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Combining *In vitro* Folding with Cell-Free Protein Synthesis for Membrane Protein Expression

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Abstract

Cell free protein synthesis (CFPS) has emerged as a promising methodology for protein expression. While polypeptide production is very reliable and efficient using CFPS, the correct co-translational folding of membrane proteins during CFPS is still a challenge. In this contribution, we describe a two-step protocol in which the integral membrane protein is initially expressed by CFPS as a precipitate followed by an *in vitro* folding procedure using lipid vesicles for converting the protein precipitate to the correctly folded protein. We demonstrate the feasibility of using this approach for the K⁺ channels KcsA and MVP and the amino acid transporter LeuT. We determine the crystal structure of the KcsA channel obtained by CFPS and *in vitro* folding to show the structural similarity to the cellular expressed KcsA channel and to establish the feasibility of using this two-step approach for membrane protein production for structural studies. Our studies show that the correct folding of these membrane proteins with complex topologies can take place *in vitro* without involvement of the cellular machinery for membrane protein biogenesis. This indicates that the folding instructions for these complex membrane proteins are contained entirely within the protein sequence.

Graphical Abstract

Integral membrane proteins account for roughly 30% of the open reading frames present in the genomes sequenced and play important roles in virtually all aspects of biology.¹ Their

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importance is underscored by the fact that they represent greater than 60% of the current drug targets.² Understanding the structure, function and dynamics of membrane proteins is an important area of study. The need for a hydrophobic environment coupled with the heterogeneity of natural membranes poses a significant challenge in the characterization of membrane proteins compared to soluble proteins. A persistent problem encountered in biochemical studies of membrane proteins is their overexpression and purification in good yields. Different strategies have been explored to overcome this problem including the use of different hosts such as *Escherichia coli*, yeast, insect and mammalian cells.³

An alternative to these *in vivo* approaches is to carry out protein expression *in vitro* using cell lysates or purified components.^{4, 5} An attractive feature of cell free protein synthesis (CFPS) is that the open nature of the system allows adjustment of many parameters of transcription and translation.⁶ The direct control of the available amino acid pool, the possibility to inhibit amino acid modifying (or scrambling) enzymes and precise control of the chemical environment makes CFPS an efficient approach for the incorporation of isotopically labelled or unnatural amino acids.⁷⁻⁹ Consequently during CFPS, unnatural amino acids do not need to be transported across the cellular membrane, are not modified by cellular enzymes and cannot be cytotoxic as long as they do not inhibit protein synthesis.¹⁰ Another advantage of CFPS is that protease inhibitors can be added during protein expression thereby allowing the production of peptides or unstable proteins including protein segments without degradation.¹¹ This enables the production of precursors for protein semisynthesis using expressed protein ligation.¹² Advantages of CFPS are visualized in the Scheme 1.

CFPS is particularly advantageous for membrane protein expression as some of the bottlenecks encountered in cellular expression such as transport of the newly expressed proteins to a specific membrane compartment are circumvented in the cell-free format. In a cell, the folding of membrane proteins takes place in the cellular membranes. Therefore, a challenge during CFPS is providing an appropriate environment for the folding of newly translated polypeptides. Attempts have been made to mimic the cellular membranes during CFPS by using detergent micelles, bicelles, liposomes or nanodiscs.^{13, 14} Although lipid bilayers (liposomes, nanodiscs) can be added during CFPS to mimic the cellular membrane, these lipid bilayers do not contain a protein insertion machinery or membrane associated chaperones, relying instead on spontaneous insertion of the newly synthesized polypeptide into the lipid bilayer.¹⁵

An alternative to the co-translational folding during CFPS is to use a two-step approach with separate synthesis and folding stages. In this approach, the CFPS of the membrane protein is carried out in the absence of a hydrophobic environment to form a protein precipitate, which in the second step is folded *in vitro* to the native state. The protein production as a precipitate usually shows very high yields. By using different N-terminal tags, we have been able to express virtually any membrane protein, including mammalian proteins, as a precipitate in large quantities.¹⁶ The bottleneck for obtaining high quality samples of membrane proteins using CFPS is therefore at the folding stage.

