

## Cell-Free Synthesis of Membrane Proteins

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### Abstract

Among the various membrane protein synthesis methods available today, the cell-free protein synthesis method is a relatively new tool. Recent technological advances in the lipid and detergent conditions for the cell-free synthesis of membrane proteins have enabled the robust production of various membrane proteins for structural biology. In this chapter, we describe representative conditions for the production of high quantities of membrane proteins by the cell-free method, with crystallization quality. We also discuss examples of membrane proteins that were successfully synthesized by the cell-free method. The crystals of these highly purified proteins resulted in solved crystal structures.

**Keywords** Cell-free protein synthesis, Membrane protein, Lipid, Crystal structure determination

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### 1 Introduction

The cell-free protein synthesis method is used to synthesize proteins *in vitro*, with the cellular translation machinery. The reaction solution for cell-free protein synthesis contains a cell extract, the template DNA or messenger RNA, substrates such as amino acids, and other components (see Fig. 1 in Chap. 5). The original cell-free protein synthesis method was developed long before the advent of recombinant protein expression with host-vector systems, and synthesis systems with rabbit reticulocyte, wheat germ, *Escherichia coli* extracts, etc. were used mainly for the small-scale preparation of proteins, particularly those labeled with a radioactive amino acid. The cell-free protein synthesis method has been drastically improved over the past 20 years, and has become one of the standard methods of protein sample preparation for structural biology, because of a variety of advantages over other expression methods (see Chap. 5). For uses in structural biology analyses by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, which require large amounts of highly pure and homogeneous proteins, the cell-free protein synthesis method

using the *E. coli* cell extract is by far the most frequently chosen, among those using various cell extracts.

The cell-free protein synthesis method is recognized as one of the best methods for the preparation of integral membrane proteins [1, 2]. Unlike recombinant protein expression in host cells, the cell-free system for protein synthesis is not encapsulated in cells, by definition, and therefore lacks the cell membrane. The function of the cell-free synthesis system may be modified, simply by adding the necessary components. Additional components required for the cell-free synthesis of integral membrane proteins are lipids and/or detergents, as the membrane proteins synthesized in the absence of lipid-detergent aggregate and precipitate, due to the hydrophobicity of their transmembrane regions [3, 4]. The membrane proteins synthesized in the cell-free system may form micelles with detergents, and liposomes and nanodiscs with lipids, by embedding their transmembrane regions in the hydrophobic environments provided by the detergents/lipids. Furthermore, certain membrane proteins may require specific boundary lipids for the formation and maintenance of their proper functional structures in the lipid bilayer. Consequently, it is important to develop techniques to supply these materials, according to their roles, to the cell-free protein synthesis system.

In both bacterial and eukaryotic cells, many integral membrane proteins are inserted into the membrane by the translocon, a membrane protein complex that translocates polypeptides through membranes. However, the common cell-free methods do not require translocons for the integration of the synthesized membrane protein into the lipid-detergent complex. In our cell-free synthesis method with both detergent and lipid, the nascent polypeptides synthesized in the cell-free system are co-translationally integrated into the lipid bilayer environment of membrane fragments, which then assemble gradually into larger membranes, such as liposomes. This observation indicated that the edges of the membrane fragments, which are possibly covered with detergents, serve as the entrance for the polypeptides to become integrated into the lipid bilayer environment, instead of the translocon. On the other hand, in eukaryotic cells, certain types of receptors and related proteins are considered to be integrated, after synthesis, into membrane domains, such as lipid rafts and detergent-resistant membranes (DRMs), which are rich in cholesterol and glycolipids [5]. These membrane proteins may interact with specific lipids such as cholesterol, which is easily included in the cell-free protein synthesis system. These aspects of the cell-free protein synthesis system have facilitated the folding of many membrane proteins into their native structures.

