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Biochimica et Biophysica Acta 1610 (2003) 57–62
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Review

In vitro folding of alpha-helical membrane proteins

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Received 7 August 2002; accepted 15 November 2002

Abstract

For large-scale production, as required in structural biology, membrane proteins can be expressed in an insoluble form as inclusion bodies and be refolded in vitro. This requires refolding conditions where the native form is thermodynamically stable and where nonproductive pathways leading to aggregation are avoided. Examples of successful refolding are reviewed and general guidelines to establish refolding protocols of membrane proteins are presented.

Keywords: Membrane protein; Inclusion body; Refolding; Detergent

1. Introduction

About one quarter of the human genes, amounting to about 10,000, are predicted to encode for integral membrane proteins (IMPs) [1]. Many of these reside in the plasma membrane and mediate flow of information or substances between the cytosol and extracellular space. Although often expressed at low levels, IMPs perform key functions in regulating the physiological state of the cell. This is especially true for receptors and ion channels that control second messenger concentrations in the cytosol as well as the transmembrane (TM) potential. These properties make them especially suitable targets for pharmaceutical drugs, because only low doses are required to achieve a desired effect. In fact, more than two thirds of today's drugs target these two protein classes.

Of the thousands of proteins in the plasma membrane of eukaryotic cells, only two are of known structure: rhodopsin [2] and aquaporin [3]. Both proteins are naturally expressed abundantly in the retina, respectively in erythrocytes. Rhodopsin is a special case, because it resides in intracellular membrane stacks of rod outer segments, but these stacks are probably derived from the plasma membrane [4]. All other eukaryotic membrane proteins of known structure are from intracellular membrane sources, that is, mitochondria, chloroplasts and sarcoplasmic reticulum. The scarcity of IMP structures is due to the well-known fact that the route from membrane protein sequences to atomic-resolution structures is not as straightforward as for their soluble counterparts. One major obstacle is the production of multi-milligram quantities of purified protein stable enough for crystallization trials at relatively high concentrations [5]. Many different expression systems have been considered and optimized to achieve this goal, each with its own drawbacks. Typical problems associated with heterologous expression of many membrane proteins are low yield, often due to toxicity, heterogeneous post-translational modification, low stability, and partial proteolysis [5]. Most known membrane protein structures result from proteins that naturally occur at high concentrations or have been overexpressed in a homologous system. However, the latter has been limited to bacterial proteins. Heterologous expression in eukaryotic cells has not yielded a single high-resolution membrane protein structure to date (Table 1).

In view of this frustrating experience, it is worthwhile to consider a radically different production method for IMPs: expression in inclusion bodies followed by refolding in vitro. In this procedure, targeting of the protein to membranes is avoided. Instead, the expression system is designed to result in the formation of cytoplasmic aggre-

Abbreviations: bR, bacteriorhodopsin; CL, cardiolipin; DAGK, diacyl glycerol kinase; DPC, dodecylphosphocholine; DPPG, dipalmitoyl phophatidylglycerol; GPCR, G protein-coupled receptor; GST, glutathione *S*-transferase; IMP, integral membrane protein; LHCP, light harvesting complex protein; OR5, olfactory receptor 5; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl phosphatidylglycerol; PS, phosphatidylserine; TM, transmembrane

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doi:10.1016/S0005-2736(02)00717-4

gates (inclusion bodies), yielding high levels of recombinant protein. In a second step, the protein is solubilized and folded in vitro into its native state. This obviously requires that the protein can spontaneously fold into its (native) energy-minimum.

The present review focuses on various aspects relevant to in vitro folding of membrane proteins. Initially, the pros and cons are summarized as to whether IMPs are thermodynamically stable in micelles and therefore amenable to in vitro folding at all. The subsequent section reviews examples of successful overproduction of membrane proteins in inclusion bodies. Finally, I will attempt to extract from the published literature some general features of successful refolding protocols.

