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ScienceDirect
Protein Expression and Purification 55 (2007) 209–224
Protein Expression & Purification
www.elsevier.com/locate/yprep

Review

Confronting high-throughput protein refolding using high pressure and solution screens

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Received 29 November 2006, and in revised form 7 May 2007
Available online 2 June 2007

Abstract

Over-expression of heterologous proteins in *Escherichia coli* is commonly hindered by the formation of inclusion bodies. Nevertheless, refolding of proteins *in vitro* has become an essential requirement in the development of structural genomics (proteomics) and as a means of recovering functional proteins from inclusion bodies. Many distinct methods for protein refolding are now in use. However, regardless of method used, developing a reliable protein refolding protocol still requires significant optimization through trial and error. Many proteins fall into the category of "Challenging" or "Difficult to Express" and are problematic to refold using traditional chaotrope-based refolding techniques. This review discusses new methods for improving protein refolding, such as implementing high hydrostatic pressure, using small molecule additives to enhance traditional protein refolding strategies, as well as developing practical methods for performing refolding studies to maximize their reliability and utility. The strategies examined here focus on high-throughput, automated refolding screens, which can be applied to structural genomic projects.

Keywords: Inclusion bodies; Aggregation; Misfolding; Solubilization; Protein recovery; Protein refolding; Small molecules assisted refolding; Fractional factorial screen; High hydrostatic pressure; Automation; High-throughput; Structural genomics

The expression of proteins in transformed microorganisms has been one of the fundamental techniques in the development and expansion of modern biological research. Numerous expression systems are available, allowing both high and low levels of protein expression in a variety of prokaryotic or eukaryotic organisms [1]. Despite the successes and growth of expression technology, significant pitfalls still exist. Expression of recombinant proteins often results in the accumulation of inactive and improperly folded proteins in the form of aggregates and occurs commonly when eukaryotic, disulfide containing, post translational modified, or multimeric proteins are expressed in insect and bacterial systems [2,3]. While some expression systems lead to high level of soluble aggregates, expression in *Escherichia coli* often leads to insoluble aggregates known as inclusion bodies. Inclusion bodies are dense structures of the misfolded expressed polypeptides that arise from the inability of cellular machinery to process and refold the polypeptide correctly [3]. In some cases, inclusion body formation is a result from the reducing environment of the cytoplasm of *E. coli* preventing native disulfide bond formation. Additionally, the cellular machinery of *E. coli* is incapable of handling the high levels of expression that occur during typical recombinant protein production and results in inclusion body formation. Optimization of soluble protein expression is often the strategy of choice when trying to obtain bioactive protein [1,4]. Numerous labor-intensive expression systems and culture conditions have been developed that attempt to prevent inclusion body formation. However, the formation of inclusion bodies is still common and often unavoidable.

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doi:10.1016/j.pep.2007.05.014

Although inclusion bodies are often considered undesirable, their formation can be advantageous. The benefits associated with inclusion bodies include: (i) high level, inexpensive, expression and homogeneity of the target protein, (ii) proteolytic resistance, and as a result, lower levels of degradation of the expressed protein, and (iii) easy isolation and purification of the inclusion bodies from host cells [5]. These benefits can only be achieved if the protein of interest can be refolded to obtain native protein with high yields. Fortunately, inclusion bodies can be solubilized and refolded to release the misfolded or aggregated protein by using high hydrostatic pressure or chaotropes. Unfortunately, there is no universal refolding method or buffer. A variety of methods may be used to refold inclusion bodies; however, the method and refolding solution conditions can greatly impact the subsequent refolding step and the cost of the overall process [6,7]. While protein refolding is often a strategy of last resort due to unpredictability, time requirement, and operational issues of the renaturation process, the literature demonstrates the applicability and success of protein refolding techniques [6]. This review summarizes the current state-of-the-art in high-throughput protein refolding, describing refolding screens that rapidly identify initial conditions that successfully result in folded protein. Additionally, new protein refolding techniques, such as high pressure refolding, have promise for improving refolding yields in many protein classes [8].

Misfolding and aggregation

The structure of proteins is maintained by a delicate thermodynamic balance of hydrogen bonding, and hydrophobic and electrostatic interactions. These interactions are weak relative to covalent bonds and proteins are inherently unstable and susceptible to the formation of non-native aggregates and precipitates. Non-native protein aggregation (described hereafter as ''aggregation'') describes the assembly of native or structurally perturbed proteins to aggregates containing non-native protein structures [9]. Aggregation occurs commonly *in vivo* and living cells have developed sophisticated mechanism to either prevent aggregation (i.e. molecular chaperones) or conduct housekeeping to degrade misfolded proteins [10]. During *in vitro* protein processing, aggregation is often irreversible at physiological conditions and may result in the formation of high levels of non-native, intermolecular β-sheet structures [11].

The reader is referred to an excellent review on protein folding, misfolding and aggregation in pharmaceutical proteins by Chi et al. [12]. Briefly, protein aggregation proceeds through specific pathways that are initiated by instability of the native protein conformation or colloid instability associated with protein–protein interactions. Conditions such as temperature, solution pH, ligands and cosolutes, salt type and concentration, preservatives, and surfactants all modulate protein structure and protein–protein interactions, and thus aggregation propensity. For aggregates that form from native protein instability, it appears that aggregates may form from protein structures present within the native state that demonstrate an expanded conformation and are often the result of non-specific hydrophobic interactions [13,14]. Consequently, aggregation is controlled by the conformational stability of the native protein relative to that of the aggregation transition state. Recently, it has been reported that proteins can form aggregates due to colloidal instability, even in solution conditions which thermodynamically greatly favor the native conformation [15]. These molecular assembly reactions are a result of intermolecular attractions. For example, GCSF at pH 7.0 has been demonstrated to have a large $\Delta G_{unfolding,}$ yet the protein aggregates readily due to colloidal instability arising from attractive electrostatic interactions [15]. Due to the myriad of aggregation mechanisms in all proteins, it is not surprising that protein aggregation is a widespread problem in all aspects of protein processing, both *in vivo* and *in vitro*. It is therefore imperative that any group wishing to refold significant numbers of proteins have access to state of the art technology and methodology.

During the process of refolding, solubilization of the protein of interest can readily be achieved by the addition of high concentrations of chaotropes, such as urea or GdHCl. The complications arise when the chaotrope is removed. The current folding theories propose that protein folding occurs along a ''free-energy funnel'', where the protein forms structure along a landscape of intermediates of lower free energy [16,17] (Fig. 1). As the protein navigates

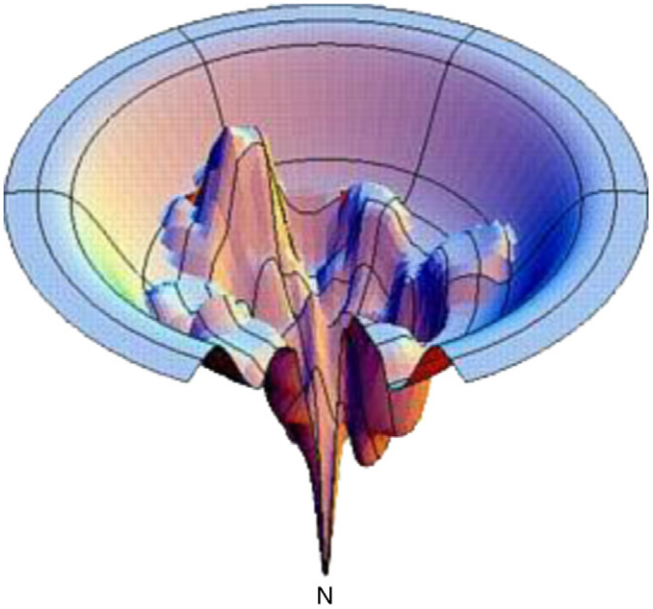

Fig. 1. Folding landscape of a protein, as depicted by Dill et al. [18]. The upper rim of the funnel is representative of high energy, denatured protein. As the thermodynamic energy state of the protein is decreased, the protein begins to fold as it migrates down the energy funnel. However, kinetic traps are often encountered which prevent the formation of native structure and can lead to reaggregation since the folding intermediates are often aggregation prone.

