Instituto Juan March
de Estudios e Investigaciones

79 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Membrane Protein Insertion, Folding and Dynamics

Organized by

J. L. R. Arrondo, F. M. Goñi, B. de Kruijff
and B. A. Wallace

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INTRODUCTION

J. L. R. Arrondo, F. M. Goñi, B. de Kruijff and B. A. Wallace

Membrane protein insertion, folding and dynamics

Biomembranes have been the object of considerable attention from the point of view of biophysical chemistry for the last three decades. Model membranes consisting of pure lipids were first developed, and integral membrane proteins were later incorporated, or "reconstituted", into lipid bilayers in the seventies. Although much effort was applied in those years to the study of lipid-protein interactions in membranes, these were treated as pre-existing, almost immutable entities. Membrane proteins could be either integral or peripheral; there were as well soluble proteins, unrelated to membranes.

In the last years, however, we are becoming increasingly aware of the dynamic aspects of membrane-protein interactions, in particular in the process of protein insertion into lipid bilayers. From many fields of molecular cell biology we are being confronted with proteins that behave at some stage as "soluble", then, after an appropriate stimulus, they get inserted into the lipid matrix. The question is no longer how lipids and proteins interact in membranes, but rather what is the conformational change that allows the protein to perform the transformation from a water-soluble to a lipid-soluble species, and what is the stimulus that triggers such a change. Other relevant questions include: to what extent do all proteins follow a similar insertion pathway, and what the protein folding mechanisms in aqueous and lipidic environments have in common.

The problem to be dealt with in the workshop had arisen simultaneously in various areas of biology, membrane biogenesis, protein folding, protein toxins and membrane fusion among others. This multiplicity of problems, with great chances of having, at least in part, a common answer, called for a joint reflection by scientists the various field involved.

But the problem of membrane insertion, folding and dynamics of proteins is a multi-faceted one also from the methodological point of view: cell physiology and pathology, protein engineering, structural biology and membrane biophysics all have a significant share in the strategy of its unraveling. Though mainly focused on the biophysical aspects, our programme intended to reflect this complexity in the variety of expertises of the invited participants. In turn, this heterogeneity of presentations appeared to be essential for a global, multidisciplinary approach to the subject.

Biophysical, molecular and cellular approaches are being combined in the study of processes that belong to membrane biogenesis, cell physiology or cell pathology. As opposed to the static view of twenty years ago, cell membranes are now seen as dynamic structures, continuously being synthesized in the endoplasmic reticulum, then processed into various organelles following the intracellular "traffick", and finally dismantled in the lysosomes, the whole process being the object of a finely tuned regulation. Intracellular membrane traffick ensures the correct distribution of membranes into the proper organelles, a process that is directly linked to the biosynthesis and insertion of the appropriate proteins in each case. Insertion is now known to be initiated by a combination of "signals" residing in the sequence of the nascent peptide. In vitro studies with synthetic peptides have been instrumental in the progress of our knowledge in this field.

Much less is known, unfortunately, of the folding of intramembranous proteins inside the hydrophobic bilayer, in spite of important insights coming out from in vitro and model studies, as summarized in the following reports. In particular, the insertion and folding of the large beta-barrels that are found in the various porins are still posing a difficult problem.

In some cases, cell physiology requires the translocation of a protein across a cell membrane. This is the case of some forms of protein secretion, or of mitochondria and chloroplast biogenesis (with some polypeptides being synthesized in the cytoplasm, others being encoded by mitochondrial/chloroplast DNA), or of the periplasmic and outer membrane proteins in gram-negative bacteria. As in other aspects of biology, convergent and divergent evolutionary phenomena appear to coexist, with hints of common patterns being discerned even in apparently dissimilar processes.

Cytotoxicity is another clear example of a phenomenon in cell biology that is conspicuous by the variety of its forms, although some common threads may be uniting many of them. Many phenomena leading ultimately to cell death start with the insertion of a protein in the target cell membrane. This occurs with the lysis of eukaryotic cells by bacterial toxins, either acting at the membrane level or through interference with intracellular mechanisms. But complement-mediated bacterial lysis requires equally the insertion of a complement subunit in the bacterial membrane. Several instances of viral infections and pathogenesis appear as well to be related to an initial event of peptide insertion into a host bilayer.

The reader will appreciate from the pages that follow that not only invited papers but also free communications presented in this Workshop, from a large variety of experimental systems and methodological approaches, contributed to enrich our view of this particular aspect of molecular cell biology, namely the insertion, folding and dynamics of membrane proteins. While many of the questions and enigmas are still to be solved, the Workshop did certainly succeed in reshaping some of our questions and putting certain enigmas under a new perspective.

José Luis R. Arrondo
Félix M. Goñi
Ben de Kruijff
Bonnie Wallace

Session I: Methods in membrane protein folding & dynamics

Chairs: Paula J. Booth
Anthony Watts

Solid state NMR

Anthony Watts,
Biomembranes Structure Unit, Department of Biochemistry,
South Parks Road, Oxford, UK

Biomembranes provide a complex environment which supports and stabilizes protein structure. Additionally, there are dynamic processes covering a wide range of timescales that couple to function. Significant gaps exist in our current understanding of both structure and dynamics of biomembranes and solid state NMR is a method with potential to fill some of those gaps.

We have devised a totally new method for resolving peptide and protein structure from previously unresolvable 1H-NMR spectra of biomembranes [Glaubitz, C. & Watts, A. (1998) J. Mag Res (in press)]. Initial studies show that 1H-NMR spectra of peptides (M13 coat protein, phospholamban) and proteins (rhodopsin, bacteriorhodopsin) can be analyzed using conventional correlation spectroscopy methods for the first time. This approach now opens up the way for structure determinations of any peptide and protein without the need for isotopic enrichment.

ANALYSIS OF PROTEIN STRUCTURE AND DYNAMICS WITH SITE DIRECTED SPIN LABELING

Wayne L. Hubbell, University of California, Los Angeles, CA, USA

Site-directed spin labeling (SDSL) is emerging as a powerful tool for determination of structure and dynamics in water soluble and membrane proteins of arbitrary size[1]. The method relies on the introduction of a nitroxide side chain at specific sites using molecular genetic techniques. The electron paramagnetic resonance (EPR) spectrum of a labeled protein can be interpreted quantitatively in terms of the side chain solvent accessibility and mobility, features that permit assignment of the side chain to one of four topographical locations in the protein fold: loops, surfaces of regular secondary structure, solvent-exposed tertiary contact sites or buried sites[1,2]. Evaluation of accessibility and mobility at multiple sites in a continuous sequence provides a means of identifying sequence-correlated secondary structure and the orientation of the secondary structural element in the fold[3] and, for membrane proteins, the depth of a side chain relative to the membrane-aqueous interface[4]. Finally, introduction of two nitroxide side chains[5-7], or the introduction of a metal ion and a nitroxide[8] allows determination of inter-residue distances through analysis of the magnetic dipole-dipole interaction.

Taken together, the above information provides a sufficient number of constraints to permit critical evaluation of models of a protein fold. In addition to determination of static structure, SDSL can be employed to reveal dynamic features of the protein, for example fluctuations about the equilibrium structure in the nanosecond regime, and real-time conformational changes associated with function in the millisecond regime. Recent applications to membrane proteins include the investigation of membrane binding and insertion processes (diphtheria toxin[9], colicin E1 and annexin XII), structure and light-activated conformational changes in retinal proteins (rhodopsin[10-12], SRI and bacteriorhodopsin[13]) structure of a bacterial K^+ channel, and structure of the lac permease transporter[14]. Examples from these systems will be used to illustrate the capabilities of the SDSL method.

References

[1]Watching your protein move using site-directed spin labeling. W. Hubbell, H. Mchaourab, C. Altenbach, M. Lietzow *Structure*, 4: 779-783 (1996).

[2]Motion of spin labeled side chains in T4 lysozyme. Correlation with protein structure and dynamics. H.S. Mchaourab, M. Lietzow, K. Hideg, W. Hubbell *Biochemistry 35:7692-7704* (1996).

[3]Transmembrane protein structure: spin-labeling of bacteriorhodopsin mutants. C. Altenbach, T. Marti, H. Khorana, and W. Hubbell *Science* 248:1088-1092 (1990)

[4]A collision gradient method to determine the immersion depth of nitroxides in lipid bilayers: Application to spin labeled mutants of bacteriorhodopsin. C. Altenbach, D. Greenhalgh, H.G. Khorana, W.L. Hubbell *Proc. Natl. Acad. Sci. 91:* 1667-71 (1994).

[5]Hustedt, E., Hustedt EJ; Smirnov AI; Laub CF; Cobb CE; Beth AH. Molecular distances from dipolar coupled spin-labels: the global analysis of multifrequency continuous wave electron paramagnetic resonance data. Biophysical Journal, 1997 Apr, 72(4):1861-77.

[6]Rabenstein and Shin,Rabenstein MD; Shin YK. Determination of the distance between two spin labels attached to a macromolecule. Proceedings of the National Academy of Sciences of the United States of America, 1995 Aug 29, 92(18):8239-43.

[7]The solution structure of T4 lysozyme: Hinge bending motion and substrate-induced conformational transition H.S. Mchaourab, K. J. Oh, C. J. Fang, W. Hubbell *Biochemistry* 36:307-316 (1997).
[8]Distance determination in proteins using designed metal ion binding sites and site-directed spin labeling: II. Application to the lactose permease of Escherichia coli. J. Voss, W. Hubbell, H.R. Kaback *Proc. Natl. Acad. Sci.* 92:12300-12304 (1995).
[9]Organization of the T domain of diphtheria toxin in membranes: a site-directed spin labeling study. K.J. Oh, H. Zhan, C. Cui, K. Hideg, R.J. Collier, W. Hubbell *Science* 273:810-812 (1996).
[10]Requirement of rigid-body motion of transmembrane helices for light activation of rhodopsin. D. Farrens, C. Altenbach, K. Yang, W. Hubbell, H.G. Khorana *Science* 274:768-770 (1996).
[11]Photoactivated conformational changes in rhodopsin:a time-resolved spin label study. Z. Farahbakhsh, K. Hideg, Hubbell WL *Science*, 262:1416-1420 (1993).
[12]Structural features and light-dependent changes in the cytoplasmic interhelical E-F loop region of rhodopsin: a site-directed spin labeling study. C. Altenbach, K. Yang, D. Farrens, Z.T. Farahbakhsh, H.G. Khorana, W. Hubbell *Biochemistry* 35:12470-12478 (1996).
[13]Time resolved detection of structural changes during the photocylce of spin labeled bacteriorhodopsin. H. Steinhoff, R. Mollaaghababa, C. Altenbach, K. Hideg, M. Krebs, H. Khorana, W. Hubbell *Science*, 266:105-107 (1994).
[14]Site-directed spin labeling demonstrates that transmembrane XII in the lactose permease of Escherichia coli is an α-helix. J. Voss, M. He, W. Hubbell, H.R. Kaback *Biochemistry* 35:12915-12918 (1996).

Circular Dichroism Studies of Membrane Proteins: Methods for Examining Structure, Function and Folding

B.A. Wallace
Dept. of Crystallography, Birkbeck College, University of London
London, U.K. WC1E 7HX

Circular dichroism (CD) is a valuable method for examining the structure, function and folding of membrane proteins. However, because membrane proteins are insoluble in aqueous solutions and require associated lipid or detergent molecules for stability, there are a number of potential artifacts associated with the particulate nature and electronic characteristics of their non-aqueous lipid environment that must be considered when measuring and interpreting their CD spectra. These include: absorption flattening (1,2), differential scattering (3), and wavelength shifts (4,5). The consequences of each of these effects will be discussed, as well as methods for overcoming them.

Examples will be presented of the use of CD spectroscopy on membrane proteins, including: a) determinations of secondary structures (and their utility for modelling studies) (6, 7, 8); b) examination of the insertion and folding of membrane proteins and peptides in phospholipid bilayers (9, 10); c) detection and quantitation of conformational changes associated with ligand binding (and their utility for mechanistic studies of function) (11); d) determination of the influence of solvent and additive effects on conformation (and the relevance of these environments for other structural studies - ie. crystallization) (12); and e) examination of the effects of chemical modification and proteolytic cleavage on the structures.

References:

1. Wallace, B.A. and Teeters, C.L. (1987) Differential Absorption Flattening Optical Effects are Significant in the Circular Dichroism Spectra of Large Membrane Fragments. Biochemistry 26, 65-70.

2. Teeters, C.L., Eccles, J., and Wallace, B.A. (1987) A Theoretical Analysis of the Effects of Sonication on Differential Absorption Flattening in Suspensions of Membrane Sheets. Biophysical Journal 51, 527-532.

3. Swords, N.A. and Wallace, B.A. (1993) Circular Dichroism Analyses of Membrane Proteins: Examination of Environmental Effects on Bacteriorhodopsin Spectra. Biochemical Journal 289, 215-219.

4. Cascio, M. and Wallace, B.A. (1995) Effects of Local Environment on the Circular Dichroism Spectra of Polypeptides. Analytical Biochemistry 227, 90-100.

5. Chen, Y. and Wallace, B.A. (1997) Secondary Solvent Effects on the Circular Dichroism Spectra of Polypeptides: Influence of Polarisation Effects on the Far Ultraviolet Spectra of Alamethicin. Biophysical Chemistry 6, 65-74.

6. Tobkes, N., Wallace, B. A., and Bayley, H. (1985) Secondary Structure and Assembly Mechanism of an Oligomeric Channel Protein. Biochemistry 24, 1915-1920.

7. Mielke, D.L. and Wallace, B.A. (1988) Secondary Structural Analyses of the Nicotinic Acetylcholine Receptor as a Test of Molecular Models. J. Biol. Chem. 263, 8177-8182.

8. Cascio, M., Gogol, E. and Wallace, B.A. (1990) The Secondary Structure of Gap Junctions: Influence of Isolation Methods and Proteolysis. J. Biol. Chem. 265, 2358-2364.

9. Woolley, G.A. and Wallace, B.A. (1993) Temperature-Dependence of the Interaction of Alamethicin Helices in Membranes. Biochemistry 32, 9819-9825.

10. Riley, M.L., Wallace, B.A., Flitsch, S.L., and Booth, P.J. (1997) Slow alpha helix formation during folding of a membrane protein. Biochemistry 36, 192-196.

11. Chen, Y. And Wallace, B.A. (1996) Binding of Alkaline Cations to the Double-Helical Form of Gramicidin. Biophysical Journal 71, 163-170.

12. Cascio, M. and Wallace, B.A. (1988) Conformation of Alamethicin in Phospholipid Vesicles: Implications for Insertion Models. Proteins - Structure Function and Genetics 4, 89-98.

INFRARED SPECTROSCOPY

José Luis R. Arrondo. Departamento de Bioquímica. Universidad del País Vasco. Apdo. 644. Bilbao. Spain

Infrared spectroscopy has proved in the past few years to be a practical tool in the study of protein structure. Even if the enormous number of normal modes causing overlapping absorption bands does not allow the extraction of detailed molecular information from the infrared spectra, the intrinsic order of repeating units provide some of the desired information on structure and interactions among subunits. Biological membranes are composed of lipids and proteins arranged in a two-dimensional array. These components give rise to separate bands in the IR spectrum allowing simultaneously the study of the structure of lipids and proteins even in intact biological membranes.

Infrared spectroscopy can study the conformation of proteins in lipid bilayers through decomposition of the amide I band. The presence of α-helix is dominated by a band around 1650 cm-1 whereas ß-sheet give rise to two bands located at 1630 and 1675-1690 cm-1 (D_2O or H_2O). One of the difficulties encountered in the application of infrared spectroscopy in the study of protein structure is the assignment of the observed bands to structural features. Because of the sensitivity of the infrared bands to changes in hydrogen bonding and dihedral angles, band assignment is made attributing spectral intervals to the same structure. Recently, we have shown that changes in the canonical wavenumber of infrared bands, are due to different environments or changes in tertiary structure. The work presented deals with the structure and assignment of two membrane proteins: cytochrome *c* oxidase from *P.denitrificans*

and sarcoplasmic reticulum ATPase. The use of temperature allows to detect an anomalous behavior in both proteins. Thermal denaturation is not a two-state process, but presents an intermediate conformation before denaturation that is associated to oligomer interactions. The use of temperature and limited proteolysis allows to detect specific features of proteins, such an α-helix structure coupled with β-sheet.