In contrast to the large body of knowledge on the folding of soluble proteins, the *in vitro* folding of membrane proteins has only been investigated for simple ones, while the *in vitro* folding of multimeric or multi-domain membrane proteins remain basically unexplored.¹⁷ Here, we investigate the feasibility of coupling *in vitro* folding of membrane proteins with the CFPS system as a widely applicable strategy for membrane protein expression. We develop a general protocol for *in vitro* folding that involves detergent solubilization of the unfolded protein followed by conversion to the native state using lipid vesicles. We demonstrate the production of functional membrane proteins by combining CFPS with *in vitro* folding using the K⁺ channel KcsA, the voltage-gated K⁺ channel MVP and the amino acid transporter LeuT as examples.

Experimental Procedures

Plasmids used for protein expression

The KcsA channel gene was cloned into the pET28b(+) vector [EMD Millipore] for CFPS and *E. coli* expression.¹⁸ Additionally, CFPS of the KcsA channel was also carried out with the gene cloned into the pET15b vector [EMD Millipore]. A synthetic gene for the MVP channel that was codon optimized for *E. coli* expression was kindly provided by Dr. Steve Goldstein (Brandeis University).¹⁹ The MVP channel gene was cloned into the pET28b(+) vector for CFPS and for *E. coli* expression. The LeuT transporter gene cloned in the pET16b vector was kindly provided by Dr. Eric Gouaux (Vollum Institute, OHSU).²⁰ The LeuT gene was transferred to the pET28b(+) vector for CFPS.

Cell free protein synthesis

CFPS was conducted in the continuous exchange cell-free configuration (CECF) using *E. coli* based S30 lysate. The *E. coli* A19 lysate and the T7 RNA polymerase were prepared according to standard protocols.^{21, 22} CFPS was done following published protocols. A detailed description including all concentrations used is given in Henrich et al.²¹

The CFPS was performed with custom built plexiglass containers²³, in combination with Slide-A-Lyzer dialysis cassettes (10 kDa MWCO, Thermo Scientific) to separate reaction mix (RM) and feeding mix (FM). For each construct, a RM volume of 3 ml and a RM: FM ratio of 1: 16 was used. The CF reactions were incubated for 16 – 20 hours with gentle shaking (200 rpm) at 30 °C. The pellets were harvested by centrifugation at 16,000 × g for 10 min. and washed twice with TBS buffer (20 mM Tris-HCl pH 7.5 and 150 mM NaCl). The washed pellet were stored on ice for direct use or flash frozen in liquid nitrogen for long-term storage.

The KcsA Channel

In vitro folding—The KcsA protein pellet obtained following CFPS was solubilized in 0.1 M Sodium phosphate pH 7.5, 0.1 M DTT and 1% SDS. *In vitro* folding of the SDS solubilized polypeptide was carried out by a 10-fold dilution into 20 mg/ml Asolecithin lipid vesicles in 50 mM 2-(4-Morpholino)-ethane sulfonic acid (MES)-NaOH pH 6.5, 200 mM NaCl and 10 mM DTT.²⁴ The *in vitro* folding reaction was incubated at 45 °C and the extent of folding was determined by SDS-PAGE. Following the folding reaction, the lipid vesicles

were dialyzed against 20 mM Tris-HCl pH 7.5 and 150 mM KCl to remove the DTT. The lipid vesicles were diluted to a lipid concentration of 10 mg/ml and solubilized using decyl- β -D-maltoside (DM, 2% w/v). The solubilized KcsA channels were purified by Co^{2+} -affinity chromatography and size exclusion chromatography (SEC) as previously described.²⁴ SEC was carried out on a Superdex S200 column using 50 mM Tris-HCl pH 7.5, 150 mM KCl and 0.25% (w/v) DM as the column buffer.

E. coli expression—The KcsA channel was expressed from a pET28b(+) vector transformed into *E. coli* BL21 (DE3) cells. Cultures were grown in LB media with 50 $\mu\text{g}/\text{mL}$ kanamycin at 37°C to an OD600 of 1.0. Protein expression was induced by the addition of 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and grown for an additional 3 hours at 37 °C. Cells were harvested by centrifugation at $4000 \times g$ at 4 °C and stored at –80°C. For preparation of membranes, the cell pellet was resuspended in buffer containing 50 mM Tris-HCl pH 7.5, 150 mM KCl, 10 $\mu\text{g}/\text{mL}$ DNase, 50 $\mu\text{g}/\text{mL}$ lysozyme, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and lysed by sonication. Cellular debris was removed by centrifugation at $7500 \times g$ and the membranes were pelleted by centrifugation at $150000 \times g$. The membranes were resuspended in 50 mM Tris-HCl pH 7.5 and 150 mM KCl. The membranes were solubilized by the addition of DM to 2% (w/v) and the KcsA channel was purified as described for the cell free expressed protein.

