Refolding of recombinant proteins

Eliana De Bernardez Clark

Expression of recombinant proteins as inclusion bodies in bacteria is one of the most efficient ways to produce cloned proteins, as long as the inclusion body protein can be successfully refolded. Aggregation is the leading cause of decreased refolding yields. Developments during the past year have advanced our understanding of the mechanism of aggregation in *in vitro* protein folding. New additives to prevent aggregation have been added to a growing list. A wealth of literature on the role of chaperones and foldases in *in vivo* protein folding has triggered the development of new additives and processes that mimic chaperone activity *in vitro*.

Addresses
Department of Chemical Engineering, Tufts University, Medford, MA 02155, USA; e-mail: edeberna@tufts.edu

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Abbreviations
DTT dithiotreitol
GdmCl guanidinium chloride

Introduction

Expression of cloned genes in bacteria is widely used both in industry, for the production of pharmaceutical proteins, and in research, for the production of proteins for structural and/or biochemical studies. Bacteria produce large quantities of recombinant proteins in rapid, often inexpensive, fermentation processes; however, the product of interest is frequently deposited in insoluble inactive aggregates or inclusion bodies. The general strategy used to recover active protein from inclusion bodies involves three steps: firstly, inclusion body isolation and washing; secondly, solubilization of the aggregated protein, which causes denaturation; and finally, refolding of the solubilized protein. While the efficiency of the first two steps can be relatively high, folding yields may be limited by the production of inactive misfolded species as well as aggregates.

When the formation of inclusion bodies was first observed almost two decades ago, existing protein folding protocols were not, in most cases, applicable to the folding of recombinant mammalian proteins, which are in most cases multidomain, oligomeric, and/or disulphide bonded proteins. Existing protein folding protocols had been developed to characterize folding intermediates and investigate folding pathways of small, monomeric proteins. When applied to the refolding of inclusion body proteins, these protocols failed to produce active proteins with significant yields. Even today, the literature on identification of protein folding intermediates and the elucidation of folding pathways deals mostly with small monomeric proteins that have either intact or no disulphide bonds [1]. For many years, eukaryotic expression hosts which produced soluble secreted recombinant proteins became favored over bacterial hosts because of the difficulties encountered when refolding inclusion body proteins; however, careful examination of the folding conditions allowed researchers to find ways to refold multidomain disulphide bonded proteins with relatively high yields. Most of the original work on inclusion body protein folding can be found in the patent literature starting around 1985 [2].

The recent literature includes many examples in which recombinant proteins have been produced by refolding from inclusion bodies. Some of these applications demonstrate the use of suboptimal refolding protocols to produce small quantities of protein for structural and/or biochemical studies. Other applications deal with commercial processes. To be acceptable for commercial applications, refolding processes must be fast, inexpensive and highly efficient. This review focuses on recent developments in the optimization of refolding processes with emphasis on methodologies applicable to large-scale protein production. Since most proteins of commercial value are secreted in their natural host and are likely to contain disulphide bonds, this review emphasizes recent progress in protein refolding with concomitant disulphide bond formation, also called oxidative protein refolding.

Inclusion body isolation and solubilization

Expression of recombinant proteins as inclusion bodies can be advantageous due to the very high levels of enriched protein produced and the protection of the protein product from proteolytic degradation. In addition, when producing a recombinant product which, when active, can be toxic or lethal to the host cell, inclusion body production may be the best available method. Cells containing inclusion bodies are typically disrupted by high pressure homogenization and the resulting suspension is centrifuged to remove the soluble fraction. Occasionally a lytic enzyme, such as lysozyme, may be added before cell disruption to increase efficiency and reduce power requirements. The resulting inclusion body-containing pellet is washed with buffers containing either low concentrations of chaotropic agents, such as urea or guanidinium chloride (GdmCl), or detergents, such as Triton X-100 [3•,4,5•] and sodium deoxycholate [4,6,7]. This washing step is designed to remove contaminants, especially proteins, that may have adsorbed onto the hydrophobic inclusion bodies during processing, and could affect protein refolding yield. Alternatively, sucrose gradient centrifugation may be performed to purify inclusion bodies and separate them from other cellular components [4,P1]. After washing,

