CHAPTER SEVENTEEN

REFOLDING SOLUBILIZED INCLUSION BODY PROTEINS

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Abstract

The vast majority of protein purification is now done with cloned, recombinant proteins expressed in a suitable host. The predominant host is \textit{Escherichia coli}. Many, if not most, expressed proteins are found in an insoluble form called an inclusion body (IB). Since the target protein is often relatively pure in a washed IB, the challenge is not so much to purify the target, but rather to solubilize an IB and refold the protein into its native structure, regaining full biological
activity. While many of the operations of this process are quite general (expression, cell disruption, IB isolation and washing, and IB solubilization), the precise conditions that give efficient refolding differ for each protein. This chapter describes the main techniques and strategies for achieving successful refolding.

1. Introduction

When proteins are expressed at high levels in *Escherichia coli* and other expression hosts, it is common to find that most of the protein is not soluble, but in an insoluble form called an inclusion body (IB). While the exact mechanism for IB formation is not fully understood and may vary with different proteins and expression conditions, it is generally thought that target proteins are being made faster than they can fold into the native structure. If a protein is partially folded or misfolded, it will generally have hydrophobic or “sticky” regions exposed that can interact with other similar proteins and form aggregates. These aggregates form IBs that are dense, refractile bodies on the order of 0.2–0.5 μm in diameter. Once in an IB, the protein is usually protected from proteolytic attack and is the predominant protein. IBs can vary from being mostly native protein, relatively easily solubilized under mild conditions to being misfolded, effectively irreversibly insoluble material that requires high concentrations of denaturants to be solubilized (see Bowden *et al.*, 1991; Ventura and Villaverde, 2006). The latter category is by far the most common and thus, the major barrier to obtaining native, active material is to find conditions under which the denatured protein can be refolded efficiently. *This refolding process and strategies to achieve it are the focus of this chapter.*

Anfinsen, in the early 1960s (see Anfinsen, 1973), observed that small protein molecules could fold spontaneously to the native state. This observation has led to a thermodynamic hypothesis: the native state has the lowest free energy. While the thermodynamic hypothesis has become a paradigm of protein folding, a simple and general model for describing how an unfolded protein molecule finds its native state is still not available. One cannot yet use computers to accurately predict protein structure from sequence.

Levinthal (1968) pointed out the paradox that an unfolded protein molecule does not have the time needed to search all possible conformations, yet it reaches the single native state rapidly. Consider a (hypothetical) small protein with 100 residues. Levinthal calculated that, if each residue can assume only three different conformations (it is probably many more than this), the total number of structures would be $3^{100}$, which is equal to $5 \times 10^{47}$. If it takes only $10^{-13}$ s to convert one structure into another, the total search time would be $5 \times 10^{47}$ times $10^{-13}$ s, which is equal to $5 \times 10^{34}$ s or $1.6 \times 10^{27}$ years! Clearly, it would take much too long for even
a small protein to fold properly by randomly trying out all possible con-
formations. This paradox has shaped many studies on the theory of refolding
(e.g., see Clark, 2004; Karplus, 1997).

A great many experimental studies and theoretical analyses have been
carried out to better understand the folding process. The consensus is that
when a denatured protein is no longer in denaturing conditions, some
secondary structure (α-helix and β-sheet) forms, a hydrophobic collapse
occurs to form a “molten globule” state with some secondary structure, but
nonnative tertiary structures, the globular states go through multiple inter-
mediates, and finally reach the lowest energy state, the native conformation.
Solution conditions can have significant effects on the folding pathway of a
given protein. If you think of the refolding energy funnel as a deep, fog-filled
 crater in the top of a volcanic mountain, then the rim is the denatured state
and the very bottom of the crater is the native state (the lowest energy state).
If you roll a ball down into the crater from one point on the rim of the crater,
then it may be able to roll all the way to the bottom. From another point on
the rim, the ball may become trapped in a local minimum and not reach the
bottom. In many ways the challenge of finding conditions for efficient
refolding is like trying to guess where on the rim to start rolling the ball.

The rate of protein refolding is rapid, with folding halftimes on the order
of seconds for proteins lacking prolines and on the order of minutes for
proteins with prolines (see Nall, 1994). The amino acid proline can consist
of two isomers, a cis and trans configuration, and the halftime of this
chemical isomerization is on the order of 1 min. In a native protein, each
proline is in a particular configuration, but in a denatured protein an
equilibrium mixture of the two isomers forms consisting of about 70%
trans and 30% cis isomer. Therefore, as a protein refolds, those prolines
that are in the incorrect conformation cannot form the native structure and
must wait for an isomerization event to occur. Thus, proline isomerization
becomes rate limiting for correct protein folding. The enzyme, protein
proline isomerase (PPI), can speed up this isomerization rate.

Even though recombinant proteins were first prepared by refolding
solubilized IBs in the late 1970s and early 1980s, and many improvements
have been made in refolding procedures and techniques, refolding of any
given protein still presents a significant challenge. During the last 10 years
the protein structure initiative (PSI), through its numerous Structural
Genomics Centers, has cloned and expressed over 110,000 proteins (as of
December 2008), but were only able to purify about 29,000 (about 26%).
On the order of 50% of Eubacterial and Archaeal proteins and only about
10% of Eukaryal proteins could be expressed as soluble proteins in E. coli
(Graslund et al., 2008). The proteins that were insoluble were generally
abandoned rather than trying to find effective refolding conditions. I believe
that many of these insoluble proteins could have been salvaged or rescued
by finding suitable refolding conditions as described below.
Many excellent reviews report approaches to protein refolding (Baneyx and Mujacic, 2004; Cabrita and Bottomley, 2004; Clark, 2001; Jungbauer and Kaar, 2006; Middelberg, 2002; Panda, 2003; Quronfleh et al., 2007; Singh and Panda, 2005; Swietnicki, 2006; Thatcher and Hitchcock, 1994; Tsumoto et al., 2003; Vallejo and Rinas, 2004). I will try to present the kind of advice below that I would give to colleagues embarking on a project that involves protein refolding.

