Archives of Biochemistry and Biophysics xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Review

Folding and stability of integral membrane proteins in amphipols

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ARTICLE INFO

Article history:
Received 30 July 2014
and in revised form 11 October 2014
Available online xxxx

Keywords:
Membrane protein
Folding
Amphipols
Kinetics
Thermodynamic stability
Outer membrane proteins
Bacteriorhodopsin
G protein-coupled receptors
A8-35

ABSTRACT

Amphipols (APols) are a family of amphipathic polymers designed to keep transmembrane proteins (TMPs) soluble in aqueous solutions in the absence of detergent. APols have proven remarkably efficient at (i) stabilizing TMPs, as compared to detergent solutions, and (ii) folding them from a denatured state to a native, functional one. The underlying physical–chemical mechanisms are discussed.

Introduction

Folding integral membrane proteins to their functionally active form: still a challenge

Integral membrane proteins – hereafter transmembrane proteins (TMPs)[1] – are key biological players because of their roles in bioenergetics, transmembrane transport, signaling, cell and tissue organization, *etc*. They are therefore primary targets for pharmaceutical drugs. Structural and pharmacological studies of TMPs are hampered by their low abundance and by difficulties in overexpressing them in a functional form. TMPs can be overexpressed either homologously or heterologously, for example in *Escherichia coli*, yeast, *etc*. Plasmid-based overexpression can be designed to target TMPs either to a membrane, which often results in low yields, or to cytoplasmic inclusion bodies, which yields larger amounts of protein, but in a misfolded and aggregated form that has to be folded to a functional state – a difficult achievement. Folding outside the cell environment is also necessary when TMPs are expressed *in vitro* in a cell-free system. Successful folding in detergent or mixed detergent/lipid micelles has been demonstrated for both known structural classes of TMPs, the α-helix bundle and the β-barrel. Over 50 α-helical TMPs have been folded or refolded *in vitro* to date [1]. Among the first ones to be studied were bacteriorhodopsin (BR) [2–4], the light harvesting complex LHCII [5,6], several G protein-coupled receptors (GPCRs) such as olfactory receptors [7,8], the BLT1 receptor of leukotriene LTB_4 [9], or the serotonin 5-$HT_{4(a)}$ receptor [10], channel proteins like the homotetramer KcsA [11], the small multidrug transporter

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[1] *Abbreviations used:* A8-35, a poly(sodium acrylate)-based amphipol comprising ~35% free carboxylates, ~25% octyl grafts, ~40% isopropyl groups, whose number-average molar mass is ~4.3 kDa; APol, amphipol; BLT1 and BLT2, two human G protein-coupled receptors of leukotriene LTB_4; BO, BR, respectively bacterio-opsin and bacteriorhodopsin from *Halobacterium salinarum*; CAC, critical association concentration; CB1, human cannabinoid receptor 1; CD, circular dichroism; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; cmc, critical micellar concentration; 2D, 3D, two- and three-dimensional; DAGK, diacylglycerol kinase; dBO, delipidated BO, obtained after solubilizing purple membranes in organic solvents; DDM, *n*-dodecyl-β-D-maltoside; DHPC, dihexanoylphosphatidylcholine; DS, dodecylsulfate; EM, electron microscopy; ESI-IMS-MS, electrospray ionization mass spectrometry coupled with ion mobility spectrometry; FomA, major outer membrane protein A from *Fusobacterium nucleatum*; GHS-R1a, human ghrelin receptor 1a; GPCR, G protein-coupled receptor; His-tag, polyhistidine tag; 5-$HT_{4(a)}$, human serotonin receptor; LDAO, *N*-lauryl-*N*,*N*-dimethylamine-*N*-oxide; LHCII, light-harvesting complex II; LPS, lipopolysaccharide; MD, molecular dynamics; OG, *n*-octyl-β-D-glucoside; OmpA and OmpT, outer membrane proteins A and T from *Escherichia coli*; OTG, octylthioglucoside; PAGE, polyacrylamide gel electrophoresis; PagP, lipid A palmytoyltransferase from *E. coli*; PDS and SDS, potassium and sodium dodecylsulfate, respectively; PM, purple membrane; SEC, size-exclusion chromatography; SUV, small unilamellar vesicles; TMP, transmembrane protein; tOmpA, transmembrane domain of OmpA; UV, ultraviolet; Z3-14, 3-[dimethyl(tetradecyl)azaniumyl]propane-1-sulfonate (also called sulfobetaine 3-14, Zwittergent 3-14).