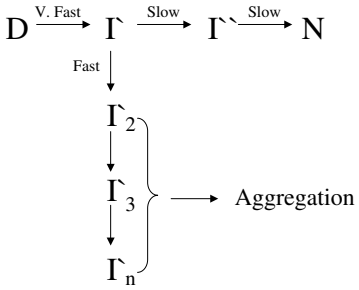

Fig. 2. Protein refolding involves intramolecular interactions and follows first order kinetics. Protein aggregation, however, involves intermolecular interactions and, thus, is a kinetic process of second or higher order, which is favored at high protein concentrations. Aggregates are often formed by non-native intermolecular hydrophobic interactions between protein folding intermediates. Prevention of aggregate-prone intermediates during the first steps of refolding is crucial to allow successful renaturation at high protein concentrations. D, denatured or unfolded; I`, partially folded intermediates; I``, monomeric intermediate; N, native.

the energy landscape, it can become kinetically trapped in an intermediate form that is aggregate prone. The competing folding and reaggregation reactions are shown in Fig. 2. The goal of any refolding strategy is to prevent the formation of aggregate-prone intermediates that lead to reaggregation and loss of yield after protein solubilization from inclusion bodies. Refolding by implementing high hydrostatic pressure has emerged as an experimentally and thermodynamically distinct alternative to traditional chaotrope-based methods. High pressure refolding methods have the advantage of solubilizing and refolding proteins without first denaturing the protein, minimizing the formation of aggregate-prone intermediates, and enabling refolding at comparatively high yields and at high protein concentration [8,18–20].

This review will summarize the methods of screening refolding additives, which minimize the formation or aggregation propensity of these refolding intermediates during chaotrope-based refolding. In addition, the review will discuss implementing high hydrostatic pressure protein refolding.

Traditional chemical-based aggregate refolding and recovery

Chemical chaotropes have been traditionally used to solubilize proteins from inclusion bodies. High concentrations of chaotropes (up to 6 M GdHCl or 8 M urea) are required to provide the chemical energy to thermodynamically dissociate the aggregates with concomitant denaturing of the protein [6,21,22]. Refolding is achieved by removing the chaotrope *via* buffer exchange after aggregate dissociation, using dilution, dialysis, diafiltration or solid-phase separation such as size exclusion and ion exchange chromatography. Several excellent reviews have detailed these and other refolding techniques [6,23,24]. A common refolding process is summarized as follows: the majority of published work suggests that inclusion bodies or aggregates are denatured and solubilized by chaotropes at protein concentrations 1–10 mg/ml. If scale up is desired this concentration can be increased. The solubilized protein solution at 1–2 mg/ml is diluted 50- to 100-fold in a solution containing low final chaotrope concentrations (0.5–1.5 M) and a thiol reducing/oxidizing environment to enable renaturation and the proper formation of disulfide bonds [6,25]. Here the final denaturant concentration is the sum of any in the solubilization and refolding buffers. Low protein concentrations, typically from 50 μg/ml to 1 mg/ml, are needed to prevent reaggregation since aggregation kinetics are greater than second order (ca. 2.6) [26]. In the ideal case, the denatured protein collapses to form folding intermediates and finally the native conformation. More commonly, folding intermediates are aggregate prone and chaotrope-based techniques result in reaggregation and a loss in protein recovery (Fig. 2). In many cases, active protein cannot be obtained, even at low protein concentrations.

To improve refolding, steps need to be taken to inhibit the reaggregation of the aggregate-prone folding intermediates. The selective choice of small molecule additives through the use of solution screens can minimize reaggregation during chaotrope-based refolding methods.

Small molecule additives

Several methods for suppressing aggregation in order to promote protein refolding have been developed. These methods generally fall into one of several categories; chaperone mediated, artificial chaperone or detergent mediated, chromatographic matrix assisted, gradient oscillation, and small molecule assisted refolding (see Table 1) [6,23,24,27–29]. This paper will focus on small molecule and detergent assisted refolding, as it is the simplest and most conducive to initial refolding trials. The technique lends itself to both miniaturization and automation resulting in reduced protein requirements and increased throughput, therefore, appealing to structural studies. However, it is important to note that no one method for protein refolding will be satisfactory for all types of application.

Small molecule assisted refolding in many ways mimics nature. *In vivo*, plants and microorganisms utilize a variety of small molecules or "osmolytes" to protect proteins as a central part of their defensive response to heat, cold, water, or salt stress. It is believed that these protective osmolytes have been evolutionarily selected for their general ability to stabilize the native structure of proteins under stress conditions without significantly affecting enzymatic activity [23]. Small molecule assisted refolding utilizes similar principles *in vitro*, where the solution environment is controlled through chemical additives in order to make aggregation less favorable.

The formation of disulfide bonds is required during many refolding reactions *in vivo*. The interconversion between free thiols and disulfides is a reduction/oxidative process, and as such, must be linked to the appropriate electron donors and acceptors [30]. Reversible formation and dissociation of disulfide bonds is facilitated by the use of the disulfide shuffling agent glutathione in cells. *In*

Table 1
Key methods in protein refolding and suppression of protein aggregation

Small molecule assisted
Denaturants: urea and GdHCl
Amino acids: Gly, Ala, Pro, and Arg
Polymers: PEG and cyclodextrin
Polyols: glycerol
Alcohol: short-chain alcohols
Reducing/oxidizing reagents: BME, BMC, TCEP, DTT, DTE, cysteine/cystine, glutathione, cysteamine/cystamine, and EDTA
Salts: NaCl, KCl, $MgCl_2$ and $(NH_4)_2SO_4$
Sugars: glucose, sucrose and trehalose
Artificial chaperones
Detergents: Triton X-100, CHAPS, Sarkosyl, SDS and CTAB
Detergents with cycloamylose or cyclodextran
Non-detergent zwitterionic agents such as Non-detergent sulfobetaines (NDSB), substituted pyridines and pyrroles, and substituted amino-cyclohexanes
Matrix assisted
Size exclusion chromatography
Affinity tag immobilization
Ion exchange chromatography
Chaperone mediated
ATP dependent: GroEL-GroES, DnaK, DnaJ, GrpE mini-chaperones (soluble form or immobilized and reusable system format)
Non-ATP dependent chaperones: DsbA and peptidyl-prolyl isomerase (PDI)
Gradient methods
Extreme gradient: sequential increase or decrease in denaturant concentration or pH
Extreme pressure or temperature

vitro, the appropriate reduced:oxidized ratio and concentration of disulfide shuffling agents must be identified for each protein, since disulfide formation is a function of the steric properties of the protein [26,30]. Conversely, if disulfide bond are not present in the native protein, reducing agents are added to prevent non-native disulfide bond formation.

A listing of the small molecules often used in a refolding reaction is shown in Table 1 and include amino acids, lipids/detergents, sugars/polyols, salts and reducing/oxidizing (redox) reagents (Table 1). To achieve refolding, a balancing point needs to be identified, as the thermodynamic forces that drive aggregation are also central to forming the native protein fold. To identify such conditions, screening experiments examining a variety of chemical and environmental factors are generally required since optimal conditions for refolding are protein specific. The probability of encountering a buffer composition favoring correct folding is likely to increase with the number of buffers/combinations tested. The following section describes the possible solution conditions and refolding factors that can be screened.

