Review

Isolation, Renaturation, and Formation of Disulfide Bonds of Eukaryotic Proteins Expressed in *Escherichia coli* as Inclusion Bodies

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Expression of recombinant proteins in *Escherichia coli* often results in the formation of insoluble inclusion bodies. In case of expression of eukaryotic proteins containing cysteine, which may form disulfide bonds in the native active protein, often nonnative inter- and intramolecular disulfide bonds exist in the inclusion bodies. Hence, several methods have been developed to isolate recombinant eukaryotic polypeptides from inclusion bodies, and to generate native disulfide bonds, to get active proteins. This article summarizes the different steps and methods of isolation and renaturation of eukaryotic proteins containing disulfide bonds, which have been expressed in *E. coli* as inclusion bodies, and shows which methods originally developed for studying the folding mechanism of naturally occurring proteins have been successfully adapted for reactivation of recombinant eukaryotic proteins.
Key words: recombinant protein • *Escherichia coli* • inclusion body • renaturation • disulfide bond

INTRODUCTION

During the last 20 years, manipulation of DNA in vitro has developed from the transfer of genetic information between procaryotic organisms[17] to a technology that facilitates efficient and controlled production of proteins in foreign hosts. A significant feature of these developments is the ability to express eukaryotic genes in prokaryotes such as *Escherichia coli*.[39] The supply of many eukaryotic polypeptides which have potential clinical or industrial use is often limited by their low natrual availability. Gene cloning and expression in *E. coli* can provide a more abundant source of these proteins.

The expression of recombinant proteins in bacteria, however, often results in the formation of inactive protein that accumulates intracellularly. The formation of inactive proteins in bacterial systems appears to be independent of the type of the protein.[63] These inactive protein species often associate to form insoluble protein aggregates called inclusion bodies (for review on inclusion bodies see refs. 56, 63, 65, 81, and 92). In cases of expression of eukaryotic proteins containing cysteines which are able to form disulfide bonds in the native active protein, often nonnative inter- and intramolecular disulfide bonds exist in the inclusion body polypeptide material as well as reduced cysteine residues.

To obtain polypeptides, the insoluble protein pellets must be separated from the other cellular components usually by homogenization, washing, and centrifugation. Inactive pellets are then solubilized in denaturants, such as guanidine hydrochloride or urea, which unfold the protein. In most examples, reducing reagents are added to reduce the polypeptide cysteines to break existing disulfide bonds to yield monomeric peptide chains.

The unfolded reduced protein must then be refolded. This includes the removal of denaturant and excess reducing reagent. As a result of renaturation the polypeptide chain can fold into its native structure and the native disulfide bonds form.

The main concepts in deciphering the folding code, a second translation of the genetic message, have been developed from in vitro refolding studies using purified proteins isolated from their natural sources. The concept states that the folding of a polypeptide chain is a spontaneous process, depending only on the amino acid sequence in a given environment, to reach a lower energy conformation.[6] This process is thermodynamically controlled and driven by the hydrophobic effect.[104] Analysis of renaturation of many proteins has proved that, during refolding, denatured polypeptides go through different intermediates: (1) unfolded polypeptide; (2) nucleation of folding; (3) formation of regular structures; (4) molten globule stage; (5) folded domains; (6) folded monomers; (7) subunit association (for a reviews on protein folding mechanisms see refs. 1, 8, 18, 19, 29, 33, 41, 44–46, 50–52, 97, and 104). Stability of the native state relative to nonnative states depends also upon specific solvent conditions.

A key reaction in refolding of reduced and denatured eukaryotic proteins is the generation of native disulfide bonds. During early studies of the renaturation of denatured polypeptides, analysis of the generated disulfide

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bonds and their locations were central tools for elucidating the folding pathways of several proteins such as lysozyme,[1,2,5,72] ribonuclease,[1] and bovine pancreatic trypsin inhibitor.[19,20]

During this time, several successful methods have been developed to study the generation of disulfide bonds from reduced, denatured polypeptides.

