

# Instituto Juan March de Estudios e Investigaciones

## 119 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Co-sponsored by

EMBO EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

Workshop on

### Pumps, Channels and Transporters: Structure and Function

Organized by

D. R. Madden, W. Kühlbrandt and R. Serrano

N. Armstrong

P. Bork

A. Engel

R. Henderson

C. Hunte

W. N. Konings

W. Kühlbrandt

J. López-Barneo

H. Luecke

D. R. Madden

E. Padrós

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## **Introduction**

**D. R. Madden, W. Kühlbrandt and R. Serrano**

Membrane proteins are implicated in the most basic physiological functions, such as nerve signalling, learning and memory, nutrient uptake, energy conversion and muscle contraction. Correspondingly, their malfunction has been linked to a wide variety of pathologies. Furthermore, determination of membrane protein structures will be an essential component of efforts to extract meaning from the human genome: approximately 30% of the new proteins discovered are predicted to be membrane bound. Yet despite their fundamental importance, membrane proteins generally remain refractory to structural characterization, and thus to a molecular understanding of their function. The difficulties begin with the production of sufficient quantities of protein for structural studies and continue through crystallization and structure determination, all of which are particularly difficult for membrane proteins. As a result, only a comparatively small number of structures have been determined from this class of proteins, predominantly from those that are naturally abundant and generally involving considerable scientific and financial resources. Nevertheless, a number of recent developments provide hope that membrane protein structure determination will accelerate, including expression, refolding as well as the two- and three-dimensional crystallization of several new membrane proteins. Coupled with molecular biological analysis of the function and regulation of such proteins, the new structural information could stimulate a much deeper understanding of their modes of action. By their nature, membrane proteins are involved in transmitting molecules or signals across lipid bilayers. The theme of transport and transporters thus provides a natural motif running throughout the program.

The goal of this workshop was two-fold: to provide an overview of the state-of-the-art in membrane protein expression, purification and crystallization, and to review the current knowledge of membrane protein structure and function. It thus included both structural presentations and technical talks, protein biochemistry and structure determination, and talks aimed at interpreting structural data in functional terms. Significant advances have been made recently in the analysis and prediction of transmembrane protein structure and function on the basis of sequence information now available from the numerous genome projects. New techniques and systems for the overexpression and two- and three-dimensional crystallization offer the prospect of direct structural and biochemical information for functionally important but previously inaccessible membrane proteins.

Channel proteins permit the passive, but regulated flow of ions and metabolites across membranes. In some cases, such channels are constitutively open, raising questions of selectivity that can be investigated, for example, in the comparison of the bacterial aquaporins and glycerol channels. In others, channels are opened or “gated” by external stimuli, such as transmembrane voltage changes or neurotransmitter binding. The conformational basis for such coupling is being investigated, among others, for the glutamate receptor ion channels responsible for excitatory synaptic signalling in the brain. Finally, both gating and flux of such channels can be modulated by protein modifications or by complex interactions between the channel and its permeant.

Pumps and transporters are involved in the active translocation of substrates across the membrane, building up electrochemical gradients or moving metabolites to the appropriate compartment in the cell. The source of energy for translocation can be provided by light, as in bacteriorhodopsin or photosynthetic proteins. It can also be chemical, as for the P-type ATPases or drug resistance proteins. The advent of structural data for such systems means that the conformational basis of such reactions can now be investigated. It can also be approached for proteins that exploit electrochemical gradients, such as secondary ion transporters or the ATP synthase.

The number of recent structures presented and the development of several promising technical advances suggest that the coming years will provide many further insights into the molecular mechanisms of active and passive membrane transport.

D.R. Madden, W. Kühlbrandt and R. Serrano

**Session 1: Channels**  
**Chair: Andreas Engel**

## Mechanisms for activation and antagonism of an AMPA-sensitive glutamate receptor

Neali Armstrong and Eric Gouaux

Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY

The ionotropic glutamate receptor (iGluR) family contains three receptor subtypes, AMPA, NMDA and kainate, which form tetrameric glutamate-gated ion channels throughout the mammalian central nervous system. Pharmacologically, AMPA receptors are characterized by a high affinity for the full agonist AMPA, a slightly lower affinity for the full agonist glutamate, and an even lower affinity for the partial agonist kainate. These receptors are competitively antagonized by the quinoxalinedione family of compounds which includes DNQX, CNQX and NBQX. For the AMPA receptor subtype, glutamate binding results in a brief opening (1-10 ms) of the ion channel which is followed by rapid and nearly complete (~95%) desensitization. Both the extent of activation and the degree of desensitization occur in an agonist-dependent manner; partial agonists, such as kainate, elicit smaller peak currents and larger steady-state currents in whole cell recordings relative to AMPA or glutamate<sup>1</sup>. NMDA and kainate receptors also activate and desensitize with varying extents and duration.

The iGluR glutamate-binding core is located in two segments, S1 and S2, which are separated by two transmembrane regions and a membrane-embedded loop. For all three iGluR subtypes a water-soluble 'mini-receptor' can be generated by genetically fusing S1 and S2 with a hydrophilic linker<sup>2,3,4</sup>. These so-called 'S1S2' constructs retain a wild-type ligand-binding profile making them valuable tools for studying the structure and biochemistry of glutamate receptors. The crystal structure of the GluR2 S1S2 core in complex with kainate revealed a bilobed structure that contains a ligand-binding site located in the cleft between the two domains<sup>5</sup>. This structure verified the predicted homology to the glutamine-binding protein, identified residues important for agonist binding, and suggested sites involved in subunit-subunit interfaces. However, for lack of an apo or full agonist-bound structure little was known about how agonist binding may trigger receptor activation.

We have now determined high-resolution crystal structures of GluR2 S1S2 in the apo state and in the presence of AMPA, glutamate, kainate and DNQX<sup>6</sup>. In the apo and antagonist-bound states the lobes of the ligand-binding core are separated. Agonist binding induces domain closure with full agonists bringing the lobes ~20° closer together while the partial agonist kainate brings them only ~12° closer. These results suggest that agonist-induced domain closure gates the ion channel and that the extent of receptor activation is dependent upon the degree of domain closure. In all of these structures, crystal packing of subunits produces a two-fold symmetric dimer. The dimer interface contains several residues that are functionally important for desensitization and is a potential binding site for allosteric effectors.

To begin testing the hypothesis that receptor activation is positively correlated to the degree of domain closure we are investigating a GluR2 S1S2 binding site mutant, L650T. The Teichberg group found that L650T (in GluR1) has a decreased kainate EC<sub>50</sub> and an increased potentiation of kainate induced currents by the desensitization blocker cyclothiazide<sup>7</sup>. We determined the crystal structure of L650T in complex with kainate and

find that the L650T mutation allows for slightly greater domain closure to occur in the presence of kainate. Structural and functional studies on L650T are in progress.

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## Electron microscopic analysis of a glutamate receptor

Willem Tichelaar, Markus Safferling, Günther Kümmerle, Kari Keinänen<sup>1</sup> & Dean R. Madden

Ion Channel Structure Research Group, MPI for Medical Research, Jahnstr. 29, D-69120 Heidelberg, Germany; <sup>1</sup>Viikki Biocenter, P.O.B. 56, FIN-00014 University of Helsinki, Finland

The ionotropic glutamate receptors (GluR) are the predominant mediators of excitatory synaptic signals in the central nervous system. They are involved in learning and memory and also appear to play an important role in neuropathologies including stroke and epilepsy. Structurally, the GluR form a family distinct from that of the acetylcholine receptor, which encompasses most neuronal ligand-gated ion channels. GluR are formed by oligomers of ~100 kD subunits. We have overexpressed the GluRB subunit in insect cells at the milligram scale and purified it to homogeneity. This subunit plays an important role in modulating the Ca<sup>++</sup> permeability of GluR ion channels and in the synaptic targeting of the receptors.

Purified GluRB homomers were negatively stained and imaged by electron microscopy. Despite the biochemical homogeneity of the preparation, individual molecules presented a heterogeneous appearance in the electron microscope. 10,000 single particles were selected for a three-dimensional reconstruction using the IMAGIC image-processing package. Following classification and Euler-angle assignment, an elongated molecular envelope was obtained. Different orientations of the model closely matched diverse single-particle images, confirming that much of the apparent EM heterogeneity was due to orientational variability.

However, although high, the orientational variability provided perspectives that were mostly confined to one plane. Furthermore, we were not confident that the bootstrap Euler angle assignment was robust for a particle of this size (ca. 500 kD) in the absence of known symmetry. Finally, we wished to have an independent assessment of possible structural heterogeneity of different orientations of the particle, e.g. due to deformation or partial staining. To address these questions, we have initiated a single-particle analysis using the random conical tilt technique. 6,000 image pairs have been selected. The untilted images were classified in IMAGIC. All further image processing used the SPIDER package. Three-dimensional reconstructions have been performed independently within each of 30 classes, and most of the different perspectives yield similar molecular envelopes, confirming the structural homogeneity of the negatively stained samples. The molecular envelopes resemble a symmetrized version of the original model, but now exhibit a prominent central ring. The reconstruction is currently being refined.

## Calmodulin interact with KCNQ channels mutated in human disease

Eva Yus-Nájera, Irene Santana, Ainhoa Etxeberria, M<sup>a</sup> Paz Regalado and Alvaro Villarroel

Instituto Cajal, Av. Dr. Arce 37, 28002 Madrid

The combination of neuronal KCNQ subunits form M-type voltage-dependent potassium channels which can be modulated by a variety of intracellular signals. Among them, calcium mediates M-current ( $I_M$ ) inhibition by B<sub>2</sub> bradykinin receptors.  $Ca^{2+}$  can suppress M-channels under conditions that do not support enzymatic activities such as phosphorylation. The action of  $Ca^{2+}$  on  $I_M$  is not compatible with a direct blocking mechanism, and appears to require a mediator, since it “desensitizes” in inside-out patches. We have screened a human brain cDNA library using the yeast two hybrid system and found that calmodulin binds to the C-terminal region of KCNQ channels. Both apo- and  $Ca^{2+}$ -calmodulin can bind to the channel. The minimal region for calmodulin binding in absence of  $Ca^{2+}$  extends about 200 amino acids. This segment is predicted to contain four alpha helix segments (A-D). Helix B contains a consensus IQ site that is necessary, but not sufficient, for calmodulin binding in absence of  $Ca^{2+}$ . In addition to helix B, helices A and D are also required for calmodulin binding in absence of  $Ca^{2+}$ . GST fusion proteins containing helices A-B, helix C or helix D bind to calmodulin in presence, but not in absence, of  $Ca^{2+}$ . These observations suggest that calmodulin is bound to the M-channel at low resting  $Ca^{2+}$  levels and that  $Ca^{2+}$  alters this interaction. Thus, calmodulin is a likely molecular substrate for  $Ca^{2+}$ -dependent modulation of M-channels.

## Variation of the rotary motors, driving ATP synthesis

H. Stahlberg<sup>1</sup>, D. J. Müller<sup>1</sup>, K. Suda<sup>1</sup>, D. Fotiadis<sup>1</sup>, T. Meier<sup>2</sup>,  
U. Matthey<sup>2</sup>, P. Dimroth<sup>2</sup>, H. Seelert<sup>3</sup>, A. Poetsch<sup>3</sup>, N. Dencher<sup>3</sup> and A. Engel<sup>1</sup>

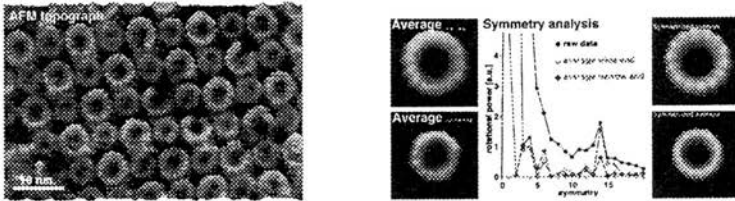
<sup>1</sup>M.E. Müller-Institute at the Biozentrum, University of Basel, CH-4056 Basel, Switzerland.

<sup>2</sup>Institute of Microbiology, ETH, Schmelzbergstr. 7, CH-8092 Zürich, Switzerland

<sup>3</sup>Technische Universität Darmstadt, Petersenstr. 22, D-64287 Darmstadt, Germany

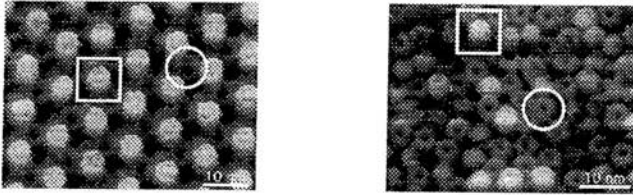
In all forms of life, ATP synthesis is driven by a transmembrane electrochemical gradient, generated by light or oxidative reactions. The flow of protons or sodium ions down the gradient propels the smallest existing rotary motor, the major membrane resident part of the ATP synthase. Rotation of these 5 to 7 nm large cylindrical structures is transmitted to a long rod which induces the conformational changes required for catalyzing the conversion of ADP and P<sub>i</sub> to ATP in the globular extramembraneous part of the enzyme. The latter exhibits a highly conserved structure comprising three catalytic sites arranged around a three-fold axis. Therefore, the proton/ATP ratio is directly linked to the stoichiometry of the rotor, which is thought to rotate by  $2\pi/n$  per translocated cation, where  $n$  is the number of  $c$ -subunits in the ring. Since such central processes have been tuned to maximum efficiency during evolution, variation in the ring stoichiometry ( $n=10$  in mitochondria (Stock *et al.* (1999)),  $n=14$  in chloroplasts, and  $n=11$  in *Ilyobacter tartaricus*) came as surprise.

