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Convenient and Efficient *In Vitro* Folding of Disulfide-Containing Globular Protein from Crude Bacterial Inclusion Bodies[1]

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We investigated how the folding yield of disulfide-containing globular proteins having positive net charges from crude bacterial inclusion bodies was affected by additives in the folding buffer. In screening folding conditions for human ribonucleases and its derivative, we found that addition of salt (about 0.4 M) to a folding buffer increased the folding yield. This suggested that electrostatic interaction between polyanionic impurities such as nucleic acids and cationic unfolded protein led to the formation of aggregates under the low-salt conditions. Since inclusion bodies were found to contain nucleic acids regardless of the electrostatic nature of the expressed protein, the electrostatic interaction between phosphate moieties of nucleic acids and basic amino acid residues of a denatured protein may be large enough to cause aggregation, and therefore the addition of salt in a folding buffer may generally be useful for promotion of protein folding from crude inclusion bodies. We further systematically investigated additives such as glycerol, guanidium chloride, and urea that are known to act as chemical chaperons, and found that these additives, together with salt, synergistically improved folding yield. This study, suggesting that the addition of salt into the folding buffer is one of the crucial points to be considered, may pave the way for a systematic investigation of the folding conditions of disulfide-containing foreign proteins from crude bacterial inclusion bodies.

Key words: chemical chaperon, electrostatic interaction, folding, inclusion body, RNase.

In producing recombinant protein, an *Escherichia coli* expression system is often chosen first because it is simple and provides extremely high yields (*1*). When the protein accumulates as inclusion bodies in the host cell, however, it is often troublesome to obtain in the properly folded form. The reduced and unfolded form of the expressed protein must be folded correctly so as to allow the formation of the native disulfide bonds. During the folding of many proteins, the aggregation of denatured protein increases, while the recovery of the properly folded protein decreases. The efficiency of protein folding *in vitro* depends on the result of competition between "folding" and "aggregation" (*2*). Therefore, the folding yield depends strongly on the folding conditions used.

Optimum conditions for folding have been reported for specific cases, and these results suggest that intermolecular non-specific hydrophobic interaction in the unfolded state is the main cause of aggregation of denatured protein (*3*). This hydrophobic interaction can be decreased by addition of a denaturant such as urea and guanidium chloride (GdmCl), although high concentration of denaturant prevents the correct folding by rendering the folded protein unstable. A high concentration of carbohydrate such as glycerol is also known to improve the folding yield by preventing the formation of precipitates, because it assists preferential hydration and leads to the net stabilization of the native structure of globular protein (*4–7*). Buchner *et al.* reported that high concentration (~0.5 M) of L-arginine improves the folding yield by suppressing aggregation (*8*).

All these strategies may be useful to select the folding conditions, but we also have to consider the large amounts of impurities that are co-precipitated from cells in the inclusion body preparations. Since impurity-containing proteins, inclusion bodies, often fail to fold but give precipitates, these impurities are usually removed prior to folding by using gel chromatography or ultrafiltration after solubilization of inclusion bodies in urea or GdmCl solution.

Here we show a convenient method to improve the yield of properly folded protein from crude inclusion bodies without purification before folding. We achieved optimal folding by addition of ~0.4 M salt, 30% glycerol, and denaturants to a redox buffer for the folding of human RNase 1 (*9*), human RNase 4 (*10*, *11*) and human RNase 1–basic FGF fused protein (*12*) from crude inclusion bodies. All of these proteins are monomeric globular proteins that form four intramolecular disulfide bonds in the native state. We found that the most important factor causing aggregates during the folding is the electrostatic interaction between

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unfolded protein and anionic contaminants such as nucleic acids contained in the inclusion bodies. From these results, we propose mechanisms for protein aggregation *in vivo* and *in vitro* and a simple strategy for folding of small globular protein from crude inclusion bodies.