inclusion bodies are solubilized using strong denaturants, such as urea, GdmCl, or thiocyanate salts, or detergents, such as SDS [8•,P1], n-cetyl trimethylammonium chloride [4], sarkosyl [6], or sodium n-laurosyl sarcosine [7], and a reducing agent, such as β-mercaptoethanol, dithiotreitol (DTT), dithioerythritol, or cysteine. Temperatures above 30°C are typically used to facilitate the solubilization process. A chelating agent, such as EDTA or EGTA, can be included in the solubilization buffer to scavenge metal ions, which could cause unwanted oxidation reactions. Solubilization can also be accomplished by the addition of acids, such as 70% formic acid [5•]. Alternatively, for periplasmic inclusion bodies the recombinant protein may be recovered by *in-situ* solubilization [P2•] in which the denaturant and reducing agent are added to the broth at the end of the fermentation process, and the cell debris is separated from the soluble material by aqueous two-phase extraction.

Solubilized inclusion body proteins can be contaminated with varying levels of host proteins, nucleic acids, and cell membrane components. It is thought that the presence of these microbial contaminants may induce aggregation during refolding, thus reducing overall yields. Maachupalli-Reddy *et al.* [9•] showed that whereas non-proteinaceous contaminants have little effect on renaturation yields, aggregation of protein contaminants can result in significant losses by triggering co-aggregation of the desired protein. Thus, some inclusion body processes include a purification step prior to refolding. Typically this step may be ion exchange [4,10], size exclusion [P3•,11], metal affinity [12], or reverse phase chromatography [P3•]. A common feature of these chromatographic steps is that they all operate with buffers that keep the protein in the denatured reduced state. If the solubilized protein is to be stored for later use, it may typically be exchanged into an acidic buffer, such as 10% acetic acid or 5–10 mM HCl [P3•,13] and freeze-dried. Exposure to low pH may result, for some proteins, in the formation of partially folded intermediates unable to refold to the native active configuration [14]. In this case, the lyophilized protein should be resolubilized using chaotropic agents or detergents, before refolding is attempted.

Renaturation of the solubilized protein

Several methods, including dilution, dialysis, diafiltration, gel filtration, and immobilization onto a solid support, may be employed to remove or reduce excess denaturing and reducing agents, allowing proteins to renature. Dilution of the denatured solution directly into renaturation buffer is the easiest process. In dialysis, the denatured protein solution is dialyzed against renaturation buffer. Because dialysis is based on the diffusion of smaller molecules and ions through membranes, it may be too slow to be used in commercial scale production of proteins. In addition, exposure of the protein to intermediate concentration of denaturants for a prolonged period of time may cause aggregation. Diafiltration is a faster, therefore, more practical membrane-based alternative because the rate of denaturant removal is not diffusion limited, the driving force being pressure difference; however, as the driving force for buffer exchange is the pressure drop across the membrane, accumulation of denatured protein on the membrane may limit its application due to excessive fouling. Gel filtration chromatography has been successfully used to renature secretory leukocyte protease inhibitor, carbonic anhydrase and lysozyme [P4,15–17]; however, problems in flow through the column may arise due to protein aggregation upon buffer exchange. Aggregation in a chromatographic column can be prevented by immobilizing individual polypeptide chains onto the matrix [12,13,18]. Potential complications may arise if folding of the protein is inhibited by binding to the solid support, which could be prevented by using fusion proteins [19,20•]. In addition to buffer exchange, column chromatography allows for some degree of purification of the desired product.

In the case of disulphide bonded proteins, renaturation buffers must promote disulphide bond formation (oxidation). The most common methods used to promote oxidation during refolding are: air oxidation; the oxido shuffling system; the use of mixed disulphides; and oxidation of sulphonated proteins. Although, oxidation with air or oxygen in the presence of trace amounts of metal ions is simple and inexpensive [P2•,21•], renaturation rates and yields can be low. Higher oxidation rates and yields can be obtained by utilizing 'oxido shuffling' reagents, low molecular weight thiols in reduced and oxidized forms, which allow for both formation and reshuffling of disulphide bonds, which can alter configurations. The most common oxido shuffling reagents are reduced and oxidized glutathione (GSH/GSSG), but the pairs cysteine/cystine, cysteamine/cystamine, DTT/oxidized glutathione, and dithioerythritol/oxidized glutathione have also been utilized. Typically a 1–3 mM reduced thiol and a 10:1 to 5:1 ratio of reduced to oxidized thiol are used to promote proper disulphide bonding [21•]. More recently, we have shown that optimum renaturation yields are obtained when the ratio of reduced to oxidized thiol is anywhere between 3:1 and 1:1 [22•]. A disadvantage of the oxido shuffling system over the use of air oxidation, is the high cost of some of the reagents, particularly oxidized glutathione.

