A c Subunit with Four Transmembrane Helices and One Ion (Na\(^+\))-binding Site in an Archaeal ATP Synthase

**IMPLICATIONS FOR c RING FUNCTION AND STRUCTURE**

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**Background:** The ATP synthase of *Pyrococcus* has an unusual gene encoding rotor subunit c.

**Results:** The c ring is made of protomers with one ion-binding site in four transmembrane helices and is highly Na\(^+\)-specific.

**Conclusion:** Unprecedented subunit c topology and ion configuration in an ATP synthase.

**Significance:** Archaeal ATP synthases are a remnant of primordial bioenergetics.

The ion-driven membrane rotors of ATP synthases consist of multiple copies of subunit c, forming a closed ring. Subunit c typically comprises two transmembrane helices, and the c ring features an ion-binding site in between each pair of adjacent subunits. Here, we use experimental and computational methods to study the structure and specificity of an archaeal c subunit more akin to those of V-type ATPases, namely that from *Pyrococcus furiosus*. The c subunit was purified by chloroform/methanol extraction and determined to be 15.8 kDa with four predicted transmembrane helices. However, labeling with DCCD as well as Na\(^+\)-DCCD competition experiments revealed only one binding site for DCCD and Na\(^+\), indicating that the mature c subunit of this A1A0 ATP synthase is indeed of the V-type. A structural model generated computationally revealed one Na\(^+\)-binding site within each of the c subunits, mediated by a conserved glutamate side chain alongside other coordinating groups. An intriguing second glutamate located in-between adjacent c subunits was ruled out as a functional Na\(^+\)-binding site. Molecular dynamics simulations indicate that the c ring of *P. furiosus* is highly Na\(^+\)-specific under in vivo conditions, comparable with the Na\(^+\)-dependent \(V_1V_0\) ATPase from *Enterococcus hirae*. Interestingly, the same holds true for the c ring from the methanogenic archaeon *Methanobrevibacter ruminantium*, whose c subunits also feature a V-type architecture but carry two Na\(^+\)-binding sites instead. These findings are discussed in light of their physiological relevance and with respect to the mode of ion coupling in A1A0 ATP synthases.

Archaea produce ATP using an ATP synthase that is distinct from the well-known F\(_1\)F\(_0\) ATP synthase found in bacteria, mitochondria, and chloroplasts (1). Archaeal A1A0 ATP synthases are evolutionary more closely related to vascular V\(_1\)V\(_0\) ATPases, notwithstanding the fact that these act as ATP-driven ion pumps and are therefore functionally different (2–4). Like F-ATP synthases and V-ATPases, A-ATP synthases comprise a membrane motor, A\(_0\), which is driven by downhill translocation of H\(^+\) or Na\(^+\), and a soluble domain, A\(_1\), where ATP is synthesized from ADP and P\(_i\). A\(_1\) and A\(_0\) are mechanically coupled by three protein stalks: one central and two peripheral. Under suitable conditions A\(_1\) can also hydrolyze ATP and function as a motor for uphill ion translocation across A\(_0\) (2, 5, 6).

The membrane-bound A\(_0\) motor contains subunits a and c (2, 7). Subunit c consists of at least two transmembrane helices and is expressed in multiple copies, which form a ring-like structure that, like the F\(_0\) motor (8), functions as a rotating turbine driven by the movement of ions across the membrane. In most A-type ATP synthases, subunit c has a single-hairpin topology as seen in F-type ATP synthases. By contrast, in V-type ATPases (9, 10) the c subunit apparently underwent gene duplication, resulting in a protein with four transmembrane helices (11). Moreover, one ion-binding site was lost during the duplication event leading to a rotor with only half the number of ion-binding sites. These missing binding sites have been seen as the reason for the inability of V-ATPases to act as ATP synthases. Instead, the rotor favors generation of large ion gradients, a function important for the cellular physiology of eukaryotes (3, 4).

In recent years, however, the determination of the genome sequences of several archaea have revealed an unexpected feature of A1A0 ATP synthases: the gene encoding for subunit c underwent duplication (12, 13), triplication (14), and even greater multiplication, so far up to 13-fold (15–17). Moreover, in some species the sequence motif characteristic of the ion-binding site is absent in one hairpin, which would result in c subunits with one ion-binding site within four transmembrane helices or two within six transmembrane helices (10, 18). In particular, the DNA data for *Pyrococcus furiosus* implies that its
c subunit has a typical V-type topology with two hairpins but only one Na\(^+\)-binding site per subunit (10, 19). The implication, according to common wisdom, would be that the enzyme lost its function as an ATP synthase. However, the A\(_1\)A\(_0\) ATP synthase from \textit{P. furiosus} is the only ATP synthase encoded in the genome and functions as an ATP synthase in \textit{vivo} (19, 20).