Only IMPs of (presumed) alpha-helical structure will be discussed here. Refolding of beta-barrel-type proteins residing in the outer membrane of bacteria, mitochondria and chloroplasts has been achieved in several cases, eventually leading to the growth of diffraction-quality crystals. The methods used in this context are reminiscent of the well-documented refolding protocols for soluble proteins and have been reviewed elsewhere [6].

2. Stability of membrane proteins

The meaning of the word "stability" in everyday language can be considerably different from its meaning in a thermodynamic context. A protein that precipitates during purification is not necessarily thermodynamically unstable. On the other hand, a thermodynamically unstable protein may remain folded for a long time under appropriate conditions simply because denaturation has slow kinetics. The following section highlights the implications of stability/instability for in vitro refolding experiments with membrane proteins.

2.1. Membrane proteins are unstable in micelles

Among protein biochemists, "membrane proteins are often thought of as delicate weaklings, unable to withstand the rigors of life outside the safety of the bilayer" [7]. This summarizes the frustrating experience of numerous graduate students who spent years on establishing a suitable purification scheme for a membrane protein, whilst their lab mates dealing with soluble proteins could proudly print a 3D structure onto the cover of their thesis. In fact, it is not clear why membrane proteins solubilized in detergent often suffer from activity loss and aggregation. No detergent can provide an environment that perfectly mimics the lipid bilayer. However, many soluble proteins in comparison remain folded and active in solvents that strongly deviate from the physiological situation, such as extreme pH, ionic strength or replacement of half of the water by glycerol. The relative instability of membrane proteins in detergent has been attributed to (1) enhanced helix movements in the micelle as compared to the bilayer, and (2) functional requirement of IMPs for flexibility to achieve optimal activity and turnover [7].

In line with these assumptions, the complete delipidation of membrane proteins often results in a loss of activity. Although specific interaction with lipids has been shown in a number of cases [8–12], it is likely that some of these interactions are promiscuous, that is, can be replaced by other lipids as long as certain structural features are present. For instance, G protein-coupled receptors (GPCRs) expressed in *Escherichia coli* have shown native pharmacology [13–15], although *E. coli* lipids are predominantly composed of phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL), whereas the natural bilayer environment of GPCRs is a mixture of mainly phosphatidylcholine (PC), cholesterol, PE and phosphatidylserine (PS). Moreover, in contrast to eukaryotic membranes, *E. coli* phospholipids lack polyunsaturated side chains and contain cyclopropane groups not found in mammalian phospholipids. This suggests that at least some IMPs are rather forgiving concerning specific lipid interactions. On the other hand, specific requirement for certain lipids has been shown in a number of cases. For instance, the oxytocin receptor and the nicotinic acetylcholine receptor require cholesterol for high-affinity ligand binding and stability [16]. Nevertheless, the physical properties of the lipid bilayer such as bilayer thickness and lateral pressure may be crucial for stability and have been shown to effect the rate of refolding of bacteriorhodopsin (bR) [17] and other membrane proteins. Mixtures of lipids and detergents, so-called mixed micelles and bicelles [18], are probably better solubilizing agents for inclusion bodies, because in such a system, the lipids tend to cluster in the center of the bicelle, close to the membrane protein and become arranged in a bilayer-like fashion.

2.2. Membrane proteins are stable in micelles

The key question regarding in vitro folding of proteins is whether the folded state corresponds to the global energy minimum. The answer is not obvious for membrane proteins, as the vectorial membrane insertion process in vivo restricts the number of accessible conformations. As a result, the native conformation may also be a kinetically trapped local energy minimum. If this were the case, a different folding pathway would be unlikely to yield the native conformation.