Another strategy employed to oxidize proteins during folding is the formation of mixed disulphides between oxidized glutathione and reduced protein before renaturation [3•]. Formation of mixed disulphides increases the solubility of the denatured protein by increasing the hydrophilic character of the polypeptide chain. Disulphide bond formation is then promoted by adding catalytic amounts of a reducing agent in the renaturation step. A similar protection of thiol groups during solubilization can be achieved by sulphonation of the denatured protein, in which a reducing agent and sodium sulphite are used

to cleave disulphide bonds and protect the resulting thiol groups as sulphonates [P3•,5•]. Under renaturation conditions, the protection groups are removed by oxidation in the presence of small amounts of a reducing agent to promote disulphide bond reshuffling.

Competition between folding and aggregation

Formation of off-pathway species, such as incorrectly folded species and aggregates, are the cause of decreased renaturation yields. Because aggregation is an intermolecular phenomenon, it is highly protein concentration dependent. The most direct means of minimizing aggregation is by decreasing protein concentration. It has been suggested that optimum recovery yields can be expected if the protein concentration is in the range of 10–50 μg/ml [21•]. Renaturation at such low protein concentrations requires large volumes of refolding buffer, driving production costs upward.

The key to a successful commercial refolding process lies in achieving high yields while refolding at high protein concentrations. One solution involves using either slow continuous or discontinuous addition of denatured protein to refolding buffer [3•]. Enough time is allowed between additions for the protein to fold past the early stages in the folding pathway, when it is susceptible to aggregation. The components of the solution containing the denatured protein must be carefully examined to avoid detrimental effects due to their accumulation in the refolding solution after multiple addition steps. Another alternative for decreasing protein aggregation while folding at relatively high protein concentrations (up to 4 mg/ml for carbonic anhydrase II) is to use the temperature-leap tactic [23], in which the protein is allowed to refold at low temperatures, to minimize aggregation, and then the temperature is rapidly raised to promote fast folding after the intermediates responsible for aggregation have been depleted. A third method involves folding by dilution to final denaturant concentrations that are high enough to solubilize aggregates but low enough to promote proper folding. We have shown that the oxidative renaturation of lysozyme can be carried out at protein concentrations of up to 5 mg/ml with very high yields in the presence of 1–2 M GdmCl [22•]. An alternative method which also exposes the refolding protein to intermediate denaturant concentrations was developed by Maeda *et al.* [24]. In this method, renaturation is started by dialysis against a buffer containing high denaturant concentration (8 M urea) and thiol/disulphide exchange reagents, and the denaturant concentration in the dialysis buffer is gradually diluted using buffer without denaturant. Using this method, Maeda *et al.* [24] were able to refold immunoglobulin G at concentrations above 1 mg/ml with yields as high as 70%. For proteins that do not tend to aggregate at intermediate denaturant concentrations, the slow dialysis method can successfully prevent aggregation by exposing the protein to a slow decrease in denaturant concentration. For proteins that aggregate at intermediate denaturant concentrations, fast or slow dilution of denatured protein into renaturation buffer, rather than slow dialysis, is the refolding method of choice.

