ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Protein Expression and Purification 28 (2003) 1–8

Protein Expression & Purification

www.elsevier.com/locate/yprep

Mini review

Practical considerations in refolding proteins from inclusion bodies

Kouhei Tsumoto,[a] Daisuke Ejima,[b] Izumi Kumagai,[a] and Tsutomu Arakawa[c,*]

[a] *Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, Sendai, Japan*
[b] *Central Research Laboratories, Ajinomoto, Inc., Kawasaki, Japan*
[c] *Alliance Protein Laboratories, 3957 Corte Cancion, Thousand Oaks, CA 91360, USA*

Received 4 September 2002, and in revised form 2 October 2002

Abstract

Refolding of proteins from inclusion bodies is affected by several factors, including solubilization of inclusion bodies by denaturants, removal of the denaturant, and assistance of refolding by small molecule additives. We will review key parameters associated with (1) conformation of the protein solubilized from inclusion bodies, (2) change in conformation and flexibility or solubility of proteins during refolding upon reduction of denaturant concentration, and (3) the effect of small molecule additives on refolding and aggregation of the proteins.

There is a strong demand, due to expansion of genomic sequence database, on a rapid, large-scale production of recombinant proteins. The proteins thus produced are used to identify their biological functions, and hence, must be in the native and active conformation. Since the biological functions of genes and corresponding proteins are unknown, whether the protein is in the native state or the non-native state cannot be evaluated by bioassay. There are a number of options for heterologous recombinant expressions. Among them *Escherichia coli* (*E. coli*) expression is most convenient and frequently used. Heterologous expression of foreign genes in *E. coli* often leads to production of the expressed proteins in insoluble inclusion bodies (IBs). IBs must then be solubilized and refolded into an active conformation. Refolding of IBs is not a straightforward process, often requiring an extensive trial-and-error approach. There are two important issues in recovering active proteins from IBs, i.e., solubilization and refolding. Solubilization must result in monomolecular dispersion and minimum non-native intra- or inter-chain interactions. Choice of solubilizing agents, e.g., urea, guanidine HCl, or detergents, plays a key role in solubilization efficiency, in the structure of the proteins in denatured state, and in subsequent refolding.

Refolding is initiated by reducing concentration of denaturant used to solubilize IBs. Protein refolding is not a single reaction and competes with other reactions, such as misfolding and aggregation, leading to inactive proteins. Rate of refolding and other reactions is determined both by the procedure to reduce denaturant concentration and the solvent condition. This review focuses on denaturant removal process and solvent additives as well as solubilizing conditions.

Idea of using small molecule additives comes from their in vivo activities. Water-stressed organisms use them to stabilize the proteins. They are hence named osmolytes [1,2]. In another instance, mutations that impair protein folding often result in malfunction of the proteins, leading to abnormal growth or function of cells harboring the mutant proteins. It has been shown that culturing these cells in the presence of certain small molecule additives can restore the function of the proteins and render the cells to grow or function normally. Since the small molecules help the mutant proteins fold correctly, they are called chemical chaperones [3,4]. Many small molecule additives are both osmolytes and chemical chaperones. Thus, it is evident that certain small molecules are effective in facilitating folding and stabilizing proteins or increasing solubility both in vitro and in vivo. Here, we outline the use

* Corresponding author. Fax: +1-805-388-7252.
E-mail address: tarakawa2@aol.com (T. Arakawa).

1046-5928/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
doi:10.1016/S1046-5928(02)00641-1

of small molecule additives to increase the recovery of active proteins and increase the efficiency of protein folding.

Solubilization

Urea, guanidine HCl, and strong ionic detergents such as *N*-lauroylsarcosine are solubilizing agents that are most frequently used. Urea and guanidine HCl lead to a flexible and disordered structure, while the unfolded structure in detergents is not well defined. It is well known that SDS–protein complex assumes a varying degree of α-helical structure [5,6]. It may be safe to say, based on the above observation, that proteins solubilized with the detergents have a more ordered structure than those with urea and guanidine HCl [7].

IBs solubilized in denaturants may be a clear, non-turbid solution, but may be aggregating as soluble oligomers. Little is known about the possibility of aggregate formation in denaturant solution. Among these denaturants, ionic detergents would be the strongest in dispersing IBs into monomolecular structure due to strong electrostatic repulsion of detergent/protein complexes.

As described above, detergent/protein complex may assume non-native secondary structure. Therefore, this system will work only when native disulfides are formed in such complex structures. Formation of native as well as non-native disulfides in Sarkosyl/protein complexes has been observed, indicating that there are native and non-native intra-molecular interactions in detergent/protein complexes (T. Arakawa and T.P. Horan, unpublished results). This is due to differences in structure and dynamics of proteins denatured by urea or guanidine HCl and by detergents, as described above.