2. General Refolding Consideration

The main problem observed in many refolding attempts is that upon dilution or dialysis of the solubilized IB to decrease the concentration of the denaturant to a nondenaturing level, you get major precipitation, especially if the protein concentration is high during refolding. In a way this is likely to be similar to the process in the cell that leads to formation of IBs in the first place. You want to go from denatured to partially folded intermediate to native structure, and bias the reaction away from aggregation of the sticky folding intermediate and precipitation. Aggregation is dependent on collision of sticky folding intermediates. At low protein concentrations the probability of collision is diminished. The best general strategy in refolding is to refold at the lowest protein concentration that is feasible. In screening refolding conditions, one searches for conditions where little aggregation or turbidity is observed. But even when there is no obvious precipitation (the dilute protein in refolding solution is not turbid), there can be significant “soluble multimer.” Soluble multimers are generally from 2- to 20-mers that are soluble and not large enough to cause much light scattering (turbidity). Usually these soluble multimers are not active and tend to bind tightly to ion exchange resins.

3. General Procedures

Given that it is very often the case that protein expressed in E. coli and other hosts is found in IBs, the following steps are almost always needed to obtain active, native protein:

1. Cell growth and protein overexpression
2. Cell lysis, isolating and washing IBs
3. Solubilizing IBs
4. Identifying suitable refolding conditions
5. Protein refolding
6. Reoxidation to form disulfide bonds where necessary
7. High-resolution ion-exchange chromatography
8. Characterization of final material to determine if refolding has been successful
4. GENERAL PROTOCOL

Below is a typical procedure that works well for many proteins. This protocol is based on and adapted from the protocol first developed by Nguyen et al. (1993) and used in the Cold Spring Harbor Protein Purification and Characterization Course section on Purification of Insoluble Recombinant Proteins (Burgess and Knuth, 1996). Other similar procedures are likely to give similar results, but this is the procedure used almost exclusively in my laboratory. The critical steps will be discussed in the following steps.

1. *E. coli* BL21(DE3)pLysS containing the target gene cloned into a pET expression vector (Studier et al., 1990) is grown in 1 L of LB in a 2-L flask at 37 °C with shaking until the $A_{600 \text{ nm}}$ reaches about 0.6.
2. IPTG is added to 0.5–1 mM to induce expression of the T7 RNA polymerase that then transcribes the target gene.
3. Three to four hours after induction, the cells are harvested by centrifugation at 15,000 rpm for 15 min, the cells are resuspended in a small volume of the culture supernatant, transferred into a preweighed, 40-ml Oak Ridge tube, and centrifuged to pellet the cells. The wet weight of the cell pellet is noted and the cells are stored frozen at −80 °C until use. It is common to get 1.5–2.0 g wet weight *E. coli* cells per liter using this procedure.
4. Cells are thawed, resuspended in 30 ml of a lysis buffer (50 mM Tris–HCl, pH 7.9, 0.1 mM EDTA, 5% glycerol, 0.1 mM DTT, 0.1 M NaCl), and then sonicated at 60% power for 3–4 intervals of 20 s each with about 1 min on ice to cool between each interval.
5. Purified Triton X-100 (from a 10%, w/v stock) is added to 1% (w/v) to dissolve membranes and solubilize membrane proteins. The lysate is incubated on ice for 10 min and then centrifuged at 15,000 rpm for 15 min to pellet the IB and remove the soluble supernatant.
6. The IB is resuspended in 30 ml of lysis buffer with 1% Triton X-100, incubated on ice for 10 min, and then centrifuged for 15,000 rpm for 15 min.
7. The drained pellet is resuspended in 30 ml of lysis buffer without Triton X-100 to remove the Triton X-100 and recentrifuged as above. The pellet is called the washed IB fraction and is usually over 90% pure.
8. The washed IB pellet is resuspended in a suitable denaturant, incubated, to allow denaturation and solubilization, and then centrifuged at 15,000 rpm for 15 min to remove any residual insoluble material. We routinely use the lysis buffer above (lacking the NaCl if diluting from GuHCl) containing either 6 M guanidine hydrochloride (GuHCl), 8 M urea, or 0.3% Sarkosyl (n-lauroyl sarcosinate) for IB solubilization.
9. More recently, we have carried out a relatively simple refolding test (see below) to identify a suitable refolding buffer.

10. Adjust the protein concentration in the denatured sample to about 1 mg/ml.

11. Dilute the denatured protein about 15–60 fold to dilute the denaturant concentration to a point where the protein can refold. We usually either flash dilute or slowly drip dilute the denatured IB into refolding buffer in a beaker with vigorous stirring to cause very rapid mixing. The dilution process is performed at room temperature and after mixing is complete, we let the solution stand for 1–2 h to allow the refolding process to complete and to allow any aggregated material to form and flocculate.

12. The refolded material is filtered through a low protein binding, 0.22 μm membrane filter (e.g., Stericup-GV 0.22 μm, 500 ml, Millipore #SCGVU05RE) to remove any particulate material (this can be preceded by a centrifugation step if there is significant precipitated material that would otherwise immediately clog the filter).

13. Pump the filtered solution onto a 10–15-ml ion-exchange column as fast as the pressure constraints of the column and system will allow (hopefully, at least 10 ml/min). Monitor the absorbance at 280, 260, and 320 nm, if possible. The absorbance at 320 nm is a measure of light scattering and for some proteins can indicate a peak that is composed of multimers.

14. Wash the column with 5–10 column volumes of Buffer A (50 mM Tris–HCl, pH 7.9, 5% glycerol, 0.1 mM EDTA, 0.1 mM DTT) plus 0.1 M NaCl and then elute with a 10-column volume, linear salt gradient in the same buffer from 0.1 to 1.0 M NaCl at a flow rate of 5 ml/min, collecting 3–4 ml fractions.

