Chapter 10

Characterization and Refolding of β-Lactamase Inclusion Bodies in *Escherichia coli*

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R_{TEM} β-lactamase was overexpressed in *E. coli* from three different plasmids resulting in the formation of cytoplasmic (plasmid pGB1) and periplasmic (plasmids pKN and pJG108) inclusion bodies. Previous work demonstrated that the inclusion bodies differ in structure and composition according to the cellular compartment in which aggregation occurred (*17*). In this study, we used inclusion bodies purified by sucrose density gradient centrifugation to investigate the effect of the *in vivo* aggregation environment on the properties of the protein within the inclusion bodies. Guanidine hydrochloride and pH solubilization experiments revealed important differences in the interactions involved in the stabilization of the aggregates. In addition, trypsin digestion results suggested a less ordered protein conformation in periplasmic inclusion bodies. The influence of the inclusion body origin on the renaturation of active protein was investigated in detail. The highest recovery was achieved with periplasmic inclusion bodies from RB791(pJG108). The yield of active β-lactamase upon refolding the material obtained from solubilized inclusion bodies was between 20% and 40% of that obtained from the renaturation of the purified protein under identical conditions. Our results suggest that the presence of certain contaminants in the inclusion bodies enhance the reaggregation of the protein during the removal of the denaturant.

High levels of expression of a cloned gene often results in the accumulation of misfolded recombinant protein into large, amorphous aggregates called inclusion bodies. Because of the complexity of intracellular events, very little is known about the mechanism of formation of inclusion bodies, but many growth parameters have been shown to affect *in vivo* aggregation. A high level of expression, and therefore high intracellular protein concentration, enhances aggregation (*1, 2*), whereas low growth temperature favors the production of recombinant proteins in a soluble form (*2-4*). The addition of non-metabolizable sugars, such as sucrose, to the growth medium has been shown to inhibit the formation of β-lactamase periplasmic inclusion bodies in *E. coli* (*1, 17*). Analogous effects of temperature, protein concentration and cosolvents have been

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observed during the *in vitro* renaturation of purified protein from denaturant solutions (*5-8*). *In vitro* aggregation has been shown to result from the intermolecular interaction of exposed hydrophobic surfaces of a folding intermediate (*9, 10*). Based on analogies with *in vitro* results and recent direct *in vivo* experimental evidence, Mitraki and King suggested that the formation of inclusion bodies follows an similar mechanism (*11, 12*).

Very little information about the conformation of the peptide chains within the aggregates is available. Recent evidence suggests that the protein can be in either a completely misfolded or an active conformation (*13*). In most cases, the recovery of active, correctly folded protein from inclusion bodies involves the complete solubilization of the aggregates under strong denaturing conditions followed by refolding of the protein by dilution or dialysis (*14, 15*). Inhibition of reaggregation during the renaturation step requires careful optimization of the refolding conditions. The properties of the inclusion bodies themselves, such as density, structure and composition, must be taken into consideration for the design of an efficient recovery procedure.

The *E. coli* β-lactamase is a good model protein for the study of both *in vivo* and *in vitro* folding and aggregation (*18*). Overexpression of this protein in *E. coli* from different plasmids has been shown to result in the formation of periplasmic (*1, 16, 17*) or cytoplasmic (*18*) inclusion bodies. The structure and protein composition of β-lactamase inclusion bodies have been shown to depend on the intracellular compartment in which aggregation occurs (*18*). The influence of several growth conditions, such as temperature (*4*), expression level and presence of non-metabolizable sugars in the medium (*1, 17*) on the formation of the periplasmic inclusion bodies have been investigated. Furthermore, the effects of protein concentration, sucrose concentration and temperature on the *in vitro* refolding of pure β-lactamase from denaturant solutions have been shown to be in good agreement with *in vivo* observations (*8*). In this study, experiments were designed to assess differences in aggregated protein conformation among the various types of inclusion bodies. The effect of the origin of the aggregated material on the efficiency of functional protein recovery upon refolding was also investigated.