1. Air oxidation: In the air oxidation of reduced proteins, oxygen is apparently used as the electron acceptor for the oxidation of reduced cysteine residues to form disulfide bonds.[1] Air oxidation is performed by aeration of the refolding solution, or simple exposure of the refolding buffer containing the reduced denatured protein to air. The main drawback of this method is the lack of precise control over the process. It has been shown that air oxidation is accelerated in the presence of 10^{-6} to 10^{-7} mol/L metal ions such as Cu^{2+}.[2,3,79] Air oxidation may be more successful if trace amounts of thiolcompounds, such as 2-mercaptoethanol, are present.[25] Using air oxidation, disulfide bond containing proteins such as ribonuclease[3,7] and lysozyme[2,25,79] have been renatured.

2. Formation of protein disulfide bonds starting from mixed disulfides: Using about 10^{-1} mol/L oxidized glutathione, almost all the reduced cysteines of the denatured proteins are converted to mixed disulfides of protein-cysteine and glutathione. After removal of excess denaturant and free glutathione, low concentrations of cysteine are added to displace the glutathione from the protein-*S*-*S*-glutathione to initiate the formation of intramolecular protein disulfide bonds. This method may be controlled by varying the ratio of added cysteine to the concentration of the mixed disulfides. Reoxidation of several serine proteases starting from mixed disulfides has been successfully developed.[24,62,68]

3. Glutathione reoxidation: An efficient system for renaturation of reduced proteins using a mixture of reduced and oxidized glutathione has been introduced by Saxena and Wetlaufer.[79] At a ratio of about 10:1 of reduced to oxidized glutathione and a concentration of reduced glutathione of about 10^{-3} mol/L, several denatured proteins such as lysozyme,[2,5,79] ribonuclease,[3] and bovine pancreatic trypsin inhibitor[18,20] have been renatured, and the correct intramolecular disulfide bonds generated. This method is controlled by the concentration of reduced and oxidized glutathione and their ratio to the protein-cysteine concentration.

4. Reoxidation by dithiothreitol: Reoxidation of reduced polypeptides by dithiothreitol has been most successfully developed by Creighton and co-workers[18,20] for studying the folding pathway of bovine pancreatic trypsin inhibitor. In a reaction of reduced protein-cysteine and oxidized dithiothreitol, an unstable intermediate of both is formed which dissolves with the release of reduced dithiothreitol and the formation of intramolecular disulfide bonds. This reaction is strongly controlled by the concentration of added oxidized dithiothreitol.

Several further chemicals have been introduced to oxidize reduced proteins; e.g., dehydroascorbic acid for the renaturation of serine proteases[85] and proinsulin.[88] None of these methods have been established as well-controlled, universally applicable protocols.

However, all these methods have been developed for studying the folding mechanism of proteins. The experiments have been started using purified material obtained from natural sources, containing naturally occurring disulfide bonds. Proteins were denatured and reduced in purified systems followed by renaturation in analytical amounts.

In contrast, biotechnology requires the renaturation of eukaryotic genes expressed in *E. coli* from inclusion bodies. The starting material differs mainly because the protein source is an inactive, aggregated, and insoluble polypeptide material. It may possess native and non-native intra- as well as intermolecular disulfide bonds, as well unusual free cysteine residues. Although it can make up to 90% of all protein in the inclusion body, it is not a pure or homogenous source.

Several previous reviews analyzed the high-level expression of recombinant proteins and the formation and properties of inclusion bodies in *E. coli*.[39,56,63,65,81,92] This study summarizes the different steps and methods of isolation and renaturation of eukaryotic proteins containing disulfide bonds, which have been expressed in *E. coli* as inclusion bodies. Those proteins have been included in this review, which have been used as representative examples in previous reviews on protein expression and inclusion body formation,[39,56,63,65,81,92] but more recent results have been added.

