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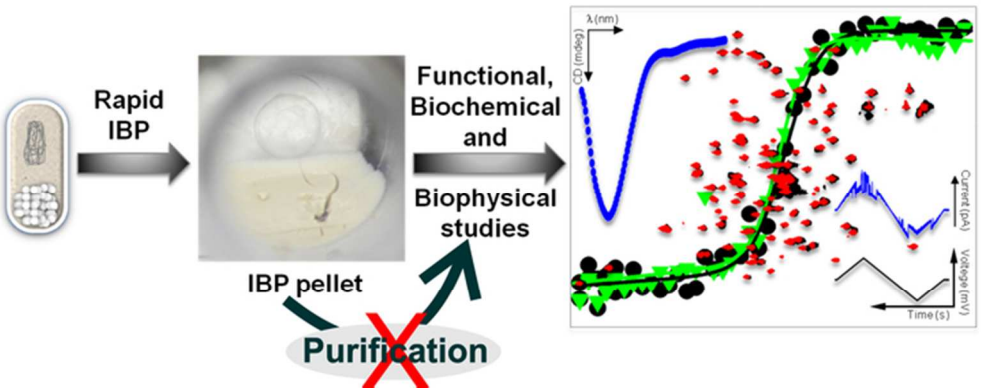

Purification-free transmembrane protein inclusion body preparations for rapid and cost-effective biophysical, functional and structural studies.
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Thermodynamic, structural and functional properties of membrane protein inclusion bodies are analogous to purified counterparts: Case study from bacteria and humans

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Structural and biophysical characterization of transmembrane proteins that require sufficiently pure protein in high amounts, are usually generated in large-scale preparations as inclusion bodies (IBPs). However, IBP and subsequent purification, is oftentimes laborious, painstaking, time-consuming, expensive and demands protein-dependent customization. We demonstrate that protein purification is dispensable if inclusion bodies are sufficiently pure; the latter can be directly used in biophysical and functional experiments. Using an assortment of membrane proteins from bacteria and humans, we validate that IBPs and their purified counterparts exhibit analogous structure, stability, thermodynamic parameters as well as channel conductance activity. Direct use of crude inclusion bodies by circumventing protein purification could find immediate application in speedy generation of high-throughput mutant libraries of transmembrane β-barrels, and possibly helical proteins.

Introduction

Transmembrane proteins constitute about 30% of the cellular proteome of any organism. They play a pivotal role in metabolite transport, signalling, quorum sensing, ion conductance, and serve as carrier proteins, receptors for hormones, enzymes essential for cellular function and are involved in cell recognition and immune response. Not surprisingly, major diseases including cancer, stroke and neurodegenerative disorders are associated with membrane proteins.[1] Membrane proteins, particularly the outer membrane proteins (OMPs), also serve as lead molecules of pharmaceutical interest and drug targeting,[2] since they account for 50% of the outer membrane mass in bacterial (Gram-negative) as well as mitochondrial and chloroplast outer membranes.[3] Owing to their instrumental role in regulating all vital cellular processes, membrane proteins are the most sought-after targets for structural and biochemical characterization.

With increasing incidence of drug-resistant pathogenic bacteria, understanding membrane protein biology is the need of the hour.[2a, 2b, 4] Unfortunately, the sparsity in our current knowledge of membrane protein structure, function and regulatory mechanisms is because membrane proteins are remarkably notorious for their amenability to structural and biochemical studies, making it exceedingly difficult to render a mechanistic understanding of their function and regulation. Generation of sufficient quantities of these proteins for in vitro studies (usual equilibrium studies under multiple conditions in triplicates generally require 100-500 mg of pure protein[5]) has always been a challenge. In such cases, over-production in the form of inclusion bodies (IBs) in laboratory expression strains of *Escherichia coli* is employed, owing to the high protein yields obtained through IBs and ease of handling, if extensive post-translational modifications are not crucial for the folding and function.[3d, 3g, 5b-d, 5f, 5g, 5j-q, 6] While protein expression in the native form and extraction from the membrane using gentle detergents is still desired to obtain functionally active membrane protein preparations, due to the limited yield from this method, biophysicists and structural biologists working towards structure determination and study of membrane protein folding choose to use IB-based preparations,[3d, 3g, 5b-d, 5f, 5g, 5j-q, 6] for the latter offers greater protein yield per unit time or resources invested.