Materials and Methods.

Materials. Guanidine hydrochloride (GuHCl) was purchased from International Biotechnologies Inc. (New Haven, CT). Dithiothreitol (DTT) and sucrose were purchased from Sigma.

Cell Growth and Inclusion Body Purification. R_{TEM} β-lactamase was produced in *E. coli* RB791 (*22*) harboring the plasmids pGB1(*18*), pJG108 and pKN (*23-24*). pGB1 expresses the protein from a *tac* promoter and contains a deletion of the region encoding the -20 to -1 amino acids of the signal sequence. In pJG108, the native signal sequence of β-lactamase was replaced by the signal sequence of the outer membrane protein A (Omp A). pKN expresses native β-lactamase from a *tac* promoter and contains a kanamycin resistance gene.

Cultures were grown at 37°C in M9 medium supplemented with 0.2% glucose and 0.2% casein. The cultures were induced with isopropylthiolgalactoside (10^{-4} M final concentration) at an optical density ($O.D._{600}$) between 0.35 and 0.4. After overnight growth, the cells were harvested by centrifugation (8,000xg for 10 min). For all experiments, the inclusion bodies were isolated from cell lysates by isopycnic sucrose gradient centrifugation as follows: cells from 50 ml of culture were resuspended in 1 ml of 10 mM Tris-HCl, pH 8.0 containing 0.75 M sucrose and 0.2 mg/ml lysozyme. After 10 min incubation at room temperature, 2 ml of ice cold 3 mM

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EDTA solution were added. The cells were then lysed in a French press at 20,000 psia. The lysates were subsequently centrifuged at 12,000xg for 30 min and the pellets, which contained the inclusion bodies, were resuspended in 1.25ml of 10 mM Tris-HCl buffer, pH 8.0, containing 0.25 M sucrose 1 mM EDTA and 0.1% sodium azide. The resuspended pellet material was layered on the top of a sucrose step gradient (40%, 53% and 67% w/w) in 1 mM Tris-HCl buffer, pH 8.0, containing 0.1% sodium azide and 1mM EDTA and centrifuged at 108,000xg for 90 min. The inclusion bodies focussed in a band at the interface between the 53% and 67% sucrose layers which was recovered and resuspended in water. The suspension was subsequently centrifuged at 12,000xg for 30 min. The pelleted material was resuspended in 0.25 M sucrose solution and applied to a second sucrose gradient as above. After recovery from the second gradient, the inclusion bodies were washed with water, resuspended in 50 mM potassium phosphate, pH 7.0 containing 0.1% sodium azide and stored at 4°C.

Solubilization Experiments. The effect of GuHCl concentration on the solubilization properties of the inclusion bodies was determined as follows. A known amount of β-lactamase aggregates was dissolved in 50 mM potassium phosphate buffer, pH 7.0 containing 5 mM DTT and various concentrations of GuHCl. The samples were incubated for three hours at room temperature and centrifuged at 8,000xg for 20 min to precipitate any remaining aggregated material. The protein concentration in the supernatant was determined using the Bio Rad assay.

To determine the effect of buffer pH on solubilization, the inclusion bodies were equilibrated with 0.45 M GuHCl and 5 mM EDTA in the following buffers (*25*): for adjusting the pH between 2.0 and 6.0, buffers were obtained by mixing 0.1 M citric acid solution with a 0.2 M dibasic sodium phosphate solution to specific ratios; buffers with pH values between 6.0 and 7.5 were obtained by mixing a 0.2 M monobasic sodium phosphate solution with a 0.2 M dibasic sodium phosphate solution; 0.2 M Tris buffers were adjusted to pH values between 7.5 and 8.5 and 0.2 M glycine buffers to pH values above 8.5 by addition of concentrated sodium hydroxide. After three hours of incubation at room temperature, the samples were centrifuged at 8,000xg for 20 min and the concentration of solubilized protein in the supernatant was determined as above.

