Functional expression of a two-transmembrane HtrII protein using cell-free synthesis

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An approach of cell-free synthesis is presented for the functional expression of transmembrane proteins without the need of refolding. The transmembrane region of the \textit{pharaonis} halobacterial transducer protein, \textit{p}HtrII, was translated with various large soluble tags added (thioredoxin, glutathione S-transferase, green fluorescent protein and maltose binding protein). In this system, all fusion \textit{p}HtrII were translated in a soluble fraction, presumably, forming giant micelle-like structures. The detergent n-dodecyl-\beta-D-maltoside was added for enhancing the solubilization of the hydrophobic region of \textit{p}HtrII. The activity of the expressed \textit{p}HtrII, having various tags, was checked using a pull-down assay, using the fact that \textit{p}HtrII forms a signaling complex with \textit{pharaonis} phorborhodopsin (\textit{ppR}) in the membrane, as also in the presence of a detergent. All tagged \textit{p}HtrII showed a binding activity with \textit{ppR}. Interestingly, the binding activity with \textit{ppR} was positively correlated with the molecular weight of the soluble tags. Thus, larger soluble tags lead to higher binding activities. We could show, that our approach is beneficial for the preparation of active membrane proteins, and is also potentially applicable for larger membrane proteins, such as 7-transmembrane proteins.

Key words: membrane protein, cell-free protein synthesis, protein-protein interaction, sensory rhodopsin

About 25–30\% of proteins are embedded in membranes. Thus, one of the most popular areas of research in biology is the functional analysis of these membrane proteins\textsuperscript{1}. However, these proteins are insoluble in aqueous solutions, and difficult to overexpress and purify. Three strategies are currently being used for the protein expression: chemical synthesis, \textit{in vivo} expression, and cell-free protein synthesis. However, the former two methods have certain limitations: chemical synthesis is not feasible for the synthesis of long peptides because of its low yield, and \textit{in vivo} expression can produce only those proteins that do not affect the physiology of the host cell\textsuperscript{2,3}. Furthermore, even if they are expressed, it requires many steps for the protein purification: membrane proteins expressed in cells have to be solubilized by a detergent, purified by an affinity column, and reconstituted into phospholipids. Cell-free translation
systems, in contrast, can synthesize proteins with high speed and accuracy, approaching \textit{in vivo} rates\textsuperscript{4}, and they can express proteins that would interfere with the cell physiology\textsuperscript{5,6}.

In previous reports, some membrane proteins have been successfully expressed in cell-free systems\textsuperscript{7,8}. However, because proteins tend to aggregate in cell-free systems, a refolding process of the proteins is, in general, required. Therefore, these proteins have been expressed in the presence of a detergent to solubilize the protein, or in the presence of lipids to reconstitute the protein\textsuperscript{9–11}. However, the yield of such expressed functional proteins was very low. Therefore, a new strategy is required for the expression of membrane proteins by using cell-free protein synthesis.

In the present study we developed a strategy for the membrane protein expression by using a cell-free protein synthesis system (Fig. 1a). A wheat germ cell-free system was chosen, because in it the translation reaction can proceed for longer than 60 hrs, and active proteins are yielded in milligram quantities per milliliter reaction volume. The membrane region of the \textit{pharaonis} halobacterial transducer protein, \(p\text{HtrII}\), was translated with various large soluble tags (thioredoxin, glutathione S-transferase, green fluorescent protein or maltose binding protein) (Fig. 1b). \(p\text{HtrII}\) has two transmembrane helices, and belongs to the family of two-transmembrane helical methyl-accepting chemotaxis proteins (MCPs)\textsuperscript{12–14}. It forms a signaling complex with \textit{pharaonis} phororhodopsin (\(p\text{pR}\), also called \textit{pharaonis} sensory rhodopsin II, \(p\text{sRII}\)) in the halobacterial membrane\textsuperscript{15}.