As aggregation is the major cause behind low renaturation yields, elucidating the aggregation pathway may hold the key to successful protein refolding at moderate to high protein concentrations. Intermediates with hydrophobic patches exposed to the solvent play a crucial role in the partition between native and aggregated conformations. Folding intermediates are believed to possess significant elements of secondary structure but little of the native tertiary structure. Due to the expanded volume of these intermediates, hydrophobic patches, which may normally be buried in the native state, are exposed to the solvent. When hydrophobic regions on separate polypeptide chains interact, intermediates are diverted off the correct folding pathway into aggregates. The so called 'molten globule' intermediate is believed to play a major role in the kinetics of folding [25] and probably plays a role in aggregation. Despite the controversy over the nature of this intermediate (on-pathway versus off-pathway) [26•] from a kinetic point of view, intermolecular association of molten globule-like intermediates may be the starting point of the aggregation pathway. On the other hand, Yon [27] suggests that intermolecular associations responsible for aggregate formation may arise from fluctuating species that precede the molten-globule state.

Pioneer work by Goldberg *et al.* [28] shed light into the nature of interactions responsible for aggregation during folding. They showed that incorrect disulphide bonding may not be the major cause of aggregation because aggregates were formed even when a carboxymethylated protein was folded, that is, all cysteines are blocked from forming disulphide bonds. They also showed that aggregation is a non-specific phenomenon. On the other hand, Speed *et al.* [29•] recently reported that in mixed folding experiments using the P22 tailspike and coat proteins, folding intermediates of the two proteins did not coaggregate, but rather that they preferred to self-associate, suggesting that aggregation is a specific phenomenon. Since they only analyzed soluble aggregates, Speed *et al.* [29•] suggest that it is possible that larger aggregates could grow by a different mechanism involving non-specific interactions.

More recently, Maachupalli-Reddy *et al.* [9•] provided new evidence of the non-specific nature of the aggregation reaction by conducting mixed oxidative renaturation studies with hen egg-white lysozyme and three other proteins: β-galactosidase, bovine serum albumin, and ribonuclease A. They found that foreign proteins that have a tendency to aggregate when folded in isolation, such as β-galactosidase and bovine serum albumin, significantly decreased lysozyme renaturation yields by promoting aggregation in mixed folding experiments. On the other hand, ribonuclease A, which does not

significantly aggregate upon folding in isolation, did not affect lysozyme renaturation yields in mixed folding experiments. We have recently conducted experiments trying to understand the role that disulphide bonding plays in the aggregation pathway [30]. We found that aggregation is fast and that aggregate concentration does not significantly increase beyond the first minute of renaturation. Hydrophobic interactions, and not disulphide bonding, were found to be the major cause of aggregation. Under renaturation conditions that promote disulphide bonding, however, aggregate size, but not concentration, was found to increase due to disulphide bond formation, resulting in covalently bonded aggregates. Based on these results, it is possible to speculate that in mixed folding experiments, in which two or more proteins are simultaneously refolded, small soluble aggregates may form due to specific interactions that are hydrophobic in nature, and large heterogeneous aggregates may grow via disulphide bonding of unpaired cysteines, thus reconciling the conflicting observations of Speed *et al.* [29•] and Maachupalli-Reddy *et al.* [9•] on the specific/non-specific nature of aggregates.

An examination of aggregation data for the P22 tailspike protein, combined with the postulation of three possible mathematical models to describe the aggregation process, led Speed *et al.* [31] to conclude that aggregates grow via a cluster–cluster multimerization mechanism in which multimers of any size associate to form a larger aggregate. Aggregation is not mediated by the sequential addition of monomeric subunits and does not stop when the concentration of monomeric subunits is depleted. This confirms the observation [30] that aggregation is fast, and that aggregate size, rather than total aggregate concentration, increases as time progresses.

Based on the hypothesis that aggregation is caused by interactions between hydrophobic patches in partially folded polypeptide chains, it is possible to envision strategies to decrease aggregate formation. A careful examination of structural and amino acid sequence data can lead to the identification of hydrophobic patches within the protein molecule that could participate in intermolecular interactions. Mutations causing the disruption of such hydrophobic patches may reduce aggregation. This strategy was tested by Plückthun and co-workers [32,33•] who identified mutations located in turns of the protein and in hydrophobic patches which led to decreased *in vitro* and *in vivo* aggregation of recombinant antibody fragments. A second strategy involves the use of antibodies which preferentially bind hydrophobic patches away from the active site to protect the protein from intermolecular associations leading to aggregation. This strategy was tested by Katzav-Gozansky *et al.* [34•] who showed that carboxypeptidase A aggregation can be prevented using specific monoclonal antibodies. Interestingly, Plückthun's group [32,33•] mutated amino acids likely to be on the surface of the native protein, while Solomon and co-workers [34•] raised their antibodies using native antigents. These results seem to indicate that intermediates responsible for aggregation may have more native-like structural features than currently speculated.