### The turbine of chloroplast ATP synthase

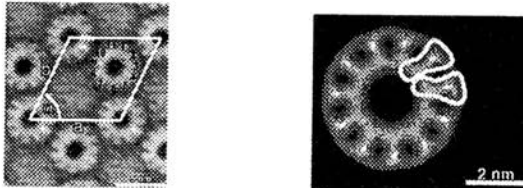


The proton driven turbine of the chloroplast F<sub>0</sub>F<sub>1</sub>-ATP synthase is built from fourteen identical subunits. Left image, AFM topograph of the subunit III oligomers assembled into a cylindrical complex. The distinct wide and narrow rings represent both surfaces of the subunit III<sub>n</sub> oligomer. Right image, The averaged topographs of the wide ( $n = 220$ ) and of the narrow ( $n = 220$ ) oligomer end were generated by reference-free translational and rotational alignment of the individual particles. For symmetry analysis the rotational power spectra were calculated from 320 topographs of individual oligomers (solid circle) and of averaged wide (circle) and narrow (diamond) rings. The spectra clearly show a fourteen-fold rotational symmetry of the oligomers. After this the averages of the wide and narrow oligomer ends were 1 fold symmetrized. Topograph was recorded in buffer solution (25 mM MgCl<sub>2</sub>, 10 mM Tris-HCl, pH 7.8) at room temperature. Full grey level range of topographs, 2 nm.

### The turbine of a bacterial Na-ATP synthase from *I. tartaricus*



AFM topographs of reconstituted  $c_x$ -oligomers from ATP synthase of *Ilyobacter tartaricus*. Left, The crystalline area is organized in a hexagonal head-to-tail packing. Right, The densely packed area allows to image the lower ends of the  $c_x$ -oligomers. The higher rings exhibit central plugs.



Cryo-electron microscopy imaging of the two-dimensional crystals. Left, The non-symmetrized projection map (6.9 Å resolution), showing a pseudo-hexagonal arrangement of the 11-fold symmetric rings. The unit cell comprises two rings, the cell dimensions are  $a=8.9 \pm 0.2$  nm,  $b=9.1 \pm 0.2$  nm and  $\alpha=65 \pm 1$ . Right, 11-fold symmetrized  $c_x$ -oligomers, showing densities of transmembrane  $\alpha$ -helices.

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## Permeating ions regulate gating in voltage-dependent K<sup>+</sup> channels

José López-Barneo, Patricia Ortega-Sáenz and Antonio Castellano

Departamento de Fisiología Médica y Biofísica and Hospital Universitario Virgen del Rocío, Universidad de Sevilla. Sevilla, Spain.

Although ion channels were classically viewed as pores with gates that move independently of the permeating ions, there are several reports indicating that occupation of the channels by the permeating ions modulates their gating properties (1, 2, see ref. 3 for a short review). Interaction of permeating ions and gating has been studied in some detail in voltage-gated potassium channels. Extracellular K<sup>+</sup> determines the occupation of sites in the channels where the cation interferes with the motion of the gates. When external [K<sup>+</sup>] decreases, some K<sup>+</sup> channels open too briefly to allow the conduction of measurable current (4, 5). It has been shown that residues T449 and D447 in the pore region of *Shaker* K<sup>+</sup> channels regulate channel activity and occupation by external cations (4-8). These residues are particularly important in determining the rate of C-type inactivation, a gating characteristic that depends on structural rearrangements in the outer mouth of the channels (5-7, 9). Given that extracellular K<sup>+</sup> is normally low, we have studied if negatively charged amino acids in the extracellular loops of *Shaker* K<sup>+</sup> channels contribute to increase the local [K<sup>+</sup>]. Surprisingly, neutralisation of the charge of most acidic residues has minor effects on gating. However, a glutamate residue (E418) in the membrane spanning segment S5 is absolutely required for keeping channels active at the normal external [K<sup>+</sup>] (10, 11). E418 is conserved in all families of voltage-dependent K<sup>+</sup> channels. Although the channel mutant E418Q has kinetic properties resembling those produced by removal of K<sup>+</sup> from the pore, the location of this residue with respect to other acidic aminoacids in the turret of the S5-S6 linker (12) suggest that E418 is not simply concentrating cations near the channel mouth but it has a direct and critical role in gating. E418 appears to stabilise the conducting vs. closed and conducting vs. C-inactivated states of the channels thus preventing the collapse of the K<sup>+</sup> conductance (10, 11). E418 seems to contribute to link channel permeation and activation and inactivation gating.

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**Session 2: Getting crystals from sequence**  
**Chair: Wil N. Konings**

## Comparative genome analysis and implications for function prediction

Peer Bork

EMBL and MDC

The classical way of function prediction from sequence is a gene by gene approach in which homology information between proteins or nucleic acids is exploited. As proteins usually have several functions, this is usually done by first identifying domains within the proteins. Several programs exist for this task but I will concentrate on our web-based tool, SMART, to illustrate the power of those approaches in i) hypotheses generation for experiments ii) identification of proteins via EST mining [1] and iii) annotation of the human genome [2]. As the human genome sequence draft is being published in February 2001, the need for entire genome comparisons becomes already obvious for gene predictions [3]. With complete genome information in hand, we could also demonstrate the importance of comparative sequence analysis in functional genomics studies, e.g. RNAi analysis of *C.elegans* [4]. Despite an enlarging number of eukaryotic genomes being sequenced (with our own genome as the latest addition), multiple genome comparisons can only be performed in prokaryotes so far with more than 30 genomes publicly available. Here, the transition from gene by gene approaches to gene context methods has already been made yielding novel concepts and methods for function predictions [5]. An example is the protein interaction prediction based on conserved gene neighborhood.

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## Overexpression of integral membrane proteins: what's the problem?

C. G. Tate

Determining the structure of an integral membrane protein is difficult and this is reflected in the lack of membrane protein structures compared to the number of structures solved for soluble proteins. One of the reasons for this is the difficulty in overexpressing polytopic membrane proteins. The majority of mammalian integral membrane proteins are currently expressed in *Escherichia coli*, yeasts, mammalian cells or in insect cells (using the baculovirus expression system) to levels of about 0.1 mg of functional protein per litre of culture, although some are expressed at slightly higher levels. The only way currently to produce 10 mg/L of a complex membrane protein is to express it in an inactive form as inclusion bodies in *E. coli* or as misfolded, membrane-associated material using viral expression systems: refolding membrane proteins is, however, extremely challenging. Why is the overexpression of membrane proteins so difficult? In my presentation I will review the main expression systems, using as an example the serotonin transporter, which has been found to be a particularly difficult membrane protein to functionally overexpress. In addition, strategies designed to improve functional expression will be discussed.

Further reading

### Introduction to expression systems:

*Protein Expression: A practical Approach.* (1999) Higgins, S.J. & Hames, B.D. (Eds) Oxford University Press, Oxford, UK

*Gene Expression Systems* (1999) Fernandez, J.M. & Hoeffler, J.P. (Eds) Academic Press, San Diego, USA

### Membrane protein overexpression:

Expression and purification of membrane proteins (colloquium of the Biochemical Society, 15 papers) (1999) *Biochem. Soc. Trans.* **27**, 883-960

Large-scale expression of receptors in yeast (1999) Reilander, H, Reinhart, C & Szmolenszky, A. in *G Protein-coupled receptors* (Haga, T & Berstein, G., Eds) CRC Press, Boca Raton, Florida.

Heterologous expression of G protein-coupled receptors (1996) Tate, C.G. & Grisshammer, R. *Trends Biotechnol.* **14**, 426-430

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## **Three-dimensional structure of the bacterial core protein translocase at 9Å resolution**

Cécile Breyton, Ian Collinson, Winfried Haase and Werner Kühlbrandt

The Sec machinery is responsible for the export and membrane integration of most secreted and membrane proteins in bacteria. In the inner membrane, a channel is formed by the association of secY, secE and secG. The complex active in protein translocation is thought to consist of up to 4 secYEG units each predicted to contain up to 15 membrane-spanning  $\alpha$ -helices. These three membrane proteins have been overexpressed in *E. coli*, purified as a complex and crystallised in two dimensions. The complex crystallises as a monomer. In the 3D map, a likely boundary for the monomer can be drawn, and densities corresponding to helices can be distinguished. Thin sections and freeze-fracture electron microscopy has confirmed that the crystals consist of two membranes, related by a crystallographic two-fold screw axis. Crystal contacts between the two membranes are likely to be mediated by the cytoplasmic domains, which are thought to be composed of the largest loops. Thus, the periplasmic side, predicted to have shorter loops, would correspond to the smooth outer surface of the crystals. This topography will be confirmed by antibody labelling.

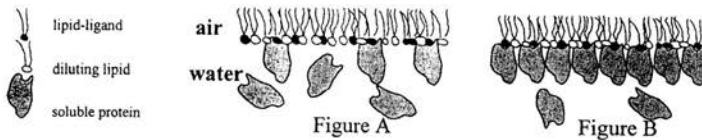
## Lipid monolayer crystallization of membrane proteins

Catherine Vénien-Bryan

The structure determination of biological macromolecules at high resolution is required for understanding their associated functions. Despite important advances in the past few years, the number of detailed structures by X-ray crystallography of three-dimensional crystals still lags far behind that of soluble proteins. In this context, electron crystallography of two-dimensional (2D) crystals represents a viable alternative to X-ray crystallography for structure determination of integral membrane proteins. Electron crystallography was originally developed by Henderson and Unwin for structure determination of 2D crystals of a membrane protein, the bacteriorhodopsin (1).

### a- Presentation of the method with soluble proteins:

Electron crystallography has been extended to 2D crystals of soluble proteins bound to a lipid monolayer. This method proposed by R. Komberg in 1983 (2; for reviews, see 3-4) exploits the electrostatic or specific interaction between a soluble protein and a ligand lipid forming a lipid monolayer at the interface air-water. Conditions required or favourable for two-dimensional crystallization include:-1) Fixation of the proteins to the lipid-ligand and concentration of the protein-lipid complexes in the plane of the lipid film. Then the mobility of the molecules within the plane allows sampling of various arrangements (figure A); 2) Self-assembly of the proteins into 2D domains and, in favourable cases, into 2D crystals (figure B).



After the 2D crystals have been transferred onto electron microscope grids, structural analysis may be performed by electron crystallography. Some 2D protein crystals are sufficiently well ordered to give near-atomic resolution (5-9).

A more recent extension of this technique exploits the specific strong interaction between metal coordinated-lipid and polyhistidine tagged protein. The nickel ion is carried by the polar head of synthesised lipids and a short stretch of contiguous histidine residues (a His-tag) is located on the C or N terminal end of the expressed proteins or naturally occurring surface histidines (10). A number of protein have been successfully crystallized using this strategy (11-14).

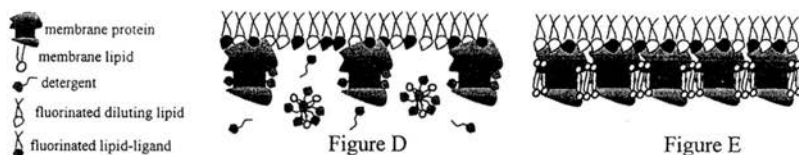
### b- Extension to membrane proteins.

Efforts to extend the approach of 2D crystallization on lipid monolayers to membrane proteins is complicated by the characteristic tendency of detergents to solubilize monolayers of regular lipids (figure C).



Figure C Effect of the addition of detergent

In collaboration with L. Lebeau and C. Mioskowski, we tackled the problem of solubilization of the lipid monolayer by designing partially fluorinated Ni-lipids which, when spread at the interface, display a high resistance towards solubilization by detergents (as monitored by ellipsometry). The dual hydrophobic and lipophobic properties of the fluorolipids exclude detergent, and the monolayer is stable allowing the His-tag membrane protein to adsorb and in some conditions to crystallize. The proton ATPase from plant plasma membrane has been successfully crystallized (15). The detergent-solubilized His-tag-ATPase binds to a nickel fluorinated lipid monolayer, accumulates, and orients at the air-water interface. The detergent-solubilized membrane lipid is then added to the subphase (figure D). 2D crystals of the ATPase, embedded in a lipid bilayer, formed as the detergent is removed by adsorption (figure E).



The 2D crystals of  $H^+$ -ATPase are large, 10  $\mu\text{m}$  or more across. These crystals are good candidates for studies in 3D.

This technique of formation of 2D crystals is quick and reproducible and requires minimal amounts of protein and functionalized lipid (approximately 7.5  $\mu\text{g}$  and 500 nmole respectively per trial). Further advantages of this technique are the possibilities of co-crystallising protein and DNA in the case of DNA binding proteins (16) and also the possibility of observing the change of conformations of protein upon variation of the composition of the subphase.

During the presentation I will show also some biophysical techniques (optical, mechanical and structural, 17-18) which have been developed in order to monitor and possibly improve the mechanism of formation and growth of 2D crystals.

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## Structure at 2.3 Å resolution of the cytochrome *bc*<sub>1</sub> complex from the yeast *Saccharomyces cerevisiae* co-crystallized with an antibody Fv-fragment

Hunte, C., Lange, C., Koepke, J., Rossmann, T., Michel, H.

Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Str. 7, D-60528 Frankfurt, Germany;  
e-mail: Carola.Hunte@mpibp-frankfurt.mpg.de

The cytochrome *bc*<sub>1</sub> complex is part of the energy conversion machinery of the respiratory and photosynthetic electron transfer chains. This integral membrane protein complex catalyzes electron transfer from ubiquinol to cytochrome *c*. It couples the electron transfer to the electrogenic translocation of protons across the membrane via the so-called Q-cycle mechanism.

The cytochrome *bc*<sub>1</sub> complex from the yeast *Saccharomyces cerevisiae* was crystallized together with a bound antibody Fv-fragment. The crystals belong to the monoclinic space group C2 and diffract x-rays up to 2.2 Å resolution using synchrotron radiation. The structure was determined at a resolution of 2.3 Å using multiple isomorphous replacement and it was refined to a crystallographic R-factor of 21.1 % ( $R_{\text{free}} = 25.4$  %). The yeast cytochrome *bc*<sub>1</sub> complex is present as a homodimer. Each "monomer" of the refined model includes 2154 amino acid residues of 9 subunits of the cytochrome *bc*<sub>1</sub> complex and of the polypeptides V<sub>H</sub> and V<sub>L</sub> of the Fv-fragment, the cofactors heme b<sub>H</sub>, heme b<sub>L</sub>, heme c<sub>1</sub>, the [2Fe-2S]-cluster, 371 water and five lipid molecules. The Fv-fragment binds to the mobile extrinsic domain of the [2Fe-2S]-"Rieske"-protein and is essential for formation of the crystal lattice. The approach to crystallize membrane proteins as complexes with specific antibody fragments appears to be of general importance.