Effective buffer components and complementing constituents for high-throughput, chaotrope-based screening

The composition of refolding buffer is strongly protein dependent. Literature precedent indicates that the choice of pH and redox reagents has the largest impact on protein refolding yields. Inclusion of co-solutes (folding enhancers or aggregation suppressors) such as arginine also promote refolding and facilitate positive synergistic interactions [23,31]. While the factors are discussed separately, a successful small molecule assisted refolding buffer generally relies on several components working together.

pH. The pH of a refolding buffer can dramatically affect the yields and rates of the refolding reaction [6]. The pH of a refolding buffer influences the charge state of the target protein, protein stability, protein solubility, kinetics of disulfide bond formation, and alters the reaggregation propensity of intermediates on the folding pathway [32,33]. Selection of a pH range of 4–9 is typical of refolding screens. In general, to minimize aggregation during refolding, the pH of a solution should be more than 1–2 pH units away from the isoelectric point of a protein. If the protein contains disulfide bonds, the optimum pH of refolding solution is further constrained due to decrease in thiol reactivity at pH < 7.0 [33]. For this class of proteins, it is recommended that alkaline conditions (pH 7.5–10) be used for initial refolding screens to enable proper disulfide bond formation. Interestingly, a few reports indicate that in many screens, pH 8.2 seem to be an optimal pH value for refolding [7,34]. Exposure to extremes of pH for extended times (<pH 3.5 and >pH 10.5) should be avoided due to the risk of chemical modification of the protein [35].

Reductive/oxidative renaturation. The redox environment used during protein refolding is dependent upon whether the native target protein contains disulfide bonds. For non-disulfide or thiol containing proteins, addition of 1–5 mM dithiothreitol (DTT)[1]-invariably is sufficient for most proteins- or 5 mM tris(2-carboxyethyl phosphine) (TCEP) to all buffers is recommended to maintain a reducing environment during refolding. TCEP, a non-thiol reductant, is a stronger reducing agent than DTT and is capable of reduction at pH values less than 7.0 [7]. Other reducing agents like 2-mercaptoethanol (BME), bis-mercaptoacetamide cyclohexane (BMC) and 2-hydroxyethyl disulfide at 5 mM could be explored. These are particularly effective in refolding disulfide-containing proteins.

For disulfide-containing proteins, a mixture of low molecular weight thiol and disulfide containing compounds (known as disulfide shuffling agents), such as reduced and oxidized glutathione (GSH/GSSG) or cysteamine/cystamine, or cysteine/cystine are added to refolding buffers to allow disulfide bond formation and shuffling [6,24]. An excellent review on the mechanism of disulfide bond formation is provided by Gilbert [30,33]. For initial refolding experiments, a 2:1, 5:1, or 10:1 ratio of reduced to oxidized reagents with the reduced reagent at 0.2–5 mM is commonly used. It should be noted that the ratio of reduced to oxidized thiol more significantly affects the redox poten-

[1] *Abbreviations used:* DTT, dithiothreitol; TCEP, tris(2-carboxyethyl phosphine); BMC, bis-mercaptoacetamide cyclohexane; NDSBs, non-detergent sulfobetaines; EDTA, ethylenediamine tetraacetic acid.

tial of the solution than the total concentration of thiol present in the refolding buffer.

In certain instances, the presence of disulfide shuffling agents fosters the formation of non-native disulfide bonds. In these cases, oxidation can be conducted in a one-step fashion which does not allow for shuffling by using air oxidation, sulfitolysis (disulfide bond breakdown), or oxidizing agents such as iodosobenzoic acid [36]. The probability of forming proper disulfide bonds using these reagents is a function of the number of thiols that are present in the protein of interest.

Denaturants. Chemicals known to destabilize protein structure often improve chaotrope-based protein-refolding yields [5,6]. Urea and GdHCl are the most commonly used denaturants in refolding buffers and should be screened at final concentrations between 0 and 2 M [26]. Note the amount of the denaturant in the final refolding mixture is the sum of any in the refolding solution plus what was diluted from the original solubilization solution. These concentrations can destabilize the interactions leading to aggregation, but will not denature the native structure. Optimal concentrations will be different for each protein and dependent on other buffer components. If robotic methods are employed for high-throughput screening, chaotropes need to be used cautiously as they may damage the pipetting valves of autopipettors.

Amino acids. Amino acids, such as arginine, lysine, proline, valine, glycine and alanine, as well as their derivatives, have been shown to enhance refolding yields [31]. As the modes of action of each of these amino acids are distinctly different, they can only loosely be categorized as a group. Of the amino acids, L-arginine is most commonly used in refolding buffers and is routinely used at concentrations between 0.2 and 1.0 M [31,37]. While L-arginine contains a guanidino group, the positive effects of L-arginine on refolding are not believed to be due to a strong denaturing effect. Rather, L-arginine has been shown to bind to tryptophan residues during chaotrope-based refolding, decreasing the extent of reaggregation [38]. Arginine concentration of 0.8 M is known to impede crystallogenesis due to its "anti-aggregation" effects and must be removed before initiating crystallization [39].

Salts. The solubility of protein of interest can increase (salting in) or decrease (salting out) as a function of the salt selected, according to the Hofmeister series [40]. Some proteins are sensitive to ionic strength, and as such, refolding must be tested as a function of ionic strengths. We recommend the use of NaCl or KCl to adjust ionic strength because of the limited or lack of adverse effect on protein solubility for these salts. A typical working salt concentration range is 0–500 mM. During screening, a utilized concentration range for NaCl is 100–200 mM and that for KCl is 50–100 mM.

EDTA and divalent cations. Chelating agents are frequently used in solubilization buffers to prevent metal-catalyzed air oxidation of cysteines and inhibit the activity of some proteases [41]. EDTA chelates free metals in solution and should be included in refolding buffers at 0.1–1 mM as (1) removal of free metals significantly stabilizes the redox environment, and (2) small concentrations of divalent cations can significantly affect the refolding of some proteins [5]. Omitting EDTA from the refolding solutions is required if divalent cations or metal containing cofactors are required for the refolding of the target protein.

Surfactants. Numerous mild denaturants, detergents, and non-detergent sulfobetaines (NDSBs) are commonly used in refolding buffers [6,7,24,42–44]. NDSBs (0.1–1 M) are shown to have a positive synergistic interaction with reductants and have been successfully used in protein crystallization experiments [7,45,46]. Surfactants such as β-octyl-glucopyranoside, Brij® 35, cholates and the buffer *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES is not a surfactant, $pK_a = 9.5$ and effective pH range 8.6–10) have been shown to minimize protein–protein hydrophobic interactions that can lead to reaggregation and protect proteins that are susceptible to instability at surfaces [43,44,47].

Polymers, polyols and sugars. Polymers, polyols, and sugars can influence refolding by modifying diffusion rates and stabilize proteins by the preferential exclusion from the protein's surface, which shifts the equilibrium toward compact states such as the native state [48,49]. Polyethylene glycol (PEG molecular weight 3500) is the most successfully used polymer in refolding buffers. Studies have shown that the success of PEG as a refolding additive may be dependent upon maintaining a specific molar ratio of PEG to protein [50]. A 2:1 to a 6:1 molar ratio of PEG to protein is recommended as a starting point in screening experiments. This optimal ratio depends on the hydrophobicity of the protein being refolded and the denaturant concentration present in the refolding buffer. In addition, other soluble polymers such as cyclodextrins or fructose-based polymers were utilized with reportedly higher refolding yields. Of the sugar family sucrose and trehalose are the most commonly used preferential excluding compounds and are generally used at concentrations of 0.3–1.0 M [51].

Ligands, cofactors, substrate analogs. For proteins containing cofactors, such as bound metals or heme groups, inclusion of these compounds in refold buffers is required. Additionally, the presence of substrate analogs has been shown to promote formation of the native fold for some proteins [52].

Temperature. Temperature affects the strength and kinetics of molecular interactions and therefore can significantly influence protein refolding. Temperatures have been shown to modulate protein stability and alter the reaction rates of slow processes required for formation of the native structure [17,53,54]. Temperature also influences the effectiveness of buffer components. Alternatively, polymers with temperature-dependent hydrophobicity can be effectively applied for protein refolding at higher temperature [24].

