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Towards a Universal Method for Protein Refolding: The Trimeric Beta Barrel Membrane Omp2a as a Test Case

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ABSTRACT: It has recently been reported that 2-methyl-2,4-pentanediol (MPD) can modulate the protein-binding properties of sodium dodecyl sulfate (SDS), turning it into a non-denaturing detergent. Indeed both alpha (the lysozyme) and beta (the carbonic anhydrase II) soluble enzymes, as well as a beta membrane protein (PagP) have been successfully refolded into their native form by using this amphiphatic alcohol. In order to support the universal character of our MPD-based technique, we have extended its transferability to the Omp2a trimeric membrane porin. The far-UV circular dichroism signature of Omp2a refolded with our original procedure is identical to that obtained by classical techniques, clearly indicating a proper refolding. Moreover, we show that the optimal SDS/MPD ratio for refolding Omp2a is similar to what has been observed for other types of proteins. While the protocol allows refolding at higher protein concentration (up to 4 mg/mL) and ionic strength (up to 1 M NaCl) than other refolding methods, it is also more efficient at basic pH values and medium temperature (20–40°C). Finally, the key role of the cosolvent was highlighted by a thorough study of the efficiency of MPD analogues, and a high variability was observed, as they can be able or unable to induce refolding at low or high salt concentrations.

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KEYWORDS: refolding; protein; circular dichroism; 2-methyl-2,4-pentanediol; sodium dodecyl sulfate

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Introduction

For decades, the structural determination of membrane proteins (MPs) has been an important challenge in structural biology due to their embedment in the lipid bilayer (Booth, 2003; Bowie, 2005; Jungbauer and Kaar, 2007). Indeed, MPs play a central role in many cellular and physiological processes but remain extremely difficult to isolate in a native form. In *Escherichia coli*, a usual strategy is to express MPs in cytosolic inclusion bodies (IB) (Bannwarth and Schulz, 2003) as it allows to overproduce in high concentration these usually membrane-toxic proteins. These IBs are resistant against degradation by proteolysis and they boost the protein expression up to several orders of magnitude, which is a significant advantage for subsequent structural investigations (Junge et al., 2008). Unfortunately, MPs in IB adopt non-native conformation, and it is a crucial issue to obtain the functional form of the proteins. Although a large number of detergents is commercially available (Tulumello and Deber, 2012), none has the optimal properties required for all steps (solubilization, purification, and refolding) involved in a structural study. As a result, the classical procedures (Seddon et al., 2004) usually include the screening of several detergents (or detergent-likes (Dill et al., 2007; Zhang et al., 2011)) mimicking the properties of the membrane as well as the optimization of the physicochemical environment inducing the best refolding, and the cost-effectiveness of such protein-wasting and time-consuming strategies is often highly questionable.

Recently, a simple and effective method has been developed to recover active (refolded) proteins. It is based on the association of an anionic detergent (the sodium dodecyl sulfate (SDS), known as denaturing agent) with an amphipathic diol solvent (the 2-methyl-2,4-pentanediol (MPD)), and it has been shown that concentrations of 1-2 M MPD can modulate the protein-binding properties of SDS

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and remodel a SDS-denatured protein into its native state. This cosolvent effect has previously been observed on soluble (human anhydrase carbonic and lysozyme) and bacterial membrane proteins (PagP, an 8-stranded beta barrel). In the presence of MPD, the SDS detergent seems to behave as a "gentle" non-denaturing detergent. Crystallographic experiments implying the lysozyme (Michaux et al., 2008b) and PagP (Cuesta-Seijo et al., 2010) confirm the recovery of the native form in presence of MPD from a SDS-denatured state. This technique presents significant advantages: (i) SDS can substitute the classical solubilizing agents (urea, guanidine chloride) interfering with biophysical measurements and refolding processes and (ii) can solubilize IB and keep proteins soluble (Boisselier et al., 2011). (iii) The use of SDS is compatible with protein crystallization, and (iv) the system is relevant for proteins with different secondary structures or sizes.

In this article, we describe how our original method was able to refold a trimeric membrane protein, Omp2a from *Brucella melitensis* (Boschiroli et al., 2001; Douglas et al., 1984; Paquet et al., 2000). To prove the successful running of the process, it has been compared to a classical protocol involving the *n*-dodecyl-β-D-maltopyranoside (DDM) (Roussel et al., 2012). Not only the efficiency of our SDS/MPD technique is demonstrated, the results also confirm again its transferability.

Materials and Methods

Bacterial Strain and Growth

Cells of *E. coli* BL21 (DE3) carrying pLysS and pET2a plasmids (containing the gene *omp2a* without peptide signal) were grown in LB medium at 37°C with constant shaking. Log cultures (OD 0.6) of 500 mL were stimulated with IPTG (0.2 μg/mL) for 3 h. Cells were then harvested by centrifugation at 4,000 rpm for 30 min, and the resulting bacterial pellets were stored at −20°C. The pET2a plasmid contains the *omp2a* coding sequence, in which the 22 first codons were removed, as previously reported for Omp2b overproduction (Paquet et al., 2001; Roussel et al., 2012).