Generating sufficiently purified protein is crucial for biophysical, functional and structural characterization. Protein preparations from inclusion bodies generally consists of two main steps, viz., isolation of inclusion bodies (referred to as inclusion body preparation, IBP) and subsequent column-based purification (involving ion exchange, affinity, gel filtration chromatography or a combination of these methods). The

literature holds several reports where purification procedures are optimized to obtain pure protein;[5e, 6d, 6g, 6j, 7] however, if the IB pellet is sufficiently pure, an intriguing possibility is to directly use this protein for biophysical and functional experiments without the need for purification. Direct use of IBPs has several advantages, since purification procedures are predominantly expensive, time consuming and labour-intensive. While the use of IBPs for biophysical studies has been attempted earlier,[5b, 5g, 8] whether this is broadly applicable to several membrane proteins has not been investigated. In this study, we report that crude IB pellet can be directly utilized, without the need for purifications, in biophysical, biochemical and structural characterization of membrane proteins. We validate the general applicability of this approach for both cysteine-containing proteins of human and bacterial origin, as well as transmembrane β-barrels from various pathogenic organisms.

The proteins we have selected have been mapped to the outer membrane of bacteria or organelles of higher eukaryotes, and act to confer protection against host defence or key transporters (channels), respectively (Table S1†). For example, Ail, a *Yersinia pestis* outer membrane protein (OMP), the causative agent of plague, is required for cell adhesion and to establish resistance from host immune response.[9] Rv1698, is a predicted OMP of *Mycobacterium tuberculosis*, implicated in transport of certain molecules (antibiotics) across the outer membrane.[10] VDAC-2, which is a found in human outer mitochondrial membrane (OMM), is the major protein of the OMM[5o] and is involved in transport of metabolites like NADH and nucleotides.[11] It also interacts with the Bcl-2 family of proteins to regulate apoptosis.[11] OmpX is an OMP of *Escherichia coli* thought to neutralize complement-mediated host response.[6p, 12] PagP is an OMP found in Gram-negative bacteria, including *Salmonella* sp., *Neisseria* sp. etc., and is required for lipid A palmitoylation; the latter leads to septic shock.[3d, 13] Many of these proteins have been examined in attempts to derive behavioural characteristics important for pathogenesis and human disease. We demonstrate, in this report, that an in-depth biophysical and functional analysis of these biologically relevant proteins can readily carried out using crude IBPs, obliterating the need for extensive and laborious purifications.

Results and discussion

In order to improve the quality of protein generated from IB preparations, we developed a unified rapid IBP protocol, by combining steps from reported methods. The details of methodology development (optimized for the OmpX-Om14 fusion construct[5l]) are available in the supplementary data (Figs. S1-S6†). The litmus test of any modified approach is in its general applicability to several membrane proteins for the production of proteins in sufficient purity for biophysical, biochemical and functional analysis. In this report, we demonstrate that IBPs of sufficient purity can be directly used for functional assays and biophysical characterization without the need for protein purification.

Crude protein derived from modified rapid IBP protocol is sufficiently pure for structural and stability studies

To test the applicability of crude protein in biophysical experiments, we chose nine proteins from various sources (Table S1†): Ail from *Yersinia pestis*;[5k, 14] Rv1698c from *Mycobacterium tuberculosis*;[10] hVDAC-2 WT from human outer mitochondrial membrane[5o], hVDAC-2 C0 (Cys-less mutant of hVDAC-2 WT,[5o]); OmpX[12] and PagP (PagP-Ec) from *E. coli*;[15] PagC,[16] PagN[17] and PagP (PagP-St)[18] from *Salmonella typhimurium*. We expressed these proteins as mature constructs in the form of inclusion bodies in *E. coli* and compared the purity of proteins obtained using our optimized protocol (Fig. S6†), with IBs prepared from reported methods. Visual inspection of the SDS-PAGE gels (Figs. S7-S8† and Table S5†) as well as densitometry analysis (Fig. 1A) suggests that crude IB pellet obtained from optimized protocol yielded us an ~5-50% improvement in the IBP purity across various proteins over the reported methods. We obtained an enrichment of the desired protein in the IBP to, by and large, ~75-85%. Based on this increase in purity level, we chose the modified IBP protocol for further biophysical characterization.

We dissolved the crude protein obtained from the modified IBP in chaotropic agents (GdnHCl or urea) and further refolded into detergents (LDAO or DPC), using reported methods, to carry out biophysical and functional analysis. On refolding, several of the 8-stranded β-barrel membrane proteins show a pronounced mobility shift on SDS-PAGE gels of unboiled samples.[3d, 3g, 5a, 5c, 5g, 5n, 19] Hence, we directly refolded crude IB pellets of Ail, OmpX and PagP in LDAO (Ail and OmpX) and DPC (PagP) micelles, and compared the results with their purified counterparts generated using the reported methods. Additionally, we probed the stability of the refolded β-barrels by subjecting them to protease digestion. The results obtained (Fig. S9†), clearly indicate that comparable refolding efficiency of these proteins can be obtained directly from the crude protein without the need for extensive purifications.