Urea and guanidine HCl show concentration-dependent binding to the proteins ([8] and S.N. Timasheff, unpublished), as shown in Fig. 1. In most cases, 6–8 M urea and 6–7 M guanidine HCl are required to achieve extensive binding sufficient to unfold and solubilize the proteins. However, even at the highest concentration intra- and inter-molecular interactions can occur [9], often resulting in non-native structure, as schematically depicted in Fig. 2. Such a non-native structure can lead to aggregation or misfolding upon removal of denaturant. For disulfide-containing proteins, native disulfides may form, even in the unfolded state with concentrated denaturant when native interactions are more favorable. In the intermediate concentrations, binding of denaturant molecules (for urea and guanidine HCl) is less (Fig. 1), and hence, the protein molecules begin to refold. Therefore, it is possible to modulate binding of urea and guanidine HCl for more efficient refolding. This is not the case for detergents. Binding of a detergent is determined by critical micelle concentration. Micelle-like binding occurs above CMC where unfolded proteins are highly soluble, while little binding occurs below CMC where protein solubility is greatly reduced [10]. This dictates that refolding must occur in the presence of detergent, since otherwise, upon removal of the detergent, the protein structure and solubility go back to those in IBs.

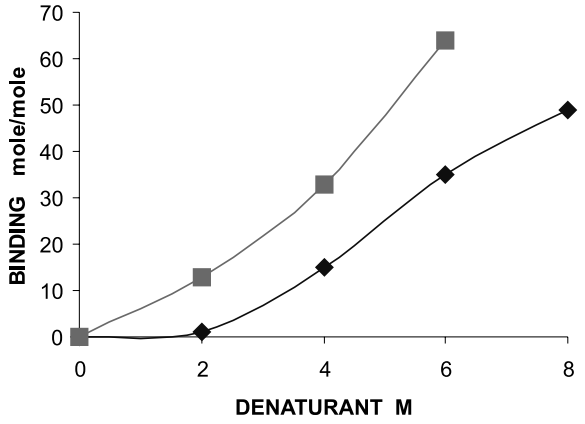

Fig. 1. Binding of urea (◆) and guanidine HCl (□) to oxidized lysozyme. Lysozyme used in this experiment has an intact disulfide structure. When disulfides are reduced (as in inclusion bodies), a larger binding of urea or guanidine HCl occurs. Binding measurements were done by equilibrium dialysis. Data are taken from T. Arakawa and S.N. Timasheff (unpublished).

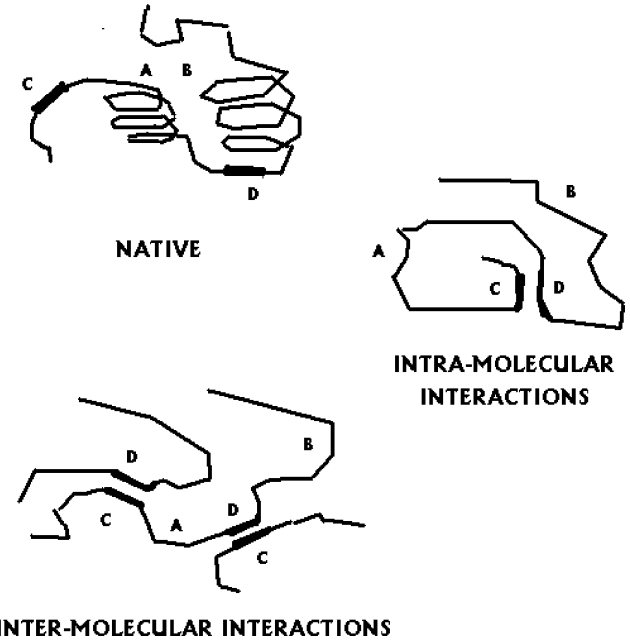

Fig. 2. Non-native intra- and inter-molecular interactions. This model protein has an inter-helical interaction (A and B) and hydrophobic regions (C and D) far apart from each other in the native state (upper panel). The helical interaction is abolished in the unfolded state, while non-native hydrophobic interaction between region C and D occurs intra-molecularly (middle panel) or inter-molecularly (lower panel).

Refolding

Refolding is a change in protein conformation from unfolded to folded state. IBs can be solubilized only by strong denaturants, whereby the proteins are unfolded, the extent of which depends on the proteins and the type of the denaturants used. The solubilized proteins from IBs are also highly flexible, solvated, and soluble. Unlike in refolding studies using purified proteins, solubilized IBs contain impurities, which may associate with the expressed protein and interfere with its refolding. Prior purification may be required to minimize such interference by impurities.

As schematically shown in Fig. 3, refolding is a process that leads to a change in protein conformation from unfolded to folded (native) state. At high denaturant concentrations, proteins are unfolded (disordered), well solvated, and flexible. In aqueous buffer, proteins are folded, rigid, and compact. Ideally, transfer of protein molecules from high denaturant concentration to aqueous buffer should lead to refolding, i.e., transfer of protein molecules from denaturant solution to aqueous solvent will force them to collapse into a compact structure. However, such a drastic process usually does not work, since it will lead to misfolding and aggregation. Once misfolded or aggregated, in the absence of denaturants protein molecules have no flexibility to disaggregate and refold into the native structure. As shown in Fig. 3, a key to refolding is in the intermediate concentration of the denaturant, where denaturant concentration is low enough to force protein molecules to collapse, yet can allow them to stay in solution and be flexible to reorganize their structures. In other words, intermediate concentration of denaturants can induce folding and still maintain solubility and flexibility during refolding. Which intermediate concentration works depends on the proteins and how the denaturant concentration is reduced.

