Refolding of *Escherichia coli* produced membrane protein inclusion bodies immobilised by nickel chelating chromatography

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Abstract Two distinctly different membrane proteins, which produced inclusion bodies in *Escherichia coli*, have been refolded to reconstitute properties appropriate to their native counterparts. The method employed utilises nickel chelating chromatography, where the solubilised inclusion bodies bind, refold and elute. Our aims were to release a large pool of membrane protein for functional, mutational and crystallisation screening studies. It is hoped that the methods described here will have a general application for other membrane proteins which have formed inclusion bodies.

Key words: Inclusion body; Refolding; Light harvesting complex II; Translocation pore; Chloroplast

1. Introduction

Large quantities of proteins intended for biophysical and structural studies are often unobtainable from native sources. These proteins or polypeptides required as engineered variants have to be produced by the recombinant route. In the case of membrane proteins this strategy faces a variety of problems (see [1] for a general review). *Escherichia coli* is the most widely and successfully used vehicle for the production of large amounts of protein for biochemical investigation. The destination, location and state of proteins produced by homologous or heterologous expression in *E. coli* varies considerably. Factors which influence the success of the expression include the host, the nature of the promoter employed, the growth conditions, and the origin and nature of the protein of interest. Ideally, the protein is expressed in its native and active state in high levels, and is then purified either conventionally or with the aid of purification tags appended to the carboxy- or amino-terminus. In other cases the protein may form insoluble aggregates, known as inclusion bodies. Inclusion bodies seem to form when highly expressed recombinant proteins cannot be tolerated as soluble proteins in the cell cytoplasm. Although inclusion bodies have often been considered an undesirable, dead end product of protein expression, their formation can be an advantage as their isolation from cell homogenates is a convenient and effective first purification step. In the worst case, the protein may not express at all or only at very low levels. In the case of membrane proteins, they may be localised in the membranes, otherwise inclusion bodies form or over-expression is not achieved.

The final location of a recombinant protein is quite difficult to predict and normally has to be determined empirically. However, a few general guidelines can be applied. Firstly, unless a protein is extremely soluble and/or benign, high expression levels tend to lead to the formation of inclusion bodies. Secondly, the further a gene is removed in evolutionary distance from the homologue of the host species, the more likely it is that problems (i.e. no over-expression or inclusion body formation) will arise during the heterologous expression of its product. Membrane protein expression, unsurprisingly, is especially prone to these problems. Obviously, the best case is the correct folding into the host membrane enabling its subsequent solubilisation and purification. More likely the protein will form inclusion bodies or the expression will be extremely low, as a result of degradation or the toxic effects of membrane protein expression in the cell [2]. Best case scenarios are often achieved during homologous expression. Variations in promoters used, growth conditions employed, in host species or in selection of a protein with a greater similarity to the host homologue, are options available in overcoming these expression problems.

To a large extent, our poor knowledge of membrane protein structures reflects the difficult biochemistry and their low abundance. Indeed, the known structures are examples of those few membrane proteins available in large quantities together with a long history of biochemical research (bacteriorhodopsin [3], bacterial photosynthetic reaction centre [4], cytochrome bc_1 [5], cytochrome *c* oxidase [6], LH2 [7], light harvesting complex II (LHC2) [8], photosystem I [9], porin [10]). The problem of low abundance has been successfully side-stepped in the case of soluble proteins by the recombinant route. In contrast, the production of large quantities of active membrane protein by over-expression is not routine. Therefore, the development of strategies to overcome this barrier will significantly boost membrane protein structural research. In this regard, membrane proteins expressed as inclusion bodies are potentially useful. Although the proteins in this form are inactive and insoluble, they could be an excellent starting point for producing membrane proteins in large quantities, provided procedures can be developed to reconstitute them in vitro. This study aims to address this problem by the reconstitution of two different membrane proteins which form inclusion bodies upon expression in *E. coli*. The refolding of globular proteins from inclusion bodies is becoming routine nowadays [11], and is frequently used as a way to produce active protein for structural and functional studies. However, in the case of membrane proteins far less examples

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Abbreviations: C_8E_4, *n*-octyltetraoxyethylene; LDS, lithium dodecylsulfate; LDAO, *N,N*-dimethyl-dodecylamine-*N*-oxide; LHC2, light harvesting complex II; LHCP, LHC2 apoprotein; OG, octyl-glucoside; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tic, translocon of the inner chloroplast envelope; Toc, translocon of the outer chloroplast envelope

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are documented. Here we describe the use of affinity chromatography on Ni^{2+}-chelating Sepharose to purify and fold complex proteins in one step.