http://dx.doi.org/10.1016/j.abb.2014.10.013
0003-9861/

EmrE [12], and the enzyme diacylglycerol kinase (DAGK) [13,14] (for reviews, see *e.g.* Refs. [1,15,16]). About 40 β-barrel TMPs have been (re)folded *in vitro* to date [1,17–20], ranging from simple 8-stranded β-barrels like OmpA [21,22], OmpX [23] or PagP [24–26] to large 22-stranded active transporters like FepA [27,28]. The list also includes eukaryotic β-barrels such as the 19-stranded VDAC human isoforms 1 [29] and 2 [30,31]. It is quite possible that all β-barrel TMPs can be folded *in vitro* without any cellular folding machinery. An unsuccessful attempt to fold a β-barrel in solution has been reported for the β-barrel domain of AIDA [32]. However even for AIDA, folding was successful when the protein was bound to a nickel-bearing column [33].

α-Helical TMPs like BR or KcsA have been folded from denatured forms in organic solvent like trifluoroacetic acid or mixtures of formic acid and ethanol [2,11]. Folding of BR was initiated by transferring denatured bacterio-opsin (BO, the apoprotein) first into micelles of the denaturing detergent sodium dodecylsulfate (SDS) and then, usually in the presence of retinal, BR's cofactor, into "mild" detergents, mixtures of detergent and lipids, or pure lipids [2–4]. Rather than diluting it into a large excess of the folding medium [2,3], dodecylsulfate (DS) can be precipitated as its potassium salt, PDS [4]. β-Barrel TMPs such as outer membrane protein A (OmpA, previously called Omp II) from *E. coli* were first refolded from their SDS-denatured forms by replacing the SDS either with lipopolysaccharide [21] or octylglucoside [22]. Subsequently, most (re)folding protocols for β-barrel TMPs have been based on their solubilization and denaturation in concentrated solutions of urea (8–10 M) [34] or other chaotropic agents (reviewed in Ref. [1]). Denatured β-barrel TMPs are generally folded by transferring them to either detergent micelles or lipid membranes under concurrent strong dilution of the denaturant.

The mechanisms of folding of TMPs of both structural classes have been the subject of extensive research. For α-helical TMPs, BR (see *e.g.* Refs. [35,36]) or DAGK [37,38] have often served as models. Folding of α-helical membrane proteins is a sequential 3-stage process, in which helices insert independently in a first stage and associate laterally in a second stage, which is followed, in a third stage, by the formation of additional tertiary structure, such as re-entrant loops, and/or by the binding of prosthetic groups (for discussions, see Refs. [35,39]). For β-barrel TMPs, a concerted mechanism has been reported for OmpA, in which folding and insertion are coupled [40,41] (for reviews, see Refs. [20,42–44].

Using detergent- or lipid-based protocols, folding yields tend to be lower than desired for many pharmacologically and physiologically interesting TMPs, *e.g.* GPCRs. For example, the leukotriene B_4 BLT1 receptor has been refolded to a moderate yield of ~30% in mixed micelles of detergent and lipid [9]. To date, this is still one of the highest yields reported using classic methods. A homolog of BLT1, the BLT2 receptor, is an example of a GPCR that cannot be folded with a decent yield (more than a few percents) in any detergent or detergent/lipid mixture that has been tried to date [45]. The development of successful refolding strategies for novel TMPs of interest using classic approaches is extremely time-consuming. It is therefore of great interest to examine new methods.