Using far-UV circular dichroism (CD) scans, we compared the secondary structure content of all the crude proteins with purified samples (representative CD scans for Ail, OmpX and PagP are shown in Fig. 1B), and estimated secondary structure by Reed's method (Table S4†). Comparable molar ellipticities for all proteins, except PagP, suggest that the IBP-generated samples attain similar secondary structure, which is independent of the purification step. Interestingly, in PagP-Ec and PagP-St, we observe that sample refolded from the purified and modified IBP stock gave similar secondary as well as tertiary CD that is different from preparations directly refolded from the reported IBPs. The tertiary or near-UV CD, observed as a positive maximum at ~231 nm in PagP (Fig. 1B), is believed to arise from a spatially positioned rigid T-shaped interaction geometry between Trp 66 and Tyr 26 (numbering from PagP-Ec;[20]), and is an indicator of a folded barrel.[20] The conspicuous absence of the 231 nm band in the reported IBP

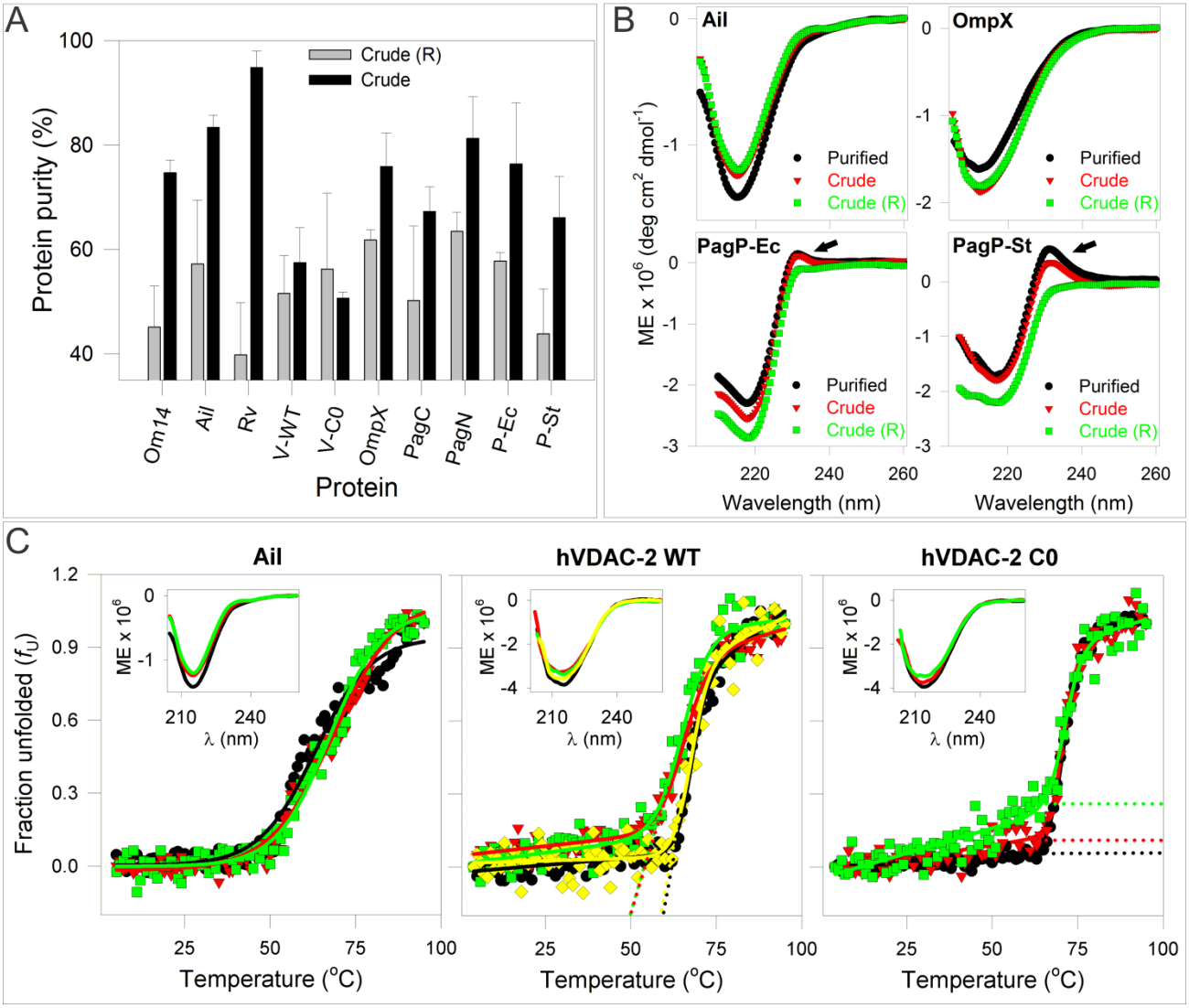


Fig. 1. Comparison of secondary structure and stability of refolded protein generated in crude and purified forms. (A) SDS-PAGE quantification of IB preparations generated using reported methods (Crude (R)) and the optimized (modified) IB protocol (Crude) discussed herein shows a ~5-50% improvement in purity of the preparation. Protein purity obtained using the modified IB preparation is, by and large, between 70-85%. Also see Figs. S7-S8† for comparison with the purified protein. (B) Far-UV CD spectra of Ail (top left) and OmpX (top right) show similar secondary structure content for the refolded protein obtained from purified (black circles) and crude preparations (reported IB protocols – green squares; modified IB protocol – red triangles). In PagP-Ec and PagP-St (bottom left and right), the CD profiles of protein obtained from purified and crude IBPs show considerable overlap, including the 231 nm near-UV CD peak (black arrow), indicative of tertiary structure, which is absent in the preparations directly refolded from the reported IBPs (Crude (R)). The conspicuous absence of the 231 nm band in the reported IBP may either indicate incorrect barrel folding or could result from substantial contribution from impurities that effectively mask the tertiary CD in this protein. See text for details. (C) Comparison of thermal denaturation profiles of Ail (left), hVDAC-2 WT (middle) and hVDAC-2 C0 (right) generated from the purified protein (reported IB protocols – black circles; modified IB protocol – yellow diamonds) and crude preparations (reported IB protocols – green squares; modified IB protocol – red triangles). Differences between the data obtained