When membrane proteins are expressed in host cells by recombinant DNA techniques, they are usually solubilized from the cellular membrane fractions with detergents. If the detergents are

too harsh, then the native structures of the membrane proteins will be destroyed. In contrast, if the detergents are too mild, the recoveries of the expressed membrane proteins in the soluble fractions will be very low. Therefore, the detergent employed for the solubilization of the membrane proteins from the cell membrane should be carefully chosen, usually by extensive screening of a variety of detergents. In particular, when membrane proteins are integrated in DRMs, which are resistant to nonionic detergents such as Triton X-100, the choice of a detergent for solubilization without denaturation is quite difficult. On the other hand, the cell-free synthesis method does not require the use of harsh detergents for solubilization and therefore allows a broader choice of the optimal detergents specific to the individual target membrane protein. This is another strong advantage of the cell-free synthesis method.

The recombinant membrane proteins expressed in cells are usually purified by chromatography, after solubilization from the membrane fractions. However, it is often difficult to remove the contaminating membrane proteins derived from the host cells. On the other hand, in the cell-free synthesis method, the targeted membrane proteins are generally the only proteins synthesized and inserted into the lipid-detergent complexes, although a number of soluble proteins exist in the cell-free reaction solution. This is the reason why the cell-free synthesized membrane proteins can be highly purified. When membrane proteins are highly overexpressed in *E. coli* cells, inclusion bodies may be generated. In these cases, the purity of the product may be very high, as long as the membrane proteins recovered from the inclusion bodies with denaturants, such as urea, guanidine hydrochloride, and sodium dodecyl sulfate, are properly refolded into the native structures. In contrast, in the case of the cell-free synthesis method, the refolding of the synthesized membrane proteins or the solubilization of precipitants with harsh denaturants is usually unnecessary.

There are other advantages of cell-free protein synthesis, as compared to cell-based expression methods. The expression levels of membrane proteins in cells are often low, probably due to the limited capacity of the cell for membrane protein accommodation and the cytotoxicity due, for example, to improper cell membrane structures generated by an excess of inserted membrane proteins. The cytotoxicity of a membrane protein in recombinant expression often prevents not only its large-scale preparation but also biochemical analyses of its functions. On the other hand, the success rate of cell-free membrane protein synthesis is rather high, since cytotoxicity is not a problem. In addition, in the cell-free synthesis system, it is easy to regulate the subunit composition for multi-subunit complexes of membrane proteins, simply by adjusting the concentrations of the DNA templates encoding the subunits to reproduce the proper subunit stoichiometry. The preparation of membrane protein samples with the full selenomethionine

substitution of methionine residues, in order to determine the phase in crystallography, is a straightforward procedure using cell-free synthesis. PCR-amplified DNA fragments may be used directly as the templates in the cell-free protein synthesis system, without cloning into plasmid vector, which is useful for high-throughput screening of conditions and/or robotic automation of synthesis.

This chapter describes the practical aspects of the cell-free membrane protein synthesis methods and their application to the structure determinations of integral membrane proteins.

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## 2 Cell-Free Synthesis Methods for Membrane Proteins

### 2.1 Cell-Free Protein Synthesis Reactions

The cell-free protein synthesis reaction can be performed in the batch, dialysis, or bilayer mode (see Fig. 1 in Chap. 5). In the batch mode, the synthesis is performed by incubating the reaction solution in a container until the reaction rate slows down, due to the exhaustion of substrates and/or energy sources. To prolong the reaction for higher yield, the dialysis (or continuous-exchange cell-free, CECF) mode [6, 7] and the bilayer mode [8] have been developed to supplement the reaction solution with components, such as substrate amino acids and energy sources, from the feeding solution. In the dialysis mode, the reaction and feeding solutions are separated by a dialysis membrane. In the case of the large-scale synthesis of a certain sample for structural biology, a dialysis bag containing the inner, reaction solution (e.g., 3–9 mL) is placed in the outer, feeding solution (tenfold larger volume than that of the reaction solution). On the other hand, the screening of a large number of constructs, with respect to checking the yield and/or the biochemical properties, can be performed by small-scale synthesis (ca. 30  $\mu$ L) in a multi-well format. In the bilayer mode, the low-density, feeding solution is laid directly on the high-density, reaction solution, and the two solutions are gradually mixed with each other during the course of the reaction. Our group primarily uses the dialysis mode for the cell-free synthesis of membrane proteins.