Thermodynamic stability not only depends on the protein and its surrounding detergent micelle, but also on the environment. Therefore, the day-to-day experience of "instability", that is, loss of activity, does not necessarily reflect an intrinsic property of the protein. It could simply mean that the detergent, pH, etc. are "wrong" in the sense that it allows for off-pathway reactions leading to kinetically trapped states, such as aggregates. The following experiments are in favor of membrane proteins being stable in

detergent at least under certain conditions: First, some 30 different proteins have yielded 3D crystals starting from the detergent-solubilized state. Crystals, however, can only form if the majority of the molecules are in a defined conformation, that is, during the days to weeks of crystallization, no significant denaturation could have occurred. Second, the following proteins have been refolded in vitro from a denatured state, proving that the folding conditions favor formation of native 3D structure: bR [19], light harvesting complex [20], a number of mitochondrial carriers [21,22], an olfactory receptor [23], and diacyl glycerol kinase (DAGK) [24]. This is encouraging, because a successful refolding experiment proves that loss of activity is not necessarily a one-way street and that membrane proteins may fold in vitro provided the proper folding conditions are known.

Rosenbusch [25] recently speculated that exposure of membrane proteins to detergent, in general, may have a deteriorating effect and that the few proteins that could be successfully crystallized are biased toward being exceptionally stable. The recent addition of structures of presumably "difficult" proteins (ion channels, rhodopsin, abc transporters) to the protein database questions this view. It is also possible that some membrane proteins fail to crystallize not because of poor stability, but because of extensive conformational fluctuations that prevent formation of an ordered array.

To summarize, practical problems in handling solubilized IMPs do not necessarily reflect thermodynamic instability. In several cases, stability of the native state in detergent could be clearly established. Therefore, major progress in IMP crystallization will result from the development of improved methods that prevent unproductive reactions such as aggregation. Whether these will simply involve synthesis of novel detergents or more sophisticated methods such as lipidic cubic phase crystallization, bicelle crystallization or crystallization from organic solvents cannot be predicted due to the low number of success cases obtained to date.

3. Practical aspects of refolding

3.1. How do IMPs fold in vivo?

How do membrane proteins adopt the native conformation in vivo and can we learn anything from these processes for in vitro folding? In the cell, the protein is inserted cotranslationally into the membrane with the help of a protein complex called the translocon. The translocon forms a tunnel that allows translocation of regions destined for the *trans* compartment as well as lateral release of hydrophobic segments into the bilayer [26]. To date, this pathway is not yet understood in enough detail to become exploited for technical applications. However, direct insertion into membranes with the help of translocase in combination with lipidic cubic phase crystallization [25] might become an option in the future to avoid the use of detergents altogether in IMP crystallization.

What happens after membrane insertion? A widely held concept explaining folding of IMPs is the two-stage model of Popot and Engelman [27]. This model postulates that in a first stage, individual TM stretches form stable alpha helices in the membrane once they become inserted. Only in the second stage do they associate to form tertiary contacts at the helix interfaces, resulting in formation of the native 3D structure [27].

The notion that individual TM stretches are domains that fold in a membrane environment has been partly questioned by a study on bR. Hunt et al. [28] synthesized peptides corresponding to the seven TM domains and investigated their structure in a lipid bilayer. Four of the seven peptides formed stable alpha helices, while the others showed various degrees of secondary structure and membrane partitioning, implying that tertiary contacts seem to be necessary for these latter regions in the protein to acquire a stable fold.

The two-stage model may serve as a guideline for in vitro folding. It is likely that even under artificial conditions such as a detergent micelle, alpha-helix formation will precede the formation of tertiary contacts. Therefore, conditions that promote the formation of alpha helix, as monitored by, for example, CD spectroscopy, might be a good starting point for further experiments aimed at obtaining the folded conformation.

3.2. Source of denatured protein

E. coli is by far the most important host of proteins produced for crystallization. Of the 31 IMPs listed in Table 1, 10 were produced in this bacterium. Notably, the only four membrane proteins expressed in a nonhomologous host and yielding an X-ray structure were produced in *E. coli*.

Table 1
Number of alpha-helical IMPs yielding high-resolution X-ray structures [40] grouped by expression host and source of gene

Source of gene	Expression host			
	E. coli	Other bacteria and archaea	Mitochondria	Other eukaryotic membranes
E. coli	6[a]	–	–	–
Other bacteria and archaea	4[b]	14[c]	–	–
Mitochondria	–	–	4[d]	–
Other eukaryotic membranes	–	–	–	3[e]

The above list contains pdb entries. If several entries exist for the same protein, only one is listed. Only the four structures listed under [b] are derived from a recombinant source (*E. coli*); all others are from natural sources.