Protein concentration. Reaggregation is more prevalent if the refolding reaction is conducted at a high protein concentration, since the aggregation reaction order has been reported to be approximately 2.6, while the folding reac-

tion is first order (Fig. 2) [26]. Historically, aggregation during refolding has been controlled by keeping the protein concentrations below 50 μg/ml, as increased concentrations favor the multi-ordered process of aggregation [55]. However, refolding at such concentrations is generally not economical or efficient for large scale recombinant protein production. Small molecule assisted refolding buffers minimize this dependence of protein concentration, and many proteins can be folded at concentrations near 1 mg/ml under optimized conditions [49]. Nevertheless, protein concentration is still a key factor in the successful refolding of a target protein. To address protein concentration it is best to perform two separate screening experiments, one at 10–50 μg/ml and one at 500–1000 μg/ml final protein concentration. Conditions for refolding a protein at low protein concentrations may be significantly different from those required for refolding at higher concentrations.

High-throughput fractional factorial refolding strategy

Understanding how different chemicals influence both the native and denatured state of a protein is complex and despite years of research, the relative utility of most chemicals for use in refolding buffers or to deter aggregation is still not completely understood. Nevertheless, the factors listed in the previous section, such as protein concentration, temperature, pH, certain amino acids, polyols, sugars, polymers, chaotropes, detergents, ionic compounds and redox reagents (Table 1) have all been independently shown to promote *in vitro* refolding of specific proteins [6,23,24,56]. Many of the compounds described as refolding enhancers work in similar ways or counteract the need for another compound. Additionally, it has been demonstrated that reagents have positive synergistic interactions and must be screened accordingly [7,56]. One can increase the chances of designing a successful refolding protocol by

(1) screening buffer components across the appropriate concentrations,
(2) examining buffer components that have the broadest range of application,
(3) using components that complement each other; for instance, NDSB 201 and BMC are synergistic as they have been shown to have significant positive effect on protein refolding in primary and secondary screens for several proteins [7], and
(4) using appropriate experimental techniques; for example, introducing appropriate quality control methods when determining protein solubility or testing protein refolding. Also, if the goal is to crystallize the protein high Arg concentration should be avoided due to anti-aggregation effect and NDSB series explored because they have been successfully used in protein crystallogenesis [7,39].

Optimal refolding conditions will be unique for each target protein, so examining numerous solutions is often required. For this, statistically designed matrix based screening experiments (statistical experimental design-SED) are generally employed. The goal of SEDs is to minimize the experimental burden while maximizing the number of conditions that are screened. However, due to the number of known refolding parameters, determining a starting point for screening experiments can be daunting. A variety of resources on statistically engineered design matrices are available [7,39].

Fractional factorial screens emerged as a way to compensate for the unpredictable nature of the refolding process and avoid using a step-by-step process that is time consuming [58]. Due to the complexity of protein folding, fractional factorial screens may provide a useful tool to systematically explore a wide range of folding conditions as suggested by Gouaux and co-workers [58]. Fractional factorial screens contain a representative subset of reagent combinations contained in full factorial screens and are designed to maximize the number of refolding variables explored while minimizing the amount of data collection [56]. In general, two fractional factorial refolding strategies are emerging. The first involves the selection of 96 different buffer conditions [7,39] while the second tests a limited subset of 8–36 buffer compositions per protein [7,34]. In both cases it is possible to miniaturize the assay reactions to a 96-well plate format for automation [39,57].

Fractional factorial screening techniques in practice

In light of the above points, in 2004 Qoronfleh and coworkers developed and reported at a CHI conference on the ProMatrix™ commercial screen, a fractional factorial protein refolding approach consisting of nine basic buffers which can be supplemented with various additives [34]. The ProMatrix™ commercial screen is available from Pierce Biotechnology. Others developed separate procedures to test several refolding conditions simultaneously [39]. Numerous designs for screening experiments are possible with ProMatrix™. Table 2 outlines a screening experiment founded on the ProMatrix™ screen designed using GdHCl, arginine and redox potential as example compounds. It is feasible to miniaturize and automate this format. Such a matrix also conserves protein consumption and is useful for initial refolding experiments as it examines compounds at three distinct concentrations and it is not overly fractional in design. Screening experiments based on small matrices such as this can be easily expanded by duplication or modification to examine additional factors. Secondary screening experiments can then be used further to optimize refolding conditions. Other fractional factorial commercial protein refolding screening kits (Novagen iFOLD™, Hampton Research Foldit™, and AthenaES QuickFold™) can serve to pinpoint optimal refolding conditions or as additional templates for the design of a refolding screen.

Devising an automated, 96 condition format refolding strategy that incorporates a fractional factorial buffer design

is perhaps the best approach if one has access to large-scale, high-throughput, screening facilities. However, this can be problematic as it produces very large screening experiments and requires significant sample and effort to analyze. Otherwise, it is generally more desirable and informative to limit the number of factors being examined (16–32 conditions). Based on our experience, for many proteins, refolding can be relatively straightforward, and the examination of a large set of compounds is unnecessary [7,39,57]. A specific protein can often refold well in two dramatically different refolding buffers [7,39,56,57]. This preliminary SED is considered a primary screen. A secondary screen (such as a five-level central composite SED) for reagent optimization is often desirable to confirm reagents which have been shown to have significant positive effect in the primary screen [7,34]. This approach provides a systematic method for optimizing the refolding process. The result is a more efficient exploration of the refolding experimental space, decreased protein requirements, and increased throughput.

As starting point for a fractional factorial primary screen, we recommend the following:

(i) A 96-well format, 100 μl reaction, low protein concentration and room temperature incubation with slight agitation.
(ii) Explore 16–32 conditions per protein.
(iii) Refolding buffer composition: Tris–HCl at 55 mM (pHs 7 and 8.5), GdHCl as a denaturant at a final concentration between 0.5 and 2 M, GSH/GSSG (2 mM/0.2 mM) for a redox environment, salts (NaCl 50–200 mM and KCl 50–100 mM) and arginine between 400–800 mM. Test refolding at 1:10 dilution.
(iv) Potential additives or substitutes TCEP and BMC at 5 mM, surfactants like NDSB at 100 mM and EDTA at 1 mM.
(v) To test effectiveness of refolding see Section III on monitoring refolding.
(vi) Results will dictate the secondary screen and/or investigating other buffer compositions.

This approach reduces the number of factors examined and facilitates screening the chosen factors at multiple concentrations/conditions [7,34]. This is essential as compounds that are effective for refolding a protein can often be ineffective or detrimental to the refolding of the same protein when present at different concentrations [7,56]. Screening at multiple concentrations ensures that the useful range of a compound is examined and adds confidence to the interpretation of results.

Experimental parameters

Inclusion body isolation. Proficient isolation of inclusion bodies can often result in a protein purity of 80–90% in a few short steps [3,22]. While high levels of purity (>90%) are not essential for the successful refolding of target proteins, contaminants within an inclusion body preparation can promote aggregation and reduce refolding yield so care should be taken with this step. Contaminants can include cell debris, DNA, lipids, and other proteins. While no one method for purifying inclusion bodies is regarded as the standard, the majority rely on achieving complete cell lysis using either lysozyme or mechanical methods such as sonication or a French press followed by successive washes with mild detergents such as 1–2% Trition X-100, and salts like 500 mM NaCl then centrifugation (12,000–25,000*g*) [3,5,6,22,55]. Low concentrations of denaturants (1–2 M urea) are sometimes employed to remove contaminant proteins as well.