1.1. The chloroplast protein import machinery

The chloroplast requires an extraordinary number of nuclear encoded proteins to be imported and targeted for its biogenesis, maintenance and function. The first step in this remarkable process is the translocation across the outer and inner envelope membranes, and is achieved by the Toc and Tic complexes (translocons of the inner and outer envelopes of chloroplasts) [12]. The outer envelope component (Toc) of this complex is a multi-subunit assembly including the core components Toc75 (putative channel), Toc86 (precursor protein receptor) and Toc34 (GTP regulatory protein). Recombinant Toc75 produced by *Escherichia coli* forms inclusion bodies [13].

For further insight into the mechanism of how proteins are targeted to and traverse a phospholipid bilayer structural information is needed. This requires the crystallisation of protein in two or three dimensions for structural analysis by electron or X-ray crystallography, which necessitates the production of large amounts of pure and active protein. In the case of the chloroplast protein import machinery these requirements rule out the possibility of utilising native proteins which can only be produced in microgram amounts.

1.2. The chloroplast light harvesting complex II (LHC2)

LHC2 is the most abundant protein in thylakoids of plant chloroplasts [14]. The 25-kDa apoprotein binds chlorophyll *a* and *b* and carotenoids, which have been localised in the 3D electron-crystallographic structure of the trimeric complex [8]. In plant cells the apoprotein of LHC2 is synthesised in the cytosol and imported into chloroplasts where it acquires pigments and folds. This process can be mimicked in vitro with a recombinantly derived precursor form of LHC2 and purified chloroplasts [15]. Another route for obtaining folded LHC2 is in vitro reconstitution of monomeric protein from polypeptide and pigments [16,17]. The kinetics of that process has been analysed by measuring the energy transfer from chlorophyll *b* to chlorophyll *a* via time resolved fluorescence spectroscopy [18]. Trimerisation and crystallisation of recombinant monomers is only possible in the presence of specific lipids, and preliminary structural maps of the recombinant complex are consistent with the native LHC2 structure [19,20].

2. Materials and methods

Sodium *N*-lauroyl-sarcosinate, also known as sarkosyl, (MicroSelect) was purchased from Fluka and Triton X-100 from Boehringer Mannheim. *n*-Octyl-β-D-glucoside and *n*-dodecyl-β-D-maltoside were purchased from Calbiochem. Chelating Sepharose Fast Flow, a prepacked Superdex 200 prep. grade gel filtration column and standard proteins for gel filtration calibration were obtained from Pharmacia Biotech. Other chemicals were obtained from Merck and Sigma.

2.1. Analytical methods

SDS-polyacrylamide gel electrophoresis was performed using 6×8×0.1-cm gels (Biorad Mini-Protean-Apparatus) with 12% acrylamide [21] and proteins were visualised by staining with Coomassie brilliant blue dye. Native gel electrophoresis was performed without SDS in the gels, with 10% glycerol in the samples. Electrophoresis was carried out at 130 V at 4°C in the dark. Chlorophyll concentration was determined according to [22] in 80% acetone. Fluorescence measurements were performed at room temperature on a Hitachi F-4500 apparatus, the excitation slit was set to 1 nm and the emission slit to 5 nm bandwidth.

2.2. Construction of Toc75 expression clones

The Toc75 expression vector (pET.24 driven by a T7 promoter utilising the lysogen λ DE3) was constructed in the laboratory of J. Soll as described elsewhere [13].