Trypsin Accessibility Experiments. Identical quantities of inclusion bodies were placed in eppendorf centrifuge tubes and dissolved in 50 mM potassium phosphate buffer, pH 7.0. Trypsin was then added to a final concentration of 0.1 mg/ml in all the tubes except the control. The samples were incubated at room temperature for various times. The digestion was then stopped by adding soybean trypsin inhibitor to a final concentration of 0.2 mg/ml. The remaining aggregated material was precipitated by centrifugation at 8,000xg for 20 min. The pellets were resuspended in 50 mM potassium phosphate buffer, pH 7.0, containing 3 M GuHCl and 5 mM DTT and incubated for three hours at room temperature to completely solubilize the aggregated polypeptides (Figure 2). The amount of soluble protein, which corresponds to the remaining undigested material, was measured using the Bio-Rad assay. The tube in which no trypsin was added was used as a reference (100% intact protein).

Refolding of Inclusion Body Protein. β-lactamase inclusion bodies were incubated for three hours at room temperature in 50 mM potassium phosphate buffer, pH 7.0 containing 5 mM DTT and various concentrations of GuHCl. The total protein concentration was either 5 mg/ml or 1 mg/ml as described in the text. The samples were subsequently dialysed against 50 mM potassium phosphate buffer, pH 6.0 for three hours at room temperature in a PIERCE Inc. model 500 microdialyzer apparatus. For all experiments, the final GuHCl concentration was 0.015 M. The remaining aggregated material was precipitated by centrifugation at 8,000xg for 20 min. The total protein concentration and the β-lactamase activity in the supernatant were measured.

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Finally, the purified inclusion bodies, the reaggregated protein and the soluble/refolded protein were loaded on a SDS polyacrylamide gel (15% acrylamide).

General Methods. Protein concentrations were measured using the Bio-Rad binding dye assay with bovine serum albumin as the standard. β-lactamase activities were determined spectrophotometrically using 0.5 g/l of penicillin G as the substrate (*19*). The dialysis tubing used in the microdialyzer apparatus was prepared by boiling in 2% sodium bicarbonate, 1 mM EDTA for 10 min, then boiling in 1 mM EDTA for another 10 min (*20*). The membrane was washed with distilled deionized water before and after each boiling. SDS-PAGE (15% acrylamide) was performed according to the method described by Laemmli (*21*). Prior to electrophoresis, the protein was denatured by boiling for 10 min in SDS electrophoresis buffer containing β-mercaptoethanol and bromophenol blue. The gels were stained with Coomassie brilliant blue.

Results and Discussion.

Inclusion Body Purification. Unlike the highly regulated cytoplasm, the periplasmic space of gram-negative bacteria is affected by the composition of the growth medium. Low molecular weight compounds (such as sucrose) can diffuse freely through the outer membrane and affect the folding and aggregation of secreted proteins (*1, 17*). To investigate the influence of the cellular environment on the formation and properties of inclusion bodies, β-lactamase was overexpressed from three different plasmids. In the plasmid pGB1, the signal sequence of β-lactamase has been deleted resulting in the expression of the mature protein preceded by the sequence Met-Arg-Ile. The absence of leader peptide prevents the translocation of the protein across the inner membrane and leads to the formation of cytoplasmic inclusion bodies consisting of the mature β-lactamase. Cells containing the plasmid pKN express β-lactamase with its native signal sequence from the *tac* promoter. Induction of the *tac* promoter results in the aggregation of mature β-lactamase in the periplasmic space. At high expression levels, a fraction of the precursor protein is unable to interact with the secretory apparatus fast enough and remains in the cytoplasm where it forms pre-β-lactamase inclusion bodies. In the plasmid pJG108, the native signal sequence of β-lactamase has been replaced by the leader peptide of outer membrane protein A. The pre-OmpA-β-lactamase gene is transcribed from the inducible lpp-lac promoter. Induction of protein synthesis leads to the formation of periplasmic inclusion bodies consisting of the mature β-lactamase

