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Amphipol-Mediated Screening of Molecular Orthoses Specific for Membrane Protein Targets

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Abstract Specific, tight-binding protein partners are valuable helpers to facilitate membrane protein (MP) crystallization, because they can i) stabilize the protein, ii) reduce its conformational heterogeneity, and iii) increase the polar surface from which well-ordered crystals can grow. The design and production of a new family of synthetic scaffolds (dubbed αReps, for "artificial alpha repeat protein") have been recently described. The stabilization and immobilization of MPs in a functional state are an absolute prerequisite for the screening of binders that recognize specifically their native conformation. We present here a general procedure for the selection of αReps specific of any MP. It relies on the use of biotinylated amphipols, which act as a universal "Velcro" to stabilize, and immobilize MP targets onto streptavidin-coated solid supports, thus doing away with the need to tag the protein itself.

Keywords HEAT repeat protein · Protein design · Phage display · Membrane protein · Amphipols · Immobilization

Abbreviations

2XTY	*E. coli* rich media
A8-35	A specific type of poly(acrylic acid)-based amphipol
APol	Amphipol
BAPol	Biotinylated A8-35
BNAPol	Biotinylated non-ionic amphipol
BR	Bacteriorhodopsin
cmc	Critical micellar concentration
DDM	Dodecyl-β-D-maltoside
$\overline{DP}_n$	Number-average degree of polymerization
EDTA	Ethylene diamine tetraacetic acid
EM	Electron microscopy
His-tag	Hexahistidine tag
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
MD	Molecular dynamics
$\overline{M}_n$	Number-average molar mass
MP	Membrane protein
MW	Molecular weight
MWCO	*MW* cut-off
NAPol	Non-ionic amphipol
NMR	Nuclear magnetic resonance
NTA	Nitrilotriacetic acid
OD_{600nm}	Optical density measured at 600 nm
βOG	*n*-octyl-β-D-glucopyranoside

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PBS	Phosphate buffer saline
PEG	Polyethylene glycol
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
TBS	Tris-buffered saline
TBST	Tris-buffered saline supplemented with Tween 20 (w/v)
Tris	Tris(hydroxymethyl)aminomethane

Introduction

Membrane proteins (MPs) are challenging targets for structural studies because of their obligatory association with a hydrophobic environment. Detergents are absolutely required to solubilize MPs, but they complicate considerably the crystallogenesis process. First, detergent-solubilized MPs are rarely stable. Second, intermolecular contacts between detergent layers do not generate the well-defined geometrical constraints that lead to the formation of crystals diffracting to high resolution. Using MPs in complex with a specific and tight-binding protein partner can considerably facilitate the challenging process of MP crystallization. This strategy is often referred to as "crystallization chaperones" (Koide 2009; Lieberman et al. 2011), even though the term "chaperone" is used here in a somewhat relaxed sense. Indeed, a chaperone is not supposed to play a role in the final association states of its transiently protected partner, whereas the "crystallization chaperone" is still present in the final crystal. Hence, we favor the term orthosis, defined as "an artificial aid to assist or improve a function" (Collins 2012), or binder. Two distinct effects can improve crystallization of MPs. First, the binder stabilizes the bound conformation of its MP partner, thereby favoring its conformational homogeneity and minimizing protein denaturation and aggregation during the crystallization process. Second, in the complex, the protein surface area eligible to establish precise intermolecular contact in the crystal lattice is greatly expanded due to the solvent-exposed surface of the binder. This approach, pioneered by H. Michel using monoclonal antibodies directed against cytochrome *c* oxidase (Ostermeier et al. 1995), found only recently increasing applicability with the advent of new types of binding partners. Indeed, monoclonal antibodies produced from hybridomas have drawbacks that seriously limit their use for this application: the conformational state of a detergent-solubilized protein cannot be controlled once injected in a living animal for immunization, and most screened antibodies are sequence specific rather than conformation specific, whereas it is absolutely required that crystallization orthoses bind specifically to the native protein conformation. Furthermore, most natural antibodies have a high aggregation propensity and disulfide bond requirement, both of which make their recombinant expression difficult in bacterial systems. Specific types of *Camelidae* antibody domains, referred to as nanobodies, represent a more favorable option, as they are usually more efficiently produced and engineered than single-chain variable fragments (ScFv) or antigen-binding fragments (Fab) (Muyldermans et al. 2009). However, this approach remains based, in most cases, on poorly controlled immunization of animals.

For these reasons, "alternative scaffolds proteins" were developed and recently became an efficient option to generate MP crystallization orthoses. A very promising class of alternative protein scaffold, namely new artificial proteins based on repeat proteins, has been introduced by Plückthun and colleagues (Binz et al. 2003; Stumpp et al. 2003). These repeat-based architectures are extremely well adapted to generate protein interactions. Repeats ($\sim$20–40 residues) fold in a periodic arrangement of secondary structures with a solenoid-like topology. Comparison of repeats of a given family shows that some strictly conserved sequence positions found within consecutive repeats correspond to residues involved in the structure of and interaction between neighboring modules. Conversely, the variable side chains oriented toward the outside surface of each repeat are juxtaposed in the protein, creating a hypervariable macrosurface that can be selected for specific interactions. Tight and highly specific "Designed ankyrin repeat proteins" (Darpins) that recognize a range of different protein targets, including integral MPs, have been selected out of these repertoires by phage or ribosome display (Sennhauser and Grutter 2008). Other type of repeats based on Armadillo, Leucine-rich repeats (LRR), or tetratricopeptide repeats (Boersma and Plückthun 2011) have also produced specific binding proteins, which, however, have not yet been used as crystallization helpers. We have recently introduced a new family of artificial repeat proteins named 'αReps,' based on the previously unexplored family of HEAT repeats. These proteins were designed from a subgroup of HEAT-like repeat found in several cytoplasmic proteins, among which four have given their initial to the acronym (Huntingtin, Elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast kinase TOR1). αReps are efficiently expressed and folded, and very stable. Large libraries have been recently described (Guellouz et al. 2013), which have allowed the selection of tight and specific binders against a range of different and unrelated soluble proteins, with dissociation constants, K_D, in the nanomolar to micromolar range. Crystallographic structures of αRep/target protein

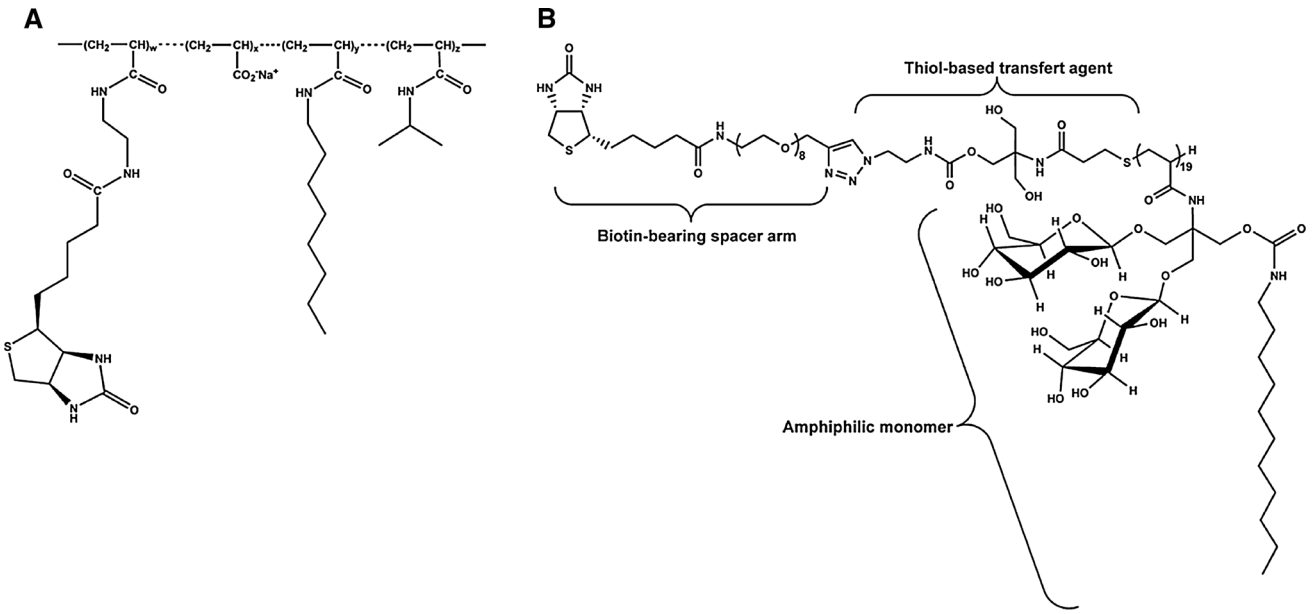


Fig. 1 Chemical structure of the two biotinylated amphipols used in this study. **a** Biotinylated A8-35 (BAPol, batch 3) (from Charvolin et al. 2009); $\overline{DP}_n \approx 35$, $\langle M_n \rangle \approx 4.6$ kDa (*cf.* Giusti et al. 2014); $w \approx 3$ %, $x \approx 41$ %, $y \approx 24$ %, $z \approx 32$ %; ~9 biotins per 40 kDa APol particle. **b** Biotinylated non-ionic amphipol (BNAPol, batch BNA14). $\overline{DP}_n$ (= $n + 1$) ≈ 35, $\langle M_n \rangle \approx 14$ kDa; ~0.56 biotin per polymer chain

complexes show conformational-specific recognition (Guellouz et al. 2013).