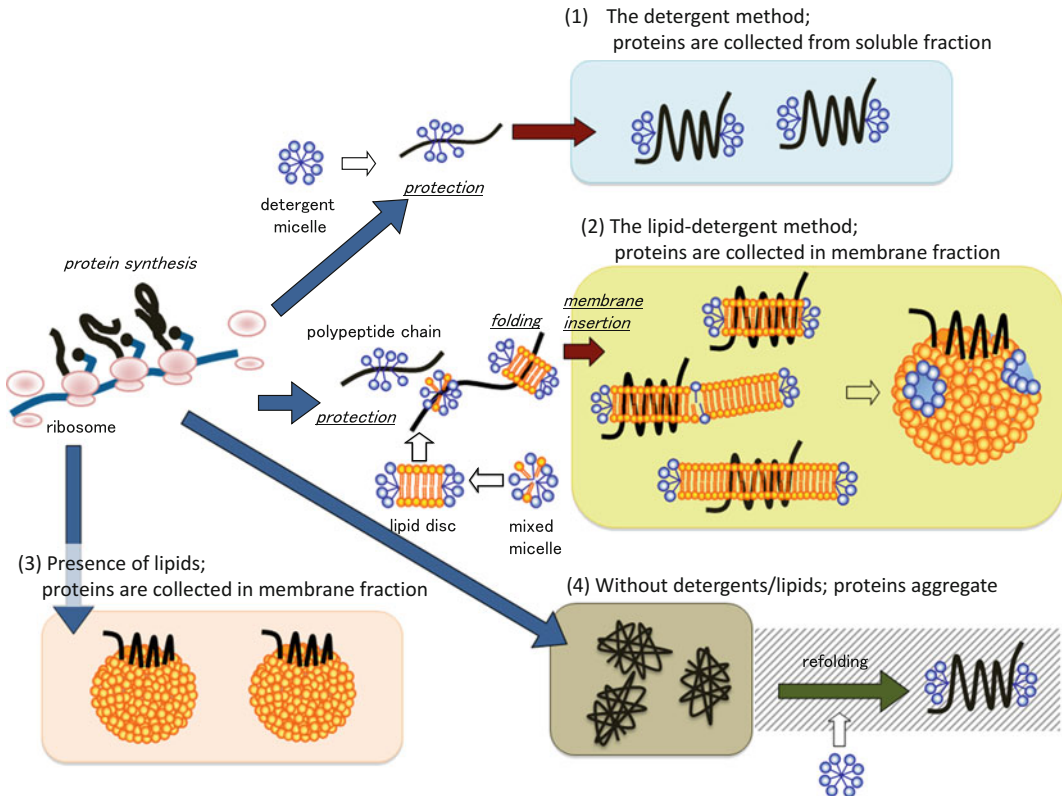
In the cell-free protein synthesis system, the extract prepared from *E. coli* cells is practically superior, with respect to both quantity and quality, to other commercially available extracts, such as the animal [9, 10], plant [11], and reconstituted systems, for the preparation of membrane proteins from not only bacteria but also eukaryotes, e.g., humans. Usually, the S30 fraction of the *E. coli* extract (the supernatant fraction obtained after cell disruption and centrifugation at  $30,000 \times g$ ) is used in the cell-free protein synthesis system. Translation is coupled with transcription by T7 phage RNA polymerase from the T7 promoter in the template DNA, encoding the target membrane protein without the signal peptide sequence, in the form of a plasmid. Detergents and/or lipids are

added to the reaction solution and/or the feeding solution for membrane protein synthesis. In the following Sects. (2.2, 2.3, 2.4, and 2.5), more details of the methods are described, according to the different uses of detergents and lipids.

**2.2 Membrane Protein Synthesis in the Presence of Detergents**

In this method, membrane protein synthesis is performed in the presence of a detergent at a concentration higher than the critical micelle concentration (CMC). In the dialysis mode, the detergent is added to both the reaction and feeding solutions. As the hydrophobic domains of the membrane protein are synthesized, they interact immediately with the hydrophobic portion of the detergent molecules. Thus, the detergent molecules surround the protein, exposing the hydrophilic portions of the molecules and generating “solubilized” membrane proteins (Fig. 1(1)). After ultracentrifugation, the protein is recovered from the supernatant. We simply refer to this synthesis method as “the detergent method.”