15. Analyze the A$_{280}$ nm peaks by SDS–PAGE to determine purity of the various fractions. If an enzyme assay is available, assay fractions to determine those with the highest specific activity.

16. Pooled peak fractions are dialyzed against lysis buffer containing 50% glycerol and stored at $-20$ or $-80$ °C.

17. Characterize the pooled peak material, if possible, by specific activity compared to a standard sample of the protein purified by a more conventional procedure that does not involve refolding.

5. Comments on this General Procedure

5.1. Overexpression of recombinant proteins in E. coli

The issue of achieving a high level of expression is a topic somewhat separate from the refolding issue and will not be discussed further here (see Chapter 12 in this volume). Many systems will allow expression levels
of the target protein of 10–40% of the total cell protein (Makrides, 1996; Murby et al., 1996; Sorensen and Mortensen, 2005; Studier et al., 1990) (see Section 9). Usually one carries out a test to see at what temperature to grow the cells, how much inducer to use, and how long after induction you should harvest the cells. After induction of the high-level expression of the target protein, the cells are usually centrifuged, and frozen at −80 °C until use. There are anecdotal reports that IBs from cells stored for weeks in the frozen state are harder to refold, but in my experience, we have gotten excellent refolding results from cells stored frozen for months. Unfortunately, to my knowledge, no systematic study has addressed this point.

5.2. Washing IBs

We originally used 2% sodium deoxycholate to wash IBs (Burgess and Knuth, 1996; Nguyen et al., 1993), but later found that 1% Triton X-100 worked better and was much easier to work with. It is highly recommended that one use what I call “gourmet” Triton X-100 (Pierce, Surfact-Amps X-100, 10% (w/v) solution) that has been purified (to remove peroxides sometimes found in old, yellowish bottles of Triton X-100) and stored under nitrogen in sealed ampoules. It is possible to test various salts and detergents to see which are particularly effective at washing out protein impurities from an IB, but not solubilizing a given target protein. Sometimes washes with 1–2 M urea can be used. In general, it is desirable to remove as much membrane material, DNA, and other proteins as possible from an IB before solubilization.

5.3. Solubilizing IBs

As mentioned earlier, some IBs are nearly native and can be solubilized by mild conditions such as nonionic detergent or even 0.5 M NaCl (Vera et al., 2006). In most cases, this will not work and much stronger denaturing conditions must be used.

The solubilizing agent/denaturant is prepared in a buffer, much like the lysis buffer above, to control pH, chelate heavy metals, and maintain a reducing environment. Usually one resuspends the IB with the solubilizing agent, lets it incubate for 30–60 min, and then centrifuges out any insoluble material. Since the material is already at least partially denatured in the IB, this solubilization can be performed at room temperature and in some cases it is even necessary to heat the solution to achieve full solubilization. For example, when native green fluorescent protein (GFP) is adjusted to 6 M GuHCl it retains its fluorescence at room temperature, but at 75 °C it denatures and loses its fluorescence within 1 min (R. Burgess and N. Thompson, unpublished results). A detailed discussion of solubilization is found in Marston and Hartley (1990).
Some comments on the use of several solubilizing agents follow:

1. **GuHCl.** This is perhaps the most commonly used solubilizing agent, usually used at 6 M in a compatible buffer. Most proteins will rapidly denature in this strong chaotropic agent, but in many cases it may help to incubate at higher temperatures to achieve complete denaturation. Once solubilization occurs, it is often possible to dilute to 3 M GuHCl without a problem, since the transition from denatured to native state often occurs in the 1–2 M GuHCl range (Pace, 1986). One usually dilutes to about 0.1 M GuHCl so that the salt concentration is low enough to allow the refolded target protein to bind to a suitable ion-exchange column (see below).

2. **Urea.** 8 M urea is a common solubilizing agent, and is especially useful if one wants to further purify the target protein in the denatured state (Knuth and Burgess, 1987). The 8 M urea is generally not as effective as 6 M GuHCl in solubilizing protein and causing complete denaturation. One must be aware of the possibility of carbamylation of proteins by the cyanate always present in urea solutions (see Chapter 44 in this volume for advice on how to minimize cyanate in urea).

3. **Sarkosyl or SDS.** Sarkosyl has been found to be a quite effective solubilizing agent that allows refolding at higher protein concentrations (see Burgess, 1996). Sarkosyl is a strong anionic detergent, much like SDS, but it seems to bind to proteins more weakly and dissociate more easily than SDS. Usually an IB pellet can be solubilized in 0.3% Sarkosyl, but you must remember that you cannot solubilize more than about an equal weight of protein per weight of Sarkosyl. For example, if you have a large washed IB that contains 150 mg of target protein, it will not be solubilized completely by 20 ml of 0.3% Sarkosyl (60 mg of Sarkosyl). You will have to add at least 50 ml of 0.3% or 30 ml of 0.5% Sarkosyl.

We have noticed that if there is much Triton X-100 present in an IB, it will take more Sarkosyl to solubilize the target protein. This is almost certainly due to the fact that Triton X-100 forms large micelles and absorbs some of the Sarkosyl to form mixed micelles that are not effective at solubilization.

If one dilutes 0.3% Sarkosyl-solubilized target protein to about 0.01% Sarkosyl, most of the Sarkosyl will dissociate from the protein and the protein will refold. The residual detergent seems to bind to the hydrophobic regions (the “sticky” sites) on partially refolded protein and prevent aggregation. It is effectively acting as a chemical chaperone. If your target protein is able to bind to a cation-exchange column such as a POROS HS, then the diluted solution can be filtered and pumped on a 10-ml column, washed with 10–20 column volumes of 0.1 M NaCl in buffer, and eluted with a salt gradient (see Section 5.5). The target protein will bind, but the free Sarkosyl will flow-through and any residual Sarkosyl bound to the protein will
dissociate during the long wash. Experimental analysis of the eluted target protein indicates essentially no (<1 molecule detergent per 10 molecules of protein) residual Sarkosyl in the protein (R. Burgess, unpublished). Do not use MonoS for this purpose since Sarkosyl binds to the column and can damage the column and contaminate your eluted protein.