The structure of the cytochrome *bc*<sub>1</sub> complex reveals the binding of the natural substrate coenzyme Q6 and the inhibitor stigmatellin. The detailed description of the binding sites gives important information to understand the mechanism of the enzyme. Furthermore, it provides the basis for molecular modelling, as this protein complex is a target for fungicides of agrochemical importance.

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**Session 3: Transporters**  
**Chair: Richard Henderson**

## Recent progress in structural and functional analysis of water oxidizing photosynthesis

H.T. Witt

Max-Volmer-Institut, Technische Universität Berlin, Str. d. 17. Juni 135, 10623 Berlin, Germany

In oxygenic photosynthesis two large membrane-integrated protein complexes photosystem I (PS I) and photosystem II (PS II) operate in series (see e.g. ref. [1] and the references therein). Following light excitation, electrons are finally transferred from the water oxidizing complex of PS II to the terminal electron acceptor of PS I. The cleavage of water catalyzed by PS II supplies the overall process with the necessary electrons and protons. For a closer understanding of the mechanism of the primary processes besides functional and biochemical analysis the elucidation of the molecular structure of PS I and PS II is indispensable.

Trimeric PS I core complexes isolated from the thermophilic cyanobacterium *Synechococcus elongatus* have been crystallized. X-ray crystallographic structure analysis has led to a comprehensive molecular model at 4 Å resolution [2]. Recently, this has been extended to 2.5 Å resolution. Very recently, also PS II core complexes were crystallized [3]. The crystals themselves are fully active in water oxidation [4] and are suitable for X-ray structure analysis at 3.8 Å resolution. The crystallizable PS II material is highly suitable also for obtaining new functional information on the mechanism of water oxidation [5]. The PS II structure at 3.8 Å resolution shows how the protein subunits and cofactors are spatially organized. First information is obtained on the position, size and shape of the Mn cluster which catalyzes the water oxidation in PS II [6]. A report is given on the mechanism of oxygenic photosynthesis taking into account results of the recent structural analysis.

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## Atomic resolution structures of bacteriorhodopsin photocycle intermediates: The role of discrete water molecules in the function of this light-driven ion pump

Hartmut Luecke

Departments of Molecular Biology & Biochemistry and Physiology & Biophysics  
3205 Biological Sciences II, University of California, Irvine, CA 92697-3900  
hudel@uci.edu

High-resolution x-ray crystallographic studies of bacteriorhodopsin have tremendously advanced our understanding of this light-driven ion pump during the last two years, and emphasized the crucial role of discrete internal water molecules in the pump cycle. In the extracellular region an extensive three-dimensional hydrogen-bonded network of protein residues and seven water molecules leads from the buried retinal Schiff base via water 402 and the initial proton acceptor Asp85 to the membrane surface. Near Lys216 where the retinal binds, transmembrane helix G contains a pi-bulge that causes a non-proline kink. The bulge is stabilized by hydrogen-bonding of the main-chain carbonyl groups of Ala215 and Lys216 with two buried water molecules located in the otherwise very hydrophobic region between the Schiff base and the proton donor Asp96 in the cytoplasmic region. The M intermediate trapped in the D96N mutant corresponds to a late M state in the transport cycle, after protonation of Asp85 and release of a proton to the extracellular membrane surface, but before reprotonation of the deprotonated retinal Schiff base. The M intermediate from the E204Q mutant corresponds to an earlier M, as in this mutant the Schiff base deprotonates without proton release. The structures of these two M states reveal progressive displacements of the retinal, main-chain and side-chains induced by photoisomerization of the retinal to 13-cis,15-anti, and an extensive rearrangement of the three-dimensional network of hydrogen-bonded residues and bound water that accounts for the changed pK<sub>a</sub>s of the Schiff base, Asp85, the proton release group and Asp96. The structure for the M state from E204Q suggests, moreover, that relaxation of the steric conflicts of the distorted 13-cis,15-anti retinal plays a critical role in the reprotonation of the Schiff base by Asp96. Two additional waters now connect Asp96 to the carbonyl of residue 216, in what appears to be the beginning of a hydrogen-bonded chain that would later extend to the retinal Schiff base. Based on the ground state and M intermediate structures, models of the molecular events in the early part of the photocycle are presented, including a novel model which proposes that bacteriorhodopsin pumps hydroxide (OH<sup>-</sup>) ions from the extracellular to the cytoplasmic side.

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Structural Changes in Bacteriorhodopsin During Ion Transport at 2 Angstrom Resolution. H Luecke, B Schobert, H-T Richter, JP Cartailler, JK Lanyi (1999) *Science* **286**, 255-260.

Atomic resolution structures of bacteriorhodopsin photocycle intermediates: The role of discrete water molecules in the function of this light-driven ion pump. H Luecke (2000) *Biochim. Biophys. Acta* **1460**, 133-156.



## The physiological role of Trk1 and Trk2 in *Schizosaccharomyces pombe*

Fernando Calero, Francisco Cabello-Hurtado and José Ramos

Trk1 and Trk2 define the major potassium transport system in fission yeast. Single mutants in any of the *trk* genes do not show significantly altered content or influx of potassium under standard conditions. However, *trk1 trk2* double mutants fail to grow at low potassium concentrations and show a dramatic decrease in rubidium (potassium) influx. In order to determine the physiological role of Trk1 and Trk2 in fission yeast, we have performed a comparative study of single mutant strains lacking *trk1* or *trk2*, under very different conditions. We will show that when grown at very low pH (3.0) the strain lacking the Trk1 protein displays impaired transport characteristics: - It requires more potassium to grow - It retains internal potassium less efficiently - It shows a lower percentage of survival when suspended in a medium without added potassium.

In addition, the *trk1*- mutants are more sensitive to lithium and to the antibiotic Hygromycin B.

All together, our results indicate that the presence of Trk1 allows a better performance of fission yeast under different stress conditions.

## The rotary mechanism of ATP synthase

John E Walker

Medical Research Council-Dunn Human Nutrition Unit, Hills Road, Cambridge, CB2 2XY,  
UK

Since the chemiosmotic theory was proposed by Peter Mitchell in the 1960's, a major objective in bioenergetics has been to elucidate the mechanism of coupling of the transmembrane proton-motive force, generated by respiration or photosynthesis, to the synthesis of ATP from ADP and phosphate. The ATP synthase found in chloroplasts, eubacteria and mitochondria is composed of two major domains, a globular  $F_1$  catalytic domain and a membrane bound  $F_0$  proton-translocating domain, linked by a central stalk. In mitochondrial  $F_1$ -ATPase, the central stalk contains the  $\gamma$ -,  $\delta$ - and  $\epsilon$ -subunits<sup>1-4</sup>. It penetrates into the  $F_1$  catalytic domain where an assembly of three  $\alpha$ -subunits and three  $\beta$ -subunits (the  $(\alpha\beta)_3$  domain) are arranged alternately around an asymmetrical anti-parallel  $\alpha$ -helical coiled-coil in the  $\gamma$ -subunit<sup>1,3,5</sup>. The  $\alpha$ - and  $\beta$ -subunits have closely related structures. They both bind nucleotides, but each  $\beta$ -subunit contains a catalytic nucleotide binding site whereas the nucleotides bound to the  $\alpha$ -subunits do not participate directly in catalysis. The structure of the bovine  $F_1$ -ATPase provides strong support for the "binding change mechanism" of catalysis in which the catalytic sites have different nucleotide affinities<sup>6</sup>. One (the "open" site) has very low affinity for substrates, the second (the "loose" site) can bind substrates reversibly and the third (the "tight" site) has a very high affinity such that ATP can form spontaneously from ADP and inorganic phosphate. In  $F_1$ -ATPase, the central stalk, which is asymmetrical, rotates during ATP hydrolysis<sup>7,8</sup> with an anticipated frequency of 100-200 Hz. The rotation of the central stalk, which proceeds in 120° steps<sup>9</sup>, changes the affinities of the catalytic sites taking each through cycles of "open", "loose" and "tight" states. Therefore, in ATP synthase, the rotating central stalk is the key coupling element in the enzyme, involved in transferring energy from the  $F_0$  membrane domain, where rotation is generated from the transmembrane proton motive force, into the catalytic sites some 100 Å above the membrane surface. A peripheral stalk linking the surface of  $F_1$  to  $F_0$ , probably acts as a stator to counter the tendency of the  $(\alpha\beta)_3$  domain to follow the rotation of the central stalk<sup>3,10</sup>.

A major unanswered question is: how is rotation of the central stalk generated by the passage of protons through the  $F_0$  membrane domain? Two  $F_0$  subunits known as subunits a and b are involved in proton translocation. An electron density map of a complex of  $F_1$  with associated c-subunits shows 10 c-subunits arranged in a ring, with the foot of the central stalk standing on the ring<sup>11</sup>. The large area of contact between the foot of the stalk and the ring suggests that the stalk-c ring subcomplex rotates as an ensemble. It is likely that the protons are translocated through the interface between the single a-subunit and the c-ring and that protonation and deprotonation of buried carboxyls in the c-subunits are involved in generation of rotation. The number of c-subunits in the ring has profound mechanistic implications. With 10 c-subunits in the ring, the number of protons translocated for each ATP synthesised is likely to be  $10/3 = 3.3$ . There is evidence that chloroplast ATP synthase has 14 subunits in its ring<sup>12</sup> and so the corresponding H<sup>+</sup>/ATP value is 14/3. It is unlikely that the mechanism of rotation will be understood fully until accurate models of the entire ATP synthase in different conformational states have been established. Determination of these structures requires either the crystallization of the intact enzyme, or the establishment of an accurate low resolution

model by electron microscopy of single complexes, which can then be used as a framework for building a molecular model from structures of subcomplexes and individual subunits.

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## Probing bacteriorhodopsin biochemistry

Esteve Padrós<sup>1</sup>, Tzvetana Lazarova<sup>1</sup>, Carolina Sanz<sup>1,2</sup>, Mercedes Márquez<sup>1,2</sup>, Samir Elouatik<sup>1</sup>,  
Francesc Sepulcre<sup>1,3</sup>, Alex Perálvarez<sup>1,2</sup>, Enric Querol<sup>2</sup>

<sup>1</sup> Unitat de Biofísica, Dept. de Bioquímica i de Biologia Molecular, Fac. de Medicina, Univ. Autònoma de Barcelona.

<sup>2</sup> Institut de Biologia Fonamental, Univ. Autònoma de Barcelona.

<sup>3</sup> Dept. d'Enginyeria Química, E.U.E.T.I., Univ. Politècnica de Catalunya, Barcelona.

Bacteriorhodopsin is one of the best-known transmembrane proton transport systems (1). In last years, several three-dimensional structures of the purple membrane have been described, in both the resting state and in some intermediates of the photocycle (2-6). In this work, different spectroscopic methods are used to probe the structure and function of the extracellular half of bacteriorhodopsin, through the use of mutants. So far, there are several aspects of the proton transfer mechanism in the extracellular side that are not yet known. The nature of the proton release group and the steps leading to proton transfers are two of the main questions. In order to get insight into them, we prepared and analysed single and multiple Glu mutants: E9Q, E74Q, E194Q, E204Q, E194Q+E204Q, E9Q+E194Q+E204Q (abbreviated 3Glu) and E9Q+E74Q+E194Q+E204Q (4Glu).

Although the most detailed structural maps place Glu194 and Glu204 within hydrogen-bonding distances (3, 4, 6), we present Fourier transform infrared spectroscopy data showing that in the M- and N-like intermediate states, the properties of the mutants E194Q and E204Q are very different, including an early deprotonation of Asp85 in E194Q at alkaline pH (7). These observations indicate that Glu194 controls the Asp85 pK<sub>a</sub> and exclude any equivalent role of these Glu side chains in the proton transport mechanism. Additionally, analyses of partially dried samples show the presence of protonated Asp85 in M and N intermediates of E194Q at alkaline pH. This corroborates that water molecules are involved in the mechanism that links the pK<sub>a</sub> of Asp85 and the pK<sub>a</sub> of the proton release group.

Differential scanning calorimetry data show that in water (pH 6.8) the single mutants E194Q and E204Q have the main transition at slightly lower temperature than wild type (for WT, T<sub>m</sub> is at about 98 °C). The E9Q and E194Q+E204Q mutants have the main transition at similar T<sub>m</sub> values (about 95 °C), whereas the quadruple mutant has the T<sub>m</sub> at about 92 °C. The decreases of about 3 and 6 °C, respectively, indicate that these mutations affect to some extent the stability of the inter-helical interactions. Anyway, these BR mutants still can be considered as stable structures against temperature increase. The pre-transition observed in the DSC scans of BR has been related to the disorganisation of the para-crystalline arrangement of the BR trimers. The E9Q mutant shows a pre-transition about 14 °C lower and less cooperative than wild type. In E194Q+E204Q, 3Glu and 4Glu mutants, the pre-transition is completely absent, indicating that these groups are involved in the organisation of the trimers. Therefore, these mutations either give rise to the disorganisation of trimers already at room temperature, or else the disorganisation is produced progressively upon temperature increase, without any cooperativity.

Thermal denaturation can also be followed by UV-Vis difference spectra. Heating wild type gives rise first to the increase of the blue form as a consequence of cation release, followed by the red form and finally by retinal denaturation. None of the multiple mutants E194Q+E204Q, 3Glu or 4Glu showed increase of the blue form, indicating either that these mutants lack cations or that they are only loosely bound, so that no further blue form appears as they are displaced.