MATERIALS AND METHODS

Materials—*E. coli* strains BL21(DE3)/pLysS (*13*) and MM294(DE3)/pLysS (*14*) were described previously. The bacterial cells expressing recombinant human RNase 1, MM294(DE3)/pLysS/pBO26 (*9*); human RNase 4, BL21-(DE3)/pLysS/pBO72 (*10, 11*), human RNase 1 and human basic FGF fused protein (RNase-FGF fused protein), MM294(DE3)/pLysS/pBO67 (*12*), and human His-cripto 1, BL21(DE3)/pLysS/pBO168 (*15*), were also described previously. Production of recombinant proteins in these transformants is regulated by the T7 expression system. Cell culture supplies were from Difco. Luria-Bertain (LB) medium contained (in 1 liter) tryptone (10 g), yeast extract (5 g), and NaCl (5 g). Bovine RNase A (type II-A) and DNase (DN-25) were purchased from Sigma.

Construction of Plasmid—The plasmid pBO234 for expression of human RNase inhibitor was generated by ligation of the open reading frame cut out from pBO145 (*16*) by restriction endonuclease *Sal*I/*Hin*dIII together with pair of linker DNAs (#124, 5'-ATGGTATATCTCCTTCTTAAAGTTAAACAAAATTATTT-3', and #125, 5'-CTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGAGC-3') into the *Hin*dIII/*Xba*I cloning site in the pET23a bacterial expression vector (Novagen). Using the resulting plasmid, transformant *E. coli* MM294(DE3)/pLysS/pBO236 was prepared for recombinant RNase inhibitor expression.

Expression of Recombinant Proteins—LB containing ampicillin (150 μg/ml) and chloramphenicol (10 μg/ml) was inoculated with a culture of each bacterial strain frozen in aqueous glycerol (20% v/v). After growth overnight at 37°C, culture was diluted 50 times with LB containing ampicillin (50 μg/ml), then incubated with vigorous shaking at 37°C. When the A_{600} of the culture reached 0.6–1.0, isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 0.4 mM and incubation was continued for 3 h. Bacterial cells were collected by centrifugation, washed once with saline (1.5% NaCl), and stored at −80°C until use.

Isolation of Inclusion Bodies—The frozen cells obtained from 1 liter of culture were suspended in 50 ml of lysis buffer comprising 10 mM Tris-HCl, pH 7.5, containing 10% sucrose and 0.2 M NaCl. The suspension was homogenized with a homogenizer (LK-21, Yamato, Japan), followed by freezing at −80°C and thawing at 37°C for complete lysis. The suspension was extensively sonicated, then centrifuged at 10,000 ×*g* for 10 min. The pellet was washed with 50 ml of 0.5% Triton X-100 containing 1 mM EDTA and 0.2 M NaCl, then centrifuged at 10,000 ×*g* for 10 min. The resulting pellet was designated as crude inclusion bodies.

Folding Assay—Solubilization of the crude inclusion bodies was achieved with 8 M urea solution containing 0.2 M Tris-HCl, pH 8.5, and the proteins were reduced with 0.1 M β-mercaptoethanol at 37°C for 90 min under a N_2 atmosphere. The folding was initiated by rapid dilution of this denatured and reduced protein to a concentration of 100 μg/ml with the folding buffer consisting of 30 mM Tris-HCl, pH 8.5, 0.5 mM oxidized glutathione (GSSG), 3 mM reduced glutathione/β-mercaptoethanol (-SH), with or without 30% (v/v) glycerol, various concentrations of salts, GdmCl, and urea. The mixture was then incubated for 24 h at room temperature. The once-purified and desalted RNase-FGF fused protein (*12*) was also denatured, reduced, and subjected to folding under the same conditions. The protein concentration of an inclusion body solution was determined by the method of Bradford (*17*), using the corresponding purified and reduced protein as a standard.

Determination of Folding Yield—The yield of folded RNase molecules was determined by measuring regenerated RNase activity using yeast RNA as a substrate according to the method of Kunitz (*18*) with some modifications. The assay was initiated by adding 5–50 μl of folded sample into 1.5 ml of substrate solution containing 0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA, and 0.06% of yeast RNA (type IV, Sigma, USA; once purified by ethanol precipitation) in a 1-cm light path quartz cell. The RNase activity was monitored by decrease in absorbance at 300 nm due to transphosphorylation with a spectrophotometer (Hitachi U-2000, Tokyo) at 25°C, and the RNase activity was determined from its initial velocity.