Equilibrium unfolding studies have demonstrated the existence of an intermediate structure (I) in the intermediate concentrations of denaturant. Such intermediate structures also form during refolding, as depicted in Fig. 4. The intermediate structure is unstable and less soluble and as a consequence readily misfolds and aggregates. It is therefore important to facilitate folding of the intermediate into the more stable native structure (N), yet maintain the solubility and flexibility of the intermediate. In connection with protein refolding, however, optimal procedure to reduce denaturant concentration and assistance of refolding by solvent additives play a key role whether protein folding goes through intermediate structure or occurs directly from unfolded to folded state.

Disulfide-containing proteins can refold even in concentrated denaturant, as depicted in Fig. 5. Namely, protein molecules in such solvent can still fluctuate between unfolded and native-like structure and can form the native disulfide bonds [U(SS)]. However, the rate and probability of native disulfide formation will be slow in concentrated denaturant. Bringing the protein to lower denaturant concentration will accelerate disulfide formation, whether conformational transition is of two-state (Fig. 5, panel A) or of three-state (panel B). There is a balance here again that low denaturant concentration will increase the rate of refolding, but may force proteins to collapse into misfolded with resultant formation of non-native disulfides. Once such a structure is formed, it may not refold to the native structure, since insufficient denaturant concentration may make proteins structure too rigid to reorganize.

Even though native disulfide bonds are formed in the presence of denaturant, it does not guarantee that the

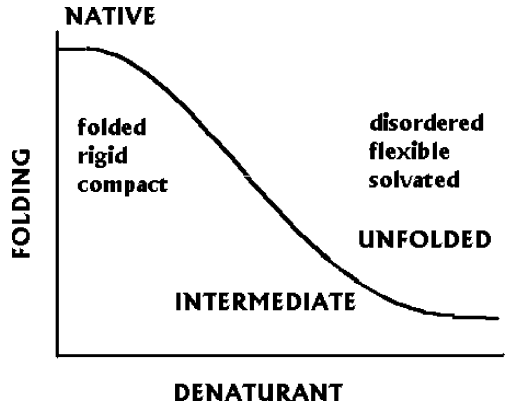

Fig. 3. Conformation, flexibility, and solubility of protein as a function of denaturant concentration. Degree of folding is plotted against denaturant concentration. Physical properties of protein solution are given at high and low sides of concentration of denaturant.

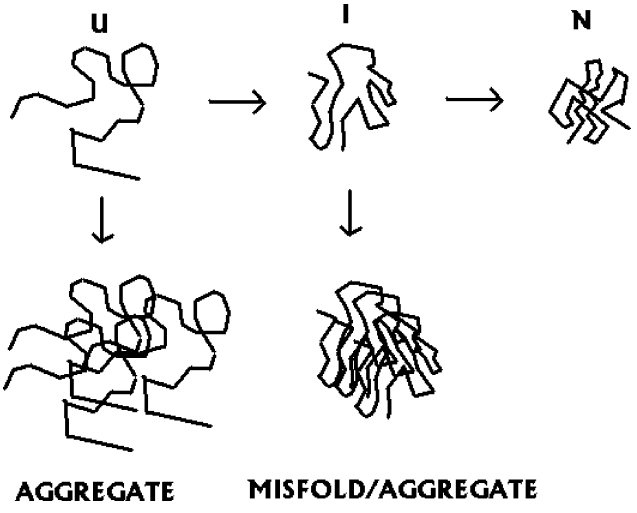

Fig. 4. Schematic diagram of refolding course. U, I, and N correspond to the unfolded, intermediate, and native state of protein, respectively.

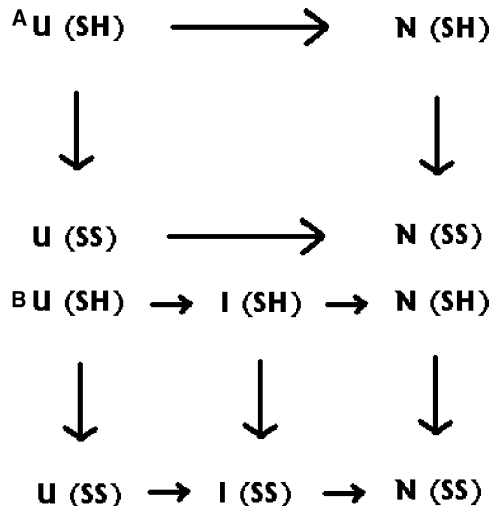

Fig. 5. Protein folding and disulfide formation. Panels A and B show the formation of disulfide bonds and conformational change during two- and three-state transition, respectively.

native conformation is formed by removing the denaturant. Proteins are still largely unfolded even with native disulfides formed and sudden removal of the denaturant may cause a collapse into a wrong conformation. The way by which the denaturant is removed can affect refolding efficiency.