After overnight growth, the cells were harvested, converted to spheroplasts by treatment with lysozyme and EDTA and then lysed. The insoluble material from the lysates was then precipitated by low speed centrifugation. The pellets contain the protein aggregates along with other contaminants such as membrane debris, nucleic acids, etc. Removal of these contaminants is typically achieved by a series of extraction steps (*26, 18*). DNase I and lysozyme were used to degrade DNA and membrane fragments respectively. Membrane material and proteins adsorbed non-specifically on the surface of the inclusion bodies can be extracted by treatment with detergents such as deoxycholate and Triton X-100. This purification procedure, however, presents some major disadvantages. The detergents can also solubilize part of the inclusion body itself and therefore affect its properties and structure. The use of detergents can be circumvented by using sucrose density gradient centrifugation to separate the inclusion bodies from the cell debris (*18*). This method exploits the density difference between the protein particles and the other cellular components present in the lysates. The protein aggregates and the membrane debris form two distinct visible bands in the gradient. Silver stained polyacrylamide gels of the inclusion bodies did not reveal the presence of any of the major outer membrane proteins in the inclusion body fraction (*18*). Scanning electron micrographs of

cytoplasmic inclusion bodies from RB791(pGB1) purified by both methods are shown in Figure 1. Inclusion bodies purified by sucrose gradient centrifugation were morphologically different from those purified by detergent extraction. The former were more homogeneous and regular in shape. Therefore all inclusion bodies used in this study were purified by sucrose density gradient centrifugation.

Solubilization and Trypsin Accessibility Experiments. The inclusion bodies purified from the three plasmids exhibit different morphologies (*18*). Cytoplasmic inclusion bodies from RB791(pGB1) are highly regular, cylindrical aggregates with a homogeneous surface. Their size can exceed 1.5 μm. Periplasmic inclusion bodies from RB791(pJG108) and RB791(pKN) are small (0.5-1 μm), semi-spherical particles having smooth and rough surfaces. Considering such disparity in structure and surface characteristics, it is tempting to suggest that the conformation of the polypeptide chains within the aggregates and the strength of interchain associations depend on the cellular environment in which the inclusion bodies are formed.

The conformation of proteins in aqueous solvents is dictated by two types of interactions: hydrophobic interactions and electrostatic interactions which include ion pairing, hydrogen bonds and Van der Waals interactions (*27*). The strength of these interactions determines the overall stability of the protein tertiary structure. The intensity of these interactions depends in turn on the environment. The addition of denaturants such as GuHCl or urea to the solvent induces unfolding by weakening the hydrophobic interactions. Changes in pH, on the other hand, affect electrostatic interactions. The extent of inclusion body solubilization in the presence of GuHCl and at different pH values was studied in order to examine the strength of association between the protein molecules within the aggregates. The GuHCl solubilization profiles of the different types of inclusion bodies are shown in Figure 2. Cytoplasmic inclusion bodies from RB791(pGB1) could not be solubilized in the presence of up to 0.75 M GuHCl. At this denaturant concentration, inclusion bodies from RB791(pKN) and RB791(pJG108) experience substantial solubilization (25% and 50% respectively). Complete solubilization occurred at 2.5 M GuHCl, regardless of the origin of the inclusion bodies. These results suggest that the hydrophobic interactions stabilizing the associated protein chains within the cytoplasmic inclusion bodies are stronger than those found in inclusion bodies from RB791(pKN) and RB791(pJG108).