SDS can also be used in a somewhat similar fashion, but it is important to use SDS that is free from higher alkane lengths such as C14, C16, etc. Refolding experiments using GFP denatured in SDS work quite well (R. Burgess N. Thompson, and R. Chumanov, unpublished), so SDS should be considered a viable solubilizing agent.

The successful use of the cationic detergent cetyltrimethylammonium chloride (CTAC) in solubilization and refolding has also been reported (Puri et al., 1992). Even distilled water at very low salt has been reported to achieve solubilization (Song, 2009).

5.4. Refolding

Assuming you have carried out refolding trials as described below, then one basically dilutes out the denaturant by diluting the solubilized protein into a suitable refolding buffer. However, there are three ways to dilute during refolding.

1. Reverse dilution: Add refolding buffer to denatured protein with mixing between each addition. This method has been successful in several cases (e.g., Gribskov and Burgess, 1983). However, on reflection, it is clear that this method of dilution results in the highest concentration of protein at the critical time when the protein is starting to refold. For example, if the solubilized protein concentration is 1 mg/ml in 6 M GuHCl, then if one adds 2–5 volumes of refolding buffer (dilutes to 1–2 M GuHCl), one is likely to go through the refolding transition between protein concentrations of 330–160 μg/ml.

2. Flash dilution: Add denatured protein to refolding buffer quickly. For example, add 10 ml of solubilized protein in 6 M GuHCl at one time with rapid mixing to 590 ml of a suitable refolding buffer to achieve a 60-fold dilution. The protein concentration will be 16 μg/ml during the refolding, much lower than with reverse dilution and less likely to result in aggregation.

3. Drip dilution: Add denatured protein to refolding buffer very slowly, drop-by-drop, over period of 1 h. In theory, this should be the optimal method, since protein is always refolding at minimal protein concentration (see Singh and Panda, 2005; Vallejo and Rinas, 2004). For example, if you add 0.1 ml of solubilized protein as above into 590 ml refolding buffer, then the protein concentration will only be 0.16 μg/ml. As you add small incremental amounts of the sample, the protein will always be
low and when the last has been added, the protein concentration will be 16 μg/ml. However, if the denatured protein is added over a period of 1 h, and significant protein refolding to native state is achieved with a refolding half-life of 1–3 min, then the concentration of partially refolded sticky protein will always be quite low and the native protein will not be available to aggregate. While adding solubilized protein over 1 h period is tedious when added drop-by-drop, one can set up a peristaltic pump at a very slow flow rate to deliver the denatured protein over 1 h period. This is more reproducible and causes considerably less graduate student burnout.

Let the protein sit after dilution for 30–60 min and then filter as described in the generic protocol. If this is not done, then as the protein solution is loaded on the column (see below) very small particulate material is likely to clog up the column, causing the pressure to rise, either aborting the run or requiring one to decrease the flow rate to such low levels that it takes many hours to load the column.

5.5. High-resolution ion-exchange chromatography

After refolding and filtering, a final, high-resolution ion-exchange column step accomplishes five important jobs:

1. *It concentrates protein* (e.g., from 600 to 4–8 ml).
2. *It removes denaturant* (in the flow-through).
3. *It removes minor impurities* (in flow-through or binding weaker or stronger than the target protein to the column). If a high-resolution anion-exchange column is used, like POROS HQ or MonoQ, any DNA contamination will bind tightly and elute at 0.6–0.9 M NaCl. If the presence of *E. coli* lipopolysaccharide (LPS) in the final product is particularly undesirable, one can wash the column with isopropanol to greatly reduce LPS before beginning the salt gradient elution.
4. *It selects for a homogeneous, active monomer*. If one has a sample of the native protein, purified by nonrefolding methods, then one can see at what salt it elutes from this same ion-exchange column. If a major peak is eluted at the same salt from the refolded material, then one has some confidence that the material is properly refolded because misfolded molecules are likely (but not necessarily) to elute slightly differently if the column is capable of high resolution.
5. *It removes soluble protein multimers*. Soluble multimers are very often found in refolded material (see Section 5.7). If you have chosen the refolding solution well, they may be minimal; if not, they may dominate. These soluble N-mers have N times as much charge as the monomer. They tend to bind tighter to the ion-exchange column and elute later due to their higher charge. Removal of multimers is
an extremely important step if the protein is being used for crystallization for structure determination, since heterodisperse material is much less likely to crystallize.

In my experience, in most cases the protein eluting in the first major peak from the column will be high quality, pure material with full biological activity.

5.6. Reoxidation to form correct disulfide bonds

If your protein has no cysteines this is not an issue. However, most proteins contain cysteines, and many have disulfide bonds as important parts of their three-dimensional structure. Since the cytoplasm of *E. coli* is very reducing, most internal proteins are in the reduced state. If you add a reducing agent like 0.1–1 mM DTT to the lysis buffer, you can usually keep the protein reduced and prevent unwanted or incorrect disulfide bonds from forming during the early steps above. However, once you refold your target protein, you must at some stage allow the reoxidation of the protein to form disulfide bonds if they can form. Proteins that have cysteines, but that do not normally contain disulfide bonds have cysteines that are not positioned in the precise geometric configuration needed to form a disulfide bond (see Anthony *et al.*, 2002). Therefore, even though there are often a very large number of possible incorrect disulfide bonds, they do not form readily. The best strategy is to have a redox buffer present during protein refolding. Such a buffer (see below) has a mixture of reducing and oxidizing agents that allow disulfide shuffling to occur (see Gilbert, 1994). Disulfide shuffling consists of allowing disulfide bonds to be formed and be reduced repeatedly. If an incorrect bond forms in a misfolded protein and cannot be reduced, the protein is frozen into an incorrect conformation and will not reach the native state. If that bond is reduced, the protein can continue to move between intermediate folding states until it reaches the correct structure. If the correct bond now forms, it stabilizes the final native protein. Even if it is occasionally reduced, the protein is in a stable state and will reform the correct bond upon reoxidation. For this reason, it is often successful merely to have reducing agent in the solubilized protein but not in the refolding buffer. After refolding, the protein is allowed to undergo slow air oxidation (exposed to air for a day or two) and often achieves the correct disulfide bonded structure. For more on reoxidation see Vallejo and Rinas (2004) and Kersteen and Raines (2003).