For the selection of binding proteins as potential crystallization helpers, it is critical that, at all stage of the selection process, the purified MPs used as targets be immobilized on a solid support under their "native" or functional state. Amphipols (APols) (Tribet et al. 1996) are specially designed amphipathic polymers that have proven able to substitute to detergents for keeping water-soluble any MP tested so far, most of which are much more stable as complexes with APols than they are in detergent solutions (reviewed in Popot 2010; Popot et al. 2011; Zoonens and Popot 2014). Because the association of APols to MPs is very strong and resist extensive dilution (Tribet et al. 1997; Tribet et al. 2009; Zoonens et al. 2007), trapping a MP with a functionalized APol effectively functionalizes the protein (reviewed in, Le Bon et al. 2014b; Zoonens and Popot 2014). Thus, an APol carrying a biotin tag can be used to attach MPs onto streptavidin-coated chips or beads (Charvolin et al. 2009). MPs thus immobilized are in their native state and can be extensively flushed with surfactant-free ligand solutions without detaching or inactivating, allowing for ligand screening (Charvolin et al. 2009; see also Basit et al. 2012; Della Pia et al. 2014a, b; Le Bon et al. 2014a). Tagged APols, therefore, may constitute powerful tools for αRep selection, as they permit to immobilize target MPs while stabilizing them in their native state throughout the lengthy screening process. The best characterized biotinylated APol to date, hereafter called 'BAPol' (Fig. 1a) (Charvolin et al. 2009), is a derivative of A8-35, a polyanionic, polyacrylate-based APol (Tribet et al. 1996). More recently, a number of chemically different APols have been developed (reviewed in Zoonens and Popot 2014), among glucose-based non-ionic amphipols ('NAPols'), whose complete absence of charges can be an asset under certain experimental circumstances (Bazzacco et al. 2012; Sharma et al. 2012). As an alternative to BAPol, we have tested the use of a biotinylated NAPol ('BNAPol') (Fig. 1b).

We introduce here a general protocol for the production of MP-specific αReps. This novel approach has been tested with model MPs of known, different structures, originating from different organisms (prokaryotes, eukaryotes, and archaebacteria) and different kinds of membranes (mitochondrion inner membrane, bacterial inner membrane, and bacterial outer membrane).

Materials and Methods

Production and Purification of Target Membrane Proteins

Bacteriorhodopsin (BR)

Halobacterium salinarum cells were grown under illumination at 37 °C in NaCl 4 M, $MgSO_4$ 150 mM, trisodium citrate 10 mM, KCl 30 mM, yeast extract 5 g L^{-1}, and

peptone 5 g L^{-1}. Purple membrane was isolated on sucrose gradient and solubilized overnight at room temperature with Triton X-100 at a 1:5 protein to detergent mass ratio as described by Gohon et al. (2008). The concentration of solubilized BR was estimated using $\varepsilon_{570\ nm} = 54{,}000\ M^{-1}\ cm^{-1}$ in potassium buffer 10 mM, NaCl 75 mM, pH 7.5.

Cytochrome bc_1

Preparation of Mitochondria Mitochondria were prepared as described by Smith (1967). Briefly, after fat and connective tissues had been trimmed from the beef heart, tissues were homogenized in a blender and immediately adjusted to pH 7.5. A first centrifugation was run to remove residual muscle tissue and lipid granules, while a second run pelleted the membranes. Pellets were washed and finally resuspended and homogenized in potassium buffer 50 mM, EDTA 0.5 mM. 50 mL of aliquots were stored at −80 °C until use.

Cytochrome bc_1 Purification The purification of cytochrome bc_1 was carried out as described by Berry et al. (1991). It is based on *n*-dodecyl-*β*-D-maltoside (DDM) extraction at a 1:1 DDM:protein mass ratio, anion-exchange chromatography on DEAE Sepharose CL6B with a 260–500 mM NaCl gradient, and size-exclusion chromatography (SEC) on Sepharose CL-6B. Pooled fractions from the last column were adjusted down to 100 µM bc_1 before titrating with a PEG precipitant solution to get rid of contaminants and aggregated materials.

MexB

MexB was heterologously expressed in a C43 (DE3) *Escherichia coli* strain (Miroux and Walker 1996). Two different constructs were produced: the first containing a C-terminal 6-histidine tag (MexB), and the second (MexBbiot) having a C-terminal tag for biotin binding followed by a 6-histidine tag (the N-terminus is required for membrane addressing). Cultures were grown at 30 °C on 2XTY *E. coli* rich culture medium containing 0.1 g L^{-1} ampicillin. Cells were induced at $OD_{600nm} = 0.6–0.8$ by the addition of 1 mM IPTG and grown for 2.5 h before centrifugation. The cell pellets were resuspended in buffer containing 20 mM Tris/HCl (pH 8.0), broken by a French pressure cell at 69 MPa, and centrifuged for 30 min at 8,500×*g* to remove inclusion bodies and unbroken cells. Membranes were collected by ultracentrifugation at 100,000×*g* for 1 h and proteins extracted by adding 1.5 g DDM per gram of protein. Purification of His-tagged MexB was performed by affinity chromatography on Ni–NTA resin followed by gel filtration on Superose 12. The yield of homogeneously purified MexB was about 0.5 mg per liter of culture. Purified proteins were concentrated using Vivaspin (VivaScience) concentrators of 100-kDa cutoff.

Biotinylation of MexB was performed as follows: a 40 µM aliquot of purified protein resuspended in 50 mM Bicine buffer, pH 8.3, 10 mM ATP, 10 mM Mg acetate, 50 mM D-biotin was incubated with 100× diluted home-made BirA, an enzyme that catalyzes covalent binding of biotin to biotin-accepting proteins (i.e., bearing a specific C-terminal sequence) in *E. coli* (Cronan 1990) for 45 min at 30 °C, after which the biotinylated protein was purified by gel filtration on a Superose 6 HR 10/300 column in 10 mM Bis–Tris buffer, pH 7, 300 mM NaCl, 0.03 % DDM, concentrated and flash frozen in liquid nitrogen.

OprM

OprM was overexpressed and purified as described by Broutin et al. (2005), but for the following modifications, after cell disruption and discarding of inclusion bodies and unbroken cells, the supernatant was deposited onto a sucrose step gradient (0.5 and 1.5 M) and centrifuged for 3 h at 4 °C at 200,000×*g*. The pellet, corresponding to the outer membrane fraction, was resuspended in a solution containing 20 mM Tris/HCl (pH 8.0), 10 % glycerol (v/v), and 2 % *n*-octyl-*β*-D-glucopyranoside (OG) (w/v) (Anatrace), and stirred overnight at 23 °C. The supernatant, containing the solubilized MPs, was fractionated by Ni–NTA affinity chromatography following standard procedures. Pure protein, in Tris/HCl 20 mM, pH 8, NaCl 200 mM, glycerol 10 % buffer, was obtained with a final yield of 5 mg protein per liter of culture.

αRep Purification

αRep variants were either produced from the library αRep 2.1 vector or sub-cloned in pQE81L (Qiagen). Expression and purification of αReps were performed as described by Urvoas et al. (2010). The plasmid coding for each protein was transformed into the expression *E. coli* strain M15 [pREP4] (Qiagen). Cells were grown at 37 °C in 2YT medium containing 200 µg L^{-1} ampicillin and 25 µg L^{-1} kanamycin to an OD_{600nm} of 0.6. Protein expression was induced by the addition of IPTG to 1 mM and the cells further incubated for 4 h at 37 °C. The cells were harvested, suspended in Tris-buffered saline (TBS) with benzonase and sonicated. His-tagged proteins were purified from the crude supernatant using nickel-affinity chromatography (Ni–NTA agarose, Qiagen) followed by size-exclusion chromatography (Hiload 16/60 Superdex 75) in phosphate-buffered saline (PBS). For each protein, the purity of the final sample was checked by SDS-PAGE with

an overloaded gel, which revealed one single, well-resolved band, with no visible contamination. For all the following experiments, the proteins were quantified by UV spectrophotometry and their concentration expressed relative to the monomer.