In this discussion, it should be noted that detergent will behave differently. Detergent binding is characterized as micelle-like binding above CMC and stoichiometric (mono-molecular) binding below CMC (Fig. 6). Proteins are quite insoluble in stoichiometric binding, while highly soluble in micelle-like binding [10]. There is no intermediate binding or structure, so if disulfides are not formed while proteins are soluble in protein–detergent complex, detergent concentration cannot be reduced below CMC to induce folding.

Refolding is determined by a balance between structure collapse and flexibility or solubility of the intermediates (or the unfolded structure). This balance affects the refolding course. Folding into native structure

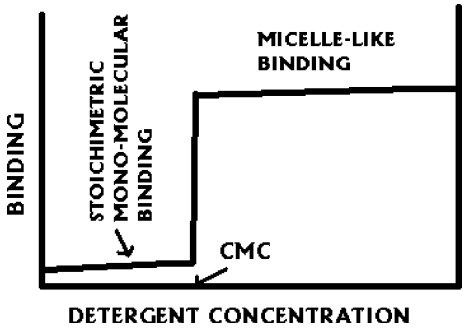

Fig. 6. Detergent binding as a function of concentration A strong detergent such as Sarkosyl exhibits a micelle-like binding above CMC and a stoichiometric binding below CMC.

competes with misfolding or aggregation. Therefore, optimal refolding can be achieved by forcing denatured protein to collapse on one hand, yet by maintaining solubility and flexibility of the molecule on the other hand. Such balance can be manipulated by two different means. One is the way by which denaturant concentration is reduced. Any procedure, for example, whether it is dialysis or dilution, must go through intermediate denaturant concentration. Both how fast the denaturant concentration is reduced and how long the protein molecules are exposed to intermediate denaturant concentration determine the rate of folding and the degree of flexibility or solubility of folding intermediates. In addition, the rate of folding vs. misfolding or aggregation can be manipulated by small molecule additives. There are certain additives that enhance structure formation or collapse, while others increase flexibility or solubility of the proteins. Here, we describe these two parameters separately, but refolding should be optimized by combining these two parameters. First, we discuss about various protocols to reduce denaturant concentration.

Refolding

One-step dialysis

Denatured, unfolded protein samples in concentrated denaturant solution are dialyzed against a refolding buffer, and hence, exposed to descending concentration of the denaturant. Denaturant concentration decreases with time to the concentration of refolding solvent (Fig. 7A). As the concentration of denaturant is decreased, the rate of folding into the intermediate and native structures increases. However, the rate of misfolding or aggregation will also increase. In particular, aggregation can be greatly enhanced, if the rate of folding is slow, since the moderate to low denaturant concentration may not be enough to keep the unfolded or intermediate structures soluble. In dialysis refolding, the intermediate structure can be exposed to intermediate denaturant concentration for a prolonged period. This protocol should have a better chance of success for those proteins which are soluble even in the unfolded or intermediate state. Note that while denaturant concentration decreases, protein concentration remains relatively constant, except for volume expansion due to high osmolality of guanidine HCl or urea. This means that the initial protein concentration in the denaturant is critical.

Step-wise dialysis

This protocol uses descending concentration of denaturant for dialysis and has been successfully used for

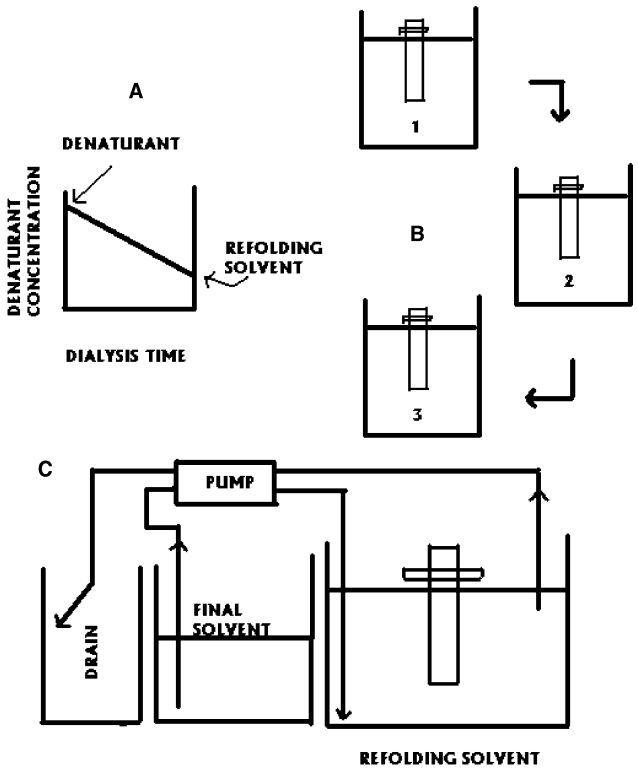

Fig. 7. Dialysis refolding. Panel A: change in denaturant concentration during dialysis. Panel B: step-wise dialysis from high denaturant concentration (1), via middle concentration (2), and to low concentration (3). Panel C: in this dialysis, unfolded protein in dialysis bag is equilibrated with solubilizing solvent (high denaturant). Final solvent is pumped in and the dialyzing solution is pumped out. This will generate a descending concentration of denaturant in refolding solvent during dialysis.