Amphipols as new tools for the refolding of integral membrane proteins

Over the last eight years, a new class of non-detergent surfactants, namely synthetic amphipathic polymers called amphipols (APols) [46], has emerged as very promising tools for folding denatured TMPs to their native state. APols are short polymers comprised of both hydrophilic groups and hydrophobic chains. They can substitute to detergents, providing a milder environment to TMPs while keeping them water-soluble (for reviews, see Refs. [47–51]). All of the three dozen TMPs tested to date form soluble complexes with APols in the absence of detergent, whatever their size, origin, function and secondary, tertiary and quaternary structure (reviewed in Ref. [51]). As compared to preparations in detergent solutions, most TMPs remain active for a much longer time when solubilized in the form of APol/TMP complexes (see *e.g.* Refs. [45,46,52–55]). APols bind to TMPs by adsorbing specifically onto their hydrophobic transmembrane surface, as demonstrated by NMR spectroscopy [56–60], electron microscopy (EM) [61–69], and molecular dynamics (MD) simulations [70]. Unless exchanged for other surfactants, APols do not dissociate from the surface of TMPs, even at very high dilution, keeping the protein water-soluble [47,71,72] (reviewed in Ref. [51]).

Several types of APols have been synthesized and validated (Fig. 1) (reviewed in Refs. [51,73]). Among these, A8-35 has been most extensively studied. A8-35 (Fig. 1A) is a poly(acrylic acid) partially amidated with octylamine and isopropylamine, leaving ~35% of the carboxylic groups free [46]. In aqueous solution at pH > 7, A8-35 assembles into small micelle-like particles with a mass of ~40 kDa [74–76]. Other APols developed more recently include glycosylated nonionic APols (NAPols) [77,78] (Fig. 1B and C), sulfonated APols (SAPols) [79] (Fig. 1D) or phosphorylcholine-based APols (PC-APols) [80,81] (Fig. 1E) (reviewed in Refs. [50,51,73]). SAPols [79] are derived from a precursor of A8-35 lacking the amidation with isopropylamine. Instead, about ~40% of the carboxyl groups are amidated by taurine (2-aminoethanesulfonic acid) (Fig. 1D). The sulfonate groups of taurine do not protonate even at pH 0, which keeps SAPols water-soluble at pH < 7, whereas under such conditions A8-35 aggregates [74,75]. Homopolymeric NAPols (Fig. 1B) are synthesized by homotelomerization of a monomer carrying two glycosyl residues [77]. Heteropolymeric NAPols (Fig. 1C) have been obtained either by cotelomerization of hydrophobic and hydrophilic monomers [82] or by randomly grafting hydrophobic chains onto a glycosylated homotelomer [83]. PC-APols are zwitterionic at neutral and basic pH, cationic at acidic pH. NAPols and PC-APols, as SAPols, remain water-soluble under acidic conditions.

APols, originally designed to keep TMPs water-soluble while preserving their activity [46], have been used for a wide range of applications (reviewed in Ref. [51]). Their properties and those of TMP/APol complexes have been reviewed in Refs. [49–51] and detailed protocols for their implementation provided in Ref. [84]. This review focuses on their use for folding TMPs and for stabilizing them against denaturation by heat or chaotropic agents. Since α-helix bundle TMPs and β-barrel TMPs fold according to very different principles (see *e.g.* Refs. [35,39,41,85]), our discussion is subdivided into separate sections for these two categories of TMPs. Historically, APol-assisted folding of TMPs was first demonstrated for three model TMPs, BR, an α-helical TMP, and two β-barrel TMPs, OmpA and FomA [86]. It was then extended to two more β-barrel TMPs and six GPCRs (Table 1).

Amphipol-assisted folding of α-helix bundle membrane proteins

Folding of BR in A8-35

BR, a light-driven proton pump [87], has served as a popular model protein in studies on TMP folding since the seminal work of Khorana and coworkers [2] (for reviews, see *e.g.* Refs. [1,35,88,89]). When overproduced, BR accumulates in the plasma membrane of *H. salinarum* in the form of 2D protein/lipid crystals, the so-called purple membrane (PM). Its covalently but loosely bound chromophore, retinal, confers it a characteristic purple color. When PM is solubilized in SDS, BR denatures to BO, releasing its chromophore, which causes an absorption peak shift from