Suggested Readings

1. Susi, H. 1969. Infrared Spectra of Biological Macromolecules and Related Systems. In Structure and Stability of Biological Macromolecules. S. N. Timasheff and L. Stevens, editors. Dekker, New York. 575-663.

2. Arrondo, J. L. R., A. Muga, J. Castresana, and F. M. Goñi. 1993. Quantitative studies of the structure of proteins in solution by Fourier-transform infrared spectroscopy. *Prog. Biophys. Mol. Biol.* 59:23-56.

3. Haris, P. I. and D. Chapman. 1993. Fourier transform infrared spectroscopy as a probe for the study of the structure of membrane proteins. *Biochem. Soc. Trans.* 21:9-15.

4. Echabe I, Haltia T, Freire E, Goñi FM, Arrondo JLR. 1995. Subunit III of cytochrome *c* oxidase influences the conformation of subunits I and II: An infrared study. Biochemistry, 34:13565-13569.

5. Arrondo JLR, Blanco FJ, Serrano L, Goñi FM. 1996. Infrared evidence of a β-hairpin peptide structure in solution. FEBS Lett, 384:35-37.

6. Echabe I, Arrondo JLR. 1997. Structural anlysis of proteins by infrared spectroscopy. In: Pifat-Mrzljak G., ed. Supramolecular Structure and Function 5. Rehovot: Balaban Publishers, pp. 9-23.

TITLE: Molecular Dynamics of Membrane Proteins

B. Roux, Physics and Chemistry Depts, University of Montreal.

Molecular dynamics is a powerful theoretical approach to gain insight into the structure and dynamics of complex biological macromolecular systems. It consists in calculating the position of all the atoms in a molecular system as a function of time, using detailed models of the microscopic forces operating between them, by integrating numerically Newton's classical equation of motion F=MA. The calculated classical trajectory, though an approximation to the real world, provides ultimate detailed information about the time course of the atomic motions, which is difficult to access experimentally. In this presentation, I will describe the main qualitative features observed from computer simulations of two systems: the gramicidin channel (GA) and melittin in the environment of a phospholipid bilayer membrane.

The location of the main binding site for sodium in the GA channel is investigated with molecular dynamics simulations using an atomic model of the channel embedded in a fully hydrated dimyristoyl phosphatidycholine (DMPC) bilayer. The results are compared with the carbonyl 13C chemical shift anisotropy solid-state NMR experimental data of Smith et al, Biochim. Biophys. Acta, 1026:161 (1990) obtained with oriented GA:DMPC samples. Predictions are made for other solid-state NMR properties that could be observed experimentally. The combined information from experiment and simulation strongly suggests that the main binding sites for sodium are near the channel's mouth, approximately 9.2 Angs from the center of the dimer channel, in good agreement with electrophysiological current-voltage measurements. The 13C chemical shift anisotropy of Leu10 is the most affected by the presence of a sodium ion in the binding site. Molecular dynamics trajectories of melittin in an explicit dimyristoyl phosphatidylcholine (DMPC) bilayer are generated to study the details of lipid-protein interactions at a microscopic level. Melittin, a small amphipatic peptide found in bee venom, is known to have a pronounced effect on the lysis of membranes. The peptide is initially set parallel to the membrane-solution interfacial region in an alpha-helical conformation with unprotonated N-terminus. Solid-state nuclear magnetic resonance (NMR) and polarized attenuated total internal reflectance Fourier-transform infrared (PATIR-FTIR) properties of melittin are calculated from the trajectory to characterize the orientation of the peptide relative to the bilayer. The residue Lys7 located in the hydrophobic moiety of the helix and residues Lys23, Arg24, Gln25 and Gln26 at the C-terminus hydrophilic form hydrogen bonds with water molecules and with the ester carbonyl groups of the lipids, suggesting their important contribution to the stability of the helix in the bilayer. Lipid acyl chains are closely packed around melittin, contributing to the stable association with the membrane. The influence of the protonation state of the N-terminus of melittin on the stability of the protein-membrane complex is examined.

REFERENCES:

Biomolecular Membranes. A molecular perspective from computation and experiment, K.M. Merz and B. Roux, editors, Birkhauser: Boston, 1996.

Woolf, T.B.; Roux, B., Proc. Natl. Acad. Sci. (USA) 1994, 91:11631-11635.

Woolf, T.B.; Roux, B., PROT. Struc. Funct. Gen. 1996, 24:92-114.

Woolf, T.B.; Roux, B., Biophys. J. 1997, 72:1930-1945.

Channels across bacterial membranes: Dynamics of solute translocation

Jurg P. Rosenbusch, Biozentrum, University of Basel, CH-4056 Basel, <rosenbusch@ubaclu.unibas.ch>

Three translocation systems will be addresses: a voltage-gated and a solute-specific channel across bacterial outer membranes, OmpF-porin and maltoporin, and a proton translocation machinery, bacteriorhodopsin. OmpF-porin was the first membrane protein obtained as three-dimensional crystals diffracting to high resolution (1). Its structure has since then been solved to 2.4 Å (2) and many site-specific mutations have been constructed (3,4). The mechanism of channel closure was modelled by molecular dynamics (5), and the proposal tested by constructing, on the basis of the coordinates, a disulfide bridge. The channel dynamics of this construct allowed that proposal to be ruled out (6), favouring multiple changes in the distribution of fixed charges. The potentials and problems of this approach will be discussed.

Maltoporin (7) bind, and catalyzes the translocation, of maltose and malto-oligosaccharides (8, 9). A greasy slide and two ionic tracks suggest, in conjunction with molecular modelling, a mechanism of translocation. Both of the porins just mentioned are highly stable transmembrane trimers and contain a β-barrel in each monomer. Most of the receptors and proteins involved in signal transduction, as well as permeases, are, however, very labile. Instead of using crystallization from detergent solutions, as they were used for the porins, we have devised a novel approach using lipidic cubic phases, which form continuous membranes in three dimensions. We have crystallized bacteriorhodopsin with this approach and attained a resolution (2.4Å), allowing the mechanism of proton translocation to be deduced (10). The potentials of this methodology (11) to other membrane proteins will be discussed.

References:

(1) Garavito, R.M. and Rosenbusch, J.P. (1980) J. Cell. Biol. **86**, 327-329.
(2) Cowan, S.W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R.A., Jansonius, J.N. and Rosenbusch, J.P. (1992) Nature **358**, 727-733).
(3) Lou, K.-L., Saint, N., Prilipov, A., Rummel, G., Benson, S.A., Rosenbusch, J.P. & Schirmer, T.(1996) J. Biol. Chem. **271,** 20669-20675.
(4) Phale, P., Schirmer, T., Prilipov, A., Lou, K.-L., Hardmeyer, A. and Rosenbusch, J. P. (1997) Proc. Natl. Acad. Sci. USA, **94**, 6741-6745.
(5) Watanabe, M., Rosenbusch, J.P., Schirmer, T., and Karplus, M. (1997) Biophys. J., **72**, 2094-2102.
(6) Phale, P., Schirmer, T., Prilipov, A., Lou, K.-L., Hardmeyer, A. and Rosenbusch, J. P. (1997) Proc. Natl. Acad. Sci. USA, **94**, 6741-6745.
(7) Schirmer, T., Keller, T.A., Wang, Y.-F. and Rosenbusch, J.P. (1995) Science **267**, 512-514.
(8) Dutzler, R., Wang, Y.-F., Rizkallah, P.J., Rosenbusch, J.P. & Schirmer, T. (1996) Structure **4**, 127-134.
(9) Wang, Y.-F., Dutzler, R., Rizkallah, P.J., Rosenbusch, J.P., and Schirmer, T. (1997) J. Mol. Biol., **272,** 56-63.
(10) Pebay-Peyroula, E., Rummel, G., Rosenbusch, J.P., and Landau, E.M. (1997) Science, **277**, 1676-1681.
(11) Rummel, G., Hardmeyer, A., Locher, K., Pedruzzi, I., Landau, E.M., and Rosenbusch, J.P. (1998) J. Struct. Biol., in press.
Pebay-Peyroula, E., Rummel, G., Rosenbusch, J.P., and Landau, E.M. (1997) Science, **277**, 1676-1681.

Session II: Membrane protein folding and assembly

Chairs: Anthony Watts

Gunnar von Heijne

Ben de Kruijff

Bonnie A. Wallace

Basic structural properties of transmembrane helices

Gunnar von Heijne
Department of Biochemistry, Stockholm University, S-106 91 Stockholm, Sweden

The membrane-embedded domain of most integral membrane proteins seems to be composed of a tightly packed bundle of transmembrane helices. The number of known high-resolution membrane protein structures is getting large enough to allow certain general architectural characteristics of such helix bundles to be defined, including the distribution of different types of amino acid both in the direction perpendicular to the membrane and between lipid-exposed and protein-facing locations. Novel experimental techniques also open up possibilities to obtain quite detailed structural information in cases where X-ray crystallography has been unsuccessful. We are currently exploring the possibilities to use a new "glycosylation mapping" technique to study the location and conformation of transmembrane segments in the lipid bilayer.

References

Wallin, E., Tsukihara, T., Yoshikawa, S., von Heijne, G. and Elofsson, A. (1997) Architecture of helix bundle membrane proteins: An analysis of cytochrome c oxidase from bovine mitochondria. Protein Sci.6, 808-815.

Abstract for the Madrid meeting, 1998

The Interplay of Structure and Energy In the Dimerization of Glycophorin Transmembrane Helices

D. M. Engelman, Z. Bu, K. G. Fleming, K. R. MacKenzie, and W.T. Russ
Department of Molecular Biophysics and Biochemistry
Yale University, New Haven, Connecticut

In biochemical studies of the folding and function of soluble proteins, great insights have been gained by combining structural and energetic views. In the case of membrane proteins, this approach has proven more difficult, since the insolubility of these molecules has limited the application of traditional methods of investigation. Our studies of the dimerization of the transmembrane helical region of human Glycophorin A (GpA) have, therefore, required the adaptation of some traditional approaches and the development of some new methods to move us toward a view of the dimerization event in terms of physical and organic chemistry.

We have now reached the point where we know the structure of the dimer and have several kinds of energy measurements. This allows us to tentatively conclude that the specific interaction of the helices depends mainly on the creation of a closely packed interface in which Van der Waals attractions and rotamer restrictions are the major terms. The large number of mutant sequences created and characterized by Lemmon (1992a,b) provides a rich database that we use to develop and test our ideas. In our presentation, we will describe the methods and findings leading to our current view of the chemistry of glycophorin dimerization.

The structure of the helix dimer has been determined in dodecylphosphocholine micelles using solution NMR (MacKenzie et al, 1997). It reveals a right handed crossing of two straight, parallel helices with axes at a crossing angle of about 40 degrees. The interface between them is closely packed, involving contacts between the polypeptide backbones as well as between side chain atoms. The motif described in the mutation studies, LIxxGVxxGVxxT, forms the interacting surface. The structure is close to the model derived from a combination of the mutagenesis data and molecular dynamics simulations (Adams et al., 1996).

Using the structure, an approach to assessing the roles of different energy factors in the dimerization was developed (MacKenzie and Engelman, 1998). The mutation data, in which the degree of disruption of dimerization seen on SDS gels is presented in a four value scale, was correlated with simplified representations of the steric clashes, attractive Van der Waals interactions, rotamer restrictions, hydrophobicity, and sidechain volume changes resulting from each mutation. No correlations were found with changes in sidechain volume or hydrophobicity, but a good relationship was seen between mutational effects on dimerization and the other variables. To test the predictive power of the method, it was applied to the recent alanine insertion data of von Heijne (Mingarro et al., 1996,1997), and an excellent correlation was found.

To measure the actual association energies and changes resulting from mutations, we are pursuing four approaches. Equilibrium ultracentrifugation in a neutrally buoyant detergent has given excellent data on the wild-type dimer and on two mutants (Fleming et al., 1997), and the free energy differences resulting from the mutations are found to correlate well with the differences seen in the analysis described above. Studies using energy transfer measurements will be reported elsewhere in the meeting (L. Fischer). Small angle x-ray scattering has been used to follow the temperature dependence of the association in detergent micelles. Finally, an assay of association in bacterial membranes using genetic reporter methods has been developed. Continued use of these methods combined with redesign of the dimer should allow critical tests of ideas about the key chemical factors in helix interactions in lipid environments, which in turn should lead to a better understanding of membrane protein folding and function.

We thank the National Institutes of Health and the National Science Foundation for support of our work.

References:

Adams, P. D., Engelman, D. M. & Brunger, A. T. (1996) Proteins 26:257-261.

Fleming, K. G., Ackerman, A. L. &Engelman, D. M. (1997) J. Mol. Biol. 272:266-275.

Lemmon, M. A., Flanagan, J. M., Hunt, J. F., Adair, B. A. Bormann, B. J., Dempsey, C. E. & Engelman, D. M. (1992a) J. Biol. Chem. 267:7683-7689.

Lemmon, M. A., Flanagan, J. M., Treutlein, H. R., Zhang, J., & Engelman, D. M. (1992b) Biochemistry 31:12719-12725.

MacKenzie, K. R., Prestegard, J. H., and Engelman, D. M. (1997) Science 276:131-133.

MacKenzie , K. R.& Engelman, D. M. (1998) Proc. Nat. Acad. Sci. In press.

Mingarro, I., Whitley, P., Lemmon, M. &von Heijne (1996) Protein Sci. 5:1339-1341.

Mingarro, I., Elofsson, A. & von Heijne, G. (1997) J. Mol. Biol. 272:633-641.

Proliferation of intracellular membranes in *Escherichia coli* upon overexpression of F_0 subunits of ATP synthase

I. Arechaga, B. Miroux, S. Karrasch, M. J. Runswick and J. E.Walker

MRC-LMB. Hills Road. Cambridge. UK.

The most widely used expression system for soluble proteins is *Escherichia coli.* However, it has been used much less extensively as a host for expressing membrane proteins. In this work we report that the overexpression of the membrane subunits b and c of the *E.coli* F- ATPase complex in C41(DE3) and C43 (DE3) mutant host cells (1) induces proliferation of intracellular membranes in the bacteria. We have isolated and characterizated the new membranes, determined lipid/protein ratios and analyzed phospholipid compositions. We have observed that the structural features of the overexpressed protein plays an important role in the amount and morphology of the new membranes. The b subunit has a hydrophobic segment of 30 residues at the amino terminus, but the rest is rich in polar charged groups. Overexpression at high levels of the subunit b induces the formation of a network of tubule-like structures in the cytoplasm of the bacteria. Subunit c is a very hydrophobic protein that forms an oligomer of 10-12 subunits in the native complex. Each monomer consists of a hairpain of two α-helixes. Although overexpression at high levels of subunit c induces proliferation of internal membranes, the amount and morpholopy of them was different that the ones observed in subunuit b overproducing cells. We believe that the lack of an extramembrane domain in subunit c could explain why no network of tubule-like structures has been observed in this case. We have produced truncated forms of subunit b in order to establish which were the structural minimal requirements neccesary to obtain the desired phenotype. We have concluded that a short transmembranar segment and an extramembrane domain rich in basic charges, in a coiled-coil structure is essential for the maximal production of membranes. This topology is very similar to the one observed in a general class of vesicle-associated proteins with a cannonical structure and topology known as SNAREs, a category of proteins that bind α–SNAP receptors. On the other hand, the general correlation between the location of positively charged amino acids and membrane protein topology -the 'positive inside' rule (von Heijne, 1994)- is now well established.

(1) Miroux, B. and Walker J. E. (1996) J. Mol. Biol. **260**, 289-298.

DjlA: a chaperone protein in the inner membrane of bacteria with an unusual transmembrane domain.