Analysis of Nucleic Acid Contaminants in Inclusion Bodies—Three kinds of variously purified inclusion bodies were prepared as follows. A bacterial cell suspension in buffer A consisting of 10 mM Tris-HCl buffer, pH 7.5, 10% sucrose, 0.15 M NaCl, and 10 mM $MgCl_2$ was lysed by freezing at −80°C and thawing at 37°C. The pellet obtained by centrifugation at this stage was designated as sample 1. The lysed cell suspension (10 ml) was further sonicated three times for 1 min each on ice by use of a TAITEC VP-5s sonicator (Japan). The pellet precipitated by centrifugation at this stage was designated as sample 2. The sonicated suspension was then treated with 2 μg/ml of DNase and 5 μg/ml of RNase A for 15 min at 37°C, and the pellet precipitated by centrifugation at this stage was designated as sample 3. Each pellet of inclusion bodies thus prepared from 15 ml of bacterial cell culture was dissolved in 0.3 ml of 8 M urea solution containing 0.2 M Tris-HCl, pH 8.5, and 1% β-mercaptoethanol, then mixed well with 0.3 ml of phenol/chloroform (1:1) and 0.9 ml of distilled water, and centrifuged at 10,000 ×*g* for 10 min at 4°C. Nucleic acids extracted in the aqueous phase were precipitated with ethanol and dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, then divided into halves. One half was treated with RNase A to hydrolyze the RNA component. Nucleic acids in each half were analyzed by 2% agarose gel electrophoresis.

RESULTS

Folding of RNase-FGF Fused Protein—In the early part of our study, we found that the folding yield of RNase-FGF fused protein from crude inclusion bodies was dramatically improved when the folding was carried out in the presence of rather high concentration of salt (Fig. 1A). On the other hand, the highly purified RNase-FGF fused protein was always efficiently folded regardless of the concentration of salt (Fig. 1B). Since the concentration dependency for Na_2SO_4 is different from those for the other salts, the folding yields were re-plotted against the ionic strength and the concentration of cation, respectively (Fig. 1, C and D). The folding yield was clearly correlated with the concentra-

tion of cation rather than ionic strength and reached the maximum at 0.4 M regardless of the kind of cation. Na^+, K^+, NH_4^+, and Arg^+ showed almost equal effects on the folding yield. Gdm^+ was about 1.5 times more effective than other cations. This may be explained by the additional effect of GdmCl as a denaturant as well as a salt, which decreases unfavorable intermolecular non-specific hydrophobic interactions and reduces the formation of precipitates of denatured protein during the folding. Since most RNase-FGF fused protein failed to fold from crude inclusion bodies under the low-salt conditions, and since highly purified RNase-FGF fused protein always folded in good yield, salt may act to prevent the electrostatic interaction that leads to unfavorable aggregation between the denatured RNase-FGF fused protein and polyanionic impurities, such as nucleic acids, which had been co-precipitated with the expressed protein in the inclusion bodies.

Analysis of Nucleic Acid Contaminants Contained in Various Inclusion Body Preparations—Nucleic acids were extracted from each of the three kinds of inclusion body preparations obtained for RNase-FGF fused protein, human RNase 1, RNase 4, His-cripto 1, and RNase inhibitor and analyzed by agarose gel electrophoresis (Fig. 2, lanes 7–9 in each panel). The components of nucleic acids were estimated by the sensitivity to RNase A digestion of the nucleic acids extracted from each inclusion body preparation (Fig. 2, lanes 11–13). Figure 2 also shows SDS-PAGE analysis of proteins of each inclusion body preparation (lanes 3–5). The first preparation was that after lysis by freezing and thawing (Fig. 2, lanes 3, 7, and 11), the second was that after further extensive sonication (Fig. 2, lanes 4, 8, and 12), and the third was that after further treatment of DNase and RNase A (Fig. 2, lanes 5, 9, and 13).

The nucleic acids originally associated with inclusion bodies were mostly RNA for RNase-FGF fused protein, human RNase 1, and His-cripto 1, but both DNA and RNA for RNase 4 and RNase inhibitor. Although the nucleic acid content decreased with each step of the three-step preparation of inclusion bodies, the protein content and composition were not greatly changed (about 90% purity) judged from SDS-PAGE analysis. Most important of all, low-molecular-weight nucleic acids always remained in the third inclusion body preparation, which was extensively digested with nucleases (Fig. 2, lane 9), regardless of the size and electrostatic nature of the expressed protein (Table I). Consequently, it is concluded that inclusion bodies contain nucleic acids that are tightly bound to the expressed unfolded protein.