Improving renaturation yields

A simpler strategy to prevent aggregation by interfering with intermolecular hydrophobic interactions is to use additives, small molecules that are relatively inexpensive and easy to remove once refolding goes to completion. A variety of additives have been tested for their ability to prevent aggregation. They may act by stabilizing the native state, by preferentially destabilizing incorrectly folded molecules, by increasing the solubility of folding intermediates, or by increasing the solubility of the unfolded state. In general, these additives do not seem to accelerate the rate of folding, but they do inhibit the unwanted aggregation reaction. Additives that have been shown to promote higher refolding yields are listed in Table 1.

As Table 1 indicates, surfactants and detergents have proven to be very efficient folding aids, and have been shown to work with a variety of proteins, in particular multimeric disulphide bonded ones. Correct disulphide bond formation by thiol/disulphide exchange using oxido shuffling systems and air oxidation have been shown to be promoted in the presence of detergents [7,8•,35•]. One drawback of the use of surfactants and detergents is that they are difficult to remove, a direct result of their ability to bind to proteins and to form micelles. Much easier to remove, low denaturant concentrations and L-arginine have shown excellent folding enhancing capabilities (Table 1); however, because they are used in the molar concentration range, they may interfere with the assembly of oligomeric proteins.

As *in vivo* folding and aggregation processes are modulated by the presence of chaperones and foldases in the cellular environment, it is not surprising that such proteins can also impact the competition between folding and aggregation in *in vitro* protein folding [36•]. Chaperones and foldases, however, are proteins that need to be removed from the renaturation solution at the end of the refolding process, and may be costly to produce unless a recovery-reuse scheme can be implemented [37]. A practical solution to this problem was proposed and implemented by Altamirano *et al.* [38•] who used immobilized mini-chaperones to promote proper folding of several proteins which proved difficult to refold by other means. The immobilized mini-chaperones consisted of fragments of GroEL attached to chromatographic resins. The technique is only applicable to GroEL substrates and has not been tested under oxidative renaturation conditions.

In an attempt to mimic the GroEL-GroES chaperonin action, Rozema and Gellman [35•,39] developed a folding strategy in which the denatured protein is first exposed

Table 1

***In vitro* folding aids.**

Additive	Protein	Reference
Non-denaturing concentrations of chaotropic agents		
GdmCl	*P. fluorescens* lipase	[10]
	Hen egg-white lysozyme	[22•]
	Carbonic anhydrase II	[41]
	Interferon-β-polypeptides	[P1]
Urea	Porcine growth hormone	[4]
	Hen egg-white lysozyme	[42•]
	IGF-I	[P2•]
	Interferon-β-polypeptides	[P1]
L-arginine	*P. fluorescens* lipase	[10]
	Fab fragments	[14]
	Hen egg-white lysozyme	[22•]
	α-gluocosidase	[20•]
Salts		
Ammonium sulphate	Hen egg-white lysozyme	[42•]
Sugars		
Glycerol	*P. fluorescens* lipase	[10]
	Hen egg-white lysozyme	[42•]
	IGF-I	[P2•]
Sucrose	IGF-I	[P2•]
Glucose	Hen egg-white lysozyme	[42•]
N-acetyl glucosamine	Hen egg-white lysozyme	[42•]
Sarcosine	Hen egg-white lysozyme	[42•]
Detergents and surfactants		
Chaps	TGF-β-like proteins	[P3•]
	Carbonic anhydrase II	[41]
Tween	Human growth hormone	[44]
SDS	Interferon-β-polypeptides	[P1]
Sarkosyl	RNA polymerase q factor	[6]
Sodium lauorsylsarcosine	Single chain Fv fragment	[7]
Dodecyl maltoside	Class II MHC	[8•]
Triton X-100	Carbonic anhydrase II	[41]
Polyethylene glycol	Carbonic anhydrase II	[41]
Octaethylene glycol monolauryl	Carbonic anhydrase II	[41]
Phospholipids	Hen egg-white lysozyme	[9•]
	TGF-β-like proteins	[P3•]
Sulphobetaines	Hen egg-white lysozyme	[43]
	β-D-galactosidase	[43]
Short chain alcohols		
n-pentanol	Carbonic anhydrase II	[41]
n-hexanol	Carbonic anhydrase II	[41]
cyclohexanol	Carbonic anhydrase II	[41]