2.3. Production of recombinant Toc75 protein (inclusion body preparation)

A colony of recently transformed *E. coli* strain c41 (DE3) [2] was inoculated into 1 l of LB growth media containing 25 μg/ml kanamycin. Cells were grown to an optical density of 0.6, whereupon IPTG (0.6 mM) was added to induce expression. After a further 3 h the cells were harvested by centrifugation and frozen. Cells were thawed and resuspended in 40 ml of cold 20 mM Tris-Cl, pH 8, 1 mM EDTA (TE) and lysed by one passage through a 40-ml pre-chilled French pressure cell. The lysate was kept on ice and PMSF was added to a final concentration of 0.001% (w/v). Inclusion bodies were pelleted by centrifugation (12 000×*g*, 20 min, 4°C) and washed by resuspension in the 40 ml of TE buffer and re-centrifuged. After resuspension in the same buffer the samples were frozen (>20 mg/ml).

2.4. Refolding of Toc75 inclusion bodies

Typically 25 mg of Toc75 inclusion bodies were centrifuged (12 000×*g*, 10 min, 4°C) and resuspended in 40 ml of 0.1 M Tris-Cl, pH 8, then re-centrifuged and resuspended in 5 ml of 0.1 M Tris-Cl, pH 8 (5 mg/ml). The isolated inclusion bodies were solubilised by addition of 20 ml of 8 M urea and 2.7 ml of 10% sodium *N*-lauroyl-sarcosinate (w/v) and after mixing the volume was brought to 50 ml with 20 mM Tris-Cl, pH 8, 0.2 M NaCl. 10% (v/v) glycerol and 0.1% (w/v) sodium *N*-lauroyl-sarcosinate (final concentration 0.5 mg/ml Toc75, 3.2 M urea, 0.6% (w/v) sodium *N*-lauroyl-sarcosinate and 90 mM NaCl). This mixture was slowly applied to a 3-ml Ni-chelated Sepharose Fast Flow column equilibrated with 20 mM Tris-Cl, pH 8, 0.2 M NaCl, 10% (v/v) glycerol and 0.1% (w/v) sodium *N*-lauroyl-sarcosinate at room temperature (23°C). The column was then washed for several hours with 100 ml of the same buffer except that the sodium *N*-lauroyl-sarcosinate had been replaced by 0.1% (v/v) Triton X-100. The column was again washed with 25 ml of the same Triton buffer including 10 mM imidazole to remove weakly bound contaminants. Toc75 was eluted in one step with 0.3 M imidazole in the same buffer. Protein was detected in the eluent by the Bradford assay (Biorad) and by SDS-PAGE. Appropriate fractions were pooled and their protein concentration was estimated by BCA (Pierce). Samples were stored at −20°C.

2.5. Size exclusion chromatography

A Superdex 200 prep. grade (60×1.6 cm i.d., 120 ml) was equilibrated in 20 mM Tris-Cl, pH 8, 0.2 M NaCl. 10% (v/v) glycerol, 5 mM DTT and 0.1% (v/v) Triton X-100. A sample of refolded and frozen Toc75 (0.5 ml, 1.5 mg/ml) was thawed and applied to the equilibrated column at 1 ml/min. Fractions were analysed by SDS-PAGE. Using identical conditions the same column was calibrated with 2 ml (ca. 2 mg/ml) of blue dextran (2000 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa) and RNase (13.7 kDa).

2.6. Construction of His-tagged LHCP expression clones

The *Cab*, AB 80 gene encoding the precursor form of pea LHC2 apoprotein (LHCP) (lhcb1*2, [24]) was modified by site-directed PCR mutagenesis using primers that generated an *Xma*I restriction site at the location corresponding to Val^{229}. With this restriction site a short synthetic DNA fragment was ligated extending the native cab gene by six histidine codons. Finally a 5′ DNA fragment of the *Cab* gene was exchanged via the *Eco*RI and *Bst*EII restriction sites with the one coding for the mature N-terminal sequence (MRKSA....) from the expression plasmid D7f.3 (Dilly-Hartwig, H., unpublished results). LHCP expression and purification of inclusion bodies was performed as described elsewhere [23].

