central stalk with about 8 nm in length. A part of the central stalk is surrounded by a collar. The collar is connected to the top of the A_1 portion via a peripheral stalk, and in addition, there is a second peripheral stalk that connects the A_0 with the A_1 domain. The A_1 headpiece is made by three copies of an A/B pair, the bottom of the central CDF-stalk domain spans the upper center of the A_0 domain, facilitating the direct contact of the rotary elements, which consists of an ensemble made from the central stalk (CDF-domain) and the membrane-embedded rotor (Coskun *et al*., 2002; Coskun *et al*., 2004; Grüber *et al*., 2001a; Lemker *et al*., 2001; Lemker *et al*., 2003). The second peripheral stalk appears to be connected to the collar domain and goes up to the A_1 headpiece. Likely candidates for this stalk are the remaining hydrophilic subunits H and E. The function of this second stalk and the collar-like structure awaits future biochemical analyses.

STRUCTURE OF *c* SUBUNITS IN A_1A_0 ATPases

c subunits have been purified and characterized from some archaea and in almost every case they were shown to be nearly the same size as *c* subunits from F_1F_0 ATP synthases, i.e. ~8 kDa with two transmembrane helices (Ihara *et al*., 1997; Inatomi *et al*., 1989; Steinert *et al*., 1997; Wilms *et al*., 1996). Furthermore, with the exceptions mentioned below genome sequences predict F_0-like *c* subunits in archaea. This F_0-like size of subunit *c* of A_1A_0 ATPases is the reason for the F_1F_0-like properties of the A_1A_0 ATPases, i.e. their function as ATP synthases.

In contrast and much to our surprise, *Methanothermobacter thermautotrophicus* and *Methanocaldococcus jannaschii* (formerly *Methanococcus jannaschii*) have duplicated and triplicated *c* subunits with two and three hairpin domains, respectively. Apparently, they arose by gene duplication and triplication, respectively, with subsequent fusion of the genes (Ruppert *et al*., 1998; Ruppert *et al*., 1999). In case of *M. thermautotrophicus*, the ion-translocating carboxylate is conserved in helix two and four, but in *M. jannaschii* it is only conserved in helix four and six, in helix two it is substituted by a glutamine residue (Fig. 1). Another triplicated *c* subunit with only two ion-binding sites is predicted from genome sequences to be present in *Methanococcus maripaludis* (Hendrickson *et al*., 2004). The genome sequence of *Methanopyrus kandleri* revealed another extraordinary feature: the A_1A_0 ATPase genes are located in one cluster, but the gene encoding subunit *c* is 13-times the size of the gene encoding the typical F_0-like *c* subunit. The sequence predicts a *c* subunit of 97.5 kDa that comprises 13 covalently linked hairpin domains (Slesarev *et al*., 2002)! These domains have a highly conserved sequence (56–86%), and the active carboxylate is conserved in helix two of every hairpin domain. However, posttranscriptional and posttranslational modifications cannot be excluded and, therefore, the extraordinary size of the proteolipid has to be verified by other means.

Another big surprise was the finding of duplicated *c* subunits in the pyrococci *Pyrococcus furiosus*, *Pyrococcus horikoshii* and *Pyrococcus abyssi* (Robb *et al*., 2001). These *c* subunits have only one ion-binding site and, therefore, are identical to the *c* subunit from a different class of enzymes, the V_1V_0 ATPases (Müller, 2004). This finding of V_0-like *c* subunits in an ATP synthase was very surprising because it was believed that the evolution of the V_0-like *c* subunits would switch the function of the