Since the type and the concentration of the detergent greatly affect the yield and folding of proteins, a systematic screening of a panel of detergents should be performed to determine the



**Fig. 1** Illustration of the detergent method (1), the lipid-detergent method (2), and the conditions in the presence of lipids (3) and without detergents/lipids (4) for cell-free membrane protein synthesis

appropriate experimental conditions. Any detergents may be tested, provided they do not interfere with protein synthesis [3]. Each detergent, at a concentration above its CMC, is mixed with ~30  $\mu$ L of a small-scale reaction solution, to assess the yield and precipitation. After suitable detergent types are identified, the optimum concentrations are investigated. The detergent used in cell-free membrane protein synthesis can be replaced by another detergent in the subsequent purification process. However, it is not always possible to completely exchange the detergents, and consequently, the residual detergent from the synthesis reaction may affect the stability of the protein during crystallography. Therefore, even in the cell-free synthesis process, it is better to select detergents that can be used in the subsequent purification and crystallization processes. Thus, we usually test digitonin, Brij-78, Brij-35, and *n*-dodecyl- $\beta$ -*D*-maltopyranoside (DDM).

After optimization of the synthesis conditions, the scale of the cell-free synthesis may be increased to 9/90 mL (reaction solution/feeding solution). The yield is usually sufficient to obtain milligram quantities of the membrane protein, if the amino acid sequence lacks problematic regions that affect the protein synthesis machinery. The specific activity of the synthesized protein should be examined, if a standard protein is available. In addition, the correctly folded proteins may be purified by ligand or substrate affinity chromatography.

### **2.3 Membrane Protein Synthesis in the Presence of Detergents and Lipids**

To synthesize membrane proteins that require either a lipid bilayer environment or a particular lipid to maintain their structure, activity, and stability, we developed a cell-free synthesis system that uses lipids with detergents [4, 12]. A suspension of lipids and one or more detergents, at concentrations above the CMCs, is added to the reaction solution, but not to the external feeding solution, at the initiation of protein synthesis. First, the synthesized membrane protein molecules form mixed micelles with the detergent and lipid molecules. The detergent in the reaction solution diffuses through the dialysis membrane to the external feeding solution, thereby reducing its concentration in the reaction solution. Therefore, the number of detergent molecules present in the detergent/lipid micelles gradually decreases, and fragments of lipid bilayer membrane (or lipid bilayer discs surrounded by a ring of detergent) are formed. Concurrently, the polypeptide chains synthesized and surrounded by micelles are incorporated into the membrane fragments, which fuse together to form larger fragments and eventually liposomes (lipid bilayer vesicles) containing the membrane proteins. The membrane proteins in this form are collected by ultracentrifugation as pellets (Fig. 1(2)). We here designate this method as “the lipid-detergent method.” In principle, it is possible to separate the target membrane proteins from the soluble proteins, including those required for transcription and translation

and those derived from the S30 fraction of the *E. coli* extract. In reality, some other lipophilic proteins bound to the membranous lipid structures are also collected, as protein contaminants. Nonetheless, the degree of contamination is markedly smaller than that of the pre-ultracentrifugation sample, and therefore, the sample is a good starting material for purification and crystallization.

Detergents should be selected based on their effects on both protein synthesis and liposome formation. In many cases, detergents derived from steroids, e.g., digitonin, sodium cholate, and CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate), are appropriate for use in the lipid-detergent method [12]. However, systematic screening is still necessary to determine the optimal concentrations.

Next, the most appropriate lipid components are selected, to prevent the target protein from losing its activity or folding incorrectly. The crystal structures of membrane proteins purified from natural materials have revealed the presence of lipids at specific locations [13]. The stability of such proteins can be improved when lipids are supplied during synthesis. If it is unclear whether lipids are needed, or which type of lipid is needed, then the best strategy is to test natural lipid extracts containing different lipid species. The first choice may be an organ extract from the organism from which the target protein was isolated. If applicable, thin-layer chromatography and mass spectrometry can be used to analyze the lipid content in the target protein isolated from the natural membrane, to identify the required lipid components. Heterologous expression systems, such as *E. coli* and insect cells, may not contain all of the lipid components needed to stabilize the target membrane protein. The limited variety of the lipid compositions in such expression systems often makes expression and purification difficult; however, the cell-free synthesis method allows the addition and assessment of various lipid compositions to mimic natural environments.