David Clarke, Christine Toutain, Annick Jacq and I. Barry Holland
Institut de Génétique et Microbiologie, Bâtiment 409, Universite Paris-Sud,
91405 Orsay cedex, FRANCE

DjlA was originally isolated, in our laboratory, as a multicopy suppressor of a mutation conferring resistance to the anticalmodulin drug, W7, in *Escherichia coli*. Sequencing of this gene revealed that the predicted amino acid sequence of DjlA had the following notable features:
1) a putative N-terminal transmembrane domain (TMD)
2) strong homology (44% similarity) with the J-region of DnaJ (Hsp40) over the last 70 amino acids of the DjlA protein. The J-region is a conserved domain that has been shown to be essential for the interaction between DnaJ and DnaK (Hsp70).

Moderate overproduction of DjlA in *E. coli* results in an increase in the expression of the genes controlling the production of the extracellular polysaccharide, colanic acid (the *cps* operon). The expression of the *cps* operon in *E. coli* is under the control of a two-component signal transduction pathway, the Rcs pathway. This pathway is composed of the membrane sensor protein, RcsC, and the cytoplasmic response regulator, RcsB. It is assumed that on receiving a signal from outside the cell the RcsC protein phosphorylates the RcsB protein which in turn activates expression from the *cps* operon. We, and others, have shown that the induction of *cps* expression observed with DjlA overproduction is dependent on the RcsC protein. Both the J-region of DjlA and the chaperone protein, DnaK, have been shown to be essential for the activation of RcsC indicating, for the first time, a role for chaperone proteins in bacterial signal transduction.

In this poster we show that DjlA is localised to the inner membrane of *E. coli* with the rare Type III topology. Thus, the small N-terminal tail of DjlA is translocated across the inner membrane resulting in the large C-terminal part of the protein, containing the J-region, being exposed to the cytoplasm. The translocation of short N-tails has been shown to be independent of the Sec pathway but dependent on the electrochemical gradient. Therefore, positive charges in this region hinder correct membrane insertion. It was noted that a lysine residue was present in the proposed translocated N-tail of DjlA, however the subsequent mutation of this residue to a glutatmate residue had no apparent affect on DjlA function (and thus correct membrane assembly) as measured by induction of the *cps* operon.

Homologues of DjlA have been identified in *Haemophilus influenzae* and *Coxiella burnetti* (the causative agent of Q fever), indicating that this protein may have an important role in bacteria. In particular, the TMD of DjlA from all these bacteria has a conserved, repeated motif of [GxxxG]3 (where G is glycine and x is any amino acid). The importance of glycine residues in the TMD of proteins has been highlighted in many recent studies with glycophorin A, M13 major coat protein and the MHC class II α and β chains. In these model systems the glycines have been shown to be essential for the specific oligomeristaion of these proteins. In this poster we will show that the activity of DjlA, with respect to the Rcs signal transduction pathway, is dependent on the TMD. Furthermore, point and multiple mutations in the TMD of DjlA (which do not affect localisation or topology) have been isolated that no longer activate RcsC, suggesting that this domain may be important for mediating specific protein-protein interactions. Finally we will present some evidence to suggest that the TMD may, in fact, be important for mediating the dimerisation of DjlA.

We propose, therefore, that DjlA is a new member of the DnaJ chaperone family, localised to the inner membrane by a single N-terminal TMD. The TMD may be reponsible for both DjlA oligomerisation and the primary interaction between DjlA and its substrates. This ensures that the J-region of DjlA will be correctly positioned to target DnaK to these substrates resulting in chaperone activity at the inner membrane.

Dimerisation of the integral membrane enzyme OMPLA monitored by FRET

I. Ubarretxena Belandia, L. Hozeman, H.M. Verheij and N. Dekker

Dept. Enzymology and Protein Engineering, Centre for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.

The outer membrane phospholipase A (OMPLA) is present as a catalytically inactive monomer in the outer membrane of *Escherichia coli*. Perturbation of this membrane results in dimerisation of the protein concurrent with the appearance of enzymatic activity. *In vitro* the monomer/dimer equilibrium is modulated by phospholipid, detergent and the essential cofactor calcium. Based on the high resolution X-ray structure of monomeric OMPLA the surface of the molecule was scanned by cysteine mutagenesis. Sulphydryl-specific chemical crosslinking was applied to determine which residues are part of the dimer interface. The cysteine mutant of the active site serine (S144C) could not be crosslinked into its dimeric state, even when crosslinkers were used of 16 Å in length. This result implies that the enzyme does not have shared active site. For the mutant H26C very efficient crosslinking was observed with full retainement of enzymatic activity, indicating that the N-termini of the subunits are in intimate contact in the active, dimeric complex. The unique cysteine in the mutant H26C-OMPLA was labelled with thiol specific fluorescent probes (IAEDANS and NBD) to allow for a characterisation of the monomer/dimer equilibrium using the fluorescence resonance energy transfer technique (FRET). FRET experiments in detergent micelles and reconstituted vesicle systems allowed us to study the dimerisation dynamics and the monomer/dimer equilibrium as a function of membrane lipid composition. The outcome of these results are discussed in view of our understanding of the molecular processes leading to the activation of this integral membrane enzyme.

Structure and function of colicin A and N

M. Cyrklaff[+], D. Massotte[+], Ingrid R. Vetter[†], Michael W. Parker[‡], Kevin Leonard*, Jeremy H. Lakey**, Franc Pattus[+]

*EMBL, Heidelberg, FRG;[†]MPI for Molecular Physiology, Dortmund, F.R.G.;[‡]St. Vincent's Inst. of Med. Res. Victoria, Australia;**Dept. of Biochem. and Gen., The University of Newcastle-upon-Tyne, UK;[+]Dept. Recepteurs et Proteines Membranaires, UPR 9050 CNRS, Illkirch, France

The lipid bilayer membrane which surrounds living cells is a very effective barrier in preventing diffusion of small molecules and macromolecules into the cytoplasm. In order to reach their site of action inside the target cells, from their site of synthesis within the producing cell, toxins must cross two to four membranes. How these macromolecules are able to cross these barriers is a central question which is still far from being understood. One large class of toxins exerts its lethal effect by inserting into the target cell membranes and forming a pore which perturbs the ionic and osmotic balance across the membrane, inducing a wide diversity of biological effects such as loss of solute, inhibition of active transport, depolarization of the membrane, and arrest of protein synthesis. These toxins are produced in a water soluble form which is able to diffuse towards the target cell in an aqueous environment. But in their final state, the pore, they become integral membrane proteins embeded in the hydrophobic core of a lipid bilayer. How a single polypeptide chain can first cross a membrane and then insert into a second membrane to form a voltage gated channel is the question we have been addressing for many years by studying the pore-forming colicins A and N.

Colicins are plasmid encoded bacteriocins produced by E. coli which kill sensitive E. coli strains and related bacteria. The cell enveloppe of gram negative bacteria with its two membranes presents a serious obstacle for the uptake of macromolecules of the size of colicins (40-60 kDa). To cross this barrier and reach their target colicins first bind to their cognate receptor on the cell surface and then translocate through the outer membrane. The pore forming colicins (E1, A, B, N, Ia, IB, K, 10, 5) then insert into the inner membrane Uptake of colicins through the outer membrane follows two alternative pathways mediated by the Ton or the Tol system. The Ton system consists of the proteins TonB, ExbB and ExbD and is also invoved in the energy-dependent uptake of vitamin B12 and iron siderophores.and the Tol system consists of 6 proteins: TolA, TolB TolQ and TolR, PAL and Orf2. Colicins B, D, Ia, IB, M, 5 and 10 (group B) are taken up via the Ton pathway, whereas the translocation of group A colicins (colicin A, the E colicins, colicin K, N and cloacin DF13) involves interaction with TolA and different combinations of the proteins from the Tol family

The structures and mode of action of colicin A and N is the topic of this presentation with special emphasis on the réceptor/colicin interaction on the outer membrane of the bacteria.

- Jeanteur, D., Schirmer, T., Fourel, D., Simonet, V., Rummel, G., Widmer, C., Rosenbusch, J. P., Pattus, F. and Pages, J. M. (1994). Structural and functional alterations of a colicin-

resistant mutant of OmpF porin from Escherichia coli. *Proc Natl Acad Sci USA* **91**: 10675-10679.

- Jeanteur, D., Pattus, F. and Timmins, P. A. (1994). Membrane-Bound Form of the Pore-Forming Domain of Colicin-A - A Neutron Scattering Study. *J Mol Biol* **235**: 898-907.

-Vetter I.R., Parker M.W., Tucker A.D., Lakey, J.H., Pattus F. & Tsernoglou D. Crystal structure of colicin N suggests a model for receptor-binding and membrane translocation. Submitted.

The Channel-Forming Protein Toxin Aerolysin and its GPI-Anchored Membrane Receptors

J. Thomas Buckley, Department of Biochemistry and Microbiology, University of Victoria, Box 3055, Victoria, B. C., Canada, V8W 3P6.

Aerolysin is secreted as a water soluble 52 kDa protoxin by bacteria in the family *Vibrionaceae*, including the pathogen *Aeromonas hydrophila*. The crystal structure of proaerolysin from *A. hydrophila* has been solved (1). The protein is a dimer in the crystal as well as in solution. Each monomer consists of a small lobe formed by the N-terminal 90 amino acids of the protein, and a large elongated lobe, formed by the remaining 380 amino acids, The fold of the large lobe is unique, whereas the small lobe is strikingly similar in structure to folds in the S2 and S3 subunits of pertussis toxin and it bears some resemblance to folds in proteins of the mammalian C-type lectin family (2).

Proaerolysin is activated by proteolytic nicking near the C-terminus of the protein, on one end of the elongated lobe. Once activated, the protein can oligomerize, forming remarkably stable heptamers. Oligomerization transforms aerolysin into an insertion competent state and the oligomers insert into the membranes of target cells forming discrete, regular channels that disrupt the permeability barrier (3).

Cells that are sensitive to aerolysin can be killed by concentrations of the toxin as low as 10^{-11}M. This is because these cells have specific receptors that bind the toxin with high affinity. Binding effectively concentrates the toxin on the cell surface, promoting the oligomerization step that leads to channel formation. We have identified aerolysin receptors on a variety of different cells. Among them are Thy-1, found in T-lymphocytes and brain, the brain surface protein contactin, a novel 47 kDa membrane protein in mammalian erythrocytes, and the variant surface glycoprotein of trypanosomes. Although these proteins appear to be unrelated in structure, they all share one unusual property-they are attached to the cell surface by glycosylphosphatidylinositol (GPI) anchors. We have shown that specific features of the glycosyl portion of the anchor are required for aerolysin binding (4) and our preliminary evidence indicates that regions of both lobes of the protein participate in the interaction with the receptor.

Aerolysin should prove to be extremely useful as a tool to detect new GPI-anchored proteins and to study their functions. The toxin may also be used in the diagnosis of diseases that affect GPI-protein metabolism. An example is the human stem cell disorder paroxysmal nocturnal hemoglobinuria, in which circulating cells no longer display GPI-anchored proteins on their surfaces. We have found that these cells are far less sensitive to aerolysin than normal cells.

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1. Parker, M. W., Buckley, J. T., Postma, J. P. M., Tucker, A. D., Leonard, K., Pattus, F., and Tsernoglou, D. (1994) Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. *Nature* 367: 292-295

2. Rossjohn, J., Buckley, J. T., Hazes, B., Murzin, A. G., Read, R. J., and Parker, M. W. (1997) Aerolysin and pertussis toxin share a common receptor-binding domain, *EMBO J.* **16**: 3426 - 3434.
3. Parker, M. W., van der Goot, F. G., and Buckley, J. T. (1996) Aerolysin-the ins and outs of a channel-forming protein. *Molec. Microbiol.* **19**: 205-212.
4. Nelson, K. L., Raja, S. M., and Buckley, J. T. (1997). The GPI-anchored surface glycoprotein Thy-1 is a receptor for the channel-forming toxin aerolysin. *J. Biol. Chem.* **272**: 12170-12174.
5 Diep, D. B., Nelson, K. L., Raja, S. M., Pleshak, E. N., and Buckley, J. T. (1998) Glycosylphosphatidylinositol anchors of membrane glycoproteins are binding determinants for the channel-forming toxin aerolysin. *J. Biol. Chem.* **273**: 2355-2360.

Membrane insertion and folding of proteins in mitochondria

Doron Rapaport and *Walter Neupert*
Institute of Physiological Chemistry, University of Munich,
Goethestr. 33, D-80336-München, Germany

Mitochondria contain several translocation machineries which facilitate transport of proteins from the cytosol into mitochondria and insertion of proteins into the outer and inner membranes. A 'preprotein translocase' (TOM complex) exists in the outer membrane and two translocases were identified in the inner membrane (TIM23 complex and TIM22 complex).

Proteins destined to the mitochondrial matrix space utilize the Tim23 complex in what was termed the general import pathway. A number of proteins of the inner membrane use also this pathway but additional steps are necessary for the insertion process. Some proteins of the inner membrane are first imported into the matrix, either completely or partially and then undergo an insertion process from the matrix. This reaction, in particular insertion of transmembrane segments accompanied by export of N-terminal tails has been studied in some detail and has turned out to be rather similar in its requirements ($\Delta\mu H^+$, negative flanking charges, matrix ATP) to the corresponding reactions in bacteria. The recently discovered inner membrane component Oxa1p is required for this reaction and probably constitutes a specific system for insertion of proteins from the matrix side. With a number of other inner membrane proteins, translocation facilitated by the TIM23 complex is halted when the transmembrane segment crosses the inner membrane and this segment is then laterally inserted into the lipid bilayer. There is yet another class of inner membrane proteins which contain internal targeting signals next to a transmembrane segment; their import is determined by a loop structure formed by these two sequence elements; the Tim23 complex is also involved in their insertion.

The TIM22 complex facilitates insertion of a subgroup of inner membrane proteins which do not contain classical cleavable targeting sequences. In particular members of the mitochondrial carrier family (e.g. ADP/ATP carrier, phosphate carrier) and TIM complex components are inserted by this pathway from the intermembrane space side into the inner membrane. Two small peripheral protein components on the intermembrane space side of the inner membrane, Tim10 and Tim12, cooperate with Tim22. Tom10 and 12 are Zn-finger-like proteins which bind and probably stabilize the precursor forms of the carrier proteins in the intermembrane space. Tim22 is a component structurally related to Tim23 and Tim17; it takes over the carrier precursors from Tim10 and

Tim12 and mediates their $\Delta\psi$-dependent insertion without the apparent participation of Tim44/mt-Hsp70.

Mitochondrial molecular chaperones are also involved in the biogenesis of inner membrane proteins. They mediate folding of these proteins and facilitate their import. mtHsp70 is also involved in the assembly of mitochondrially encoded proteins, subunits 9 and 6, in the F_1F_0-ATPase complex.

Insertion of mitochondrially encoded proteins such as subunits of cytochrome oxidase, in particular subunit II has also been studied. Oxa1p is involved in the insertion of this component which contains two membrane spanning segments with N- and C-termini exposed in the intermembrane space.

In conclusion, mitochondria harbor a considerable diversity of pathways and components for the insertion of proteins into membranes and all of them are only poorly understood so far.

References

Stuart, R.A. and Neupert, W. (1996) Topogenesis of proteins of the inner membrane proteins of mitochondria. *Trends Biochem. Sci.* **21**, 261-267.

Bauer, M.F., Sirrenberg, C., Neupert, W., and Brunner, M. (1996) Role of Tim23 as voltage sensor and presequence receptor in protein import into mitochondria. *Cell* **87**, 33-41.

Sirrenberg, C., Bauer, M.F., Guiard, B., Neupert, W. and Brunner, M. (1996) Import of carrier proteins into the mitochondrial inner membrane mediated by Tim22. *Nature* **384**, 582-585.

Neupert. W. (1997) Protein import into mitochondria. *Annu. Rev. Biochem.* **66**, 863-917.

Arnold., I., Bauer, M.F., Brunner, M., Neupert, W., and Stuart, R.A. (1997) Yeast mitochondrial F_1F_0-ATPase: the novel subunit e is identical to Tim11. *FEBS Lett.* **411**, 195-200.

Hell, K., Herrmann, J.M., Pratje, E., Neupert, W., and Stuart, R.A. (1997) Oxa1p mediates the export of the N- and C-termini of preCoxII from the mitochondrial matrix to the intermembrane space. *FEBS Lett.* **418**, 367-370.