Folding of RNase 1 and RNase 4—Based on the analysis for folding of RNase-FGF fused protein (Fig. 1), we also

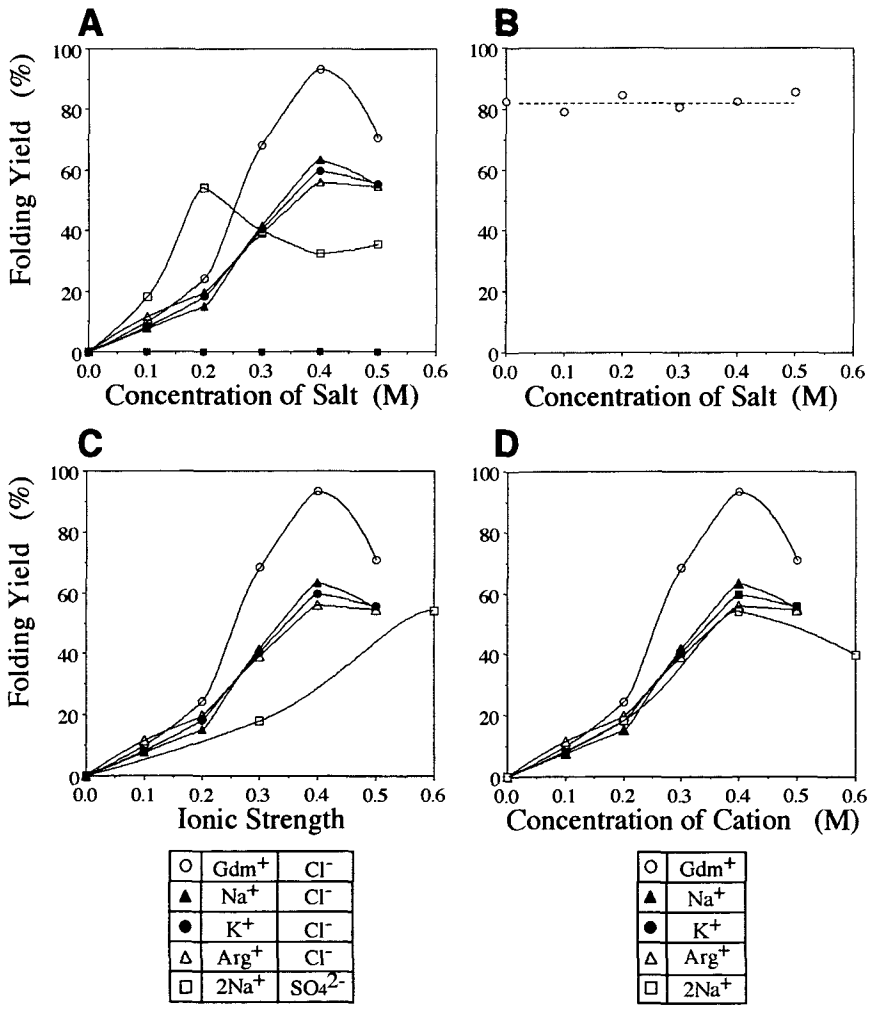

Fig. 1. **Folding of RNase-FGF fused protein from crude inclusion bodies (A) and from once completely purified protein (B).** All redox folding buffers consisted of 3 mM –SH/0.5 mM GSSG, 30 mM Tris-HCl, pH 8.5, and 30% glycerol. The salts added to the folding buffer were GdmCl (open circles), NaCl (closed triangles), KCl (closed circles), Na_2SO_4 (open squares), L-arginine-HCl (open triangles), and glycine (closed squares). The results of (A) were replotted against ionic strength (C) and concentration of cation (D).