This complex transmits a light signal to the sensory system which is called the two-component system in the cytoplasm\textsuperscript{13}. \(p\text{HtrII}\) activates phosphorylation cascades which modulate flagellar motors\textsuperscript{14}. By using these signaling systems, \textit{Natronomonas pharaonis} cells avoid harmful near-UV light, a behaviour called negative phototaxis. It is known that both, \(p\text{HtrII}\) and \(p\text{pR}\), are stable in membranes as well as in detergent micelles\textsuperscript{16–18}, and both have been well characterized over the past few years using various methods\textsuperscript{15,19}. Therefore, we chose to use \(p\text{HtrII}\) as a model for membrane proteins.

**MATERIALS AND METHODS**

**Protein synthesis**

Commercially available tag genes, Trx, GST, GFP and MBP, were used here. They are cloned into a plasmid vector and all constructed plasmids were analyzed using an automated sequencer to confirm the expected nucleotide sequences. In the presence of 16 mM Mg\textsuperscript{2+}, mRNAs of various soluble tagged \(p\text{HtrII}s\) were synthesized with a SP6 RNA polymerase, using the plasmids as templates. These synthesized mRNAs were precipitated with ethanol, dissolved in a dialysis buffer, and then mixed with the wheat germ extract for protein synthesis. The purification of the wheat embryos, and the preparation of the cell-free extract were performed as described previously\textsuperscript{20}. Fusion \(p\text{HtrII}\), displayed in Figure 1b, were expressed as a soluble fraction (Fig. 2), to which 0.1% DDM was added for 1 hour for solu-
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Bilization at room temperature under gentle stirring.

Analysis of the activity of the soluble tagged pHtrII

An in vitro pull-down assay was performed using a Ni-NTA column, essentially, as previously described\textsuperscript{21–23}. ppR and pHtrII\textsuperscript{1-114}His were expressed in \textit{Escherichia coli}\textsuperscript{21,24}. Here, His denotes a tag with six histidine residues attached at the C-terminus of the protein. It was previously shown that truncated pHtrII, expressed from position 1 to position 159 (pHtrII\textsuperscript{1-159}), is sufficient to permit the interaction with ppR\textsuperscript{21,25,26}. Furthermore, Engelhard and coworkers reported that also pHtrII, expressed from position 1 to position 114, had a full binding activity with ppR, and that pHtrII\textsuperscript{1-114} is a more hydrophobic protein than pHtrII\textsuperscript{1-159}. Therefore, we used mainly pHtrII\textsuperscript{1-114} in this study as a model for membrane proteins. The preparation of crude membranes, and the purification of proteins were performed as previously described\textsuperscript{21,24}. The sample medium was exchanged by Amicon Ultra (Millipore, Bedford, MA) filtration, and the samples were suspended in buffer S (300 mM NaCl, 50 mM MES, 5 mM Imidazole, pH 6.5) containing 0.1% DDM. Purified untagged ppR (0.8 mM) and histidine tagged pHtrII (0.08 mM), with various soluble tags, were mixed in the molar ratio of 1:10 in buffer S containing 0.1% DDM for 1 hour at room temperature under gentle stirring. The Ni-NTA resin was washed extensively with buffer W (0.1% DDM, 300 mM NaCl, 50 mM MES, 50 mM Imidazole, pH 6.5) to remove non-specifically bound proteins. The ppR/pHtrII complexes were eluted with buffer E (0.1% DDM, 300 mM NaCl, 50 mM Tris-HCl, 150 mM Imidazole, pH 7.0), and the absorbance at 498 nm was measured (V-560 spectrophotometer, Japan, Spectroscopic, Tokyo, Japan). As ppR maximally absorbs light at 498 nm\textsuperscript{27}, which is not affected by the binding of pHtrII\textsuperscript{21,28}, the concentration of ppR could be easily detected by the color and the absorbance.