Planar lipid bilayer measurements—For functional measurements, the KcsA channel was reconstituted into lipid vesicles (10 mg/ml) composed of a 3: 1 ratio of 1-palmitoyl-2-oleoyl-glycero-3-phosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoylglycero-3-phosphatidylglycerol (POPG) at a protein to lipid ratio of 1:400.²⁴ For measurements of channel activity, the lipid vesicles were fused with planar lipid bilayers composed of POPE (15 mg/ml) and POPG (5 mg/ml) painted over a 300 μ hole in a polystyrene bilayer cuvette (Warner Instruments, Hamden, CT) with 10 mM succinate pH 4.0, 150 mM KCl as the internal solution and 10 mM HEPES (pH 7.0) and 150 mM KCl as the external solution.²⁴ The membrane voltage was controlled and the current recorded by an Axopatch 200B amplifier with a Digidata 1322A analogue-to-digital converter and electrophysiological data was analyzed using pCLAMP software (Axon Instruments, Union City, CA).

Crystallization and structure determination—For structure determination of the *in vitro* folded KcsA channel, the N-terminal His tag was removed using by proteolysis using trypsin (1: 50 ratio) and the C-terminal 35 amino acids was removed by proteolysis with chymotrypsin (1: 15 ratio). The truncated KcsA was purified by SEC and then complexed with a Fab fragment from anti-KcsA antibody for crystallization. Crystallization and structure determination was carried out as previously described.²⁵

The MVP Channel

In vitro folding—For *in vitro* folding, the MVP protein pellet obtained by CFPS was solubilized in 0.1 M sodium phosphate pH 7.5, 0.1 M DTT and 1% (w/v) SDS and then diluted 10- fold into Asolecithin vesicles (8 mg/ml in 50 mM HEPES-KOH, pH 7.5, 150 mM KCl and 30 mM DTT). The folding reaction was sonicated in a water bath sonicator (2 $\times$ 30 seconds) to facilitate the incorporation of the MVP polypeptide into the lipid vesicles. The *in*

vitro folding reaction was carried out at 45 °C and analyzed by SDS-PAGE. The folding of the MVP channel was indicated by the appearance of a tetrameric species on an SDS-PAGE gel. Following *in vitro* folding, the lipid vesicles were dialyzed against 20 mM Tris-HCl, pH 7.5, 150 mM KCl for the removal of DTT. The vesicles were solubilized using 2% (w/v) dodecyl α -D-maltoside (DDM) and the MVP channel was purified by Co²⁺-affinity chromatography. The folded tetrameric MVP channels were then purified from any unfolded monomeric proteins by SEC on a Superdex S200 column with 50 mM HEPES-KOH pH 7.5, 150 mM KCl and 0.25% (w/v) DM used as the column buffer.

E. coli expression—The MVP channel gene cloned into pET28b(+) was transformed into BL21(DE3) pLysS cells. Cells were grown in Terrific Broth medium containing 1% glucose to an OD₆₀₀ of 0.8–0.9 and protein expression was induced by 0.5 mM IPTG for 18 hours at 22 °C. Membrane vesicles were prepared as described for the KcsA channel and MVP channel purification was carried out as described for the cell free expressed protein.

Planar lipid bilayer measurements—For functional studies, the MVP channel was reconstituted into lipid vesicles made of POPE: POPG (3:1) 10 mg/ml. The lipids were solubilized using 10 mM DM followed by the addition of protein. Lipid vesicles were formed by the removal of the detergent by dialysis against 10 mM HEPES-KOH pH 7.5 and 450 mM KCl. The proteoliposomes obtained were aliquoted and snap frozen in liquid nitrogen and stored at –80 °C and used within 1–2 months of reconstitution.