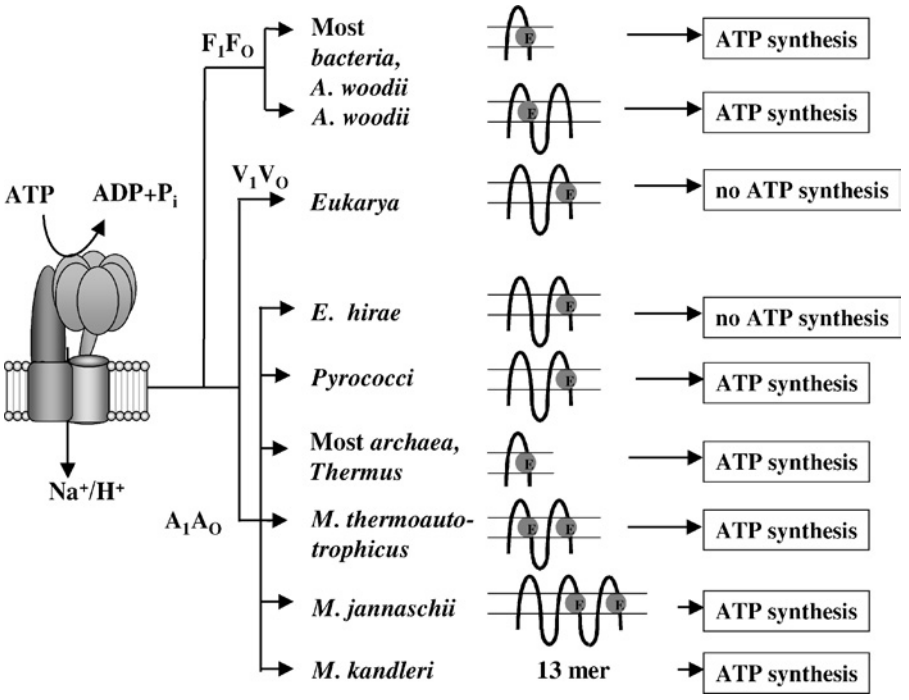

Fig. 1. Diversity of rotor subunits in the three classes of ATPases. *E. hirae, Enterococcus hirae*. The ATPases of *Thermus thermophilus* and *E. hirae* are often referred to as (bacterial or prokaryotic) V_1V_0 ATPases, but phylogenetic analyses demonstrate that they are of archaeal origin and, therefore, should be considered as A_1A_0 ATPases (for discussion, see (Müller and Grüber, 2003)).

enzyme from ATP synthase to ATPase. However, there is indirect experimental evidence that the A_1A_0 ATPase from *Pyrococcus furiosus* acts as an ATP synthase (Sapra *et al*., 2003), despite its V_0-like *c* subunit. How can this apparent discrepancy be explained? Apparently, a third reversal of function, back from ATPase to ATP synthase must have occurred (Cross and Müller, 2004). This could involve the evolution of a second ion-conductiong channel or a rotor with an increased number of monomers. The elucidation of the structural basis for this reversal of function has to await high-resolution structures of A_1A_0 ATPases.

THE ROTOR OF THE F_1F_0 ATPase FROM *ACETOBAC-TERIUM WOODII* CONTAINS F_0-LIKE AND V_0-LIKE *c* SUBUNITS

The anaerobic acetate-forming bacterium *Acetobacterium woodii* contains a Na^+ F_1F_0 ATPase (Reidlinger and Müller, 1994). 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 three *c* subunits (Forster *et al*., 1995; Rahlfs *et al*., 1999). AtpE$_2$ (subunit c_2) and AtpE$_3$ (subunit c_3) are 100% identical at the amino acid level; only 18 base substitutions occur at the DNA level (Rahlfs *et al*., 1999). This pattern is strong evidence for a duplication of an ancestral gene. The deduced molecular mass of the subunits c_2 and c_3 is 8.18 kDa and they are very similar to "F_0-like" *c* subunits. 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 at the DNA level, indicating a duplication of a precursor and subsequent fusion of the two gene copies. The deduced molecular mass of subunit c_1 is 18.37 kDa with four predicted transmembrane helices arranged in two hairpins but like the *c* subunit from V_1V_0 ATPases it contains only one ion-translocating residue. The purified Na^+ F_1F_0 ATPase contains both types of *c* subunits and is, therefore, the first ATP synthase found in nature that has mixture of "F_0-like" 8- and "V_0-like" *c* subunits (Aufurth *et al*., 2000). The stoichiometry of the different *c* subunits in the rotor has not yet been determined.