Interaction between the un-tagged pHtrII and ppR

MBP-pHtrII (0.1 mM) was incubated with PreScission protease (5 μg/mL) for 5 hrs at 10°C. The reaction was stopped by the addition of a protease inhibitor (APMSF), and the digested fragments were separated by Ni-affinity chromatography. Purified histidine tagged ppR (0.01 mM) and pHtrII, derived from MBP-pHtrII (0.1 mM), or pHtrII\textsuperscript{1-114} (0.1 mM) were mixed in buffer S, the Ni-NTA resin was washed extensively with buffer W, and the ppR/pHtrII complexes were eluted with buffer E. These proteins were separated by 12% SDS-PAGE.

Activity of pHtrII solubilized by various detergents

We used 0.1% DDM, 1% n-octyl-β-D-glucoside (OG), 1% n-octyl-thio-β-D-glucoside (OTG), 20 mM cholic acid, 15 mM SDS or 12 mM CHAPS as a detergent for the solubilization of pHtrII. Translated MBP-pHtrII was solubilized by DDM, OG, OTG, cholic acid, SDS or CHAPS for 1 hour at room temperature under gentle stirring. Then, the suspending medium was completely exchanged by dialysis against buffer S containing 0.1% DDM for one week, because ppR loses its activity in detergent solutions, except for those containing DDM, OG or OTG.

Expression of large soluble tagged pHtrII in cells and its interaction with ppR

GST-pHtrII was expressed in \textit{E. coli} BL21 (DE3) star by induction initialized by the addition of 1 mM IPTG. The expressed proteins were suspended in buffer A (5 mM MgCl\textsubscript{2}, 50 mM Tris-Cl, pH 8.0) containing 1 mM dithiothreitol (DTT) and 8 M urea, and refolded by the addition of 50-fold diluted buffer A. A detergent (0.1% DDM) was added after this rapid dilution at 15°C for the solubilization of the membrane helical region of GST-pHtrII. The Y199A

![Figure 2](image-url) SDS-PAGE analysis of cell-free protein synthesis of tagged pHtrII. T, S and P represent total translational product, soluble fraction and pellet after low speed centrifugation, respectively. The bands marked by a star in the gel represent the tagged pHtrII\textsuperscript{1-114} obtained by cell-free translation.
mutant of the ppR gene was prepared by PCR using the QuickChange method. The preparation of crude membranes and the purification of proteins were performed as previously described21. The sample medium was exchanged by Amicon Ultra filtration, and the samples were suspended in buffer A containing 0.1% DDM. Purified ppR (0.8 mM) and GST-pHtrII (0.08 mM) were mixed in buffer A containing 0.1% DDM, and incubated for 1 hour at room temperature under gentle stirring. The samples were applied to a glutathione sepharose 4B resin, filled into a chromatography column, and extensively washed with buffer A containing 0.1% DDM to remove non-specifically bound proteins. The ppR/GST-pHtrII complex was eluted with buffer GE (0.1% DDM, 5 mM MgCl2, 50 mM Tris-Cl, pH 8.0, 30 mM glutathione (reduced form)). The absorbance of the elution fractions was measured at 498 nm.

RESULTS

Translational products

In our system, translation products were mainly soluble in the reaction solution (Fig. 2). Furthermore, for example, GST-pHtrII was even soluble in the absence of a detergent, and formed large complexes of a size of about 10 Mda, estimated by dynamic light scattering. The crude cell-free translation mixture without detergent was used in these experiments (i.e., soluble fraction in Fig. 2). This could be explained by the formation of hydrophobic and hydrophilic clusters by the hydrophobic (membrane) and hydrophilic (tag) regions of the proteins, respectively, leading to structures such as giant micelles or lipid bilayers (Fig. 1). However, because of the instability of ppR in the absence of the detergent, 0.1% DDM, a comparably mild detergent, was added to all these soluble tagged pHtrII, used in the experiments.