Structural alterations were also noted in the extracellular mutants. Multiple Glu mutants show increased accessibility of ions and small molecules such as hydroxylamine to the Schiff base environment (8). Thus, whereas the 4Glu mutant has an absorption maximum of about 510 nm at neutral pH, Cl<sup>-</sup> anions at molar concentrations restore the wild type absorption maximum of 558 nm. On the other hand, the multiple mutants E194Q+E204Q, 3Glu and 4Glu show increased accessibility of hydroxylamine in the dark as compared with wild type. We interpret these results in the way that some negative charges are needed at the locations of the Glu side chains and at high concentration, Cl<sup>-</sup> anions provide them; further, in the native protein cations are bound through Glu194/Glu204 and Glu9. In the absence of these Glu side chains, the cations are of low affinity and the region loses compactness.

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**Session 4: Techniques and examples**  
**Chair: Hartmut Luecke**

## A structural mechanism for proton pumping by bacteriorhodopsin

Richard Henderson

MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, UK

I will present an atomic resolution model [1] for the protein and retinal structural changes that are involved in the vectorial, light-driven transport of protons by bacteriorhodopsin. The “switch” mechanism which ensures the vectorial nature of pumping consists of two elements. First, unbending of retinal following the release of a proton from the Schiff base results in the displacement of the Schiff base nitrogen towards the cytoplasm, and second, a protein conformational change opens up a path to the cytoplasmic surface for reprotonation.

Electron crystallographic determination of the structure of a triple mutant (D96G/F171C/F219L) locked in this conformationally altered state reveals major rearrangements of helices F and G by up to 3.5 and 2.0 Angstroms respectively, which provide an “opening” of the half-channel between the Schiff base and the cytoplasmic surface of the protein. The relevance of the structure of the triple mutant to the structural changes which normally occur during the photocycle of wild-type bacteriorhodopsin is based on projection structure analyses of wild-type and a variety of mutant proteins [2] in which the photochemical intermediates are trapped by rapid freezing following a light flash. The retinal unbending is inferred [1] from a comparison of 8 accurate, small molecule X-ray structures of retinals and retinal derivatives, which shows that the polyene chain becomes more curved when retinal forms a protonated Schiff base and that the curvature is greater when the charge is more delocalised.

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## Structure of primary and secondary membrane transporters by electron cryo-microscopy

Werner Kühlbrandt

Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Str. 7,  
D-60528 Frankfurt am Main, Germany  
e-mail: [kuehlbrandt@mpibp-frankfurt.mpg.de](mailto:kuehlbrandt@mpibp-frankfurt.mpg.de)

Structural studies of membrane proteins still lag far behind soluble proteins, due to the difficulty of growing well-ordered crystals. Electron cryo-microscopy of two-dimensional (2D) crystals is a promising approach for determining the structure of membrane proteins. We have used this technique to determine the structure of two proteins active in membrane transport: the plasma membrane proton pump ( $H^+$ -ATPase) from *Neurospora* (1,2) and *Arabidopsis* at  $\sim 8 \text{ \AA}$  resolution, and of the sodium/proton antiporter NhaA from *E. coli* at  $4 \text{ \AA}$  in projection (3) and at  $\sim 7 \text{ \AA}$  resolution in 3D (4).

The  $H^+$ -ATPase is a prototype of the P-type ATPases, a large family of primary ion pumps that use ATP to create electrochemical gradients across membranes of all cell types, cycling between two structurally distinct states, known as E1 and E2, with a phosphorylated intermediate. It has a single polypeptide chain of  $\sim 950$  amino acids. The 3D map shows the ATPase in the unliganded, open E1 conformation. The 10 membrane-spanning  $\alpha$ -helices are clearly resolved (1). The large cytoplasmic portion of the ATPase, comprising  $\sim 70\%$  of its molecular mass, contains the phosphorylation site and undergoes a large conformational change in the ion pumping cycle (2). We used the recently determined x-ray structure of the related  $Ca^{2+}$ -ATPase (Toyoshima et al, *Nature* **405**, 647-655) to build an atomic model of the  $H^+$  ATPase which has been fitted to the  $8 \text{ \AA}$  map, revealing major changes in position of the N and A domains which may be related to ion pumping.

NhaA is a secondary transporter, an extremely large and diverse class of membrane proteins that make use of ion gradients to transport ions, sugars and amino acids across the plasma membrane of bacterial and eukaryotic cells. Most are predicted to have 12-14 membrane-spanning helices. NhaA is a dimer which may be its functional state in the membrane (3). The 3D map of NhaA (4) is the first structure of a secondary transporter. It shows 12 membrane spanning helices, arranged with no apparent internal symmetry. Work towards higher resolution is in progress.

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## The (cbb<sub>3</sub>)-type oxidase from *Pseudomonas stutzeri*: A characterization and crystallization study

Andrea Urbani and Matti Saraste

European Molecular Biology Laboratory (EMBL), Structural Biology Programme,  
Heidelberg

The *Pseudomonas stutzeri* strain ZoBell is a gram negative bacteria belonging to the family of the Pseudomonaceae ( $\gamma$  subdivision). This family includes a number of soil and water living species as well as pathogen bacteria for animals and plants. The (cbb<sub>3</sub>)-type cytochrome oxidases are heme copper oxidases in the respiratory chain of gram negative bacteria. In N<sub>2</sub> fixing bacteria, such as *Bradyrhizobium japonicum*, this enzyme has been shown to be involved in the respiration at low oxygen tension. A common feature to all these enzymes is the absence of the Cu-A centre, the electron entry primary site in the mitochondria like. This class of enzymes has been predicted to be composed by a complex of four integral membrane proteins ccoNOQP (FixNOQP). The major catalytic subunit (ccoN, FixN) is homologous to subunit I of the aa<sub>3</sub>-type oxidase and it has been predicted to be a 12 helix transmembrane protein. The six histidine residues involved in chelating of the low, high spin hemes and Cu-B site are strictly conserved. Nevertheless, the catalytic binuclear center consists of a unique association of cofactors in the cytochrome c oxidase family, Cu atom (Cu-B site) and a b-type heme. Two smaller membrane bound cytochromes ccoO (FixO) and ccoP (FixP) are part of the enzyme complex. While ccoO is a monoheme cytochrome c, the ccoP subunit presents a peculiar diheme cytochrome c arrangement. Despite the amount of information available on the (cbb<sub>3</sub>)-type oxidases belonging to the alpha subgroup of the proteobacteria, little research has been focused on this class of enzymes from the  $\gamma$  subdivision.

We have developed a method for the purification of the (cbb<sub>3</sub>)-type oxidase in high yield from the soil bacteria *Pseudomonas stutzeri*. The protein has been purified as a three subunit complex (ccoNOP). The enzyme is active with catalytic efficiencies (kcat/Km) of 10<sup>6</sup> s<sup>-1</sup> M<sup>-1</sup> in the oxidation of cytochrome C-551 from *Pseudomonas stutzeri*. The protein complex contains b and c hemes in a ratio 2 to 3 and it behaves as a monomer with an overall molecular weight of 108 kDa as determined by analytical ultracentrifugation. The contribution of lipids and detergent has been evaluated and compared with analytical gel filtration data. A novel colorimetric method for the determination of the detergent bound has been applied successfully. Crystals diffracting at 5.0 Å resolution have been grown by the sitting drop method to an average size of 0.1x0.1x0.3 mm. Preliminary X-ray data analysis highlights a hexagonal lattice, with a space group P6<sub>1</sub>/522 and cell dimensions of 101x101x717 Å. A possible model of the molecular shape based on the hydrodynamic properties is presented and compared with preliminary X-ray diffraction data on 3D crystals.

Andrea Urbani, Ph. D.  
European Molecular Biology Laboratory (EMBL),  
Structural Biology Programme,  
D-69112, Heidelberg, Postfach 102209  
e-mail: [urbani@EMBL-Heidelberg.de](mailto:urbani@EMBL-Heidelberg.de)  
tel: +49-6221-387270  
FAX +49-6221-387519

## Aquaglyceroporins exhibit variable surfaces but highly conserved channels

A. Engel<sup>1</sup>, S. Scheuring<sup>1</sup>, D. Fotiadis<sup>1</sup>, T. Braun<sup>1</sup>, B. de Groot<sup>2</sup>, P. Agre<sup>3</sup>, Y. Fujiyoshi<sup>4</sup>, and H. Stahlberg<sup>1</sup>.

<sup>1</sup>M. E. Müller-Institute for Microscopy at the Biozentrum, University of Basel, Basel CH-4056, Switzerland

<sup>2</sup>Max-Planck-Institute for Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen, Germany

<sup>3</sup>Departments of Biological Chemistry and Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2185, USA

<sup>4</sup>Department of Biophysics, Faculty of Science, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto, 606-8502, Japan

Osmoregulation in bacteria, plant, and animals requires the presence of membrane channels, specific for water and small non-ionic solutes. Expression of aquaporin-1 (AQP1, the water channel of human erythrocytes) in *Xenopus* oocytes demonstrated its water permeation capacity (Preston *et al.*, 1992) and initiated many studies of the rapidly growing aquaporin superfamily. Phylogenetic analyses revealed the existence of two clusters of subfamilies, the aquaporins (AQPs) and glycerol facilitators (GLPs; Park and Saier, 1996). Sequence based structure prediction provided a model comprising six membrane spanning helices, while sequence analyses suggested strategic residues that may be important for structure and function (Heymann and Engel, 2000).

The surface topography of several AQPs has been mapped by atomic force microscopy, revealing different features that could be correlated with differences in the loops connecting the transmembrane helices. In addition, the sidedness of these AQPs has been determined by comparing the topography before and after proteolytic digestion (Fotiadis *et al.*, 2000; Scheuring *et al.*, 1999).

The 3D structures of AQP1 and GlpF have been determined by electron cryo-microscopy. The 3.8 Å density map allowed the first atomic model of AQP1 to be built (Murata *et al.*, 2000), taking into account data from sequence analyses (Heymann and Engel, 2000) and fitting of short helical segments to the map (de Groot *et al.*, 2000). This model provides some insight into the permeation of water through a channel that blocks the passage of protons. GlpF has been resolved to 6.9 Å, revealing helices that are similar to those of AQP1 (Stahlberg *et al.*, 2001). Additional densities indicate domains that correlate with the additional residues in loop C and loop E as compared to the sequence of AQP1 and the N-terminal deca-his tag. Homology modeling, however, shows the channel region of these distant aquaglyceroporins to be rather similar.

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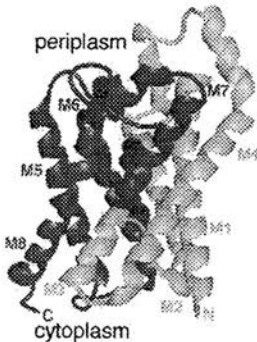
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## Structure of a glycerol conducting channel and the basis for its selectivity

Robert M. Stroud (stroud@msg.ucsf.edu)

UCSF

Membrane channel proteins of the aquaporin family are highly selective for permeation of specific small molecules, with absolute exclusion of ions including protons or OH<sup>-</sup> anions and charged solutes, and without dissipating the electrochemical potential across the cell membrane. The family includes channels that conduct water called aquaporins, and channels that conduct glycerol or other linear polyalcohols (aquaglyceroporins), and ion conducting channels. The *E. coli* glycerol facilitator (GlpF) was cloned, expressed, purified, and crystallized with its primary permeant substrate glycerol. The crystal structure of GlpF was determined by multiple isomorphous replacement at 2.2Å resolution and shows aspects of the channel that illustrate the basis for its selectivity. Glycerol molecules G1, G2, G3 line up in an amphipathic channel in single file. In the narrow selectivity filter of the channel the glycerol alkyl backbone is wedged against a hydrophobic corner and successive OH groups form hydrogen bonds with a pair of acceptor, and donor atoms. Two conserved -Asn Pro Ala-



motifs met in the center of the membrane and form a conserved interface between two gene duplicated segments that each encode three and one half membrane spanning helices around the channel. This structure helps to elucidate the mechanism of selective permeability for linear carbohydrates and suggests how ions and water may be excluded. A functional assay was developed, and shows that the channel is both stereo, and enantio selective. The conservation of sequence suggests how the water channels of the same family that conduct water, may exclude glycerol. A comparison of features of the channel itself, and of the space through the centre of the tetrameric arrangement of channels leads to conclusions about what is required of ion conducting channels.

**Session 5: Pumps**  
**Chairs: Werner Kühlbrandt/David L. Stokes**

## Crystal structure of calcium pump of sarcoplasmic reticulum

Chikashi Toyoshima

Institute of Molecular and Cellular Biosciences  
The University of Tokyo

P-type ATPase is a family of ion transporting ATPases that include  $\text{Na}^+\text{K}^+$ -ATPase, sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$ -ATPase and gastric  $\text{H}^+\text{K}^+$ -ATPase among others. As a family of ATPases, P-type ATPase has been regarded as a peculiar one, because it lacks the P-loop commonly found in other ATPases and GTPases. The simplest and a best studied mammalian P-type ATPase is SR  $\text{Ca}^{2+}$ -ATPase. It consists of a single polypeptide of 110 kDa and transports up to 2  $\text{Ca}^{2+}$  per ATP hydrolysis against concentration gradient from muscle cells to SR. Thus it is responsible for relaxing muscle cells after contraction.

We have been working on this ATPase using electron and X-ray crystallography and determined its structure at 2.6 Å resolution with two calcium ions bound in the transmembrane domain consisting of 10  $\alpha$ -helices (*Nature* 405: 647-655, 2000). The two calcium ions are located side by side surrounded by 4 transmembrane helices, two of which are unwound to realise efficient co-ordination geometry. The cytoplasmic region consists of 3 well-separated domains, with the phosphorylation site in the central catalytic domain and the adenosine binding site on another domain. The phosphorylation domain has the fold of L-2-haloacid dehalogenase, confirming the proposal by Aravind *et al.* (*Trends Biochem. Sci.* 23: 127-129, 1998). Thus it is now established that P-type ATPase belongs to a much larger family including enzymes that have apparently nothing to do with nucleotides.