Electrophysiological measurements were carried out using planar lipid bilayers composed of POPE: POPG (3:1, 20 mg/ml) with the same setup used for recording the KcsA channel. The recordings were carried out with 10 mM HEPES-KOH (pH 7.5) and 150 mM KCl both inside and outside the bilayer cuvette. Open probability (NP_o) was determined for bilayers containing 1–3 MVP channels using pCLAMP software. For the purpose of comparison between different membranes, the values reported at the various voltages are normalized to the NP_o measured at –200 mV for the same membrane. The midpoint for activation of the MVP channel is reported to be –175 mV and we were therefore unable to observe maximal activation of the MVP channel.¹⁹

The LeuT transporter

In vitro folding—The LeuT protein pellet obtained by CFPS was solubilized in 20 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 1% (w/v) SDS and 0.1 M DTT and *in vitro* folding was carried out by a 10-fold dilution of the SDS solubilized polypeptide into Asolecithin lipid vesicles (20 mg/ml in 20 mM HEPES-NaOH pH 7.5, 100 mM NaCl and 10 mM DTT). The *in vitro* folding reaction was incubated for 4–5 hours at 55–60 °C and then dialyzed against 20 mM HEPES-NaOH pH 7.5, 100 mM NaCl and 0.5 mM DTT. Following dialysis, the lipid vesicles were diluted by an equal volume of dialysis buffer, solubilized using DDM (2% w/v) and the folded LeuT was purified by Ni²⁺-affinity chromatography followed by SEC (Superdex S200 column using 20 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 1 mM DTT and 1mM EDTA as the column buffer).

E. coli expression—The LeuT gene cloned into pET16b was transformed into C41(DE3) cells and protein expression was carried out using Terrific Broth medium as described.²⁰ Membrane vesicles were prepared as described for the KcsA channel and LeuT purification was carried out as described for the cell free expressed protein.

Flux assays—The purified LeuT was reconstituted into lipid vesicles composed of 3:1 E. coli polar lipids and 1-palmitoyl-2-oleoyl-glycero-3-phosphatidylcholine (POPC) as previously described.²⁶ The proteoliposomes obtained were snap frozen in liquid N₂ and stored at –80 °C. Proteoliposomes were used within 1–2 months of reconstitution. ¹⁴C-L-Alanine uptake assays were carried out as previously described.²⁷

Results

Strategy for *in vitro* folding

Multiple approaches have been previously described for the *in vitro* folding of membrane proteins.¹⁷ In all these approaches, the folding procedure starts with the unfolded protein in denaturant and folding is initiated by a lowering of the denaturant concentration. Multiple means such as dialysis, rapid dilution or precipitation have been used to lower the denaturant concentration to enable protein folding.¹⁷ A folding strategy that we have previously described consists of rapidly diluting the unfolded membrane protein in SDS with a solution of lipid vesicles.²⁴ We have applied this approach for the *in vitro* folding of the ion channels, KcsA, NaK, K_vAP and the glutamate transporter homolog, Glt_{ph}.^{24, 28–30} Here, we sought to determine whether a similar approach could be applied for converting membrane protein precipitates obtained by CFPS into their native state. Scheme 1 shows a summary of the described approach.

The KcsA channel

We initially established that the coupled CFPS/*in vitro* folding strategy is a viable approach for membrane protein expression by using the KcsA channel (Fig. 1A). We selected the KcsA channel as the *in vitro* folding of this protein has been previously described.²⁴ KcsA was produced in the precipitate form by CFPS in the absence of a membrane mimetic. For *in vitro* folding, the KcsA precipitate was solubilized using SDS and then diluted into Asolecithin vesicles (Scheme 1). This dilution step lowers the SDS concentration below the critical micellar concentration thereby allowing folding of the KcsA channel. We used SDS-PAGE to assay the folding of the KcsA channel. SDS-PAGE provides a convenient assay as the folded KcsA channel migrates as a tetramer, while the unfolded KcsA migrates as a monomer (Fig. 1B).³¹ CFPS expressed KcsA was readily converted from the monomeric to a tetrameric state on dilution into lipid vesicles (Fig. 1C). The tetrameric KcsA obtained by the *in vitro* folding (iv-KcsA) was purified from the lipid vesicles (Fig. 1D). In total, we were able to obtain ~0.4 mg of the purified iv-KcsA channel from a 3.0 ml CFPS reaction.