Synthesis and Purification of Amphipols

A8-35

Amphipol A8-35 (batch FGH29) was synthesized as described by Gohon et al. (2004, 2006). Briefly, A8-35 results from the hydrophobic modification of a short poly(acrylic acid) (PAA) precursor carried out in two steps in *N*-methylpyrrolidone (NMP) in the presence of dicyclohexylcarbodiimide (DCI) as a coupling reagent. *n*-Octylamine and isopropylamine were successively grafted onto the polymer's backbone at molar ratios of ~25 and ~40 %, respectively, leaving ~35 % of the carboxylates free (Tribet et al. 1996). A8-35 was purified by three cycles of precipitation at acidic pH followed by dissolution at basic pH, and lyophilized as its sodium salt. Its chemical composition was analyzed by nuclear magnetic resonance (NMR) and elemental analysis (Gohon et al. 2004, 2006). Its behavior in aqueous solution was checked by SEC as described by Gohon et al. (2004, 2006). $\overline{DP}_n$, the number-average degree of polymerization of A8-35, is ~35, corresponding to a number-average molecular mass of 4.3 kDa (Giusti et al. 2014).

BAPol

BAPol (batch BAPol-3), a biotinylated version of A8-35 (Fig. 1a), was synthesized as described in ref. (Charvolin et al. 2009). In brief, the mono-biotinylated ethylene diamine is incorporated at low molar ratio (~1–3 % of PAA units), simultaneously with *n*-octylamine, during the first step of PAA modification. Grafting with isopropylamine and purification are then carried out as for untagged A8-35. The chemical composition and physicochemical properties of BAPol in aqueous solution were characterized as described above.

NAPol

NAPols are synthesized by free radical telomerization of acrylamide monomers in the presence of a thiol-based transfer agent. Homopolymeric NAPols result from telomerization of an amphiphilic monomer carrying two glucose moieties and a single undecyl alkyl chain (Sharma et al. 2012). For the NAPol used in the present study, NA13 (batch MB155), the average molecular mass is ~13 kDa and the number-average degree of polymerization $\overline{DP}_n \approx 20$.

BNAPol

A detailed protocol for the synthesis of biotinylated non-ionic amphipols will be published elsewhere (Bosco et al., *in preparation*). In brief, BNAPols are synthesized by free radical homotelomerization of an amphiphilic monomer carrying two glucose moieties and a single undecyl alkyl chain in the presence of a thiol-based transfer agent bearing a single azido group. The biotin function is subsequently connected to the polymer through a Huisgen cycloaddition reaction with the azido group catalyzed by copper. For the BNAPol used in the present study, BNA14 (batch MB129) (Fig. 1b), the average molecular mass, is ~14 kDa, and the number-average degree of polymerization is ~20. The extent of grafting of the biotin group was estimated to be ~0.56 per polymer chain by means of a combination of ^{1}H NMR and spectrophotometric test using 4-hydroxyazobenzene-2-carboxylic acid, an avidin-binding dye that can be stoichiometrically displaced by biotin (Batchelor et al. 2007).

Preparation of Membrane Protein/Amphipol Complexes

Trapping of MP in APols was achieved as described in Zoonens et al. 2007, 2014. Briefly, APols were supplemented to the protein from a 100 g L^{-1} solution in water and the mixture incubated for 2 h at 4 °C. The detergent was removed by adding wet Bio-Beads (20 × the mass of detergent present in the solution), incubating for 3 h at 4 °C under slow agitation, diluting 5–10 × in buffer, and incubating overnight. Samples were concentrated using Centricon or Amicon devices of appropriate molecular weight cut-off (MWCO). For details regarding the trapping of BR and cytochrome bc_1 and the solution behavior of the resulting complexes, see i) Dahmane et al. (2013) and Gohon et al. (2008), and ii) Charvolin et al. (2009, 2014), respectively.

Selection of αRep Binders Specific for each Membrane Protein Target, Using Phage Display Libraries

Optimization of the Panning Procedure

The library used for the selection of binders is αRep library 2.1, described in refs. (Guellouz et al. 2013; Urvoas et al. 2010). Phages from each library were prepared using XL1-Blue MRF' bacteria transformed with the phagemid libraries and infected with the helper phage Phaberge (Soltes et al. 2003). Phages were allowed to replicate overnight at 30 °C.

The cultures were centrifuged at 5,000×*g* for 30 min, and the phage-containing supernatants recovered and dialyzed against the protein's buffer using a 300 kDa MWCO dialysis membrane to eliminate free proteins from the phage solution. Selection of αRep binders was performed as described in refs. (Guellouz et al. 2013; Urvoas et al. 2010) except for the following modifications.

For the selection against the biotinylated MexB target solubilized in detergent, a DDM concentration of 0.1 % was maintained throughout the selection procedure to avoid aggregation of the protein. Regarding the selection against MexB trapped in BNAPol, it was checked that the protein remains bound to the streptavidin-coated plate after more than 20 extensive washing steps. This experiment was carried out by ELISA, following the same procedure as within the selection, except that phages were not added in this control experiment: the protein MexB trapped in BNAPol was immobilized on the streptavidin-coated wells of an ELISA plate. Extensive washing steps were performed as for the selection procedure, and the remaining bound protein was detected by an anti-His horseradish peroxidase (HRP)-conjugated antibody.

For the selection against targets immobilized via biotinylated APols, attempts were made to deplete the dialyzed phage population (1–2 × 10^{10} particles/well) of non-specific binders. To that purpose, phages were first pre-incubated with the APol alone, in order to deplete the suspension from phages exposing APol binders. To this end, the wells of an immunoplate (Nunc Maxisorp) were coated with streptavidin (20 mg L^{-1}) for 4 h at 4 °C and then blocked overnight with TBS + Tween 20 containing bovine serum albumin (BSA) (4 %). The phage solution was then pre-incubated for 2 h at 4 °C on a series of BAPol-coated wells and unbound phages transferred to the blocked (BSA-treated) target-coated wells for 1 h at 4 °C. Plates were then washed with 40 volumes of the buffer used for the purification of the target protein. Bound phages were subsequently eluted using, unless otherwise indicated, acidic conditions (glycine 0.1 M, pH 2.5 for 10 min at RT). The eluted phages were used to infect 5 mL of XL1-Blue cell suspension and plated onto large agar plates containing ampicillin (200 mg L^{-1}), tetracyclin (12.5 mg L^{-1}), and glucose (1 %, w/v). The recovered bacteria were used for the next selection rounds.

Screening for Target Binding: Clonal Phage ELISA

After three selection rounds, individual clones were systematically screened for target binding by phage ELISA essentially as described by Urvoas et al. (2010). Individual colonies were randomly picked and grown overnight at 37 °C in a 96-well plate in 2XTY medium (150 µL) containing ampicillin (200 mg L^{-1}), tetracyclin (12.5 mg L^{-1}), and glucose (1 %, w/v). This master plate was used as a pre-culture plate for phage production and stored at −80 °C in the presence of glycerol (20 % w/v). Exponentially, growing cells were infected for 1 h at 37 °C with 10^{10} particles of helper phage and transferred into 2YT medium (1.5 mL) containing ampicillin (200 mg L^{-1}) and kanamycin (50 mg L^{-1}) in a deep-well culture plate. The phage particles were produced overnight at 30 °C. A maxisorp ELISA plate (Nunc) was coated with streptavidin (20 mg L^{-1}) in PBS overnight at 4 °C and with the target MP (10 mg L^{-1}) in target buffer during 2 h at 4 °C. The plates were blocked with target buffer BSA for 3 h at 4 °C, washed with target buffer, and 100 µL of the phage supernatant from each well was added and incubated for 2 h at 4 °C. The plates were washed with target buffer. The presence of bound phages was revealed with an HRP-conjugated anti-M13 monoclonal antibody (Amersham) and detected at 450 nm using BM Blue POD as a substrate (Roche Diagnostic) after the addition of HCl. For each clone, a negative control with a well blocked and coated with streptavidin but not with the protein was performed on the same ELISA plate.

Secondary Screening for αRep Expression and Binding: Bacterial Soluble Fraction ELISA

For some targets, an additional screen was performed for positive clones in phage ELISA. This screen relies on the detection of the interaction between the target and the whole of the soluble protein population (among which the desired binder) expressed by the bacteria. Individual clones were grown at 37 °C, and expression induced upon addition of 1 mM IPTG and further incubated for 4 h at 37 °C. The bacteria were recovered and concentrated to $OD_{600\ nm} = 8.0$ and lysed with the B-PER reagent with benzonase (Thermo Scientific) for 30 min at 37 °C. The soluble fractions were diluted 5–10 × and transferred on a previously target-coated and blocked ELISA plate. Binding of αRep proteins to the target was revealed using a horseradish peroxidase-conjugated anti-flag M2 monoclonal antibody (Sigma). This makes it possible to test both the soluble expression of the αRep and its binding efficiency against the MP target. Clones screened as positive were further sequenced and the corresponding αRep genes sub-cloned in a pQE81L vector for αRep protein production and purification on Ni–NTA resin.

