Closing Report on the NKFI (OTKA) grant K101633

entitled

The rotary mechanism of a molecular engine, the vacuolar proton-ATPase, working in a biomembrane

Results on the vacuolar proton-ATPase (V-ATPase)

There is a class of membrane-attached macromolecular assembly, which plays a crucial role in living processes and disease, whose action involves true, full-cycle rotation of certain parts, the rotor, relative to other parts, the stator (Forgac 2007; Morales-Rios et al. 2015; Nakanishi-Matsui 2010; Nishi and Forgac 2002, Stock et al. 2000; Zhao et al. 2015). Rotary enzymes are complex, highly challenging biomolecular machines whose biochemical working mechanism involves intersubunit rotation. Direct measurement of rotation in such rotary enzymes was so far impossible without genetic, chemical and even physical modification. In all such studies the rotary enzyme had to be removed from its native membrane environment, and in most cases some subunits were also removed (Nishio et al. 2002; Noji et al. 1997; Omote, et al. 1999; Rondelez et al. 2005; Sabbert et al. 1996; Sambongi et al. 1999; Stock et al. 2000; Yasuda et al. 2001). Even the (so far) least invasive and least perturbing single molecule fluorescence resonance energy transfer approach to detect the rotation required the chemical modification of the rotary enzyme, namely binding of fluorescent donor and acceptor groups and reconstitution of the enzyme into artificial liposomes as "quasi-native environment" (Diez et al. 2004). Therefore, the true intrinsic rate of rotation of any rotary enzyme was not known in a native, unmodified state.

As a family, the normal function of V-ATPase is to pump protons across specific biomembranes. It is a key rotary enzyme in all eukaryotic cells, acidifying intracellular compartments and the extracellular space in some tissues (Forgac 2007; Nakanishi-Matsui 2007; Nishi and Forgac 2002; Zhao et al. 2015). A class of proteins including the proteolipid c-ring subcomplex has functions independent from V-ATPase activity (Arechaga et al. 2002; Couoh-Cardel et al. 2016; El Far et al. 2011; Holzenburg et al. 1993). The V-ATPase is also a potential therapeutic target for several diseases (Bowman et al. 2006; Farina and Gagliardi 1999; Forgac 2007; Nishi and Forgac 2002). Functionally, the V-ATPase works in the opposite sense to the better known F-ATP synthase (Bustamante et al. 2001; Morales-Rios et al. 2015; Nakanishi-Matsui et al. 2010; Stock et al. 2000; Nishio et al. 2002; Rondelez et al. 2005; Sambongi et al. 1999; Stock et al. 1999; Yasuda et al. 2001). Proton transport is energised by the chemical energy of ATP, which is transduced into mechanical force to rotate a special group of subunits relative to the rest of the protein complex. Both F- and V-ATPases are true molecular engines (Bustamante et al. 2001; Grabe et al. 2000; Nakanishi-Matsui et al. 2010; Rondelez et al. 2005; Stock et al. 1999). A complementary arrangement of proton-binding sites in the V-ATPase, namely glutamic acid (Glu) on the rotor – one Glu on each c-ring subunit – and proton-conducting hemichannels at the interface with a stator subunit, ensures active proton transport across the lipid bilayer driven by protein rotation (Grabe et al. 2000; Nishi and Forgac 2002; Zhao et al. 2015). As in the F-ATPase, ATP hydrolysis and proton transport are strongly coupled via the rotary mechanism: there is no active proton transport without ATP hydrolysis and blocking protonation-deprotonation of the unique Glu residue stops ATP hydrolysis (Bowman et al. 2006; Farina and Gagliardi 1999; Forgac 2007; Rondelez et al. 2005; Sambongi et al. 1999; Stock et al 2000; Zhao et al. 2015).

We have invented two approaches to measure the rotation rate in native V-ATPase and to gain more information about its rotary mechanism:

1. (**Ferencz et al. 2013**) Membrane vesicles were formed spontaneously after exposing purified yeast vacuoles to osmotic shock. The fraction of total ATPase activity originating from the V-ATPase was determined by using the potent and specific inhibitor of the enzyme, concanamycin A (concA). Inorganic phosphate liberated from ATP in the vacuolar membrane vesicle system, during ten minutes of ATPase activity at 20 °C, was assayed spectrophotometrically for different concA concentrations. A fit of the quadratic binding equation, assuming a single inhibitor binding site on a monomeric V-ATPase (see argument below) to the inhibitor titration curve determined the concentration of the enzyme. Combining this with the known ATP/rotation stoichiometry of the V-ATPase and the assayed concentration of inorganic phosphate liberated by the V-ATPase, led to an average rate of ~10 Hz for full 360° rotation (and a range of 6–32 Hz, considering the ± standard deviation of the enzyme concentration), which, from the time-dependence of the activity, extrapolated to ~14 Hz (8–48 Hz) at the beginning of the reaction. These were lower-limit estimates. To our knowledge, this was the first estimate of the rotation rate in a V-ATPase that was not subjected to genetic or chemical modification and was not fixed to a solid support; instead it was functioning in its native membrane environment. The picture on concA binding to V-ATPase was not clear in the literature, and our data gave further restrictions to localise the binding site of this inhibitor. Our data was compatible only with a single binding site per monomeric enzyme. Because concA most likely binds to intramembranous subunits (Bowman et al. 2006; Dixon et al. 2008; Huss et al. 2002; Pali et al. 2004; Whyteside et al. 2005;), our results suggest that an interaction with a single c-subunit, of which three or four copies are present in the c-ring, is not sufficient for high-affinity concA binding: either the binding site consists of more than one c-subunit or it includes either subunit a or c', of which there is only one copy each in Vo. In the future, we plan more titration experiments with different inhibitors and under different conditions in order to localise the inhibitor binding sites, and determine stoichiometry and binding constants.

2. (**Ferencz et al. 2017**) The inherent limitations of the current approaches prompted us to explore another very different route. Our idea was straightforward: because the periodicity of vectorial charge movement relates directly to that of rotation, an oscillating transmembrane potential with frequency matching the turnover rate of charge movement, should have maximum effect on enzyme activity. As above, we used spherical single-layer membrane vesicles, with a mean diameter of 300 nm, formed from yeast vacuoles. These vesicles contained high concentration of properly oriented V- ATPase, on which we measured ATPase activity (as in Ferencz et al. 2013). In addition, since ATP was added to pre-formed stable vacuolar vesicles, reversely (inside-out) oriented V-ATPases, if any, remained inactive and did not contribute to the measured ATPase activity. An oscillating, sinusoidal electric (AC) field was applied to the vesicle suspension in a rectangular cuvette with platinum electrodes. The challenge was to optimise the conditions to obtain significant effects. For the first time: (i) we applied an AC field to V-ATPase; (ii) we revealed a narrow, resonance-like frequency response to the AC field that has never been seen before for any enzyme, and cannot be interpreted within the framework of electro-conformational coupling theory (Astumian 1994; Robertson and Astumian 1991; Tsong et al. 1989); and (iii) the resonance-like effect of the AC field on transmembrane proton movement allowed us to determine the intrinsic mean rotation rate in native V-ATPase directly: it was 13.2±0.5 Hz (under well defined conditions). We observed a linear dependence, in a linear-log plot, over the frequency range 1 Hz to 20 kHz, excluding the narrow peak, with lower frequencies being the more inhibitory and frequencies above 10 kHz having no effect. In all previous experiments on frequency dependence of the effect of an AC field on activity

of other enzymes very broad peaks were observed, even orders of magnitude wide, and the curves were fitted according to electro-conformational coupling theory (Robertson and Astumian 1991; Robertson and Astumian 1992; Tsong et al. 1989). However, the ratio of width to position of the narrow peak was outside the region of applicability of that theory. The reason is most certainly that proton transport is driven by ATP hydrolysis, even at the resonance peak, and not by the AC field. Indeed, as opposed to other studies (Robertson and Astumian 1991; Tsong et al. 1989; Robertson and Astumian 1992; Witt et al. 1976) the AC field in our system inhibits rather than stimulates ATP hydrolysis, except for the resonance peak. In the paper, we present animated mechanistic models of the AC effect on the V-ATPase and propose a hold-and-release mechanism, which explains both the resonance phenomenon and the effect in the low- and high-frequency limits. The hold-and-release effect of the AC field changes the stochastic stepping motion of the rotor (Sabbert and Junge 1997) into a more regular periodic motion. The **on-line videos** (that will be available in Nature's web site as supplementary material to the paper) give simple, mechanistic illustrations for the timing of binding ATP, releasing ADP and inorganic phosphate, protonation-deprotonation steps, and proton transport as a function of rotation under normal conditions, and under influence of a transmembrane AC field with frequency either matching, or being slower or faster than, the intrinsic rate of rotation of the V-ATPase with excess ATP and assuming 6 c-ring subunits. (Subject to clarifying copyright issues, we plan that the original, interactive version of the **animations will be available in our group's web page**.) Using the Lorentzian fits, the mean frequency of the full-cycle 360°-rotation is 13.2±0.5 Hz. It should be noted that this result is independent of the presence of any inactive (e.g. reversely oriented) V-ATPases. This mean rotation rate agrees very well with our indirect estimate of ~14 Hz on the same experimental system but using the inhibition curve of ATP hydrolysis of V-ATPase by concA (see above). In addition, the average width of the Lorentzian yields a relaxation rate constant of 19±12 Hz, which can be interpreted as the rate of protonation of any single Glu residue, which is again the frequency of full-cycle 360°-rotation. These agreements give us full confidence that we have indeed measured the intrinsic mean rotation rate in an intact and unmodified rotary enzyme in its native membrane environment, for the first time. We expect that this paper will be a well cited reference of rotary enzymes. This paper is the most significant result of the grant, also because only colleagues of our Institute participated in it, and it was purely Hungarian funded project.