to a detergent-containing solution to prevent aggregation, followed by stripping of the detergent with cyclodextrin to promote folding. The technique has been named 'artificial chaperone-assisted refolding' and has been applied to the refolding of carbonic anhydrase B [39], and the oxidative renaturation of lysozyme [35•]. This procedure has also been shown to work in the refolding MM-creatine kinase [40].

Conclusions

Inclusion body protein refolding used to be considered a difficult task. A protocol that worked for one protein did not work for others. Finding the right conditions to fold a given protein was a trial and error process in which existing methods were tried until a successful one was found. This was in part due to our lack of understanding of the competition between folding and aggregation in *in vitro* protein folding. Despite this lack of knowledge, many efficient refolding process have been developed in which aggregation is reduced by the use of additives that interfere with intermolecular interactions responsible for aggregation. As more and more additives are added to the list, there is a pressing need to characterize the aggregation process at the molecular level in order to select the right additive. Advances in our understanding of the aggregation pathway combined with knowledge on the role that chaperones play in *in vivo* protein folding provide the tools that will allow us to develop efficient refolding processes. Among these developments, finding sites on the protein molecule that interact with molecular chaperones, and identifying protein regions involved in intermolecular interactions will provide a rational basis for finding specific mutations and designing small binding molecules that prevent aggregation.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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This chapter covers relevant topics and techniques in protein unfolding/folding. It contains protocols for cell lysis, isolation of inclusion bodies, and renaturation of solubilized proteins with special emphasis on the renaturation of disulphide bonded proteins. It also covers monitoring of protein folding, kinetics and equilibrium considerations, folding and association of oligomeric proteins, the effects of ligands and the effect of auxiliary proteins assisting protein folding.

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E. coli derived recombinant human proinsulin produced in inclusion bodies was solubilized with 70% formic acid and cleaved with cyanogen bromide. After solvent evaporation and freeze-drying, the lyophilized protein was dissolved in 7 M urea and subjected to oxidative sulphitolysis. The sulphonated material was purified by anion exchange, and refolded at pH 10.5 in the presence of air and β-mercaptoethanol. The refolded proinsulin contained the correct disulphide bond pattern.

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An in depth discussion of the mechanism of detergent-mediated protein folding of a heterodimeric disulphide bonded protein with four domains. The contributions of detergent headgroup and aliphatic tail to the stabilization of folding intermediates are dissected. Optimal detergent concentration decreases with increasing critical micelle concentration. Formation of secondary structure occurs early in the folding pathway when the denaturing detergent SDS is replaced by a mild detergent. Tertiary structure formation and heterodimer

association occurs later in the folding pathway concomitantly with disulphide bond formation.

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This paper shows that non-proteinaceous contaminants, such as plasmid DNA, ribosomal RNA, and lipopolysaccharides, have little effect on protein renaturation. Phospholipids improve folding yields by about 15%. Proteinaceous contaminants, on the other hand, can have a significant detrimental effect on folding yields by causing co-aggregation of the protein of interest. The paper also shows that contaminants affect the overall rate of the aggregation reaction without affecting the folding rate.

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The authors demonstrate a strategy to prevent aggregation upon folding by physically isolating protein molecules from each other through immobilizing onto a solid support. In this technique, the protein of interest is produced as a fusion protein with either an amino- or a carboxy-terminal hexa-arginine peptide. The denatured protein is attached to a solid support containing polyanionic groups and renaturation on the immobilized protein is performed by removal of the denaturant. The effect of ionic strength, addition of ethylene glycol and support material are thoroughly investigated.