Inclusion body solubilization. Treating inclusion bodies with high concentrations (6–8 M) of GdHCl or urea in the presence of reducing agents is generally sufficient to solubilize fully inclusion bodies. GdHCl is generally preferred over urea as it is a stronger denaturant and degradation of urea solutions can cause carbamylation of free amino groups, chemically modifying the protein. A recommended solubilization buffer is 6 M GdHCl, 50 mM Tris (pH 8.0), 1–20 mM dithiothreitol (DTT), and 1 mM ethylenediamine tetraacetic acid (EDTA). Inclusion bodies should be solubi-

Table 2
Example of a fractional factorial screening experiment founded on the ProMatrix™ refolding buffer set

Refolding buffer #[a]	(1) GdHCl (M)	(2) L-Arginine (M)	(3) Redox environment	Factor 4 user defined
1	0.4	0.0	5 mM DTT	a
2	0.4	0.4	2 mM GSH:0.2 mM GSSG	b
3	0.4	0.8	2 mM GSH:0.4 mM GSSG	c
4	0.9	0.0	2 mM GSH:0.2 mM GSSG	b
5	0.9	0.4	2 mM GSH:0.4 mM GSSG	c
6	0.9	0.8	5 mM DTT	a
7	1.4	0.0	2 mM GSH:0.4 mM GSSG	c
8	1.4	0.4	5 mM DTT	a
9	1.4	0.8	2 mM GSH:0.2 mM GSSG	b

Each buffer contains the indicated denaturant concentrations as well as 55 mM Tris, 21 mM NaCl, 0.88 mM KCl adjusted to pH 8.2. This is an open-design as it allows user defined additives. This allows customization and optimization of matrix conditions for each target protein as well as identification of unique small molecule interactions that promote refolding. The right most column is a specific refolding additive defined by the user. Protein dilution is 1:10. Appropriate controls should be carried out. A blank (negative control) that is a buffer mixture without protein to subtract background absorbance when measuring turbidity and denatured protein solution to reference protein precipitation.

[a] Each refolding buffer is supplied as a 1.1× stock solution.

lized preferably at a concentration of 10 mg/ml for a minimum of 2–4 h at room temperature. After incubation the solution should be centrifuged at 5000–15,000*g* for 20 m to remove residual insoluble material [23,55]. If the protein to be refolded contains disulfide bonds, the concentration of DTT must be sufficiently low to enable oxidation during the refolding step. Researchers accomplish this either by dilution of the DTT during the initiation of the refolding reaction or by removing the DTT in the solubilization buffer. Researchers have removed DTT by dialyzing against 4 M GdHCl (pH 4) or by passing the sample through a desalting column equilibrated in DTT free solubilization buffer. Affinity-tag or size exclusion based chromatographic separations under denaturing conditions can be used for additional purification after solubilization of the inclusion bodies if needed [59,60]. Alternatively, non-detergent sulfobetaines can be used to partially solubilize inclusion bodies and preserve some elements of secondary structure that can aid in the refolding of some proteins or detergents like *N*-lauroylsarcosine which is easier than SDS to remove from proteins [6,24,46,61–63].

Initiation of refolding. Protein refolding is initiated by reducing the concentration of the denaturant [6]. Dilution, dialysis, and complex chromatographic methods have been developed to initiate this process step [6,23,24,29,55,64]. Of these methods, dilution is the simplest and most economical to use, however, other methods may be beneficial for specific proteins [55,65,66].

To initiate refolding by dilution, the denatured protein solution is added directly to a 10- to 50-fold excess refolding buffer. If possible, dilution should be carried out with high concentrations of solubilized protein (1–2 mg/ml) to minimize the dilution volume needed to achieve the desired final protein concentration. For a structural genomic project, a typical reaction 5 μl of solubilized protein into 95 μl refolding buffer. This allows greater control of the refolding environment by preventing dramatic changes in buffer conditions from the beginning to the end of sample addition. Dilution should be performed with immediate mixing to avoid pockets of high protein concentration [23]. Additionally, Middelberg and coworkers have shown that pulse dilution (dilution is made in aliquots, rather in continuous mode) of denatured protein can be used to increase the final protein concentration in a refolding reaction while reducing reaggregation [23]. A sequential addition of small aliquots of solubilized protein allows intermediates to refold between additions, effectively lowering the population of the refolding intermediates that are involved in reaggregation.

PreEMT™—hydrostatic pressure aggregate refolding and recovery

High hydrostatic pressures (1–3 kbar) have been demonstrated to solubilize and refold protein aggregates [8,19,20,67–70]. High pressure prevents protein reaggregation during refolding since aggregates can be dissociated and refolded at conditions that favor the native protein conformation, inhibiting the formation of aggregate-prone intermediates [18,19]. This property differentiates PreEMT™ high hydrostatic pressure refolding techniques from chaotrope-based methods, which require protein denaturation prior to refolding. Some of the key advantages of the high pressure method are: (1) performs solubilization and refolding/disaggregation of protein simultaneously, (2) requires little or no chaotropic agents, (3) is often independent of protein concentration, and (4) can disaggregate and refold proteins rapidly.

Minimizing the reaggregation of protein intermediates is critical. The goal of small molecules solution screens during chaotrope-based refolding is to develop solution conditions that curtail reaggregation of protein intermediates [49]. It is important to note that high pressure can solubilize aggregates while favoring the native protein conformation. Consequently, reaggregation is further minimized relative to chaotrope-based refolding methods. Combining high pressure techniques with high-throughput solution screens could offer superior results particularly with proteins designated as "challenging" or "difficult-to-express" [67,68]. This approach could be proven useful in the case of structural genomics projects where a significant number of proteins have an unknown function or to satisfy the need to obtain a monodispersed protein sample in order to attain diffraction quality protein crystals.

Thermodynamics of high hydrostatic pressure protein refolding

Pressure is a useful, although generally unfamiliar, thermodynamic variable that may be manipulated to probe protein self-association, both in terms of kinetics and thermodynamic equilibrium. The thermodynamics of pressure-induced protein transitions have been known for some time. An interested reader can obtain a more complete description of the underlying thermodynamics supporting the mechanism for pressure-based protein disaggregation and refolding from Seefeldt et al. [18,19,70,71].

High hydrostatic pressures have been shown to unfold native proteins, typically at pressures above 4000 bar [72–74] (Fig. 3). Moderate pressures of approximately 2000 bar have been shown to dissociate native oligomers [75,76]. Aggregates behave like multimers in the sense that they are readily dissociated with moderate pressure, at conditions that do not denature the native conformation [8]. It has been hypothesized that pressure-modulated refolding is often more effective than chaotrope refolding due to the presence of a "refolding window" [19,77]. The "refolding window" is defined as the pressure conditions where the native conformation is thermodynamically favored, while higher-order aggregate structures are disfavored. A depiction of the refolding window is shown in Fig. 4. Properly folded lysozyme retains its native structure for pressures up to 4500 and 3000 bar in the presence of 1 M GdHCl [18]. Lysozyme was refolded effectively (70% yield), inde-

pendent of high protein concentrations (up to 2 mg/ml) in 1 M GdHCl at pressures of 2000 bar [8]. High (~96%) refolding yields were observed for the refolding of placental bikunin at pressures that favored the native conformation [78] while disfavoring aggregation.

High hydrostatic pressure has been reported to disrupt both ionic and hydrophobic interactions within protein aggregates [79–82]. In contrast to chaotropic agents, pressure does not affect hydrogen bonding significantly [76]. This observation may explain the higher refolding efficiencies reported using high hydrostatic pressure methods for biotherapeutic proteins such as recombinant human growth hormone (>98% yields), interferon-gamma (>98% yield), and bikunin (>95% yield). These refolding conditions were optimized using approaches outlined below [19,83,84]. Of interest are the reports for ''difficult-to-express' proteins [67,68]. Shoner et al. reported that using a single set of pressure/buffer conditions successfully reconstituted activity of three insoluble nuclear receptor–ligand binding domain proteins (NR-LBD's). This same group of investigators reported the results extended to ten different NR-LBD's [85]. Recently, Kim's lab has reported the use of high hydrostatic pressure and a limited set of buffer conditions to successfully obtain yields of >40% of active, refolded protein from inclusion bodies of five proteins not successfully refolded by any other method to date. These proteins included gram negative binding proteins, as well as human phosphatases [67]. Table 3 lists a summary of published proteins that have been refolded using high hydrostatic pressure, the best-case refolding conditions, and comparisons to chaotrope-based refolding when available. BaroFold has also refolded over a hundred proteins commercially.

