2P.24 Kinetics of oxidative phosphorylation catalyzed by inside-out plasma membrane vesicles of *Paracoccus denitrificans*

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Coupled inside-out plasma membrane vesicles of *Paracoccus denitrificans* are capable of proton-motive force (*pmf*)-required, *pmf*-generating ATP hydrolysis [1]. The steady-state ATP hydrolysis, catalyzed by *P. denitrificans* $F_O \cdot F_1$ ($PF_O \cdot F_1$) proceeds via compulsory sequential mechanism where ADP leaves the enzyme–products (ADP and Pi) complex first followed by irreversible release of Pi [2]. Inasmuch operation of $PF_O \cdot F_1$ ATPase (synthase) is macroscopically reversible it seems imperative to reconcile the steady-state kinetics of *pmf*-generating ATP hydrolysis with that of *pmf*-utilising ATP synthesis. To reach this goal the dependencies of the initial steady-state rates of oxidative phosphorylation on ADP and Pi concentrations and on *pmf* were measured. ATP synthesis rate showed simple hyperbolic dependence on either substrate (within the concentration ranges of 1.5–50 μM for ADP and 10–500 μM for Pi) with no mutual dependence of apparent K_m values thus suggesting random formation of the enzyme–ADP·Pi complex. When the 'third substrate', *pmf* was varied by limitation of succinate or NADH oxidation rates an apparent 'ping-pong' mechanism was evident: a decrease of the maximal rates caused by a decrease of *pmf* resulted in proportional decrease of apparent K_m values for either Pi or ADP. This pattern suggests that an irreversible step, presumably ATP formation at the enzyme active site, precedes the involvement of *pmf* in the overall reaction (likely at the product release step). Comparison of the steady-state kinetics of ATP hydrolysis [2] and synthesis shows that macroscopic reversibility of the $PF_O \cdot F_1$ ATPase (synthase) reaction cannot be ascribed to operation of single microscopically reversible enzyme species. We propose that apparent equilibrium between any given *pmf* and intracellular (intramitochondrial) phosphoryl potential ([ATP]/[ADP]·[Pi]) is maintained by 'futile cycle' of ATP synthesis and hydrolysis catalyzed by kinetically (and structurally) distinct $F_O \cdot F_1$ species.

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2P.25 Structural basis for the ion selectivity of F-ATP-synthase c-ring rotors

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F_1F_O ATP-synthases are the most prominent ATP-producing enzymes across all life forms, from microbes to humans. They synthesize ATP in eukaryotic mitochondria and chloroplasts, as well as in the bacterial cytoplasm, by catalyzing the reaction between ADP and inorganic phosphate. To derive the energy required for catalysis, ATP synthases use the electrochemical gradient of protons or sodium ions across the membranes where they reside. Conversely, F_1F_O ATP synthases can hydrolyze ATP and thus serve as membrane ion pumps. Because ATP is the main energy source in living cells, this coupled process of synthesis/hydrolysis of ATP and Na^+/H^+ transport is of great importance, but remains poorly understood. In this work, we analyze the selectivity for H^+ or Na^+ of the membrane-embedded ion-binding subunit in the F_O domain, referred to as the c-ring rotor. We focus on the c_{11}-ring from *Ilyobacter tartaricus* and the c_{15}-ring from *Spirulina platensis*, and estimate their binding selectivity through DCCD labelling as a function of pH and salt concentration. While the c_{15}-rotor is shown to be highly proton selective, the c_{11}-ring is able to bind both Na^+ and H^+. Using molecular dynamics simulations and free energy calculations, we elucidate the structural and energetic basis for the distinct selectivity of these rotors, as well as of a series of mutants in which that selectivity is reversed towards H^+ or Na^+ binding.

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2P.26 Single, double and triple alanine to glycine replacements in the AxAxAxA motif of alkaliphilic *Bacillus pseudofirmus* OF4 *c*-subunits affect *c*-ring stability, change both monomer and *c*-ring mobility in SDS-PAGE and lead to deficits in ATP synthesis

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The F_1F_O-ATP synthases/ATPases that are used to support ATP synthesis during non-fermentative growth of some extremely alkaliphilic *Bacillus* species have special features that distinguish them from ATP synthases of neutralophiles or sequestered organelles, non-respiratory alkaliphiles or less extreme alkaliphiles [1]. One of the major deviations observed in the *c*-subunit, which forms the oligomeric *c*-ring rotor of the synthase, is the replacement of a GxGxGxG motif that is near the center of the N-terminal helix of most other c-subunits [2] with an AxAxAxA motif in alkaliphilic *Bacillus pseudofirmus* OF4. Replacement of the alkaliphile version with a consensus motif, GxGxGxG, resulted in severe deficits in malate growth and ATP synthase activity at both pH 7.5 and 10.5, without a comparable deficit in detergent-stimulated ATPase activity (i.e. total ATPase activity). Single mutants and sample double and triple mutants were shown to have more modest defects in malate growth than the quadruple mutants [2]. Here, we took advantage of the stability of the wild-type *B. pseudofirmus* OF4 *c*-ring to investigate an enlarged panel of ^{16}AxAxAxA^{22} mutants of *B. pseudofirmus* OF4 to assess changes in content and mobility of the rings on SDS-PAGE gels. ATP synthases from 4 single, 4 double, 1 triple and the 1 quadruple mutant were his-tagged on the β-subunit, purified and fractionated on SDS-PAGE gels with no treatment, treatment with trichloroacetic acid (TCA) to monomerize the ring, or after extraction with lauryl sarcosine to purify the *c*-ring away from most of the other synthase subunits. A striking finding was the doublet *c*-ring band observed in the single A16G mutant on the gel; the less intense band was the approximate size of the wild-type ring and the more intense bottom band was below the wild-type. All mutants containing A16G mutations along with other mutations exhibited only the lower band. In addition, *c*-subunit monomers from mutants with A16G

mutations exhibited somewhat diverse mobility in SDS-PAGE but were all below the wild-type monomer band. All of the AxAxAxA mutants exhibited reduced stability of the *c*-ring; the A18G mutant was least affected and the quadruple mutant was among the most affected. These observations are discussed in the context of catalytic properties and emerging structural information about the alkaliphile c-ring.