The structural rationale for the ATP synthesis activity of the \textit{P. furiosus} enzyme, despite the predicted V-type c subunit, is unknown. It could involve post-transcriptional modifications as well as additional, yet hidden ion-binding sites in the mature protein. Because the primary structure and number of ion-binding sites are assumed based on predictions from the DNA sequence, it was important to isolate the mature c subunit and determine experimentally its primary structure, molecular mass, and ion-binding sites. These studies culminate in a three-dimensional computational model of the c ring from \textit{P. furiosus}, which provides a structural interpretation for our biochemical experiments, and enables us to assess the physiological ion specificity of this and other archaeal A\(_1\)A\(_0\) ATP synthases.

### EXPERIMENTAL PROCEDURES

**Strain and Cultivation Condition—** \textit{P. furiosus} DSM 3638 was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany) and was grown anaerobically in a 300 liter enamel-coated fermenter at 98 °C in medium without sulfur, but with yeast extract, starch, and pepton as energy source and N\(_2\)/CO\(_2\) (80:20, v/v) as described before (21). The cells were harvested and stored at −80 °C until further use.

**Membrane Preparation and Protein Determination—** 20–40 g of \textit{P. furiosus} cells (wet weight) were resuspended in buffer A (25 mM Tris, pH 7.5, 5 mM MgCl\(_2\), 0.1 mM PMSF) containing 0.1 mg DNase I/ml. The cells were homogenized and disrupted by three passages through a French pressure cell (Aminco) at 3000 p.s.i. Cell debris and the thermosome, a cytoplasmatic protein solubilization. Triton X-100 was added to a concentration of 3% (v/v) (1 g of Triton X-100/g of membrane protein), and membranes were incubated for 2 h at 40 °C and then over-night at room temperature under shaking. The membranes were collected by ultracentrifugation (Beckman Optima L90-K, TFT 65.13 rotor; 42,000 rpm for 2 h at 4 °C), and contaminating proteins were precipitated with PEG 6000 (4.1%, w/w) for 30 min at 4 °C. The precipitated proteins were removed by centrifugation (Beckman Optima L90-K, TFT 65.13 rotor; 38,000 rpm for 2 h at 4 °C), and the supernatant was loaded onto a sucrose gradient (20–66%) and centrifuged for 19 h in a vertical rotor (Beckman Optima L90-K, VTI50 rotor; 43,000 rpm at 4 °C). ATP hydrolysis activity of each sucrose gradient fraction was tested as described before (21). Fractions with the highest ATPase activity were pooled and applied to anion exchange chromatography using DEAE-Sepharose, which was equilibrated with buffer D (50 mM Tris, pH 7.5, 5 mM MgCl\(_2\), 10% glycerol, 0.1 mM PMSF, 0.1% (v/v) reduced Triton X-100). A salt gradient (0–1 M NaCl) in buffer D was used for protein elution at a flow rate of 0.5 ml/min. Fractions with the highest ATPase activity were pooled, concentrated (molecular mass cutoff, 100 kDa), and applied to gel filtration using a Superose 6 column (10/300 GL; GE Healthcare). Gel filtration was performed in buffer E (50 mM Tris, pH 7.5, 5 mM MgCl\(_2\), 10% glycerol, 0.1 mM PMSF, 0.05% n-dodecyl-β-D-maltoside) at a flow rate of 0.2 ml/min. Again, fractions with the highest ATP hydrolysis activity were pooled.

**Chloroform/Methanol Extraction of Subunit c of Membranes from \textit{P. furiosus}—** The membranes resuspended in buffer C were mixed with 20 volumes of chloroform/methanol (2:1, v/v) for 20 h at 4 °C and filtered. 0.2 volume of H\(_2\)O was added to the filtrate and mixed for another 20 h at 4 °C. The organic phase was separated from the aqueous and interphase using a separation funnel and was washed twice with 0.5 volume of chloroform/methanol/H\(_2\)O (3:47:48, v/v/v). The washed organic phase was filled up with 1 volume of chloroform. Methanol was added until the turbid solution cleared up. The volume of the solution was reduced to 1 ml using vacuum evaporation. Protein was precipitated with 4 volumes of diethyl ether at −20 °C for 12 h and sedimented by centrifugation (Eppendorf 5417R, FA-45–24-11 rotor; 8,000 rpm at −8 °C). Sedimented protein was resolved in 1 ml of chloroform/methanol (2:1, v/v).

