Protein refolding for industrial processes

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Inclusion body refolding processes are poised to play a major role in the production of recombinant proteins. Improving renaturation yields by minimizing aggregation and reducing chemical costs are key to the industrial implementation of these processes. Recent developments include solubilization methods that do not rely on high denaturant concentrations and the use of high hydrostatic pressure for simultaneous solubilization and renaturation.

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Abbreviations

CTAB	n-cetyl trimethylammonium bromide
DTE	dithioerythritol
DTT	dithiothreitol
GdmCl	guanidinium chloride
PDGF	platelet-derived growth factor
SDS	sodium dodecyl sulfate
SEC	size-exclusion chromatography

Introduction

The need for the efficient production of genetically engineered proteins has grown and will continue to grow as a consequence of the success of the human genome project. A variety of hosts may be used to produce these proteins, with expression in bacteria poised to play a major role, particularly when the biological activity of the protein product is not dependent on post-translational modifications. Expression of genetically engineered proteins in bacteria often results in the accumulation of the protein product in inactive insoluble deposits inside the cells, called inclusion bodies. Faced with the prospect of producing an insoluble and inactive protein, researchers usually attempt to improve solubility by a variety of means, such as growing the cells at lower temperatures, co-expressing the protein of interest with chaperones and foldases and using solubilizing fusion partners, among others [1]. However, expressing a protein in inclusion body form can be advantageous. Large amounts of highly enriched proteins can be expressed as inclusion bodies. Trapped in insoluble aggregates, these proteins are for the most part protected from proteolytic degradation. If the protein of interest is toxic or lethal to the host cell, then inclusion body expression may be the best available production method. The challenge is to take advantage of the high expression levels of inclusion body proteins by being able to convert inactive and misfolded inclusion body proteins into soluble bioactive products [2–5].

The recent literature includes many examples of the refolding of genetically engineered proteins. A significant number of these publications deal with the expression and purification of small amounts of proteins for structure/function relationship and biophysical characterization studies. Although valuable, the processes described in these publications are usually inefficient, include multiple unnecessary steps and have very low recovery yields. A second significant fraction of the refolding literature deals with understanding the folding pathway of a variety of proteins and, in particular, early folding events. These studies are performed with purified proteins that are subjected to unfolding under a variety of conditions, followed by carefully designed and monitored refolding experiments. A third fraction of the refolding literature, and the focus of this review, deals with the development of more efficient refolding methods that can be used for the commercial production of genetically engineered proteins

The general strategy used to recover active protein from inclusion bodies involves three steps: inclusion body isolation and washing; solubilization of the aggregated protein; and refolding of the solubilized protein (Figure 1a). Although the efficiency of the first two steps can be relatively high, renaturation yields may be limited by the accumulation of inactive misfolded species as well as aggregates. Because the majority of industrially relevant proteins contain one or more disulfide bonds, this review focuses on recent advances in oxidative protein refolding, that is, refolding with concomitant disulfide-bond formation.

Inclusion body isolation, purification and solubilization

Inclusion bodies are dense, amorphous protein deposits that can be found in both the cytoplasmic and periplasmic space of bacteria [1,6•]. Structural characterization studies using ATR-FTIR (attenuated total reflectance Fourier-transformed infrared spectroscopy) have shown that the insoluble nature of inclusion bodies may be due to their increased levels of non-native intermolecular β-sheet content compared with native and salt-precipitated protein [7,8]. Cells containing inclusion bodies are usually disrupted by high-pressure homogenization or a combination of mechanical, chemical and enzymatic methods [6•,9•]. The resulting suspension is treated by either low-speed centrifugation or filtration to remove soluble proteins from the particulate containing the inclusion bodies. The most difficult to remove contaminants of inclusion body protein preparations are membrane-associated proteins that are released upon cell breakage. Washing steps are performed to remove membrane proteins and other contaminants. Methods used to solubilize prokaryotic membrane proteins can be adapted to wash inclusion bodies. The most common washing steps utilize EDTA, and low concentrations of denaturants and/or weak detergents such as Triton X-100, deoxycholate and octylglucoside [6•,9•,10,11•,12,13,P1,P2].

Batas, Schiraldi and Chaudhuri [10] recently compared centrifugation and membrane filtration for the recovery and washing of inclusion bodies. Two membrane pore sizes (0.1 and 0.45 μm) were compared; the larger pore size membrane gave better solvent flux and protein purity. Centrifugation resulted in higher protein purity, probably because it takes advantage of the density differences between cell debris and inclusion bodies.