Characterization of the Formation of αREP/Membrane Protein Complexes

Isothermal Titration Calorimetry

The binding parameters were monitored with a VP–ITC microcalorimeter (MicoCal). For titration of APol binding

to selected αReps, 10 µL aliquots of APol (0.4 g/L) were injected from a computer-controlled 200 µL microsyringe at intervals of 180 s into the solution of αRep (10 µM) dissolved in the same standard buffer (PBS) while stirring at 600 rpm. The heat released upon dilution of the binder was determined from the peaks measured after full saturation of the αRep by the APol. The data were integrated to generate curves where the area under the injection peaks was plotted against the ratio of injected sample-to-cell content. Analysis of the data was performed using the MicroCal Origin software provided by the manufacturer according to the one-binding-site model. Changes in the free energy and entropy upon binding were calculated from determined equilibrium parameters using the the following equation: $-RT\mathrm{Ln}(K_A) = \Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$, where R is the universal gas constant (1.9872 cal mol^{-1} K^{-1}), T is the temperature in Kelvin, K_A is the association constant, ΔG° is the standard change in Gibbs free energy, ΔH° is the standard change in enthalpy, and ΔS° is the standard change in entropy. The binding constant of each interaction is expressed as $1/K_A = K_D$ (in mol L^{-1}).

Analytical Size-Exclusion Chromatography

Analytical SEC was done with an Äkta Purifier (GE Healthcare) system using a Superdex 200 10/300 column (flow-rate 1 mL min^{-1}) equilibrated in Bis/Tris 10 mM, pH 7.4, NaCl 300 mM,, DDM 0.03 %, and glycerol 10 %. For MexB protein, 100 µL of protein sample (10 µM) was injected in the presence or in the absence of αRep (24 µM). For each elution profile, $A_{280\ \mathrm{nm}}$ was normalized relatively to its maximum.

ELISA

OprM, MexB, cytochrome bc_1, and BR (100 µL at 1 µM) were coated on an ELISA plate for 1 h at 4 °C. After three washes with target buffer, each well was blocked with target buffer with BSA (4 %) during 1 h at 4 °C, and then washed three times with protein buffer. Each purified, Flag-tagged, αRep (100 µL at 10 µM) was incubated with the target protein. The presence of αRep proteins was revealed using a HRP-conjugated anti-flag M2 monoclonal antibody (Sigma).

Results

Choice of Membrane Protein Targets

The design of αRep library 2.1 has been recently described, as well as the selection by phage display of specific αRep binders for various soluble protein targets (Guellouz et al. 2013). The present study aims at defining ways to select binders against MPs. MexB and OprM from the Gram-negative bacteria *Pseudomonas aeruginosa*, the cytochrome bc_1 complex from beef heart mitochondria, and bacteriorhodopsin from the archaebacterium *Halobacterium salinarum* were selected as targets, as they cover a wide range of structures, sizes, functions, and biological origins (see Fig. 2).

Efflux pumps have a central role in the resistance against antibiotic therapy. In the Gram-negative bacteria *Pseudomonas aeruginosa*, which is protected by an outer membrane, efflux transporters are organized as tripartite systems (Pos 2009), where MexB, the efflux pump, located in the inner membrane, works in conjunction with MexA, a periplasmic protein, and OprM, an outer membrane channel. MexB (3 × 113 kDa) acts as an energy-dependent pump with broad substrate specificity, OprM (3 × 50 kDa) as a porin. The cytochrome bc_1 complex is a large multicomponent system, which contains eleven different proteins and numerous prosthetic groups and is organized as a superdimer (2 × 240 kDa). It carries out electron and proton transfer reactions that allow to establish the transmembrane proton gradient necessary for synthesizing ATP in the mitochondrial respiratory chain (Berry et al. 2000). In certain archaebacteria, this gradient is built by bacteriorhodopsin (27 kDa), an integral MP that extrudes protons from the cytosol upon illumination by light of its cofactor, retinal (Neutze et al. 2002). The trapping and immobilization of MexB and OprM using APols have not been reported previously. Trapping of BR and bc_1 and the solution properties of the resulting complexes have been extensively studied in refs. (Dahmane et al. 2013; Gohon et al. 2008) and (Charvolin et al. 2009, 2014), respectively. Their immobilization using a biotinylated APol and the study of the immobilized complexes by surface plasmon resonance and fluorescence microscopy have been described in refs. (Charvolin et al. 2009; Della Pia et al. 2014a, b). Among those proteins, we first focus on MexB in order to show that the αRep library 2.1 is indeed suitable to select binders against a MP.

Selection Against Biotinylated MexB in Detergent

In the selection process, the target protein is immobilized onto a solid support and incubated with the phages produced from the library, each phage exposing a different αRep protein at its surface; several washing steps are then carried out to wash off the phages that bind non-specifically, and the strongly bound phages eluted using an acidic glycine solution (see Materials and Methods). MexB is produced in a recombinant form and purified from DDM-solubilized membranes (Mokhonov et al. 2005). A new plasmid construction was designed, in which an Avi tag is

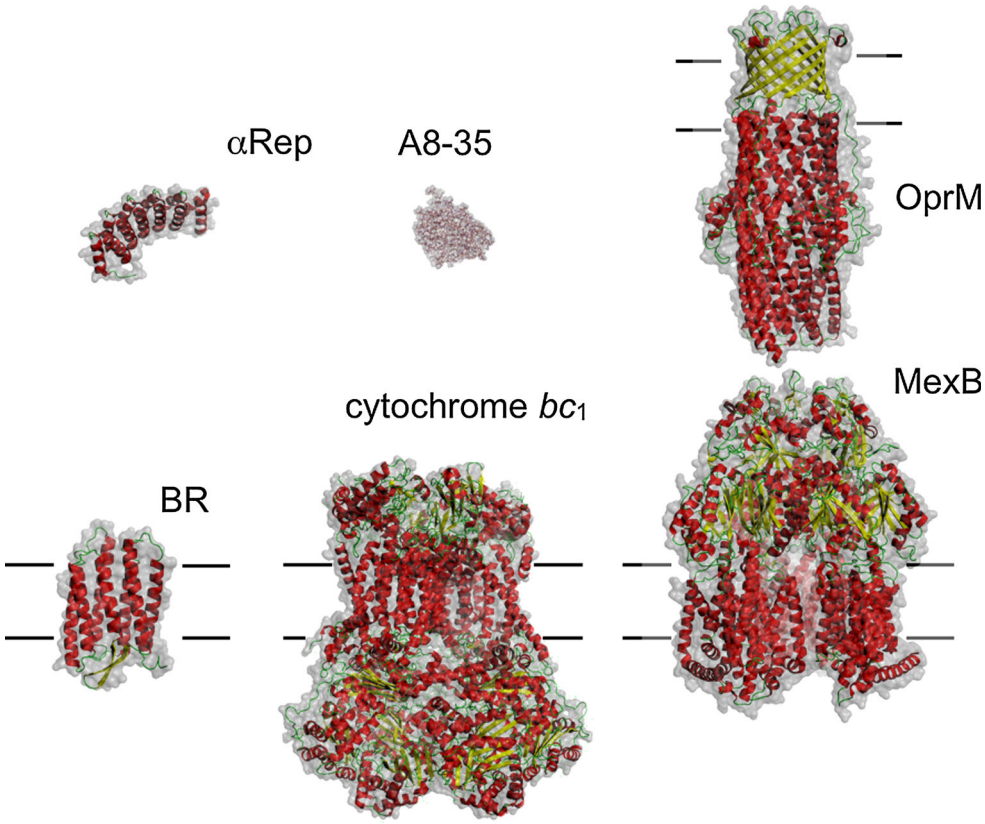


Fig. 2 Representation of a particle of amphipol A8-35 and of the proteins used in the study. Protein secondary structures and surface representation were displayed and calculated using Pymol (DeLano 2002). Atomic coordinates: BR: PDB accession number 1AT9; cytochrome bc_1: PDB accession number 2A06; MexB: PDB accession number 2V50; OprM: PDB accession number 3D5 K; αRep: PDB accession number 3LTJ; particle of A8-35: atomic coordinates computed from a molecular dynamics model kindly provided by J.D. Perlmutter and J.N. Sachs (from Perlmutter et al. 2011)

fused at the C-terminal end of the protein. The Avi tag is a small sequence that can be specifically biotinylated in vitro by the BirA enzyme in the presence of free biotin. Avi-tagged MexB-Biot was recovered from DDM-solubilized membranes, purified using Ni–NTA affinity chromatography followed by SEC, and biotinylated in vitro (see Materials and Methods). The phage library was screened against MexB-biot immobilized in wells coated with streptavidin. Three rounds of phage display selection were performed using the purified DDM-solubilized MexB-Biot as a target. A detergent concentration of 0.1 %—well above the cmc (critical micellar concentration)—was maintained throughout the selection procedure in order to prevent protein precipitation. Individual clones obtained from the second and third selection rounds were analyzed by clonal phage ELISA. Positive signals were found for 19/48 (39 %) clones from round 2 and 31/48 (64 %) clones from round 3, indicating a significant enrichment in binders of the phage population. No binding signal was found in wells coated with streptavidin alone, suggesting that the binders were indeed specific for MexB-Biot. A series of 20 clones were further analyzed in a secondary ELISA experiment by incubating the bacterial soluble fractions. with immobilized MexB-Biot in DDM ("Soluble fraction Phage ELISA"; see Materials and Methods). A specific positive binding signal was obtained in all cases, confirming the phage-ELISA results. Sequence analysis of the 20 clones revealed redundant sequences, and only two distinct binders were eventually found: bMexB-B9, found for 18 clones; and bMexB-E11, found for two clones. Each of these two αReps contains three internal repeats, but no other common feature obviously emerged from the analysis of the amino acids selected in the variable positions.