Overexpression and Non-Native Purification of Omp2a

The bacterial pellets were thawed and treated with 8 mL of TEN lysis buffer (50 mM Tris–HCl pH 8, 1 mM EDTA, 17 mM NaCl, 125 μM PMSF, 250 μg/mL lysozyme) for 20 min at 25°C. Harvested cells were further broken by addition of 10 mg of sodium deoxycholate for 60 min at 37°C with constant shaking, and 2 mg of DNase I (Sigma AMPD1-1KT) for 60 min at 25°C. The suspension was then centrifuged at 14,000*g* for 20 min at 4°C. The resulting pellet underwent a washing buffer (2 M urea, 20 mM Tris–HCl pH 8, 500 mM NaCl, 2% Triton X-100) and centrifuged at 14,000*g* for 20 min at 4°C. The inclusion bodies (IB) were solubilized with 8 mL of TEN buffer (50 mM Tris–HCl pH 8, 1 mM EDTA, 17 mM NaCl, 8 M urea). The solubilized proteins were then applied onto an anion-exchange DEAE column (1.5 cm × 5 cm) previously equilibrated with 25 mL of buffer A (50 mM Tris–HCl pH 8, 17 mM NaCl, 8 M urea). Omp2a was eluted with a 50 mL linear gradient of NaCl from 17 to 500 mM whereas the protein profile was further analyzed using SDS–PAGE. Fractions containing 39 kDa proteins were then pooled and stored at 4°C. The protein concentration was estimated using the Nanodrop® system by measuring the A_{280} ($\varepsilon_{calculated} = 85{,}300\ M^{-1}\ cm^{-1}$, calculated from ProtParam (Gill and Von Hippel, 1989)).

Omp2a Refolding in SDS–MPD System

To refold Omp2a, the protein solution (1 mg/mL protein, 250 mM NaCl, 50 mM Tris–HCl pH 8 and 8 M urea) was eluted onto a PD-10 column to exchange the buffer (150 mM NaCl, 50 mM Tris–HCl pH 8, and 120 mM SDS which is 15 times the critical micellar concentration, CMC). SDS-unfolded sample were then diluted 1:1 in a refolding solution (50 mM Tris–HCl pH 8, 150 mM NaCl, 3 M MPD). The protein solution was then incubated at room temperature (RT). The samples were stored at −20°C to stop the refolding reaction.

SDS–PAGE Analysis

Samples (20 μL) were loaded on 15% acrylamide SDS–PAGE gels without boiling. After electrophoresis, the gels were stained with Coomassie Blue or silver nitrate and digitally scanned. Densitometry was performed using ImageJ software (Collins, 2007). The linear regions in the densitometry profile were determined by measuring the density of standards with known protein amounts. The refolded fraction was estimated by dividing the intensity of the trimer band by the sum of the intensities of both monomeric bands. The error bars associated with each data point represent the standard deviation of three independent experiments.

Circular Dichroism Measurements

CD measurements were performed at 20°C, with a Jasco J-810 spectropolarimeter, using a protein concentration of ~0.1 mg mL^{-1} and a 0.1 cm cell path length. The buffer was 150 mM NaF, 50 mM H_2PO_4 pH 8 with 60 mM SDS, and 1.5 M MPD. Spectra were acquired at a scan speed of 10 nm min^{-1}, with a 0.2 nm data pitch, using a 1 nm bandwidth and a 4 s digital integration time. The spectra were averaged after four accumulations and corrected by subtraction of the buffer spectrum obtained under the same conditions. Ellipticity measurements in mdeg were converted in molar mean residue ellipticity by considering a mean residue weight value of 107.85 Da.

Results and Discussion

Background

The refolding effect of MPD in a SDS environment has already been observed on a wide range of proteins including soluble and membrane proteins, and millimolar and molar concentrations of detergent and cosolvent, respectively, are required to induce the renaturation. The activity and structure of both the hen egg-white lysozyme and the human carbonic anhydrase II vanish in SDS concentrations above 1 mM (that is below the expected CMC of 8.4 mM) but are recovered after addition of 2 M MPD. With respect to soluble enzymes, integral membrane proteins need higher SDS concentrations to be unfolded. For instance, PagP is unfolded in 70 mM SDS (above the CMC) while MPD concentrations in the range of 1–2 M induce the refolding.

Optimization of the Detergent/Cosolvent Ratio for the Refolding of Omp2a

SDS and MPD concentrations were scanned in the millimolar and molar range, respectively, to determine the optimum concentrations for Omp2a refolding. Protein samples were prepared directly from inclusion bodies in 50 mM Tris–HCl pH 8, 150 mM NaCl at room temperature (RT), while SDS and MPD were added by the dilution method (Jungbauer and Kaar, 2007). During electrophoresis, folded Omp2a (trimers, 115 kDa) that is SDS-resistant (Manning and Colo, 2004) migrates to a different position than denatured species (monomers, 39 kDa). This shift in the apparent molecular mass was used to determine by densitometry the fraction of refolded proteins after 72 h under given conditions. Figure 1 shows the refolded fractions of Omp2a in SDS and MPD concentrations respectively going from 0 to 100 mM and from 0 to 2.5 M.

As expected in the SDS-only buffer, no refolding takes place, that is, no trimers can be observed. Indeed, this detergent is well known to induce unfolding at millimolar concentrations (Manning and Colo, 2004; Nielsen et al., 2007). On the other hand, MPD was unable to induce the refolding of Omp2a in absence of SDS, and one can conclude that the performance of the system depends on the actual balance between the SDS and MPD concentrations. That is, the combination of SDS (between 40 and 100 mM) and MPD (from 1.0 to 2.5 M) enables the refolding with an optimum at 60 mM SDS and 1.5 M MPD. This observation is in agreement with what has already be pointed out for the monomeric β-barrel membrane enzyme PagP, where 70 mM SDS and 1 M MPD could optimally refold the protein. It also confirms the key role of the millimolar/molar ratio of SDS and MPD in the process. This is the very first time our method successfully achieved a proper refold of a multimeric membrane protein, as demonstrated by the CD signature of the SDS–MPD refolded Omp2a that perfectly matches the classical method results (see CD spectra below).