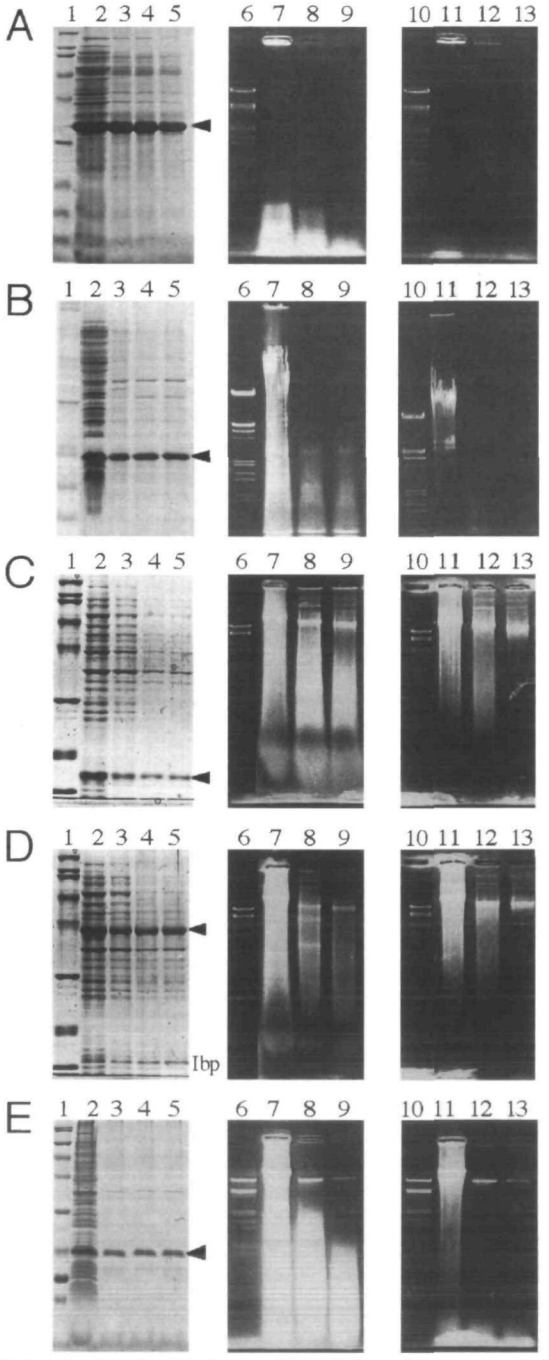

Fig. 2. **Analysis of proteins and nucleic acids co-precipitated in the inclusion bodies for RNase-FGF fused protein (A), RNase 1 (B), RNase 4 (C), RNase inhibitor (D), and His-cripto 1 (E).** The left panels show SDS-PAGE analysis of proteins. Arrows indicate the positions of expressed proteins. The band indicated by Ibp in (D) was inclusion body-binding protein A/B (SWISS-PROT P29209, P29210) judged from the result of amino terminal sequence analysis. The middle panels show agarose gel electrophoretic analyses of total nucleic acids extracted from the respective inclusion body preparations, and the right panels show those of remaining nucleic acids after RNase A treatment of the extracted nucleic acids. Lane 1 is broad range protein molecular weight maker (Bio-Rad) and lanes 6 and 10 are lamda phage DNA digested by *Hin*dIII and *Eco*RI. Lane 2 is total cellular protein. Proteins or nucleic acids contained in the three kinds of inclusion body preparations are analyzed in lanes 3, 7, and 11 for sample 1, lanes 4, 8, and 12 for sample 2, and lanes 5, 9, and 13 for sample 3. See "MATERIALS AND METHODS" for details.

attempted to fold RNase 1 and RNase 4, both basic proteins, from crude inclusion bodies (Fig. 3A). The folding yield was improved in a salt concentration–dependent manner, and the folding conditions optimized for RNase-FGF fused protein were also optimal for RNase 1. On the other hand, urea was found to be very important for the folding of RNase 4, but not favorable for RNase 1, although the presence of salt was equally important for both of them (Fig. 3B). Furthermore GdmCl did not promote the folding of RNase 4 at all (data not shown).