2.7. LHC2 refolding

Inclusion bodies were dissolved in 1 ml of 8 M urea at a protein concentration of 1 mg/ml and loaded on a 2-ml Ni affinity column at room temperature at 0.6 ml/min. The column was then washed with

5 ml of LDS buffer (see below) followed by 2 ml of OG buffer at 4°C. Chlorophyll (1 mg, $a/b = 1.5$) and 0.3 mg of carotenoids were dissolved in 70 μl ethanol and mixed with 0.7 ml of OG buffer, applied to the column and incubated for 30 min (with the flow stopped). Afterwards the flow was continued with 1 ml of OG buffer, 4 ml of TX buffer and finally with ELUATE buffer. Buffers: LDS: 2% (w/v) Li-dodecylsulfate, 0.1 M Tris, pH 9.0, 0.1 M NaCl, 20 mM imidazole; OG: 1% (w/v) octyl-glucoside, 0.1 M Tris, pH 9.0, 12.5% (w/v) sucrose; TX: 0.05% (v/v) Triton X-100, 0.1 mg/ml L-phosphatidyl-D,L-glycerol dipalmitoyl (PG), 0.1 M Tris, pH 7.5; ELUATE: 0.05% (v/v) Triton X-100, 0.1 g/l PG, 10 mM Tris, 0.3 M imidazole, pH 7.5.

3. Results

3.1. Production of recombinant Toc75 protein (inclusion body preparation)

The yield of inclusion bodies was around 70 mg of protein per litre of cell culture (BCA assay, Pierce). At this stage Toc75 was already ca. 80% pure (Fig. 1, lane A).

3.2. Refolding of Toc75 inclusion bodies

Analysis of fractions by SDS-PAGE revealed that pure Toc75 had eluted from the Ni-column (Fig. 1, lanes B and C) indicating that the protein is now soluble in a mild detergent solution. Activity measurements were performed in a manner described elsewhere [13]. The protein was incorporated into phospholipid membranes, which subsequently formed aqueous pores blocked specifically by the precursor small subunit of Rubisco (and not by the mature subunit) [13]. The material exhibited the same properties except that the apparent efficiency of reconstitution was much higher than previously described [13] (Wagner and Collinson, unpublished results). The protein was recovered from the column in 4 ml at 0.5 mg/ml (BCA assay) from 25 mg of protein, which is an 8% yield. Taking into account that inclusion body protein determinations are usually over-estimated, the efficiency is probably somewhat higher. An estimation by band intensities of SDS-PAGE of material before and after refolding indicates the yield is about twice as much.

3.3. Size exclusion chromatography of Toc75

Gel filtration (Fig. 2A) was used as a test for the oligomeric state of the sample. No protein was recovered at the void volume indicating that there is no aggregation. Indeed,

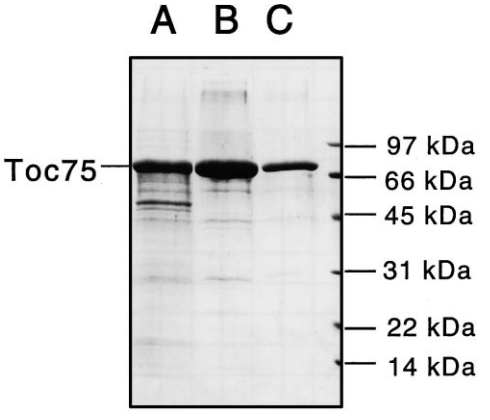

Fig. 1. Polyacrylamide gel analysis of Toc75 before and after Ni^{2+}-column chromatography. Samples were applied to a 12% gel. Lane A: 20 μl (from 50 ml) of solubilised inclusion bodies prior to loading onto the Ni^{2+}-column; lane B, C: 10 μl (each from 2 ml) of consecutive protein containing fractions from the Ni^{2+}-column.