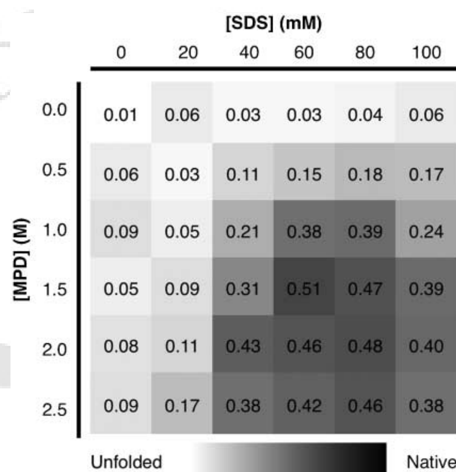

Figure 1. Folded fraction of Omp2a in various SDS and MPD concentrations. Omp2a was incubated 72 h at room temperature in various amounts of SDS and MPD. The buffer was 50 mM Tris–HCl pH 8, 150 mM NaCl. After electrophoresis, the relative folded fraction was determined by densitometry and values represent the mean of three independent experiments.

Influence of Physicochemical Parameters on Refolding

To determine the best conditions for optimal yields of trimeric Omp2a and to understand how the physicochemical parameters influence the SDS–MPD system, the effects of protein concentration, temperature, pH, and ionic strength were individually investigated (Fig. 2 A–D).

Typically, membrane proteins refolding process is limited to protein concentrations ranging from 0.1 to 1 mg/mL due to the predisposition of this class of protein to form hydrophobic patches and aggregates (Batas et al., 1999; Goldberg et al., 1991). In our case, the best folding efficiencies (>55%) were obtained at higher protein concentrations (2–7 mg/mL) in 150 mM NaCl, RT, 50 mM Tris–HCl pH 8, 60 mM SDS, and 1.5 M MPD (Fig. 2A) with an optimum at 4 mg/mL (59 ± 9%). This observation differs from results obtained in DDM were refolding was more efficient at low protein concentrations (from 0.1 to 1.0 mg/mL), probably due to the membrane protein solubilization properties of the SDS (Chuang et al., 2011; Keller et al., 2006; le Maire et al., 2000; Rath et al., 2009). Working at high protein concentrations with SDS could increase the contacts between monomers and their assembly in trimers. This potential advantage of higher concentrations will also allow to ease further structural studies by X-ray diffraction.

A higher incubation temperature increases the thermal energy available for the refolding. The temperature effect is most critical on high molecular weights MPs (Burgess et al., 2008) such as OmpF and Omp85, for which the aggregation occurs at temperature above 50°C (Surrey and Jähnig, 1995). We therefore investigated the effect of this parameter on Omp2a renaturation by repeating the folding experiments at

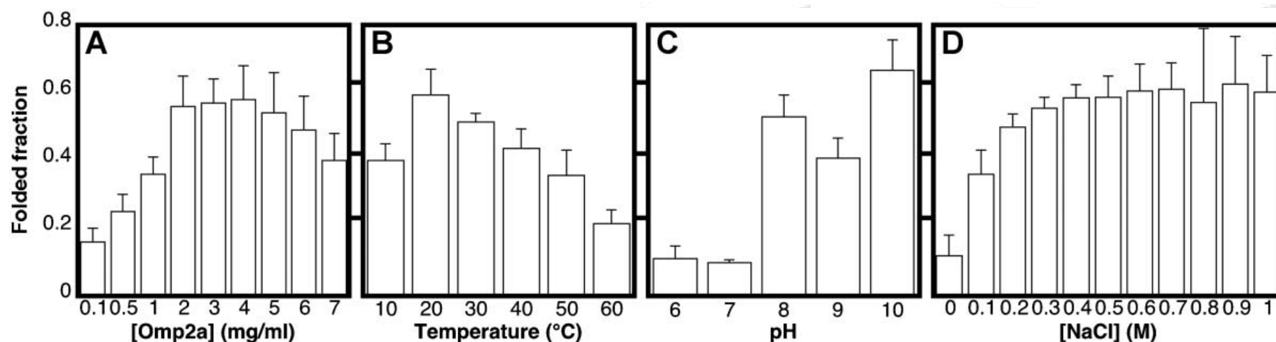

Figure 2. Influence of physico-chemical parameters on SDS–MPD refolding. Omp2a was incubated 72 h under given sets of conditions. We used SDS–PAGE and densitometry to determine the relative folded fraction of three independent experiments. **A**: Effect of the protein concentration. Omp2a was refolded at RT in 50 mM Tris–HCl pH 8, 150 mM NaCl, 60 mM SDS, and 1.5 M MPD. **B**: Effect of the incubation temperature. Omp2a was refolded in 50 mM Tris–HCl pH 8, 150 mM NaCl, 60 mM SDS, and 1.5 M MPD. **C**: Effect of pH buffer values. The protein was refolded at RT in 50 mM Tris–HCl, 150 mM NaCl, 60 mM SDS, and 1.5 M MPD. **D**: Effect of ionic strength. Omp2a was refolded at RT in 50 mM Tris–HCl pH 8, 60 mM SDS, and 1.5 M MPD.

temperatures ranging from 10 to 60°C in 150 mM NaCl, 50 mM Tris–HCl pH 8, 60 mM SDS, and 1.5 M MPD and 1 mg/mL protein. Figure 2B shows that refolding is optimal at 20°C (59 ± 8%) and the renaturation efficiency fades away at higher temperatures, similarly to other high molecular weights MPs. Nevertheless, a minor refolding (as in DDM) is still observed at 60°C, suggesting that the folded Omp2a is probably quite stable in comparison to other MPs.