for hVDAC-2 preparations are highlighted by dotted lines. See text for details. In the case of hVDAC-2 C0, protein purities calculated using both IB preparations are comparable. However, we observe a difference in the unfolding cooperativity in thermal denaturation measurements. The interference here is from lipid impurities, which are retained in samples prepared using reported methods, but are substantially reduced in our IBPs. Solid lines represent fits to a sigmoidal equation for Ail, and two-state equation[21] for hVDAC-2 WT and C0. Insets show the far-UV CD wavelength scans for the refolded protein prepared using the different proteins and are color coded to match the thermal denaturation profiles. ME is in deg cm^2 $dmol^{-1}$.

may either indicate incorrect barrel folding or could result from substantial contribution from impurities that effectively mask the tertiary CD in this protein.

Our experiments indicate that if impurity levels exceed ~40%, significant interference is anticipated in the CD spectra of such membrane protein preparations due to contributions of these impurities to the measured wavelengths. The modified IBP does not pose such difficulties, supporting the direct utilization of this unpurified preparation for biophysical studies. Nevertheless, it is recommended to exercise caution in carrying out experiments with previously uncharacterized proteins; here, a preliminary analysis comparing experiments performed with both purified and crude protein preparations, for data reproducibility, is useful.

Temperature-dependent structural measurements indicate that IBPs provide similar thermal denaturation profiles as purified proteins

In order to distinguish whether the modified IBP-generated protein is stably refolded as its purified counterpart, we subjected the refolded protein to thermal denaturation measurements from 4 °C to 95 °C, and monitored the change in secondary structure content using far-UV CD at 215 nm (Fig. 1C). We observe that there is no influence of the IBP processing on the T_m (mid-point of thermal denaturation) of Ail, which suggests that protein has been stably refolded to the same extent in both the purified protein as well as samples generated directly from the modified IBP. In hVDAC-2, both WT and C0 constructs do not display a gel mobility shift upon refolding.[5e] Hence, we compared the secondary structure content of both refolded protein samples using CD wavelength scans (insets in Fig. 1C). The secondary structure content for both samples is compared in Table S4†, which shows that the refolded protein prepared from reported and our modified IBP pellets adopt similar structure and are comparable to the refolded protein prepared from purified stocks.