What might be the selective advantage of having F_0 and V_0 rotor subunits in an ATPase? It is reasonable to assume that an organism could, depending on its cellular needs, alter the function of the ATPase between

ATP synthesis and ATP hydrolysis by varying the number of ion-translocating residues per rotor. V_0-like *c* subunits favor ATP hydrolysis, F_0-like ATP synthesis. *A. woodii* could produce a Na^+ F_1F_0 ATPase with only "F_0-like" *c* subunits during autotrophic growth on $H_2 + CO_2$, this life style does not include substrate-level phosphorylation and relies solely on chemiosmosis. In contrast, by incorporation of more c_1 subunits, the F_1F_0 ATPase could work, like the V_1V_0 ATPase, as an efficient, ATP-driven ion pump during fermentation. This would not only generate a transmembrane ion potential but more important, an effective means for pH regulation in this acid-producing anaerobe. Proton pumping could be achieved by the F_1F_0 ATPases that can also translocate protons or by a secondary Na^+/H^+ antiporter (Müller *et al*., 2001; Müller, 2003). Testing the hypothesis of a substrate-dependent regulation of the Na^+ F_1F_0 ATPase of *A. woodii* is a challenging task for future experiments.

EVOLUTION OF ATPases

The ATPases arose from a common ancestor that underwent structural and functional changes leading to three distinct classes of enzymes present in the three domains of life, the F_1F_0, V_1V_0, and A_1A_0 ATPases (Hilario and Gogarten, 1998; Müller *et al*., 1999). A_1A_0 ATPases combine features of both, the F_1F_0 and V_1V_0, and were considered for some time to be V_1V_0 ATPases. This was based on the fact that subunits A and B are more similar to V_1V_0 than to F_1F_0 (Schäfer and Meyering-Vos, 1992). Of course, this is an insufficient criterium for classification and, therefore not surprisingly, phylogenetic analyses demonstrate that archaeal ATPases form a distinct group (Hilario and Gogarten, 1998). In addition to phylogenetics, functional differences also apply. The A_1A_0 ATPases are relative insensitive to bafilomycin (Becher and Müller, 1994). V_1V_0 ATPases are regulated by a substrate dependent assembly/disassembly of V_1 and V_0 that renders V_1 inactive and involves subunits C and H (Kane and Smardon, 2003). This has so far not been observed in A_1A_0, they lack homologues of subunits C and H of V_1V_0 ATPases, and in sharp contrast to V_1 the isolated A_1 domain is catalytically active (Fig. 2).

It was always believed that the diversion of F_1F_0 ATP synthases and V_1V_0 ATPases took place by evolution of the V_0-like proteolipid (Nelson and Taiz, 1989).