bMexB-B9 and bMexB-E11 were produced from an overexpression plasmid (pQE81L), purified, mixed with purified MexB-Biot, and the two mixtures subjected to analytical SEC (Fig. 3). The elution profile of MexB displays two peaks, corresponding to the trimer (elution volume $V_e = 9.9$ mL) and the monomer ($V_e = 11.8$ mL), previously described to coexist in equilibrium in detergent solutions (Stroebel et al. 2007). Each of the two αReps elutes in a single peak, at 15.9 mL for E11 and 17.5 mL for B9. The elution profiles of the MexB-Biot/αRep mixtures (1:2) show three peaks, corresponding to the MexB-Biot trimer (9.8 mL), the MexB-Biot monomer (11.7 mL), and the free αRep (15.9 mL for E11 and 17.5 mL for B9). A slight increase of the absorbance of the MexB-Biot trimer peak and a slight decrease of that of the αRep one are consistent with the formation of a MexB-Biot trimer/αRep complex, even though no change in the elution volume of

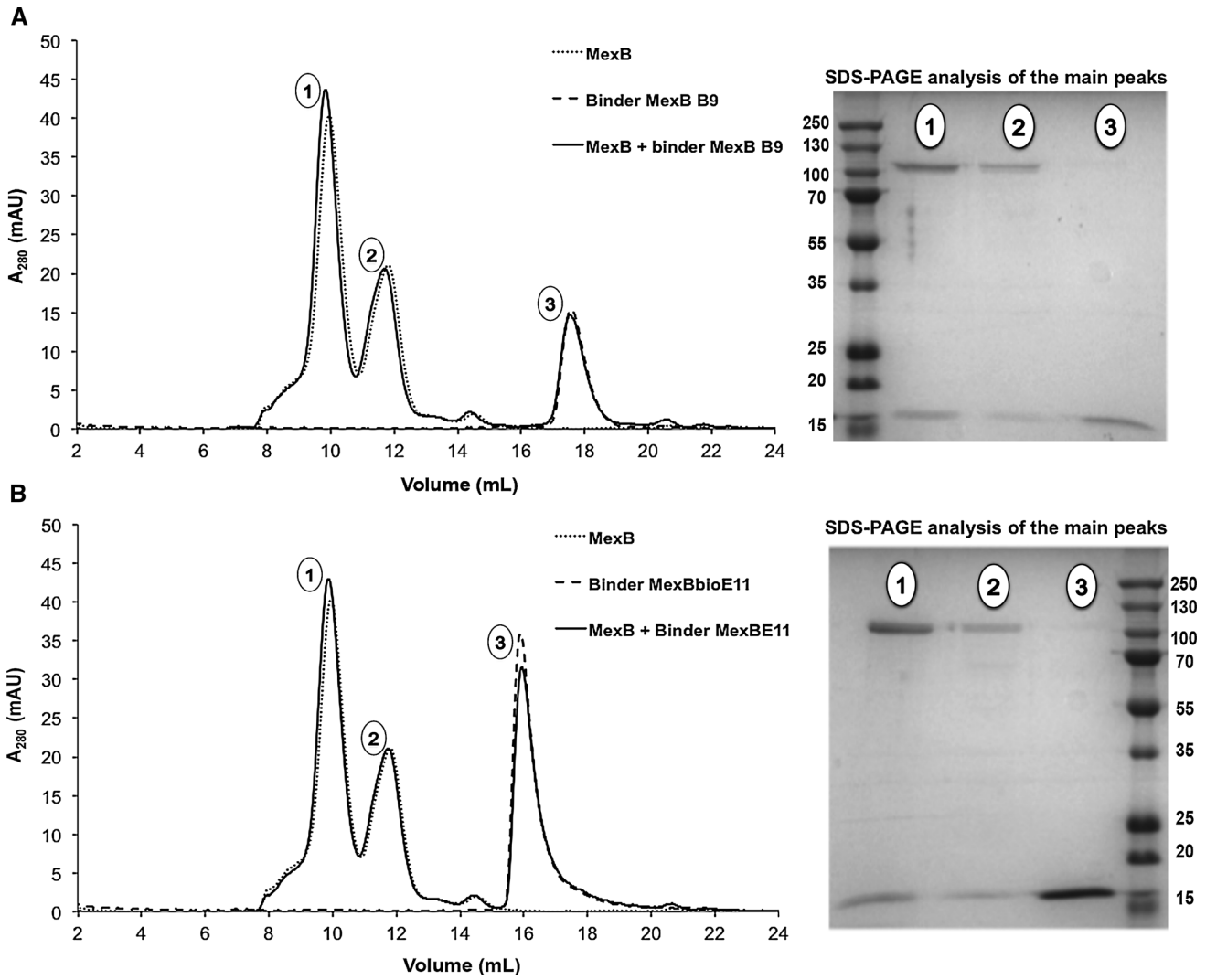


Fig. 3 Size-exclusion chromatography analysis of the interaction between MexB and a specific αRep. **a** SEC analysis on a Superdex 200 10/300 column of the interaction between MexB and αRep MexB B9. *Solid line* elution profile of a mixture of MexB (10 μM) and MexB B9 (24 μM); *dashes* elution profile of MexB B9 alone (18 μM); *dotted line* elution profile of MexB alone (24 μM). Each peak of the elution profile of the mixture of MexB (10 μM) and MexB B9 (18 μM) was analyzed by SDS-PAGE. **b** SEC analysis, under the same conditions, of the interaction between MexB and αRep MexB E11. *Solid line* elution profile of a mixture of MexB (10 μM) and MexB B9 (24 μM); *dashes* elution profile of MexB E11 alone (24 μM); *dotted line* elution profile of MexB alone (24 μM). Each peak of the elution profile of the mixture of MexB (10 μM) and MexB E11 (24 μM) was analyzed by SDS-PAGE

the trimer could be detected. SDS-PAGE analysis of each eluted fraction confirmed the presence of MexB-Biot/αRep complexes (Fig. 3).

The results show that αReps can be selected from library 2.1 against at least one MP immobilized as a MP/detergent complex. This protocol of selection, however, implies that the protein be biotinylated. This is not always possible and, when it is, the biotinylation procedure has to be optimized, as well as conditions ensuring the stability and homogeneity of the protein, which can often be elusive and tedious. In the αRep selection process, a key step is the immobilization of the protein target, a procedure during which the protein must remain folded and functional. A promising route to stabilizing MPs is to resort to APols instead of detergents (see "Introduction"). Biotinylated APols have recently been developed (Basit et al. 2012; Bosco et al. in preparation; Charvolin et al. 2009). In the following, two forms of biotinylated APols have been used to stabilize and immobilize target MPs in their native conformation, one of them ('BAPol'; Fig. 1a) derived from the polyanionic APol A8-35 (Tribet et al. 1996), the other ('BNAPol'; Fig. 1b) from a non-ionic APol (Sharma et al. 2012).

Selection Against a Membrane Protein Immobilized with Biotinylated A8-35 (BAPol)

In a first set of experiments, unbiotinylated MexB was trapped with biotinylated A8-35 (BAPol) (see Materials

and Methods). A 50:50 mixture of biotinylated/non-biotinylated APol was used in order to diminish the number of biotin moieties per MexB/BAPol complex. The rationale behind this dilution is to avoid that MP-associated APol belts carry too many biotins, the interaction of which with the support could possibly distort the complex, and compromise the stability of the protein. Before immobilization, the oligomeric state of the complexes in solution was assessed by SEC and BN-PAGE. The elution profiles showed that BAPol-stabilized MexB retains an oligomeric state comparable to that observed in detergent solution, indicating that the protein is in its native state (data not shown). The complexes were immobilized on streptavidin-coated plates and used as targets in a selection procedure.

Because MPs are trapped with an excess of APol over that which actually binds to the protein (for discussions, see Zoonens et al. 2007, 2014), preparations of MexB/BAPol contain both MexB/BAPol complexes and protein-free BAPol particles, both of which attach to streptavidin. Both the protein-bound BAPol belt and protein-free BAPol particles are the potential targets during the selection process. Prior to the actual positive selection process, a pre-screening step was, therefore, added with the view of removing eventual APol binders from the library. To this end, phages from the αRep library were first pre-incubated on wells containing immobilized BAPol. Non-adsorbed phages were then incubated on MexB/BAPol-coated wells for three rounds of selection performed as described in Materials and Methods. After the selection process, individual clones were screened by clonal phage ELISA. A significant number of clones gave a positive signal for MexB/BAPol (33/96; 34 %). Individual proteins were then expressed, and soluble bacterial fractions prepared and used in a secondary ELISA experiment: The bacterial extracts were incubated with MexB/BAPol complexes, with BAPol alone, with a decoy MP/BAPol complex, or with streptavidin alone. For each clone, a positive signal was obtained whenever BAPol was present in the well, whereas only a few (4/40) were found to bind streptavidin (data not shown). This result suggested that the αReps selected were more APol binders than MexB binders.