The state of the lipids added to the reaction solution is also important. To form a finely dispersed lipid suspension, the lipids must be ultrasonically dispersed and mixed with the optimal detergent. If the lipid suspension is poorly prepared, then the synthesized membrane protein will not be properly integrated within the membrane bilayer. The simple addition of liposomes into the cell-free reaction solution will not allow the proper and efficient integration of the synthesized membrane protein into the lipid bilayer. In the lipid-detergent method, the efficiencies of protein folding and integration into the membrane are drastically increased, due to liposome formation concurrent with protein synthesis.

#### **2.4 Membrane Protein Synthesis in the Presence of Lipids**

Inverted membrane vesicles and natural membrane vesicles, such as microsomes, have been used in place of liposomes (Fig. 1(3)). This method is different from that described in Sect. 2.3, because no detergent is used. In a previous study, inverted membrane vesicles from *E. coli* were used to synthesize a functional form of tetracycline transporter, a membrane protein derived from *E. coli*, under cell-free conditions [14]. To prepare a protein sample for crystallography, the use of lipid fractions with no protein contaminants, such as artificial liposomes, is advantageous for subsequent purification. The PURE system, a reconstituted cell-free protein synthesis system containing liposomes and factors that promote membrane insertion, such as signal recognition particle (SRP), is also available [15].

Although it is not a lipid-only system, nanodiscs (also known as nanolipoprotein particles) have been attracting attention. The synthesis of many functional G protein-coupled receptors (GPCRs) by the nanodisc method has been reported [16]. The large amount of apolipoproteins, which comprise nanodiscs, is problematic for crystallography. However, this nanodisc-based cell-free synthesis method can be used for some structural analyses of membrane proteins, e.g., nuclear magnetic resonance imaging [17].

#### **2.5 Membrane Protein Synthesis Without Detergents/Lipids**

This method synthesizes membrane proteins in aggregates without using any detergents or lipids, and the synthesized proteins are obtained from a pellet after low-speed centrifugation (Fig. 1(4)). It is unlikely that the proteins collected in the pellet fraction are correctly folded, because of nonspecific hydrophobic interactions between the hydrophobic domains of the proteins. However, the original protein activity can be reconstructed if an appropriate detergent is used to solubilize the protein and form liposomes [18], indicating that the correct protein folding depends on the experimental conditions. According to the review by Katzen et al., unlike inclusion bodies in *E. coli* [19], the protein aggregates formed by cell-free synthesis can be easily solubilized by detergent, because the protein-protein interactions are relatively weak. However, when a structural analysis is planned, the detergent method and the lipid-detergent method, which both enhance correct protein folding during synthesis, are more advantageous for synthesizing the proteins than the synthesis without detergent/lipid method, as the latter requires an extensive search for the optimal refolding conditions.

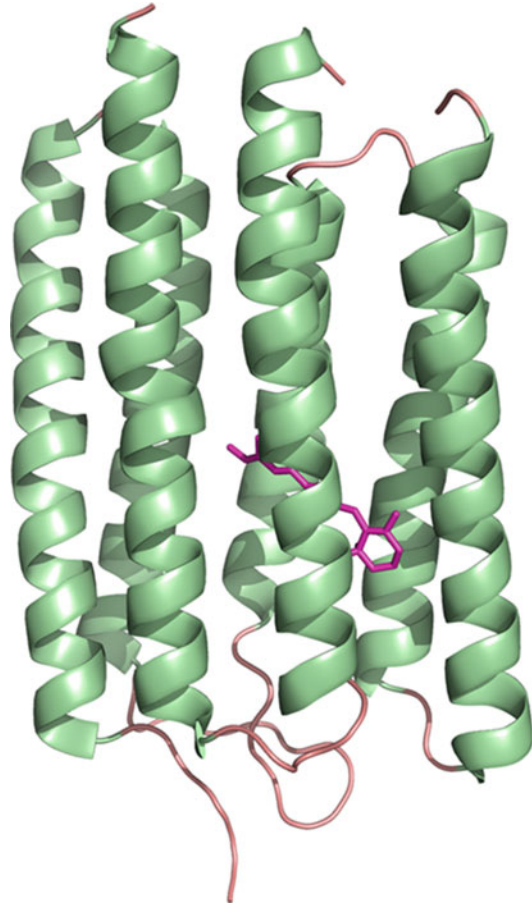
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### **3 Crystallography of Proteins Synthesized by the Cell-Free Synthesis Methods**