The atomic model was fitted to an 8 Å-resolution density map of the enzyme derived by electron microscopy of tubular crystals formed in the absence of  $\text{Ca}^{2+}$  and the presence of decavanadate (Zhang *et al.*, *Nature* 392: 835-839, 1998). In the tubular crystals the enzyme is considered to be in a state analogous to the phosphorylated (E2P) state. The density map was very well explained by large domain movements in the cytoplasmic region. By comparing the two models, it has become clear that the enzyme has a mechanism that converts the movements of the cytoplasmic domains to those of transmembrane helices. This seems to be the mechanism how calcium ions are transported. So it looks as if ion transporting ATPases work like mechanical pumps at atomic scale.

## Structural mechanisms for regulating $\text{Ca}^{2+}$ -ATPase

David L. Stokes

Skirball Institute, NYU School of Medicine, 540 First Ave, New York, NY 10016

$\text{Ca}^{2+}$ -ATPase is a charter member of the family of P-type ATPases, which carries out ATP-dependent cation transport across virtually every type of cell membrane. A recent x-ray crystal structure for  $\text{Ca}^{2+}$ -ATPase has at last revealed its atomic architecture (1). Furthermore, comparisons of this structure with an 8-Å structure from cryoelectron microscopy reveal large-scale conformational changes, which have been associated for many years with particular steps in the reaction cycle. In fact, these conformational changes are thought to provide the structural mechanism for coupling the energy of ATP hydrolysis in the cytoplasmic domains to ion binding and transport across the transmembrane domain. We have been studying the structural basis for  $\text{Ca}^{2+}$ -ATPase regulation by both thapsigargin (TG) and phospholamban (PLB). TG is a very specific inhibitor of  $\text{Ca}^{2+}$ -ATPase from endoplasmic/sarcoplasmic reticulum and is widely used by cell biologists to empty the internal cellular stores of  $\text{Ca}^{2+}$  from the endoplasmic reticulum. TG inhibits by binding to a particular conformation of  $\text{Ca}^{2+}$ -ATPase and preventing the next step in the reaction cycle: i.e.,  $\text{Ca}^{2+}$  binding (2). The precise mechanism for this inhibition is not known. PLB is a small protein from cardiac sarcoplasmic reticulum that suppresses  $\text{Ca}^{2+}$ -ATPase activity at low  $\text{Ca}^{2+}$  concentrations. In a physiological context, this inhibition is relieved by a signaling cascade initiated by beta-adrenergic stimulation, leading to stronger, quicker heart contractions and relaxations, which is beneficial during exercise (3). The mechanism for PLB regulation is still unclear, though numerous mutations on both PLB and  $\text{Ca}^{2+}$ -ATPase have defined several likely sites of interaction.

We have addressed the structural mechanisms of inhibition by PLB and TG by using cryoelectron microscopy to solve the structure of co-crystals of  $\text{Ca}^{2+}$ -ATPase with each inhibitor. Both inhibitors appear to interact with the  $\text{E}_2$  conformation of  $\text{Ca}^{2+}$ -ATPase and the tubular crystals induced by vanadate appear to stabilize this conformation. Indeed, TG is particularly effective at promoting crystallization and stabilizing these crystals to the normally disruptive effect of  $\text{Ca}^{2+}$ . Various lines of evidence indicate the PLB also binds to the  $\text{E}_2$  form of the enzyme, though the stoichiometry of this interaction is not clear. For both inhibitors, we solved several independent structures representing both controls (no ligand bound) and co-crystals. For PLB, we used several different genetic constructs that differ in their oligomeric properties and their affinities for  $\text{Ca}^{2+}$ -ATPase. In particular, wild-type PLB has a strong tendency to form a pentamer, whereas the L37A mutant is predominantly monomeric and has a slightly higher affinity for  $\text{Ca}^{2+}$ -ATPase (4). Finally, the N27A mutant has the highest affinity and retains the pentameric character (5).

By calculating difference maps between PLB containing crystals and controls, we identified a difference density that we believe corresponds to the cytoplasmic domain of PLB. No density was seen for the transmembrane helix of PLB, possibly because of disorder or displacement from its binding site by a crystal contact. This difference density breaks the two-fold symmetry innate to these tubular crystals and appears to link adjacent  $\text{Ca}^{2+}$ -ATPase molecules into dimers. This is consistent with the documented tendency of PLB to aggregate  $\text{Ca}^{2+}$ -ATPase within both sarcoplasmic reticulum and reconstituted vesicles. Based on the

interaction between this cytoplasmic domain and  $\text{Ca}^{2+}$ -ATPase, we suggest that PLB prevents the large conformational changes that drive the reaction cycle.

Likewise, difference maps between TG containing crystals and controls reveal a difference density that we attribute to TG. This binding site is between two luminal loops of  $\text{Ca}^{2+}$ -ATPase, which is contrary to conclusions based on site-directed mutagenesis of a segment near the cytoplasmic side of the membrane (6). We suggest, instead, that the mutagenesis may cause a conformational change in  $\text{Ca}^{2+}$ -ATPase, which indirectly affects the TG binding site. We further suggest that TG inhibits  $\text{Ca}^{2+}$  by holding the luminal loops in a fixed configuration, thus preventing necessary movements of the transmembrane helices.

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## Structure-function relationships in plasma membrane H<sup>+</sup>-ATPase

Michael Gjedde Palmgren, Morten J. Buch-Pedersen, Jens Bukrinsky, Kees Venema, Ramon Serrano, Sine Larsen

Department of Plant Biology, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark; e-mail: palmgren@biobase.dk

Most transport proteins in fungal and plant cells are energized by electrochemical gradients of protons across the plasma membrane. The formation of these gradients is due to the action of plasma membrane H<sup>+</sup>-ATPase. This enzyme shares a membrane topography and general mechanism of action with other P-type ATPases, but differ in regulatory properties. Recent advances in the field include the identification of the complete H<sup>+</sup>-ATPase gene family in *Arabidopsis*, analysis of H<sup>+</sup>-ATPase function in plants by the methods of reverse genetics, an improved understanding of the posttranslational regulation of pump activity by 14-3-3 proteins, novel insights into the H<sup>+</sup> transport mechanism, and progress in structural biology. Furthermore, the elucidation of the three-dimensional structure of a related Ca<sup>2+</sup> pump has implications for understanding of structure-function relationships in plasma membrane H<sup>+</sup>-ATPase.

We have used the 2.6 Å structure of the sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase SERCA1a [Toyoshima, C., Nakasako, M., Nomura, H. and Ogawa, H. (2000) *Nature* 405, 647-655] to build models by homology modelling of two plasma membrane H<sup>+</sup>-ATPases, *Arabidopsis thaliana* AHA2 and *Saccharomyces cerevisiae* PMA1. We propose that in both yeast and plant PM H<sup>+</sup>-ATPases a strictly conserved aspartate in M6 (D684<sub>AHA2</sub>/D730<sub>PMA1</sub>)[1] and three backbone carbonyls in M4 contribute to a binding site for H<sub>3</sub>O<sup>+</sup>, suggesting a previously unknown mechanism for transport of protons. In addition, a possible receptor region is suggested for the C-terminal auto-inhibitory domain extending from the P- and A-domains into the transmembrane region.

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## **LmrA, a P-glycoprotein homologue in *Lactococcus lactis*, confers resistance to a broad spectrum of antibiotics**

Rik van Veen, Gerrit Poelarends, Abelardo Margolles and Wil N. Konings

Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

The lactic acid bacterium *Lactococcus lactis* possesses two multidrug resistance systems, LmrA and LmrP, which prevent the entrance in the cell of a wide variety of antibiotics and other cytotoxic agents. LmrP is a secondary transporter while LmrA belongs to the ATP-binding cassette (ABC) transporters. LmrA is closely related to the human P-glycoprotein and extrudes drugs at the expense of ATP hydrolysis. LmrA has been overexpressed in *L. lactis* to 30 - 35 % of the membrane proteins, solubilised, purified to homogeneity and functionally reconstituted in liposomes. LmrA can substitute for P-glycoprotein in human lung fibroblast cells and both proteins have very similar drug and modulator specificity. LmrA functions as a vacuum-cleaner system by which the lipophilic substrates are picked up from the inner leaflet of the membrane and removed directly to the external water phase. Equilibrium binding experiments, photoaffinity labeling and drug transport assays indicated that the homodimeric LmrA mediates drug transport by an alternating two-site transport (two-cylinder engine) mechanism. The transporter possesses two drug-binding sites: a transport-competent site on the inner membrane surface and a drug-release site on the outer membrane surface. The interconversion of these two sites, driven by the hydrolysis of ATP, occurs via a catalytic transition state intermediate in which the drug transport site is occluded. The mechanism proposed for LmrA may also be relevant for P-glycoprotein and other ABC transporters.

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## **A family of protein kinases dedicated to the regulation of pumps, channels and transporters**

Ramón Serrano, Alain Goossens, Jose M. Mulet and Francisco Portillo

Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-C.S.I.C.

Camino de Vera, 46022 Valencia, Spain

The regulation of ion homeostasis, as many biological phenomena, is better investigated under stress conditions. A mutational analysis of genes which upon gain or loss of function improve tolerance to  $\text{Na}^+$  and  $\text{Li}^+$  stress has uncovered several protein kinases which regulate the major yeast ion transporters. The Pma1  $\text{H}^+$ -ATPase is regulated by the Ptk2 and Hrk1 kinases while the Trk1,2  $\text{K}^+$  transporter is regulated by the Hal4 and Hal5 kinases. Ptk2 and Hrk1 transduce a glucose metabolic signal which activate the  $\text{H}^+$ -ATPase in conditions of fast growth. Hal4 and Hal5 transduce a  $\text{K}^+$  starvation signal. Interestingly, all these kinases belong to a subfamily of yeast protein kinases, the Npr1 group, which seems dedicated to the regulation of pumps, channels and transporters. Hal1, a salt-inducible determinant of salt tolerance, has been shown to work through Hal4,5, providing a logical salt stress response pathway. The mechanism by which Pma1 and Trk1,2 activities modulate toxic cation tolerance is indirect, based on the setting of the electrical membrane potential which determines the uptake of toxic cations such as  $\text{Na}^+$ ,  $\text{Li}^+$ , tetramethylammonium, polyamines and aminoglycosidic antibiotics.



# POSTERS

## The two major plant plasma membrane H<sup>+</sup>-ATPases display different regulatory properties

Stéphanie Dambly and Marc Boutry

The major plant plasma membrane H<sup>+</sup>-ATPases fall into two gene subfamilies (I and II) (Moriau et al., 1999; Ouffatole et al., 2000). However, in many plant tissues, their expression overlaps and this has precluded their individual characterization (Moriau et al., 1999; Ewing et al., 1994). Yeast expression of PMA2 and PMA4, representatives of the two plasma membrane H<sup>+</sup>-ATPase subfamilies in *Nicotiana glauca*, has previously shown that (i) these isoforms have distinct enzymatic properties (Luo et al., 1999), and that (ii) PMA2 is regulated by phosphorylation of its penultimate residue (Thr) and binds regulatory 14-3-3 proteins, resulting in the displacement of the auto-inhibitory C-terminal domain (Maudoux et al., 2000). To obtain insights into regulatory differences between the two subfamilies, we have constructed various chimeric proteins in which the 110 residue C-terminal region of PMA2 was progressively substituted by the corresponding one from PMA4. The PMA2 auto-inhibitory domain was localized to a region between residues 851 and 915 and could not be substituted by the corresponding region of PMA4. In contrast to PMA2, PMA4 was poorly phosphorylated at its penultimate residue (Thr) and bound 14-3-3 proteins only weakly. The only sequence difference around the phosphorylation site is located two residues upstream of the phosphorylated Thr, this being Ser in PMA2 (as in most members of subfamily I) and His in PMA4 (as in most members of subfamily II). Substitution of His by Ser in PMA4 resulted in an enzyme showing increased phosphorylation status, 14-3-3 binding, and ATPase activity, as well as better yeast growth. The reciprocal substitution of Ser by His in PMA2 resulted in the failure of this enzyme to complement the absence of yeast H<sup>+</sup>-ATPases. These results show that the two plant H<sup>+</sup>-ATPase subfamilies differ functionally in their regulatory properties.

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## **Two-dimensional crystallization of recombinant plant plasma membrane H<sup>+</sup>-ATPase on detergent-resistant monolayers**

Jens Dietrich (1), Luc Lebeau (2), Franck Lach (2), Catherine Vénien-Bryan (3), Anne Renault (4), Thomas Jahn (5), Michael G. Palmgren (5), Charles Mioskowski (2) and Werner Kühlbrandt (1)

(1) Max-Planck-Institut für Biophysik, 60528 Frankfurt am Main, Germany. (2) Laboratoire de Synthèse Bioorganique associé au CNRS, Université Louis Pasteur, 67401 Illkirch, France; (3) Institut de Biologie Structurale CEA-CNRS, 38027 Grenoble Cedex 1, France (present address: Laboratory of Molecular Biophysics, South Parks Road, Oxford OX1 3QU, UK); (4) Laboratoire de Spectrométrie Physique, Université Joseph Fourier, 38402 Saint Martin d'Hères, France; (5) The Royal Veterinary and Agricultural University, 1871 Frederiksberg C, Copenhagen, Denmark

P-type ATPases are relatively small ion pumps with a single catalytic subunit that are characterized by forming a phosphorylated (hence P-type) intermediate during the reaction cycle. The plant plasma membrane H<sup>+</sup>-ATPase is responsible for the ATP fueled ejection of protons from the cell, and is an important element in generating the transmembrane H<sup>+</sup>-gradient and membrane potential in plant cells. The plant plasma membrane ATPase from *Arabidopsis thaliana* was expressed in yeast with a C-terminal His-tag and purified in amounts suitable for protein crystallization. A new strategy for the two-dimensional (2D) crystallization of membrane proteins was employed. The method is based on the use of ligand-functionalized, partially fluorinated lipids spread on the air-water interface. By binding to the functionalized lipid, the detergent-solubilized membrane protein accumulates and orients at the lipid monolayer prior to forming a crystal lattice. Specifically fluorinated lipid compounds were designed to spread into stable monolayers at the air-water interface even in the presence of high concentrations of various detergents. They thus provide a robust ligand-functionalized planar surface onto which the membrane protein can anchor in a particular orientation. If detergent-solubilized lipid is present in the subphase, a lipid bilayer forms around the membrane protein as the detergent is removed by absorption. This creates a favorable membrane-like environment for the membrane protein. The efficiency of the new strategy is illustrated by the fact that we obtained large well-ordered 2D crystals of a plasma membrane H<sup>+</sup>-ATPase yielding a projection map to a resolution of 9 Å.