Estimation of Contributions of Individual Additives to the Folding—The combined effects of additives on the folding yields of RNase-FGF fused protein, RNase 1, and RNase 4 from crude inclusion bodies were examined in terms of: (i) reduction of "electrostatic interaction" between unfolded protein and nucleic acid contaminants by addition of 0.4 M salt, either NaCl or GdmCl; (ii) reduction of intermolecular non-specific "hydrophobic interaction" between unfolded proteins by addition of denaturant, either 0.4 M GdmCl or 2 M urea; and (iii) assisting "preferential hydration" by addition of 30% glycerol (Fig. 4). The disruption of

TABLE I. **Properties of recombinant RNase-FGF, RNase 1, RNase 4, RNase inhibitor, and His-Cripto 1.**[a]

Molecule	Number of amino acids	M.W. (kDa)	Isoelectric point (native/reduced)	Residues in native molecule (S-S/-SH)
RNase-FGF	276	31.2	9.8/9.4	4/4
RNase 1	129	14.7	9.8/9.0	4/4
RNase 4	120	14.0	10.3/9.2	4/0
RNase inhibitor	455	49.3	4.7/4.7	0/32
His-Cripto 1	172	19.3	–/8.6[b]	–/13[b]

[a]Details are described in the text. [b]Native structure is not known.

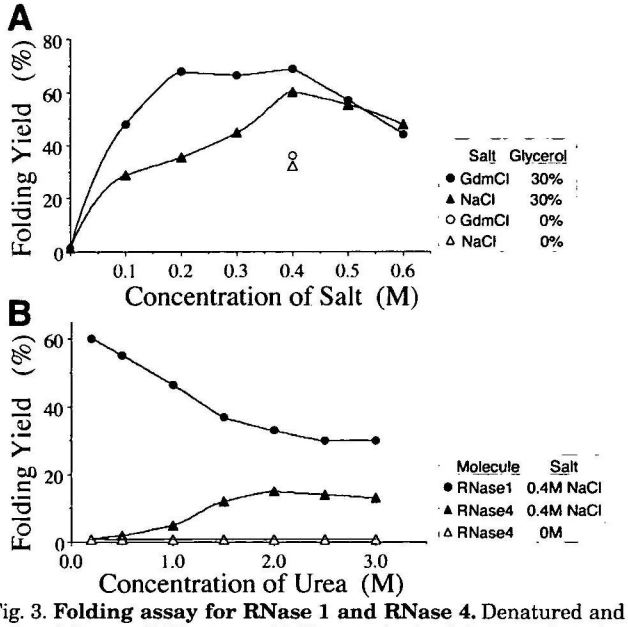

Fig. 3. **Folding assay for RNase 1 and RNase 4.** Denatured and reduced forms of RNase 1 and RNase 4 obtained from crude inclusion bodies were rapidly diluted into redox folding buffer (3 mM –SH/0.5 mM GSSG at pH 8.5) at a protein concentration of 100 μg/ml. Effects of salt on the folding of RNase 1 were assessed by addition of various concentrations of GdmCl or NaCl with or without 30% glycerol (A). Effect of urea on the folding of RNase 1 or RNase 4 in the presence of 30% glycerol with or without 0.4 M NaCl (B).

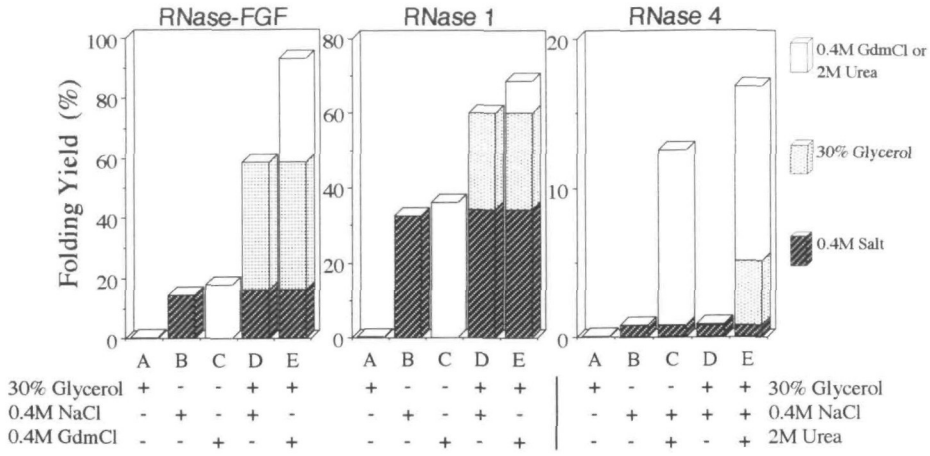

Fig. 4. **Contributions of additives to the folding of RNase-FGF fused protein, RNase 1, and RNase 4.** Five typical folding conditions were compared in terms of effects of 0.4 M salt (NaCl or GdmCl), denaturant (0.4 M GdmCl or 2 M urea), and 30% glycerol, respectively. GdmCl is a salt as well as denaturant.