Considering that the strong contribution of BAPol to the binding signal could mask the contribution of the protein, the clones selected against MexB/BAPol complexes were subjected to an ELISA test for the binding of MexB-Biot/DDM complexes. No specific binding signal for the biotinylated protein was detected (data not shown). In order to assess if the presence of BAPol biased the selection process specifically with the MexB target, a second MP trapped in BAPol was submitted to the same selection process. Cytochrome bc_1 was chosen in this context, as this multi-component complex cannot be obtained in a recombinant form, and thus in vivo biotinylated, whereas its trapping with BAPol is well established (Charvolin et al. 2009). The panning process was identical to that used with MexB/BAPol complexes, and similar results were obtained, with a large fraction of clones screened positive in Phage ELISA (43/96; 45 %). However, positive signals were also observed, as for MexB/BAPol, when BAPol alone was present in the wells.

These results confirmed that, although much care had been taken to optimize the screening procedure, interaction with BAPol contributed predominantly to the binding of the selected αReps. These observations suggest that the fraction of potential BAPol binders in the library is so high that their selection is favored, swamping the emergence of rare putative MexB and cytochrome bc_1 binders.

Non-specific BAPol Binders are most Probably Selected Through Electrostatic Interactions

The data just described show that a protein target stabilized and immobilized in BAPol and subsequently used for αRep screening leads to the selection of APol binders, even though, when the same procedure is applied to its detergent-solubilized, biotinylated counterpart, protein-specific binders can be obtained. This suggests that the presence of the BAPol belt surrounding the transmembrane domain of MPs (see e.g. Althoff et al. 2011; Perlmutter et al. 2014; Liao et al. 2013, 2014) and/or the protein-free BAPol particles adsorbed to streptavidin present characteristics that favor the selection of binders.

In order to understand the propensity of the selected binders to interact with BAPol, the sequences of all motifs were aligned. They exhibit an enrichment in arginine residues at all variable positions (18, 19, 22, 23, and 26) (Fig. 4a), pointing to electrostatic interactions as a likely factor in the interaction between αReps and the polyanionic BAPol.

ITC experiments were performed to characterize more precisely the interaction between A8-35 (from which BAPol is derived) and αReps obtained during the selection against cytochrome bc_1/BAPol complexes. For three binders giving a positive phage ELISA signal against BAPol, an interaction profile was obtained with K_D values in the micromolar range (respectively 120 nM, 1, and 420 μM for binders bBAPol-G1, bBAPol-A8, and bBAPol-F10) and a stoichiometry of one APol particle for two αRep molecules (Fig. 4b). Given the relative size of the two partners (Fig. 2), this stoichiometry suggests that two αReps more or less completely surround a particle of A8-35. In a control ITC experiment, the titration with A8-35 of an unrelated αRep (αRep-A3, described by Guellouz et al. 2013) showed no significant signal (Fig. 4c). These results indicate that BAPol binders are selected from the αRep library, probably thanks to strong electrostatic interactions.

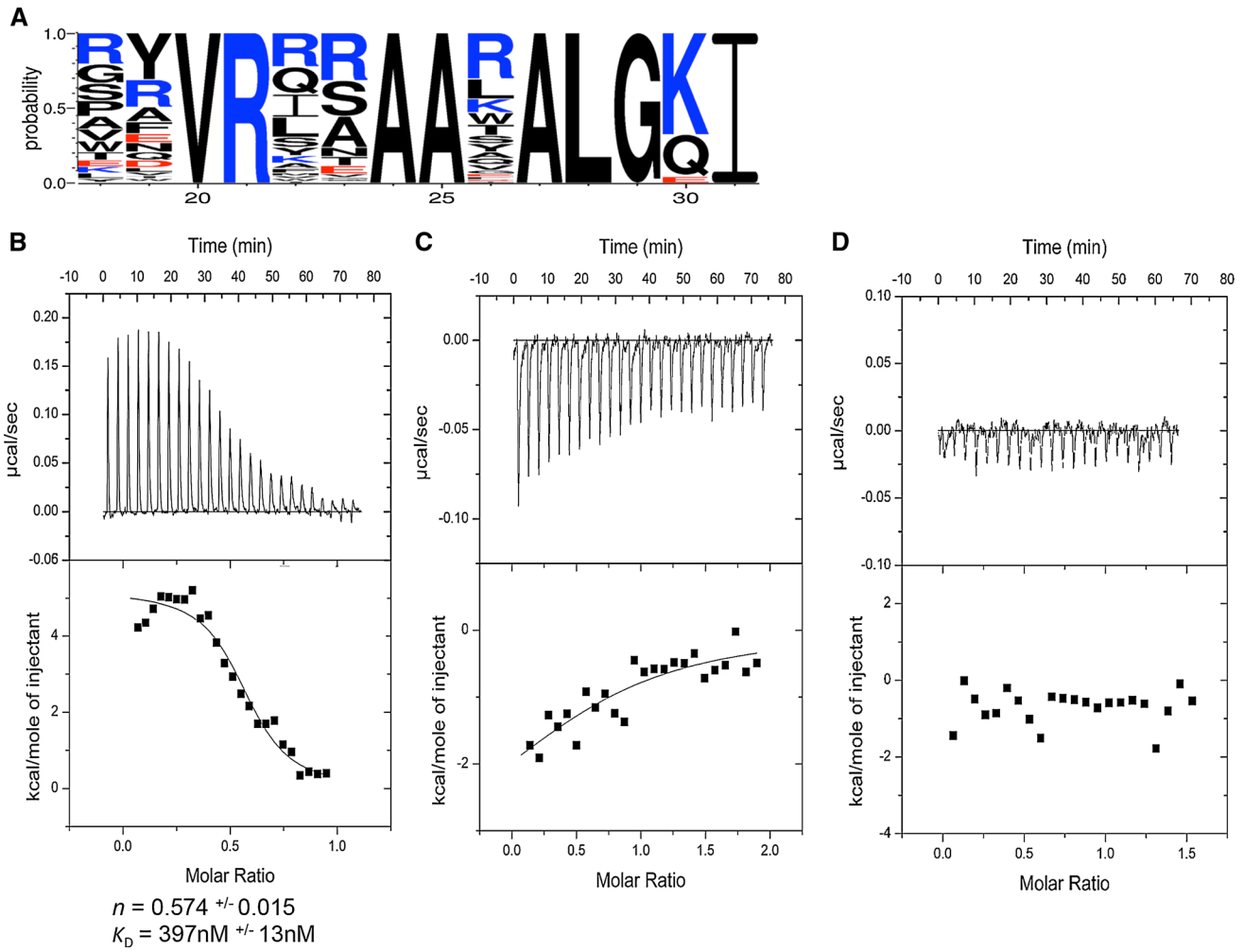


Fig. 4 **a** Alignment of all motifs of αReps selected against MexB/BAPol and cytochrome bc_1/BAPol complexes, shown as a sequence logo using the sequence numbering in each motif family as the abscissa. Hydrophobic amino acid residues are shown in *black*, basic ones in *blue*, acidic ones in *red*. **b–d** ITC titrations of **b** αRep bBAPol-F10 (10 µM) by A8-35 (100 µM); **c** αRep-A3 (10 µM) by A8-35 (100 µM); and **d** bBAPol-F10 (10 µM) by NAPol (100 µM) (Color figure online)

The negative screening against BAPol alone during the panning process is obviously not sufficient to avoid the selection of such binders. It thus appeared critical to minimize non-specific interactions during the selection process to obtain protein-specific binders rather than APol binders. As electrostatic interactions seem to be involved, MP immobilization via non-ionic APols was explored. To this end, a biotinylated non-ionic APol (BNAPol) was synthesized (Fig. 1b), derived from recently described homopolymeric NAPols (Bazzacco et al. 2012; Sharma et al. 2012). An ITC titration of αRep bBAPol-F10, a binder that does interact with A8-35 (see above), did not reveal any interaction with NAPol (Fig. 4d). A new set of panning experiments were, therefore, performed, using BNAPol to immobilize target MPs. For these experiments, we turned to a particularly challenging MP, bacteriorhopsin (BR), a deeply membrane-integrated protein that exposes only small extramembrane regions for interaction with soluble binders (Fig. 2; cf. the model of BR/NAPol complexes derived from neutron scattering data in Sharma et al. 2012).