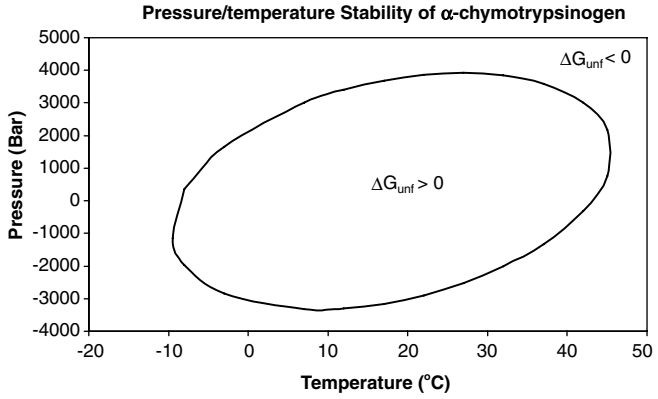

Fig. 3. Temperature–pressure stability contours for chymotrypsinogen, derived using the model and parameters of Hawley [74]. Outside of the contour, the native state is unstable, within the contour it remains thermodynamically stable. Pressures of 4 kbar denature many proteins. Refolding is typically carried out at non-denaturing pressures near 2 kbar.

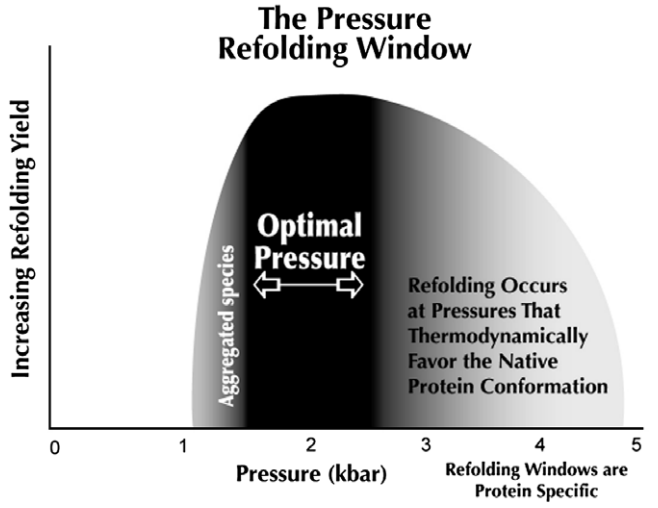

Fig. 4. The pressure refolding window for protein refolding. Pressures of 1000 bar begin to dissociate some protein aggregates. However, maximum aggregate dissociation and refolding yields are typically observed to be near 2000 bar for many proteins. Within the shaded region, the native conformation remains thermodynamically stable. Consequently, high pressure refolding occurs without requiring denaturation, differentiating it from traditional chaotrope-based methods.

What is the thermodynamic basis that supports the results reported in the literature for the use of high hydrostatic pressures to obtain higher yields of refolded protein? High pressures favor protein structures that result in overall decreases in system volume. Aggregates of rhIL-1ra resulting from incubation at elevated temperatures have been shown to be less dense than native rhIL-1ra. Consequently, a pressure of 1.5 kbar was effective in refolding the aggregates of this protein with recovery of active structures [70]. The refolding of disulfide-crosslinked aggregates of recombinant human placental bikunin was found to have a $\Delta V_{refolding}$ of −28 ml/mol, further demonstrating that aggregates are less dense relative to native proteins [19]. This general property is the fundamental driving force for pressure being an effective refolding tool across all protein classes.

PreEMT™ high hydrostatic pressure methods in practice

High pressure refolding does not require the use of chaotropes to solubilize protein from aggregates/inclusion bodies. As a result, this allows a much greater degree of freedom in choosing the appropriate protein refolding conditions or buffer ingredients that would maximize refolding. Many of the same protein-specific factors important to traditional protein refolding are also important for pressure-modulated refolding: pH, amino acids, ionic strength, ligands/cofactors, preferential excluders, and detergents [8,19,67,86].

Equipment and screening

PreEMT™ high pressure chambers are commercially available (2–100 L) that include a license to the appropriate patents (BaroFold, Boulder, CO). Research scales chambers accommodate up to 28 individual samples of up to 1 ml or single samples up to 150 ml. Each individual sample is contained within its own variable volume high pres-

Table 3
Summary of published proteins refolded by high pressure

Protein	Type	MW	Aggregate form	Disulfides	Solubilization yield	Activity yield	Comparison to Chaotrope-based refolding yield	Best-case high pressure refolding condition	Protein concentration	Reference
Bikunin	Kunitz-type protease inhibitor	23	Soluble	6	100%	100%	55% at 0.375 mg/ml	2000 bar, pH 8.0, 24 h, 25 °C, 4 mM DTT, 2 mM GSSG, 157 mM NaCl, slow depressurization	0.0625–2 mg/ml	Seefeldt et al., Protein Science, v13, 2639–2650 (2004)
Lysozyme	Hydrolase	14	Urea shock diluted, disulfide crosslinked	4	90%	80%	80% at lower protein concentration	2000 bar, pH 8.0, 2 mM DTT, 6 mM GSSG, 1 M guanidine, 50 h, 10 bar/min depressurization	0.25–2 mg/ml	St. John et al., Biotechnology Progress, v18, 565–571 (2002)
IL-1ra	Interleukin receptor antagonist	17	Benzyl alcohol, guanidine, pH, thermal-induced	0	60%	60%	Not tested	1500 bar, pH 7.0, 31 °C, 10 mM β-mercaptoethanol, 16 h, rapid depressurization	0.5–5 mg/ml	Seefeldt et al., Journal of Biotechnology and Bioengineering (in press)
P22 Tailspike	P22 bacteriophage	60	Urea shock diluted aggregates	0	100%	75%	Not tested	2400 bar, pH 7.6, 1 mM EDTA, 5 min, 4 °C, rapid depressurization	0.1 mg/ml	Lefebvre et al., Biotechnology Progress, v20, 623–629 (2004)
Estrogen receptor–ligand binding domain	Nuclear receptor–ligand binding domain	29	Inclusion body	0	80%	Confirmed activity	Low—<1%	2500 bar, 16 h, 25 °C, 200 bar/15 min depressurization—refolding buffer not specified	1 mg/ml	Shoner et al., Molecular Genetics and Metabolism, v85, 318–322 (2005)
Farnesoid X receptor–ligand binding domain	Nuclear receptor–ligand binding domain	30	Inclusion body	0	40–50%	Confirmed activity	Very low expression	2500 bar, 16 h, 25 °C, 200 bar/15 min depressurization—refolding buffer not specified	Not specified	Shoner et al., Molecular Genetics and Metabolism, v85, 318–322 (2005)
Liver receptor homologue 1–Ligand binding domain	Nuclear receptor–ligand binding domain	34	Inclusion body	0	Not published	Confirmed activity	Very low expression	2500 bar, 16 h, 25 °C, 200 bar/15 min depressurization—refolding buffer not specified	Not specified	Shoner et al., Molecular Genetics and Metabolism, v85, 318–322 (2005)
Prion protein PrP	Prion precursor protein	24	Inclusion body	0	>90%	Monomeric by SEC-HPLC	Not tested	2000 bar, pH 7.0, 25 °C	App. 1 mg/ml	Torrent et al., Biochemistry, v42, 1318–1325 (2003)
Interferon-γ	Human interferon	17	Thermal, guanidine-induced aggregate	0	100%	Similar 2 D-UV structure to native IFN-γ	Not tested	2500 bar for 30 min, followed by 1000 bar for 1 h to enable dimer formation, 5 mM succinate, pH 5.0	20 mg/ml	John Webb, University of Colorado-Boulder, Dept. of Chemical and Biological Engineering Thesis, 2000