The ionic strength can change the properties of detergents, affect the protein-detergent interactions (Negin and Carbeck, 2002) and consequently the solubility of the protein (Ruckenstein and Shulgin, 2006). It is well known that NaCl plays an important role on the distribution of negative charges by shielding the positive headgroup (Chen et al., 1986). This results in negligible electronic interactions between SDS monomers at salt concentrations higher than 400 mM (Corti, 1981). The addition of NaCl induces an increase in the micellar size and promotes a shape transition from spherical micelles to prolate ellipsoids and flexible rods (Nielsen et al., 2007; Otzen, 2002). We have consequently modulated the ionic strength of the refolding solution by increasing the concentration of NaCl up to 1 M, in 50 mM Tris–HCl pH 8, 60 mM SDS, and 1.5 M MPD at RT (Fig. 2D). As in DDM, Omp2a renaturation is gradually favored by salt concentration up to a maximum (58 ± 4%) at 400 mM but while yields decrease in DDM at high salt concentrations, SDS keeps the protein in a soluble state leading to higher refolding rates. That is, our method is efficient in a larger range that the classical protocol.

In vivo, OMPs fold in the periplasm of bacteria where the pH is determined by the extracellular environment that is mainly basic (Wilks and Slonczewski, 2007). As a result, β-barrel proteins, like PagP, OmpA, OmpX, Omp85, and OmpF all efficiently fold at high pH values (8–10) (Huysmans et al., 2007; Surrey et al., 1996; Tamm et al., 2004). To focus on the electrostatics of protein-detergent interactions at constant ionic strength, the refolding was followed while varying pH values from 6 to 10 in 150 mM NaCl, RT, 50 mM Tris–HCl, 60 mM SDS, and 1.5 M MPD, and it has been observed to be much more efficient (up to 67 ± 9%) at basic pH values (see Fig. 2C). Omp2a has a theoretical p*I* between 4.5 and 5 (calculated using ExPASy (Wilkins et al., 1999)) and therefore bears a negative charge in the tested pH range. This suggests that the strong pH-dependence observed is not correlated to the protein but more likely to the SDS–MPD system itself.

Efficient Refolding of Omp2a in SDS/MPD

By combining all the optimal settings, we achieved the full refolding of Omp2a (4 mg/mL) within 4 days at 20°C in 50 mM Tris–HCl pH 10, 800 mM NaCl, 60 mM SDS, and 1.5 MPD (Fig. 3A). Although the rate of the reassembly is slower than in vivo experiments (range of the second (Reid et al., 1988)), the present in vitro kinetics is similar to other MPs (Kleinschmidt, 2003; Kleinschmidt and Tamm, 1996; Watanabe and Inoko, 2009) and perfectly matches the results obtained in DDM (Roussel et al., 2012).

As shown by far-UV CD measurements (Fig. 3B), the native structure of Omp2a was recovered after 4 days. The spectrum of the SDS-unfolded Omp2a (solid line; sample directly frozen after addition of MPD) shows an α-helix profile with a double minimum at 208 and 222 nm, typical for SDS-unfolded proteins (Manning and Colo, 2004; Mattice et al., 1976; Nielsen et al., 2007). Little is known about the mechanism by which SDS-unfolding occurs (Bhuyan, 2010; Dutta et al., 2010). In contrast, the refolded sample (dashed line; after 4 days refolding) displays spectral features that are compatible with predominantly β-stranded proteins (Venyaminov and Yang, 1996). These

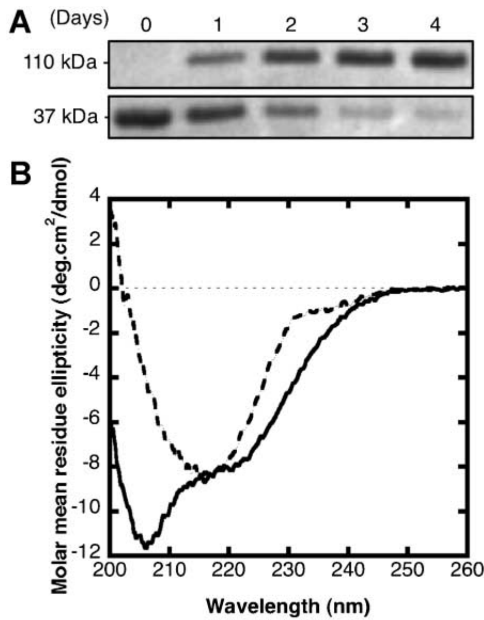

Figure 3. Characterization of totally folded Omp2a. Omp2a (4 mg/mL) was incubated at 20°C in 50 mM Tris–HCl pH 10, 800 mM NaCl. **A**: Time-course of reassembly. Refolding was quenched by freezing samples after incubation for the period shown in the figure. Monomers migrate at 39 kDa while trimers show an apparent molecular weight of 110 kDa. **B**: Far-UV spectrum of a SDS-unfolded sample (day 0, bold line) and totally folded sample (day 4, dashed line).

results are similar to spectra obtained for other porins (Siritapetawee et al., 2004; Visudtiphole et al., 2005) and reproduce what can be obtained in DDM. The transition from an α-helix structure to a β-stranded structure during the refolding is likely due to the presence of MPD, and it has been hypothesized that the cosolvent interacts more strongly with SDS than with the protein (Michaux et al., 2008a). As a consequence, the strong denaturing interactions between SDS and the protein (Gudiksen et al., 2006; Reynolds et al., 1967; Valstar et al., 1999) are altered and the refolding of the protein is enabled.