Selection Against Membrane Proteins Immobilized with Biotinylated Non-ionic Amphipols (BNAPols): A way Toward a Universal Binder Selection Procedure

Three rounds of selection were performed as described above, but now using MPs trapped in BNAPol. Again, a pre-incubation step of the phages on BNAPol-coated wells was performed to deplete the phage library from potential BAPol binders. During the third round of screening, phages selected for BR were eluted following different strategies: specific elution by incubation of bound phages with a solution of detergent-solubilized BR or pre-elution of APol binders by incubation with a mixture of A8-35 and NAPols. In either

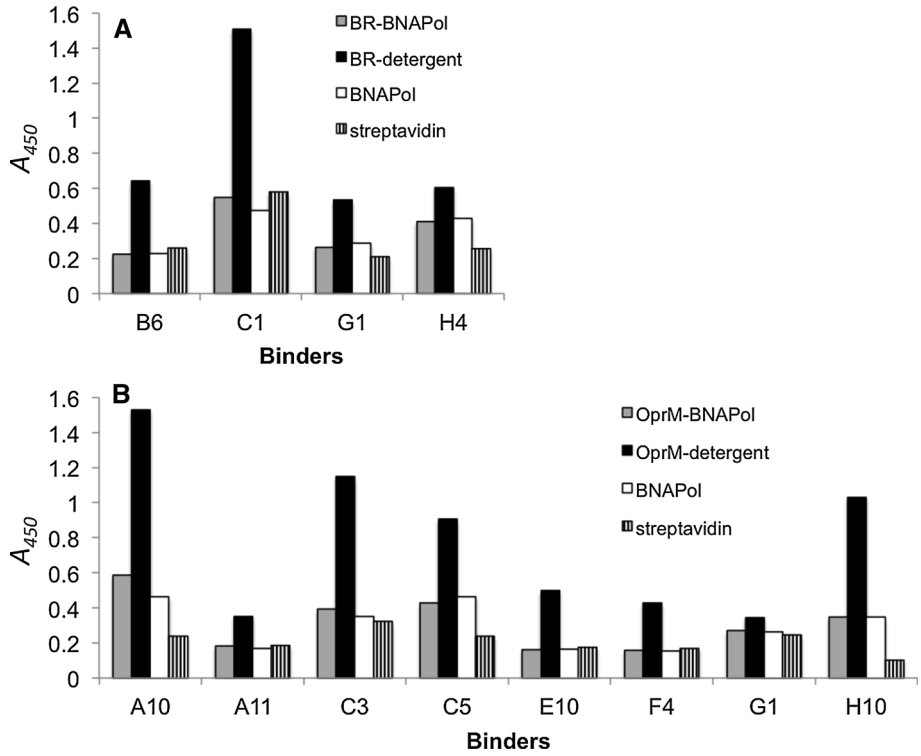
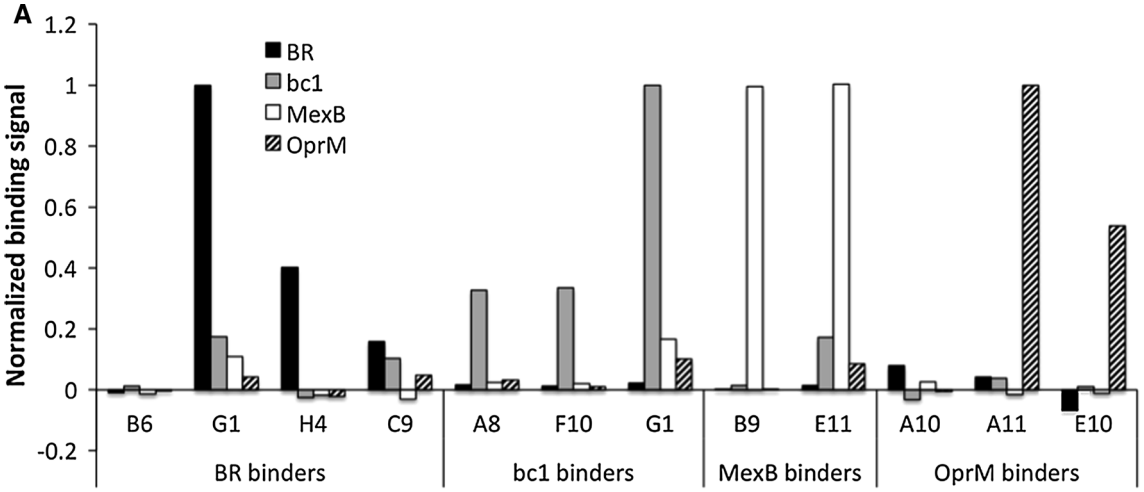


Fig. 5 ELISA analysis of the binding specificity of αRep binders selected against BR/BNAPol (**a**) and OprM/BNAPol (**b**) complexes. Positive phage ELISA clones obtained after the selection against BR (**a**) and OprM (**b**) were further analyzed. Crude bacterial extracts obtained after IPTG induction were diluted 5× in the buffer used for the purification of the protein and transferred on a previously coated and blocked ELISA plate. Different coating conditions were used: Streptavidin followed by the target immobilized in BNAPol (*gray*), streptavidin followed by BNAPol alone (*white*), streptavidin alone (*stripes*), or the target purified in detergent (*black*), immobilized by adsorption onto the plastic walls of the wells. The presence of αRep proteins bound to the ELISA plate was revealed using a horseradish peroxidase-conjugated anti-flag M2 monoclonal antibody

case, the remaining phages were sequentially eluted by an acidic buffer. Clones were obtained in all four cases, and a series of 96 of them were screened by Phage ELISA. To discriminate target-specific αReps from non-specific ones, each clone was systematically analyzed on the basis of the signal obtained on wells coated with i) streptavidin alone, ii) BNAPol alone, iii) BR/BNAPol complexes, or iv) BR/detergent complexes. All clones displayed a positive signal on BR/BNAPol-coated wells, as well as for BNAPol-coated wells. No binding signal was observed for streptavidin-coated wells, suggesting that, once more, the polymer alone contributed significantly to the binding signal. However, at variance with the observations made following selections performed using BAPol, for 45 % of the clones (33/96), a significant binding signal was also detected on wells coated with BR/detergent complexes, meaning that the protein contribution was now significant. Among those selected clones, some of them turned out to be protein specific, having been selected on the basis of their non-reactivity toward NAPols or NAPol-stabilized proteins and their interaction with the detergent-solubilized target. The best clones in phage ELISA (18 clones for BR) were produced in soluble bacterial fractions and further analyzed by ELISA. Four of these clones displayed a significant specific binding signal for detergent-solubilized BR, clearly indicating that, in addition to the APol, BR itself contributed to the binding (Fig. 5a). The other 14 αReps, expressed independently of the phage, displayed too low an affinity for BR/detergent complexes.

The fact that BNAPol can be used to obtain binders truly specific for BR being established, the procedure was adapted to an external membrane β-barrel protein (OprM), so as to complete the exploration of target MPs with different architectures (α-helical or β-barrel) and from various origins (mitochondrial, cytoplasmic, eukaryotic, and prokaryotic). Eight OprM-specific binders were screened and characterized, showing a significant binding signal with detergent-solubilized OprM (Fig. 5b). OprM and BR binders were subjected to size-exclusion chromatography (SEC) in the presence of their target in order to isolate stable complexes in detergent conditions. Most of the binders migrate as monomers. No complexes could be observed, suggesting that target/αRep interactions are too weak, leading to dissociation upon SEC.



B

BR-G1
MRGSHHHHHHTDPEKVEMYIKNLQDDSRHVRNDAAIALGKI
GDERAVEPLIKALKDEDAEVRIRAAYALGKI
GDERAVEPLIKALKDEDGYVRLRAATALGKI
GDERAVEPLIKALKDEDVRVRRAAATALGKI
GDERAVEPLIKALKDEDWRVRRNAAKALGKI
GGERVRAAMEKLAETGTGFARKVAVNYLETHKSLIS

BR-H4
MRGSHHHHHHTDPEKVEMYIKNLQDDSARVRADAATALGKI
GDERAVEPLIKALKDEDWQVRQRAAKALGKI
GGERVRAAMEKLAETGTGFARKVAVNYLETHKSLIS

BR-C9
MRGSHHHHHHTDPEKVEMYIKNLQDDSQTVRNFAATALGKI
GDERAVEPLIKALKDEDPDVRISAATALGKI
GGERVRAAMEKLAETGTGFARKVAVNYLETHKSLIS

BC1-A8
MRGSHHHHHHTDPEKVEMYIKNLQDDSRQVRTNAAYALGKI
GDERAVEPLIKALKDEDDAVRRVAAAALGEI
RDERAVEPLIKALKDEDTVVRRAAARALGQI
GDERAVEPLIKALKDEDPNVRSSAASALGKI
GDERAVEPLIKALKDEDPWVRYRAARALGKI
GGERVRAAMEKLAETGTGFARKVAVNYLETHKSLIS

BC1-F10
MRGSHHHHHHTDPEKVEMYIKNLQDDSRAVRATAAIALGKI
GDERAVEPLIKALKDEDSRVRRAAAWALGKI
GDERAVEPLIKALKDEDWQVRKTAARALGQI
GDERAVEPLIKALKDEDRAVRQEAARALGKI
GDERAVEPLIKALKDEDWQVRKEAAWALGQI
GGERVRAAMEKLAETGTGFARKVAVNYLETHKSLIS