ISOLATION OF THE INCLUSION BODIES

After fermentation of bacterial host cells, expressed gene products have to be isolated. Inclusion bodies seems to be very compact and stable.[56,63,65,81,92] They are localized in the cytoplasm[56,65,71] of the bacterial cells. To obtain inclusion bodies, *E. coli* cells were disintegrated by mechanic forces such as French pressing,[9,10,27,67,83] homogenization,[15,21,59,89] sonication,[23,30,34,43,48,53,57,58,76,78,82,87,90,95,96,100] or by a combination of lysozyme treatment and sonication.[4,22,31,40,42,64,66,94,99,101,102] There is no apparent correlation between the expressed protein and the isolation method. After disruption of *E. coli* cells, inclusion bodies are sedimented from the homogenate by low-speed centrifugation at 5,000 to 20,000*g* for about 15 min.

In addition to the plasmid encoded protein, inclusion bodies can contain other proteins, such as the four subunits of RNA polymerase; some combination of the outer membrane proteins OmpC, OmpF, and OmpA; 16S and 23S rRNA; plasmid DNA; or other enzymes.[64,81,82] To purify inclusion bodies from adhering impurities they can be washed several times with buffer containing sucrose, Triton-100, deoxycholate or urea.[9,30,54,59,64,67,70,73,78,82,84,86,89,102] The majority of pro-

teins associated with the inclusion bodies, such as membrane proteins[64] or kanomycin phosphotransferase,[82] have been substantially solubilized without solubilization of the polypeptide material of interest. Table I shows several examples of different washing media used for the purification of isolated inclusion bodies. Investigations showed that inclusion bodies containing different proteins resisted up to 5 mol/L urea.[23,54,69,78]

SOLUBILIZATION OF INCLUSION BODIES

After isolation of inclusion bodies the polypeptides have to be solubilized. In most cases, the addition of a reducing reagent is necessary to break existing nonnative intramolecular and intermolecular disulfide bonds. Tables II and III summarize several of the conditions used for the solubilization of recombinant proteins from inclusion bodies by use of either guanidine hydrochloride or urea. However, less frequently, detergents such as sodium dodecylsulfate have been used to solubilize inclusion bodies.[52,69] 2-Mercaptoethanol as well as dithiothreitol are commonly used to reduce the proteins. In most reports on inclusion body solubilization and renaturation of proteins, Tris buffer, at a concentration of about 0.1 mol/L, has been used. A comparison of the data presented in Tables II and III shows that solubilization and reduction is performed around pH 8. Concentrations of about 8 mol/L urea or 6 to 8 mol/L guanidine HCl are widely used to solubilize proteins from inclusion bodies. Investigations on inclusion bodies containing proteins, such as bovine and human growth hormone and interleukin, showed that they resisted up to 5 mol/L urea.[52,69,82] In comparison to 2-mercaptoethanol, dithiothreitol is used at lower concentrations, due to its stronger reducing potential.

Results from Tables II and III show that there is no apparent correlation between the number of disulfide bonds in the native proteins and the method of solubilization/reduction. However, the temperature and time of exposure to denaturants vary drastically between different, and even within, the same protein. This is obviously caused by the incomplete knowledge and experience in this field; most denaturation conditions have been selected empirically. Several proteins, such as immunoglobulins, growth hormones, and tissue-type plasminogen activator, have been solubilized by both urea and guanidine HCl. For most of these proteins, advantages and disadvantages of different solubilization/reduction methods have not been analyzed in comparative studies to investigate their influences on the final yields. In addition, further research has to show if inclusion bodies are solubilized even at lower denaturant concentrations. If different solubilization/reduction methods yield similar results for the same protein, this will enable selection of the method of choice to reduce expense in reagents, materials, and time.