Effect of the Alcohol Nature on Refolding

How MPD regulates the SDS properties is still unclear. It is well known that addition of alcohol can strongly influence the shape of the SDS micelles and increase or decrease the micellar size depending on the hydrophilic/hydrophobic character of the alcohol (Akhter, 1999; Bolkhus et al., 2004; Candau et al., 1982; Forland et al., 1994; Stephany et al., 1994), but the intricated behavior of the mixed (detergent/cosolvent) micellar aggregates makes it difficult to predict any variation of the system. Hints can be sought by testing analogues of MPD (1.5 M) for their ability to change the denaturing effect of SDS and to induce the refolding. Figure 4 compares SDS–PAGE results for the refolding of SDS-denatured Omp2a in the presence of various cosolvents in the optimal conditions (Fig. 4A, 800 mM NaCl, 50 mM Tris–HCl pH 10, 60 mM SDS, and 1.5 M cosolvents) with previously described results (Michaux et al., 2008a) (Fig. 4B, 150 mM NaCl, 50 mM Tris–HCl pH 8, 60 mM SDS). Four situations can be observed: (i) cosolvents that are ineffective to change the denaturing properties of SDS at low or high ionic strength; (ii) alcohols able to (completely or partially) induce the refolding of SDS-denatured Omp2a at any ionic strength; cosolvents inducing refolding only; (iii) at low ionic strength; or (iv) at high salt concentrations. At low ionic strength we observe a correlation between the

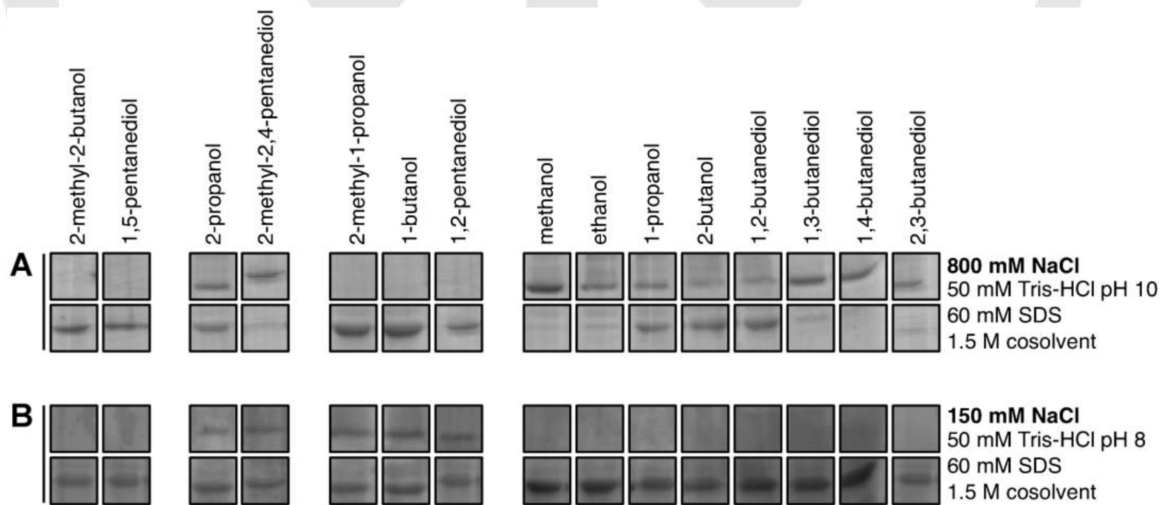

Figure 4. Effect of various cosolvents on SDS refolding. Omp2a (4 mg/mL) was incubated 4 days at 20°C in the presence of SDS (60 mM) and different cosolvents (1.5 M) and (**A**) 50 mM Tris–HCl pH 10, 800 mM NaCl, 60 mM SDS or (**B**) 50 mM Tris–HCl pH 8, 150 mM NaCl, and 60 mM SDS (below). **(i)** Ineffective cosolvents. **(ii)** Effective cosolvents. **(iii)** Effective cosolvents at a low ionic strength **(iv)** Effective cosolvents at high ionic strength.

ability to induce refolding from a SDS-unfolded state and the hydrophobicity of the cosolvent, similarly to what was observed for PagP (Michaux et al., 2008a). As an extra complexity level in the refolding process, the behavior of the SDS/MPD mixture is however strongly affected by adding salt. Though some cosolvents can induce renaturation at low ionic strength, most of the cosolvents need a significant increase in NaCl concentration to turn the SDS into a gentle detergent and to allow the renaturation of the protein from the SDS-unfolded state. Amongst the cosolvents able to induce refolding at low or high salt concentration, MPD gives the best result (complete refolding in 4 days at 800 mM NaCl). This observation clearly underlines the key role of the SDS/MPD binary and its specificity to induce refolding, making it an essential issue when optimizing new renaturation protocols.

Conclusions

Our original procedure combining SDS and MPD was successfully applied to recover the native state of a protein, as a millimolar/molar ratio detergent/cosolvent ratio was able to induce the refolding of a trimeric membrane protein. This result demonstrates the transferability of this protocol to complex MPs. By tuning the temperature, protein concentration, pH and ionic strength, we were able to completely refold our Omp2a sample and the comparison with a method involving a classical detergent confirms the recovery of a native-like state. This new refolding method based on the SDS/MPD system avoids the preliminary screening of detergents coming with the classical methods, and with respect to other detergents it is efficient in a larger range of protein concentrations and ionic strength.

Combining SDS with various cosolvents, we confirm that the MPD is the most efficient universal cosolvent able to induce the refolding of a multimeric MPs.

The large ionic strength dependence of the system efficiency emphasizes its complexity and the intricated behavior of the mixed micellar aggregates makes it difficult to predict any variation in the system. A complete description of this system requires the understanding of the complex interplay between protein-SDS, protein-cosolvent, and SDS-cosolvent interactions.

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References

Akhter MS. 1999. Effect of solubilization of alcohols on critical micelle concentration of non-aqueous micellar solutions. Colloids Surf A Physicochem Eng Aspects 157:203–210.

Bannwarth M, Schulz GE. 2003. The expression of outer membrane proteins for crystallization. Biochim Biophys Acta, Biomembr 1610:37–45.

Batas B, Schiraldi C, Chaudhuri JB. 1999. Inclusion body purification and protein refolding using microfiltration and size exclusion chromatography. J Biotechnol 68:149–158.

Bhuyan A. 2010. On the mechanism of SDS-induced protein denaturation. Biopolymers 93:186–199.

Boisselier E, Audet ML, Cantin L, Salesse C. 2011. A strategy for purifying glutathione S-transferase in the presence of sodium dodecyl sulfate. Biotechniques 51:193–194.