Some typical methods for forming disulfide bonds are

1. *Air oxidation:* expose to air, without reducing agent for several days.
2. *Redox buffer:* for example, reduced/oxidized glutathione = 10/1 (3 mM GSH/0.3 mM GSSG). Various redox pairs are used including reduced
and oxidized cysteine, dithiothreitol, and glutathione. The molar ratio of reduced to oxidized form is sometimes varied to achieve optimal disulfide reoxidation.

3. **Protein disulfide isomerase (PDI):** this is an enzyme that catalyzes disulfide shuffling (see review by Kersteen and Raines, 2003). One can also use small molecule PDI mimics (see Woycechowsky et al., 1999).

### 5.7. Characterization

One of the most common problems I see when reviewing papers involving refolding is that one has no idea if the material at the end of the procedure is correctly refolded, monodisperse, or has full biological activity. It is common to see an enzyme or biological assay, but often without any standard. You see phrases like “since the final product has activity it is shown that it is correctly folded and fully active.” Only a comparison with a known, fully active standard will allow an estimation of the percent of the final product that is active. Without this comparison you know there is activity, but do not know if 100%, 10%, 1%, 0.1%, or 0.01% of the refolded material is active. Such a determination of specific activity of the final product is essential in a refolding procedure, if possible.

Another important characterization is to determine the size of the refolded material. Is it monomeric (monodisperse) or does it contain large amounts of soluble multimer (heterodisperse)? Crystallographers routinely use dynamic light scattering (DSL) to determine if purified protein, refolded or not, is monodisperse. Another method, developed by Mark Knuth at GNF in La Jolla, CA is to analyze the final product by analytical size exclusion chromatography (ANSEC). We have found this to be very useful since very small amounts of protein can be analyzed. Typically, we run 20–50 μg of protein on a 12-ml Shodex KW-802.5 column at 0.5–1.0 ml/min in a buffer containing 0.25 M NaCl, monitoring absorbance at 280 and 215 nm. When the column is calibrated with suitable MW markers, one can quickly determine if most of the protein is in a single peak (monodisperse) of the size expected for the monomer (this is good), or if much of the protein elutes earlier indicating that it is much larger (heterodisperse) due to formation of multimers (this is bad). Use of this procedure is an important part of a thorough set of refolding test trials (see below).

One cannot use circular dichroism (CD) or Western blot analysis to determine activity or monodispersity. There are plenty of examples of proteins whose CD spectra are very similar or identical to native standard material and which react just fine in a Western blot analysis that are not monodisperse or active, respectively.
6. Performing a Protein Refolding Test Screen

6.1. Systematic refolding screens

In the general procedure and discussion earlier, it was assumed that you knew a suitable refolding solution for your protein. This, however, is the most essential and the most difficult part in designing an efficient refolding protocol. In early refolding papers, usually a single refolding solution was chosen and it either worked or did not work. The ones that worked were published and the unsuccessful attempts were often abandoned. As a result, the early successes were usually proteins that refolded readily under many different conditions. In recent years, it has become increasingly clear that many proteins can only be refolded under very specific conditions. The challenge has become how to screen the very many possible conditions to find one that is effective in promoting refolding. An important advance was the use of fractional factorial approaches to systematically determine the effects of many different variables (Armstrong et al., 1999; Cowieson et al., 2006; Quronfleh et al., 2007; Trésaugues et al., 2004; Vincentelli et al., 2004; Willis et al., 2005).

Several commercial products have been developed to aid the researcher in identifying suitable refolding conditions. Much more information on these refolding screens and related protocols are available at the company web sites.

AthenaES QuickFold\textsuperscript{TM} (15-solution kit); (http://www.athenaes.com/QuickFoldProteinRefolding Kit).
EMD/Novagen’s iFOLD\textsuperscript{1TM}, iFOLD\textsuperscript{2TM}, and iFOLD\textsuperscript{3TM} (96-solution kit); (http://www.novagen.com).
Pierce Biotechnology’s ProMatrix\textsuperscript{TM} (96-solution kit components); (http://www.fishersci.com).

It is quite likely that this list will grow and that the screens will evolve as more experience is gained in the use of this type of screen and new refolding aids are identified. It should be noted that nothing prevents a researcher who cannot afford to buy these rather expensive kits from designing a set of test solutions of their own and customizing the refolding screen to fit their special needs. The key concept here is the systematic, parallel screening of multiple refolding conditions.

6.2. Variables in refolding

There are many solution variables (such as pH, temperature, salt concentration, redox environment, and the presence of divalent ions) and additives that have been reported to improve the efficiency of refolding of solubilized proteins from IBs. Such variables and additives have been taken into
account in designing the commercial refolding screens mentioned above and serve as examples for those wishing to design their own refolding screen. These variables are discussed below. Other reviews on protein refolding go into greater detail on some of these (Armstrong et al., 1999; Cowieson et al., 2006; Middelberg, 2002; Quronfleh et al., 2007; Schein, 1991; Singh and Panda, 2005; Trésaugues et al., 2004; Vallejo and Rinas, 2004; Vincentelli et al., 2004; Willis et al., 2005).

**pH**: Most refolding is done in the range of pH 5–9 and, in our experience, most proteins refold best at about pH 8–8.5. In general it is a good idea to use a pH that is at least one pH unit away from the isoelectric point or pI (the pH at which the protein has zero net charge and is most prone to precipitation).