Rojo, E.E., Guiard, B., Neupert, W., and Stuart, R.A. (1998) Sorting of D-lactate dehydrogenase in the cytosolic domain of TOM22 in the import of precursor proteins into mitochondria. *J. Biol. Chem.*, in press.

Sirrenberg, C., Endres, M., Fölsch, H., Stuart, R.A., Neupert, W., and Brunner, B. (1998) Zinc finger-like proteins Tim10/Mrs11p and Tim21/Mrs5p mediating import of carrier proteins into mitochondria. *Nature*, in press.

Hell, K., Herrmann, J.M., Pratje, E., Neupert, W., and Stuart, R.A. (1998) Oxa1p, an essential component of the novel N-tail protein export machinery in mitochondria. *Proc. Natl. Acad. Sci. USA*, in press.

The Secreton : a complex machinery for transporting folded proteins across the outer membrane of Gram-negative bacteria.

Anthony P. Pugsley
Unité de Génétique Moléculaire, CNRS, Institut Pasteur, 25, rue du Docteur Roux, 75724 Paris cedex 15, France. Tel : 33/1.45688494 ; Fax : 33/1 45688960 ; E-mail : max@pasteur.fr

One of the best examples of the transport of folded polypeptides across lipid bilayer membranes is provided by the main terminal branch of the general secretory pathway (GSP) in Gram-negative bacteria such as *E. coli*. The transport of the folded and often multimeric proteins across this membrane is energy-dependent and requires a complex machinery, the secreton, components of which are associated with one or both of the two membranes that compose the cell envelope.

In the model system studied in the laboratory, the pullulanase secretion pathway of *Klebsiella oxytoca*, a total of 14 genes are specifically required to reconstitute the secreton in *E. coli* K-12. Two of these genes code for outer membrane proteins that associate to form a stable multimeric complex in the outer membrane. High resolution cryomicroscopic analysis of the purified complex reveals features predicted for a pore-forming structure, consistent with the ability of the purified complex to form pores in artificial lipid bilayers. Features of the regulation of pore activity and its association with other components of the secreton will be presented.

The work that will be presented was carried out by Ingrid Guilvout, Nico Nouwen and Odile Possot at the Institut Pasteur, Paris, France, by Alexandre Ghazi at the University of Orsay, France, by Neil Ransom and Helen Saibil at Birkbeck College of the University of London, UK and by Bettina Fedtke, S.A. Müller and Andreas Engel at the Biozentrum of the University of Basle, Switzerland.

Modulation of the rate of folding of a membrane protein

Paula J. Booth[1], A. Rachael Curran[1] and Richard H. Templer[2]

Departments of [1]Biochemistry and [2]Chemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY

Intermolecular interactions within lipid membranes seem to play a key role in modulating the activity of a wide range of important membrane-bound receptors, signal transducers and ion channels. It also seems likely that these membrane forces should affect and control the folding of proteins embedded in the membrane. We show that by altering these interactions we are indeed able to modulate the folding of a trans-membrane protein [1]. The recent novel crystallisation methods reported for bacteriorhodopsin also seems to be dependent on manipultaion of such membranes forces [2].

The membrane spanning regions of many integral membrane proteins consist of transmembrane α helices which are thermodynamically stable and can be considered as autonomous folding domains. We have been investigating the forces which drive association of these helices using the 7-transmembrane helix protein, bacteriorhodopsin as a model system. We have identified a rate-limiting step in the folding of this protein, and been able to control the rate of this step. The refolding system used contains two lipids with the same phosphatidylcholine headgroup but different chain lengths, C_{14} DMPC and C_6DHPC. The rigidity to bending of the DMPC bilayer is reduced by the incorporation of the shorter chain DHPC lipid, which appears to cause a sharp reduction in the pressure near the middle of the bilayer. Increasing the proportion of DMPC in this mixed DMPC/DHPC bilayer, slows the rate-limiting folding step. We postulate that this step involves packing of the central regions of the 7 transmembrane helices. Increasing the proportion of DMPC increases the bilayer torque tension and the lateral pressure which the lipid chains impose on the protein. As a result helix migration in the bilayer is impeded.

In order to quantify the above hypothesis and overcome problems inherent in using micelles with lipids of different chain length, we are developing a new refolding system using two lipids of equal chain length. In this case the lateral pressure in the chain region is altered by varying headgroup composition or the degree of chain unsaturation.

1. Booth, P.J., *et al. Biochemistry* **36**, 197-203 (1997).
2. Landau, E.M. & Rosenbusch, J.P. *Proc. Natl. Acad. Sci. USA* **93**, 14532-14535 (1996).

Session III: Membrane protein insertion and translocation

Chairs: Bonnie A. Wallace

Gunnar von Heijne

José Luis R. Arrondo

Félix M. Goñi

Design of functional channel proteins

M. Montal

Department of Biology, University of California San Diego, La Jolla, CA 92093-0366, USA.

Channel proteins, a special class of membrane proteins which mediate cell signaling, are pivotal control elements of cellular homeostasis. Their dysfunction leads to disease processes and they constitute a prime target for drug intervention. As a tractable approach to investigate the sequence-structure determinism, we set out to establish the occurrence of minimum units of structure with specific functional attributes The novelty of the strategy resides in the notion of a discrete modular assembly based on the premise that small, independently folded modules may be stable in the absence of the entire channel protein and that structure determination of such isolated modules reconstituted in lipid bilayers is feasible and realistic (1). Channel properties are characterized by single channel recordings from reconstituted lipid bilayers. Protein structure is determined by multidimensional NMR spectroscopy of isotopically labeled proteins in deuterated detergent micelles and by solid-state NMR in oriented phospholipid bilayer lamellae. Structure-function relations are developed by the convergence of structural information with the characterization of channel function. Progress achieved on the design and structural characterization of the channel proteins that imitate the inner bundle that lines the pore of the nicotinic acetylcholine receptor and the NMDA receptor will be presented (2,3).

1. Montal, M. **Curr. Opin. Struct. Biol.** 6: 499-510 (1996).
2. Oblatt-Montal, M., T. Iwamoto, J.M. Tomich and M. Montal. **FEBS Lett.** 320:261-266. (1993).
3. Opella, S.J., J. Gesell, A.P. Velente, F.M. Marassi, M. Oblatt-Montal, A.V. Ferrer-Montiel, W. Sun and M. Montal. **CHEMTRACTS** 10:153-174 (1997).

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email:montal@biomail.ucsd.edu

The Interaction of Synthetic α-Helical Transmembrane Peptides with Lipid Bilayers

Yuan-peng Zhang, Ruthven N.A.H. Lewis, Robert S. Hodges and Ronald N. McElhaney*.
Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

The conformation and amide proton exchangeability of the hydrophobic transmembrane model peptides P_{24} (Ac-K_2-G-L_{24}-K_2-A-amide), L_{24} (Ac-K_2-L_{24}-K_2- amide) and $(LA)_{12}$ (Ac-K_2-$(LA)_{12}$-K_2-amide) have been studied in various solvents, detergent micelles and phospholipid dispersions (1,2,4,5). Although all three peptides are predominantly α-helical in all media, the polyleucine-based peptides P_{24} and L_{24} are more strongly so and exhibit less conformational plasticity than $(LA)_{12}$. The exchangeability of the amide protons indicate that there are two populations of amide protons, a rapidly exchanging populated located near the peptide terminae (the frayed ends of the helices) and a slowly exchanging portion arising from the hydrophobic core of the peptide. In the thicker gel state dipalmitoylphosphatidylcholine (DPPC) bilayers, virtually no amide proton exchange takes place, while in liquid-crystalline DPPC bilayers only the exposed terminal lysines and one or two adjacent leucine (or alanines) undergo proton exchange, suggesting that the hydrophobic core of the DPPC bilayer effectively shields that portion of the peptide buried within it from water.

We also studied the interaction of these peptides with a homologous series of linear saturated phosphatidylcholines (PCs). Although the stoichiometry of interaction and general effects of these peptides on the thermotropic phase behavior of PC bilayers were similar, P_{24} and L_{24} exhibited the characteristic shifts in phase transition temperature predicted by hydrophobic mismatch theories while this effect was markedly attenuated with $(LA)_{12}$, probably due to its greater conformational flexibility. In addition, $(LA)_{12}$ was generally more perturbing to the organization of the host PC bilayer than the polyleucine-based peptides, perhaps due to its rougher surface topology and/or decreased surface hydrophobicity. In addition, the enthalpy of the PC hydrocarbon chain-melting phase transition was not abolished even at very high peptide concentrations, indicating that these peptides reduce but do not abolish the cooperative gel/liquid-crystalline phase transition in the host bilayer. In all cases, the width of the lipid phase transition decreases with increasing PC hydrocarbon chain length and lipid/peptide lateral phase separation occurs in very thin or very thick PC bilayers at higher peptide concentrations. As well, P_{24} and L_{24} undergo small distortions in the α-helical structures in response to hydrophobic mismatch with the host PC bilayer, while $(LA)_{12}$ exhibits more pronounced conformation alterations.

We also studied the interaction of P_{24} and $(LA)_{12}$ with members of the homologous series of *n*-saturated diacylphosphatidylethanolamines (PEs) (3,6). Although the stoichiometry of peptide/lipid interactions is the same as in PC bilayers, these peptides always disorder gel and liquid-crystalline PE bilayers and virtually no effects of variations in hydrocarbon chain length on PE thermotropic phase behavior are observed, in contrast to the PC system. We postulate that peptide incorporation into PE bilayers disrupts the relatively strong electrostatic and hydrogen-bonding interactions at the bilayer surface, masking the effects of hydrophobic mismatch between the peptide and the host PE bilayer. Variations in PE bilayer hydrophobic thickness do, however, again alter the conformation of the peptide, and these peptide show decreasing miscibility in PE bilayers at low temperatures, particularly as hydrocarbon chain length increases. These results demonstrate that the chemical structure of the host lipid bilayer can markedly influence protein-lipid interactions.

In conjunction with Dr. David Thomas, we have examined the rotational mobility of L_{24} with a terminal cysteine residue on which a nitroxide spin label has been placed. In PC bilayers having a considerable range of hydrocarbon chain lengths and degrees of unsaturation, the rotational mobility of this peptide is considered greater in liquid-crystalline than in gel-state bilayers, as expected. However, no significant aggregation of the peptide takes place in either phase state, indicating that the positively charged dilysine caps, and the topologically smooth polyleucine core of these peptides, favor lipid-peptide over peptide-peptide interactions, even in the gel state (unpublished work). These results were confirmed in recent studies of unlabelled L_{24} in spin-labeled POPC bilayers (7), which also showed that this peptide orders rather than disorders the hydrocarbon chains of adjacent phospholipids, in contrast to natural membrane proteins. Moreover, the presence of this peptide actually increases the hydrophobicity of the POPC bilayer both near the membrane surface and in the center of the hydrocarbon core.

We also found that unlike the other three peptides studied to date, A_{24} will not insert into, or remain associated with, phospholipid bilayers. This peptide partitions almost exclusively into the aqueous phase where it exists only partially in the α-helical conformation. This result indicates that, by itself, the polyalanine core of this peptide is not sufficiently hydrophobic to enable stable interactions with the hydrophobic domains of lipid bilayers, a conclusion also reached in studies published by Thompson and coworkers and by Deber and coworkers. This finding is also compatible with data published by White and coworkers, who demonstrated that the affinity of alanine residues for lipid bilayers is significantly less than their affinity for water. In this regard, the relevance of recent molecular modeling studies of polyalanine-based transmembrane peptides in lipid bilayers to the transmembrane segments of real membrane proteins must be reevaluated.

Major References

1. Zhang, Y.-P., R.N.A.H. Lewis, R.S. Hodges and R.N. McElhaney. *Biochemistry* **31**: 11572-11578 (1992).
2. Zhang, Y.-P., R.N.A.H. Lewis, R.S. Hodges and R.N. McElhaney. *Biochemistry* **31**: 11579-11588 (1992).
3. Zhang, Y.-P., R.N.A.H. Lewis, R.N. McElhaney and R.O. Ryan. *Biochemistry* **32**: 3942-3952 (1993).
4. Zhang, Y.-P., R.N.A.H. Lewis, G.D. Henry, B.D. Sykes, R.S. Hodges and R.N. McElhaney. *Biochemistry* **34**: 2348-2361 (1995).
5. Zhang, Y.-P., R.N.A.H. Lewis, R.S. Hodges and R.N. McElhaney. *Biochemistry* **34**: 2362-2371 (1995).
6. Zhang, Y.-P., R.N.A.H. Lewis, R.S. Hodges and R.N. McElhaney. *Biophys. J.* (1998) (submitted for publication).,
7. Subczynski, W.K., R.N.A.H. Lewis, R.N. McElhaney, R.S. Hodges, J.S. Hyde and A. Kusumi. *Biochemistry* **37**: (1998) (in press).

Differential use of the SRP-translocon targeting pathway for membrane protein assembly in *Escherichia coli*

Jan-Willem L. de Gier

Department of Biochemistry, Arrhenius Laboratory, Stockholm University, S-106 91 Stockholm University, Sweden

Assembly of the inner membrane proteins leader peptidase (Lep), a Lep mutant (Lep-inv) that inserts with an inverted topology compared to the wild-type protein, and the phage M13 procoat protein has been studied in *Escherichia coli* strains that are conditional for the expression of either Ffh or 4.5S RNA, the two components of the *E. coli* SRP, or SecE, an essential core component of the *E. coli* translocon. Membrane insertion has also been tested in a strain that does not express the SecB chaperone.

Both Lep and Lep-inv are known to require the SRP for correct assembly into the inner membrane (de Gier *et al.*, (1996) FEBS Letters 399, 307-309); in contrast, we find that the M13 procoat protein does not. Furthermore, both Lep and, surprisingly, Lep-inv fail to insert properly when SecE is depeleted, whereas insertion of M13 procoat is unaffected under these conditions. None of the three proteins depend on SecB for assembly. These observations indicate that inner membrane proteins in *E. coli* can assemble either by a mechanism where SRP delivers the protein at the SecYEG translocon (Lep and Lep-inv) or by what appears to be a direct integration into the lipid bilayer (M13 procoat).

DORON RAPAPORT

Institut für Physiologische Chemie, Gothestraße 33,
D-80336 München (Germany).
Tel.: 49 89 5996 289. Fax: 49 89 5996 270.
E-mail: RAPAPORT@BIO.MED.UNI-MUENCHEN.DE

Dynamics of the TOM complex of mitochondria during binding and translocation of preproteins

Translocation of mitochondrial preproteins across the outer membrane is mediated by the TOM complex. This complex consists of receptor components for the initial contact with preproteins at the mitochondrial surface and membrane-embedded proteins which promote translocation and form the translocation pore. In order to understand the interplay between the translocating preprotein and the constituents of the TOM complex, we analysed the interactions of the essential component Tom40 within the TOM complex during translocation of preproteins across the outer membrane of *Neurospora crassa* and yeast mitochondria. Tom40 exists in a homo-oligomeric assembly and dynamically interacts with Tom6. The Tom40 assembly is influenced by a block of negatively charged amino acid residues in the cytosolic domain of Tom22 indicating a dynamic cross-talk between preprotein receptors and the translocation pore. Preprotein or presequence binding to specific sites on either side of the outer membrane induces distinct structural alterations of Tom40. Our data show that the TOM complex comprises a dynamic protein-conducting channel whose structure is modulated by the interaction with mitochondrial targeting sequences. We propose that such targeting sequence-induced adaptations of the translocase are crucial for the movement of preproteins across membranes.

Membrane insertion of a bacterial toxin, *E. coli* α-haemolysin

Félix M. Goñi

Departamento de Bioquímica, Universidad del País Vasco, Aptdo. 644, 48080 Bilbao.