Bolkhus AM, Høiland H, Gilje E. 2004. The effect of inorganic salt on the solubilization of alcohols in aqueous surfactant solutions. J Colloid Interface Sci 124:125–129.

Booth PJ. 2003. The trials and tribulations of membrane protein folding in vitro. Biochim Biophys Acta Biomembr 1610:51–56.

Boschiroli ML, Foulongne V, O'Callaghan D. 2001. Brucellosis: A worldwide zoonosis. Curr Opin Microbiol 4:58–64.

Bowie JU. 2005. Solving the membrane protein folding problem. Nature 438:581–589.

Burgess NK, Dao TP, Stanley AM, Fleming KG. 2008. Beta-barrel proteins that reside in the Escherichia coli outer membrane in vivo demonstrate varied folding behavior in vitro. J Biol Chem 283:26748–26758.

Candau S, Hirsch E, Zana R. 1982. Effect of alcohol on the properties of micellar systems: IV. Effect of the isotopic composition of the system on the micellar properties in the presence and absence of 1-pentanol. J Colloid Interface Sci 88:428–436.

Chen JM, Su TM, Mou CY. 1986. Size of sodium dodecyl sulfate micelle in concentrated salt solutions. J Phys Chem 90:2418–2421.

Chuang JH, Kao YJ, Ruderman NB, Tung LC, Lin Y. 2011. Optimal concentrations of N-decanoyl-N-methylglucamine and sodium dodecyl sulfate allow the extraction and analysis of membrane proteins. Anal Biochem 418:298–300.

Collins T. 2007. ImageJ for microscopy. Biotechniques 43:S25–S30.

Corti M. 1981. Quasi-elastic light scattering study of intermicellar interactions in aqueous sodlum dodecyl sulfate solutions. J Phys Chem 85:711–717.

Cuesta-Seijo JA, Neale C, Khan MA, Moktar J, Tran C, Bishop RE, Pomès R, Privé GG. 2010. PagP crystallized from SDS/cosolvent reveals the route for phospholipid access to the hydrocarbon ruler. Structure 18:1210–1219.

Dill KA, Ozkan SB, Weikl TR, Chodera JD, Voelz VA. 2007. The protein folding problem: When will it be solved? Curr Opin Struct Biol 17:342–346.

Douglas JT, Rosenberg EY, Nikaido H, Verstreate DR, Winter AJ. 1984. Porins of Brucella species. Infect Immun 44:16–21.

Dutta A, Kim TY, Moeller M, Wu J, Alexiev U, Klein-Seetharaman J. 2010. Characterization of membrane protein non-native states. 2: The SDS-unfolded states of rhodopsin. Biochemistry 49:6329–6340.

Forland G, Samseth H, Hoiland H, Mortensen K. 1994. The effect of medium chain length alcohols on the micellar properties of sodium dodecyl sulfate in sodium chloride solutions. J Colloid Interface Sci 164:163–167.

Gill S, Von Hippel P. 1989. Calculation of protein extinction coefficients from amino acid sequence data. Anal Biochem 182:319–326.

Goldberg ME, Rudolph R, Jaenicke R. 1991. A kinetic study of the competition between renaturation and aggregation during the refolding of denatured-reduced egg white lysozyme. Biochemistry 30:2790–2797.

Gudiksen KL, Gitlin I, Whitesides GM. 2006. Differentiation of proteins based on characteristic patterns of association and denaturation in solutions of SDS. Proc Natl Acad Sci USA 103:7968–7972.

Huysmans GHM, Radford SE, Brockwell DJ, Baldwin SA. 2007. The N-terminal helix is a post-assembly clamp in the bacterial outer membrane protein PagP. J Mol Biol 373:529–540.

Jungbauer A, Kaar W. 2007. Current status of technical protein refolding. J Biotechnol 128:587–596.

Junge F, Schneider B, Reckel S, Schwarz D, Dötsch V, Bernhard F. 2008. Large-scale production of functional membrane proteins. Cell Mol Life Sci 65:1729–1755.

Keller S, Heerklotz H, Jahnke N, Blume A. 2006. Thermodynamics of lipid membrane solubilization by sodium dodecyl sulfate. Biophys J 90:4509–4521.

Kleinschmidt H, Tamm LK. 1996. Accelerated publications folding intermediates of a barrel membrane protein. Kinetic evidence for a multistep membrane insertion mechanism. Biochemistry 35:12993–13000.

Kleinschmidt JH. 2003. Membrane protein folding on the example of outer membrane protein A of Escherichia coli. Cell Mol Life Sci 60:1547–1558.

le Maire M, Champeil P, Moller JV. 2000. Interaction of membrane proteins and lipids with solubilizing detergents. Biochim Biophys Acta 1508:86–111.

Manning M, Colo W. 2004. Structural basis of protein kinetic stability: Resistance to sodium dodecyl sulfate suggests a central role for rigidity and a bias toward-sheet structure. Biochemistry 43:11248–11254.

Mattice WL, Riser JM, Clark DS. 1976. Conformational properties of the complexes formed by proteins and sodium dodecyl sulfate. Biochemistry 15:4264–4272.

Michaux C, Pomroy NC, Privé GG. 2008a. Refolding SDS-denatured proteins by the addition of amphipathic cosolvents. J Mol Biol 375:1477–1488.

Michaux C, Pouyez J, Wouters J, Privé GG. 2008b. Protecting role of cosolvents in protein denaturation by SDS: A structural study. BMC Struct Biol 8:29.

Negin RS, Carbeck JD. 2002. Measurement of electrostatic interactions in protein folding with the use of protein charge ladders. J Am Chem Soc 124:2911–2926.