[a] 1FFT, 1FUM, 1FX8, 1KPL, 1KQF, 1L7V.

[b] 1H68, 1BL8, 1MSL, 1KPK.

[c] 1PRC, 4RCR, 1EYS, 1JBO, 1BRX, 1E12, 1H68, 1KZU, 1LGH, 1IJD, 1QLE, 1AR1, 1EHK, 1E7P.

[d] 1OCR, 1QRC, 1BCC, 1KB9.

[e] 1F88, 1J4N, 1EUL.

The reasons for its popularity include the vast choice of expression vectors, rapid cell growth, standardized scaling-up procedures as well as low cost per milligram of protein. Auxotrophic strains enabling selenomethionine labeling are of additional value in obtaining phase information of diffraction data.

Because many attempts to functionally overexpress eukaryotic membrane proteins in bacteria resulted in toxicity and consequently low yields, alternative strategies have been envisaged. A promising improvement of this situation was the selection of *E. coli* mutants that could tolerate expression of normally toxic membrane proteins [29,30]. The selected strains markedly increased expression levels of some proteins. However, for other proteins, no improvement could be obtained. When successful, induction of protein production led to the formation of membrane stacks and the increase in expression levels correlated with an increase in membrane surface area.

Alternatively, the toxicity could be abolished when the membrane protein was not inserted into the membrane but instead deposited in the cytoplasm in an aggregated state denoted "inclusion bodies". For some proteins, for example, mitochondrial transporters [22], cytoplasmic aggregation happens spontaneously. Others, such as GPCRs, can be preferentially directed to the cytoplasm by including an N-terminal fusion and/or by introducing positively charged amino acids into the loop regions to prevent translocation [23,31]. In any case, inclusion body formation correlates with a dramatic increase in expression levels, often by a factor of 1000 to 10,000 as compared to functional expression in the membrane.

Inclusion body formation is thought to depend on the kinetic competition between protein folding (in the case of membrane proteins, by membrane insertion) and the aggregation of unfolded protein. Therefore, inclusion bodies can be promoted (1) by slowing down the folding and membrane insertion kinetics, for example, by reducing the growth temperature (unpublished observations); and (2) by increasing the protein concentration, because aggregation is a higher order reaction. An N-terminal fusion can help in several ways. First, if the N-terminal domain alone is expressed at high levels, it is likely to improve expression of the C-terminal fusion partner. Second, slow folding of the fusion protein will promote aggregation. Third, if the N terminus of the native membrane protein is extracellular, the fusion will preclude translocation and reduce the fraction of membrane-inserted protein. For the expression of GPCRs, an N-terminal glutathione *S*-transferase (GST) fusion has been proven to increase protein production by a factor of 1000 to 10,000. Other soluble proteins fused to the N terminus (lacZ, malE, ZZ domain of protein A, thioredoxin) were much less efficient (our unpublished data). These proteins tend to confer better solubility to their C-terminal fusion partners, which, in the present case, is not desired.

Even eukaryotic organisms such as yeast [32] or insect cells [33] have been shown to yield inclusion body protein in some cases. However, it is not known whether proteins can be driven to form aggregates in those systems. An obvious advantage would be that codon usage problems resulting from transfer of mammalian genes to the host are less likely to occur in eukaryotic cells than in bacteria. However, I am not aware of any literature exploiting this possibility.

Finally, cell-free synthesis of membrane proteins leading to aggregation could possibly yield higher quantities than the attempt to express directly into detergent micelles or membranes, because the added detergents might interfere with the transcription/translation system. However, in cell-free protein synthesis, like in eukaryotic expression systems, aggregation is generally considered a nuisance and avoided whenever possible.

A successful system yielding high protein levels has been described by Smith and Johnson [34]. It has been used by us to overproduce some 50 different GPCRs with yields ranging from 0.5 to 50 mg/l cell culture, with 5–10 mg being the typical yield [31].

