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3L.4 Structure based study of the functionality of NhaA in pH and Na^+ homeostasis

Etana Padan
Biological Chemistry, Institute of Life Sciences, Hebrew University, Jerusalem 91904, Israel
E-mail: etana@vms.huji.ac.il

Na^+/H^+ antiporters are essential for homeostasis of Na^+, H^+ and volume and are critical to cell viability. The crystal structure of *Escherichia coli* NhaA determined at pH 4 has provided insights into the mechanism of activity of a pH-regulated Na^+/H^+ antiporter [1,2]. The structural fold of NhaA is novel; six of the twelve transmembranes (TM) form an inverted repeat of which two TMs are interrupted in the middle of the membrane forming a unique electrostatic organization, important for activity. Two funnels, a deep cytoplasm-facing and a shallow periplasm-facing are separated by a barrier. The functional unit of NhaA is a monomer [3] with a "pH sensor" separated from the active site. Novel structure-based experimental and computational approaches demonstrate that amino acid residues in TM II contribute to the cation pathway of NhaA and its unique pH activation between pH 6 and 8.5 [4,5]: 1) the highly conserved residues of TM II are located on one side of the helix facing either the cytoplasmic or periplasmic funnels of NhaA. 2) Cys replacements of the conserved residues and measuring their antiporter activity in everted membrane vesicles identified new functional important residues. 3) Several of the Cys replacements were significantly alkylated by a membrane permeant probe implying the presence of water-filled cavities in NhaA. 4) Several Cys replacements were modified by MTSES and/or MTSET, membrane impermeant, negatively and positively charged reagents, respectively, that could reach the Cys replacements only via water filled funnel(s). Remarkably, MTSES but not MTSET repaired the mutant D65C implying the importance of Asp65 negative charge for pH activation of NhaA. The crystal structure of NhaA allowed to model the eukaryotic NHE1 [6] and NHA2 [7].

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3L.5 Novel secretory pathway pumps

Lisbeth Rosager Poulsen, Rosa Laura López-Marqués, Danny Mollerup Sørensen, Thomas Pomorski, Morten Buch Pedersen, Michael Gjedde Palmgren
Centre for Membrane Pumps in Cells and Disease—PUMPKIN, Danish National Research Foundation, Department of Plant Biology and Biotechnology, University of Copenhagen, DK-1871 Frederiksberg C, Denmark
E-mail: palmgren@life.ku.dk

The super family of P-type ATPases constitutes a large class of membrane proteins involved in the active transport of ions and lipids across biological membranes. Among the prominent members are the Na^+/K^+-ATPase, the Ca^{2+}-ATPase of sarcoplasmic reticulum and the plasma membrane H^+-ATPases of plants and fungi. The family can be divided phylogenetically into five distinct subfamilies (P1–P5), each divided into additional subgroups (A, B etc.). The importance of these biological pumps is underlined by the fact that its members are found in all forms of life, from bacteria to man and are involved in fundamental physiological processes, ranging from ion homeostasis and signal transduction to heavy metal and lipid transport across membranes. P4 ATPases constitute the largest P-type ATPase subfamily in eukaryotes but are absent from prokaryotes. They are found in all membranes of the secretory pathway, except the endoplasmic reticulum. P4 ATPases have been associated with flipping of lipids across membranes, a process likely to be the initial event in vesicle budding in the secretory pathway. However, our understanding of lipid translocation, vesiculation and the involvement of P4-type ATPases in these processes is just beginning to emerge and further biochemical characterization of P4-ATPases is required in order to clarify whether these transporters indeed are capable of directly catalyzing transmembrane phospholipid flipping. The β-subunit of P4-ATPases shows unexpected similarities between the β- and γ-subunits of the Na^+/K^+-ATPase. It is likely that these proteins provide a similar solution to similar problems, and might have adopted similar structures to accomplish these tasks. P5 ATPases remains the least characterized group of P-type ATPases. They evolved at the branching point between eukaryotic and prokaryotic organisms and thus are associated with the event of compartmentalization in eukaryotes. Localization studies indicate that they reside in internal membrane systems, a hallmark of eukaryotic cells. As no P4-ATPases have been identified in the endoplasmic reticulum, where P5-ATPases are present, it remains an intriguing possibility that in this compartment P5A-ATPases are functional homologues of P4-ATPases.

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3P.1 Probing the conformation of the yeast ADP/ATP carrier by fluorescent probes

Valerie L. Brandt, Edmund R.S. Kunji
MRC Mitochondrial Biology Unit, Wellcome Trust/MRC Building, UK
E-mail: vlb@mrc-mbu.cam.ac.uk

The mitochondrial ADP/ATP carrier exchanges cytosolic ADP for ATP synthesised in the mitochondrial matrix. During the transport cycle the carrier opens the central substrate binding site to the intermembrane space in the cytoplasmic state and to the mitochondrial matrix in the matrix state [1,2]. The charged residues of the PX[DE]XX[RK] motifs form a salt bridge network on the matrix side of the cavity, when the carrier is in the cytoplasmic state [3,4], whereas the charged residues of the [FY][DE]XX[RK] motifs, which are present on the cytoplasmic side of the cavity, could form a salt bridge network when the carrier is in the matrix state [2]. A structure of the

aborted cytoplasmic state of the carrier has been determined [4], but no structures are available for the empty or substrate binding states. The aim is to probe the substrate binding and conformational changes of the ADP/ATP carrier by measuring changes in labelling efficiency of single-cysteine residues with membrane-impermeable sulphydryl reagents.