Activity of soluble tagged pHtrII

In an effort to determine if the soluble tagged pHtrII is functional, we performed an in vitro pull-down assay (see Materials and Methods, and refs 21–23). ppR was adsorbed onto a Ni-NTA resin containing immobilized histidine tagged fusion pHtrII proteins. Figure 3 shows the adsorbed fraction of ppR in the absence (lane 1) and presence of Trx-pHtrII (lane 2), GST-pHtrII (lane 3), GFP-pHtrII (lane 4), MBP-pHtrII (lane 5) and pHtrII1-159 (lane 6). pHtrII1-159 was expressed in E. coli cells, purified by the column chromatography and used as a positive control. Because ppR maximally absorbs light at 498 nm, the concentration of ppR can easily be determined by the color and the absorbance (Fig. 3). Specifically adsorbed ppR was detected in the presence of all fusion pHtrII, indicating that all tagged pHtrII have a certain binding activity to ppR. As already mentioned, in these experiments, purified untagged ppR (0.8 mM) and all histidine tagged pHtrII (0.08 mM) were mixed in the molar ratio of 1:10. Therefore, the bound fraction of ppR to pHtrII represents the activity related to the folded fraction of soluble tagged pHtrII.

It has been reported that pHtrII forms a complex with ppR with a 2:2 stoichiometry under a number of conditions, such as in detergents, in crystals and lipids21,22,26,29–31. Therefore, pHtrII, expressed in E. coli, could be used as a control having the full binding activity to ppR with folded structures. As can be seen in figure 2, three of the soluble tagged pHtrII (Trx, GST and GFP) had a binding activity of only about 50% that of pHtrII, while the forth tagged form (MBP) acts similar to the wildtype. This suggests that an unfolded fraction exists in these three tagged pHtrII (Trx, GST and GFP), although the translated proteins were mainly soluble in solution (Fig. 2). From these analyses, we could conclude that all four soluble tagged pHtrII, expressed in the cell-free system, were functional, although the fraction of functional proteins depended on the tag used. As already mentioned, the MBP-tagged pHtrII has the highest activity. This is interesting given that MBP is the largest tag among the soluble tags used showing the highest activity. Both values, binding activity and the molecular weight of the tags seem to be positively correlated (Fig. 3). As the soluble tag surrounds the hydrophobic region of pHtrII, the soluble

![image](https://example.com/image.png)
tag may support the exact folding of \( \text{pHtrII} \), and prevent the aggregation of the hydrophobic regions of \( \text{pHtrII} \), which is more effective when the size of the tag is large.

**Activity of MBP-digested pHtrII**

For the preparation of un-tagged membrane proteins, it is necessary to digest the fusion soluble tag. Then, the binding activity to \( \text{ppR} \) was checked using a pull-down assay. For this, \( \text{pHtrII} \) was adsorbed onto a Ni-NTA resin containing immobilized histidine tagged fusion \( \text{ppR} \). Figure 4 shows the adsorbed fraction of \( \text{pHtrII} \), in the presence of histidine tagged \( \text{ppR} \) without \( \text{pHtrII} \) (lane 1), in the presence of both, \( \text{ppR} \) and MBP-digested \( \text{pHtrII} \) (lane 2), and for MBP-digested \( \text{pHtrII} \) in the absence of \( \text{ppR} \) (lane 3). As expected, specifically adsorbed \( \text{pHtrII} \) was only detected in the presence of both, \( \text{ppR} \) and \( \text{pHtrII} \). The results show that also tag-digested \( \text{pHtrII} \) is functional.

**Detergent dependency on the activity of MBP tagged pHtrII**

In this report, we used DDM as a detergent for the solubilization of \( \text{pHtrII} \). However, the kind of detergent used might have an influence on the activity of the tagged protein, and therefore, we examined the detergent dependency on the activity of the MBP tagged \( \text{pHtrII} \) (Fig. 5). \( \text{pHtrII} \) solubilized in non-ionized detergents (DDM, OG and OTG) had a much higher activity than in ionic (cholic acid and SDS) and amphoteric (CHAPS) detergents. Thus, in a cell-free protein synthesis system, non-ionized detergents might be the best choice for solubilization of tags, as it is at least in the present system. These results are consistent with previous results showing that \( \text{ppR} \) and \( \text{pHtrII} \) are not stable in the presence of cholic acid, SDS and CHAPS (ref 32 and unpublished data).