3.3. Analysis of published refolding protocols

For practical applications, the necessary requirement is that conditions are found that thermodynamically favor the native conformation over the denatured state. Moreover, pathways leading to the formation of trapped, nonproductive states such as aggregates have to be avoided. The folding pathway, however, can be very different from the in vivo situation.

It is instructive to compare the protocols describing successful renaturation of membrane proteins from *E. coli* inclusion bodies (references cited in Section 2.2). Although the procedures differ in detail, the following steps are common to all of them: (1) Solubilization in either a strong detergent, a chaotrope or in organic solvent; (2) transfer into a strong detergent, if not already achieved in (1); (3) addition of mixed phospholipid detergent micelles; and (4) removal of detergent. Folding as assessed by functional measurements, occurred either at step 3 or 4. In the following, the individual steps are analyzed in more detail.

3.3.1. Solubilization

The relatively small proteins light harvesting complex (LHCP) [20] and DAGK [24] were soluble in the chaotrope urea and could be coupled to a Ni-column in the urea-solubilized state. Mitochondrial transporters [35] as well as the rat olfactory receptor OR5 [23] were solubilized in the strong, negatively charged, detergent *N*-lauroyl sarcosine. Even for DAGK, solubilisation in the detergent dodecylphosphocholine (DPC) was eventually considered more successful than urea solubilisation. bR [19], finally, was extracted and completely denatured, in organic solvent, that is, a mixture of chloroform, methanol, water and trimethylamine.

Alkyl ionic detergents such as SDS and sarkosyl are considered "strong" or "harsh" due to their denaturing

effect on most proteins [36]. DPC is a zwitterionic detergent and therefore possibly less disruptive. Literature describing its use in solubilizing native membrane proteins is scarce, though, indicating that DPC should be classified as a harsh detergent as well.

3.3.2. Transfer into harsh detergent

Proteins solubilized in urea or organic solvent were transferred into a strong detergent. This was achieved either by simple solvent exchange of the matrix-bound protein (LHCP), or by adding SDS to bR dissolved in organic solvent, removing the solvent in vacuo, and finally resuspending the protein in aqueous buffer.

3.3.3. Addition of mixed lipid/detergent micelles

In the case of LHCP, this was preceded by exchanging the detergent SDS for OG. Then, mixed micelles of Triton X-100 and dipalmitoyl phophatidylglycerol (DPPG) were added. For OR5, sarkosyl was first replaced by digitonin. Subsequently, mixed micelles of dodecyl maltoside and 1-palmitoyl-2-oleoyl PC (POPC) and 1-palmitoyl-2-oleoyl PG (POPG) were added. To bR, the mixed micelles consisted of CHAPS and DMPC, whereas for DAGK, a mixture of DPC and POPC proved to be most successful. Finally, mitochondrial transporters were transferred into a mixture of Triton X-100 and phospholipids. In all cases, the molar ratio of detergent to lipid was between 5 and 10, while the mass ratio of detergent to protein was 5 or higher, corresponding to a minimum molar ratio of 500 detergent molecules plus 50 phospholipid molecules per protein. In three cases, it could be established by functional measurement that the protein refolded into the native state in the detergent/lipid mixture, namely, for bR, LHCP and OR5. Interestingly, bR did not require removal of SDS to become functional; instead, a ca. 8-fold excess of the CHAPS/DMPC mixture induced refolding. The remaining proteins (mitochondrial transporters and DAGK) required complete removal of detergent (sarkosyl/DPC) before any function could be detected.

3.3.4. Removal of detergent

This procedure was carried out either by dialysis (DAGK/DPC, bR) or by treatment with a hydrophobic matrix adsorbing the detergent (mitochondrial carriers: Amberlite; DAGK: Biobeads; OR5: Calbiosorb). LHCP was not reconstituted into liposomes.