Nielsen MM, Andersen KK, Westh P, Otzen DE. 2007. Unfolding of beta-sheet proteins in SDS. Biophys J 92:3674–3685.

Otzen DE. 2002. Protein unfolding in detergents: Effect of micelle structure, ionic strength, pH, and temperature. Biophys J 83:2219–2230.

Paquet J, Vinals C, Wouters J, Letesson J, Depiereux E. 2000. Topology prediction of Brucella abortus Omp2b and Omp2a porins after critical assessment of transmembrane beta strands prediction by several secondary structure prediciton methods. J Biomol Struct Dyn 17:747–757.

Paquet JY, Diaz MA, Genevrois S, Grayon M, Verger JM, De Bolle X, Lakey JH, Letesson JJ, Cloeckaert A. 2001. Molecular, antigenic, and functional analyses of Omp2b Porin size variants of Brucella spp. J Bacteriol 183:4839–4847.

Rath A, Glibowicka M, Nadeau VG, Chen G, Deber CM. 2009. Detergent binding explains anomalous SDS–PAGE. Proc Natl Acad Sci USA 106:1760–1765.

Reid J, Fung H, Gehrings K, Klebbag PE, Nikaido H. 1988. Targeting of porin to the outer membrane of Escherichia coli. Rate of trimer assembly and identification of a dimer intermediate. J Biol Chem 263:7753–7759.

Reynolds JA, Herbert S, Polet H, Steinhardt J. 1967. The binding of divers detergent anions to bovine serum albumin. Biochemistry 6:937–947.

Roussel G, Matagne A, De Bolle X, Perpète EA, Michaux C. 2012. Purification, refolding and characterization of the trimeric Omp2a outer membrane porin from Brucella melitensis. Protein Expr Purif 83:198–204.

Ruckenstein E, Shulgin IL. 2006. Effect of salts and organic additives on the solubility of proteins in aqueous solutions. Adv Colloid Interface Sci 123–126:97–103.

Seddon AM, Curnow P, Booth PJ. 2004. Membrane proteins, lipids and detergents: Not just a soap opera. Biochim Biophys Acta 1666:105–117.

Siritapetawee J, Prinz H, Krittanai C, Suginta W. 2004. Expression and refolding of Omp38 from Burkholderia pseudomallei and Burkholderia thailandensis, and its function as a diffusion porin. Biochem J 384:609–617.

Stephany SM, Kole TM, Fisch MR. 1994. Light scattering study of the effects of 1-Pentanol on solutions of sodium dodecyl sulfate in NaCl-H20 solutions. J Phys Chem 98:11126–11128.

Surrey T, Jähnig F. 1995. Kinetics of folding and membrane insertion of a beta-barrel membrane protein. J Biol Chem 270[Q1]:28199–22203.

Surrey T, Schmid A, Jähnig F. 1996. Folding and membrane insertion of the trimeric beta-barrel protein OmpF. Biochemistry 35:2283–2288.

Tamm LK, Hong H, Liang B. 2004. Folding and assembly of beta-barrel membrane proteins. Biochim Biophys Acta 1666:250–263.

Tulumello DV, Deber CM. 2012. Efficiency of detergents at maintaining membrane protein structures in their biologically relevant forms. Biochim Biophys Acta 1818:1–8.

Valstar A, Brown W, Almgren M. 1999. The lysozyme−sodium dodecyl sulfate system studied by dynamic and static light scattering. Langmuir 15:2366–2374.

Venyaminov SY, Yang JT. 1996. Determination of protein secondary structure. In: Fasman GD, editor. Circular dichroism and the conformational analysis of biomolecules. New York: Plenum Press. p 69–104.

Visudtiphole V, Thomas MB, Chalton DA[Q2], Lakey JH. 2005. Refolding of Escherichia coli outer membrane protein F in detergent creates LPS-free trimers and asymmetric dimers. Biochem J 392:375–381.

Watanabe Y, Inoko Y. 2009. Reassembly of an integral oligomeric membrane protein OmpF porin in n-octyl beta-D-glucopyranoside-lipids mixtures. Protein J 28:66–73.

Wilkins MR, Gasteiger E, Bairoch A, Sanchez JC, Williams KL, Appel RD, Hochstrasser DF. 1999. Protein identification and analysis tools in the ExPASy server. Methods Mol Biol 112:531–552.

Wilks JC, Slonczewski JL. 2007. pH of the cytoplasm and periplasm of Escherichia coli: Rapid measurement by green fluorescent protein fluorimetry. J Bacteriol 189:5601–5607.

Zhang Q, Tao H, Hong W-X. 2011. New amphiphiles for membrane protein structural biology. Methods 55:318–323.

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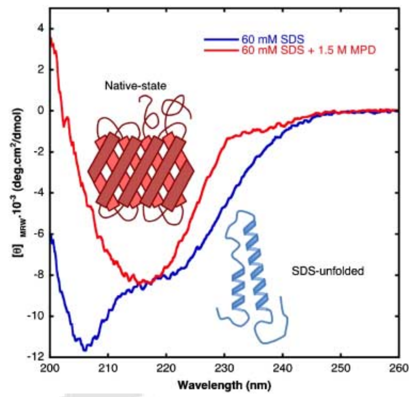

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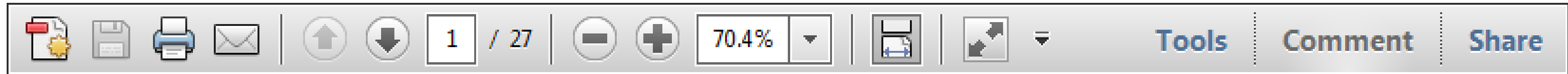

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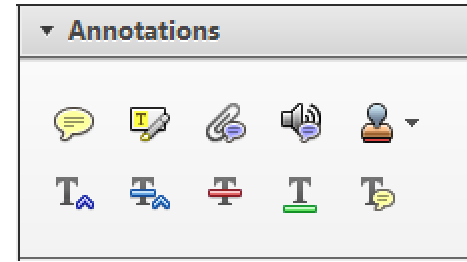

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Strikes a line through text and opens up a text box where replacement text can be entered.