#### **3.1 Acetabularia Rhodopsins I and II**

*Acetabularia* rhodopsins I (ARI) and II (ARII) are microbial-type rhodopsins, membrane proteins with seven  $\alpha$ -helical transmembrane domains, derived from a eukaryotic unicellular organism, the marine alga *Acetabularia acetabulum*. It was difficult to over-express ARI and ARII in *E. coli*. However, we used the lipid-





**Fig. 2** Model of ARII from *Acetabularia acetabulum*, based on the crystal structure. ARII, synthesized by the cell-free method, was purified and its structure was solved by crystallography (PDB ID: 3AM6)

detergent method containing the essential pigment all-*trans* retinal, to achieve large-scale cell-free synthesis, biochemical and biophysical analyses, and protein crystallography [20]. The synthesis was performed in the presence of 0.05–0.8 % digitonin as the detergent source and egg yolk lecithin (L- $\alpha$ -phosphatidylcholine, 6.7 mg/mL) as the lipid source. The ARII protein was isolated from the pellet fraction after ultracentrifugation, because the majority of ARII was present in the membrane fraction, rather than the soluble fraction. In addition, with 0.4 % digitonin, the largest fraction of the synthesized protein was incorporated into the liposomes. At lower detergent concentrations, the protein production was high, but the synthesized proteins precipitated without being incorporated into the liposomes. The membrane fraction was solubilized using DDM, and ARII was purified and crystallized in the presence of DDM. Although the crystal structure (Fig. 2, PDB ID: 3AM6) was similar to the previously determined structure of

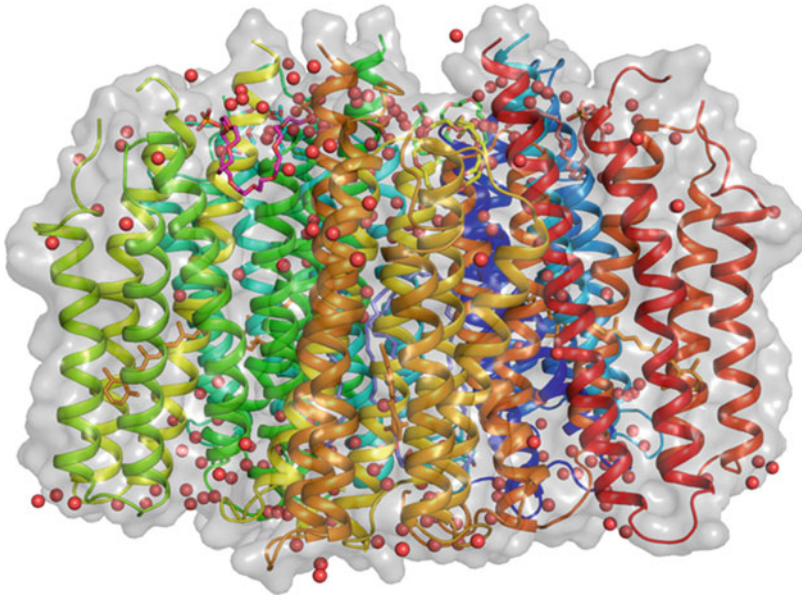
bacteriorhodopsin, we observed several structural features specific to ARII. This is the first crystal structure of a membrane protein that was synthesized by our lipid-detergent method. We tested the ARII protein in biochemical experiments and confirmed its proton transport activity [21].