However, it was found for human interleukin-4 that solubilization in guanidine-hydrochloride increased the recovery of this protein, whereas, after solubilization in urea, no active peptide could be recovered.[53]

Table IV shows additional methods that have been used successfully to isolate polypeptides from inclusion bodies without the use of denaturants and reducing reagents. A comparison of the proteins listed in Table IV with proteins summarized in Tables II and III reveals that both recombinant bovine growth hormones and huma interleukins were isolated also from inclusion bodies by urea or guanidine HCl treatment. However, in several preparations, no reducing reagents were added to solubilize these proteins. Solubilization of several further proteins was achieved without the addition of reducing reagent (Tables II and III). This points to the fact that these polypeptides existed in inclusion bodies without intermolecular disulphide bonds. It was found for recombinant bovine growth hormone that this protein was stored in inclusion bodies in its complete, reduced form.[59] Further, several of these proteins, such as growth hormones and interleukins, have been renatured without a specific renaturation/reoxidation method (see below). However, a correlation between the nature of these proteins and their specific renaturation behavior has not been analyzed so far.

By contrast, when tissue-type plasminogen activator was isolated for the first time from inclusion bodies without a reducing reagent,[40] the yield was very poor, although the gene was expressed at high levels in *E. coli*. Later investigations on this enzyme showed that solubilization was achieved with high yields by adding 2-mercaptoethanol or dithiothreitol, which demonstrated that this protein with a molecular weight of about 57,000 and 34 cysteine residues forms high molecular weight aggregates held together by intermolecular disulfide bonds in the inclusion bodies.[30,73,74,78.]

Table I. Purification of inclusion bodies.

Expressed protein	Addition to washing buffer	Refs.
Eel GH/salmon GH	1 mol/L sucrose	89, 84
Eel GH/salmon GH	4% Triton X-100	89, 84
Bovine GH	2% deoxycholate	59
Bovine GH	5 mol/L urea	82
Pro-urokinase	0.1% Triton X-100	70
T-PA	2.5% Triton X-100	73
T-PA	5 mol/L urea/2% Triton X-100	78, 30
Human IL-2	0.375 mol/L sucrose	95
Human IL-2	4 mol/L urea	54
Bovine pancreatic RNase	5% Triton X-100	67
Porcine phospholipase A_2	1% Triton X-100	9
Human M-CSF	2% Triton X-100	102
Horseradish peroxidase C	2 mol/L urea	86
Prochymosin	0.5% Triton X-100	64

GH, growth hormone; T-PA, tissue-type plasminogen activator; IL, interleukin; RNase, ribonuclease; M-CSF, macrophage-colony stimulating factor.

Table II. Solubilization of polypeptides from inclusion bodies by guanidine hydrochloride.

Protein	Number of disulfide bonds in active protein	Guanidine HCl concentration (mol/L)	Reducing reagent (mmol/L)	pH	Period of solubilization (h)	Temperature (°C)	Refs.
Immunoglobulin fragment	5	6	DTT: 300	8.5	1	RT	12
Immunoglobulin chain	15	7.6	2-ME: 100	8	1	37	13
Immunoglobulin fragment	2	7.6	2-ME: 100	8	1	37	27
Creatine kinase	2	6	DTT: 100	8	1	RT	8
T-PA	17	7	2-ME: 50	7.5	24	4	78
T-PA	17	6	DTE: 400	8.6	3	25	73
T-PA	17	6	DTE: 200	8.6	2.5	25	74
T-PA mutant	12	7	2-ME: 100	7.5	24	4	30
T-PA kringle-2 domain	3	7	DTT: 200	8	12	RT	99
T-PA kringle-2 domain	3	6	DTT: 5	8	12	RT	42
Low-molecular-weight urokinase	6	5	—	8	12	4	100
Pro-urokinase	12	6	2-ME: 50	8.5	12	RT	70
Human serum albumin	17	7	2-ME: 100	7.5	nr	4	60
Insulin	3	6	2-ME: 140	nr	1	nr	35
Insulin	3	7	DTP: 1	7.9	nr	nr	98
Chicken GH	2	6	—	8.5	nr	nr	34
Bovine GH	2	6	—	8.5	nr	nr	34
Bovine GH	2	6	—	8	80	RT	59
Eel GH	2	5	—	8	2	4	89
Human IL-2	1	8	DTT: 10	8.5	1	37	96
Human IL-2	1	7	—	7	1	4	49
Human IL-2	1	6	2-ME: 14	8	2	RT	95
Human IL-4	3	6	2-ME: 14	8	2	RT	57
Human IL-4	3	5	GSH: 2 GSSG: 0.2	8	1	RT	53
Human IL-6	2	6	—	8.3	nr	nr	103
Porcine phospholipase A_2	7	6	Na_2SO_3: 300	8	0.3	4	9
M-CSF	9	7	2-ME: 25	7.5	4	RT	102
Human angiogenine	3	7	2-ME: 100	7.5	nr	nr	23
TGF-α	3	7	—	nr	nr	nr	101
IGF-I	3	6	2-ME: 10	nr	nr	nr	76