α-Haemolysin (HlyA) is an extracellular protein toxin (M = 107 kDa) secreted by *E. coli*, that acts at the level of the plasma membranes of target eukaryotic cells. The nature of the toxin interaction with the membrane is not known at present, although it has been established that receptor-mediated binding is not essential. Our studies using model membranes (large unilamellar liposomes) as targets have shown that calcium ions are essential for HlyA action, and that the protein must bind calcium prior to its interaction with the lipid bilayer for membrane lysis to occur. HlyA can either reversibly adsorb on the membrane surface or irreversibly insert into the bilayers. Lysis requires the irreversible insertion of the calcium-bound toxin. The calcium-bound form has an increased tendency to membrane insertion but also an increased tendency to self-aggregation, so that both phenomena compete with eachother. Membrane insertion is favoured by lipids in fluid, disordered states. A number of studies on the perturbation produced by HlyA on lipid bilayers, under conditions in which the toxin has been shown to induce vesicle cleavage, indicate that the toxin becomes inserted in the target membranes in the way of intrinsic or integral proteins. An equally important conclusion from these experiments is that inserted α-haemolysin appears to occupy only one of the membrane phospholipid monolayers, i.e. it would not be a transmembrane protein. In addition, structure prediction studies suggest the presence of as many as ten amphipathic α-helices, appropriate for protein-lipid interaction. However, no hydrophobic transmembrane helices are predicted in α-haemolysin. These observations and predictions have important consequences for the mechanism of cell lysis by α-haemolysin; in particular, a non-transmembrane arrangement of the toxin in the target membrane is not compatible with the concept of α-haemolysin as a pore-forming toxin.

References

Ostolaza, H., Bartolomé, B., Ortiz de Zárate, I., de la Cruz, F., and Goñi, F.M. (1993). Release of lipid vesicle contents by the bacterial protein toxin α-haemolysin. Biochim. Biophys. Acta 1147, 81-88.

Bakás, L., Ostolaza, H., Vaz, W.L.C., and Goñi, F.M. (1996). Reversible adsorption and nonreversible insertion of *Escherichia coli* α-hemolysin into lipid bilayers. Biophys. J. 71, 1869-1876.

Soloaga, A., Ramírez, J.M., and Goñi, F.M. (1998). Reversible denaturation, self-aggregation and membrane activity of *E. coli* α-haemolysin, a protein stable in 6M urea. Biochemistry (in press).

Complement-mediated Killing of Gram-negative Bacteria: C9 translocation through the envelope and insertion into the inner membrane. Alfred F. Esser, Division of Cell Biology & Biophysics, School of Biological Sciences, University of Missouri, Kansas City, MO 641110.

The serum complement system plays a major role in defense against microbial invasion of the host by two different mechanisms: (i) C3-mediated opsonization of cells and (ii) direct killing as a result of C5b-9 complex formation on the target. Without doubt, the former mechanism is of great importance in many infectious diseases. However, epidemiological studies of complement-resistant bacteria in systemic infections, as well as studies on individuals with genetically determined complement deficiencies have indicated that C5b-9-mediated mechanisms play an important role in the control of Gram-negative infections. Death of sensitive strains after exposure to serum complement is extremely rapid, with rates of viability loss following first-order kinetics and reaching a level greater than 99.99% [1,2].

Our own studies [2] have demonstrated that C5b-8 on the outer membrane (OM) acts as the receptor for C9 and although the C5b-8 complex can *transiently* collapse the membrane potential $\Delta\Psi$ in *E. coli* cells it is the addition of C9 which rapidly and irreversibly dissipates $\Delta\Psi$ and causes cell death. In contrast, addition of native C9 to whole cells, protoplasts or IM vesicles has no effect on $\Delta\Psi$. However, the purified C-terminal thrombin fragment of C9 (i.e., amino acids 245 - 538, or C9b) inhibits generation of $\Delta\Psi$ in right-side-out and inside-out IM vesicles. The isolated C9b fragment also produces single ion-conducting (~ 11 pS) channels across bilayer lipid membranes (BLM). These results confirm and extend earlier work with photolabeling techniques by demonstrating that the membrane interaction domain is located in the C-terminal fragment of C9. The importance of these combined results was emphasized by the observation that the presence of the C5b-8 receptor on the OM is not an absolute requirement for C9-mediated killing. When C9 is osmotically shocked into the periplasm of cells *not bearing a C5b-8 complex* it kills the cells whereas C7 and C8 are ineffective [3]. Of additional importance was the observation by Bhakdi and Tranum-Jensen [4] that the $(C5b\text{-}8)_1C9_1$ complex is sufficient to lyse erythrocytes fully whereas such a complex is completely devoid of bactericidal activity. This result has been confirmed and extended to show that a $(C5b\text{-}8)_1C9_4$ complex is completely bactericidal and that incorporation of more C9 molecules does not improve bacterial killing [5].

Based on these observations we asked the question, would C9 kill if it were to be introduced into the periplasm from *inside* the cell, for example by secretion of recombinant C9, rather than from the *outside* by osmotic shock? To get an answer we cloned the gene for mature C9 into commercial vectors that provide signal peptides for export of recombinant proteins into the

periplasm. Induction of protein expression from these plasmids caused immediate loss of cell viability. Significantly, when C9 was cloned without any signal peptide and expressed in the cytoplasm no toxic effects were observed. Although these results suggested that C9 was exported into the periplasm and exhibited its toxic effects just like external C9 shocked into the periplasm we could not exclude the possibility that loss of viability was caused by merely expressing a foreign gene in *E. coli* since many gene products are known to harbor such *general* cytopathic effects. To test for this possibility we took advantage of the fact that C9 is a heavily disulfide-linked protein which is inactive when reduced. Disulfide bonds are formed in *E. coli* in the periplasm by the products of the *dsb A, B, C* genes. The DsbA protein is a protein disulfide isomerase(PDI) in the periplasm which introduces cystines into secreted proteins and becomes reduced, and the DsbB protein is an inner membrane protein which reoxidizes reduced DsbA. In mutants lacking functional DsbA, disulfide bond formation can be obtained by simply adding an OM permeable redox reagent such as glutathione or N-acetyl cysteine (NaC) to the cell culture. Using *dsbA*$^-$ *E. coli* mutants we have observed that such cells continue to grow after induction of expression but when NaC is added to the culture medium an immediate viability loss is observed. Cells harboring a plasmid which expresses C9 in the cytoplasm, that is without a signal peptide, are not affected by NaC addition demonstrating that the effect is specific for periplasmic C9. We believe that these results demonstrate that periplasmic rC9 is toxic to host cells just like extracellular C9, and that in addition, some folding and disulfide bond formation is required for toxicity. Furthermore, since exposure of native C9 has no effect on protoplast functions we postulate that constituents in the bacterial envelope participate in C9-mediated cell killing in analogy to the role played by Ton and Tol proteins in colicin-mediated killing.

References:

1. P.W. Taylor (1983) *Microbiol. Rev. 47*: 46-83; and
(1992) *Exp. Clin. Immunogenet.* 9: 48-56.
2. A.F. Esser (1994) *Toxicology* 87: 229-247.
3. J.R. Dankert and A.F. Esser (1987). *Biochem. J.* 244: 393-399.
4. S. Bhakdi and J. Tranum-Jensen (1986) *J. Immunol.* 136: 2999-3005.
5. S.L.D. MacKay and J.R. Dankert (1990) *J. Immunol.* 145: 3367-3371.

JAMMING OF MEMBRANE TRANSPORT CAUSED BY Helicobacter pylori CYTOTOXIN VacA

C. MONTECUCCO, E. PAPINI, M. de BERNARD, M. MOLINARI, B. SATIN, V. PELICIC, G. VITALE
CNR Center for Biomembranes and Dept. Biomedical Sciences, University of Padova, Via G. Colombo 3, PADOVA, ITALY tel. 049.8276058, fax 8276049, e.mail: cesare@civ.bio.unipd.it

Membrane trafficking inside cells is a highly coordinated phenomenon for the for the uptake of extracellular ligands, nutrients and receptors and for the delivery of newly synthesized molecules to different cell compartments and to the extracellular medium and. Following endocytosis, some membrane portions are recycled to the plasma membrane, whereas other proceed to late endosomes (LE), a cross point which also receives vesicles from TGN (1). Membrane trafficking at the LE/prelysosmal level is specifically altered by VacA, a 95 kDa protein, which is the major virulence factor produced by pathogenic strains of Helicobacter pylori (2,3). This toxin shows the peculiar property of being activated and to resist to pH 2 in the presence of pepsin (4). The acid-activated toxin exerts its effect on stomach cells, but can as well reach the duodenum and cause damages in the absence of bacteria. The neutral pH form of the toxin is characterized by an oligomeric rosette-shaped structure, whereas the acid-activated toxin is largelly present in forms of monomers (5,6). This structural change can be monitored with various spectroscopic techniques and the activated toxin is able to insert into lipid bilayers (4, Molinari et al., submitted) and to form ion channels in planar lipid bilayers (our unpublished results).
When added to sparse cells in culture, VacA causes the formation of vacuoles that grow in size to occupy all the cell cytosol (2,7). Vacuoles originate from fusion of late endosomal compartments among themselves and with lysosomes and by fusion of internal membranes with the organnelles' limiting membranes. The vacuolar membrane is rich in rab7 and v-ATPase and contain lysosomal membrane markers (8). Vacuoles enlarge because they accumulate membrane permeant weak bases, which accumulate in the lumen follwing protonation. Vacuolated cells recycle transferrin normally but are defective in degradation of external ligands such as EGF and in processing of cathepsin precursors, as expected on the basis of the alteration and (9). Pre-pro-cathepsin D and other acidic hydrolases are secreted in the medium following VacA intoxication and this phenomenon may be involved relevant to the damage of the stomach mucosa because acid hydrolases are particularly active in this environment. VacA also inhibits antigen processing and presentation, as expected because the antigen processing compartment has a late endosomal/lysosomal nature (10). This effect may well contribute to H. pylori growth via depression of the host immune response.
At variance, epithelial cell monolayers do not vacuolate and show a specific lowering of their trans-epithelial monolayer resistance with increased paracellular permeability of metabolites and ions. These various cell alterations induced by VacA are interpreted in terms of H. pylori survival strategy in the sub-mucus apical niche, where it is preferentially found, because the supply of nutrients and ions from the underlying mucosa would support effectively its growth (Papini et al., submitted) .

An active research is under way to identify the target of the toxin and its biochemical activity. It is likely that the target is a cell component facing the cytsol because VacA expressed in the cytosol causes the formation of vacuoles of the same nature of those induced by externally added toxin (11). This result together with the fact that VacA is frequently found in the supernatant in two chains, associated via non covalent interactions, and that it forms channels in model membranes, suggests that it belongs to the A-B type toxins. These toxins bind to cell via the B protomer, which also assists the A domain in the membrane translocation into the cytosol.

References

1. Mukherjee S, Gosh RN & Maxfield FR (1997) Endocytosis . Physiol. Rev. 77: 759
2. Cover TL (1996):The vacuolating cytotoxin of Helicobacter pylori . Mol. Microbiol. 120:241
3 . Papini E, Satin B, Bucci C, de Bernard M, Telford JL, Manetti R, Rappuoli R, Zerial M, Montecucco C: The small GTP binding protein rab7 is essential for cellular vacuolation induced by Helicobacter pylori cytotoxin. EMBO J 1997, 16: 15-24.
4. De Bernard, M., Papini, E., de Filippis, V., Gottardi, E., Telford., J., Manetti, R., Fontana, A., Rappuoli, R. and C. Montecucco. Low pH activates the vacuolating toxin of Helicobacter pylori, which becomes acid and pepsin resistant. J. Biol. Chem, 1995, 270:23937-
5. Lupetti P, Heuser JE, Manetti R, Massari P, Lanzavecchia S, Bellon PL, Dallai R, Rappuoli R, Telford JL: Oligomeric and subunit structure of the Helicobacter pylori vacuolating cytotoxin. J Cell Biol 1996, 133:801-807.
6. Cover T L, Hanson PI, HeuserJE: Acid-induced dissociation of VacA, the Helicobacter pylori vacuolating toxin, reveals its pattern of assembly. J. Cell Biol. 1997, 138: 759-769.
7. Leunk RD, Johnson PT, David BC, Kraft WG, Morgan DR: Cytotoxin activity in broth-culture filtrates of Campylobacter pylori. J Med Microbiol 1988. 26: 93-99.
8. . Molinari M,Galli C, Norais N, Telford JL, Rappuoli R, Luzio JP, Montecucco C: Vacuoles induced by Helicobacter pylori toxin contain both late endosomal and lysosomal markers. J. Biol. Chem. 1997, 272: 25339-25344.
9. Satin B, Norais N, Telford JL, Rappuoli R, Murgia M, Montecucco C, Papini E: Vacuolating toxin of Helicobacter pylori inhibits maturation of procathepsin D and degradation of Epidermal Growth Factor in HeLa cells through a partial neutralization of acidic intracellular compartments. J. Biol. Chem. 1997, 272:25022-25028.
10. Molinari M, Salio M, Galli C, Norais N, Rappuoli R, Lanzavecchia A, Montecucco C: Selective inhibition of Li-dependent antigen presentation by Helicobacter pylori toxin VacA. J. Exp. Med., 1998, 87:135-140.
11. de Bernard M, Aricò B, Papini E, Rizzuto R, Grandi G, Rappuoli R, Montecucco C: Helicobacter pylori VacA induces vacuole formation by acting in the cell cytosol. Mol Microbiol 1997, 26:665-674.

Lipid specific insertion of proteins into membranes.

B. de Kruijff

Proteins or polypeptides can bind to membranes via electrostatic interactions. Alternatively, they can insert into the lipid head group region or more deeply into the hydrophobic phase of the membrane. Each of these possibilities is frequently encountered in cellular processes as membrane biogenesis, flow and signalling. Also in the mode of action of many polypeptide toxins membrane insertion plays a crucial role.

The type of membrane interaction a protein is involved in depends on the structure of that protein and the way it can by modulated by the membrane. Equally important are the characteristics of the membrane itself. Its properties, including the presence of specific type of lipids will determine the insertion process.

The aim of this contribution is to illustrate the importance of specific lipid-protein interactions in a number of protein insertion processes. Anionic phospholipids are shown to the essential for control of topology of leader peptidase in the *E. coli* inner membrane [1]. These lipids determine also the mode of action of nisin by their influence on membrane association, insertion and translocation of this bacteriocide [2,3]. Phoshatidylethanolamine (PE) will be shown to play an important role in anchoring the periplasmic domain of leader peptidase to the membrane [4]. The importance of PE as a non-bilayer lipid for function of preprotein translocase in *E. coli* will be indicated [5]. Together with other recent data [6] a general hypothesis on the importance of non-bilayer lipids for non-bilayer lipids for protein function will be presented [7].

References

1. W. van Klompenburg, I.-M. Nilsson, G. von Heijne and B. de Kruijff, EMBO J. 16 (1997) 4261-4266. Anionic phospholipids are determinants of membrane protein topology.

2. E.J. Breukink, C. van Kraaij, R.A. Demel, R.J. Siezen, O.P. Kuipers and B. de Kruijff, Biochemistry 36 (1997) 6968-6976. The C-terminal region of nisin is responsible for the initial interaction of nisin with the target membrane.

3. E.J. Breukink, C. van Kraaij, et. al., (1998) submitted,
- The orientation of nisin in the membrane.
- Pore formation by nisin involves translocation of the C-terminal part across the membrane.

4. W. van Klompenburg, M. Paetzel, J.M. de Jong, R.E. Dalbey, R.A. Demel, G. von Heijne and B. de Kruijff (1998) submitted. The catalytic domain of leader peptidase inserts in a phosphatidylethanolamine dependent way into the outer leaflet of the E. coli inner membrane.

5. A. Rietveld, M.C. Koorengevel and B. de Kruijff, EMBO J. 14 (1995) 5506-5513. Non-bilayer lipids are essential for efficient protein translocation across the *E. coli* plasma membrane.

6. B. de Kruijff, Current opinion in Chemical Biology 1 (1997) 564-569. Lipid polymorphism and biomembrane function.

7. B. de Kruijff, Nature 386 (1997) 129-130. Lipids beyond the bilayer.

POSTERS

The interaction of phospholipase C with phospholipid bilayers.

M.B. Ruiz-Argüello, F.M. Goñi and A. Alonso. Grupo Biomembranas (Unidad Asociada al C.S.I.C.), Departamento de Bioquímica, Universidad del País Vasco, Aptdo. 644, 48080 Bilbao, Spain.