Figure 3 shows the solubilization profiles of the three types of inclusion bodies as a function of the buffer pH. The inclusion bodies were resuspended in buffers of different pH values containing 0.45 M GuHCl and 5 mM DTT. The experiment was conducted in the presence of a moderate concentration of GuHCl to amplify the effect of pH on the stability of the protein aggregates. While the aggregates from RB791(pGB1) show little solubilization over a wide pH range, the inclusion bodies from RB791(pJG108) and to a lesser extent from RB791(pKN) exhibit pH-dependent solubilization. The ionic strength of the buffer also appears to affect the extent of solubilization of the periplasmic inclusion bodies. At pH 7.0, in 50 mM potassium phosphate buffer, the extent of solubilization in 0.45 M GuHCl, 5 mM DTT was 10% for RB791(pKN) inclusion bodies and 30% for RB791(pJG108) aggregates (Figure 2). Under the same conditions but in 0.2 M sodium phosphate buffer, the extent of solubilization was 20% and 50% respectively (Figure 3). The sensitivity of the periplasmic inclusion bodies from RB791(pKN) and especially RB791(pJG108) to pH and ionic strength suggest that electrostatic interactions are involved in stabilizing the aggregate structure. On the other hand, the cytoplasmic inclusion bodies from RB791(pGB1) were not affected by the pH and ionic strength. Therefore it appears that hydrophobic interactions play a dominant role in protein association in the cytoplasm.

The susceptibility of proteins to proteolytic degradation depends on two factors: the presence of the sequences recognized by the protease and the steric accessibility of

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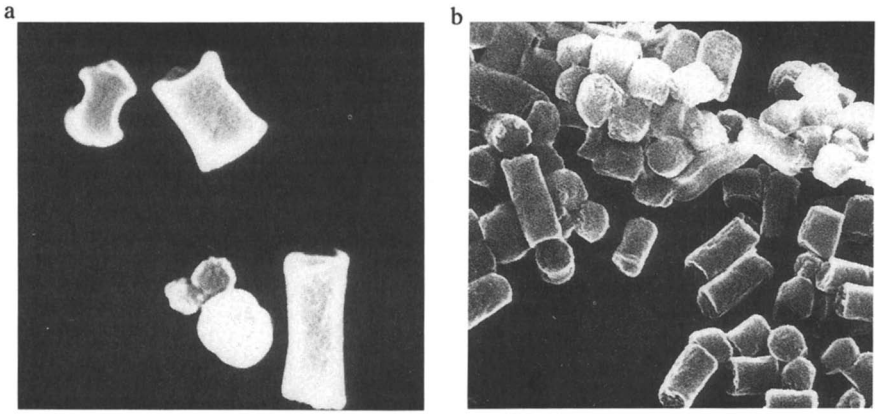

Figure 1. Scanning Electron Microscopy of cytoplasmic inclusion bodies from RB791(pGB1). a. Purified by detergent extraction. b. Purified by sucrose density gradient centrifugation.

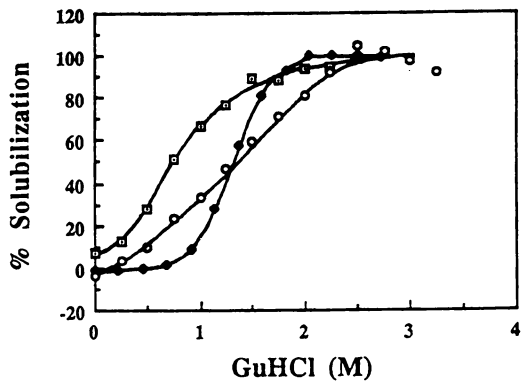

Figure 2. Extent of solubilization of sucrose density gradient centrifugation purified inclusion bodies by incubation with different concentrations of GuHCl. Inclusion bodies from RB791(pGB1) (◆), RB791(pKN) (○), RB791(pJG108) (⊡) were resuspended in 50 mM potassium phosphate, pH 7.0 containing 5 mM DTT and various concentrations of GuHCl. After three hours of incubation at 23°C the remaining aggregates were precipitated by centrifugation and the concentration of soluble protein was determined.