refolding antibodies [11,12]. Unfolded protein sample is first brought to equilibrium with high denaturant concentration (Fig. 7B-1), then with middle concentration (Fig. 7B-2), and with low concentration (Fig. 7B-3). Difference from the one-step dialysis is the establishment of equilibrium at each denaturant concentration. This does not work if the rate of misfolding or aggregation kmis/agg (Fig. 8) is faster than the rate of refolding, kref. An advantage in this protocol is that the return to correct refolding pathway kex may occur, in particular for disulfide-containing proteins in redox disulfide exchange reaction, at intermediate denaturant concentration. At each denaturant concentration, folding intermediate may form the misfold or aggregate. However, intermediate concentration of denaturant may allow the protein molecule to fluctuate and convert to the native structure with correct disulfides formed. Another advantage may be afforded on multi-domain proteins, if the folding or stability of each domain is different. Equilibration at higher denaturant concentration may result in folding of the most stable domain. It is possible that folding of this particular domain is more favorable at higher denaturant concentration than at lower concentration and simultaneous refolding with less stable domains, in one-step dialysis, may cause misfolding.

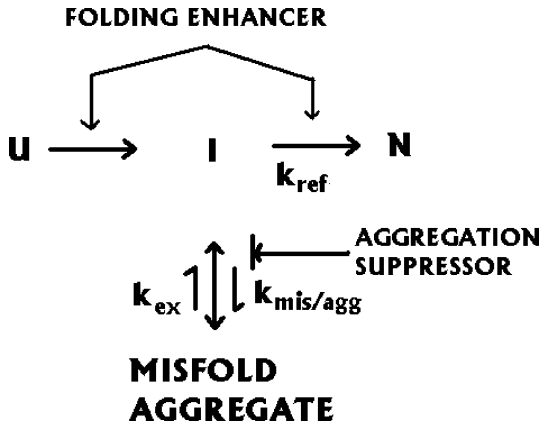

Fig. 8. Refolding and aggregation. The parameters, kref, kmis/agg, and kex, are the rate of refolding from I to N, the rate of misfolding or aggregation, and the rate of the reverse reaction from misfold or aggregate to I. Folding enhancer enhances the reaction of both U to I and I to N, while aggregation suppressor reduces the reaction rate from I to misfold or aggregate.

Descending denaturant concentration dialysis

As shown in Fig. 7C, this is one-step dialysis against descending concentration of denaturant [13]. The unfolded protein sample at high denaturant concentration is placed in dialysis bag and immersed in the denaturant solution. This dialyzing solvent is pumped out and the final buffer (refolding solvent) is pumped in. The rates of pumping-out and -in determine the gradient of denaturant concentration reduction. If the rate is fast, it is similar to one-step dialysis, while, in slow rate, it resembles multi-step dialysis.

Buffer-exchange by gel filtration

Gel filtration column is equilibrated with the final refolding buffer. Unfolded protein sample in denaturant is applied to the column and run through it with the refolding buffer. Use of desalting column will separate proteins from denaturant, while use of protein-sizing column will fractionate protein species. In any case, gradual change in denaturant concentration occurs as in one-step dialysis. The same problems that occur in dialysis refolding may be encountered here. If the unfolded or intermediate folded structure converts more slowly to the native state than to the misfolds or aggregates, there may not be enough time for the misfolds to exchange into the native structure in the descending concentration of denaturant. Alternately, a prolonged exposure to intermediate denaturant concentration may cause protein aggregation or misfolding. A difference from dialysis is the environment of column matrix surrounding proteins during folding. Column matrix may

interact with the proteins, via hydrogen boding or hydrophobic interaction, during folding under the intermediate denaturant concentration that may prevent misfolding or aggregation. Column matrix may also help proteins disperse, reducing aggregation. This protocol was successfully used for refolding IL-6 from guanidine HCl [14]. In this case, IL-6 was oxidized to form correct disulfides in guanidine HCl and then refolded by gel filtration on G-25 column equilibrated with refolding buffer. This also demonstrates that correct disulfide formation is prerequisite, but insufficient for IL-6 refolding. An optimal procedure to remove guanidine HCl is essential to lead to a native structure, even after the correct disulfides are formed.

Dilution

Protein samples at high denaturant concentration are delivered into a large volume of refolding buffer. Dilution brings the unfolded sample into a rapid collapse, whereby bypassing the intermediate denaturant concentration. There are a few parameters to be considered. First, as shown in Fig. 9 (normal dilution), both the denaturant and protein concentrations increase, as the unfolded protein in concentrated denaturant is delivered, e.g., from 0 (refolding solvent) to 1 M (final concentration) if a protein in 6 M guanidine HCl is diluted 6-fold (at the end of dilution) into a buffer. This means that earlier part of dilution (where denaturant concentration is close to zero) is very different from later part (where denaturant concentration is close to 1 M). Dilution into a buffer means a collapse into a rigid structure which cannot fluctuate or convert to the native structure, without the presence of low concentration of denaturant. It is therefore recommended to include some level of denaturant, the concentration of which depends on the stability of the protein to be refolded. Second, for oligomeric proteins, the earlier part of dilution means low protein concentration during refolding. Therefore, slow dilution may result in insufficient concentration of refolded, monomeric state for a prolonged period, and hence, rapid dilution may be recommended. Third, if lower protein concentration is required to avoid aggregation, pulsed-dilution may work better, as described below.