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3P.2 Identification of the mammalian mitochondrial pyruvate carrier

Chloe LS. Burns[1], Andrew P. Halestrap[2]
[1]*University of Bristol, Department of Biochemistry, England*
[2]*Bristol Heart Institute, England, UK*
E-mail: Chloe.Burns@bristol.ac.uk

The existence of a specific mitochondrial carrier for pyruvate was first demonstrated in this laboratory by the use of the specific inhibitors, α-cyano-4-hydroxycinnamate (CHC) and its more potent analogue UK-5099, that reversibly modify a thiol group on the carrier. The yeast mitochondrial pyruvate carrier (MPC) was subsequently identified by measuring inhibitor-sensitive pyruvate uptake, from 18 different *Saccharomyces cerevisiae* mutants, each lacking a member of the mitochondrial carrier family (MCF). Only mitochondria from the YIL006w deletion mutant were transport deficient and this gene encodes a 41.9 kDa member of the mitochondrial solute transporter family [1]. However, others have reported this protein to be the NAD^+ transporter [2]. The nearest mammalian homologues to YIL006w are the mitochondrial folate carrier SLC25A32 (30% identity), SLC25A33 (28% identity) and SLC25A36 (28% identity). We have used two techniques to try and identify which, if any, of these is the mammalian MPC. First we have investigated whether CHC and UK-5099 can protect a specific mitochondrial inner membrane protein from labelling by fluorescent maleimide derivatives. Fluoranthyl maleimide and pyrene maleimide were identified as the most appropriate maleimide derivatives since these were found to inhibit mitochondrial pyruvate transport. However, no consistent protection of a protein was identified on 2D gels although this might reflect the recognised problems associated with separating members of the MCF by this technique. Progress in resolving these problems will be presented. Second, we are using siRNA to knockdown the candidate genes for the MPC in cultured cell lines (SiHa cells). This has required the development of an assay for mitochondrial pyruvate transport in these cells which is a technical challenge because the yield of mitochondria is too small to perform radioactive transport assays. However, we have developed an indirect assay that can detect small changes in MPC activity *in situ* and we will present data that uses this technique to screen for the mammalian MPC.

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3P.3 Determining the oligomeric state of mitochondrial carrier proteins by blue native gel electrophoresis

Paul G. Crichton, Edmund R.S. Kunji
MRC Mitochondrial Biology Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge, CB2 0XY, UK
E-mail: pgc@mrc-mbu.cam.ac.uk

Mitochondrial carriers were widely believed to be homo-dimers comprised of two about 32 kDa subunits. In blue native polyacrylamide gel electrophoresis (BN-PAGE) studies mitochondrial carriers from detergent-solubilised mitochondria migrate in the range of 70–120 kDa, which was interpreted to represent homo-dimers. Work from our laboratory has shown that the ADP/ATP carrier has an apparent mass in the range of 67–134 kDa when purified by size exclusion chromatography in the alkyl-maltoside detergent series. However, the carrier is a monomer, but the mass contribution of the associated detergent–lipid micelles varies. The effects of lipids and detergents on the migration of mitochondrial carriers in BN-PAGE have not been determined. Here, BN-PAGE was used to determine the apparent mass of the yeast ADP/ATP carrier AAC3. Our experiments show that AAC3 in dodecyl maltoside migrates at about 130 kDa species when solubilised from mitochondrial membranes, but at about 60 kDa species when purified. Importantly, the difference in apparent mass does not relate to a difference in oligomeric state, as the detergent micelle of the carrier solubilised from mitochondria has higher lipid content than the purified protein. When solubilised from mitochondrial membranes at higher detergent concentrations, the apparent mass of AAC3 decreases to about 70 kDa as the associated lipid is diluted away from the protein by detergent. The stepwise re-introduction of mitochondrial lipid to the purified protein leads to an increase in the apparent mass of AAC3 to about 130 kDa. Importantly, the incremental changes in mass are too small to account for a change in the oligomeric state of AAC3. When purified in various alkyl maltoside detergents, AAC3 decreases in apparent size with decreasing length of the detergent alkyl chain (from about 65 kDa in tri-decyl maltoside to about 45 kDa in decyl maltoside). We conclude that in previous studies the observed masses were incorrectly interpreted in favour of the dimer, as the contribution of lipid and detergent to the migration of the mitochondrial carriers in BN-PAGE had not been fully appreciated.

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3P.4 The new type of uncouplers which selectively interact with non-equilibrium membrane bounded protons

S.A. Eremeyev[1], K.A. Motovilov[2,3], L.S. Yaguzhinsky[2,3]
[1]*Lomonosov Moscow State University, Bioengineering and Bioinformatics Department, Russia*
[2]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Russia*
[3]*Moscow Institute of Physics and Technology, Scientific Educational Center "Bionanophysics", Russia*
E-mail: s.eremeev@gmail.com

In the previous work [1] we detected some interesting properties of a derivative of 2,4,6-threechlorine-3-pentadecylphenol (TCP-C15). This compound has very high affinity to membranes as compared to most of classical uncouplers of oxidative phosphorylation. TCP-C15 at concentrations of 10–60 μM was shown to selectively interact with the proton fraction [2,3] interacting with membrane under non-equilibrium conditions, as distinct from classical uncouplers which have more hydrophilic properties. It should be referred to a new class of uncouplers. Meanwhile, the question if the action of this compound was due to the detergent effect (the consequence of