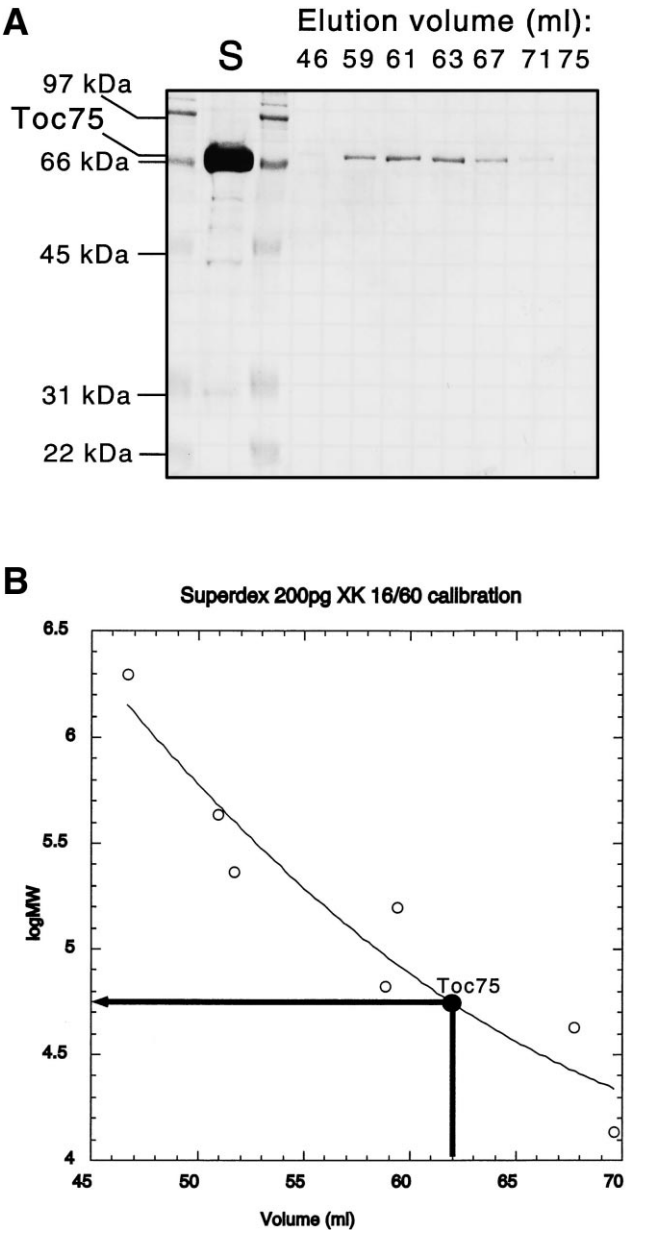

Fig. 2. Analysis of refolded Toc75 by size exclusion chromatography. A: Fractions from the gel filtration column were visualised by SDS-PAGE. S denotes the starting material before column chromatography of which 20 μl was loaded onto the gel. Also 20 μl of fractions at a given elution volume were applied. The void volume of the column corresponds to the first elution lane at 46 ml. B: The Superdex 200 column was calibrated with molecular weight standards and the elution volume was plotted against $\log_{10}$ MW.

Toc75 was recovered at a volume corresponding to 60 kDa (Fig. 2B) which would suggest a monomeric form in Triton X-100.

3.4. Tryptophan fluorescence of Toc75

To assess the folding state of Toc75, tryptophan fluorescence spectroscopy was employed (Fig. 3). The results obtained were similar to those observed for the porin OmpA [29]. Folded Toc75 emission peaks at 342 nm, while the same sample unfolded in urea emits with a decrease in intensity and a shift to 353 nm; unfolded inclusion bodies exhibited

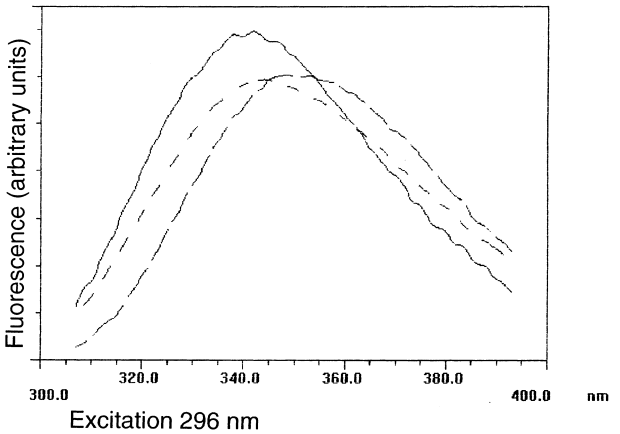

Fig. 3. Fluorescence spectra of refolded and denatured Toc75. The excitation wavelength was set at 296 nm for absorption by tryptophan. The full line represents the emission for refolded Toc75 (0.25 mg/ml protein, 20 mM Tris-Cl, 0.2 M NaCl, 0.05% (w/v) Triton, 0.05% (w/v) dodecyl maltoside, 10% (w/w) glycerol) and the long dashed line is the same sample with 8 M urea added; the short dashed line shows the emission for the equivalent quantity of inclusion bodies (8 M urea). In all cases the buffer blank was subtracted.