**N,N’-Dicyclohexylcarbodiimide Labeling Experiments—** For labeling experiments with N,N’-dicyclohexylcarbodiimide (DCCD, dissolved in ethanol),\(^3\) purified A\(_1\)A\(_0\) ATP synthase was used. 1 ml of ATP synthase was dialyzed in a dialysis tube (molecular mass cutoff, 3.5 kDa) against 1000 ml of buffer F (25 mM Tris, 25 mM MES, 5 mM MgCl\(_2\), 10% glycerol) adjusted to pH 5.5, 6.0, or 6.5 with HCl or KOH at 12 h at 4 °C. 20 µl of ATP synthase (9 µg of protein) was incubated with 250 or 500 µM DCCD at pH levels of 5.5, 6.0, or 6.5 for 60 min at room temperature. For competition experiments between DCCD and NaCl or KCl, the salts were added to the ATP synthase solution in concentrations of 1.25, 2.5, 5, or 25 mM, directly before labeling. After labeling with DCCD, the ATP synthase was purified using C\(_4\) Zip Tips to remove excessive DCCD and salts. The C\(_4\) matrix (bed volume, 0.6 µl) of a 10-µl Zip Tip was first equilibrated with 20 µl of 100% acetonitrile and 20 µl of 0.1% trifluoroacetic acid. ATP synthase was coupled to the equilibrated matrix and washed with 30 µl of 0.1% trifluoroacetic acid. The A\(_1\)A\(_0\) ATP synthase was eluted with 10 µl of 90% acetonitrile in 0.1% trifluoroacetic acid. To desintegrate the ATP synthase and the c ring of \textit{P. furiosus} into c monomers, 10 µl of chloroform/methanol (2:1, v/v) was added and mixed.

\(^3\) The abbreviations used are: DCCD, N,N’-dicyclohexylcarbodiimide; FEP, free energy perturbation; CHES, 2-(cyclohexylamino)ethanesulfonic acid.
The solution containing the c monomers were dried by vacuum evaporation for 1.5 h at room temperature. The dried protein pellet was mixed with 1 μl of 2,5-dihydroxycetophenone matrix and applied to MALDI-TOF-MS as described below.

**MALDI-TOF-MS Measurements**—Chloroform/methanol extracts for protein m/z determination were mixed in a 1:1 (v/v) ratio with matrix 2,5-dihydroxycetophenone (15 mg/ml 2,5-dihydroxycetophenone in 75% ethanol in 20 mM sodium citrate; Bruker Daltonics) or 2,5-dihydroxybenzoic acid (30 mg of 2,5-dihydroxybenzoic acid/100 μl of TA solution (0.1% trifluoroacetic acid/acetonitrile, 1:2 (v/v); Bruker Daltonics)) and spotted on ground steel target plates (Bruker Daltonics). MALDI mass spectra were recorded in a mass range of 5–20 kDa using a Bruker Autoflex III Smartbeam mass spectrometer. Detection was optimized for m/z values between 5 and 20 kDa and calibrated using calibration standards (protein molecular weight calibration standard 1; Bruker Daltonics).