Interestingly, in hVDAC-2 WT, we observe a marginal lowering of T_m (from ~69 °C for the purified protein to ~65 °C for the refolded protein prepared directly from IBP) and a loss in the overall unfolding cooperativity (compare the extended transition lines in Fig. 1C, middle panel). This is accompanied by an ~10% reduction in secondary structure content (compared to purified protein), in the thermal denaturation profile, before nucleation of the unfolding transition. Upon purification of hVDAC-2 WT (from reported or our modified IBP), we observe that the cooperativity and protein stability are regained (Fig. 1C, middle panel). We have previously reported that hVDAC-2 WT has a higher affinity for lipids.[5o] We believe that some lipid molecules are likely to be retained even in the case of our modified IBPs of hVDAC-2 WT, despite the methanol treatment, and interfere in the thermal denaturation property. Cys-less hVDAC-2 (C0), which shows lowered protein-lipid affinity,[5o, 22] exhibits comparable thermal denaturation profiles (T_m and cooperativity) for our modified IBP and purified proteins (Fig. 1C, right panel). The crude hVDAC-2 C0 preparations generated from the reported protocols, show an

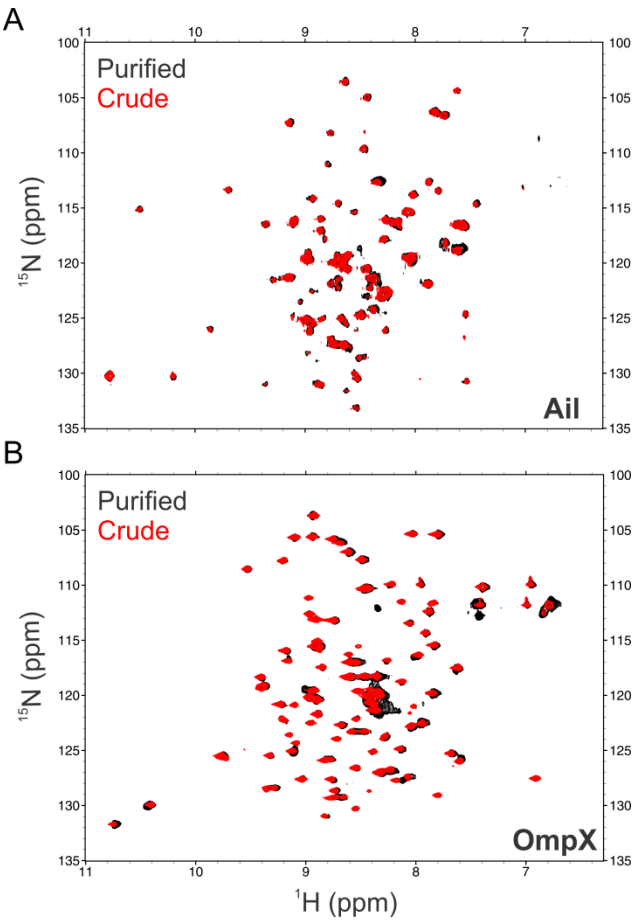


Fig. 2. ^{1}H-^{15}N HSQC spectra of uniformly ^{15}N labelled refolded Ail and OmpX. (A) Spectrum of ~0.1 mM Ail refolded directly from the crude IB protein (red) superposed on purified Ail (black) spectrum. Both proteins were refolded in ~175 mM LDAO micelles re-suspended in 20 mM Tris-HCl pH 8.5 and recorded at 40 °C. (B) Spectral dispersion of ~0.2 mM OmpX (crude IB protein; red) refolded in 100 mM LDAO prepared in 20 mM Tris-HCl pH 9.5, and acquired at 40 °C. This spectrum is overlaid on the HSQC of the refolded protein obtained after purification (black), and is adapted with permission from reference [5n].

~20% loss in protein structure (see the extended pre-transition baseline in Fig. 1C, right panel) due to interference from the impurities retained in this preparation indicating marginally unstable behaviour of the crude protein. This instability is no longer observed in our modified IBP-generated crude protein (Fig. 1C, right panel).

Unlike in the case of PagP (described in the previous section), the protein purities obtained from the various IBPs are comparable (Fig. 1A, Figs. S7-S8† and Table S5†). Differences in unfolding cooperativity observed in the CD thermal melts could arise due to interference from lipid impurities in the preparations. Since our IB protocol utilizes an organic solvent wash, such lipidic impurities are readily removed, providing us with protein preparations whose biophysical behaviour is similar to protein preparations that have been purified

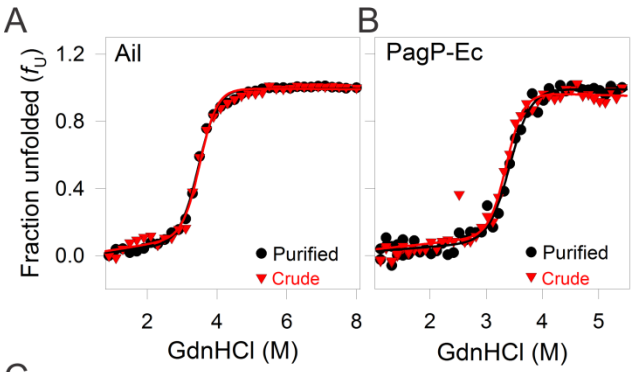
chromatographically. Hence, we deduce that impurities associated with the protein may lead to a considerable difference in the observed secondary structure content and biophysical characteristics of the protein under investigation. Based on our observations, if sufficiently pure protein can be generated from IBPs, these proteins can demonstrate biophysical characteristics that very closely resemble their purified counterparts, thereby eliminating the need for extensive chromatographic separation.