A variety of methods may be used to solubilize inclusion bodies; however, the choice of solubilizing agent can greatly impact the subsequent refolding step and the cost of the overall process. The most commonly used solubilizing agents are denaturants, such as guanidinium chloride (GdmCl) and urea. Using these denaturants, solubilization may be accomplished by the complete disruption of the protein structure (unfolding) or by the disruption of intermolecular interactions with partial unfolding of the protein. The latter approach has the advantage that it requires lower amounts of denaturant to succeed. Although proteins have been successfully refolded from the denatured state, it may prove to be difficult to fold proteins from a partially folded state. Key to the development of an efficient and economic denaturant-based solubilization step is the determination of the minimum amount of denaturant needed to solubilize the protein and to allow for full bioactivity recovery in the refolding step. The majority of the published work on inclusion body protein refolding has used relatively high denaturant (6–8 M) and protein (1–10 mg/ml) concentrations in the solubilization step [5,9•,10,11•,12–14].

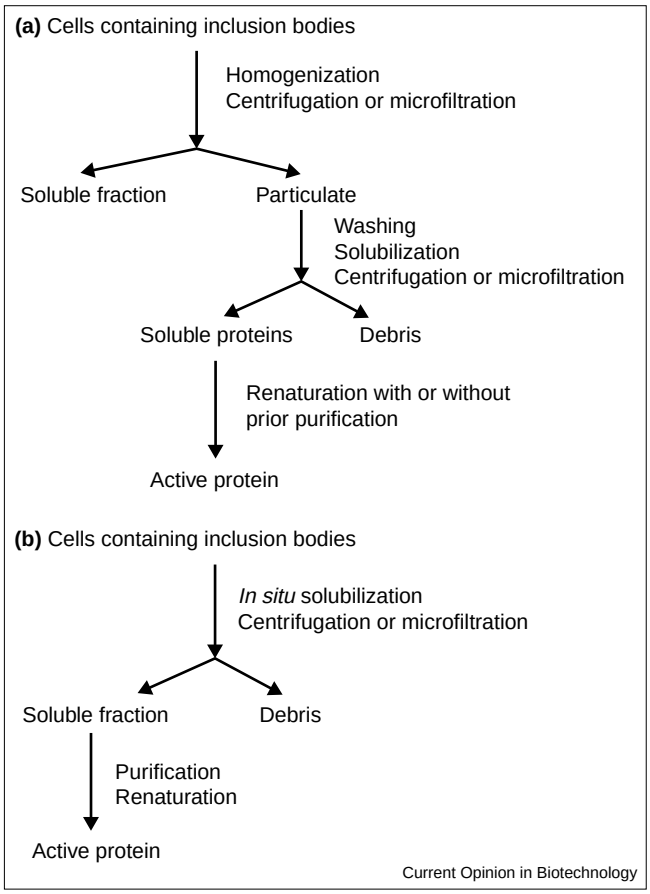
Lower denaturant concentrations (1–2 M) have been used to solubilize cytokines from *Escherichia coli* inclusion bodies [P3]. The purity of the solubilized protein was much higher at GdmCl concentrations of 1.5–2 M compared with the more commonly used 4–6 M concentrations, because at the higher GdmCl concentrations contaminating proteins were also released from the particulate fraction. No information was provided about the efficiency of this solubilization process or the range of inclusion body protein concentrations for successful solubilization.

Extremes of pH have also been used to solubilize inclusion bodies. Gavit and Better [15] used a combination of low pH (≤ 2.6) and high temperature (85°C) to solubilize antifungal recombinant peptides from *E. coli*. Lower temperatures and higher pH values resulted in increased solubilization time. Reddy and coworkers [16] utilized 20% acetic acid to solubilize a maltose-binding protein fusion from inclusion bodies. These low pH solubilization processes may not be applicable to many proteins, particularly those that undergo irreversible chemical modifications at these conditions or those susceptible to acid cleavage.

High pH (≥ 12) has been used to solubilize growth hormones [17,18] and proinsulin [P4]. Exposure to elevated pH conditions for extended periods of time may also cause irreversible chemical modifications to the protein. Thus, this high pH solubilization method, although attractive for its simplicity and low cost, may not be applicable to most pharmaceutical proteins. More effective solubilization methods for growth hormones combine high pH with low denaturant concentrations [17,18], 20–40% isopropyl or n-propyl alcohol solutions [P1] or acyl glutamate detergents [P5].