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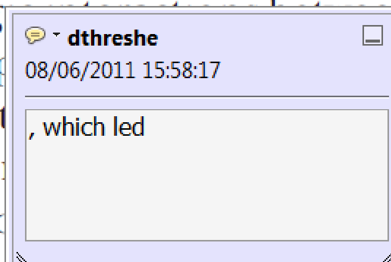

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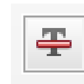

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

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- Click at the point in the proof where the comment should be inserted.
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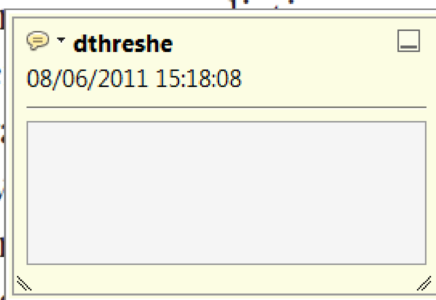


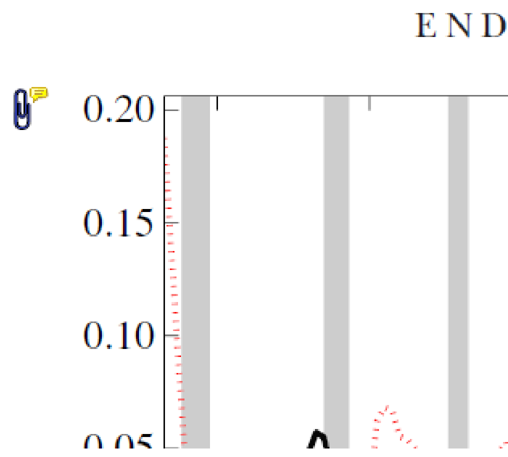
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.



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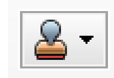

Inserts a selected stamp onto an appropriate place in the proof.

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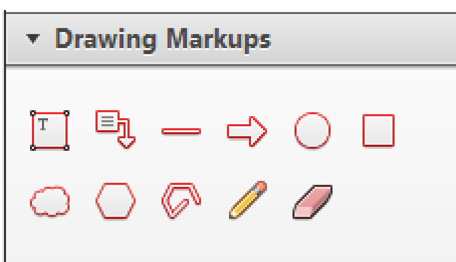

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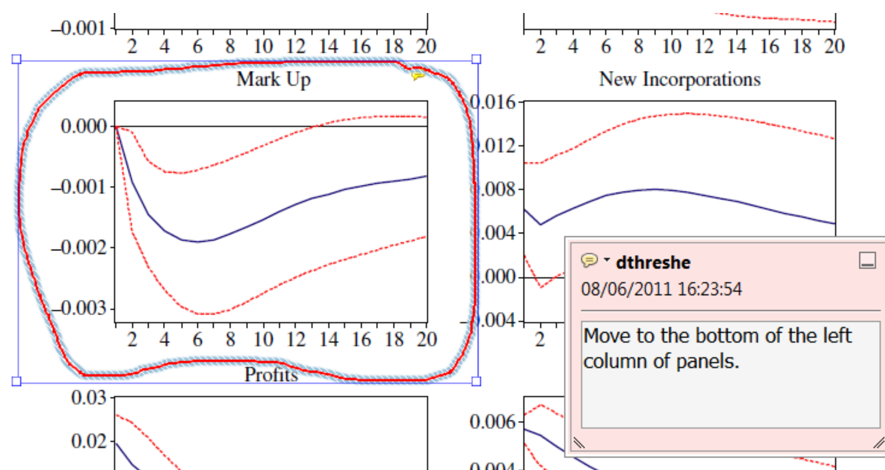

How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
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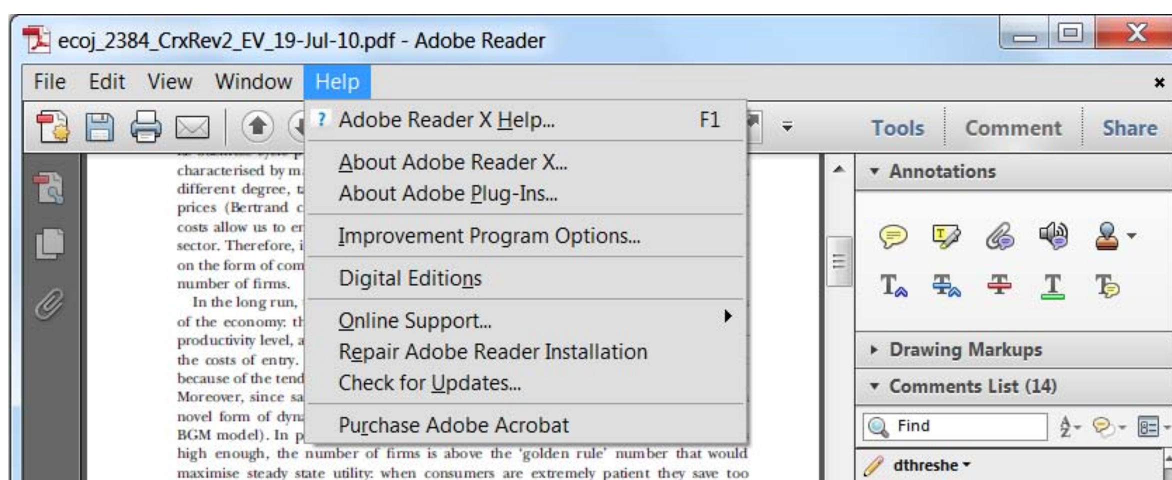

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