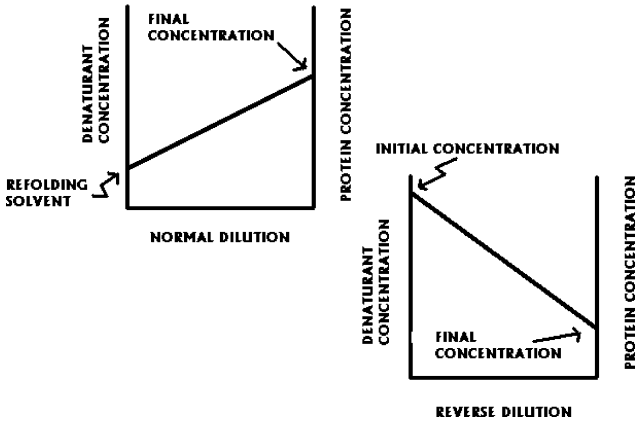

Fig. 9. Dilution refolding. Left panel, normal dilution. Right panel, reverse dilution.

Reverse dilution

Reverse dilution is done by adding refolding buffer into an unfolded protein containing concentrated denaturant such that both the denaturant and protein concentrations decrease simultaneously (Fig. 9. reverse dilution). This results in exposure of unfolded or intermediate protein molecules to descending denaturant concentration for a prolonged period. Unlike dilution, protein concentration is high at intermediate denaturant concentration. Such conditions result in aggregation and precipitation. However, if the intermediate structure is soluble in the intermediate denaturant concentration and refolding requires slow structure rearrangement, this protocol may be desirable.

Mixing

Refolding solvent and unfolded protein solution are mixed at a constant ratio. With this procedure, both protein and denaturant concentrations during refolding are maintained constant (Fig. 10, upper panel), unlike dilution or reverse dilution. The course of protein folding is similar to that in dilution procedure, i.e., mixing leads to a rapid collapse of the protein into an intermediate structure.

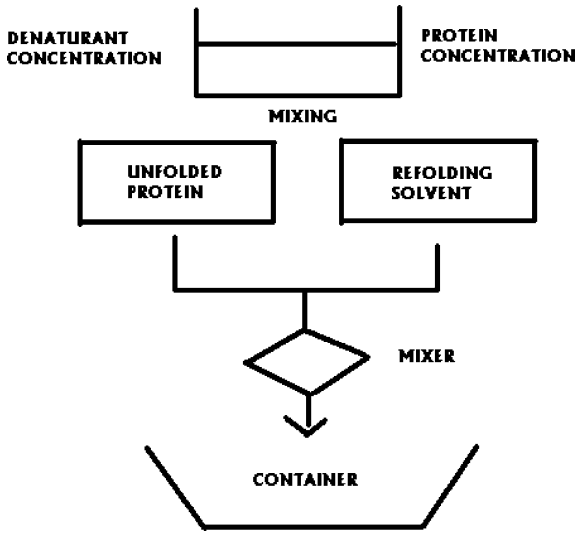

Fig. 10. Refolding by mixing. Unfolded protein and refolding solvent are delivered, e.g., via pump, into a mixer at a constant ratio. Mixing at constant ratio maintains both denaturant and protein concentrations constant during refolding.

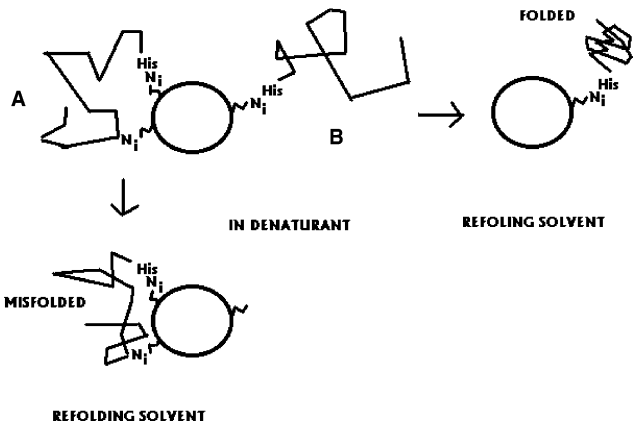

Fig. 11. Refolding on column. Two types of protein binding in denaturant solution are shown. In type A, unfolded protein has two contacts with the solid matrix, one through terminal His-tag, and another through an amino acid residue in the polypeptide. Such multiple contacts can lead to misfolding. In type B, unfolded protein binds only through terminal His-tag and is refolded into the native structure.