BC1-G1
MRGSHHHHHHTDPEKVEMYIKNLQDDSQHVRNNAANALGKI
GDERAVEPLIKALKDEDVRVRYRAARALGKI
GDERAVEPLIKALKDEDGYVRRAAARALGQI
GDERAVEPLIKALKDEDVNVRQEAALALGKI
GDERAVEPLIKALKDEDTDVRLRAARALGKI
GDERAVEPLIKALKDEDTDVRIAAARALGKI
GGERVRAAMEKLAETGTGFARKVAVNYLETHKSLIS

MexB-B9
MRGSHHHHHHTDPEKVEMYIKNLQDDSGLVRVYAAYALGKI
GDERAVEPLIKALKDEDPWVRREAALALGEI
GDERAVEPLIKALKDEDSAVRLRAADALGKI
GDERAVEPLIKALKDEDSRVRSSAATALGEI
GGERVRAAMEKLAETGTGFARKVAVNYLETHKSLIS

MexB-E11
MRGSHHHHHHTDPEKVEMYIKNLQDDSMRVRYNAATALGKI
GDERAVEPLIKALKDEDGYVRLEAAEALGEI
GDERAVEPLIKALKDEDPDVRSEAALALGKI
GDERAVEPLIKALKDEDRYVRMAAAWALGKI
GGERVRAAMEKLAETGTGFARKVAVNYLETHKSLIS*

OprM-A11
MRGSHHHHHHTDPEKVEMYIKNLQDDSNRVRYDAATALGKI
GDERE--PLIKALKDEDGYVRRAAAWALGKI
GDERE--PLIKALKDEDGYVRRAAAWALGKI
GDERE--PLIKALKDEDGYVRRAAAWALGKI
GDERE--PLIKALKDEDGYVRRAAAWALGKI
GDERAVEPLIKALKDEDARVRRVAARALGKI
GDERAVEPLIKALKDEDANVRIEAALALGKI
GDERAVEPLIKALKDEDWRVRRLAARALGKI
GGERVRAAMEKLAETGTGFARKVAVNYLETHKSLIS

OprM-E10
MRGSHHHHHHTDPEKVEMYIKNLQDDSPSVRYNAATALGKI
GDERAVEPLIKALKDEDGYVRQAAAKALGQI
GDERAVEPLIKALKDEDVRVRARAAEALGKI
GDERAVEPLIKALKDEDRYVRIRAAFALGKI
GGERVRAAMEKLAETGTGFARKVAVNYLETHKSLIS

Fig. 6 **a** Binding specificity comparison of selected binders as analyzed by ELISA. The wells of an ELISA plate were coated with each membrane target and blocked using BSA (4 %). Code for the nature of the target protein immobilized: cytochrome bc_1, *gray*; BR, *black*; MexB, *white*; OprM, *stripes*. Purified αReps obtained after selection against their respective targets (BR-B6, BR-G1, BR-H4, BR-C9, cytochrome bc_1-F10, bc_1-A8, bc_1-G1, MexB-E11, MexB-B9 and OprM-A11, OprM-A10, OprM-E10) were tested against each of the four proteins. The binding of each αRep was revealed using a horseradish peroxidase-conjugated anti-flag M2 monoclonal antibody. For each series of binders, the maximal absorbance was used to normalize the signal. **b** Primary sequence of the different binders obtained in this study. In *bold*, variable positions in the repeats

Analysis of Membrane Protein Specificity for Selected αRep Binders

In order to address the question of the specificity of each selected αRep, the most promising binders to each MP were incubated with the four other targets and their binding efficiencies compared in a single ELISA test (see Fig. 6). The rationale behind this experiment was to distinguish the protein contribution in the interaction, if any, from that mediated by the APol. Hence, the ELISA test was

performed with detergent-solubilized protein adsorbed onto the plastic wall of the wells. Such an immobilization mode is rather harsh, although it is standard procedure for the screening of soluble proteins, but it makes it possible to qualitatively compare the respective binding potency and specificity of each binder. For each αRep binder (except BR-B6 and OprM-A10), a higher binding signal is observed for the target against which it had been selected. This experiment clearly indicates that, although APols can contribute to the affinity of αReps for target MPs, part of the energy of interaction does involve binding to the protein itself.

Discussion

The present work describes a procedure for selecting αReps specific for MPs. The selection is based on a panning process during which the target MP is immobilized onto a solid support using a biotinylated APol.

Detergent-solubilized proteins are appropriate for selection provided that i) they are sufficiently stable during the time frame required for three rounds of phage display selection, namely more than one week, and ii) they can be tagged. Using of a biotinylated APol is a priori attractive, because it combines MP stabilization with a very mild mode of immobilization onto solid supports. Unfortunately, when using biotinylated A8-35 (BAPol), the panning process systematically selects αReps specific of the APol itself, and not of the protein target. The mechanism of recognition of the APol by the αRep is not known, but the frequency of basic side chains found in the sequence of A8-35 binders strongly suggests that ionic interactions play an essential role. This can be alleviated by resorting to a non-ionic biotinylated APol (BNAPol). The use of BNAPol lets MP-specific binders emerge during the selection process, even though interaction with the polymer is systematically present.

Although it had been anticipated that phages expressing αReps that interact with APols could be eliminated by a pre-screening procedure, our results clearly show that such negative selection steps are insufficient to prevent the selection of composite binders. The failure of this preliminary counter-selection may have various origins. For instance, one can imagine that APols immobilized at the bottom of the well during the negative selection (protein-free particles) do not present exactly the same interface with the solution as that displayed during the actual, positive selection step (APol belt wrapping a MP). This artifact could be circumvented by performing the negative selection with a decoy MP trapped and immobilized with BAPol or BNAPol. Perhaps more likely, it is well known that all phages containing a plasmid copy coding for a given αRep in a library will not necessarily express it at their surface in a given round of selection but they may do so during a subsequent screening step. Hence, a significant proportion of phages coding for APol binders might escape the negative selection step if it is not repeated.

It is, therefore, crucial to check a posteriori that αReps selected by screening against a given APol-trapped MP indeed recognize the same MP when it is kept soluble by a detergent. In other words, one should make sure that the protein contribution is strong enough to give rise to specific interactions. It is well established, based on NMR (see e.g. Catoire et al. 2010; Etzkorn et al. 2014; Planchard et al. 2014; Zoonens et al. 2005), electron microscopy (see e.g. Althoff et al. 2011; Huynh et al. 2014; Liao et al. 2013, 2014), and MD data (Perlmutter et al. 2014), that in MP/A8-35 complexes, the polymer interacts almost exclusively with the hydrophobic transmembrane surface of the protein (reviewed by Planchard et al. 2014; Popot et al. 2011; Zoonens and Popot 2014). One should, therefore, expect that this surface is inaccessible during the selection process, and that specific binders should be directed toward MP polar surfaces.

In the case of Darpin selection against detergent-solubilized MPs, where the same situation should hold, an enrichment of unspecific hydrophobic binders has been observed. In that case, the group of Grütter resorted to a new-generation library with reduced hydrophobicity (Seeger et al. 2013). This does not seem to be necessary in the case of αReps, the variability of side chains in library 2.1 having been tuned to match their frequency in αRep-like natural repeats, with the consequence that highly hydrophobic variable surfaces combinations are relatively uncommon. The approach may nevertheless be worth exploring for αReps.

Although crystallization chaperones are a direct application of αRep binders, αReps specific for MPs could also prove useful in fields outside structural biology, for instance i) in purification procedures, taking advantage of their highly efficient expression and their ability to select well-folded proteins; ii) for use as molecular interactants (potential destabilizers of macromolecular assemblies); or iii) for *in cell* Protein Interference (perturbation of cellular process). On the longer term, co-assembly of multiproteic complexes via multifunctional scaffolding repeats assembled one with another through flexible links may emerge as an attractive strategy—for instance, in our case, for the stabilization of MexA/MexB/OprM assemblies. As a matter of fact, natural proteins of these families are often recruited in vivo to act as scaffolding proteins of multimolecular complexes (Skerra 2007).

To conclude, using neutral APols instead of charged ones did not prevent the αReps thus selected from binding to the polymer. However, in these ternary target/NAPol/

αRep complexes, the polymer contribution was not overwhelming, preserving a degree of a specificity of the binders for their proteic targets sufficient for some of them to recognize their targets in detergent solution. The poor stability of many MPs in detergent solutions can be a limitation to the phage display panning, which is a lengthy procedure including several immobilization and washing steps. For MPs that can be biotinylated and are stable in the presence of detergent, resorting to APols presents, in the current state of the technology, more drawbacks than advantages. On the contrary, the use of biotinylated APols during the phage display process seems to be a promising approach to obtain binders against MPs that have limited stability in detergents or cannot be easily tagged.

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