Crude protein refolded directly from the IB pellet is structurally similar to the purified protein

While gel mobility shifts and thermal denaturation measurements serve as excellent tools to determine the structural scaffold and stability of transmembrane β-barrels, residue-wise information at the atomic level provides deterministic verification of subtle variations in the protein structure. NMR-based HSQC measurements offer a quick means to estimate local structural changes arising from variations in the protein preparation and omission of the purification step in our IBP. HSQC spectra have previously been reported by us and other groups for Ail, OmpX and PagP-Ec. We therefore directly refolded uniformly ^{15}N-labeled protein generated from the IBP, without purification, into LDAO micelles. We then acquired ^{1}H-^{15}N HSQC spectra of these samples and compared the spectral dispersion and peak overlap with the purified protein, folded under identical conditions. The superimposed spectra for Ail and OmpX are provided in Fig. 2. It is evident that most of the peaks in the HSQC spectra of the crude and purified protein overlap with each other, again inferring equal extent of refolding and similar barrel structures for the crude as well as purified proteins.

Fluorescence-based equilibrium thermodynamic experiments substantiate use of rapid IBPs in biophysical studies

Understanding the folding and unfolding process of β-barrel proteins to derive molecular information on membrane protein behaviour and key stabilization factors are crucial to engineer these proteins as drug targets and in vaccine development. Using tryptophan fluorescence, we investigated whether residual protein and lipid impurities retained in the crude IB preparation affects the (un)folding pathways of these transmembrane proteins. Figs. 3A and 3B show the superposition of the observed refolding pathways of Ail and PagP-Ec, respectively; corresponding (representative) changes in fluorescence emission profiles are shown in Fig. S10†. The presence of protein impurities that are particularly enriched with Trp residues could significantly interfere in equilibrium refolding experiments that employ Trp fluorescence measurements. While there may be minor difference in the observed emission intensities between our crude IBPs and purified proteins, we do not perceive any observable difference in the corresponding folding thermodynamics data. As our protein preparations are ≥75% pure, at these purity levels, we do not observe interference from protein impurities on equilibrium fluorescence measurements.



C

Protein	Protocol	$\Delta G_F^{0,H_2O}$ (kcal/mol)	*m* value (kcal/mol.M)	C_m (M)
Ail	Purified	-7.53 ± 0.4	2.17 ± 0.1	3.48
	Crude	-8.44 ± 0.7	2.40 ± 0.2	3.52
PagP-Ec	Purified	-10.81 ± 1.0	3.16 ± 0.3	3.42
	Crude	-12.90 ± 1.7	3.84 ± 0.5	3.36

Fig. 3. Equilibrium folding of Ail and PagP-Ec. Normalized Trp fluorescence for Ail monitored at 330 nm (A) and PagP-Ec at 339 nm (B) is plotted against GdnHCl concentration. Both proteins show superposed folding pathways for the purified (black circles) and the IBP-generated crude proteins (red triangles). Thermodynamic parameters ($\Delta G_F^{0,H2O}$, *m* value and C_m), derived from fits (solid lines in A and B) to a two-state equation, [5i] are tabulated in panel C. Errors represent goodness of fit.

We also derived the thermodynamic parameters such as $\Delta G_F^{0,H2O}$, *m* value and C_m (Fig. 3C), which only reflect minute differences in protein stability, folding cooperativity and folding efficiency between the folding pathway of the purified protein and crude IB protein. These fine observations suggest that either folding pathway of protein is not influenced by trace impurities present in the IB pellet or the protein is sufficiently pure for this type of studies. We propose herewith that the crude IBP product can be readily used for rapid and exhaustive screening of mutant libraries of any membrane protein, without the need for extensive purification procedures.