ABSTRACT: Phosphatidylcholine phospholipase C (EC 3.1.4.3) from *Bacillus cereus* has been assayed with substrates in the form of large unilamellar vesicles. Phosphatidylcholine, phosphatidylethanolamine (also a substrate for the enzyme), sphingomyelin and cholesterol have been mixed in various proportions, in binary, ternary and quaternary mixtures. A lag period, followed by a burst of enzyme activity, has been found in all cases. The activity burst was always accompanied by an increase in turbidity of the vesicle suspension. Varying lipid compositions while keeping constant all the other parameters leads to a range of lag times extending over two orders of magnitude (from 0.06 to 38.0 min), and a similar variability is found in maximal enzyme rates (from 0.24 to 55.9 min^{-1}). Meanwhile, the proportion of substrate that is hydrolyzed during the lag period remains relatively constant at $\approx$10% moles of total lipid, in agreement with the idea that enzyme activation is linked to vesicle aggregation through diacylglycerol-rich patches. Phosphatidylethanolamine and cholesterol enhance the enzyme activity in a dose-dependent way: they reduce the lag times and increase the maximal rates. The opposite is true of sphingomyelin. These lipids exert each its own peculiar effect, positive or negative, either alone or in combination, so that the susceptibility of a given mixture to the enzyme activity can be to some extent predicted from its composition. The modulation of phospholipase C activity by the various lipids is not related to the formation of nonlamellar structures. Rather the mere presence of "nonbilayer lipids", phosphatidylethanolamine or cholesterol, even under conditions at which purely lamellar phases exist, appears to stimulate the enzyme, and the opposite happens with sphingomyelin, a well-known stabilizer of the lamellar phase. Thus the enzyme, while responding in a predictable way to the presence of specific lipids, appears to do so through changes in the global properties of the bilayer.

IMPALA : A simple restraint field to simulate the biological membrane in molecular structure studies.

Ph. Ducarme[0], M. Rahman and R. Brasseur

Centre de Biophysique Moléculaire Numérique
Faculté des Sciences Agronomiques de Gembloux
Passage des Déportés 2
B5030 Gembloux
Belgium
pducarme@fsagx.ac.be

The lipid bilayer is crucial for the folding of integral membrane proteins. This poster presents an empirical method to account for water-lipid interfaces in the insertion of molecules interacting with bilayers.

The interactions between the molecule and the bilayer are described by restraint functions designed to mimic the membrane effect. These functions are calculated for each atom and are proportional to the accessible surface of the latter.

The membrane is described as a continuous medium whose properties are varying along the axis perpendicular to the bilayer plane.

The insertion is analyzed by a Monte Carlo procedure applied to the restraint functions

The method was successfully applied to small α peptides of known configurations. It provides insights of the behaviors of the peptide dynamics that cannot be obtained with statistical approaches (e.g. hydropathy analysis).

The Effect of Environmental Variables on the Dimerization Free Energy of the Glycophorin A Transmembrane Domain

Lillian E. Fisher[1,2] and Donald M. Engelman[2]
[1]Departments of Chemistry and [2]Molecular Biophysics and Biochemistry
Yale University, New Haven, CT.

We are using resonance energy transfer to monitor the association of the transmembrane domain of human Glycophorin A, (GpATM), in a variety of detergent and lipid environments. Adair *et al.* had used a donor-quencher system to measure the oligomeric state in DMPC bilayers. However, the results did not distinguish between monomer/dimer and dimer/tetramer interactions. In this study, a peptide corresponding to the transmembrane domain of Glycophorin A, (GpATM), was synthesized and labeled with either a donor or an acceptor fluorophore. The label was attached specifically at either the N-terminus or at one of two lysine residues near the C-terminus. We selected pyrene as a donor, (D), and N,N-dimethyl-aminocoumarin as an acceptor, (A), because they are likely to produce only small perturbations to the structure yet still give a strong enough signal to measure the association constant at nanomolar concentrations. We present the preparation and spectroscopic characterization of the labeled peptides and demonstrate their utility in monitoring the GpA exchange reaction: DD + AA → DA. Conditions have been found where artifactual tetramerization can be avoided and the monomer-dimer reaction is reversible with concentration and temperature. As expected, the equilibrium constant does not depend on the location of the fluorophores but does depend on the identity and concentration of the surfactant. The rate of the exchange reaction depends on the identity and concentration of the surfactant as well as the concentration of the peptide. Thus we can measure thermodynamic and kinetic parameters, which describe the dimerization reaction of Glycophorin A, in a variety of detergents and lipids. With this system we can begin to explore the relative importance of environmental factors that are postulated to influence transmembrane helix association, such as bilayer composition, thickness and fluidity.

Structural and thermodynamic features of the interaction of PDC-109, the major protein from bull seminal plasma. with phosphatidylcholine membranes.

María Gasset[1], Leticia Magdaleno[1], Teresa González-Gallego[1], José Laynez[1], Edda Töpfer-Petersen[2] & Juan J. Calvete[2]

[1]Inst. Quimica-Fisica "Rocasolano", CSIC, Madrid, Spain; [2]Inst. Reproduktionsmedizin, Tierärztliche Hochschule, Hannover, Germany.

At ejaculation PDC-109, the major protein of bull seminal plasma, binds to spermatozoa plasma membrane and modulates capacitation promoted by glycosaminoglycans during sperm residence in the female genital tract. It is composed of a single polypeptide chain of 109 amino acids organized into a 23-residue acidic N-terminal polypeptide strecht followed by two tandemly arranged fibronectin type II-like domains. In solution, PDC-109 behaves as a polydispersed solute whose size and heterogeneity can be modulated by medium composition as evidence by analytical ultracentrifugation experiments. In the presence of phosphatidylcholine model membranes PDC-109 becomes lipid-soluble, due to the specific recognition of choline groups. Protein binding alters vesicle contour and membrane permeability. Complex formation occurs with increases of lipid acyl chain disorder and of bilayer interface hydration which result in the sequestering of molecules from participating in the gel to liquid crystalline thermotropic transition as evidenced by FTIR and DSC. Upon choline lipid binding, PDC-109 undergoes a conformational change characterized by an increase in the unordered structure percentage at expenses of a reduction in the turn content, a decrease in the polarity of Trp environment and an overall rearrangement of the tertiary structure. When the interaction is modelled with o-phosphorylcholine discrete entities, PDC-109 oligomers disassemble by shielding of the hydrophobic solvent-exposed surface of the fibronectin type II domain

NICOTINIC ACETYLCHOLINE RECEPTOR INDUCES LIPID PHASE SEPARATION IN COMPLEX RECONSTITUTED MEMBRANES.

J.M. González-Ros, A.M. Fernández, J.A. Encinar and J.A Poveda.
Centro de Biología Molecular y Celular.
Universidad Miguel Hernández, 03206 Elche (Alicante), Spain.

The nicotinic Acetylcholine Receptor (AcChR) from Torpedo is a large transmembrane glycoprotein composed of four different polypeptide subunits (α, β, γ and δ) in a 2:1:1:1 stoichiometry. Binding of cholinergic agonists to extracellular domains on the α subunits, causes the formation of a transient cation channel within the protein, responsible for the initiation of postsynaptic membrane depolarization.

Studies of reconstitution of the purified AcChR protein into multicomponent artificial liposomes have shown that the presence of certain lipids, namely cholesterol and phosphatidic acid, is required to preserve the ability of the AcChR to exhibit an optimal cation channel activity. The maintenance of such functional response by the AcChR can be correlated with the preservation of a high percentage of α-helical components in the protein secondary structure. On the other hand, the absence of such lipids results in inactive reconstituted samples, in which the AcChR protein exhibits a significant loss of α-helical structure, concomitant with an increase in non-ordered structural components.

Fourier-transform infrared spectroscopy and differential scanning calorimetric studies on the effects of the protein on phospholipid dynamics within the reconstituted samples show that in the absence of protein, the complex population of membrane lipid components remain ideally mixed. On the contrary, the presence of the AcChR directs the formation of specific lipid domains, that become segregated from the bulk lipid matrix.

Our results suggest that a protein-induced, lipid phase separation phenomenon underlies the lipid modulation of AcChR structure and function referred above.

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Abstract Title: Study of the mechanism of membrane permeabilization induced by the protein Sticholysin II from the sea anemone Stichodactyla helianthus.

Abstract Authors: Mancheño, J.M., de los Ríos, V., Lanio, M.E (*)., Oñaderra, M., and Gavilanes, J.G.

Abstract Address: Dept. Bioquímica y Biología Molecular I. Fac. Químicas. Universidad Complutense. 28040 Madrid. SPAIN.
* Dept. Bioquímica. Fac. Biología. Universidad de la Habana. CUBA.

Abstract Body: Sticholysin II (St-II) is a single polypeptide chain protein composed of 175 amino acid residues isolated from the sea anemone Stichodactyla helianthus, characterized by its high hemolytic activity. This protein promotes leakage of aqueous contents from model membranes composed of phosphatidylcholine (PC) or sphingomyelin (SM), either when binary mixtures (PC:SM) are employed or when cholesterol is included in the bilayers. Kinetic analysis of the leakage, as studied by the ANTS/DPX assay, suggests the involvement of protein-protein interactions in the mechanism of permeabilization. This result together with the the dependence of the maximum extent of leakage on the molecular size of the encapsulated solute, point to the formation of discrete pores. The conformation of the St-II has been studied by far-UV circular dichroism spectroscopy. Conformational changes are detected after interaction with leakage-susceptible membranes. Interestingly, under certain acidic conditions St-II shows conformational properties typical from partly folded polypeptides, i.e., secondary structure content similar to the native state, absence of rigid tertiary structure, as detected by near UV-CD, and binding of hydrophobic probes as 2,6-ANS.

INTERACTION OF α-THIONIN WITH LIPIDIC VESICLES

J. M. Martínez-Caaveiro, F. M. Goñi and J. M. González-Mañas.
Department of Biochemistry and Molecular Biology, Faculty of Sciences, University of the Basque Country, P.O.Box 644, 48080 Bilbao, Spain.

Thionins are cystine-rich, basic polypeptides with a molecular mass of about 5000, which are quite abundante in the endosperms of many Graminae [1]. They were the first plant peptides for which an antimicrobial activity had been reported [2], and have been shown to alter cell membrane permeability [3] and to interact with artificial liposomes containing phosphatidylserine [4].

In our work, we have used large unilamellar vesicles with different lipid compositions and a whole battery of fluorescent probes to study in detail diverse aspects of the interaction of wheat α-thionin with model membranes, i.e. the release of encapsulated fluorophores, the mixing of the lipids, the aggregation of the vesicles and the fusion of the vesicles.

Our results indicate that the interaction of the protein with the vesicles is electrostatic, and that there is a threshold value of negative charge surface density that ensures the binding of the required amount of protein to exert any effect. Binding of the protein is followed by the rapid aggregation of the vesicles. Once the bilayers come into close contact, we failed to observe vesicle fusion as other authors have suggested [4]. Instead, we observed that the mixing of the lipids and the release of the encapsulated vesicles followed similar kinetics, suggesting that both processes take place simultaneously and are the result of the protein-induced desorganization of the bilayer, which can no longer function as a permeability barrier.

References

[1] Balls, A. K., Hale, W. S. and Harris, T. H. "A crystalline protein obtained from a lipoprotein of wheat flour". *Cereal Chem.* **19** (1942): 279-288.

[2] Stuart, L. S. and Harris, T. H. "Bactericidal and fungicidal properties of a crystalline protein isolated from unbleached wheat flour". ". *Cereal Chem.* **19** (1942): 288-300.

[3] Carrasco, L., Vázquez, D., Hernández-Lucas, C., Carbonero, P. and García-Olmedo, F. "Thinins: plant peptides that modify membrane permeability in cultured mammalian cells". *Eur. J. Biochem.* **116** (1981): 185-189.

[4] Gasanov, S.E., Vernon, L. P. and Aripov, T. F. "Modification of phospholipid membrane structure by the plant toxic peptide Pyrularia thionin". *Arch. Biochem. Biophys.* **301** (1993): 367-374.

Conformation of nascent polypeptide chains during membrane translocation.

Ismael Mingarro & Gunnar von Heijne
Arrhenius Labs, Dept. Biochemistry, Stockholm University, SWEDEN

Protein translocation through the membrane of the endoplasmic reticulum (ER) is the initial step for proteins that are to be secreted or are destined to membrane compartments along the secretory pathway of eukaryotes. This process is achieved by a complex multisubunit translocation machinery comprising cytoplasmic, integral membrane, and lumenal components. It has been proposed that the integral membrane components of this machinery -the so called "translocon"- form a water accessible channel in the membrane through which nascent polypeptide chains can pass.

In the cotranslational pathway of protein translocation, signal (anchor) sequences direct nascent polypeptides to the ER membrane where they are further elongated by membrane-bound ribosomes and simultaneously transported across the membrane through the translocon.

The architecture of the translocation machinery is further increased in complexity by proteins such as signal peptidase and oligosaccharyl transferase (OST) that are associated with the translocon but are not directly involved in the translocation process. It has been shown that the active site of the OST is positioned at a well defined distance above the surface of the ER membrane and can be used as a fixed point to measure the minimum length of polypeptide chain required to bridge the distance between the ribosomal peptidyl transferase site on the cytoplasmic side of the ER membrane and the OST active site on the lumenal side.

The nascent chain is thought to resemble an unstructured random coil and have no defined conformation at least inside the ribosome. In our studies the minimum distance has been measured for nascent polypeptide chains harbouring specific peptide sequences located both at the translocon and at the ribosome and the results will be discussed in terms of possible conformational structure already at these very early events of protein translation/translocation.

Turning an integral membrane protein into a water soluble protein: metamorphosis of bacteriorhodopsin

Kakoli Mitra

In 1994 Creamer and Rose put forth the Paracelsus challenge which called for the design of a protein with 50% sequence identity to a protein with a different fold. Dalal *et al.* met this challenge in 1997 by redesigning a beta-sheet protein into a native like four helix bundle. One might ask a similar but slightly different question of membrane proteins: Is it feasible that mutating the lipid exposed surface of a helical membrane protein to resemble that of a soluble protein would render the 'membrane' protein soluble in water with the structure of its interior still intact, i.e. retaining its function?

The system used to address this question is the seven transmembrane helix protein bacteriorhodopsin (bR). bR is well-suited for this study since its structure has been solved by Henderson *et al.* to 3.5 Å resolution by electron microscopy, which facilitates identifying lipid exposed residues for subsequent modification. In addition a visible assay for correct folding exists: the bR apoprotein turns purple upon uptake of the chromophore retinal. Two different design strategies are being employed. The first aims to preserve the native subunit contacts that hold the bR trimer together in the lipid bilayer (sbR). The second series of designs endeavours to abolish the trimer contacts to result in a monomeric 'solubilized' bR (mbR). When expressed in *E. coli* sbR is expressed but proteolyzed completely while in the cell, indicating that perhaps the oligomer is not stable in a 'solubilized' state. Characterization of the potentially monomeric mbR is in progress.

A MODEL FOR THE INTERACTION OF THE HIV-1 FUSION PEPTIDE WITH ELECTRICALLY NEUTRAL MEMBRANES

Francisca B. Pereira[1], José M. Valpuesta[2], Gorka Basañez[1,3], Félix M. Goñi[1], Arturo Muga[1], Aitziber Aguirre[1], Tatiana Suárez[1], and José L. Nieva[1].

[1]Grupo de Biomembranas (Unidad Asociada al C.S.I.C.), Departamento de Bioquímica, Universidad del País Vasco, Aptdo. 644, 48080 Bilbao, Spain.

[2]Centro Nacional de Biotecnología, C.S.I.C., Universidad Autónoma de Madrid, 28049 Madrid, Spain.

[3]Present address: The Laboratory of Cellular and Molecular Biophysics, National Institutes of Health, Bethesda, Maryland 20892, USA.