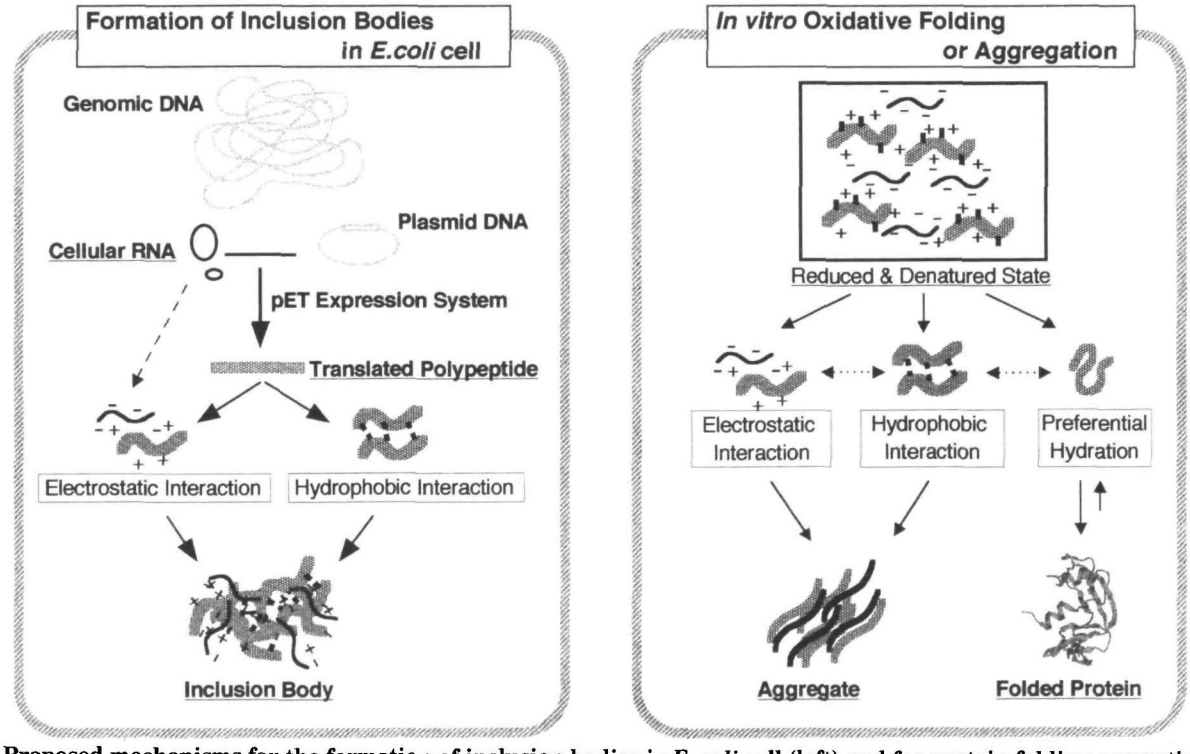

Fig. 5. **Proposed mechanisms for the formation of inclusion bodies in *E. coli* cell (left) and for protein folding competing with aggregation during the folding reaction of inclusion bodies *in vitro* (right).**

"electrostatic interaction" was found to be essential for all proteins. As for denaturants which decrease "hydrophobic interaction", RNase-FGF fused protein and RNase 1 preferred GdmCl, but RNase 4 preferred urea (Fig. 3). Addition of glycerol alone did not appreciably assist the folding of the proteins. However, it appeared to strengthen the improving effects of salt and denaturant. Although the effect of 30% glycerol is unclear at the molecular level, it would assist the folding of protein by shifting the equilibrium away from denatured state and toward the native state by promoting unfavorable "preferential hydration" around the denatured protein molecule (*4–7*), and this effect would become evident only when the "electrostatic interaction" and "hydrophobic interaction" are successfully decreased.