**Protein Identification and Quantification Using Mass Spectrometry (Peptide Mass Fingerprinting)**—Chloroform/methanol extracts of *P. furiosus* membranes were mixed in a 1:1 (v/v) ratio with 20 mM MES, pH 5.5, containing 0.5% n-octyl-β-D-glucopyranoside, and the organic phase was removed by a gentle N2 stream until the turbid solution was getting clear. The protein extract was then submitted to 12.5% SDS-PAGE (22) and stained with silver, suitable for mass spectrometry (23). Bands of interest were excised, reduced, alkylated, and digested using trypsin, chymotrypsin or both proteases according to trypsin/chymotrypsin, however, carries one Na+ coordinated by Glu-140 in each c subunit and another coordinated by Glu-59 in between adjacent c subunits. The protein/membrane systems were equilibrated using constrained all-atom molecular dynamics simulations. The strength of the constraints on the protein were gradually weakened over 12 ns for the c10 rings and 7 ns for the c3 construct. Subsequently, unconstrained simulations were carried out for 40 and 10 ns, respectively. The conformations obtained after the constrained equilibrations were used as input of all-atom free energy perturbation (FEP) calculations of the exchange between Na+ and H+ in each ring and vice versa. The FEP calculations were performed in the forward and backward direction, in 32 intermediate steps; each of these steps consists of 500 ps of sampling time, including 100 ps of equilibration. Both molecular dynamics and FEP calculations were carried out with NAMD2.7 (38) using the CHARMM27 force field for proteins and lipids (39, 40). All simulations were at constant pressure (1 atmosphere) and temperature (298 K), and with periodic boundary conditions in all directions. The dimensions of the simulation box in the plane of the membrane (150 × 150 Å for the c10 rings and 72 × 96 Å for the c3 construct) were kept constant. The particle mesh Ewald method was used to compute the electrostatic interactions, with a real space cutoff of 12 Å. A cutoff of 12 Å was also used for van der Waals interactions, computed with a 6–12 Lennard-Jones potential. During the molecular dynamics and FEP simulations of the *M. ruminantium* c3 construct, the conformations of the first (residues 6–77) and last hairpins (residues 86–161) were preserved using a weak harmonic restraint on the root mean square deviation of the backbone, relative to the initial model.
RESULTS

Purification of Subunit c and Mass Determination—Subunit c of ATP synthases/ATPases is a very hydrophobic protein that can be isolated from membranes using organic solvents such as chloroform/methanol (12, 41, 42). Membranes of *P. furiosus* were thus extracted by chloroform/methanol, and the extract was applied to an SDS gel. As can be seen in Fig. 1, this procedure yielded two bands with apparent molecular masses of 16 and 10 kDa. To identify these proteins, peptide mass fingerprinting was used, and their molecular masses were determined by MALDI-TOF-MS. Analysis of the 10-kDa band showed that it actually consists of two proteins. One is the subunit K of the RNA polymerase (PF1642) with an apparent molecular mass of 6,269 Da, and the other is subunit F of a putative monocation/H\(^+\)/H\(^+/H\(^+\) antiporter (PF1452) with an apparent mass of 9,076 Da. The 16-kDa band in the SDS gel was identical to subunit c of the A\(_{1}\)A\(_{O}\) ATP synthase (PF0178). Its apparent molecular mass was 15,853 Da, which matches almost exactly the mass deduced from the genome sequence (M\(_r\) \(\approx\) 15,806). The difference in the molecular mass, of around 50 Da, is likely caused by a low signal intensity of the peak and multiple nonresolved oxidations of subunit c. Nevertheless, this is evidence that the mature c subunit of *P. furiosus* is indeed a duplication of the “classical” 8-kDa c subunit of F-type ATP synthases.

Validation of the Amino Acid Sequence Predicted from DNA Data—Peptide mass fingerprinting was used to verify the predicted sequence of the *P. furiosus* subunit c (Fig. 2). Subunit c was digested by trypsin, chymotrypsin, and a combination of both, and the fragments were analyzed by electrospray ionization-MS. The sequence coverage was 78.6%, and only one large fragment, from Ser-109 to Phe-131, was not resolved. The experimental data not only verified the predicted start codon but also unequivocally confirmed the predicted amino acid sequence. As will be discussed later, the absence of a glutamine at position 26 (replaced by valine) and a glutamate at position 55 (replaced by methionine) are particularly noteworthy. Of special interest is the presence of a second glutamate at position 51.

Quantitative DCCD Labeling Indicates That Each c Subunit Carries a Single Na\(^+\) Site—DCCD inhibits ATP synthases/ATPases by covalently binding to a key carboxylate side chain found in the ion-binding sites in the c subunit. In H\(^+/H\(^+\)-driven ATP synthases, this carboxylate is the site of H\(^+/H\(^+\) binding, through protonation (43). In Na\(^+/H\(^+\)-coupled c subunits, this side chain can also be protonated in the absence of Na\(^+\), but otherwise it is deprotonated and coordinates the Na\(^+\) directly (30, 44, 45). DCCD reacts with this carboxylate side chain only in its protonated state; therefore, in Na\(^+/H\(^+\)-driven c subunits, DCCD and Na\(^+\) compete for this common binding site, in a manner that is pH-dependent (46, 47). Thus, a DCCD labeling assay can in principle be used to quantify the number of ion-binding sites in the c subunit, as well as to reveal whether or not they bind Na\(^+\).