hVDAC-2 channel activity assessed in planar lipid bilayers

hVDAC-2 forms ion channels that exist in an open state (single channel conductance of ~3.5 nS) and show selectivity to anions at low voltages (till ± 30 mV); this protein shifts into a closed sub-conductance state and exhibits cation selectivity at higher voltages.[11] Fig. 4 shows the behaviour of purified hVDAC-2 channels (WT and the Cys-less mutant C0) in response to a voltage ramp ranging from 0 mV to ± 60 mV. The steep slope at low voltages and the shallow slope at higher voltages depict the open and closed state of the channel, respectively. Fig. 4 also presents the response of hVDAC-2 refolded without purification from the IB pellet (of the modified IBP protocol). Unlike the thermal denaturation measurements described earlier (Fig. 1C), we do not observe any significant differences between the purified protein prepared using the reported

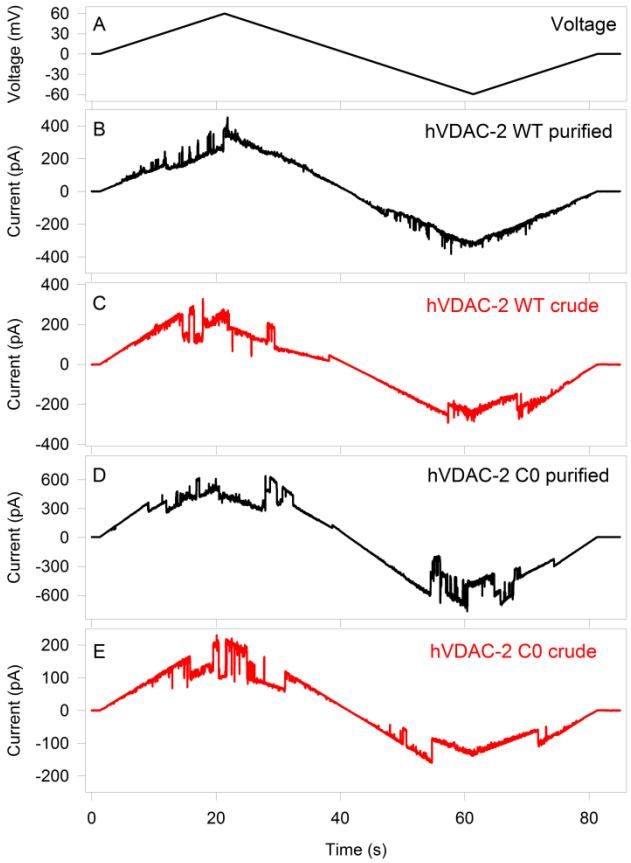


Fig. 4. Voltage ramps monitoring channel conductance of hVDAC-2 WT and C0. Representative responses of hVDAC-2 WT (B and C) and hVDAC-2 C0 (D and E) to an applied voltage ramp (A) from 0 mV to ± 60 mV at a rate of 3 mV/s. Data from the purified proteins are in B and D (black), while C and E (red) represent data from the crude protein refolded directly from the IBP. Both the purified and crude IB proteins show similar response to voltage, with steeper slopes at lower voltages (indicating open channels) and shallower slopes at higher voltages (indicating closed channels). 2-4 active channels were present in the membrane during each recording. Data in panels B and D are adapted with permission from reference [22].

protocols,[5o] and the IBP pellet obtained using the modified protocol for both WT (Figs. 4B and 4C) and C0 (Figs. 4D and 4E). To the best of our knowledge, this is the first successful evidence for the direct use of a mammalian protein refolded from inclusion bodies in biochemical studies as well as meaningful electrophysiology measurements, without any chromatographic purification. Use of the proposed rapid IBP protein pellet directly for biochemical studies and functional assays provides us with similar results as the purified protein, and further validates the advantages of this approach.

Conclusions

With increasing interest in the structural and functional characterization of membrane proteins, it is imperative to generate sufficient protein amounts to screen refolding conditions and biophysical experiments.[23] Additionally, producing sufficiently homogeneous protein preparations, rapid generation of mutant libraries and systematic studies that demand extensive protein preparations and purifications involve significant time and are labour-intensive.

Our rapid IBP protocol can not only be widely used for several membrane proteins, but can work as an effective tool for generating protein preparations of sufficient purity, which can be directly utilized in several biophysical studies, saving cost, labour and considerable amount of time – in our studies, the complete protein preparation is completed in about half a day. Crude IBPs of transmembrane barrels from bacteria and humans provide us with largely indistinguishable biochemical, functional and biophysical results with respect to their purified counterparts – indeed, we observe that 70-80% protein purity is sufficient for these experiments.

The use of crude protein preparations for structural and functional studies is expected to draw greater usefulness and widespread application in facilitating extensive, yet effortless, characterization of newly discovered membrane proteins.