**Temperature**: So far no obvious trend has developed as to any generally optimal temperature to use during refolding. Most people carry out the refolding process at near room temperature. This is a low enough to prevent thermal damage to the protein and high enough to increase the thermal motion of the molecules that is likely important in melting out transient misfolded conformations and reaching the native state. One could argue that higher temperatures will strengthen the hydrophobic interactions that likely lead to aggregation, but then again it should also strengthen the hydrophobic interactions needed to bury hydrophobic residues in the interior of a native protein.

A very interesting paper by Xie and Wetlaufer (1996) reported the refolding of carbonic anhydrase II at 4 mg/ml in 1 M GuHCl and 50 mM Tris sulfate, pH 7.5 at different temperatures. It appears that refolding at low temperatures (4–12 °C) for 120 min followed by a “temperature-leap” to 36 °C for 30 min gave excellent recovery of activity (>90%). They argue that hydrophobic interaction is diminished at the low temperature, minimizing aggregation and allowing slow conversion to an intermediate that is not active, or aggregation prone. Upon temperature-leap from 4 to 36 °C, this intermediate converts to a highly active, native form.

**Salt concentration**: Some salt is probably desirable to cause “salting in” to increase solubility of the native protein (see Chapter 20 in this volume). Often 50–100 mM salt is used. In the case of a 60-fold dilution of 6 M GuHCl, the final concentration of GuHCl is 0.1 M. Additional salt is often avoided to keep the salt low enough to allow protein binding to an appropriate ion-exchange column.

**Redox agents**: The use of redox buffers to aid in correct disulfide bond formation is discussed in the section on reoxidation above. In some of the commercial kits there are solutions with no reducing agent, with reducing agent and redox buffers with several ratios of reducing to oxidizing agents.

**Divalent cations**: This variable has not been thoroughly explored, but it is clear that native proteins often have divalent cations such as Mg$^{2+}$ or Zn$^{2+}$ as part of their structure. Obviously, anything that will stabilize the native
structure will bias refolding toward native and away from aggregated protein.

**Arginine and other amino acids**: There is an extensive literature on the use of arginine to promote refolding of solubilized protein and how it functions to diminish aggregation (Arakawa et al., 2007; Baynes et al., 2005; Das et al., 2007; Dong et al., 2004; Reddy et al., 2005). It appears that arginine decreases aggregation by slowing the rate of protein–protein interactions. Das et al. argue that this is because arginine forms supramolecular assemblies in solution. One of its drawbacks is that it is usually used and is most effective at concentrations of 0.5–1.0 M. At these concentrations, it interferes with subsequent chromatography on Ni²⁺-chelate affinity columns and will prevent the binding of most protein to an ion-exchange column without further dilution or dialysis. The natural osmoprotectant proline has also been found to be effective in some cases at increasing solubility both *in vitro* and *in vivo* (Ignatova and Gierasch, 2006).

**Glycerol and sugars (sucrose, mannitol, sorbitol, and trehalose)**: We have found glycerol to be an excellent refolding additive in many cases, usually used in the 5–30% range. One extreme method by Shimamoto et al. (1998) involves solubilization of IB protein to 2–10 mg/ml by 6 M GuHCl, addition of glycerol to 50% (v/v), dialysis into 75% (v/v) glycerol to remove denaturant, and then rapid dilution to lower glycerol to 5–10%. Several sugars in the 0.5–1.5 M range have been seen to promote successful refolding (Bowden and Georgiou, 1988).

**Other chemical additives**: A large variety of papers have reported the use of materials such as polyethylene glycol (PEG) (Cleland et al., 1992), cyclodextrin (Rozema and Gellman, 1996), and various nondetergent sulfobetaines (NDSBs) (Expert-Bezancon et al., 2003) to increase refolding for certain proteins. The effects of certain ionic liquids (i.e., organic salts with melting points below 100 °C) such as N’-alkyl-N-methylimidazolium chlorides as refolding additives have been reported (Lange et al., 2005). The ethyl and propyl derivatives, at concentrations of 0.5–1.0 M, showed effects in increasing activity and refolding yields comparable to l-arginine.

**Detergents**: In theory, detergents should be helpful in preventing aggregation during refolding. At low concentrations they bind weakly to exposed hydrophobic or sticky regions and mask them, preventing aggregation. As their concentration decreases they dissociate and allow reformation of native structure. This is thought to be why Sarkosyl works as well as it does. At high concentration it is a denaturant, but at low concentration it acts as an artificial chaperone and promotes refolding without aggregation.

**Chaotropic agents (denaturants at high concentrations)**: Since aggregation is due to interactions among partially folded intermediates, it has sometimes been useful to have a nondenaturing amount of a chaotrope in the refolding solution to dissociate aggregates, but not the more stable, native structures. 1 M urea and 0.5 M GuHCl have been used.
**Target protein-specific additives:** The same argument given above for the potential benefit of certain divalent cations can be used to suggest that the presence of appropriate substrates, or cofactors, or essential heme groups, for example, would stabilize native structure and improve refolding yield.

### 6.3. A practical, inexpensive rational approach

With all the refolding screens, you still have to obtain some sort of a readout that indicates which condition works best. The best situation is when your target enzyme is a known enzyme and can be easily assayed. You merely make your dilutions from solubilized protein into the various refolding solutions, wait some time for refolding (often several hours), and then assay a portion of the dilute solution for enzyme activity.

It is even easier if you want to examine the effects of conditions on refolding of a fluorescent protein such as GFP since the fluorescence is restored when the GFP refolds correctly and can very conveniently be measured during and after the refolding with an appropriate fluorescence plate reader. This makes an excellent exercise for teaching protein refolding and is used at the Protein Course at Cold Spring Harbor (R. Burgess, N. Thompson, R. Chumanov, unpublished results).