Detergents have also been used to solubilize inclusion bodies. Commonly used detergents are sodium dodecyl sulfate (SDS) and n-cetyl trimethylammonium bromide (CTAB) [3,18,19]. Detergents offer the advantage that the solubilized protein may already display biological activity, thus avoiding the need for a refolding step. If this is the case, it is important to remove contaminating membrane-associated proteases in the inclusion body washing step to avoid proteolytic degradation of the solubilized inclusion body protein [6•]. One acknowledged drawback of the use of detergents as solubilizing agents is that they may interfere with downstream chromatographic steps. Extensive washing may be needed to remove solubilizing detergents [P5]. Alternatively, detergents may be extracted from refolding mixtures by using cyclodextrins [20], linear dextrins [21] or cycloamylose [22].

Figure 1



Processes for the recovery of inclusion body proteins. **(a)** Inclusion body isolation followed by solubilization. **(b)** The *in situ* solubilization of inclusion bodies.

Patra and coworkers [18] compared several solubilization methods for the recovery of human growth hormone from *E. coli* inclusion bodies. They observed similar solubilization efficiencies when using 8 M urea, 6 M GdmCl, 1% SDS or 1% CTAB (all at pH 8.5) or 2 M urea (at pH 12.5). Refolding for the first four solubilization conditions required a dilution step resulting in increased process volumes. Solubilization in 2 M urea at pH 12.5 was simple, economical and efficient, and refolding could be accomplished by a simple pH adjustment without dilution. However, this high pH solubilization method may not be applicable to proteins that might undergo irreversible chemical modifications under these conditions.

A key to the solubilization process is the addition of a reducing agent to maintain cysteine residues in the reduced state and thus prevent non-native intra- and interdisulfide bond formation in highly concentrated protein solutions at alkaline pH. Typically used reducing agents are dithiothreitol (DTT), dithioerythritol (DTE), and 2-mercaptoethanol [2,3]. These reducing agents should be added in slight excess to ensure complete reduction of all cysteine residues. Chelating agents are added to the solubilization solution to prevent metal-catalyzed air oxidation of cysteines. Alternatively, reduced cysteines may be protected from oxidation by the formation of S-sulfonate derivatives [23,P6,P7] or mixed disulfides [9•,P7].

When expression levels are very high, a competitive alternative is to add the solubilizing agents directly to the broth at the end of the fermentation process. This *in situ* solubilization method has been used to recover insulin-like growth factor using urea under alkaline conditions [P8] and antifungal recombinant peptides using a combination of low pH (< 2.6) and high temperature (85°C) [15]. The main disadvantage of *in situ* solubilization concerns the release of both proteinaceous and nonproteinaceous contaminants that may have to be removed before renaturation is attempted. It has been shown that protein refolding in the presence of impurities may result in decreased yields [6•,24]. The main advantage of this method is the elimination of time-consuming and energy-consuming mechanical disruption methods and of one centrifugation and/or filtration step (Figure 1b).

Solubilization may also be accomplished by applying high hydrostatic pressures (1–2 kbar) in the presence of reducing agents and low concentrations of solubilizing agents [25•,P9].

Renaturation of the solubilized protein

When inclusion bodies have been solubilized using a combination of reducing agents and high concentrations of denaturants, renaturation is then accomplished by the removal of excess denaturants by either dilution or a buffer-exchange step, such as dialysis, diafiltration, gel-filtration chromatography or immobilization onto a solid support. Because of its simplicity, dilution of the solubilized protein directly into renaturation buffer is the most commonly used method in small-scale refolding studies. The main disadvantages of dilution refolding for commercial applications are the need for larger refolding vessels and additional concentration steps after renaturation. The key to successful dilution refolding is to control the rate of the addition of denatured protein to renaturation buffer and to provide good mixing in order to maintain low protein concentration during refolding and thus prevent aggregation. Dilution refolding can also be accomplished in multiple steps, also known as 'pulse renaturation', in which aliquots of denatured reduced protein are added to renaturation buffer at successive time intervals [2,9•], or semicontinuously via fed-batch addition of the denatured reduced protein to refolding buffer [26]. Recently, Katoh and Katoh [26] developed a continuous refolding method in which denatured reduced protein is gradually added from the annular space of a membrane tube to renaturation buffer flowing continuously through the inner space of the membrane tube. Refolding yields obtained using this continuous refolding method were similar to those obtained using fed-batch dilution and about 10% higher than those using batch dilution [26].

Buffer exchange to remove high denaturant concentrations can also be accomplished by diafiltration [27] and dialysis [28] using ultrafiltration membranes. Renaturation yields using these membrane-based methods can be significantly affected by protein binding to the membranes. Binding can be minimized by using highly hydrophilic materials, such as cellulose acetate, which are more compatible with unfolded protein molecules. With typical hydrophobic membrane materials, such as polyether sulfone, the majority of the denatured protein was found bound to the membrane [28]. Significant losses of unfolded protein occurred via transmission through the membrane. These losses could be reduced by dialysis against lower denaturant concentrations that lead to molten-globule or native configurations [28].