β-Lactamase	Hydrolase	28	Inclusion body	0	90%	85%	Not tested	2000 bar, pH 8.0, 37 °C, 48 h, 10 bar/min depressurization	0.1 mg/ml	St. John et al., Proceeding of the National Academy of Sciences, v96, 13029–13033 (1999)
Human growth hormone	Hormone	22	Agitation induced aggregate	0	100%	Similar 2 D-UV structure to native rhGH	Not tested	2000 bar, pH 6.0, 0.1% azide, 1 mM EDTA, with either 0.75 M guanidine or at 65 °C, 24 h, 10 bar/min depressurization	Up to 8.7 mg/ml	St. John et al., Journal of Biological Chemistry, v276, 46856–46863 (2001)
3 Gram-negative binding proteins	Gram-negative binding proteins (fruit fly)	~50	Inclusion body	6, 7, and 6 cysteines	80%	55–75%	No active protein had been obtained (no structure)	2000 bar, pH 8.0, 150 mM NaCl, 0.05% azide, 1 mM EDTA, 0.5 M arginine, 24 h, 25 °C, 10 bar/min depressurization	1 mg/ml	Lee et al., Protein Science, v15, 304–313 (2006)
2 Human phosphotases	Human phosphatase	35 & 64	Inclusion body	11 & 9 cysteines	80%	Activity confirmed	No active protein had been obtained (no structure)	2000 bar, pH 8.0, 150 mM NaCl, 0.05% azide, 1 mM EDTA, 0.5 M arginine, 24 h, 25 °C, 10 bar/min depressurization	1 mg/ml	Lee et al., Protein Science, v15, 304–313 (2006)
Malaria pfs48	Unknown	48	Inclusion body	12	>95%	Activity confirmed	<1%	2650 bar, pH 10.5, 12 mM GSH, 4 mM GSSG, 7°C, 16 h, 10 bar/min depressurization	1 mg/ml	Matthew Seefeldt, University of Colorado-Boulder, Dept. of Chemical and Biological Engineering Thesis, 2004

sure 'caisson' (ProVENT™) that is specifically designed for use at high pressure while eliminating any air which contains oxygen and compromises the oxidation/reduction potential of the solution. Inclusion bodies (or aggregated protein) are prepared in 1.5 ml micro-centrifuge tubes, an equal volume of a 2× pressure treatment refolding buffer is added, well mixed, and then loaded directly into the caisson. Inclusion bodies will settle, consequently care must be taken to pipette a homogeneous solution and that pipetting does not filter protein aggregates. The Caissons are then loaded into the chamber and it is sealed and pressurized to the appropriate level, usually ~2 kbar (1 bar = 14.7 psi). It should be noted that inclusion bodies would settle during pressure refolding. Since PreEMT™ methods are effective at high concentrations (discussed below), refolding is still achieved.

For convenience the chamber is often pressurized overnight, but published kinetics for solubilization of the aggregated proteins show that this step usually requires less than 6 h [19,83]. Refolding of a specific protein is dependent on a number of unique characteristics for that protein. The pressure is released in a controlled manner and the protein solution is expelled from the caisson so the appropriate analysis can be performed.

Buffer components and complementing constituents

A variety of factors has been observed to have a significant effect on protein refolding. In general, these factors are protein specific and the stabilizing effects are not altered by high pressure methods. BaroFold has developed HiPER-FOLD™ reagent kits that facilitate the rapid screening of pH, redox reagents, surfactants, preferential excluding compounds, amino acids, and chaotropes.

Protein concentration. An important observation has been that yields of soluble, monomeric rhGH (recombinant human growth hormone) from aggregates after 24 h incubation at 2 kbar were essentially independent of protein concentration in the range 0.87–8.7 mg/ml [8], likely owing to the low population of aggregate-prone intermediates due to the moderate pressure used. This suggests that under pressure, the intermediates in the disaggregation/refolding pathway are at a lower population or not as prone to aggregation, allowing high refolding yields independent of rhGH concentration. This is in sharp contrast to observations at atmospheric pressure, where a molten globule intermediate of rhGH formed during dilution from concentrated GdHCl solutions aggregates readily [44].

PreEMT™ methods are performed at protein concentrations in excess of 1 mg/ml, up to the inherent solubility limit for the specific proteins. In general, cytokines and growth factors have been refolded at concentrations in excess of 5 mg/ml. Other proteins have been solubilized and refolded at concentrations in excess of 25 mg/ml. None of the inherent limitation of chaotrope methods (and the dilution required to initiate refolding) are present with pressure-modulated refolding.

pH. The pH of the buffer can dramatically affect the rates/yield for refolding. As with chaotrope-based methods, the pH of a refolding buffer influences the charge state of the target protein, protein stability, protein solubility, and rate of disulfide bond formation. It should be noted that at <pH 8, disulfide bond formation will be significantly slowed or prevented. The HiPER-FOLD™ protein refolding kits allow for a screen across a wide range of pH (4–10) that accommodates the diversity of proteins and their requirements. Many common buffers will shift in pH under pressure, due to the property of electrostriction [81]. This phenomenon is associated with the higher water density that surrounds charged molecules, in comparison to bulk water. Thus, pressure favors ionization. As a result, buffers need to be specifically selected which do not shift pK_a's at elevated pressure. For instance, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) is used in place of phosphate buffer within certain pH ranges.

Reductive/oxidative renaturation. Redox reagents are selected in a similar manner as chaotrope-based methods, as pressure has no effect on disulfide thermodynamics. The redox environment is dependent upon whether the native target protein contains disulfide bonds. For non-disulfide containing proteins, addition of DTT or TCEP is recommended to maintain a reducing environment during refolding. For disulfide-containing proteins, a mixture of low molecular weight thiol and disulfide containing compounds, such as reduced and oxidized glutathione (GSH/GSSG), cysteine/cystine, or other traditional redox agents are added to refolding buffers to allow disulfide bond formation and shuffling [19,77]. When refolding at high protein concentrations (>10 mg/ml), calculations should be conducted to ensure the concentration of disulfide shuffling agents is not rate limiting.

Temperature. Temperature affects the strength and kinetics of molecular interactions and therefore can influence protein refolding. In practice, the majority of proteins refold adequately at room temperature while under pressure. Either increased [83,84] or decreased [19] temperatures have improved yields as a function of the hydrophobicity and T_m of the specific protein.

Denaturants: High pressure methods often do not require the use of any chemical denaturants such as urea or GdHCl. It is recommended that these be avoided for most proteins since they can destabilize the native protein confirmation.

Detergents. Numerous mild detergents are available, and non-detergent sulfobetaines are also commonly used in refolding buffers. Generally, it is best to stay higher than the critical micelle concentration (CMC) for these molecules. A variety of these detergents are included in the HiPER-FOLD™ high pressure solution screening kits and can be helpful when optimizing conditions for difficult-to-refold proteins.

Amino acids. Amino acids, such as arginine, lysine, and glutamic acid, as well as their derivatives, have been shown to enhance refolding yields [67,86]. In the case of arginine, there is some evidence that it improves aggregate dissociation during pressure incubation.

EDTA and divalent cations: Pressure has no specific effect on EDTA. The use of this additive is protein dependent, as in chaotrope-based methods.

Polymers, polyols and sugars. Molecules in these categories can be considered as a class of preferential hydrators. The effect of this class of molecules can be observed in the system volume; as a result they can be affected by pressure. The molecules can be screened to look for effects, but may demonstrate inhibitory effects on certain proteins, while enhancing the yields for others [67]. In general, this class of molecules is detrimental to aggregate dissociation.

Ligands, cofactors, substrate analogs. For proteins containing cofactors, such as bound metals or heme groups, inclusion of ligands and cofactors in refold buffers is required, as in chaotrope-based methods. Additionally, the presence of substrate analogs has been shown to promote formation of the native fold for some proteins [86]. Pre-EMT™ methods have been demonstrated to refold even difficult proteins (for example, estrogen receptor β-ligand binding domain) in the absence of ligand [68].

Experimental methodology

Inclusion body isolation. Standard methods for inclusion bodies isolation and washing are routinely used with high pressure methods. It is important to include protease inhibitors in the buffers if the protein of interest is susceptible. Proteases are generally stable to moderate pressures and can remain active under many buffer conditions [74,87].

Inclusion body solubilization. The simple act of pressurizing the chamber will provide the thermodynamic tool to solubilize the protein aggregate due to density differences between the aggregated and native confirmation.

Protein refolding: High pressure methods provide the conditions such that solubilization and refolding can be occurring concurrently. No specific initiation event is needed.

