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In vitro selection and characterization of DARPins and Fab fragments for the co-crystallization of membrane proteins: The Na^+-citrate symporter CitS as an example

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Abstract

The determination of 3D structures of membrane proteins is still extremely difficult. The co-crystallization with specific binding proteins may be an important aid in this process, as these proteins provide rigid, hydrophilic surfaces for stable protein–protein contacts. Also, the conformational homogeneity of the membrane protein may be increased to obtain crystals suitable for high resolution structures. Here, we describe the efficient generation and characterization of Designed Ankyrin Repeat Proteins (DARPins) as specific binding molecules for membrane proteins. We used both phage display and ribosome display to select DARPins *in vitro* that are specific for the detergent-solubilized Na^+-citrate symporter CitS of *Klebsiella pneumoniae*. Compared to classical hybridoma technology, the *in vitro* selection systems allow a much better control of the structural integrity of the target protein and allow the use of other protein classes in addition to recombinant antibodies. We also compared the selected DARPins to a Fab fragment previously selected by phage display and demonstrate that different epitopes are recognized, unique to each class of binding molecules. Therefore, the use of several classes of binding molecules will make suitable crystal formation and the determination of their 3D structure more likely.

Keywords: Co-crystallization; Designed ankyrin repeat proteins (DARPins); *In vitro* selection; Membrane protein; Multi-angle (static) light scattering (MALS); Na^+-citrate symporter CitS; Phage display; Recombinant antibody Fab fragment; Ribosome display; Protein engineering

1. Introduction

Multitopic membrane proteins, such as channels, transporters or receptors, are involved in many fundamental biological processes and today, the majority of drug targets are integral membrane proteins. Therefore, there is an immediate and growing need for high-resolution structure information to gain detailed insight into the function of membrane proteins at the atomic level.

In the different genomes analyzed to date, 20–30% of all open reading frames encode integral membrane proteins (Wallin and von Heijne, 1998). As of September 2006, only about 100 membrane protein structures[2,3] have been deposited in the Protein Data Bank (Berman et al., 2000), and this even includes all homologs from different species and a number of relatively robust bacterial outer membrane proteins with β-barrel topology. The even smaller number of non-redundant α-helical membrane proteins remains in stark contrast to the about 12,000[4] solved structures of non-redundant[5] soluble proteins. This contrast points out the difficulties in membrane protein structure determination.

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[2] www.mpibp-frankfurt.mpg.de/michel/public/memprotstruct.html
[3] http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html
[4] http://www.pdb.org/pdb/holdings.do
[5] Proteins with less than 70% identity.

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1.1. Crystallization of membrane proteins

A major bottleneck in structure determination of membrane proteins is the production of high quality crystals. The difficulties are mainly attributed to the inherent protein flexibility and conformational inhomogeneity of the detergent-solubilized membrane protein–detergent complex. Additionally, the polar surface of those membrane proteins having only very short solvent-exposed loops cannot reach beyond the detergent layer wrapped around the hydrophobic surface, and therefore stable protein–protein contacts essential for crystal packing are not formed.

1.2. Co-crystallization

A relatively new approach to overcome these problems is the co-crystallization of membrane proteins with antibody fragments (reviewed in Hunte and Michel, 2002). For successful co-crystallization a stable complex of an antibody fragment bound to a structural epitope present in the native conformation of the membrane protein is needed. Thereby, the bound antibody fragment reduces the protein flexibility and increases the conformational homogeneity of the membrane protein–detergent complex since it recognizes—ideally—only the native and functional conformation of the membrane protein. This specificity can also be exploited during membrane protein purification to increase the homogeneity of the protein sample (Kleymann et al., 1995). Equally important, the bound antibody fragment provides additional polar surfaces to mediate stable protein–protein contacts for well-ordered crystal packing. However, this specificity comes at a cost: a new binding molecule fulfilling all the above requirements has to be generated for each membrane protein structure to be solved.

Published co-crystals of membrane proteins and antibody fragments include cytochrome *c* oxidase from *Paracoccus denitrificans* (PDB entries 1QLE/1AR1), cytochrome bc1 complex from *Saccharomyces cerevisiae* alone (PDB entries 1EZV/1KB9) and in complex with cytochrome *c* (PDB entry 1KYO), potassium channel KcsA from *Streptomyces lividans* (PDB entries 1K4C/1K4D/1R3I/1R3J/1R3K/1R3L/2BOB/2BOC), and the ClC chloride channel from *Escherichia coli* (PDB entries 1OTS/1OTT/1OTU). Interestingly, in all crystal structures the antibody fragment fills the gap between adjacent membrane proteins in the crystal lattice and mediates important protein–protein interactions for well-ordered packing.

1.3. Monoclonal antibodies

In all published examples the antibody fragments used for co-crystallization were ultimately derived from monoclonal antibodies, and Fab fragments were either produced by proteolysis of the IgG or the antibody fragment genes from hybridomas were cloned and expressed in *E. coli*.

Several fundamental problems are encountered, however, in the generation of monoclonal antibodies with the desired properties from animals. When the solubilized membrane protein is injected into the animals, the detergent is diluted and the further fate of the protein and its conformational integrity cannot be controlled. The use of adjuvants such as mineral oil casts an additional shadow of doubt on maintaining the native structure for a long time. The membrane protein is processed by antigen-presenting cells and at the same time, some molecules need to be bound to IgM on the surface of B-cells, which triggers the antibody response in the animal. It is at least doubtful whether the conformational epitopes would still be intact at this stage, unless the protein is very stable. Subsequent screening of hybridomas for reactivity with the native state of the protein will detect those antibodies that bind to epitopes present in the folded structure—if such antibodies have been elicited at all. However, when producing antibodies against less rigid molecules, e.g. GPCRs, it is highly likely that most binders that do crossreact with the native protein will be directed against exposed N- or C-terminal tails (Niebauer et al., 2006) or extracellular compact domains, rather than that binders recognize the loops connecting the helices in their native conformation. If the protein denatures during the immunization process, many "real" conformational epitopes will be lost and conformation-specific antibodies are not found.

1.4. Our approach

Here, we demonstrate the use of *in vitro* selection methods to overcome the above limitations and we report a fast downstream screening process to efficiently identify suitable binding partners of membrane proteins for co-crystallization. After we showed in a previous study that conformation-specific, high-affinity antibody Fab fragments that bind to the detergent-solubilized Na^+-citrate symporter CitS can be generated by phage display (Röthlisberger et al., 2004), we now expand this approach to another selection system as well as to another class of binding proteins. With the use of a different class of binding proteins the shape of the binding module can be varied and we intended by using different binding molecules to obtain binders to different epitopes. Both factors can lead to different crystal packing, which should clearly increase the chance of crystal formation suitable for high resolution structure determination.

1.5. Recombinant Fab fragments and Designed Ankyrin Repeat Proteins

The two classes of binding proteins investigated and compared here are antibody Fab fragments and Designed Ankyrin Repeat Proteins (DARPins). The heterodimeric Fab fragment (Fig. 1a) consists of the entire light chain (V_L and C_L domains) and the Fd fragment (V_H and C_H domains) of the heavy chain, which—in the format used in this study—are not disulfide-linked to each other (Röthlisberger et al., 2004). The antigen binding site is formed by