**DISCUSSION**

In the past, membrane proteins without soluble tags have been successfully expressed in cell-free system, however, the protein formed aggregates, and thus, a refolding processes were required\(^7\)\(^8\). In this report, we succeeded in designing an approach of cell-free protein expression in which refolding is not needed. The translation products in our system were soluble in the reaction solution forming micelle-like or lipid bilayer-like structures. Among others, the detergent DDM was added into the solution for the solubilization of the hydrophobic regions. Using various non-ionized detergents, we could obtain functional \( \text{pHtrII} \) (Fig. 5). It is well known that the electro-neutral detergents, DDM, OG and OTG are mild agents for the solubilization of native cell membranes because the lengths of their alkyl chains are all similar, and moreover, almost identical to the hydrophobic chains of lipids\(^33\)\(^34\). In contrast, ionic and amphoteric detergents are not mild agents, and lead to a comparably low activity of the proteins. Therefore, non-ionized detergent may be the best choice for the maintenance of the protein structure. However, the detailed struc-
ture of the soluble tagged pHtrII in solution in the absence of detergents is still not known, and thus, further studies are necessary. Nonetheless, in all cases, our membrane protein synthesis system did not lead to irreversibly aggregated states of the proteins, and thus, it was not necessary to refold the proteins. Moreover, MBP-digested pHtrII could be shown to be as functional as pHtrII obtained by an in vitro expression system (Fig. 4). Therefore, the high-throughput functional expression of pHtrII was easily achievable.

In this study, we demonstrated that a larger soluble tagged pHtrII has a higher pHtrII activity (see Figs. 1 and 3). It can be expected that membrane protein which have even larger transmembrane (hydrophobic) regions, as e.g., seven-transmembrane helix proteins, as is the G-protein coupled receptor (GPCR), can be only expressed in the cell-free protein synthesis by adding even more hydrophilic tags. While, in the case of pHtrII, whose molecular weight is about 10 kDa, a 40 kDa soluble tag was sufficient for the preparation of a fully active pHtrII (Fig. 3), for seven-transmembrane proteins with much larger hydrophobic region whose molecular weight is about 20 kDa, it will be necessary, for ensuring the functional expression in the cell-free protein synthesis, to add the soluble tags whose molecular weight is about 80 kDa. Such a 80 kDa soluble tag could be, for example, possible prepared by combining a MBP tag with four Trx or two GFP tags.

It can be assumed that the soluble tags may enhance the protein expression level not only in the cell free protein synthesis but also in the cell protein synthesis. To verify this, GST-tagged pHtrII, including the transmembrane region of pHtrII, was expressed in E. coli. The obtained expression levels and yields were comparably high (100 mg/L culture), however, because of the high protein concentration in the cells, GST-tagged pHtrII forms aggregation (inclusion body), therefore a refolding process was required. In order to determine if the obtained pHtrII is functional, a GST pull-down assay was performed (see Materials and Methods, and refs 21–23). Figure 6 shows the relative Optical Density (OD) at 498 nm from the adsorbed fractions of pHtrII, for seven-transmembrane proteins with much larger hydrophobic region whose molecular weight is about 20 kDa, it will be necessary, for ensuring the functional expression in the cell-free protein synthesis, to add the soluble tags whose molecular weight is about 80 kDa. Such a 80 kDa soluble tag could be, for example, possible prepared by combining a MBP tag with four Trx or two GFP tags.

Figure 6 Binding activity of GST-pHtrII expressed in a cell to ppR. The relative binding activities of pHtrII to ppR without pHtrII (left), the mixture containing the ppR Y199A mutant and pHtrII (center) and the mixture containing ppR and pHtrII (right) are shown. The circle display the color of the sample obtained after elution with buffer GE (for details, see Materials and Methods). The color originates from ppR which absorbs at ~500 nm. About 100 mg functional protein was produced in a 1 liter culture.
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