We first measured DCCD labeling to individual c subunits extracted with chloroform/methanol from *P. furiosus* membranes. The c subunits were transferred from the organic phase to a water phase with different pH levels of 5.5, 7.0, and 10.0 (25 mM MES, pH 5.5, Tris, pH 7.0, CHES, pH 10.0, containing 1% n-octyl-\(\beta\)-D-glucopyranoside) by mixing both phases and removing the chloroform/methanol by a N\(_2\) stream, because the DCCD labeling reaction does not proceed readily in this organic solvent. After the addition of 500 \(\mu\)M DCCD, samples (1...
μl of sample mixed with 1 μl of 2,5-dihydroxybenzoic acid) were taken at 0, 30, 60, 90, and 150 min and examined with MALDI-TOF-MS. This analysis revealed one DCCD molecule bound to each c subunit, as is evident from the increase in molecular mass by 206 Da, which corresponds to one molecule of DCCD. DCCD labeling was time-dependent (34% after 30 min, 46% after 60 min, and 53% after 90 min) and dependent on pH. Labeling was only observed at pH 5.5 and not at pH 7.0 or 10.0.

Unfortunately, DCCD labeling of c subunits isolated by chloroform/methanol was not protected by NaCl, even when smaller amounts of DCCD (50 or 100 μM) were used. This is likely due to the partial unfolding of subunit c as a result of the harsh purification procedure in chloroform/methanol. Instead, we labeled the purified A1AO ATP synthase with DCCD, and the c subunit was isolated by chloroform/methanol afterwards. Upon incubation of the enzyme with DCCD, the molecular mass of subunit c increased from 15,803 to 16,010 Da (for the unoxidized protein), from 15,818 to 16,026 Da (for the one time oxidized protein), and from 15,835 to 16,042 Da (for the two times oxidized protein), indicating again that one c subunit had bound one DCCD molecule (Fig. 3). The extent of DCCD labeling was clearly dependent on the pH used (Fig. 4). The labeling efficiency with 500 μM DCCD after 60 min was roughly twice that observed with 250 μM, for the same pH. For the same DCCD concentration, the labeling efficiency at pH 6.5 was one-fourth of that at pH 5.5. Again, only one DCCD-reactive site was identified. Crucially, DCCD labeling was prevented by Na⁺, but not K⁺ (Fig. 5). The competing effect of Na⁺ on DCCD modification was clearly pH-dependent: the higher the pH, the less Na⁺ was required to prevent DCCD labeling.

**Structural Model of the c Ring of P. furiosus with Its Na⁺-binding Sites**—After the primary structure predicted from the DNA sequence had been verified, we generated a structural model of the c ring of P. furiosus. The model is based on the structure of the c ring from E. hirae, which also consists of V-type c subunits. Supplemental Fig. S1 shows the alignment of the c subunit sequences from the V-type ATPase from E. hirae and the A-type ATP synthase from P. furiosus. The known secondary structure and the transmembrane spans (TM1 to TM4) of the E. hirae c subunit match well those predicted for the P. furiosus sequence. Furthermore, given that laser-induced liquid beam ion desorption-mass spectroscopy (LILBID-MS) data suggest that the c ring of P. furiosus assembles as decamer (7), it is reasonable to employ the crystallographic structure of the c₁₀ rotor from E. hirae (30) as a template to model the archaeal ring.
**c Ring of AₐAₒ ATP Synthases**

As explained under “Experimental Procedures,” we produced a large ensemble of tentative models and ranked them according to two independent scoring functions, namely DOPE and GA341. Among these, we selected the two models with top ranks according to either score, plus a third one that was also highly ranked in both scoring schemes. For all of them, the GA341 score was >0.8 (the closer to 1, the better is the model). All these three models are highly similar in their transmembrane region (Co-trace root mean square deviation, ~1.2 Å) but differ elsewhere (root mean square deviation, ~9 Å). Mostly they vary in the long loop that connects the second and third transmembrane spans, where there is a gap in the target-template alignment. Importantly, the ion-binding sites, clearly located within the membrane domain, do not vary significantly in the different models. In sum, given the high confidence in the sequence alignment, the quality of the template structure, and the convergence in the calculations toward a unique prediction, we expect these models of the *P. furiosus* c ring to be very realistic, particularly in the transmembrane domain.