Pulsed dilution

Dilution is made in aliquot, rather than in continuous mode. In pulsed dilution, after an aliquot of denatured protein solution is diluted into a refolding solvent, refolding is allowed to occur for some period before addition of the next aliquot. This will avoid accumulation of high concentration of folding intermediates that occur in one-step dilution. This has an advantage when the folded structure does not aggregate with the unfolded or folding intermediates.

Solid phase refolding

Denatured protein is first non-covalently bound to solid matrix such as Ni-resin or ion-exchange resin in the presence of denaturant (Fig. 11). Denaturant concentration is then decreased to initiate refolding. Since protein molecules are bound to resin, this procedure minimizes aggregation of unfolded protein or folding intermediates. Binding of proteins to solid phase can be multivalent, which renders protein folding impossible (Fig. 11, lower panel). Even when it is monovalent, folding may be interfered with due to steric hindrance or binding sites on protein molecule being important for folding. To overcome this problem, refolding may be carried out under weakly dissociating conditions, where protein to be refolded fluctuates between bound and unbound states in the resin.

Co-solute assistance

Small molecules (co-solutes) are usually added to refolding solvent to facilitate refolding. In general, in particular for refolding by dilution, low concentration of urea or guanidine HCl is included in refolding solvent. This concentration is low enough for efficient refolding, yet high enough to maintain solubility and flexibility of folding intermediates. However, inclusion of urea or guanidine HCl alone is insufficient and addition of co-solutes is often essential. Without it, refolding generates a varying degree of aggregates or misfolds. Co-solutes may be classified into two groups, folding enhancer and aggregation suppressor as summarized in Table 1. Such distinctive effects are schematically depicted in Fig. 8. These two groups may be exclusive, since folding enhancer in principle enhances protein–protein interactions, while aggregation suppressor reduces side chain interactions. Aggregation suppressor reduces association of folding intermediates without interfering with refolding process. It encompasses polyethylene glycol [15], cyclodextrin [16], arginine HCl [17,18], and proline [19–21]. Polyethylene glycol and cyclodextrin bind to the hydrophobic region of the folding intermediate. Among these co-solutes, arginine HCl is most frequently used. It is not clear, however, how arginine HCl reduces aggregation of folding intermediates. It is clear from its effect on protein stability that arginine HCl is not a protein stabilizer nor folding enhancer. There are many polar small molecule additives that enhance protein stability [22–27] and also in vivo protein folding [28–30]. These encompass sugars, polyols, certain salts such as ammonium sulfate and magnesium chloride, and certain amino acids such as glycine and alanine. Although these will enhance protein to collapse into a compact structure, they may also enhance misfolding and aggregation. Such collapsed structure may be too compact and rigid,

Table 1
Classification of small molecule additives

Classification	Model co-solute	Effect on protein stability	Effect on protein–protein interaction
Folding enhancer	Sucrose Ammonium sulfate	Stabilizer	Enhance
Aggregation suppressor	Arginine Mild detergent	Neutral	Reduce
Denaturant	Urea Guanidine HCl Strong detergent	Destabilizer	Reduce

rendering the misfolded structure unable to reorganize into the native state. Reduced flexibility by protein stabilizers, i.e., sucrose, has been shown by H-D exchange experiments [31]. They may be useful when the unfolded or folding intermediates are too soluble and cannot be readily converted to a more compact structure. It has been shown that α-synuclein, highly soluble even in the unfolded state, can attain a certain folded structure in the presence of trimethylamine-N-oxide, a strong protein stabilizer [32].

References

[1] P.H. Yancey, M.E. Clark, S.C. Hand, R.D. Bowlus, G.N. Somero, Living with water stress: evolution of osmolyte systems, Science 217 (1982) 1214–1222.

[2] G. Somero, Protons, osmolytes, and fitness of internal milieu for protein function, Am. J. Physiol. 251 (1986) R197–213.

[3] C.R. Brown, L.Q. Hong-Brown, J. Biwersi, A.S. Verkman, W.J. Welch, Chemical chaperones correct the mutant phenotype of the δF508 cystic fibrosis transmembrane conductance regulator protein, Cell Stress Chaperones 1 (1996) 117–125.

[4] E. De Bernardez Clark, E. Schwarz, R. Rudolph, Inhibition of aggregation side reactions during in vitro protein folding, Methods Enzymol. 309 (1999) 217–236.

[5] T. Takagi, K. Kubo, Sodium dodecyl sulfate–protein polypeptide complexes in 8 M urea with special reference to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Biochim. Biophys. Acta 578 (1979) 68–75.

[6] T. Takagi, K. Tsuji, K. Shirahama, Binding isotherms of sodium dodecyl sulfate to protein polypeptides with specific reference to SDS–polyacrylamide gel electrophoresis, J. Biochem. (Tokyo) 77 (1975) 939–947.

[7] L.M. Gierasch, J.E. Lacy, K.F. Thompsosn, A.L. Rockwell, P.I. Watnick, Conformations of model peptides in membrane-mimetic environments, Biophy. J. 37 (1982) 275–284.

[8] B.K. Muralidhara, V. Prakash, Molten globule state of human serum albumin, Curr. Sci. 72 (2001) 831–834.