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The authors refolded a mixture of P22 tailspike and coat proteins under conditions which promote aggregation of both proteins. Co-aggregation of the proteins was not observed indicating that aggregation is due to specific interactions. As only soluble multimeric species were analyzed, the possibility of formation of heterogeneous insoluble aggregates cannot be excluded.

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33. • Nieba L, Honegger A, Krebber C, Plückthun A: **Disrupting the hydrophobic patches at the antibody variable/constant domain interface: improved *in vivo* folding and physical characterization of an engineered scFv fragment.** *Protein Eng* 1997, **10**:435-444.

Amino acid mutations away from the antibody fragment binding site resulted in decreased aggregation rates *in vitro*. Mutations were performed on exposed hydrophobic residues which are adjacent to another exposed hydrophobic residue in order to disrupt hydrophobic patches. The paper also explores the role of these mutations on *in vivo* folding and aggregation of the resulting scFv fragments.

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This paper shows that certain monoclonal antibodies that bind to the antigen at locations removed from the active site, are capable of promoting proper folding by decreasing aggregation. Thus, monoclonal antibodies can be used as folding additives to prevent aggregation, and as tools to identify sites where protein aggregation may be initiated.

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The artificial chaperone strategy consists of refolding by dilution in the presence of a detergent followed by stripping of the detergent using methyl-β-cyclodextrin. Only ionic detergents are effective in the role of artificial chaperones. Lysozyme is not active in the presence of detergent, and activity is recovered only after the stripping step. Thiol/disulphide reagents can be added either in the dilution step (in the presence of detergent) or in the stripping step. Higher yields are obtained when thiol/disulphide exchange occurs in the presence of detergents.

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The authors demonstrate the practical use of the GroEL apical domain (residues 191–376) and the core of the apical domain (residues 191–345) as folding enhancers in the renaturation of several difficult to refold proteins. The mini-chaperones are immobilized on chromatographic resins and

chromatography can be conducted in both elution and batch modes. The technique only works with proteins that are known to be GroEL substrates. Its applicability in the oxidative renaturation of proteins is not demonstrated.

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Increasing concentrations of additives such as sarcosine, glycerol, ammonium sulphate, glucose and N-acetyl glucosamine resulted in improved refolding rates (except for glycerol) and yields in the oxidative renaturation of lysozyme. Sarcosine was the most effective folding enhancer.

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44. Bam NB, Cleland JL, Randolph TW: **Molten globule intermediate of recombinant human growth hormone: stabilization with surfactants.** *Biotechnol Prog* 1996, **12**:801-809.

Patents

P1. Dorin G, McAlary P, Wong K: **Bacterial production of hydrophobic polypeptides.** *World (WO) Patent* 1996, 96/39523.

P2. • Builder S, Hart R, Lester P, Reifsnyder D: **Refolding of misfolded insulin-like growth factor-I.** *US Patent* 1997, 5 663 304.

A method is disclosed in which the oxidative renaturation of insulin-like growth factor-I (IGF-I) is conducted using oxygen and in the presence of low copper or manganese concentrations, an alcoholic or polar aprotic solvent (such as 20% ethanol), an effective amount of chaotropic agent (such as 2 M urea), an effective amount of an alkaline earth, alkali metal, or ammonium salt (such as 1 M NaCl), an optional osmolyte (such as glycerol), and a reducing agent (such as 1 mM DTT). An extensive factorial design analysis of the effects of IGF-I concentration, salt type and concentration, urea, ethanol and glycerol concentrations on folding yield is also included.

P3. • Cerletti N, McMaster GK, Cox D, Schmitz A, Meyback B: **Process for refolding recombinantly produced TGF-β-like proteins.** *US Patent* 1997, 5 650 494.

Methods to improve the oxidative renaturation yields of dimeric forms of transforming growth factor β-like proteins are disclosed. The procedure requires the use of mild detergents, such as 3-(3-chlolamidopropyl) dimethylammonio-1-propane sulphonate. Several methods for thiol/disulphide exchange are disclosed. These include the use of reduced and oxidized glutathione, thioredoxin, and folding from a S-sulphonate monomer.

P4. Seely R, Ladisch M: **Process for protein refolding by means of buffer exchange using a continuous stationary phase capable of separating proteins from salt.** *World (WO) Patent* 1997, 97/04003.