these sequences. Consequently, proteolytic degradation is highly dependent on the protein conformation. The mature β-lactamase has been shown to be extremely resistant to digestion by trypsin whereas the β-lactamase precursor and mutants exhibiting altered stability are readily degraded (*28-30*). Purified inclusion bodies were incubated in a 0.1 mg/ml trypsin solution for various times. The digestion was stopped by adding soybean trypsin inhibitor to a concentration of 0.2 mg/ml. The remaining aggregated material was precipitated by centrifugation, resuspended in potassium phosphate buffer containing 3 M GuHCl and 5 mM DTT and the concentration of solubilized protein was measured by the Bradford assay. Figure 4 shows that inclusion bodies from cells containing the plasmid pGB1 were the most resistant to degradation. The periplasmic inclusion bodies from RB791(pJG108), on the other hand, were digested extremely rapidly. After 20 min of incubation in presence of trypsin, 50% of the initial amount of protein present in the cytoplasmic inclusion bodies (from plasmid pGB1) was digested, compared to more than 70% for RB791(pKN) and about 95% for RB791(pJG108). SDS-PAGE of the aggregated protein remaining after trypsin digestion of the RB791(pKN) inclusion bodies revealed that the precursor was resistant to degradation. On the other hand, the degradation of the mature protein present in periplasmic inclusion bodies was extensive (*18*). These results suggest that the conformation of the polypeptide chains within the periplasmic aggregates is more accessible to trypsin compared to cytoplasmic inclusion bodies.

Raman spectroscopy can provide information on the conformation of insoluble proteins in membranes in the precipitated state (*31*). Preliminary studies showed that the protein in cytoplasmic inclusion bodies from RB791(pGB1) exhibits a high degree of α-helicity similar to the native soluble protein. The periplasmic inclusion bodies, on the other hand, contain a mixture of α-helical, β-sheet and random conformations (T. Przybycien, preliminary results). The higher level of organization of the cytoplasmic inclusion bodies is consistent with their greater resistance to GuHCl solubilization and trypsin digestion.

London et al. (*9*) demonstrated that *in vitro* aggregation of *E. coli* tryptophanase results from the specific intermolecular interactions between exposed hydrophobic surfaces of a folding intermediate. If, as proposed by Mitraki and King (*11, 12*), *in vivo* aggregation proceeds along a similar pathway, then the conformation of the polypeptide chains inside the inclusion bodies should reflect the conformation of the intermediates responsible for aggregation. Our studies show that the conformation of β-lactamase within the inclusion bodies depends on the cellular compartment in which aggregation occurs. This suggest that different association pathways may be responsible for the formation of different inclusion bodies. *In vitro*, β-lactamase aggregation from denaturant solutions has been shown to depend on the redox potential, the pH and the cosolvent composition of the renaturation buffer. Large variations of these three parameters occur across the membranes separating the different compartments of a cell. The cytoplasm is a highly regulated reducing environment of nearly constant pH and composition. On the other hand, the pH and the concentration of low molecular weight solutes in the periplasmic space are very sensitive to external conditions. Small molecular weight compounds can diffuse freely through the outer membrane and directly affect the formation of inclusion bodies (*1*). Differences in the protein composition within cellular compartments might also play an important role. The interaction of folding intermediates with cytoplasmic cellular components such as chaperonins (*32-38*) and other folding catalysts (*10*) could dictate the protein association pathway that leads to inclusion body formation. Other proteins could enhance aggregation by interacting non-specifically with the nascent polypeptide. In addition, cellular components such as nucleic acids and phospholipids could also affect the formation of inclusion bodies.