To confirm the correct folding of the iv-KcsA channel, we investigated the functional and the structural similarity to the KcsA channel obtained by cellular expression. iv-KcsA was reconstituted into planar lipid bilayers for measurement of channel activity. We observed that the single channel conductance of the iv KcsA channel was similar to the cellular

expressed channel (Fig. 1E, F). We also crystallized the iv-KcsA channel as a complex with a Fab fragment and determined the structure at 2.85 Å resolution (Supplementary Table 1).²⁵ Electron density corresponding to the selectivity filter of the KcsA channel is shown in Fig. 2A. An overlay of the structure of the selectivity filter of the iv-KcsA channel to the cellular expressed channel shows that the structures are similar (Fig. 2B). These results indicate that the coupled CFPS/*in vitro* folding approach is capable of providing good yields of folded and functional membrane proteins.

The MVP channel

To determine the applicability of coupling CFPS with *in vitro* folding for the expression of other ion channels, we investigated the voltage-gated K⁺ (K_v) channel MVP.¹⁹ MVP, like most K⁺ channels is tetrameric with each subunit consisting of 6 transmembrane helices that are arranged in two distinct domains.³² The first four transmembrane helices form the voltage sensor domain while the last two transmembrane helices form the pore domain. The MVP channel is therefore a multimeric multi-domain protein, which presents a substantial challenge for *in vitro* folding. The MVP channel is also of particular interest as it opens in response to hyperpolarization of the membrane potential, unlike the well-studied K_v channels, K_vAP or Shaker that open on depolarization of the membrane potential (Fig. 3A).¹⁹

For *in vitro* folding studies, we used CFPS to express the MVP channel in the unfolded form as a precipitate. Following the folding protocol that we developed, the MVP precipitate was solubilized in SDS and then diluted into lipid vesicles. Folding of the MVP channel was assayed by SDS-PAGE because the folded MVP channel migrates as a tetramer while the unfolded channel migrates as a monomer (Fig. 3B).³² On dilution into lipid vesicles, we observed rapid and almost quantitative conversion of the MVP polypeptide from the unfolded monomeric state to the folded tetrameric state (Fig. 3C). The *in vitro* folded MVP (iv-MVP) channel was purified from the lipid vesicles. The iv-MVP channel showed an elution profile on SEC that was similar to the cellular expressed channel confirming the tetrameric nature of the iv-MVP channel (Fig. 3D). The purified iv-MVP channel was reconstituted into planar lipid bilayers for measurement of channel activity. These functional studies showed that the ion permeation properties of the iv-MVP channel were similar to the cellular expressed channel (Fig. 3E and F). The open probability of the iv-MVP channel was dependent on the voltage applied across the membrane indicating that the voltage gating properties are preserved (Fig. 3G). These experiments show that the iv-MVP channel obtained using the coupled approach is biochemically and functionally similar to the channel obtained from *E. coli* expression. Using this procedure, we obtained ~0.4 mgs of the iv-MVP channel from a 3.0 ml CFPS reaction.

The LeuT transporter

To further explore the scope of our approach, we chose the bacterial amino acid transporter LeuT (Fig 4A). LeuT is a homolog of mammalian neurotransmitter-sodium symporters and has been extensively used as a model for understanding the mechanism and pharmacology of this important class of transporters.³³ LeuT is a monomer with twelve transmembrane segments.²⁰ There is an internal structural repeat within the first ten transmembrane

segments with the five N-terminal helices related to the latter five helices by a pseudo two-fold symmetry. The nature of the LeuT fold makes it an excellent test case to determine whether CFPS coupled with *in vitro* folding can be used for membrane proteins with complex topologies.

We expressed the unfolded LeuT protein by CFPS in the precipitate mode. The LeuT precipitate was solubilized by SDS and *in vitro* folding was tested by dilution into lipid vesicles. We used SEC to assay for the folding of LeuT. The SEC profile of LeuT after dilution into lipid vesicles (and subsequent purification) was similar to the cellular expressed transporter, indicating the successful *in vitro* folding of LeuT (Fig. 4B). A similar SEC elution profile was not observed in the absence of lipid vesicles when the SDS-solubilized LeuT was directly diluted into a DDM solution indicating that the step of incorporation into lipid vesicles is necessary for the *in vitro* folding of LeuT. We purified the *in vitro* folded LeuT (iv-LeuT) and reconstituted it into lipid vesicles for assaying transporter activity. Vesicles containing the iv-LeuT showed amino acid uptake at a rate that was similar to vesicles containing the cellular expressed transporter indicating that the iv-LeuT was functionally similar (Fig. 4C). Using this approach, we were able to obtain ~0.2 mgs of the iv-LeuT transporter, starting with the protein precipitate obtained from a 1.0 ml CFPS reaction.