3.4. A rational approach toward membrane protein folding?

What is the rational basis of these refolding protocols? To understand each process in some detail and to be able to design rational screens allowing to find and optimize refolding conditions for new proteins, the following questions would require an answer: (1) What is the structure of membrane protein in inclusion bodies? (2) Why is the ‘‘harsh’’ detergent required? (3) What is the conformation of the protein in the harsh detergent? (4) What happens upon folding?

The answer to question (1) is still pending. Proteins in inclusion bodies are not easily amenable to biophysical investigation, especially not if other contaminating proteins are present as is often the case. For inclusion bodies formed from soluble proteins, considerable secondary structure has been detected by CD spectroscopy, but similar experiments have not been published for membrane protein inclusion bodies. It is clear though that IMPs can aggregate in different ways leading to aggregates that do become soluble in strong detergents, such as IBs, and ‘‘irreversible’’ aggregates that do not, such as precipitates obtained, for example, from heating solubilized IMPs (unpublished observation). As long as the structure of protein in inclusion bodies is unknown, better solubilization methods will be a result of trial and error even in the future.

Concerning the second question, the most likely answer is that harsh detergents are required to prevent proteins from aggregating before folding is induced. The negative surface charge on SDS or sarkosyl micelles will electrostatically prevent close contact between protein molecules. At the same time, these detergents induce alpha-helix formation upon binding to the hydrophobic TM segments. Therefore, in a mixed protein/SDS (or sarkosyl) micelle, the protein will probably have considerable secondary structure and be in a good starting position for subsequent formation of tertiary contacts. In a later folding step, these detergents will no longer be required and have to be replaced by milder detergents, respectively a lipid bilayer. The situation is reminiscent of the use of detergents as ‘‘artificial chaperones’’ to refold soluble proteins [37]. Here, detergents are needed temporarily to prevent aggregation. The folded protein no longer requires any detergent as stabilizer.

The third question relates to the conformation of IMPs solubilized in harsh detergents. When analyzed, alpha-helical structure could be detected and mostly corresponded to the expected fraction of alpha helix in the folded protein. Still, the function of proteins in SDS or sarkosyl is impaired: the visible spectrum of bR indicates that the chromophore is exposed to solvents, DAGK does not trimerize and therefore has no enzymatic activity, lactose permease (LacY) loses its ability to bind substrate and so do many GPCRs. It seems therefore that these proteins in SDS or sarkosyl are in a molten globule-like state that contains considerable secondary structure but not the native tertiary fold. This view is supported by a number of IMPs running abnormally, that is, with a lower than expected molecular mass, on SDS-PAGE, indicating that they are more compact than their soluble, completely unfolded, counterparts. This, in combination with the fact that multimeric proteins are dissociated in SDS, favors the assumption that SDS-solubilized IMPs are molten globules.

Finally, what happens upon folding? The denaturing detergent is removed and replaced either directly by lipid

or by a milder detergent, respectively by detergent/lipid mixtures. In either case, functional measurements were used to quantify the folded state. The portion of the protein that becomes functional must have formed proper helix–helix contacts upon transfer into the more bilayer-like environment. bR is the only IMP where refolding kinetics has been studied thoroughly [38], indicating that helix–helix assembly precedes binding of the chromophore retinal. With the exception of bR, 100% refolding yield could never be achieved, though.

In lack of a detailed theoretical understanding of the various factors affecting refolding yields and tendency to aggregate, the most straightforward strategy to find suitable folding conditions is to vary all the parameters, especially the composition of detergent/lipid micelles, in a systematic way and quantify the refolding yield. A proper functional assay that works in detergent is therefore a crucial success factor. Obviously, this is easier to achieve for IMPs with ligand binding properties than for proteins that require membrane reconstitution to become active such as, for example, voltage-gated ion channels.

Future progress in membrane protein refolding could come from automation, allowing screening of many more folding conditions than can be routinely achieved to date. Also, physical methods that distinguish between folded and nonfolded conformation, such as CD or light scattering, could contribute to an increase in throughput and speed. Finally, methods that stabilize membrane proteins such as random mutagenesis and selection of more stable mutants [39] might help to make even the weakest weaklings amenable to crystallization and, eventually, structural analysis.

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