GH, growth hormone; T-PA, tissue-type plasminogen activator; IL, interleukin; M-CSF, macrophage-colony stimulating factor; TGF-α, transforming growth factor-α; IGF, insulin-like growth factor; DTT, dithiothreitol; DTE, dithioerythritol; 2-ME, 2-mercaptoethanol; DTP, dithiopropanol; GSH, reduced glutathione; GSSG, oxidized glutathione; RT, room temperature; nr, not reported.

Table III. Solubilization of polypeptides from inclustion bodies by urea.

Protein	Number of disulfide bonds in active protein	Urea concentration (mol/L)	Reducing reagent (mmol/L)	pH	Period of solubilization (h)	Temperature (°C)	Refs.
M-CSF	9	8	DTT: 10	nr	0.5	RT	38
Prochymosin	3	8	—	8	1	RT	64
Prochymosin	3	7.5	—	7.5	nr	nr	87, 90
Goat α-lactalbumin	3	8	DTT: 1	7.5	nr	nr	58
T-PA	17	8	—	8.5	nr	nr	40
Peroxidase C	4	8	DTT: 30	nr	1	30	86
EGF	3	8	DTT: 1	8.3	nr	nr	4
Immunoglobulin chain	15	7	DTT: 2	8	nr	nr	11
Immunoglobulin fragment	2	9	2-ME: 20	10.8	nr	nr	10
Chicken lysozyme	4	8	2-ME: 500	8.6	2	40	43
Bovine pancreatic RNase	4	8	2-ME: 25	8	1	nr	67
Salmon GH	2	7	—	8	nr	nr	84

M-CSF, macrophage-colony stimulating factor; T-PA, tissue-type plasminogen activator; EGF, epidermal growth factor; GH, growth horman; RNase, ribonuclease; RT, room temperature; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; nr, not reported.

RENATURATION AND REOXIDATION OF SOLUBILIZED POLYPEPTIDES

After solubilization of inclusion bodies, the polypeptides are completely denatured and usually reduced. To obtain the native conformation and to generate correct disulfide bond formation, excess denaturant and reducing reagents have to be removed, and the polypeptide changed into a buffer under oxidizing conditions. The most frequently

Table IV. Alternative methods of polypeptide isolation from inclusion bodies.

Protein	Number of disulfide bonds in active protein	Method of solubilization	Refs.
Human IL-1β	1	Extraction at acidic pH	55
Human IL-2	1	Incubation with Q-Sepharose	42
Human renin	2	Repeated vortex mixing	83
Bovine GH	2	Solubilization at pH 12	34

GH, growth hormone; IL, interleukin.

used way to reduce the concentration of denaturant and reducing reagents is to dilute the polypeptide directly into the refolding buffer.[4,12,21,23,38,40,42,53,64,70,73,74,100,102] It is also possible to dialyse the protein against different buffers[11,30,35,74,76,89,99] or to remove the chemicals by gel filtration.[86] Starting the refolding by dilution changes the solvent properties surrounding the denatured protein instantly. This makes the process more kinetically dependent. By contrast, dialysis of the denatured protein against decreasing denaturant concentrations allows the transformation to approach equilibrium conditions.