We also constructed a system to overproduce correctly folded ARI by the cell-free protein synthesis method, using very similar conditions to those for ARII synthesis. We were able to obtain a large amount of the highly purified protein by the above-described simple purification methods. We performed the biophysical analysis of the light-driven proton pump mechanism during the photochemical reaction of ARI, using the cell-free synthesis product, and also crystallized ARI by the lipidic mesophase method. As the result of the X-ray crystallography analysis, the structure has been determined at 1.52–1.80 Å resolution [22] (PDB IDs: 5AWZ, 5AX0, 5AX1), which was the third highest-resolution structure, among the numerous structures of microbial rhodopsins in the dark state. The existence of abundant water molecules was confirmed in the large cavity on the proton-releasing side, which explained the relatively low  $pK_a$  of the proton-releasing residue. These results indicated that for membrane proteins, the cell-free protein synthesis methods, and particularly the lipid-detergent method, provide large amounts of high-quality samples. Thus, we were able to obtain the high-resolution crystal structure. This is a good example of the utility of the cell-free synthesis methods for structural-functional studies of membrane proteins.

### 3.2 *Proteorhodopsin*

Proteorhodopsin (PR), which was first discovered in the metagenomic uncultivated SAR86 group prokaryotes (*γ-proteobacteria*) in a DNA library from Monterey Bay, California, contains at least seven transmembrane  $\alpha$ -helices and a retinal molecule that is covalently bound via a Schiff base to the side chain of a lysine residue [23, 24]. Currently, over 4,000 PR gene sequence variants have been deposited in the GenBank database [25–29]. The sequence identity between PR and bacteriorhodopsin is approximately 30 % [23]. Proteorhodopsin is a light-harvesting proton pump and thus could play an important role in solar energy transduction in the biosphere [24, 30–35]. However, biochemical and electrophysiological investigations have progressed slowly. Moreover, the structural analysis of PR was not performed until recently.

We have embarked on research toward the structural analysis of the marine  $\gamma$ -proteobacterium PR protein, from an ocean isolate, by the *E. coli* cell-free synthesis method. The lipid-detergent method was used for the cell-free synthesis of the PR protein. Almost all of the PR protein was embedded within liposomes. PR was purified by affinity chromatography, protease digestion of the affinity tag and gel filtration. We thus obtained about 10 mg of purified PR from a 9/90 mL cell-free synthesis reaction. Crystals



**Fig. 3** Pentameric structure of proteorhodopsin determined at 2.0 Å resolution

suitable for X-ray diffraction were obtained from the purified PR samples, and the crystal structure was solved at 2.0 Å resolution (Fig. 3, Hosaka et al. manuscript in preparation).

### **3.3 Microbial Multidrug Efflux Protein EmrE, Purified from the Insoluble Fraction**

Chen et al., of the Scripps Research Institute in the United States, determined the crystal structure of EmrE, a four-transmembrane multidrug transporter from *E. coli*, using a cell-free expression system that enabled the facile labeling of proteins with selenomethionine. The protein was synthesized in the absence of detergents, but was solubilized using *n*-nonyl- $\beta$ -*D*-glucopyranoside (NG), followed by purification, crystallization, and crystallography [36]. They also used an *E. coli* cell-based expression system to synthesize non-labeled EmrE, which was purified and crystallized in a similar manner. Their study revealed that the crystal structures of the cell-free and cell-based expressed EmrE are nearly identical. Furthermore, the substrate-binding activity and affinity are similar between the two proteins, suggesting that the EmrE solubilized in NG folded correctly.

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## **4 Conclusion**

We have reviewed the methods currently used for the cell-free synthesis of integral membrane proteins for structural biology. We also described some of the successful crystallographic studies performed with proteins generated by the cell-free system derived from *E. coli* cells. Several *E. coli* cell-free protein synthesis kits are

now commercially available, including the Remarkable Yield Translation System Kit (ProteinExpress, Chiba, Japan). The cell-free protein synthesis methods provide a variety of advantages in terms of both quantity and quality for structural biology, as compared to the conventional recombinant expression in host cells, including *E. coli*, insect, and mammalian cells. Therefore, we believe that the cell-free protein synthesis methods will be more extensively used for the preparation and crystallography of integral membrane proteins, including human GPCRs and channels.

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