One of these equivalent models is depicted in Fig. 6. The model is perfectly consistent with the notion that ATP synthesis in this archaeon is driven by Na⁺ gradients. The ion-binding sites are located within each c subunit, flanked by TM2 and TM4 (Fig. 6B). The Na⁺ is coordinated by the side chains of Glu-142 (TM4), Gln-113 (TM3), Thr-56 (TM2), and Gln-57 (TM2) and by the backbone of Leu-53 (TM2). In addition, the side chain of Tyr-60 (TM2) forms a hydrogen bond with Glu-142 and contributes to stabilize the geometry of the ion coordination shell. This network of interactions is identical to that revealed by the crystal structure of the c₁₀ rotor from the *E. hirae* V-type ATPase, which has been established to function as a Na⁺ pump under physiological conditions (48).

As mentioned, the c subunit from the *P. furiosus* ATP synthase also resembles that from the *E. hirae* ATPase in that it consists of four transmembrane helices. It is therefore reasonable to ask whether Na⁺-binding sites may be found not only within each c subunit, but also in between them, as occurs in rotor rings whose c subunits have a two-helix topology (49). Our structural model suggests that this is highly unlikely, because this region is markedly hydrophobic, namely Val-26 (TM1), Leu-48 (TM2), Met-55 (TM2), and Met-140 (TM4) (Fig. 6C). Such an environment could not possibly counter the cost of dehydration incurred upon Na⁺ binding within the membrane domain. Consistently, the analogous location in the crystal structure of the *E. hirae* rotor lacks a bound Na⁺; in that structure, all of these hydrophobic residues are conserved, except for Met-55, which is substituted by Gly-63.

**Molecular Dynamics Simulations of the P. furiosus c Ring in the Membrane**—To further assess the verisimilitude of the c₁₀ model of the *P. furiosus* c ring, we carried out a molecular dynamics simulation of this model embedded in a phospholipid membrane and compared the outcome with an analogous simulation of the c ring from the *E. hirae* ATPase (Fig. 7A). The rationale here is that if the model is a realistic approximation of...
the actual structure, its behavior in simulation ought to be comparable with that of an experimentally determined structure. What we observe is that the dynamical range of the individual c subunits is essentially identical when comparing the model from *P. furiosus* and the structure from *E. hirae* (Fig. 7B). Likewise, the structure and dynamics of the Na\(^+\) -binding sites in both c rings are largely undistinguishable (Fig. 7C). These results indicate that the internal structure of the c subunits in the *P. furiosus* model is indeed very plausible. The relative orientation of the c subunits in the initial model of the ring, however, seems to be somewhat suboptimal. In the first half of the simulation, the structure of the ring as a whole departs from the starting model more than the c ring from *E. hirae* does, i.e., more than the magnitude of the natural room temperature fluctuations. Nevertheless, also this overall structural arrangement becomes stable in the second half of the simulation (Fig. 7B).

A Second, Constitutively Protonated Glutamate within the Membrane Domain—A noteworthy feature of the *P. furiosus* sequence is the presence of a second glutamate side chain in TM2/H11032 (Glu-51), one helix turn toward the cytoplasmic side of the rotor (Fig. 6C). Could this be a second ion-binding site? Our model suggests that this side chain is constitutively protonated and that it contributes to the stability of the interface between adjacent c subunits in the assembled ring, by forming a hydrogen bond with the carbonyl group of residue Phe-137 (in TM4). Consistently, this nonconserved side chain is replaced by glutamine in homologous sequences, for example in *E. hirae*. Indeed, in the crystal structure of the *E. hirae* rotor, this glutamine side chain is seen to form the same interaction, across from TM2 to TM4 of the adjacent c subunit. Therefore we hypothesize that protonation of Glu-51 is structurally important, but not functionally relevant.
be transiently protonated at low Na⁺ concentrations, rather than the modification of Glu-51. Indeed, as shown in Fig. 8, DCCD modification is structurally viable in the case of Glu-142, upon an outwards rotation of the carboxylate group. This minor but necessary rearrangement is essentially identical to that seen in crystal structures of DCCD-modified c rings (50, 51). In the case of Glu-51, however, we find that DCCD modification would be sterically impossible in all rotamers of the side chain (in X₀, X₁, and X₂). Thus, Glu-51 cannot be modified in the context of the assembled rotor. Consistent with this interpretation, increasing concentrations of Na⁺ inhibit DCCD labeling of the rotor (Fig. 5), because Na⁺ binding precludes protonation of Glu-142 (but not Glu-51).