Discussion

CFPS has emerged as a promising methodology for protein expression. While polypeptide production is very reliable and efficient using CFPS, the correct co-translational folding of membrane proteins during CFPS is still a challenge. Here, we describe a two-step protocol in which the integral membrane protein is initially expressed by CFPS as a precipitate followed by an *in vitro* folding procedure for converting the protein precipitate to the correctly folded protein. We demonstrate the feasibility of using this approach for the K⁺ channels KcsA and MVP and the amino acid transporter LeuT.

The *in vitro* folding procedure used consists of solubilization of the protein precipitate using the harsh detergent SDS, followed by incorporation into lipid vesicles. We observe that the presence of an intact lipid bilayer is important for the folding process because the dilution of the denaturant in the absence of lipid vesicles does not support folding. It is surprising that a lipid bilayer is required for the folding as the proteins investigated in this study are very stable in the absence of a lipid bilayer (in detergent micelles). The KcsA channel and the LeuT transporter have been crystallized and structure determined in detergent micelles.^{20, 25} We speculate that the requirement of the lipid bilayer for the *in vitro* folding of the KcsA and the MVP channels is related to the tetramerization required for forming the native state. Subunit association required for forming the native state is facilitated following incorporation into lipid bilayers as the diffusion of the subunits is limited to the plane of the lipid bilayer. We also observe a lipid bilayer requirement for the *in vitro* folding of the monomeric LeuT. In this case, the lipid bilayer requirement might be related to the complex topology involving the internal repeat in LeuT. Further studies will be necessary to elucidate the mechanism by which the lipid bilayer facilitates the *in vitro* folding reaction.

The unfolded protein used for membrane protein folding studies is routinely obtained by unfolding the native protein. In these cases, the unfolded protein might still contain residual native-like structure which could act as a nucleation point for the *in vitro* folding reaction.³⁴ The cell free expressed protein precipitates were however never in the native state prior to incorporation into the lipid vesicles. Interestingly, solid state NMR experiments have shown that membrane proteins in the precipitate obtained from CFPS show α -helical content, in contrast to proteins obtained from *E. coli* inclusion bodies that show a β -sheet content.^{35–37} We have successfully also carried out *in vitro* folding of the KcsA and the MVP channels after expression in *E. coli* as inclusion bodies indicating that the *in vitro* folding procedure is not dependent on the source of the unfolded membrane protein.

Our experiments show that the *in vitro* folding of these membrane proteins only requires the presence of a lipid bilayer. This result suggests that the cellular factors such as chaperones and the membrane protein insertion machinery are dispensable (*in vitro*) for the correct folding of our investigated proteins. We can therefore conclude that these membrane proteins with complex folds such as the MVP channel or the LeuT transporter follow the Anfinsen's principle, which postulates that the directions for folding are encoded entirely within the primary sequence.³⁸

In conclusion, we have developed a strategy of combining CFPS with *in vitro* folding for the expression of membrane proteins. Applications of this combined procedure include protein production for structural studies. NMR investigations in particular will benefit from the possibilities of labelling the expressed membrane proteins with stable isotopes in an amino acid specific manner largely without metabolic scrambling.^{39–41} In addition, our procedure allows us to produce protein segments that can be used for semisynthesis using expressed protein ligation.¹² In this way specific parts of the protein can be modified or labeled and then be incorporated into the full length protein.⁴² Furthermore, the buffer conditions during *in vitro* folding can be chosen at will which allows us to use, for example, redox shuffling systems to provide an environment for forming disulfide bridges. The *in vitro* folding procedure also allows us to choose the desired lipid composition without any contamination or carry over from host membranes, therefore allowing us to use deuterated or labeled lipids for investigating lipid-protein interactions. The strategy of membrane protein production by combining CFPS with *in vitro* folding is therefore likely to be of great utility in membrane protein investigations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CFPS	Cell free protein synthesis
CECF	continuous exchange cell-free configuration
RM	reaction mix
FM	feeding mix
MES	2-(4-Morpholino)-ethane sulfonic acid
DM	decyl- β -D- maltoside
SEC	size exclusion chromatography
PMSF	phenylmethylsulfonyl fluoride
POPE	1-palmitoyl-2-oleoyl-glycero-3-phosphatidylethanolamine
POPG	1-palmitoyl-2-oleoylglycero-3-phosphatidylglycerol
DDM	dodecyl- β -D-maltoside
POPC	1-palmitoyl-2-oleoyl-glycero-3-phosphatidylcholine.