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2P.27 Cyclophilin D interaction with the ATP synthase oligomeric forms

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The mitochondrial F_OF_1ATP synthase is a rotary enzyme organized into a catalytic (F_1) and a membranous moiety (F_O) linked by central and peripheral stalks. The core mammalian enzyme consists of 16 conserved subunits, the F_1 subunits α–ε, the inhibitor protein IF_1 and the F_O subunits a–g, OSCP, A6L and F_6. The complexity of such structural organization requires accessory factors, whose definition is still under investigation. We recently demonstrated that in mitochondria cyclophilin (CyP) D, a well characterized mitochondrial chaperone, is associated to ATP synthase interacting with the enzyme lateral stalk, which is formed by the F_O subunits b, d, F_6 and OSCP. CyPD function does not appear to be related to enzyme assembly, which is not affected in CyPD-null mitochondria. Conversely, the ATP synthase–CyPD interactions have functional consequences, so that increased CyPD binding decreases enzyme catalysis and *vice versa* [1]. Involvement of the lateral stalk raises the question to which form of ATP synthase CyPD is bound. In fact, although this complex is commonly isolated as a functional monomer, different lines of evidence support that dimers/oligomers of ATP synthase exist in membrane and are stabilized by subunits of both F_O, i.e. subunits a, b, c, e, g, and F_1, i.e. IF_1 [2]. Treatment of whole mitochondria or submitochondrial particles from bovine heart with 2–10 mM oligomycin, which binds to the F_O subunit a, affected the supra-molecular organization of ATP synthase and the percentage of dimers/oligomers purified by digitonin and separated by Blue-native PAGE were inversely related to oligomycin concentration. Immunoprecipitation showed a parallel reduction of the amount of CyPD associated to ATP synthase. Accordingly, treatment of the membranes with *n*-dodecyl-maltoside, which destabilized the oligomeric forms during extraction and generated only monomers in Blue-native PAGE, significantly reduced CyPD bound to ATP synthase with respect to the treatment with digitonin in the absence of oligomycin. These data demonstrate that the dimer/oligomer formation strongly stabilizes CyPD binding to the lateral stalk, supporting a functional role of CyPD as modulator of the dimers/oligomers, which would represent the physiological form of ATP synthase [2].

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2P.28 Structural studies of the membrane-embedded c-ring of the F_1F_O-ATP synthase from a thermoalkaliphilic bacterium reveal a strategy for adaptation to alkaline environments

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F_1F_O-ATP synthases are bipartite molecular motors able to convert an electrochemical membrane potential into ATP by a rotational mechanism. F_1 ($\alpha_3\beta_3\gamma\delta\varepsilon$) contains the catalytic subunits while F_O (ab_2c_{10-15}) includes those embedded in the membrane. Within the rotor ($c_{10-15}\gamma\varepsilon$), the c-subunits are assembled in a ring of variable size in different species, comprising from 10 to 15 subunits. The c-ring is crucial as it transduces the energy gained from ion translocation across the membrane into conformational changes in the $\alpha_3\beta_3$-headpiece, thus catalyzing ATP synthesis. The stoichiometry of the ring determines the number of translocated ions required per ATP synthesized and therefore is an important parameter of the cell bioenergetics. Here, we have used *E. coli* to heterologously express the ATP synthase from the thermoalkaliphilic *Caldalkalibacillus thermarum* (formerly *Bacillus* sp.) strain TA2.A1, and show that it is fully assembled and functional. From this high-yield expression, we have isolated the c-ring and determined its stoichiometry to be 13, using various techniques. These include a novel mass-spectrometry method, termed "laser-induced liquid bead ion desorption" (LILBID), which allows the mass determination of non-covalently assembled, detergent-solubilized membrane protein complexes. In addition, AFM imaging, cryo-EM of 2D crystals and X-ray diffraction of 3D crystals also demonstrate that this c-ring harbors 13 c-subunits. Analysis of the c-ring isolated from wild-type cells is consistent with this result, demonstrating that the stoichiometry of a c-ring is a property of its primary sequence. Our structural analysis also reveals a special feature of this c-ring, namely an unusually distant packing of the inner helices. A conserved GxGxGxGxG motif, known to permit close packing of transmembrane helices, is altered to AxGxSxGxS in this strain, and appears to change the inter-c-subunit contacts, thereby enlarging the ring diameter to host a greater number of c-subunits. Comparative sequence analysis shows that this altered glycine-motif is also a feature of c-subunit sequences in many other alkaliphilic members of the *Bacillaceae* family. We propose that the enlargement of the c-rings in H^+-dependent F-ATP synthases is an adaptation to facilitate ATP synthesis at low proton-motive-force, which typically occurs in bacteria growing at alkaline pH. Further structural studies of the holo-enzyme by X-ray crystallography and single-particle EM are underway.

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