DISCUSSION

Most heterologous secretory proteins expressed in *E. coli* accumulate as inclusion bodies. To obtain the recombinant protein in an active soluble form, attempts have been made to secrete these proteins into the periplasm with the help of a signal peptide of a membrane or secretory protein. So far, such attempts have met with only limited success due to the complex nature of heterologous proteins and their secretory mechanism (*19*). The formation of inclusion bodies offers several advantages: the expressed protein is less likely to be toxic toward the host cells and is itself protected from proteolysis by bacterial proteases. However, the folding of the protein into the native conformation from inclu-

sion bodies is often technically difficult. Since other cell components included in inclusion bodies often interfere with folding of the expressed proteins, the inclusion bodies are usually solubilized by use of denaturants, and the proteins are partially purified by chromatographic procedures before folding (*19*). It may be convenient if the protein can be folded from inclusion bodies without tedious chromatographic purification procedures. To facilitate the folding of proteins from inclusion bodies, it is necessary to understand how inclusion bodies are formed *in vivo* and why a particular procedure for folding of proteins from inclusion bodies is apt to lead to aggregation instead of renaturation *in vitro*.

We have addressed these questions here and suggest the mechanisms for the formation of inclusion bodies in *E. coli* cells *in vivo* and for the folding and/or aggregation *in vitro* as summarized in Fig. 5. In the mechanisms, aggregation due to electrostatic interaction between nucleic acids and unfolded protein is newly proposed. Inclusion body pellets are known to contain varying proportions of impurities derived from the host, such as proteins, nucleic acids, and lipids. Since the population of impurities is considered to vary depending on the isolation conditions of inclusion bodies, we employed several isolation methods and analyzed the nucleic acids included in each inclusion body preparation. Most of the nucleic acid contaminants were found to be decreased by extensive sonication, nuclease treatment (Fig. 2), and washing with a solution of rather high concentration of salt (data not shown). These results suggest that most nucleic acid contaminants are deposited on the surface of insoluble particles during the isolation procedure of inclusion bodies, and can be removed by the above treatments (Fig. 2). However, some portion of the nucleic acids could not be removed by these treatments in all cases, including that of RNase inhibitor, an acidic protein (Fig. 2 and Table I). These nucleic acids appear to be tightly bound to the inclusion bodies, and they might be tangled with expressed proteins, probably due to electrostatic interaction between the negative charges of their phosphate moieties and the positive charges of basic amino acid residues of the denatured protein, regardless of whether the net charge of expressed protein is positive or negative, during the formation of inclusion bodies in *E. coli* cytoplasm (Fig. 5).

Proteins and impurities included in inclusion bodies were able to dissociate and enter solution in the presence of strong denaturants, and therefore proteins were usually purified at this stage by using chromatographic procedure (*19*). In the cases of RNase 1 and RNase-FGF fused protein, however, they were able to be folded in good yields without such a purification step from the crude inclusion bodies if the folding was simply carried out in the presence of 0.4 M salt. On the other hand, the folding yield of RNase 4 from crude inclusion bodies was only about 18% even in the presence of 0.4 M NaCl as well as 2 M urea and 30% glycerol, although it was less than 1% in the absence of salt (Fig. 3). However, folding after purification by use of TAPS-sulfonate [Wako Chemicals, Osaka (*15*, *20*)], gave about 50% yield of the folded RNase 4 under similar conditions (*11*). These observations suggest that host cell–derived impurities other than nucleic acids also affect the protein folding to some extent. Some proteins may be still difficult to fold from crude inclusion bodies, and in this case, it would be better to purify the protein before folding.

In conclusion, we have demonstrated that protein folding from crude inclusion bodies can be synergistically improved by addition of three types of additives, denaturant, glycerol, and salt as chemical chaperons. The addition of salt, newly proposed here, may enhance the folding efficiency of other small globular secretory proteins containing disulfide bonds from inclusion bodies expressed in *E. coli*, and decrease the time and cost of purification.

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