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Figure 1. *In vitro* folding of the KcsA protein obtained by CFPS

A) The tetrameric KcsA channel is depicted in ribbon representation. **B)** SDS-PAGE gel showing the native KcsA channel (lane 1) and the KcsA polypeptide obtained by CFPS (Lane 2). **C)** SDS-PAGE gel showing the KcsA protein before (lane 1) and after incorporation into lipid vesicles (lane 2). In B and C, the monomer (M) and the tetramer (T) bands are indicated. **D)** Size exclusion chromatography of the *in vitro* folded KcsA (iv-KcsA) channel. Inset: SDS-PAGE gel with lane 1) cellular expressed KcsA and 2) iv-KcsA. **E)** A representative single-channel trace for the iv-KcsA channel recorded at +150 mV in 10 mM succinate/150 mM KCl (pH 4.0) inside and 10 mM HEPES/150 mM KCl (pH 7.0) outside. **F)** Single channel currents as a function of voltage for iv-KcsA (○) and the cellular expressed KcsA (□) channels in the above solutions. The error bars represent the SD for 3 or more measurements.

Figure 2. Structure of the selectivity filter of the *in vitro* folded KcsA channel

A) Structure of the selectivity filter of iv-KcsA channel. The $2 F_o - F_c$ electron density of the selectivity filter of iv-KcsA channel contoured at 3.0σ is shown with residues 71–80 as sticks, and the K⁺ ions in the selectivity filter shown as purple spheres. **B)** Superimposition of residues 71–80 of the iv- KcsA (red) and the cellular expressed KcsA channel (blue).

Figure 3. *In vitro* folding of the MVP protein obtained by CFPS

A) Schematic diagram showing the gating of the MVP channel. The MVP channel opens on membrane hyperpolarization and closes on depolarization. **B)** The native MVP channel migrates as a tetramer on SDS-PAGE (lane 1) while the unfolded MVP channel migrates as a monomer (lane 2). The MVP polypeptide obtained by CFPS was folded using lipid vesicles. **C)** SDS-PAGE gel showing the MVP protein before (lane 1) and after incorporation into lipid vesicles (lane 2). The monomer (M) and the tetramer (T) bands are indicated. **D)** Size exclusion chromatography of the *in vitro* folded MVP channel (iv-MVP).

Inset: SDS-PAGE gel showing the purified iv-MVP channel. **E)** Representative single-channel traces for the iv-MVP channel recorded at -200 mV in a POPE: POPG (3:1) bilayer with 10 mM HEPES/150 mM KCl (pH 7.5) on both sides of the membrane. **F)** Single channel currents as a function of voltage for iv-MVP ($\circ$) and the cellular expressed MVP ($\square$) channels in the above solutions. **G)** The voltage dependence of the open probability of the iv-MVP channels. The open probability is normalized to the value observed at -200 mV. The data are plotted as mean $\pm$ SD for 3 or more measurements.

Figure 4. CFPS of the LeuT transporter

A) Structure of the LeuT transporter shown in ribbon representation. **B)** SEC of the cellular expressed (blue), the unfolded (black) and the *in vitro* folded (red) LeuT transporter (iv-LeuT). The elution profile is observed for iv-LeuT is similar to the cellular expressed LeuT and is very distinct from the elution profile observed for the unfolded LeuT. **C)** Alanine uptake assay by the cellular expressed and iv-LeuT transporter. Alanine uptake assays were used to examine the functionality of LeuT. Time course of [¹⁴C]-Ala uptake by iv-LeuT (red circles) and the cellular expressed LeuT (blue squares) in the presence of a Na⁺ gradient is shown. [¹⁴C]-Ala uptake is not observed in the absence of a Na⁺ gradient for the *in vitro* folded (red open circles) or the cellular expressed transporter (blue open squares). Error bars indicate the SD for 3 measurements.

Scheme 1. Strategy for membrane protein expression by combining cell-free synthesis with *in vitro* folding

The various advantages of cell-free synthesis of membrane proteins is described.