[9] N.S. Bhavesh, S.C. Panchal, R. Mittal, R.V. Hosur, NMR identification of local structure preferences I HIV protease tethered heterodimer in 6 M guanidine hydrochloride, FEBS Lett. 509 (2001) 218–224.

[10] T. Arakawa, J. Philo, W.C. Kenney, Structure and solubility of interleukin-2 in sodium dodecyl sulfate, Int. J. Pept. Protein Res. 43 (1994) 583–587.

[11] I. Kumagai, K. Tsumoto, Generation of novel functional antibody molecules by in vitro selection system, Tanpakushitsu Kakusan Koso 43 (1998) 201–205.

[12] K. Tsumoto, K. Shinoki, H. Kondo, M. Uchikawa, T. Juji, I. Kumagai, Highly efficient recovery of functional single-chain Fv fragments from inclusion bodies overexpressed in *Escherichia coli* by controlled introduction of oxidizing reagents—application to a human single-chain Fv fragment, J. Immunol. Methods 219 (1998) 119–129.

[13] Y. Maeda, H. Koga, H. Yamada, T. Ueda, T. Imoto, Effective renaturation of reduced lysozyme by gentle removal of urea, Protein Eng. 8 (1995) 201–205.

[14] D. Ejima, M. Watanabe, Y. Sato, M. Date, N. Yamada, Y. Takahara, High yield refolding and purification process for recombinant human interleukin-6 expressed in *Escherichia coli*, Biotechnol. Bioeng. 62 (1999) 301–310.

[15] J.L. Cleland, D.I.C. Wang, Cosolvent assisted protein refolding, Biotechnology 8 (1990) 1274–1278.

[16] N. Karuppiah, A. Sharma, Cyclodextrins as protein folding aids, Biochem. Biophys. R. 211 (1995) 60–66.

[17] D. Arora, N. Khanna, Method for increasing the yield of properly folded recombinant γ interferon from inclusion bodies, J. Biotechnol. 52 (1996) 127–133.

[18] M. Suenaga, H. Ohmae, S. Tsuji, T. Itoh, O. Nishimura, Renaturation of recombinant human neurotrophin-3 from inclusion bodies using a suppressor agent of aggregation, Biotechnol. Appl. Biochem. 28 (1998) 119–124.

[19] W.B. Ou, Y.D. Park, H.M. Zhou, Effect of osmolytes as folding aids on creatine kinase refolding pathway, Int. J. Biochem. Cell Biol. 34 (2002) 136–147.

[20] F. Meng, Y. Park, H. Zhou, Role of proline, glycerol, and heparin in protein folding aids during refolding of rabbit muscle creatine kinase, Int. J. Biochem. Cell Biol. 33 (2001) 701–709.

[21] D. Samuel, T.K. Kumar, G. Ganesh, G. Jayaraman, P.W. Yang, M.M. Chang, V.O. Trivedi, S.L. Wang, K.C. Hwang, D.K. Chang, C. Yu, Proline inhibits aggregation during protein refolding, Protein Sci. 9 (2000) 344–352.

[22] T. Arakawa, S.N. Timasheff, Stabilization of protein structure by sugars, Biochemistry 21 (1982) 6536–6544.

[23] T. Arakawa, S.N. Timasheff, Preferential interaction of proteins with salts in concentrated solution, Biochemistry (1982) 6545–6552.

[24] T. Arakawa, S.N. Timasheff, Preferential interaction of proteins with solvent components in amino acid solutions, Arch. Biochem. Biophys. 224 (1983) 169–177.

[25] T. Arakawa, S.N. Timasheff, The stabilization of proteins by osmolytes, Biophys. J. 47 (1985) 411–414.

[26] J.C. Lee, S.N. Timasheff, The stabilization of proteins by sucrose, J. Biol. Chem. 256 (1981) 7193–7201.

[27] K. Gekko, S.N. Timasheff, Mechanism of protein stabilization by glycerol: preferential hydration in glycerol–water mixtures, Biochemistry 20 (1981) 4667–4676.

[28] R.R. Kopito, Biosynthesis and degradation of CFTR, Physiol. Rev. 79 (1999) S167–173.

[29] T. Ohnishi, K. Ohnishi, X. Wang, A. Takahashi, K. Okaichi, Restoration of mutant TP53 to normal TP53 function by glycerol as a chemical chaperone, Rad. Res. 151 (1999) 498–500.

[30] S. Bourot, O. Sire, A. Trautwetter, Glycine betaine-assisted protein folding in a lysA mutant of *Escherichia coli*, J. Biol. Chem. 275 (2000) 1050–1056.

[31] B.S. Chang, R.M. Beauvais, T. Arakawa, L.O. Narhi, A. Dong, D.I. Aparisio, J.F. Carpenter, Formation of an active dimer during storage of interleukin-1 receptor antagonist in aqueous solution, Biophys. J. 71 (1996) 3399–3406.

[32] V.N. Uverski, J. Li, A.L. Fink, Trimethylamine-N-oxide-induced folding of α-synuclein, FEBS Lett. 509 (2001) 31–35.