Size-exclusion chromatography (SEC) is an alternative buffer-exchange method to remove high denaturant concentrations and promote renaturation [11•,13,29•]. Fahey, Chaudhuri and Binding [13] examined the effect of gel type on renaturation yields and found that as the fractionation range of the gel matrix increases from Sephacryl (S)-100 to S-400, aggregation decreases but the resolution between protein and denaturant decreases. Thus, optimum renaturation yields were obtained with the S-300 gel. In a separate study, Fahey, Chaudhuri and Binding [29•] compared batch dilution and SEC refolding. Dilution also takes place during SEC but, for similar dilution factors, SEC resulted in higher refolding yields when compared with batch dilution as long as the dilution factor was below 40. Sample application conditions were found to have a strong effect on the efficiency of SEC refolding, because rapid structural collapse takes place during sample application that can lead to aggregation. Renaturation yields decreased with higher protein concentrations and sample

volumes and lower flow rates [29•]. Muller and Rinas [11•] circumvented the problem of aggregation during sample application by allowing the denatured protein to penetrate the column under denaturing conditions and then changing the buffer to renaturation conditions. They successfully refolded the complex heterodimeric protein platelet-derived growth factor (PDGF) using a combination of SEC for refolding of the monomeric species followed by prolonged incubation under renaturation conditions to promote dimerization.

Buffer exchange to remove high denaturant concentrations can also be achieved by transiently binding the denatured protein to a solid support. Intermolecular interactions leading to aggregation are minimized when the refolding molecules are isolated through binding to the support. Freedom for structure formation during renaturation is facilitated by binding through fusion partners, such as a His-tag [30] or the cellulose-binding domain [31], which retain their binding capabilities under the denaturing conditions required for loading the solubilized inclusion body protein onto the column. *In situ* purification is achieved by washing the bound protein before elution.

Disulfide-bond formation during folding

In the case of disulfide-bonded proteins, renaturation buffers must promote disulfide-bond formation (oxidation). The simplest and most inexpensive oxidation method uses air in the presence of a metal catalyst and a reducing agent to facilitate disulfide-bond reshuffling [P1,P8]. The rate of disulfide-bond formation through air oxidation may be limited by the slow mass transfer rate of oxygen in aqueous solutions. Increased agitation, which can be used to improve mass transfer rates, may also lead to aggregation due to increased shear and interfacial stresses [32].

Oxidation rates can be accelerated using an oxido-shuffling system, which consists of mixtures of reduced and oxidized low molecular weight thiol reagents. The most commonly used oxido-shuffling reagents are reduced and oxidized glutathione (GSH/GSSG) but the pairs cysteine/cystine, cysteamine/cystamine, DTT/GSSG and DTE/GSSG have also been utilized [2,3,9•]. Molar ratios of reduced to oxidized thiol of 3:1 to 1:1 and total thiol concentrations between 5–15 mM have been found to be optimal [14,33]. Disulfide-bond formation using the oxido-shuffling system can be accelerated by using a small-molecule mimic of protein disulfide isomerase [34,P10]. A disadvantage of the oxido-shuffling system is the high cost of some of the reagents, particularly glutathione.

A third oxidation method uses a two-step approach: the formation of mixed disulfides between glutathione and the denatured protein before renaturation, followed by refolding in the presence of catalytic amounts of a reducing agent to promote disulfide-bond formation and reshuffling [9•,P7]. The refolding yield of recombinant human tissue plasminogen activator could be increased sixfold when the oxido-shuffling refolding system was replaced with the mixed disulfide approach [9•]. Alternatively, cysteines in the denatured protein may be protected by sulfonation, followed by the addition of a reducing agent such as cysteine [P7] and 2-mercaptoethanol [P6] or a thiol/disulfide mixture such as cysteamine/cystamine [23].

Improving renaturation yields

The formation of incorrectly folded species, and in particular aggregates, is usually the cause of decreased renaturation yields. A very efficient strategy to suppress aggregation is the inhibition of the intermolecular interactions leading to aggregation by the use of low molecular weight additives. These small molecules are relatively easy to remove when refolding is complete. Numerous additives have been shown to prevent aggregation [3,9•]. The mechanism of action of additives is still unclear. They may influence both the solubility and the stability of the native, denatured and intermediate state(s), they may act by changing the ratio of the rates of proper folding and aggregate formation or they might simply act by solubilizing aggregates. The most commonly used low molecular weight additives are L-arginine (0.4–1 M), low concentrations of denaturants such as urea (1–2 M) and GdmCl (0.5–1.5 M) and detergents (Chaps, SDS, CTAB and Triton X-100). In a recent review, De Bernardez Clark, Schwarz and Rudolph [9•] discussed different approaches to inhibiting aggregation during refolding and provided a detailed list of low molecular weight additives and the concentration ranges needed to increase renaturation yields.