## **Does the 14-3-3/H<sup>+</sup>-ATPase complex generate both a high- and low-affinity Fusicoccin binding site?**

Katrine Drumm, Anja T. Fuglsang and Michael G. Palmgren

A search for the receptors of the fungal phytotoxin fusicoccin led to the identification of 14-3-3 proteins (Marra et al. 1994). Fusicoccin binding studies in plant microsomal vesicles reveal the existence of two populations of fusicoccin binding sites (Meyer et al. 1989; Drabkin et al. 1997): High and low affinity sites have a  $K_D$  of around 1 to 2 nM and 40 to 200 nM fusicoccin, respectively. We have shown that high affinity sites are generated when 14-3-3 proteins interact with a 14-3-3 binding site involving a phosphorylated threonine residue in the C-terminal region of the plasma membrane H<sup>+</sup>-ATPase (Fuglsang et al. 1999). We are currently investigating whether low affinity binding sites result from the formation of a different type of complex between 14-3-3 and the H<sup>+</sup>-ATPase.

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**Molecular identification of the E-ATPase from electric organ of *Torpedo marmorata*. A stretch-activated and hyperpolarization-induced ionic channel**

Antonio Felipe<sup>1</sup>, Artur Escalada<sup>2</sup>, Jordi Aleu<sup>2</sup>, Mireia Martín-Sauté<sup>2</sup>, Jordi Marsal<sup>2</sup> and Carles Solsona<sup>2</sup>

<sup>1</sup>Departaments de Bioquímica i Biologia Molecular and <sup>2</sup> Biologia Cel·lular i Anatomia Patològica. Universitat de Barcelona, 08071 Barcelona, Spain.

The electric organs of fish have been used as an experimental model of neuromuscular synapses. The electric organs of *Torpedo* fish are made up of dozens of prisms surrounded by connective tissue and filled with thousands of cells placed on parallel planes. The cells are disk shaped and embryologically derived from skeletal muscle. This special skeletal fibers are called electrocytes because they are responsible for the generation of the electric field. The electrocytes are polynnervated and covered by cholinergic nerve terminals (Whittaker, V.P. 1977, *Naturwissenschaften* 64:606-611). There is a release of ATP not only during synaptic transmission in from motor nerve terminal (Silinsky, E.M. 1975, *J. Physiol.* 247:145-162) but also, from the postsynaptic stimulated muscle cell in mammals (Forrester, T. 1966, *J. Physiol.* 186:107P-109P). The same two physiological phenomena have been described in the electric organ of *Torpedo* (Meunier, F., Israel, M., Lesbats, B. 1975, *Nature* 257:407-408). *Xenopus* oocytes injected with RNA from electric organ from a *Torpedo marmorata* showed similar characteristics that those described in electric organ and in motor nerve terminal and postsynaptic stimulated muscle cell. During hyperpolarizing pulses the permeability for ATP<sup>4-</sup> increases and the sensitivity to dadolinium ions of the ionic current, ATP release and E-ATPase activity suggest their dependence on stretch activated ion channels (Bodas, E., Aleu, J., Pujol, G., Martín-Satué, M., Marsal, J. and Solsona, C. 2000. *J. Biol. Chem.* 275:220268-20273). The pharmacological profile of the ionic current coincides with the inhibition of ecto-ATPase activity that is encoded by CD39 mRNA. This protein was first described as CD39 lymphoid cell antigen in mouse B-lymphocytes (Gayle, R.B. 3rd, Maliszewski, C.R., Poindexter, K., Birks, K., Delespesse, G.J.T., Schoenborn, M.A., Armitage, R.J. Fanslow, W.C., Nakajima, T., Baker, E., Sutherland, G., Alpert, A., Friend, D., and Gimpel, S.D. 1994, *J. Immunol.* 153:3574-3583) and is highly conserved among mammalian species.

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## **C2 domains of protein kinase Cs: Structure and interaction with phospholipids**

Wendy Fernández-Ochoa

Protein Kinase C is a family of related kinases which includes at least eleven different mammalian isoforms. PKCs can be classified into three groups based on their structure and cofactor regulation. The first group includes the classical isoforms (a, bI, bII and g) which are distinguished because their function is regulated by acidic phospholipids, diacylglycerol (DAG), phorbol esters and calcium ( $\text{Ca}^{2+}$ ). The second group corresponds to the novel PKCs (d, e, h and q) that are also activated by acidic phospholipids, DAG and phorbol esters but their function is not regulated by calcium. The third group comprises the atypical PKC isoform (x, l and m) that are regulated neither by DAG nor by  $\text{Ca}^{2+}$ . PKC molecules consist of a conserved catalytic C-terminal region and a variable regulatory region constituted by two types of molecular motifs, the C1 and C2 domain. These two domains are targets for PKC activators. The C2 domain interacts with acidic phospholipids and in classical PKCs also binds  $\text{Ca}^{2+}$ . The crystal structures of two C2 domains of the two different isoforms, PKCa and PKCe have now been resolved by X-ray crystallography both in absence and presence of 1,2-dicaproyl-sn-phosphatidyl-L-serine, 1,2-diacetyl-sn-phosphatidyl-L-serine and 1,2-dicaproyl-sn-phosphatidic acid. A calcium-dependent mechanism is proposed for the interaction of the PKCa-C2 domain with model membranes. And a calcium-independent mechanism is also proposed for the PKCe, which retains many of the structural features of the docking of the classical PKCs. The important differences in membrane-binding and activation reported between PKCa and PKCe appear to originate in a few specific structural differences which include the replacement, in PKCe, of most of the residues that coordinate with calcium in classical PKCs.

## **The activity of the ATP synthase from *E. coli* is regulated by the transmembrane protonmotive force**

Susanne Fischer, Peter Graeber, Paola Turina

The ATP synthase from *E. coli* is an intensely studied enzyme. For interpretation of functional data it is important to distinguish between catalytic and regulatory phenomena. Therefore we have checked for the possibility that the *E. coli* enzyme might be activated by the proton motive force. The enzyme was reconstituted into liposomes and energized by an acid-base transition and a  $K^+$ /valinomycin diffusion potential. One second after energization the electrochemical proton gradient was dissipated by uncouplers and measurement of ATP hydrolysis was started. In the presence of ADP and  $P_i$  the rate of ATP hydrolysis was up to 9-fold higher when the proteoliposomes had been pre-energized. This higher rate decayed to the non-pre-energized level within about 15 s. In the absence of ADP and  $P_i$  the rate of ATP hydrolysis was already high and could not be increased significantly by pre-energization. It is concluded that ATP hydrolysis is inhibited when ADP and  $P_i$  are bound to the enzyme and that a high proton motive force is required to release ADP and  $P_i$  and to convert the enzyme into a high activity state. Thus, the proton motive force does not only supply energy for ATP synthesis but also regulates the fraction of active enzymes. Since activation phenomena have earlier been found in ATP synthases from chloroplasts, mitochondria and photosynthetic bacteria it is now very likely that this type of regulation is a common feature of all ATP synthases.

## Structure-Function analysis of fungal TRK-transporter

R. Haro and A. Rodríguez-Navarro

Dpto. Biotecnología.E.T.S.I.Agrónomos. Universidad Politécnica de Madrid. 28040 Madrid. Spain.

Two K<sup>+</sup> transporters have been identified in *Saccharomyces cerevisiae*: TRK1 and TRK2. TRK1 is a large protein of 1235 amino acids and 141 kDa, containing 12 hydrophobic segments(1). TRK2 is a protein 55% identical in sequence overall, but the hydrophobic segments show higher homology (70-90%)(2). TRK1 is involved in the high affinity K<sup>+</sup> uptake and TRK2 shows moderate affinity and its role in K<sup>+</sup> uptake is not clear (3). K<sup>+</sup> transporters of TRK type have been found in other fungi (*Saccharomyces uvarum*(4), *Schizosaccharomyces pombe* (5), and *Neurospora crassa* (6)). The sequence analysis of TRK transporters reveals that all of them are well conserved and exhibiting the maximum sequence homology in the hydrophobic segments. The fungal TRK family is related to K<sup>+</sup>-transporters of organisms as distant as higher plants and eubacterias. In plants, HKT1 was the first identified transporter related to TRK1(7). In bacteria, a large number of K<sup>+</sup>-transporters, now called KtrB(8), are also related to TRK1. The HKT transporter of wheat and the KtrB of *Vibrio alginoliticus* are K<sup>+</sup>-Na<sup>+</sup> symporters, but for the other members of the family the transport mechanism is unknown. The resolution of the structure of the K<sup>+</sup> channel KscA of *Streptomyces lividans* allowed the development of a structural and functional model for this channel (9). Sequence analysis by Durrell et al (10, 11) suggests a common structure for the superfamily of K<sup>+</sup> channel proteins and the four TRK-type transporters: fungal TRK, plant HKT, bacterial trkG and KtrB. All these prokaryotic and eukaryotic K-transporters are proteins formed by four repetitions of a structure evolved from the bacterial K-channel KscA type. The structure of a TRK type transporter is not 12 transmembrane fragments, but 4 sequential MPM elements. The M elements are the transmembrane fragments and P is a loop which enters the membrane forming a hairpin. The P-loop of K<sup>+</sup> transporters keeps only a G residue of the selectivity filter residues GYG, which are absolutely required for the K<sup>+</sup> transport of K<sup>+</sup> channel. The residue G-290 from the third P-loop of ktrB from *Vibrio alginoliticus* was mutated by S, D, or A, residues and the activity of the transporter was null or reduced considerably (12). We have followed a similar approach with the TRK1 of *S. cerevisiae*. We have identified several mutants which show a reduced capacity of transport and a change in the K<sup>+</sup>/Na<sup>+</sup> discrimination. We are studying these mutants whose changes map in the P-loop fragments. Our results support the model of Durrell et al about the structure of the TRK-type K<sup>+</sup> transporters.

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## Mutagenesis studies on the plant $\text{Na}^+/\text{H}^+$ antiporter AtNhx1

Agustín Hernández and José M. Pardo

Instituto de Recursos Naturales y Agrobiología de Sevilla (CSIC). Dpto. de Biología Vegetal.  
Avda. Reina Mercedes 10. Sevilla 41012. Spain

Sodium/proton exchangers are a ubiquitous class of transporters both with respect to life form or intracellular location. The product of the AtNHX1 gene is the first of its kind to be described in plants. It corresponds to a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter that confers salt resistance upon overexpression in *Arabidopsis* [1] though whether its main function is pH control or salt compartmentation is still unclear. These kind of proteins consists typically of 10-12 transmembrane segments and a long C-terminal cytosolic tail. However, both the catalytic cycle and the amino acids involved in it are still unresolved. Knowledge on the regulation of this family of transporters comes primarily from studies on mammalian NHE exchangers. From these studies, it has been found that the regulation is highly particular for each isoform and relies mainly on domains found at the C-terminal tail [2]. Our group has started a mutagenic approach towards understanding the regulation of AtNhx1p using yeast cells as a tool. Deletion of stretches of similar length on the C-terminal tail revealed the presence of at least two important domains: a proximal autoinhibitory domain and a distal activating domain. Furthermore, site-directed and random mutagenesis have revealed amino acids important for function that modulate salt resistance. In particular, a single mutation in a putative phosphorylation site abolishes salt tolerance without apparent loss of basal activity, whereas five other mutations confer a higher salt resistance to yeast cells. From these data, AtNhx1p seems to have a dual function (pH control and salt compartmentation). The bias on each function may be intrinsic to AtNhx1p or dependent on the interaction with other proteins.

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## Native interactions of the bacterial outer membrane transporter FhuA with an Lipopolysaccharide (LPS) molecule

Andrew D. Ferguson, Wolfram Welte, Eckhard Hofmann, Buko Lindner, Otto Holst, James W. Coulton and Kay Diederichs

During X-ray analysis of the integral outer membrane protein FhuA from *Escherichia coli* K-12 we identified an Lipopolysaccharide (LPS) molecule bound to the outer barrel wall. As the lipid is copurified along with the protein from membrane extracts the observed binding position in the crystal most likely represents the contact between the protein and the membrane components in vivo.

We solved the X-ray structures FhuA/LPS complexes of two different *Escherichia coli* strains, AW740 and DL41, at 2.5Å and 2.7Å respectively. In both structures we were able to model the Lipid A region and as the inner and outer core regions of the lipids. To confirm the identity and composition of the LPS molecules we conducted a complete chemical analysis of samples extracted from both strains and from purified FhuA/LPS preparations.

After identification of a binding motif for LPS on FhuA we searched for a similar pattern in all structurally known proteins. This was motivated by the fact that LPS recognition triggers the immune response to a bacterial infection in humans and is responsible for the septic shock syndrome. We could identify a subset of 4 positively charged residues which are conserved in structurally known eukaryotic LPS-binding proteins.

Details of the LPS-Protein interactions will be shown and implications for the analysis of proteins involved in the human innate immune response to LPS will be discussed.