Abstract: The HIV-1 fusion peptide has the capacity to induce leakage and lipid mixing adopting an extended structure in vesicles composed of dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine and cholesterol (molar ratio, 1:1:1) [Pereira, F.B., Goñi, F.M., Muga, A., and Nieva, J.L. (1997) *Biophys. J.* 73, 1977]. When examined by means of cryo-transmission electron microscopy, peptide-treated vesicles showed two characteristic morphologies, membrane protrusions in isolated vesicles and highly bent lamellae in large lipidic aggregates. ^{31}P-nuclear magnetic resonance data were compatible with the existence of isotropic structures. Tryptophan fluorescence quenching by vesicles containing brominated phospholipids indicated that the peptide penetrates to the acyl chain level and locates in the proximity of the interface. A model is proposed according to which the differential partition and eventual penetration of the peptide into the external monolayer may account for the observed vesicle destabilization processes.

DIFFERENT INSERTION LEVELS OF SURFACTANT PROTEIN SP-B IN PHOSPHATIDYLCHOLINE BILAYERS. POSSIBLE IMPLICATIONS IN SURFACTANT DYNAMICS AT THE ALVEOLAR SPACES

J. Pérez-Gil[1], A. Cruz[1], C. Casals[1], K.M.W. Keough[2], and D. Marsh[3]

[1]Dpto. Bioquímica, Fac. Biología, Universidad Complutense, Madrid (SPAIN)
[2]Dpt. Biochemistry, Memorial University of Newfoundland, St. John's (CANADA)
[3]Abt. Spektroskopie, Max-Planck-Institut für biophysikalische Chemie, Göttingen (GERMANY)

Pulmonary surfactant-associated protein SP-B has been isolated from porcine lungs and reconstituted in bilayers of dipalmitoylphosphatidylcholine (DPPC) or egg yolk phosphatidylcholine (PC) in order to characterize the extent of insertion of the protein into phospholipid bilayers. The depth of penetration of SP-B into phospholipid bilayers has been estimated by the parallax method, which compares the relative efficiencies of quenching of the fluorescence of the single protein tryptophan by a shallow or a deeper spin-labelled phospholipid probe. SP-B tryptophan was found to be located 10-13 Å from the center of bilayers, which is consistent with a superficial location of SP-B in phosphatidylcholine membranes. Parallax experiments, as well as resonance energy transfer from SP-B tryptophan to an acceptor probe located in the center of the bilayer, indicate that there are significant differences in the extent of insertion of the protein, depending on the method of reconstitution. SP-B reconstituted from lipid/protein mixtures in organic solvents inserts more deeply in PC or DPPC bilayers than does the protein reconstituted by addition to preformed phospholipid vesicles. These differences in the extent of insertion lead to qualitative and quantitative differences in the effect of the protein on the mobility of the phospholipid acyl chains, as studied by spin-label electron spin resonance (ESR) spectroscopy.

On the other hand, compression of DPPC/SP-B monolayers formed at an air-liquid interface by adsorption of DPPC/SP-B bilayers conducted to protein squeeze-out from the lipids at surface pressures around 45 mN/m. All these results indicate that different extents of insertion of SP-B in surfactant bilayers and monolayers could modulate the biophysical properties of the tensoactive material in the respiratory dynamics.

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MEMBRANE INSERTION OF PF3 MAJOR COAT PROTEIN

A.N.J.A. Ridder, J. Stam, A. Kuhn, J.A. Killian and B. de Kruijff.

Department of Biochemistry of Membranes, CBLE, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.

The major coat protein of the filamentous bacteriophage Pf3 is a simple membrane protein containing only one transmembrane segment. It has a negatively charged N-terminus of 18 amino acids, a hydrophobic transmembrane segment of 18 amino acids and a positively charged C-terminus of 8 amino acids. Pf3 inserts into the *E. coli* inner membrane in an N_{out}-C_{in} orientation. This insertion depends on the membrane potential, but not on the Sec-machinery. An *in vitro* translocation system has been developed in which the N- and C-termini of Pf3 can be differentially labelled and thus specifically detected (1). This system and the simple topology of the protein make it an attractive model protein for studying membrane protein insertion.

Using this *in vitro* system, the insertion of Pf3 in liposomes with different lipid compositions and in the presence or absence of a membrane potential was investigated. The importance of tryptophan residues on the insertion of Pf3 was also studied. Tryptophans are frequently found on the membrane-water interface of bacterial membrane proteins and are more abundant on the periplasmic side of the membrane (2,3). It is not known whether they can play a role in determining the topology of membrane proteins. In order to answer this question, we studied the membrane insertion of a series of mutants of the Pf3 protein, with either 2, 1 or 0 tryptophans at one or both sides of the transmembrane segment. The results of these studies will be presented.

(1) : D. Kiefer, X. Hu, R. Dalbey and A. Kuhn, *EMBO J.* **16** : 2197 - 2204 (1997).

(2) : G. von Heijne, *EMBO J.* 5 : 3021 - 3027 (1986).

(3) : B. Persson and P. Argos, *Prot. Sci.* 5 : 363 - 371 (1996).

STRUCTURAL STUDIES ON THE CYTOCHROME C550: PROTEIN THAT STABILIZES OXYGEN EVOLUTION IN CYANOBACTERIA BY INTERACTING WITH THE PHOTOSYNTHETIC MEMBRANES.

Javier De Las Rivas & Pedro Heredia

Department of Biochemistry and Molecular Biology. Faculty of Science. University of the Basque Country. P.O.Box 644. 48080 - BILBAO - SPAIN. E-mail: gbpdesaj@lg.ehu.es

ABSTRACT:

Cytochrome c550 is a membrane-bound low potential c-type cytochrome that has been recently discover to be associated with photosystem II in cyanobacteria. (Enami et al., 1995). Moreover, this cytochrome has been probed to play a significative role in maintaining photoinduced oxygen evolution in cyanobacteria cells, since when its gene (*psb*V) was deleted together with the *psb*O gene (which encodes the 33 kDa manganese stabilizing protein) all the photosynthetic activity of this mutated cyanobacteria did disappeared (Shen et al., 1995). The structure of this cytochrome is not known. We have carried out a structural study of the protein using modern computational methods for protein structural prediction. So, we present a multiple sequence alignment of the known sequences of this cytochrome c550 present in algae and in cyanobacteria (6 full sequences). The alignment allows the drawing of a simple phylogenetic tree of these proteins. Using homology modelling we have also produced a 3D model of the cytochrome based on a found similarity to another cytochrome c of known 3D structure. This cytochrome c has 103 aa and is used as a template to build up a 3D model of the cytochrome c550 from the cyanobacteria *Synechocystes sp.* (cytc550-syn3) , that has 135 aa. The PDB file of the template is named 3CYT and the model was produced using the program WHATIF. For the construction of the model we obtained the best alignment leaving out 23 aa in N-terminal and 8 aa in the C-terminal of cyt c550 (23+8=31). We also have to include two deletions in the sequence of the model between His18 and Gly19 and between Gly76 and Tyr77, since the template 3CYT has only 103 aa and cyt c550 has 135 aa. The first deletion is of 2 aa and corresponds to Leu42 and Gln43 in the sequence of cyt c550 from *Synechocystes*. The second deletion corresponds to 16 aa from Glu85 to Ile100 in the sequence of cyt c550. Therefore, the model includes 135 – 31 – 18 = 86 aa. These deletions in the modelled sequence are compensated with a deletion of 17 aa in the template (103 – 17 = 86 aa) in two gaps of 15 and 2 aa. With this modifications we obtained a model where all the key residues that ligate the heme are conserved in the correct 3D position: Cys37 and Cys40 covalent ligands to the heme; His41 and Met104 axial ligands to the iron of the heme (the numbers of the aa correspond to the cyt c550 sequence of *Synechocystes*). Also the 4 α-helix present in the template 3CYT are conserved in the model without any insertion or deletion that could disturb these important secondary structure features.

REFERENCES

Enami I., et al. (1995) *Biochim. Biophys. Acta* 1232:208-216.
Shen J. R., et al. (1995) *Biochemistry* 34:12661-12668.

"Interaction of TrwD protein of R388 plasmid with model membranes"

Rivas, S., de la Cruz, F. & Goñi, F.M.

trwD is an essential gene for the conjugative process of R388 plasmid (1). The predicted amino acid sequence of TrwD protein shows significant homology with PulE/VirB11 protein superfamily of potential ATPases involved in several macromolecular transport processes in gram-negative bacteria (2).

Subcellular fractions were obtained from cell cultures expressing TrwD and the protein was found in both the soluble and the outer membrane fractions (3). This observation may be indicative of a peripheral association of TrwD to the bacterial membrane. Besides, the yield of TrwD purification increases significantly in the presence of 0.5 M NaCl (3). This is typical of peripheral or extrinsic membrane proteins and in our case indicates the release of the membrane-bound fraction. A related observation is the fact that the ATPase activity displayed by TrwD protein was increased by pure detergent (at concentrations above cmc values) or detergent-phospholipid vesicles but not by phospholipid in bilayer form (3). This is suggestive of TrwD possessing hydrophobic patches on its surface, which would allow association to micelles or even a peripheral binding to bilayers (perhaps to intrinsic proteins in bilayers) but not insertion in the hydrophobic matrix of a membrane. Finally, we used a model system in order to further study this interaction of TrwD with membranes. TrwD was able to induce aggregation of liposomes, lipid mixing and leakage of the vesicle contents.

REFERENCES

(1) Bolland, S., Llosa, M., Ávila, P. & de la Cruz, F. (1990) *J. Bacteriol.* **172**, 5795-5802.

(2) Possot, E., d'Enfert, C., Reyss, I. & Pugsley, A. (1992) *Mol. Microbiol.* **6**, 95-105.

(3) Rivas, S., Bolland, S., Cabezón, E., Goñi, F.M. & de la Cruz, F. (1997) *J. Biol. Chem.* **272**, 25583-25590.

VACCINIA VIRUS 21K AND 15K PROTEINS ARE INCORPORATED INTO CELLULAR MEMBRANES AND PLAY AN ESSENTIAL ROLE IN VIRAL MEMBRANE FORMATION

Rodríguez, J.R.,Esteban, M., Risco, C., Carrascosa, J.L., Rodríguez, D.

Centro Nacional de Biotecnología. C.S.I.C. Madrid. Spain

Vaccinia virus (VV) morphogenesis is a complex multistep process that involves numerous viral elements. The molecular events that dictate the ordered incorporation of the viral structures during the assembly of the viral particles remain to be elucidated. Recent studies indicate that viral membranes are derived from the cellular intermediate compartment (IC) located between the rough endoplasmic reticulum (RER) and the Golgi apparatus. However, very little is known about the key viral factors involved in the recruitment, modification, and organization of these IC derived membranes to generate the mature viral envelope. We have identified two membrane proteins with apparent molecular weights of 21 and 15 Kilodaltons (kDa), encoded respectively by the A17L and A14L viral genes, that form a stable complex with a previously characterized 14kDa (A27L gene) envelope protein of VV. The intracellular transport of these two proteins defined by immunoelectron microscopy (RER- IC- viral envelope) suggest that they may participate in the initial sequence of events in the formation of VV membranes. Through the generation of recombinant viruses that inducibly express the 21kDa or the 15kDa proteins we demonstrate that these two proteins are essential for VV morphogenesis. Our results suggest that 21kDa protein is involved in organizing the IC-derived membranes recruited to the locations of VV assembly (viral factories), while the 15kDa protein appears to be necessary for the correct assembly and attachment of the membrane precursors on the periphery of the viral factories.

Insertion of glycosylphosphatidylinositol (GPI)-anchored proteins into liposomes. Effects of cholesterol and glycosphingolipids on the reconstitution of proteoliposomes.

O. Nosjean, A. Coleman* and B. Roux Laboratoire de Physico-Chimie Biologique, UPRESA CNRS 5013, UCB-Lyon I 43 bd du 11 novembre 1918 69622 Villeurbanne France
*Institut de Biologie et Chimie des Protéines CNRS UPR 412

Membranes that contains GPI-anchored proteins are enriched in sphingolipids and cholesterol. To understand the organization of biologic membranes and the interactions between proteins and lipids, we have reconstituted proteoliposomes containing cholesterol, lactocerebroside and alkaline phosphatase (AP). The protein was purified with GPI-anchor from bovine kidney and incorporated into preformed liposomes by incubation in presence of detergent. Then detergent was eliminated by dialysis. AP insertion was confirmed by centrifugation on sucrose gradient and by antibody-mediated proteoliposomes precipitation.
We have studied the influence of glycosphingolipid and cholesterol on the stability and the morphology of these reconstituted proteoliposomes as follows : AP stability in the membranes was evaluated by heat treatment and accessibility of the GPI-anchor to specific phospholipase hydrolysis. AP localization on the liposomes and liposomes morpholoy wre studied by atomic force microscopy.

DIFFERENTIAL SCANNING CALORIMETRY OF THE BIOLOGICAL MEMBRANES

Galina Zhadan and Valery Shnyrov

Departamento de Bioquímica y Biología Molecular, Universidad de Salamanca, Salamanca, SPAIN

The advent of calorimetric methods has resulted in a qualitative step forward in the thermodynamic characterization of biological macromolecules and an understanding of process involving these species. In particular, differential scanning calorimetry (DSC) has become a very powerful and convenient technique to study the temperature induced conformational transitions in biological systems.

Biological membranes and related model systems are currently being investigated by DSC. The application of DSC to these systems has been primarily directed to the study of the thermotropic phase behaviour of lipids. On the other hand, there have been much fewer DSC studies on the thermal denaturation of membrane proteins.

The aim of this presentation is to review what has been carried out so far in DSC of membrane proteins.

MEMBRANE BINDING MODULATES THE CONFORMATION AND STABILITY OF PLASTOCYANIN

S.G. Taneva, F. Goñi, A.A. Donchev*, M.I. Dimitrov* and A. Muga

Department of Biochemistry, University of Basque Country, P.O. Box 644, Bilbao, Spain and *Institute of Biophysics, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria

The water-soluble copper protein plastocyanin acts as an electron transporter in the light reactions of plant photosynthesis. Its redox state is regulated by the interaction with two membrane-embeded complexes; it is reduced by cytf in the $cytb_6/f$ complex and oxidized by photosystem I reaction center P700. Two electron transfer sites in the redox reactions of plastocyanin, the hydrophobic northern patch important for the interaction with photosystem I and the acidic east patch essencial for binding with cytf and photosystem I, has been proposed.
The structure and stability changes of plastocyanin associated with the binding of its reduced and oxidized forms to lipid vesicles was studied by FTIR. Plastocyanin binding to positively charged lipid vesicles, via electrostatic interactions, destabilizes both the reduced and oxidized form with respect to the protein in solution without significant variation of the protein conformation. This is evident from the reduction of the midpoint denaturation temperature of the lipid-associated protein as compared with that of the protein in solution. The lumenal side of thylakoid membrane experiences a pH decrease to approx. pH 5 upon illumination that regulates the electron transfer rate. Therefore, the interaction of reduced and oxidized plastocyanin with neutral lipid vesicles at pH 5 was studied. Interestingly, under these experimental conditions, the membrane-binding of plastocyanin is markedly influenced by the protein redox state. Once bound, the reduced plastocyanin can adopt several conformations depending on the protocol used for sample preparation, revealing a strong structural plasticity.

STUDY OF THE INTERACTION BETWEEN THE HIV-1 ENVELOPE PROTEIN AND CELLULAR CORECEPTORS.

Florence Verrier, Andrew Borman and Marc Girard. Unité de Virologie Moléculaire (CNRS URA 1966), Département de Virologie, Institut Pasteur, Paris, FRANCE.

The HIV-1 Env glycoprotein (gp160) comprises an external surface polypeptide (gp120) and a transmembrane anchor polypeptide (gp41) which remain associated in a non-covalent fashion. The primary receptor for HIV is CD4. Binding of HIV to CD4 induces conformational rearrangements in the gp120/gp41 molecule that lead to fusion of the virus and cell membranes by a process mediated by the N-terminal fusion peptide of gp41. Recent evidence indicates that certain chemokine receptors collaborate with CD4 to facilitate HIV-1 entry into $CD4^+$ target cells : T-cell line-adapted (TCLA) strains and T-cell-tropic, 'syncytium-inducing (SI)', primary virus isolates use CXCR-4 (LESTR, fusin) as their coreceptor, whereas macrophage-tropic, so-called 'non-syncytium-inducing (NSI)' primary isolates principally use CCR-5. The cellular tropism of HIV-1 is determined by the structure of the envelope protein and particularly by specific sequence within the V3 loop of gp120.