an intermediate peak of 346 nm. Perhaps the intermediate result for the inclusion bodies signifies a resistance to unfolding of inclusion bodies relative to the folded counterpart; alternatively, it could be a result of contaminating proteins of inclusion bodies which are stable in urea. These observations (a decrease in intensity and a shift to longer wavelengths) were also noted in the case of OmpA [29].

3.5. LHC2 refolding

The solubilised inclusion bodies were applied to the Ni-column and were subjected to a buffer change from the harsh detergent (LDS) to a milder one (OG); upon the addition of pigments folding proceeds. Washing with buffer removed most of the pigments, but the column retained a pale green colour, which represents the protein bound pigment. This material was eluted with high imidazole (Fig. 4). The green native gel is partially denaturing, therefore the trimeric as well as monomeric form of LHC2 is observed.

The established and most accurate method for LHC2 quantification is by measuring the chlorophyll content. Therefore, the yield of refolding is conveniently expressed as the amount of chlorophyll in the elution peak relative to chlorophyll used in the reconstitution mixture. This gives a value of 3%, which

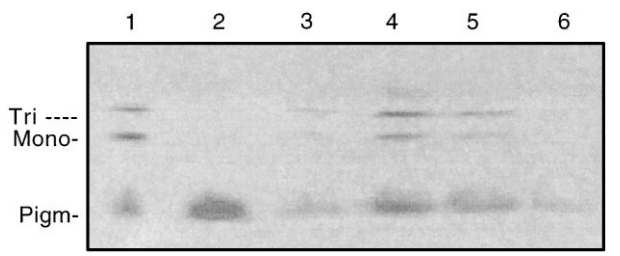

Fig. 4. Green native gel of LHC2 eluting from Ni^{2+}-column. Lane 1: reference native LHC2 (pea); lane 2: unbound pigments washed off with OG and TX-buffer; lanes 3–6: elution of refolded LHC2 with ELUATE buffer.

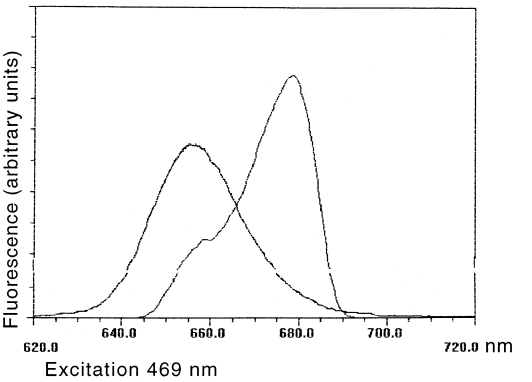

Fig. 5. Fluorescence spectra of refolded LHC2 and pigments. The excitation wavelength is set to chlorophyll *b* at 469 nm. Left peak at 655 nm: pigment mixture used for refolding in OG buffer, 50-fold dilution. Right peak at 679 nm: recombinant LHC2 fraction eluted from Ni-column.

is calculated as 5% with respect to protein. The chlorophyll which is not incorporated into LHC2 is not bound to the column and is washed off. The non-folded protein elutes together with the folded one and can be easily separated by further purification steps. In short, the overall yield is comparable with the published protocol, while the method described here is more conveniently performed in a single step. The quality of material is also improved, as the ratio of trimer to monomer is higher than in samples produced by the old method [31].

When the peak from the column is loaded on a sucrose density gradient, a discrete band forms. The chlorophyll *a* to *b* ratio of the band corresponds to the eluting fractions from the column; with the value of 1.3 it has marginally decreased in relation to the initial reconstitution conditions.