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***In vitro* reconstitution and biophysical characterization of recombinant OEP16, a pore protein of the chloroplast outer envelope, from *E.coli* inclusion bodies**

Dirk Linke\*, Joachim Frank# and Petra Fromme\*

\* Max Volmer Institut für Biophysikalische Chemie und Biochemie, TU Berlin, Straße des 17. Juni 135, 10623 Berlin, Germany # Fritz-Haber-Institut der Max-Planck-Gesellschaft, Faradayweg 4-6, 14195 Berlin, Germany

Four channel proteins have been identified and functionally characterised in pea chloroplast outer membranes. Besides the preprotein conducting channel (1), they were named according to their molecular weight OEP16, OEP21, OEP24 (2,3,4). The channel characteristics of OEP24 closely resemble those described for general diffusion pores (4). OEP21 has been shown to form an anion channel which is regulated by ATP and triosephosphates (3). OEP16 on the other hand forms a high conductance channel with a remarkable selectivity for amino acids and amines (2). Moreover, OEP16 shows homologies to LivH, an amino acid transporter in *E.coli* (5). The amino acid sequence of OEP16 suggests no structural resemblance of OEP16 to other membrane pores. Here we describe the *in vitro* reconstitution of overexpressed OEP16, which has been purified from *E.coli* inclusionbodies. Reconstitution in detergent micelles was monitored by CD and fluorescence spectroscopy (6). As a reference, OEP16 was reconstituted into liposomes, where the protein function can be monitored by single channel conductance measurements (2). Differential scanning calorimetry gave an estimate of the enthalpy of protein folding and first protein crystals were obtained that have to be further refined for X-ray structural analysis (6). The described methods of membrane protein reconstitution and biophysical analysis might prove helpful in the study of other membrane proteins that occur only in very low amounts *in vivo*.

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## The *Escherichia coli* ammonium transporter, AmtB

Mike Merrick, Gavin Thomas and Dan Blakey

Dept of Molecular Microbiology, John Innes Centre, Colney Lane, Norwich, NR4 7UH, UK

Genes encoding high-affinity ammonium transporters were first isolated from *Saccharomyces cerevisiae* and *Arabidopsis thaliana* in 1994 (2,4). The Amt family of proteins is both unique and ubiquitous, being found in eubacteria, archaeobacteria, fungi, plants, nematode worms and insects (1,2,4,5). The Rhesus proteins, which are found in mammals, nematodes, slime moulds and marine sponges also show homology to Amt proteins (3). The physiological function of Rh proteins remain unknown but they may also function as ammonium transporters. We are studying *Escherichia coli* AmtB in order to investigate fundamental properties of Amt proteins. We have generated an *in silico* topological model for the structure of AmtB which predicts twelve transmembrane helices with both the N and C-termini cytoplasmically located. This model was tested by the construction of AmtB-PhoA and AmtB-LacZ fusions and the *in vivo* data agree with the proposed topology (6).

In order to investigate the biochemistry and mode of action of AmtB we have constructed a histidine-tagged derivative expressed from an inducible T7 promoter. The protein has been over-expressed and purified from isolated membranes by solubilisation in dodecylmaltoside using nickel affinity and ion exchange chromatography. We will describe characterisation of the protein by a variety of biophysical techniques and implications for the oligomeric nature of AmtB and related proteins.

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## **A family of protein kinases dedicated to the regulation of pumps, channels and transporters**

Ramón Serrano, Alain Goossens, José M. Mulet and Francisco Portillo

Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-C.S.I.C.

Camino de Vera, 46022 Valencia, Spain

The regulation of ion homeostasis, as many biological phenomena, is better investigated under stress conditions. A mutational analysis of genes which upon gain or loss of function improve tolerance to  $\text{Na}^+$  and  $\text{Li}^+$  stress has uncovered several protein kinases which regulate the major yeast ion transporters. The Pma1  $\text{H}^+$ -ATPase is regulated by the Ptk2 and Hrk1 kinases while the Trk1,2  $\text{K}^+$  transporter is regulated by the Hal4 and Hal5 kinases. Ptk2 and Hrk1 transduce a glucose metabolic signal which activate the  $\text{H}^+$ -ATPase in conditions of fast growth. Hal4 and Hal5 transduce a  $\text{K}^+$  starvation signal. Interestingly, all these kinases belong to a subfamily of yeast protein kinases, the Npr1 group, which seems dedicated to the regulation of pumps, channels and transporters. Hal1, a salt-inducible determinant of salt tolerance, has been shown to work through Hal4,5, providing a logical salt stress response pathway. The mechanism by which Pma1 and Trk1,2 activities modulate toxic cation tolerance is indirect, based on the setting of the electrical membrane potential which determines the uptake of toxic cations such as  $\text{Na}^+$ ,  $\text{Li}^+$ , tetramethylammonium, polyamines and aminoglycosidic antibiotics.



**A potassium-stimulated vacuolar-type H<sup>+</sup>-pyrophosphatase from the hyperthermophilic bacterium *Thermotoga maritima***

Pérez-Castiñeira, J.R., López-Marqués, R.L., Losada, M., Serrano, A.

Instituto de Bioquímica Vegetal y Fotosíntesis (Universidad de Sevilla-CSIC)  
Avda Americo Vespucio s/n  
41092 Sevilla, Spain

Vacuolar type membrane-bound proton-translocation inorganic pyrophosphatases (V-PPases) belong to a recently identified category of proton pumps which utilize pyrophosphate hydrolysis as the driving force for H<sup>+</sup> movement across biological membranes. V-PPases have been identified in a wide variety of organisms such as higher plants, photosynthetic bacteria, archaeobacteria and parasitic protists. Current evidence suggest the occurrence of two types of protein distinguishable by their sensitivity to potassium. Thus, whereas potassium-insensitive V-PPases seem to occur both in eukaryotes and prokaryotes, potassium-stimulated proteins have been identified to date only in eukaryotes. In this communication, we report the isolation of a full-length gene coding for a vacuolar pyrophosphatase in the anaerobic hyperthermophilic bacterium *Thermotoga maritima* (TVP) and the functional characterization of the heterologously-expressed protein in yeast. TVP has an optimal temperature between 65 and 75°C and its activity is increased up to seven-fold by potassium. To our knowledge, this is the first report of a potassium-sensitive V-PPase in a prokaryotic organism. TVP seems to be resistant to yeast proteases and its thermostability depends on the binding of magnesium ions to the protein. Computer-assisted phylogenetic studies performed with sequences of V-PPases from different organisms consistently ascribed TVP to the potassium-stimulated family of V-PPases.

### Three efflux pumps are required to provide efficient tolerance to toluene in *Pseudomonas putida* DOT-T1E

A. Rojas, E. Duque, G. Mosqueda, G. Golden, A. Hurtado, J.L. Ramos and A. Segura

In *Pseudomonas putida* DOT-T1E multidrug efflux pumps of the RND family make a major contribution to solvent resistance. Two pumps have been identified, TtgABC, expressed constitutively, and TtgDEF induced by aromatic hydrocarbons. A double mutant in both efflux pumps was able to survive a sudden toluene shock if and only if preinduced with low amounts of toluene supplied via the gas-phase. We have identified and characterized a third efflux pump in this strain named TtgGHI. The *ttgGHI* genes form an operon that is expressed constitutively at high levels from a single promoter. In the presence of toluene the operon is expressed at an even higher level from two promoters, the constitutive one and another new overlapping inducible one. By site-directed mutagenesis we constructed a single mutant in TtgH, which was shown to be unable to survive sudden toluene shocks regardless of the preculture conditions. The mutation was transferred to the above described single and double mutant so that mutant strains were constructed where the two or the three pumps had been knocked out. Survival analysis of induced and non-induced cells revealed that the TtgABC and TtgGHI extrude toluene, styrene, m-xylene, ethylbenzene and propylbenzene, whereas the TtgDEF only pumps toluene and styrene. The triple mutant was hypersensitive to toluene since it was unable to grow with toluene supplied via the vapor phase. Southern blot and PCR analysis of total DNA from various pseudomonads showed that toluene-resistant strains possessed the three efflux pumps, whereas in the toluene sensitive ones, only one or two of these pumps were present.

## The projection structure of EmrE, a proton-linked multidrug transporter from *Escherichia coli*, at 7Å resolution

Christopher G. Tate<sup>1\*</sup>, Edmund R.S. Kunji<sup>1,2</sup>, Mario Lebendiker<sup>3</sup> and Shimon Schuldiner<sup>3</sup>

1. MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

2. MRC Dunn Human Nutrition Unit, Hills Road, Cambridge CB2 2XY, UK

3. Institute of Life Sciences, Givat Ram, Hebrew University, Jerusalem 91904, Israel

\* Corresponding author: email: [cgt@mrc-lmb.cam.ac.uk](mailto:cgt@mrc-lmb.cam.ac.uk)

EmrE belongs to a family of eubacterial multidrug transporters that confer resistance to a wide variety of toxins by coupling the influx of protons to toxin extrusion. EmrE was purified and crystallized in two-dimensions by reconstitution with dimyristoylphosphatidylcholine into lipid bilayers. Images of frozen hydrated crystals were collected by cryo-electron microscopy and a projection structure of EmrE was calculated to 7Å resolution. The projection map shows an asymmetric EmrE dimer with overall dimensions ~31Å x 40Å, comprising an arc of highly tilted helices separating two helices nearly perpendicular to the membrane from another two helices, one tilted and the other nearly perpendicular. There is no obvious two-fold symmetry axis perpendicular to the membrane within the dimer, suggesting that the monomers may have different structures in the functional unit.

## **Tripartite ATP-independent periplasmic (TRAP) transporters: an expanding group of functionally and structurally related systems**

Gavin Thomas

Characterisation of the Dct transport system for C4-dicarboxylates in *Rhodobacter capsulatus*, revealed a completely novel type of transport system (1,2). Essentially, this transport system is a secondary transporter that also has a requirement for an extracytoplasmic solute receptor protein; a component previously thought to only occur in the ABC family of primary transporters. These 'hybrid' transporters have three domains that were defined on the basis of the three proteins that constitute the DctPQM system in *R. capsulatus* (3). The DctP family are extracytoplasmic solute receptor proteins involved in substrate capture. The DctQ and DctM families are integral membrane proteins of 4 and 12 transmembrane helices, respectively.

Using an *in silico* approach we have defined an additional family of TRAP transporters which are the only type so far identified in archaea. These retain the tripartite nature except that the two integral membrane proteins are always fused (DctQM homologues). These systems have an extracytoplasmic solute receptor protein that is not homologous to the DctP family, which we have designated the TAXI protein family. We have also defined a subfamily of DctP homologues that potentially bind mannitol. Additionally we have identified genes encoding a novel protein family that are genetically linked to TRAP transporter operons. These are found in archaea, Gram negative and Gram positive bacteria (4).

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## Vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters in *Arabidopsis* and tomato

Kees Venema

Existence of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters in plants has been inferred from biochemical studies (1), and the first genes encoding putative vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters have been cloned recently (2,3). These genes belong to the Na<sup>+</sup>/H<sup>+</sup> exchanger family (PFAM-A PF00999). AtNHX1 can complement salt-sensitive phenotypes of NHX1 null mutants in yeast (2,4,5) and unregulated overexpression in *Arabidopsis* improves salt tolerance (6). However, a role in pH regulation or osmoregulation is also indicated (5,7). We purified the *Arabidopsis* AtNHX1 protein from yeast microsomes. A 6xHistidine tag was inserted in the C-terminal end which allowed purification using a resin with immobilized Ni<sup>2+</sup> ions (Ni-PDC). The purified protein will be used to show transport activity after reconstitution, as well as possible interactions, with other proteins. We also cloned two new isoforms of this gene from tomato (LeNHX2 and LeNHX3). These isoforms were expressed in shoots and roots, but very little in leaves. We did not observe increased expression by prolonged growth on NaCl, but are currently testing induction by short term NaCl treatment (osmotic shock). We expressed both genes in a yeast strain devoid of endogenous Na<sup>+</sup> transport systems (Den1-4, Dnha1, Dnhx1) to show complementation of the salt sensitive phenotype and to perform a biochemical characterization of the transport activities of the proteins. Finally we are investigating the expression levels and induction of these genes in salt sensitive and tolerant species of tomato and plan to make transgenic plants overexpressing histagged versions of these genes or antisense constructions.

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## Structure determination of secondary transport proteins by electron-cryo microscopy

C. Ziegler and W. Kühlbrandt

MPI of Biophysics, Department of Structural Biology, Heinrich-Hoffmann Strasse 7, D-60528 Frankfurt

Electron crystallography is a powerful tool to determine the structure of membrane proteins that form well ordered two- dimensional (2D) crystals. To produce high-quality 2D crystals the protein must be purified and solubilized in detergent. The pure protein can be reconstituted into a lipid bilayer by decreasing the detergent concentration in the presence of additional lipids. An alternative approach for 2D crystallization is the use of functionalized lipid monolayers where partially fluorinated Ni-NTA lipids form stable, detergent resistant monolayers at an air-water interface. The detergent solubilized His-tagged membrane protein attaches to the Ni-NTA complex. The protein is concentrated and orientated at the interface and large crystalline areas may form upon detergent removal.

In the last few years 2D crystals have been obtained for many membrane proteins yielding in projection maps at intermediate resolution of 6-9 Å. However, only a few of these have yielded structures at near atomic resolution in three dimensions.

NhaA, a sodium/proton antiporter of *E.coli* is the first ion-coupled membrane transport protein where a 3D structure was determined from 2D crystals using electron cryo-microscopy [1]. NhaA was expressed with a C-terminal His<sub>6</sub>-tag and purified by passing over a Ni<sup>2+</sup> -NTA column. 2D crystallization was performed by reconstitution into proteoliposomes. Large, tubular 2D crystals were obtained which diffract to better than 4 Å [2].

The size and the quality of the tubular NhaA crystals is found to be dependent on the characteristics of the head group and fluidity of the reconstituting lipids. By using synthetic phosphatidylcholine lipids with different fatty acid chains a crystal size of about 3 microns in width can be obtained.

The crystals are grown at pH but can be transferred to pH 6-9 where the protein is highly active without loss of order to investigate pH induced conformational changes.

NhaA was also crystallized directly at pH 8 using partially fluorinated Ni-NTA lipids. Image analysis has indicated a different unit cell and crystal packing compared to the low pH form. 2D crystals have been grown from fully active mutants with only one cysteine residue left at a defined location in the protein which allows labeling of this site with a heavy metal cluster. Based on the experiences with NhaA, 2D crystallization of other ion-coupled transport proteins is currently being performed.