Ideally, the renaturation procedures employed both in research and industrial applications would use high protein concentrations. However, a common observation from renaturation experiments of nonrecombinant proteins has been that the final yield of renatured protein decreases, sometimes dramatically, with increasing concentrations of solubilized protein undergoing renaturation; concomitantly, the percentage of insoluble inactive protein aggregates increases.[71,93] This is due to the formation of unstable intermediate products.[25,75,79] Caused by hydrophobic interactions between the normally inaccessible core residues that become exposed on the surface of folding intermediates[75] aggregation occurs. As a result, refolding of nonrecombinant proteins has been performed at a low protein concentration of about 10^{-6} mol/L corresponding to about 1 to 20 μg/mL.[25,36,39,47,61,62,71,79,85,88,93] Although the renaturation of recombinant proteins is dependent on protein concentration, this has not been documented in publications to the same degree as has been done with purified natural proteins. It is likely though, that at these low protein concentrations the final yield of renatured active protein will be increased.[12,13,27,30,43,60,73,74,78]

Most successful methods for renaturation/reoxidation of recombinant eukaryotic proteins, i.e., air oxidation, the glutathione renaturation system, and the reoxidation starting from mixed disulfides, have been used. In the air oxidation system the reduced cysteine residues are oxidized by oxygen and disulfide bonds are formed. Table V shows a summary of different conditions used for the reoxidation of recombinant proteins by this method. Process control during air oxidation should include dissolved oxygen control and precise control over oxidation catalyst concentration. However, no studies have been reported in which an attempt was made to specifically control these variables.

In the glutathione reoxidation system the formation of the disulfide bonds is generated by the redox-couple of reduced and oxidized glutathione. By varying the ratio of reduced to oxidized glutathione in relation to the concentration of the protein-cysteine residues, the optimal refolding conditions for each polypeptide can be determined (Table VI). This makes the glutathione system more flexible in comparison with the air oxidation system. To prevent air oxidation in the glutathione system, the buffer solution must be deaerated and kept under an inert gas such as nitrogen. Tables V and VI show that in both renaturation systems the reoxidation is performed mainly between pH values of 8 and 9. The temperature during renaturation and the period of reoxidation varies considerably. The ratio of reduced and oxidized glutathione used varies from 10:1 to 1:1 at concentrations of reduced glutathione of about 10^{-3} mol/L.

Table V. Renaturation and reoxidation of recombinant proteins isolated from inclusion bodies by air oxidation.

Protein	Number of disulfide bonds to be formed	Addition	pH	Temperature (°C)	Time (h)	Refs.
Human IFG-I	3		9.0	37	16	77
Creatine kinase	2		nr	4	12	8
Human serum albumin	17		8.5	4	24	60
Prochymosin[a]	3	1 mol/L urea	10.7	nr	0.5	64
Human IL-6[a]	2	6 mol/L GnHCl	8.5	nr	nr	103
Human IL-2	1	1.5 μmol/L Cu_2SO_4/0.6 mol/L GnHCl	8.5	20	3	96
Angiogenine	3		8.5	4	24	23
Bovine GH[a]	2	6 mol/L GnHCl	8.0	20	20	59
Pro-urokinase	12	2.5 mol/L urea	8.0	15	24	70

IGF, insulin-like growth factor; IL, interleukin; GH, growth hormone; GnHCl, guanidine hydrochloride; nr, not reported.
[a] Isolation from inclusion bodies without addition of a reducing reagent.

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