Experimental Section

Cloning, protein expression and purification

Genes for all proteins described herein (Table S1†) were cloned in pET-3a vector under an IPTG-inducible promoter, overexpressed as inclusion bodies in modified *E. coli* BL21 cells, and purified using reported protocols.[5k, 5n, 5o, 12] Details are available in supplementary data (ESI†).

Gel quantitation and protein folding

Densitometry analysis of Coomassie-stained bands on SDS-PAGE was carried out using MultiGauge v2.3.[5m] Intensities of background-subtracted bands were used to calculate the percent purity of the band of interest, using:

% purity = (Intensity of desired band / Intensity of total lane) x 100

Refolding of OmpX, Ail and hVDAC-2 were carried out in lauryldimethylamine oxide (LDAO) micelles as reported earlier.[5k, 5n, 5o, 9b] PagP refolding was carried out using minor modifications of reported methods.[5n] Briefly, PagP protein denatured in 8.0 M urea was rapidly diluted 10-fold into 100 mM dodecylphosphocholine (DPC) micelles prepared in 20 mM Tris-HCl pH 9.5, and heat shock was administered, as reported.[5n] All 8-stranded β-barrels were tested for successful refolding using gel mobility shifts of unboiled samples, on 15% SDS-PAGE gels.[3d, 3g, 5a, 5c, 5g, 5k, 5n, 19] Refolding efficiency was calculated by densitometry using the formula:

% folded = [(Folded band intensity) / (Folded band intensity + Unfolded band intensity)] x 100

CD measurements

Far-UV circular dichroism (CD) spectra of the refolded protein was recorded from ~200 - 280 nm, using sample concentrations of 0.5 μg/μl in 25 mM LDAO for Ail,[9b] 0.5 μg/μl in 20 mM

LDAO for OmpX,[5m] 0.16 μg/μl in 13 mM LDAO for hVDAC-2,[5o] and 0.06 μg/μl in 10 mM DPC for PagP-Ec and PagP-St. These protein and detergent (LDAO or DPC) concentrations were chosen based on reported studies or independent stability screens (not shown). The parameters for CD spectra acquisition, thermal denaturation and processing were carried out using reported protocols.[5n] The data was normalized using reported methods,[21] and fit to a two-state equation[21] or a sigmoidal function, to derive the T_m.

HSQC measurements

^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) data of uniformly ^{15}N-labeled samples were recorded on a Bruker Avance III 700 MHz FT-NMR instrument using a cryoprobe, and standard pulse programs available in the Bruker library. Spectra for OmpX were acquired using ~0.2 mM protein refolded in 100 mM LDAO micelles at 40 °C in 20 mM Tris pH 9.5, as reported.[5n] Ail HSQCs were acquired at 40 °C using ~0.1 mM protein refolded in 176 mM LDAO containing 20 mM Tris-HCl pH 8.5. A total of 1024 points in t2 and 256 t1 increments were acquired and data were processed using NMRPipe,[24] with a linear prediction to 512 t1 points. Plots were generated using Sparky.[25]

Equilibrium refolding experiments

GdnHCl-mediated equilibrium refolding experiments were carried out by monitoring Trp fluorescence at 25 °C, using reported methods.[5i] In the case of Ail, 2.5 μg/μl protein in 8.0 M GdnHCl and 20 mM Tris pH 8.5 was diluted 5-fold into a GdnHCl gradient (8.0 M - 1.0 M) containing final concentrations of 25 mM LDAO.[9b] In the case of PagP-Ec, 0.6 μg/μl protein in ~8.0 M GdnHCl, 0.8 M urea, 20 mM Tris pH 9.5 was 10-fold diluted to the GdnHCl gradient (~5.5 M - 1.0 M) containing 10 mM DPC (final concentration). Unfolded fractions (f_U) were calculated,[5i] using Trp fluorescence intensity at 330 nm (for Ail) and 339 nm (for PagP). Data was fitted to a two-state equation,[5i] to derive thermodynamic parameters.

Channel conductance measurements

hVDAC-2 channel activity was studied on a black lipid membrane system, as described previously.[6k] Briefly, freshly refolded protein in 65 mM LDAO (0.1% cholesterol and 1% triton X-100 (TX100), supplemented after refolding), was incorporated in DiPhPC planar bilayers painted across a 200 μm aperture that separated two chambers containing 10 mM HEPES pH 7.4, 5 mM $CaCl_2$ and 1 M KCl. The channels were subjected to a triangular voltage ramp from +60 mV to -60 mV at 3 mV/s, and analysed as reported earlier.[6k]

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Notes and references

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† Electronic Supplementary Information (ESI) available: Detailed IBP optimization protocol, supplementary figures and tables. See DOI: 10.1039/b000000x/

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