P. furiosus Is Not the Only Archaeon That Has a 16-kDa c Subunit with Only One Na⁺-binding Site—An alignment of all c subunit sequences available for archaea (supplemental Fig. S2) indicates that c subunits with four transmembrane helices are found in Crenarchaeota and Euryarchaeota, but not in Korarchaeaota and Thaumarchaeota. Pyrococci and Thermococci are the only archaea of the Euryarchaeota with a c subunit containing four transmembrane helices and a single Na⁺-binding site between TM2 and TM4 of the same subunit, like P. furiosus. In the Crenarchaeota phylum, the Desulfurococcaceae and Staphylothermus species, as well as Ignisphaera aggregans, also feature a duplicated c subunit with a single Na⁺-binding site. Interestingly, among the archaeal c subunits with four transmembrane helices, only those from methanogens contain two Na⁺-binding sites per c subunit. Methanobrevibacter, Methanothermobacter, and Methanobacterium species, as well as Methanospirillum stadtmanae, feature a binding site analogous to that in P. furiosus, i.e., formed within the c subunit, and a second one, identical in its amino acid composition, which would appear between adjacent c subunits in the assembled ring, i.e., mediated by a glutamate in TM2, a glutamine in TM1 and the prototypic set of additional coordinating groups in TM3’ and TM4’. A close-up view of the structure of these two binding sites, derived from simulations of a homology model of the M. ruminantium c ring is shown in Fig. 9 (A and B) (see also supplemental Fig. S3).

The Rings of E. hirae, P. furiosus, and M. ruminantium Have Equivalent Na⁺ Specificity—Although most ATP synthases are driven by transmembrane gradients of either protons or Na⁺, recent studies of the methanogenic archaeon Methanosarcina acetivorans have revealed that its c ring is coupled to both gradients, i.e., its c subunit is effectively nonspecific under typical in vivo concentrations of H⁺ and Na⁺ (52). This is to say that the ion-binding sites in the M. acetivorans c ring are sufficiently H⁺ selective to counter the large physiological excess of Na⁺ over H⁺, but not so much as to preclude Na⁺ binding altogether. Because methanogenesis in this cytochrome-containing organism is coupled to primary Na⁺ and H⁺ translocation, the ability of M. acetivorans ATP synthase to use both seems to be a very efficient bioenergetic adaptation. However, the generality of this solution is unclear. It has been suggested that the c ring from M. ruminantium might also be able to utilize both gradients (53), but this methanogen does not have cytochromes and therefore does not have a primary proton but only a Na⁺ gradient generated by the methyltetrahydrodihydroxypterin-coenzyme M methyltransferase, and thus its ATP synthase should be Na⁺-specific.

To clarify this question, we used molecular dynamics simulations to compute the free energy of selectivity for H⁺ over Na⁺ of the binding sites in the c rings of P. furiosus and M. ruminantium, relative to the selectivity of the c ring of the Na⁺-pumping ATPase from E. hirae (Fig. 9C). From this analysis, we conclude that the ion specificity of the c rings in these three species is largely identical, consistent with the similarity in the amino acid make-up of their ion-binding sites. That is, the M. ruminantium ATP synthase is very likely to be coupled exclusively by Na⁺ under in vivo conditions. The H⁺ selectivity of the M. acetivorans c ring is, by contrast, much more pronounced. As mentioned, this enables this ATP synthase to utilize the proton gradient even under conditions of Na⁺ excess. Organisms such as the cyanobacterium Spirulina platensis and the alkaliphilic bacterium Bacillus pseudoferus have c rings with an even greater H⁺ selectivity (43, 54), so much so that Na⁺ binding is no longer viable, despite its excess, and therefore these ATP synthases are exclusively coupled to H⁺.

**DISCUSSION**

Archaea not only inhabit environments with extreme temperatures, pH, and/or salinity, but some can also live autotrophically. They are believed to be early life forms (55), implying that also their bioenergetics is ancient. Methanogenesis (and acetogenesis), processes in which carbon dioxide is...
Sugar degradation. Thus, in vivo, such a ring may be an adaptation to growth at thermodynamic equilibrium.

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