The aims of this study were to characterise the CCR-5-dependent cell-to-cell fusion mediated by the envelope from a primary HIV-1 isolate, and to probe the domains in gp120/gp41 involved in the interaction with the CD4 and CCR-5 molecules using a panel of anti-gp120/gp41 monoclonal antibodies. To study the requirements for HIV-1 Env-mediated fusion, we developed an assay system based on the use of Semliki Forest virus (SFV) recombinants that express Env gp120/gp41 either from the TCLA HIV-1_{LAI} strain, or from a primary, macrophage-tropic isolate, HIV-1_{BX08}. The ability of these recombinants to induce syncytium formation in CD4+ HeLa cells or CD4+/CCR5+ HeLa cells was examined in the presence and absence of a variety of potential inhibitors of HIV infection.

In cells expressing the appropriate co-receptor, gp120/gp41 of the 'non syncytium-inducing', primary, macrophage-tropic HIV-1_{BX08} isolate, was at least as fusogenic as that of the 'syncytium-inducing' HIV-1_{LAI} strain. BX08 Env-mediated fusion was inhibited by the β-chemokines RANTES and MIP-1β and by antibodies to CD4, whereas LAI Env-mediated fusion was insensitive to these chemokines. In contrast, sCD4 significantly inhibited LAI, but not BX08 Env-mediated fusion, suggesting that the primary isolate envelope glycoprotein has a lower affinity for CD4.

The domains in gp120/gp41 involved in the interaction with the CD4 and CCR-5 molecules were probed using monoclonal antibodies (Ab) or gp120 peptides. The greatest inhibition of fusion was observed with Ab directed to conformation-dependent epitopes. Efficient inhibition of fusion was not restricted to epitopes in any one domain of gp120/gp41. The assay was sufficiently sensitive to distinguish between Ab- and β-chemokine-mediated fusion inhibition using serum samples from patient BX08, suggesting that the system may be useful for screening human sera for the presence of biologically significant antibodies. Peptides corresponding to the LAI, MN or BX08 V3 loops efficiently blocked fusion mediated by the homologous viral Env protein. In addition, the MN and LAI peptides showed activity against fusion mediated by both TCLA viral envelopes, but little or none against BX08 Env-mediated fusion. We examined the effects of BX08-related V3 peptides mutated at 1, 2 or all 3 of the positions reported to be important in determining viral tropism[2]. BX08-related peptides which contained combinations of the 3 mutations were incapable of blocking BX08 env-mediated fusion, but became increasingly efficient in inhibiting fusion mediated by the TCLA LAI envelope. The capacity of these mutant peptides to inhibit primary or TCLA Env-mediated fusion in our system correlated very closely with the tropism of viral isolates containing the equivalent V3 loop mutations. Overall, the results of these peptide inhibition studies suggest strongly that the BX08, LAI and MN V3 loop sequences alone are sufficient to determine coreceptor usage by the corresponding viruses.

[1] Verrier et al, 1997. PNAS, 94,9326-31.
[2]de Jong et al, 1992. J.Virol. 66,6777-80.

LIST OF INVITED SPEAKERS

José Luis R. Arrondo — Departamento de Bioquímica, Universidad del País Vasco, Apdo. 644, 48080 Bilbao (Spain). Tel.: 34 4 464 77 00. Fax: 34 4 464 85 00. E-mail: GBPROARJ@LG.EHU.ES

Paula J. Booth — Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY (U.K.). Tel.: 44 171 594 51 93. Fax: 44 171 225 09 60. E-mail: p.j.booth@ic.ac.uk

J. Thomas Buckley — Department of Biochemistry and Microbiology, University of Victoria, Box 3055, Victoria, B.C. V8W 3P6 (Canada). Fax: 1 250 721 88 55. E-mail: tbuckley@uvic.ca

Donald M. Engelman — Department of Molecular Biophysics and Biochemistry, Yale University, 266 Whitney Avenue, New Haven, CT. 06520-8114 (USA). Fax: 1 203 432 63 81. E-mail: ENGELMAN@IBPC.FR

Alfred F. Esser — Division of Cell Biology & Biophysics, School of Biological Sciences, University of Missouri, Kansas City, MO. 641110 (USA). Tel.: 1 816 235 53 16. Fax: 1 816 235 15 03. E-mail: aesser@cctr.umkc.edu

Félix M. Goñi — Departamento de Bioquímica, Universidad del País Vasco, Apdo. 644, 48080 Bilbao (Spain). Tel.: 34 4 464 77 00. Fax: 34 4 464 85 00. E-mail: GBPGOURF@LG.EHU.ES

Wayne L. Hubbell — Jules Stein Eye Institute, School of Medicine, University of California, Los Angeles, CA. 90095-7008 (USA). Fax: 1 310 794 21 44. E-mail: hubbellc@jsei.ucla.edu

Ben de Kruijff — Dept. of Biochemistry of Membranes, Centre for Biomembranes and Lipid Enzymology, Utrecht University, 3508 TB Utrecht (The Netherlands). Tel.: 31 30 253 16 07. Fax: 31 30 253 91 71. E-mail: b.dekruijff@chem.ruu.nl

Ronald N. McElhaney — Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7 (Canada). Tel.: 1 403 492 24 13. Fax: 1 403 492 00 95. E-mail: rmcelhan@gpu.srv.ualberta.ca

Maurice Montal — Department of Biology, University of California San Diego, 9500 Gilman Drive, La Jolla, CA. 92093-0366 (USA). Tel.: / Fax: 1 619 534 09 31. E-mail: montal@biomail.ucsd.edu

Cesare Montecucco — CNR Center for Biomembranes and Dept. Biomedical Sciences, University of Padova, Via G. Colombo 3, Padova (Italy). Tel.: 39 49 827 60 58. Fax: 827 60 49 . E-mail: cesare@civ.bio.unipd.it

Walter Neupert — Institute of Physiological Chemistry, University of Munich, Goethestr.33, D-80336-München (Germany). Tel.: 49 89 59 96 313. Fax: 49 89 599 62 70. E-mail: Neupert@bio.med.uni-muenchen.de

Franc Pattus — Dept. Recepteurs et Proteines Membranaires, UPR 9050 CNRS, Illkirch (France). Tel.: 33 3 88 65 52 78. Fax: 33 3 88 65 52 49. E-mail: pattus@esbs1.u-strasbg.fr

Anthony P. Pugsley — Unité de Génétique Moléculaire, CNRS, Institut Pasteur, 25, rue du Docteur Roux, 75724 Paris cedex 15 (France). Tel.: 33 1 45 68 84 94. Fax: 33 1 45 68 89 60. E-mail: max@pasteur.fr

Jurg P. Rosenbusch — Biozentrum, University of Basel, Klingelbergstr. 70, CH-4056 Basel (Switzerland). Tel.: 41 61 267 21 10. Fax: 41 61 267 21 18. E-mail: rosenbusch@ubaclu.unibas.ch

Benoit Roux — Depts. of Physics & Chemistry, Université de Montreal, C.P. 6128, succ. Centre-Ville, H3C 3J7 Montreal, PQ. (Canada). Tel.: 1 514 343 71 05. Fax: 1 514 343 75 86.

Gunnar von Heijne — Department of Biochemistry, Stockholm University, S-106 91 Stockholm (Sweden). Tel.: 46 8 16 25 90. Fax: 46 8 15 36 79. E-mail: gunnar@biokemi.su.se

Bonnie A. Wallace — Dept. of Crystallography, Birkbeck College, University of London, Malet Street, London WC1E 7HX (U.K.). Tel.: 44 171 631 68 00. Fax: 44 171 631 68 03.

Anthony Watts — Oxford Biomembrane Structure Unit, Biochemistry Department, University of Oxford, South Parks Road, Oxford OX1 3QU (U.K.). Tel.: 44 1865 27 52 68. Fax: 44 1865 27 52 34. E-mail: awatts@bioch.ox.ac.uk

LIST OF PARTICIPANTS

Alicia Alonso — Dept. de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad del País Vasco, Apdo. 644, 48080 Bilbao (Spain). Tel.: 34 4 464 77 00. Fax: 34 4 464 85 00. E-mail: gbpaliza@lg.ehu.es

Ignacio Arechaga — MRC-LMB, Hills Road, Cambridge, CB2 2QH (U.K.). Tel.: 44 1223 40 22 54. Fax: 44 1223 41 21 78.

Cristina Casals — Dept. de Bioquímica y Biología Molecular. Facultad de Ciencias Biológicas, Universidad Complutense, 28040 Madrid (Spain). Tel.: 34 1 394 42 61. Fax: 34 1 394 46 72. E-mail: casals@solea.quim.ucm.es

David Clarke — Institut de Génétique et Microbiologie, Bâtiment 409, Universite Paris-Sud, 91405 Orsay cedex (France). Tel.: 33 1 69 15 66 99. Fax: 33 1 69 15 78 08. E-mail: Clarke@Igmors.U-psud.fr

Philippe Ducarme — Centre de Biophysique Moléculaire Numérique, Faculté des Sciences Agronomiques de Gembloux, Passage des Déportés 2, B5030 Gemblux (Belgium). Tel.: 32 81 62 25 21. Fax: 32 81 62 25 22. E-mail: pducarme@fsagx.ac.be

Antonio Ferrer — Dept. de Neuroquímica, Universidad Miguel Hernández, c/Monovar s/n. 03206 Elche, Alicante (Spain). Tel.: 34 6 665 87 61. Fax: 34 6 665 86 80. E-mail: aferrer@umh.es

Lillian E. Fisher — Dept. of Chemistry, Yale University, 225 Prospect St., New Haven, CT. 06511 (USA). Fax: 1 203 432 31 04. E-mail: fisher@paradigm.csb.yale.edu

María Gasset — Instituto Química-Física "Rocasolano", CSIC, c/Serrano 119, 28006 Madrid (Spain). Tel.: 34 1 561 94 00. Fax: 34 1 584 24 31. E-mail: mgasset@iqfr.csic.es

Jan-Willen L. de Gier — Dept. of Biochemistry, Arrhenius Laboratory, Stockholm University, S-106 91 Stockholm (Sweden). Tel.: 46 8 16 42 76. Fax: 46 8 15 36 79. E-mail: degier@mail.biokemi.su.es

Juan M. González Mañas — Dept. of Biochemistry and Molecular Biology, Faculty of Sciences, University of the Basque Country, P.O. Box 644, 48080 Bilbao (Spain). Tel.: 34 4 464 77 00. Fax: 34 4 464 85 00. Gbpgomaj@lg.ehu.es

José M. González-Ros — Centro de Biología Molecular y Celular, Universidad Miguel Hernández, c/Monóvar s/n, 03206 Elche, Alicante (Spain). Tel.: 34 6 665 87 57. Fax: 34 6 665 86 80. E-mail: gonzalez.ros@umh.es

Aitziber López Cortajarena — Dept. de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad del País Vasco, Aptd. 644, 48080 Bilbao (Spain). Tel.: 34 4 464 77 00. Fax: 34 4 464 85 00. E-mail: gbblocoa@lg.ehu.es

José M. Mancheño — Dept. de Bioquímica y Biología Molecular I, Facultad de Químicas, Universidad Complutense, 28040 Madrid (Spain). Tel.: 34 1 394 42 58. Fax: 34 1 394 41 59. E-mail: jmmg@solea.quim.ucm.es

José M. Caaveiro — Dept. of Biochemistry and Molecular Biology, Faculty of Sciences, University of the Basque Country, P.O. Box 644, 48080 Bilbao (Spain). Tel.: 34 4 464 77 00. Fax: 34 4 464 85 00. E-mail: gbbmacaj@lg.ehu.es

Ismael Mingarro — Dept. Bioquímica, Facultad de Biología, Universidad de Valencia, Dr. Moliner 50, 46100 Burjassot, Valencia (Spain). Tel.: 34 6 386 43 85. Fax: 34 6 386 46 35. E-mail: mingarro@uv.es

Kakoli Mitra — Dept. of Molecular Biophysics & Biochemistry, Yale University, 266 Whitney Ave., New Haven, CT. 106520-8114 (USA). Tel.: 1 203 432 55 99. Fax: 1 203 432 63 81. E-mail: mitra@csbmet.csb.yale.edu

José Luis Nieva — Dept. de Bioquímica y Biología Molecular,, Facultad de Ciencias, Universidad del País Vasco, Apdo. 644, 48080 Bilbao (Spain). Tel.: 34 4 464 77 00. Fax: 34 4 464 85 00. E-mail: GBPNIESJ@lg.ehu.es

Jesús Pérez-Gil — Dept. de Bioquímica y Biología Molecular, Fac. de Biología, Universidad Complutense, 28040 Madrid (Spain). Tel.: 34 1 394 46 20. Fax : 34 1 394 46 72. E-mail: peregil@solea.quim.ucm.es

Doron Rapaport — Institut für Physiologische Chemie, Gothestraße 33, D-80336 München (Germany). Tel.: 49 89 5996 289. Fax: 49 89 5996 270. E-mail: RAPAPORT@BIO.MED.UNI-MUENCHEN.DE

Mariasun Requero — Martin-Luther-Universität, Halle-Wittenberg, Fachbereich Chemie, Inst. für Physikalische Chemie, 06099 Halle (Germany). Fax: 49 345 552 71 57. E-mail: requero@chemie.uni-halle.de

A.N.J.A. Ridder — Department of Biochemistry of Membranes, CBLE, Utrech University, Padualaan 8, 3584 CH Utrech (The Netherlands). Tel.: 31 30 253 33 42. Fax: 31 30 252 24 78. E-mail: a.n.j.a.ridder@chem.ruu.nl

Javier de las Rivas — Department of Biochemistry and Molecular Biology, Faculty of Science, University of the Basque Country, P.O. Box 644, 48080 Bilbao (Spain). Tel.: 34 4 464 88 00. Fax. 34 4 464 85 00. E-mail: gbpdesaj@lg.ehu.es

Susana Rivas — John Innes Centre, The Sainsbury Laboratory, Norwich Research Park, Colney Lane, Norwich NR4 7UH (U.K.). Tel.: 44 1603 45 25 71. Fax: 44 1603 45 68 44. E-mail: rivas@bbsrc.ac.uk

Dolores Rodríguez — Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 585 45 53. Fax: 34 1 585 45 06. E-mail: DRODRIG@cnb.uam.es

Bernard Roux — Laboratoire de Physico-Chimie Biologique, UPRESA CNRS 5013, UCB-Lyon I 43 bd du 11 november 1918, 69622 Villeurbanne (France). Tel.: 33 472 44 80 78. Fax: 33 472 43 15 43. E-mail: rouxsy@cismibrn.univ-lyon1.fr

Hildgund Schrempf — Universität Osnabrück, FB Biologie/Chemie, Barbarastraße 11, D-49069 Osnabrück (Germany). Fax: 49 541 969 28 04.

Valery L. Shnyrov — Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de Salamanca, Avda. Campo Charro s/n., 37007 Salamanca (Spain). Tel.: 34 23 29 44 65. Fax: 34 23 29 45 79. E-mail: shnyrov@gugu.usal.es

Stefka Taneva — Department of Biochemistry, University of Basque Country, P.O. Box 644, Bilbao (Spain). Tel.: 34 4 464 77 00. Fax: 34 4 464 85 00. E-mail: gbbvealm@lg.ehu.es

Iban Ubarretxena Belandia — Dept. of Enzymology and Protein Engineering, Centre for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Utrecht University, Padualaan 8, 3508 CH Utrecht (The Netherlands). Tel.: 31 30 253 34 23. Fax: 31 30 252 24 78. E-mail: I.ubarretxena@chem.ruu.nl

Florence Verrier — Unité de Virologie Moléculaire (CNRS URA 1966), Dpt. de Virologie, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris, cedex 15 (France). Tel.: 33 1 40 61 36 50. Fax: 33 1 40 61 30 45. E-mail: fverrier@pasteur.fr

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