Concerning future studies and biotechnological application, we note that a transmembrane AC field is capable of synchronising rotation in individual V-ATPase molecules by blocking them in the "wrong" and releasing them in the "right" phase, which is a hold-and-release mechanism. Synchronisations of V-ATPase (and other enzymes) could be used to amplify weak electric or chemical signals, also of biological origin. The present method could also work on other transmembrane ion-transporting systems, not only rotary enzymes, to determine the intrinsic rate of ion pumping. We plan to build an experimental setup to measure the spontaneous ATPase activity and proton pumping by V-ATPase in real-time for kinetic studies, under different conditions. In addition, we plan spin- and fluorescence spectroscopic and atomic force microscopic experiments to reveal further details of the rotary mechanism of V-ATPase. Since the most sensitive frequency region of V-ATPase to the AC field falls in the audio range, these experiments brought audio engineering aspects into the project. Most recently, we have investigated the effect of AC field of musical origin and the V-ATPase reacted differently to different genres of music. This work has not yet been finished but we believe we can explain the effect with the frequency dependence of ATPase activity on sinusoidal AC field. This latter result has great potential in popular science and art-and-science presentations.

In an international collaboration, we studied the role of the protein-water interface in protein dynamics from the thermodynamics point of view. We contributed to this project with calorimetric measurements on bacteriorhodopsin (bR). Recently, the same team have elaborated a thermodynamic theory that could coherently interpret the diverse effects of Hofmeister ions on proteins, based on the protein–water interfacial tension (Der et al. 2007). This theory predicts changes in protein conformational fluctuations upon addition of Hofmeister salts (containing either kosmotropic or chaotropic anions) to the medium. In our recent paper (**Szalontai et al. 2013**) we report experimental tests of this prediction using a complex approach by applying methods especially suited for the detection of protein fluctuation changes (neutron scattering, micro-calorimetry, and Fourier-transform infrared spectroscopy). It is demonstrated that Hofmeister salts, via setting the hydrophobic/hydrophilic properties of the protein–water interface, control conformational fluctuations even in the interior of the typical membrane transport protein bacteriorhodopsin, around its temperature-induced, unusual α(II) → α(I) conformational transition between 60 and 90°C. We found that below this transition kosmotropic ($COOCH^{-3}$), while above it chaotropic (ClO^{-4}) anions increase structural fluctuations of bR. It was also shown that, in each case, an onset of enhanced equilibrium fluctuations presages this phase transition in the course of the thermotropic response of bR. These results are in full agreement with the theory, and demonstrate that predictions based on protein–water interfacial tension changes can describe Hofmeister effects and interpret protein dynamics phenomena even in unusual cases. This approach is expected to provide a useful guide to understand the principles governing the interplay between protein interfacial properties and conformational dynamics, in general. These results urged us to plan experiments on V-ATPase in the presence of different Hofmeister ions, since most of the V1 domain and some of the Vo domain is permanently in contact with the aqueous phase.

When studying the literature on the inhibitor binding, medical relevance of different ATP-driven transmembrane ion pumps and the electric field effect on them, we gained enough knowledge to write three sections in an e-book for educational purpose (**Pali 2015; Sebok-Nagy and Pali 2015a,b**). In addition, we have organised a conference, edited a special issue of the European Biophysics Journal and wrote an editorial on membrane proteins, with strong focus on ATP-ases (**Pali and Szalontai 2013**). The Satellite Conference "Structure, Function, Folding and Assembly of Membrane Proteins—Insight from Biophysics" was a direct continuation of the 8th European Biophysics Congress, but with themes centred around membrane proteins. These papers in our Special Issue were representative of the following themes: membrane proteins and their environment; membrane transport and channels; advanced structural biology techniques for membrane proteins; and folding and assembly of proteins in membranes.