3.6. Measurement of LHC2 fluorescence transfer

The fluorescence spectrum of the complex which elutes from the column, shows efficient energy transfer from chlorophyll *b* to chlorophyll *a* as in native LHC2 (Fig. 5). The broadening of the peak at low wavelength is indicative of some incomplete transfer due to free chlorophyll or partially misoriented chlorophylls. For comparison an emission spectrum of the pigment suspension as used for refolding in OG buffer is shown, which yields almost no transfer.

4. Discussion

Here we report the effective refolding of two quite different membrane proteins by a related procedure: Toc75 and LHC2, a pigment binding oligomeric assembly.

Recombinant Toc75 and LHCP from inclusion bodies bind by virtue of their His-tags to the Ni-column. Subsequently, exchange from chaotrope to mild detergent leads to refolding, and in the case of LHC2 to pigment binding and trimerisation by exposing the complex to mixed lipid-detergent micelles [25]; trimerisation presumably occurs as the monomers are released from the column.

In each case the samples seem to represent the native structures and are therefore suitable for further structural and functional analysis. The advantage of using such a refolding approach where the proteins are immobilised on a column

may be that aggregation is prevented. Proteins are immobilised so that the hydrophobic faces exposed in partially folded and folding proteins are then not free to associate with one another. In this respect the column material may have a chaperone-like function in keeping folding proteins separated.

A few membrane proteins have been refolded from inclusion bodies; a G-protein coupled receptor involved in olfaction [26] was refolded using a similar approach. Inclusion bodies were solubilised in *N*-lauroyl-sarcosinate and were then exchanged into digitonin while the protein was bound to a nickel chelated column. An odorant binding capability was subsequently demonstrated. However, no data on the yields of refolding were given nor was the aggregation state of the detergent solubilised protein assessed. Refolding of recombinant porins from inclusion bodies has also been reported. Porins from *Rhodopseudomonas blastica* [27] and a type-b porin from *Haemophilus influenzae* [28] seem to be quite amenable to refolding into their trimeric state. The former example makes use of an anion exchange column to exchange from chaotrope to LDAO and to C_8E_4, which seems similar to the approaches applied here. Further, the reconstituted trimers have been shown to readily form crystals identical to those from the native source. In the latter case refolding occurred by dialysis of the chaotrope employed to solubilise the inclusion bodies and in the presence of 3,14-zwittergent and the correct salts, then trimers could form. It should be stated that porins are particularly robust proteins which fold spontaneously even when unfolded protein is diluted with detergent solution or lipid suspension [29]. This approach does not work with Toc75 or LHC2. The oxoglutarate carrier from bovine heart mitochondria over-expressed in *E. coli* as inclusion bodies has also been refolded and a reconstituted activity demonstrated [30]. The method employed also utilised the detergent sarkosyl, and the activity was reconstituted by addition of Triton and lipids, followed by detergent removal by adsorption onto amberlite beads. It is also possible to reconstitute bacteriorhodopsin inclusion bodies by sequential exchanges form harsh (SDS) to milder (Triton X-100) detergents, and finally to octyl-glucoside also by anion exchange chromatography [31]. Another interesting refolding strategy utilises a column with a bound fragment of GroEL (a mini-chaperone) [32]. This column was reported to have considerable refolding activity. However, its application as a general tool for processing large amounts of inclusion bodies needs to be tested, as does its application towards membrane proteins requiring detergents.

To understand the folding, pigment binding and trimerisation process in LHC2, biochemical and mutational perturbations have been applied [33,34]. With the new method described here future experiments can be performed in an improved and accelerated manner. Likewise, structural information of Toc75, as an example of a protein translocation pore, will require extensive screens consuming large amounts of protein. These experiments are underway using reconstituted, inclusion body derived material.

The approach of refolding membrane protein inclusion bodies while immobilised on chelating Sepharose (or other resins) has been shown to be useful for both α-helical and β-barrelled membrane proteins. Moreover, in the case of LHC2 in one step the polypeptide has folded, bound the required pigments and lipid, and trimerised into its functional state. Therefore, we believe this approach will be more generally applicable.

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