However, most proteins being studied these days are either not enzymes or have a very difficult biological assay. How do you know which refolding condition worked best? The following protocol has been used with great success (R. Chumanov and R. Burgess, unpublished results). This procedure is quite general and takes advantage of the turbidity of solutions where protein aggregation has occurred (see Trésaugues et al., 2004; Vincentelli et al., 2004).

1. Prepare washed IBs solubilized in several different chaotropic agents as listed above and centrifuged to remove insoluble material. Protein concentration should be 3–5 mg/ml.
2. Prepare a set of test solutions based on variables and additives that you think might be effective.
3. Pipette 10 μl of solubilized protein into the appropriate number of wells of a 96-well plate microtiter dish (Linbro, flat bottomed, polystyrene).
4. Carry out a 20-fold flash dilution by quickly adding 190 μl of the test refolding solution and mixing the solution up and down several times in the pipette at room temperature.
5. Wait 15–60 min and then read the absorbance of the plate at 320 nm. The solution is not absorbing the light, but rather the protein aggregates scatter light and decrease the amount of transmitted light measured. This works well because light scattering increases as you go to lower wavelengths, proteins do not absorb at 320 nm, and 320 nm is about a low as you can go in wavelength before absorbance of the plate gets too great.
At 320 nm, the absorbance of the empty well due to the plastic is about 0.2 and can be subtracted from the apparent absorbance readings due to solution turbidity to give a corrected turbidity.

6. The results of the absorbance readings with the plate absorbance subtracted can be graphed versus the solution number and one can see which solutions give the lowest turbidity. Corrected turbidity values range from 0.0 to about 0.4. We often find several conditions that give low- or zero-corrected turbidity. One often gets somewhat different results with protein denatured using different solubilizing agents such as 6 M GuHCl, 8 M urea, or 0.3% Sarkosyl.

7. Choose the best 2–3 conditions that are compatible with loading directly (after filtration) onto an analytical size exclusion column (ANSEC, see Section 5.7). To be really useful for large-scale refolding, it must also be compatible with loading onto an appropriate ion-exchange column at a salt concentration of about 0.1 m NaCl. The solution (about 200 μl) can be removed from the corresponding well, filtered through a syringe filter or centrifuged in a microfuge tube and loaded onto a 12-ml ANSEC column. A refolding condition that gives low turbidity and mostly monomeric material on the ANSEC column is the condition that is likely to be useful in the large-scale refolding of the target protein.

7. Other Refolding Procedures

Although the main focus of this chapter has been on refolding of solubilized IB protein by dilution into a suitable refolding solution, there are several other significant approaches to protein refolding that need to be described briefly.

1. On-column refolding. This is a large and active area of research and application. The basic principle is that denatured proteins when bound to a column resin or residing within the pores of a gel filtration resin are tethered or sequestered and if they experience a gradual decrease in the concentration of denaturant, they will refold, but be less likely to aggregate. This idea was first proposed by Tom Creighton in the late 1980s and is now widely used in various column modes such as ion exchange, affinity, metal chelate affinity, hydrophobic interaction, and gel filtration chromatography. For example, one can load an IB protein solubilized with 8 M urea onto an ion-exchange column, wash away some impurities with 8 M urea, wash with a gradually decreasing urea gradient, and elute with a linear salt gradient. Another approach is to preload a gel filtration column with a reverse gradient of denaturant. The top of the column is 8 M urea, the gradient decreases linearly to 0 M urea.
in the middle of the column, and the bottom half of the column is 0 M urea. One then loads the denatured protein and denaturant is applied at a slow flow rate to the column. The protein will move down the column faster than the buffer (because it is partially or completely excluded from the bead) and move gradually into lower and lower concentrations of denaturant until it has refolded and elutes from the column. The difficulty here is to decide on the steepness and volume of the reverse gradient. The various protocols and applications are summarized nicely in several recent reviews and articles (Jungbauer and Kaar, 2006; Jungbauer et al., 2004; Oganesyan et al., 2005; Swietnicki, 2006; Veldkamp et al., 2007). While this on-column refolding method seems ideal, it is often the case that as the denaturant concentration is decreased, the protein precipitates on the column and is lost. This is usually due to the tendency of many researchers to overload their column or to load from one end that becomes saturated with protein. In these cases, the fact that the protein is tethered to the resin does not help because the bound proteins are close enough together on the column that they can still aggregate during refolding. One general procedure that sometimes helps is to bind the protein in denaturant to the column in batch (obviously not applicable to size exclusion chromatography) at well below saturating amounts, then load the resin into a column, and finally carry out the subsequent washing and elution steps in column.

2. Use of folding catalysts such as protein and chemical chaperones, PPI, PDI. Another nice approach is to covalently attach various enzymes and chaperones to a gel filtration column resin and then refold in column. The enzymes that have been immobilized, either individually or perhaps better in combinations are: PPI (catalyze proline isomerization), PDI (promote disulfide shuffling), DsbA and DsbC to catalyze disulfide formation, and the chaperones GroEL/GroES to aid in refolding (Baneyx and Mujacic, 2004; Jungbauer et al., 2004; Paul et al., 2007; Swietnicki, 2006).

3. High-pressure refolding. A number of papers, summarized by Quronfleh et al. (2007) report the successful solubilization of IBs by exposure to high hydrostatic pressure (1.5–2.5 kbar). It is claimed that exposure of IBs to high pressure within a certain range will disaggregate the aggregated protein but not denature it. The process of pressurizing, holding at high pressure, and slowly decreasing the pressure in a variety of buffers has been reported to result in high recovery of soluble and active enzymes. While this method has been commercialized by Barofold (Boulder, CO) using a specialized pressure cells (PreEMPTM high-pressure chamber), the equipment is not widely available. In some cases, while the IB is solubilized, the protein is largely in the form of soluble multimers. Like the solubilization with denaturants and refolding discussed for most of this chapter, one must find the right conditions for optimal recovery of active protein by screening multiple conditions.
4. **Alkaline pH shift to solubilize and refold.** Singh and Panda (2005) argue that proteins in IBs are largely folded and can more effectively be refolded if not subjected to high concentrations of GuHCl or urea. They state that IB protein (recombinant human growth hormone) was solubilized efficiently at pH 12.5 in 2 M urea and a concentration of 2 mg/ml, but retained significant secondary structure. The solubilized protein was then diluted into 2 M urea, pH 8 with a 40% recovery of activity.