Low concentrations of urea and GdmCl, although widely used to inhibit aggregation [14,29•,P2,P3,P7], are not always effective folding enhancers. GdmCl concentrations as low as 0.25 M were found to inhibit the oxidative dimerization of PDGF [11•]. Similarly, bone morphogenetic protein-2 proved difficult to refold in the presence of low concentrations of denaturants [35].

Detergents such as Chaps [12,35], CTAB [20,21], Triton X-100 [20] and SDS [19] have been successfully used to improve renaturation yields. As noted earlier under solubilization methods, one drawback of the use of detergents is that they may be difficult to remove and may affect downstream chromatographic steps. Detergents have been extracted from refolding mixtures using cyclodextrins [20], linear dextrins [21], cycloamylose [22] and ion-exchange chromatography in the case of ionic detergents [19].

The *in vivo* competition between folding and aggregation is modulated by chaperones and foldases [1]. It is not surprising that these proteins can also affect the *in vitro* competition between folding and aggregation [36]. Because chaperones and foldases are proteins that need to be removed from the renaturation solution at the end of the refolding process and as they may be costly to produce, their commercial use will require a recovery–reuse scheme. Altamirano and coworkers [37] developed a

Table 1

Most frequently used oxidative renaturation methods.

Solubilization method	Renaturation method*	Comments	References
High denaturant concentrations: urea (≥ 8 M) or GdmCl (≥ 6 M) in the presence of a reducing agent (DTT, DTE, 2-mercaptoethanol)	Removal of the denaturant by dilution or buffer exchange (dialysis, diafiltration, size-exclusion chromatography or binding to a matrix) with concomitant disulfide-bond formation†	Residual denaturant concentrations may interfere with the assembly of oligomeric proteins	[9•,11•,12–14, 18,29•,P2]
Detergents: SDS, CTAB in the presence of a reducing agent (DTT, DTE, 2-mercaptoethanol)	Oxidation† followed by detergent removal by extensive washing or stripping	Residual detergent concentrations may interfere with downstream purification processes	[18–22]
Extremes of pH in the presence of low concentrations of denaturants	pH adjustment to 7.5–9.5 to promote disulfide-bond formation†	Extreme pH may cause irreversible chemical modifications	[15–18]

*Low molecular weight additives may be added to prevent aggregation. †Disulfide-bond formation may be accomplished by air oxidation in the presence of a metal catalyst and a reducing agent or by using an oxido-shuffling system.

reusable molecular chaperone system for oxidative refolding chromatography that utilizes a GroEL minichaperone, which can prevent aggregation, the oxido-shuffling catalyst DsbA, and peptidyl-prolyl isomerase, all immobilized on an agarose gel. Recently, Kohler, Preuss and Miller [38] developed a chaperone-assisted refolding bioreactor that uses a stirred-cell membrane system to immobilize the GroEL–GroES complex. In its current design, the bioreactor could only be used for three cycles of refolding. Further design improvements will be needed before this bioreactor can be considered as a commercially viable refolding alternative.

Interestingly, high hydrostatic pressures (1–2 kbar) in combination with low concentrations of denaturants have been used for the simultaneous solubilization and refolding of inclusion body proteins [25•,P9]. Similarly, high hydrostatic pressures can be used in the refolding process to prevent aggregation [39,40].

Conclusions

The recovery of bioactive proteins from inclusion bodies is a complex process. Despite its complexity, there are clear guidelines on how to proceed when faced with the task of refolding an inclusion body protein (Figure 1; Table 1). As with other protein recovery processes, however, optimum conditions have to be determined on a case by case basis. The key to a commercially viable renaturation process lies in minimizing the number of steps (to increase the overall yield) and the amounts and costs of chemicals needed. This can be accomplished by eliminating unnecessary buffer-exchange steps, by exploring the use of alternative solubilization methods that do not rely on high denaturant concentrations, and by developing efficient oxidation methods that do not require the use of expensive oxido-shuffling systems. Future developments in protein refolding will benefit from a more fundamental understanding of inclusion body solubilization methods, and on the role that additives play in the inhibition of aggregation.

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