Effective starting conditions for high pressure refolding

High pressure refolding has been conducted on over one hundred proteins to date. Based on this experience, effective starting conditions for the screening of a new protein using high pressure are: 2000 bar, 25 °C, 16-h incubation, depressurization rate of 250 bar/5 min at a protein concentration of 0.5–1 mg/ml. The pH should be screened as a priority (pH 4–10) in redox environments that foster disulfide bond formation (e.g. 4 mM reduced glutathione and 2 mM oxidized glutathione) or prevent non-native disulfide bond formation (e.g. 5 mM TCEP) as a function of the protein disulfide requirements. As with all proteins, solution effects are protein specific and consequently must be screened.

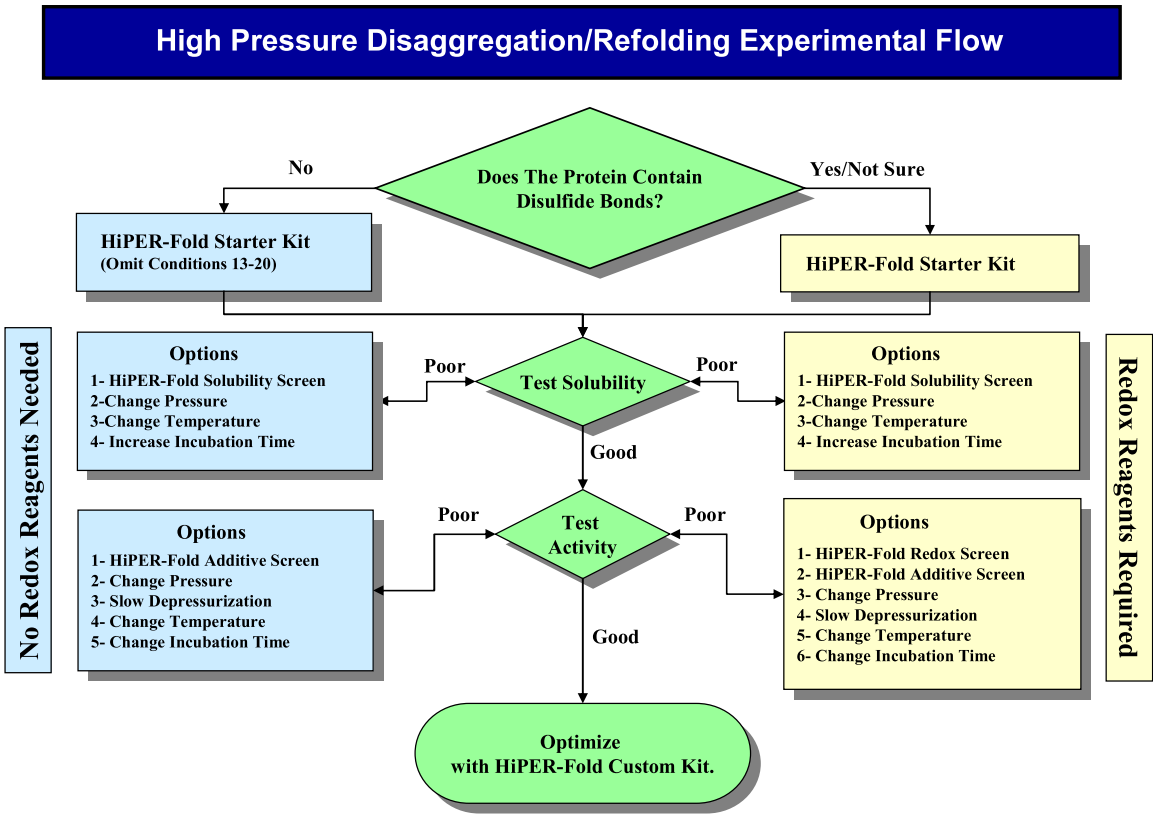

Fig. 5. Experimental methodology used to explore the PreEMT™ refolding space. Four reagent kits can be used to rapidly screen solution conditions for developing refolding conditions. The flow chart for the use of these kits is shown. After significant conditions for a given protein are identified through the reagent kits, custom statistical experimental designs are developed to optimize a specific process and take into account interacting experimental conditions.

High pressure is a valuable tool for modulating protein structure and it has been BaroFold's experience that for the majority of proprietary proteins tested, high pressure is effective in refolding proteins at yields higher than those obtained through chaotrope-based processes, a result supported by literature [19,77]. Additionally, BaroFold has observed that high pressure refolding can be an "enabling" technology, recovering active protein in instances where chaotrope-based methods have previously failed [67,68].

High-throughput studies

For individual proteins, a series of four sets of reagents (kits) are to be used sequentially, as depicted in Fig. 5. The kits include the initial starting conditions described above as well as downstream solution screens to optimize refolding based on the additive effects discussed previously. The pre-formulated reagents combine pressure-neutral buffers, salts, redox reagents and small molecules in a manner to combine the advantages that high pressure offers with those offered by small molecule additives. A schematic describing the experimental methodology that can be employed with the PreEMT™ refolding system is shown in Fig. 5. BaroFold Inc. is in the process of developing 96-well plates that can be pressure-treated to further aid high-throughput studies.

Monitoring refolding

Protein overexpression or identification of protein of interest is routinely carried out by SDS–PAGE, Western blots, and more recently by mass spectrometry methods [88]. In order to determine if a target protein has been correctly refolded, a variety of generic (e.g., solubility and folding) or specific (e.g., activity) methods that vary in protein requirements can be used [57]. Functional assays (bioassays, immunoassays, etc.) are the most informative and obvious choice. However, in the post-genomic era, one is often dealing with genes encoding proteins with either unknown or putative function, therefore functional tests for each of the targets are frequently lacking. Alternately, analytical techniques such as intrinsic fluorescence, circular dichroism (CD, secondary structure), dynamic light scattering (DLS, turbidity) measurements, or chromatographic analysis can be used as indicators to ascertain refolding success [7,39,57]. The two most wide spread biophysical methods are CD (measures protein secondary structure) and DLS (measures protein aggregation-state) since they can be applied to any protein in a

structural genomic project. A word of caution, protein solubility is not a sufficient indicator of protein refolding since native protein structure might not yet be formed. In practice, protein solubility tests based on DLS are implemented particularly during high-throughput automated refolding screening. Absorbance at wavelengths of 340 nm (manual procedure) and 350 nm (automated procedure) provide best results by minimizing false negatives and giving highest signal-to-noise ratio [7,56]. Therefore, quality control methods such as CD (prognostic indicator) and crystallogenesis are introduced [39,57]. Crystallogenesis is a valid criterion because only properly folded proteins with an uniform aggregation state (monodispersed sample) yield well-ordered crystals. While the method of choice will depend upon the target protein or project it is generally recommended that more than one method be employed.

Conclusions

Small molecule assisted refolding buffers have a long history in the literature and have been used successfully for high yield refolding of numerous proteins. The common link between all of these methods is the minimization of the aggregation of intermediates. Small molecule refolding protocols require no specialized equipment and are easy to customize in scale and composition making them a method of choice for initial refolding trials. While refolding protocols still have to be developed on a per protein basis, following some general guidelines can minimize time invested in screening experiments and make protein refolding a practical laboratory procedure. These methods however have limitations, as a result, 'challenging' and 'difficult' proteins have not refolded effectively with traditional chaotrope-based methods. PreEMT™ high hydrostatic pressure methods have recently been developed and have demonstrated some exceptional results with challenging and difficult proteins that were not amenable to refolding with small molecule chaotrope-based methods. If the equipment is available, the method offers an effective and simple alternative to traditional methods. Identifying conditions for high yields of correctly folded biotherapeutic or industrial scale proteins may require more effort than biochemical and/or structural studies where only a few milligrams are needed. For larger scale biotherapeutic production, the ability to both solubilize and refold proteins to high yield in a single step at a high protein concentration has been reported to provide large cost savings of the production costs of biotherapeutic proteins.

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