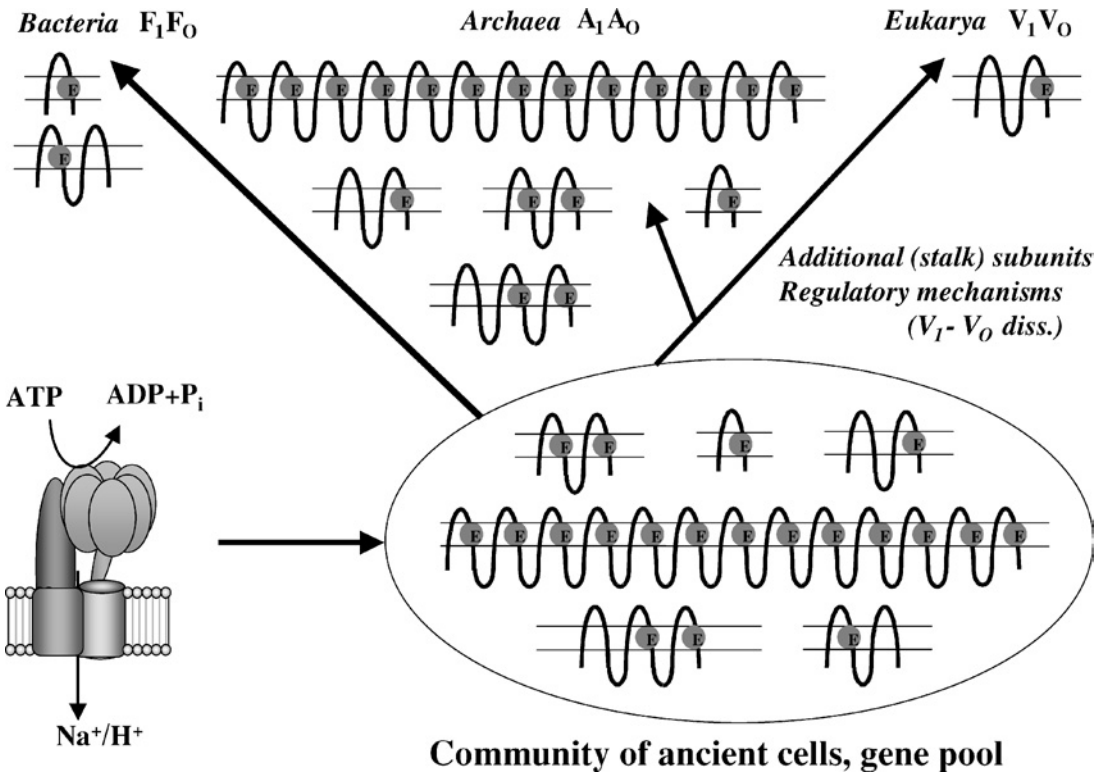

Fig. 2. Evolution of ATPases. For explanations, see text.

It is apparent that the rotor subunits seen today in the three classes of ATPases were already invented in early evolution and are still present in members of the archaea. The different rotor subunits seen today in A_1A_0, F_1F_0 and V_1V_0 were already present in early history of life. F_1F_0 and V_1V_0 ATPases took a distinct *c* subunit out of the already present genes in the universal gene pool as an adaptation of their enzymes to either chemiosmostic energy generation or membrane energization by ATP hydrolysis, and the evolution of F_1F_0 and V_1V_0 ATPases involved invention of additional subunits that, for example, allowed regulation of enzyme activity.

Two different driving forces may be responsible for the diversity of rotors present in A_1A_0 ATPases. Life at high temperatures with energy-limited substrates may have driven the formation of rotors with fewer monomers per rotor, i.e. more covalently linked rotor domains. Decreasing temperatures eliminated the need for monomeric rotors and led to the generation of multimeric rotors with the number of monomers per ring increasing with decreasing temperatures. Multimeric rotors have the advantage that the coupling efficiency and thus the physiological function of the ATPases may be changed by changing the number of (different) *c*-subunits in the rotor. That such a layer of regulation of ATPases exists is supported by some experiments (Schemidt *et al*., 1998) and might also be true for the Na^+ F_1F_0 ATP synthase from *A. woodii* with its different F- and V-type *c* subunits. However, its elucidation is still a challenging task for future studies.

FUTURE PERSPECTIVES

The rotor subunits of some A_1A_0 ATPases as well as the Na^+ F_1F_0 ATP synthase of *A. woodii* have exceptional features and predict rotors with unusual subunit composition. For *A. woodii*, determining the actual subunit composition of the rotor and testing the hypothesis of a substrate-dependent regulation of the Na^+ F_1F_0 ATPase is of prime interest and can now be addressed. In archaea, these rotors should lock the enzyme in an ATP hydrolysis mode, but apparently, they still work as ATP synthases. Structural analyses must be used to verify or falsify the predicted rotor compositions and to determine their structures. As the first complete A_1A_0 ATPase (from *M. jannaschii*) is now available, it should be possible to analyze its structure and especially the structure of its membrane-embedded motor. Moreover, functional analyses in a reconstituted system are now feasible. This will open a new road to structure/function analyses of A_1A_0 ATPases.

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