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**LIST OF INVITED SPEAKERS**

- Neali Armstrong** Dept. of Biochemistry, Columbia Univ. 650 West 168th St., New York, NY. 10032 (USA). Tel.: 1 212 305 4062. Fax: 1 212 305 8174. E-mail: naal5@columbia.edu
- Peer Bork** EMBL and MDC, Meyerhofstr. 1, 69012 Heidelberg (Germany). Tel.: 49 6221 387 526. Fax: 49 6221 38 75 17. E-mail: Peer.Bork@EMBL-Heidelberg.de
- Andreas Engel** Biozentrum, Univ. of Basel, Klingelbergstrasse 70, 4056 Basel (Switzerland). Tel.: 41 61 267 2262. Fax: 41 61 267 21 09. E-mail: Andreas.Engel@unibas.ch
- Richard Henderson** MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH (UK). Tel.: 44 1223 402 215. Fax: 44 1223 249 565. E-mail: rh15@mrc-lmb.cam.ac.uk
- Carola Hunte** Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Str. 7, 60528 Frankfurt (Germany). Tel.: 49 69 96 76 93 89. Fax: 49 69 96 76 94 23. E-mail: Carola.Hunte@mpibp-frankfurt.mpg.de
- Wil N. Konings** Dept. of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, Univ. of Groningen, Kerklaan 30, 9751 NN Haren (The Netherlands). Tel.: 31 50 363 21 52. Fax: 31 50 363 21 54. E-mail: w.n.konings@biol.rug.nl
- Werner Kühlbrandt** Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Str. 7, 60528 Frankfurt am Main (Germany). Tel.: 49 69 96769 399. Fax: 49 69 96769 359. E-mail: kuehlbrandt@mpibp-frankfurt.mpg.de
- José López-Barneo** Dpto. de Fisiología Médica y Biofísica y Hospital Universitario Virgen del Rocío, Univ. de Sevilla. Avda. Sánchez Pizjuán, 4, 41009 Seville (Spain). Tel.: 34 95 455 17 68. Fax: 34 95 455 17 69. E-mail: lbarneo@cica.es
- Hartmut Luecke** Departments of Molecular Biology & Biochemistry and Physiology & Biophysics, 3205 Biological Sciences II, Univ. of California, Irvine, CA. 92697-3900 (USA). Tel.: 1 949 824 1605. Fax: 1 949 824 3280. E-mail: hudel@uci.edu
- Dean R. Madden** MPI for Medical Res. Jahnstr. 29, 69120 Heidelberg (Germany). Tel.: 49 6221 486 286. Fax: 49 6221 486 437. E-mail: madden@mpimf-heidelberg.mpg.de
-

- 
- Esteve Padrós** U. de Biofísica, Dept. de Bioquímica i de Biologia Molecular, Fac. de Medicina. Univ. Autònoma de Barcelona, 08193 Bellaterra, Barcelona (Spain). Tel.: 34 93 581 18 70. Fax: 34 93 581 19 07. E-mail: Esteve.Padros @uab.es
- Michael Gjedde Palmgren** Department of Plant Biology. The Royal Veterinary & Agricultural Univ. Thorvaldsensvej 40, 1871 Frederiksberg C, Copenhagen (Denmark). Tel.: 45 35 28 25 92. Fax: 45 35 28 33 33. E-mail: palmgren@biobase.dk
- Ramón Serrano** Instituto de Biología Molecular y Celular de Plantas. Universidad Politécnica de Valencia-C.S.I.C. Camino de Vera, 46022 Valencia (Spain). Tel.: 34 96 387 78 60. Fax: 34 96 387 78 59. E-mail: rserrano@ibmcp.upv.es
- Henning Stahlberg** Biozentrum. Univ. of Basel. Klingelbergstr. 70, 4056 Basel (Switzerland). Tel.: 41 61 267 09 49. Fax: 41 61 267 21 09. E-mail: Henning.Stahlberg @unibas.ch
- David L. Stokes** Skirball Institute. NYU School of Medicine. 540 First Ave, New York, NY. 10016 (USA). Tel.: 1 212 263 15 80. Fax: 1 212 263 15 80. E-mail: stokes@mcabi-34.med.nyu.edu
- Robert M. Stroud** Biochemistry Department. University of California, San Francisco, CA. 94143 (USA). Tel.: 1 415 476 4224. Fax: 1 415 476 1902. E-mail: stroud@msg.ucsf.edu
- Chris G. Tate** MRC Laboratory of Molecular Biology. Hills Road, Cambridge CB2 2QH (UK). Tel.: 44 1223 40 22 91. Fax: 44 1223 21 35 56. E-mail: cgt@mrc-lmb.cam.ac.uk
- Chikashi Toyoshima** Inst. of Molecular and Cellular Biosciences. The Univ. of Tokyo. 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032 (Japan). Tel.: 81 3 58 41 84 92. Fax: 81 3 58 41 84 91. E-mail: ct@iam.u-tokyo.ac.jp
- Catherine Vénien-Bryan** Lab. of Molecular Biophysics. South Parks Road, Oxford OX1 3QU (UK). Tel.: 44 1865 27 53 77. Fax: 44 1865 27 51 82. E-mail: venien@biop.ox.ac.uk
- John E. Walker** MRC-Dunn Human Nutrition Unit. Hills Road, Cambridge, CB2 2XY (UK). Tel.: 44 1223 25 27 01. Fax: 44 1223 25 27 05. E-mail: walker@mrc-dunn.cam.ac.uk
- Horst T. Witt** Max-Volmer-Inst. Technische Univ. Berlin. Str. d. 17. Juni 135, 10623 Berlin (Germany). Tel.: 49 30 314 22245. Fax: 49 30 314 21122. E-mail: pitts@struktur.chem. tu-berlin.de
-



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**LIST OF PARTICIPANTS**

- Armando Albert** Instituto Química Física Rocasolano (CSIC). Serrano 119, 28006 Madrid (Spain). Tel.: 34 91 561 94 00. Fax: 34 91 564 24 31. E-mail: xalbert@iqfr.csic.es
- Gregor Anderluh** University of Newcastle upon Tyne, NE2 4HH Newcastle upon Tyne (UK). Tel.: 44 191 222 7442. Fax: 44 191 222 7424. E-mail: gregor.anderluh@ncl.ac.uk
- Cécile Breyton** MPI für Biophysik. Heinrich Hoffmann Str., 7, 60528 Frankfurt am Main (Germany). Tel.: 49 69 96 769 357. Fax: 49 69 96 769 359. E-mail: Cecile.Breyton@mpibp-frankfurt.mpg.de
- Stéphanie Dambly** Université catholique de Louvain. Croix du Sud 2, bte 20, 1348 Louvain-la-Neuve (Belgium). Tel.: 32 10 45 36 18. Fax: 32 10 47 38 72. E-mail: dambly@fysa.ucl.ac.be
- Jens Dietrich** Max-Planck-Institut für Biophysik. Heinrich-Hoffmann-Str. 7, 60528 Frankfurt am Main (Germany). Tel.: 49 69 967 69357. Fax: 49 69 967 69359. E-mail: dietrich@mpibp-frankfurt.mpg.de
- Katrine Drumm** The Royal Veterinary and Agricultural University. Thorvaldsensvej 40, 1871 Copenhagen (Denmark). Tel.: 45 3528 2594. Fax: 45 3528 3365. E-mail: kd@staff.kvl.dk
- Antonio Felipe** Departaments de Bioquímica i Biologia Molecular. Univ. de Barcelona. Avda. Diagonal 645, 08071 Barcelona (Spain). Tel.: 34 93 403 46 16. Fax: 34 93 402 15 59. E-mail: afelipe@porthos.bio.ub.es
- Wendy Fernández-Ochoa** Instituto de Biología Molecular (CSIC). Jordi Girona, 18-26, 08034 Barcelona (Spain). Fax: 34 93 204 59 04. E-mail: wfocri@cid.csic.es
- Susanne Fischer** EMBL. Meyerhofstrasse 1, 69117 Heidelberg (Germany). Tel.: 49 6221 387 265. Fax: 49 6221 387 306. E-mail: sfischer@embl-heidelberg.de
- Anja T. Fuglsang** Biochimie et Physiologie Moleculaires des Plantes. UMR ENSA-M / INRA / CNRS, 34060 Montpellier cedex 1 (France)

- 
- M<sup>a</sup> Rosa Gómez-Villafuertes** Facultad de Veterinaria. Universidad Complutense de Madrid. Avenida Puerta de Hierro, s/n, 28040 Madrid (Spain). Tel.: 34 91 394 38 90. Fax: 34 91 394 39 09. E-mail: marosa@eucmos.sim.ucm.es
- Rosario Haro** Dpto. Biotecnología. E.T.S.I.Agrónomos. Univ. Politécnica de Madrid. Ciudad Universitaria s/n, 28040 Madrid (Spain). Tel.: 34 91 336 57 55. Fax: 34 91 336 57 57. E-mail: rharo@bit.etsia.upm.es
- Agustín Hernández** Instituto de Recursos Naturales y Agrobiología de Sevilla (CSIC). Dpto. Biología Vegetal. Avda. Reina Mercedes 10, Sevilla 41012 (Spain). Tel.: 34 95 462 47 11. Fax: 34 95 462 40 02. E-mail: ahernan@cica.es
- Eckhard Hofmann** Ion Channel Structure Group, Max Planck Institute for Medical Research. Jahnstrasse 29, 69120 Heidelberg (Germany). Tel.: 49 6221 486 154. Fax: 49 6221 486 437. E-mail: eckhard.hofmann@mpimf-heidelberg.mpg.de
- Chaim Kahana** Department of Molecular Genetics. Weizmann Institute of Science, 76100 Rehovot (Israel). Tel.: 972 8 9342745. Fax: 972 8 9344199. E-mail: chaim.kahana@weizmann.ac.il
- Dirk Linke** Max Volmer Institut für Biophysikalische Chemie und Biochemie. TU Berlin. Str. des 17. Juni 135, 10623 Berlin (Germany). Tel.: 49 30 31426403. Fax: 49 30 31421122. E-mail: linke@phosis1.chem.tu-berlin.de
- Luis Martínez-Martínez** Universidad de Sevilla. Avda. Sánchez Pizjuan s/n, 41080 Sevilla (Spain). Tel.: 34 95 500 82 87. Fax: 34 95 437 74 13. E-mail: lmartin@cica.es
- Mike Merrick** Department of Molecular Microbiology. John Innes Centre. Colney Lane, Norwich, NR4 7UH (UK). Tel.: 44 1603 45 0749. Fax: 44 1603 45 0778. E-mail: mike.merrick@bbsrc.ac.uk
- José M. Mulet** Instituto de Biología Molecular y Celular de Plantas, UPV-C.S.I.C.. Camino de Vera, 14, 46022 Valencia (Spain). Tel.: 34 96 387 78 60. Fax: 34 96 387 78 59. E-mail: jmmulet@ibmcp.upv.es
- George L. Orriss** Biozentrum. Klingelbergstrasse 70, 4056 Basel (Switzerland). Tel.: 41 61 267 2092. Fax: 41 61 267 2109. E-mail: George-Leighton.Orriss@unibas.ch
-

- 
- José R. Pérez-Castiñeira** Instituto de Bioquímica Vegetal y Fotosíntesis (Universidad de Sevilla-CSIC). Avenida Américo Vespucio s/n, 41092 Sevilla (Spain). Tel.: 34 95 448 95 24. Fax: 34 95 446 00 65. E-mail: jroman@cica.es
- José Ramos** ETSI Agrónomos y de Montes. Universidad de Córdoba. Apdo. 3.048, 14070 Córdoba (Spain). Tel.: 34 957 218 521. Fax: 34 957 218 563. E-mail: mi1raruj@uco.es
- Antonia Rojas** Estación Experimental del Zaidín (CSIC). Apdo. 419, 18080 Granada (Spain). Tel.: 34 958 12 10 11. Fax: 34 958 12 96 00. E-mail: antoniarojas@hotmail.com
- Roc Ros** Fac. de Ciencias Biológicas. Univ. de Valencia. Doctor Moliner, 50, 46100 Burjassot, Valencia (Spain). Tel.: 34 96 386 46 34. Fax: 34 96 386 43 72. E-mail: roc.ros@uv.es
- Aurelio Serrano** Instituto de Bioquímica Vegetal y Fotosíntesis (CSIC). Universidad de Sevilla. Américo Vespucio s/n, 41092 Sevilla (Spain). Tel.: 34 95 448 95 24. Fax: 34 95 446 00 65. E-mail: aurelio@cica.es
- Gavin H. Thomas** Univ. of Sheffield. Firth Court, Western Bank, S10 2TN Sheffield (UK). Tel.: 44 1142 222 846. Fax: 44 1142 728 697. E-mail: g.h.thomas@sheffield.ac.uk
- Andrea Urbani** European Molecular Biology Laboratory (EMBL), Struct. Biology Programme. Meyerhofstr. 1, 69012 Heidelberg (Germany). Tel.: 49 6221 387 270. Fax: 49 6221 387 519. E-mail: Andrea.Urbani@EMBL-Heidelberg.de
- Kees Venema** Estación Experimental del Zaidín (CSIC). Apdo. 419, 18008 Granada (Spain). Tel.: 34 958121011. Fax: 34 958 129 600. E-mail: kev@eez.csic.es
- Alvaro Villarroel** Instituto Cajal (CSIC). Avda. Dr. Arce 37, 28002 Madrid (Spain). Tel.: 34 91 585 47 18. Fax: 34 91 585 47 54. E-mail: av@cajal.csic.es
- Christine Ziegler** MPI of Biophysics, Department of Structural Biology. Heinrich-Hoffmann Strasse 7, 60528 Frankfurt (Germany). Tel.: 49 69 96 769371. Fax: 49 69 96 769 359. E-mail: Ziegler@mpibp-frankfurt.mpg.de
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