### 8. Refolding Database: Refold


This database, developed by S. Bottomley and his colleagues at Monash University, contains over 1000 proteins that have been successfully refolded and presents protocols and statistics on the frequency of use of various refolding techniques, disruption methods, fusion proteins, and preparation prior to refolding (Chow et al., 2006). For example, almost 85% of the examples utilize some form of dilution or dialysis to remove denaturant, while about 12% utilize on-column refolding.

### 9. Strategies to Increase Proportion of Soluble Protein

The standard method for determining the proportion of soluble and insoluble material is to prepare a cell lysate and then centrifuge to sediment insoluble material (unbroken cells, cell debris, and IBs). The total (crude lysate), soluble (supernatant), and insoluble (pellet) fractions are analyzed by SDS–PAGE and the intensity of the target protein band in the three fractions is compared by stain intensity or by Western blot analysis. If the vast majority of the target protein is in the soluble fraction, then you say that the protein is soluble. If the majority is in the pellet, then you say the protein is insoluble. Often target protein is in both soluble and insoluble fractions. While this is often an accurate estimate of the proportion of the protein that is soluble, I have seen two cases where the results can be misleading. The first is when cell breakage is incomplete and the SDS gel pattern of the pellet looks much like that of the lysate. This suggests that more effective lysis conditions be used (see Chapter 18 in this volume). Sometimes one sees significant amounts of target protein in the soluble fraction due to soluble multimers or to ineffective pelleting of IBs. If sonication is too vigorous, sometimes the IBs are broken up into smaller particles that may not be completely sedimented. This can be remedied by longer, harder
centrifugations. Also sedimentation of IBs can be incomplete if the viscosity of the lysate is high due to the presence of too much large DNA. Treatment of the lysate with a nuclease such as Benzonase can decrease this viscosity.

Even though I believe it is possible to find effective refolding conditions for most expressed proteins, many researchers strongly prefer to decrease IB formation and increase the proportion of soluble product if possible. In many expression systems so much protein is produced that even if only 20% of the protein is soluble there can be quite significant amounts of protein available for purification by more standard methods. Some of the strategies for increasing the amount of soluble product are listed below (see Schein, 1989; Chapters 11 and 12 in this volume).

1. *Induce overproduction at lower temperatures, for example, 20 °C.* It was observed in the late 1980s (Schein, 1989) that one could increase the proportion of soluble material by inducing the target protein overexpression in *E. coli* grown below the normal growth temperature of 37 °C. Temperatures of 20–25 °C are commonly used, although cell growth is quite slow at the lower temperature. The basic rationale seems to be that at the lower temperature, transcription and translation are slower, so that proteins have more time to refold into native structures and the internal concentration of the partially folded, sticky intermediate is lower, resulting in less aggregation, and IB formation (see Vera *et al.*, 2006).

2. *Add 0.4 M sucrose to the culture medium.* This approach has been reported by Bowden and Georgiou (1988). This phenomenon may be due to the high osmolyte concentration inducing the osmotic shock response, which increases the internal level of glutamate, proline, and trehalose, thus providing conditions more favorable for refolding.

3. *Coexpression of molecular chaperones.* Since molecular chaperones such as DnaK/J and GroEL/ES are known to suppress misfolding and aggregation in the cell, one can increase the levels of these proteins by introducing a separate plasmid that contains inducible genes for these chaperones.

4. *Subject cells to brief heat shock at 42 °C, then shift to 20 °C, and induce.* This is an elegant approach that avoids the need to coexpress chaperones as above, but rather takes advantage of the natural heat shock response where DnaK/J and GroEL/ES levels increase. A reasonable procedure is to grow cells at 35 °C to A600 nm of about 0.5, shift the cells rapidly to 42 °C for 15–20 min to induce the heat shock response, decrease the temperature to 20–25 °C, and then induce expression of target protein for 4–8 h.

5. *Coexpression of several subunits of a complex.* It has been observed that if two proteins (say protein X and Y) that form a stable heterodimer are expressed in *E. coli* individually they are mostly insoluble, but if they are coexpressed they refold, form their normal dimer (XY) and are soluble. Vectors for such coexpression are marketed by EMD/Novagen as the Duet vectors.
6. Fuse to easily refolded protein, for example, a “solubility tag” such as NusA, Trx, MBP, GST, SUMO, and HaloTag (see Chapter 16 in this volume).
7. Form disulfide bonds in the *E. coli* cytoplasm. The environment in the *E. coli* cytoplasm is reducing, preventing most disulfide bond formation. Mutations in the glutathione reductase (*gor* gene) and thioredoxin reductase (*trx*B gene) enhance formation of disulfide bonds in the *E. coli* cytoplasm (Bessette *et al.*, 1999). Strains AD494 (DE3) and BL21trxB(DE3) are *trx*B deficient. Novagen Origami strains are *trx*B and *gor* deficient.

10. Conclusion

Recombinant proteins overexpressed in *E. coli* often form insoluble IBs in the cell. The IBs are easy to purify and solubilize, but finding suitable conditions for efficient refolding of the solubilized protein is sometimes difficult. The best general strategy seems to be to screen many different refolding conditions in parallel to find ones that give the highest recovery of enzyme activity or the lowest turbidity due to aggregation and that show the least amount of soluble multimers. Many additives may prove useful in refolding, but the surest way to prevent aggregation and precipitation upon refolding is to refold at low protein concentration.

REFERENCES