Other related results

We have published three papers with topics broadly related to, and with partial or no financial support from this project:

1. (**Pali and Kota 2013**) Since about two third of the rotor of the V-ATPase is in direct, probably non-covalent interaction with the lipids of the host membrane, the composition and state of the lipid environment could be of key significance in the regulation of V-ATPase. Despite of this notion, there have not been many studies in the literature on the lipid-protein interaction of V-ATPase (e.g., Pali et al. 1995). Spin label electron paramagnetic resonance (EPR) of lipid–protein interactions reveals crucial features of the structure and assembly of integral membrane proteins. We planned to

use it in the present project and plan to use it in the future as well, because spin label EPR spectroscopy is the technique of choice to characterise the protein-solvating lipid shell in its highly dynamic nature. The EPR spectra of lipids that are spin labeled close to the terminal methyl end of their acyl chains display two spectral components, those corresponding to lipids directly contacting the protein and those corresponding to lipids in the bulk fluid bilayer regions of the membrane. In this book chapter, we describe typical spin label EPR procedures that allow determination of the stoichiometry of interaction of spin-labeled lipids with the intra-membranous region of membrane proteins or polypeptides, as well as the association constant of the spin-labeled lipid with respect to the host lipid. Stoichiometry and selectivity are directly related to the structure of the intra-membranous sections of membrane-associated proteins or polypeptides and can be used to study the state of assembly of such proteins in the membrane. The importance of our technique is well demonstrated by the fact that our first figure made it to the front cover of the book.

2. (**Marsh and Pali 2013**) In this theoretical work, we evaluate orientational order parameters and individual dihedral torsion angles for phospholipid and glycolipid molecules that are resolved in X-ray structures of integral transmembrane proteins in crystals. The order parameters of the lipid chains and glycerol backbones in protein crystals are characterised by a much wider distribution of orientational order than is found in fluid lipid bilayers and reconstituted lipid–protein membranes. This indicates that the lipids that are resolved in crystals of membrane proteins are mostly not representative of the entire lipid–protein interface. Much of the chain configurational disorder of the membrane-bound lipids in crystals arises from C–C bonds in energetically disallowed skew conformations. This suggests configurational heterogeneity of the lipids at a single binding site: eclipsed conformations occur also in the glycerol backbone torsion angles and the C–C torsion angles of the lipid head groups. Conformations of the lipid glycerol backbone in protein crystals are not restricted to the gauche C1–C2 rotamers found invariably in phospholipid bilayer crystals. Lipid head-group conformations in the protein crystals also do not conform solely to the bent-down conformation, with gauche–gauche configuration of the phosphodiester, that is characteristic of phospholipid bilayer membranes. Stereochemical violations in the protein-bound lipids are evidenced by ester carboxyl groups in non-planar configurations, and even in the cis configuration. Some lipids have the incorrect enantiomeric configuration of the glycerol backbone, and many of the branched methyl groups in the phytanyl chains associated with bacteriorhodopsin have the incorrect S configuration. Unfortunately, we could not purify native V-ATPase to test whether it contains bound lipids. Also, since no X-ray structure is available for V-ATPase, we could not include it in the above analysis.

3. (**Csonka et al. 2015**) In this paper, we compared different methods for measuring nitric oxide (NO) in biological samples. Although the physiological regulatory function of the gasotransmitter NO (a diatomic free radical) was discovered decades ago, NO is still in the frontline research in biomedicine. NO has been implicated in a variety of physiological and pathological processes; therefore, pharmacological modulation of NO levels in various tissues may have significant therapeutic value. NO is generated by NO synthase in most of cell types and by non-enzymatic reactions. Measurement of NO is technically difficult due to its rapid chemical reactions with a wide range of molecules, such as, for example, free radicals, metals, thiols, etc. Therefore, there are still several contradictory findings on the role of NO in different biological processes. In this review, we briefly discuss the major techniques suitable for measurement of NO (electron paramagnetic resonance, electrochemistry, fluorometry) and its derivatives in biological samples (nitrite/nitrate, NOS, cGMP, nitrosothiols) and discuss the advantages and disadvantages of each method. We conclude that to obtain a meaningful insight into the role of NO and NO modulator

compounds in physiological or pathological processes, concomitant assessment of NO synthesis, NO content, as well as molecular targets and reaction products of NO is recommended.

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