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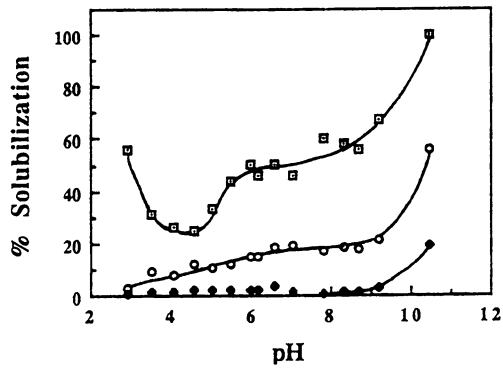

Figure 3. Solubilization of inclusion bodies from RB791(pGB1) (◆), RB791(pKN) (○), RB791(pJG108) (⧈) in buffers of various pH containing 0.45 M GuHCl and 5 mM DTT.

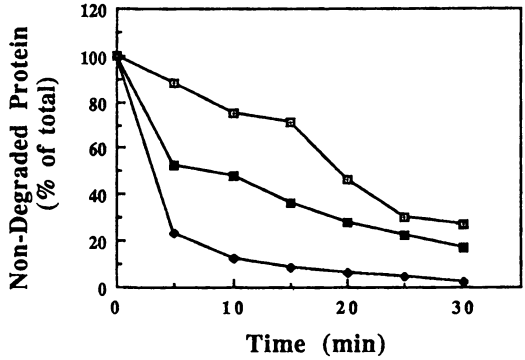

Figure 4. Digestion of inclusion bodies from RB791(pGB1) (⧈), RB791(pKN) (■), RB791(pJG108) (◆) by trypsin. The aggregates were incubated for various times in 0.1 mg/ml trypsin. The degradation was stopped with 0.2 mg/ml of soybean trypsin inhibitor.

Denaturation-Renaturation Experiments. The expression of recombinant protein into inclusion bodies is sometimes considered to be advantageous for several reasons. The aggregates can be easily precipitated from crude cell lysates by centrifugation. They contain the protein of interest at relatively high purity levels (40% or higher). Also aggregated protein is often more resistant to proteases within the cell. The limiting factor in deciding whether a protein should be expressed in an soluble form or as inclusion bodies resides in the difficulty to recover the native functional protein. The efficiency of the refolding process depends on the properties of the inclusion bodies such as density, solubilization characteristics and composition. SDS polyacrylamide gel electrophoresis shows that the protein composition of purified inclusion bodies varies according to their origin (Figure 7). We have showed that the solubilization characteristics of these inclusion bodies is also plasmid dependent. We therefore investigated in some detail the effect of the origin of the inclusion bodies on the efficiency of β-lactamase recovery upon renaturation. In this study, β-lactamase inclusion bodies were incubated for three hours at room temperature in 50 mM potassium phosphate buffer, pH 7.0, containing 3 M GuHCl and 5 mM DTT. The samples were subsequently dialyzed for three hours at room temperature against 50 mM potassium phosphate buffer, pH 6.0. This denaturation-renaturation procedure was completely reversible when purified β-lactamase was used at a concentration lower than 5 mg/ml. The protein recovery profiles for inclusion bodies from all three plasmids are shown in Figures 5 and 6. In all cases, the highest recoveries, both in term of activities and total soluble protein, were obtained with periplasmic inclusion bodies from RB791(pJG108). The activity recovered from RB791(pGB1) inclusion bodies was roughly half those obtained from RB791(pJG108). The lowest activity recoveries were obtained from RB791(pKN). Figures 5a and 6a also reveal that activities recovered at a total protein concentration of 1 mg/ml were about one fifth the activities obtained at 5 mg/ml. Also, the percent recovery of soluble protein obtained after renaturation at a total protein concentration of 1 mg/ml were practically identical to those obtained with 5 mg/ml (between 15% and 45% recovery depending on the plasmid). Our previous work on the refolding of purified β-lactamase clearly showed that the extent of aggregation depends on the protein concentration (*8*). When refolding of denatured β-lactamase was performed at concentrations greater than 5 mg/ml, the activity recovery decreased linearly decreasing as the protein concentration was increased. This type of behavior has been observed with numerous other proteins (*5-7*). The fact that the percent yields obtained from renaturation of inclusion body polypeptide at total protein concentrations of 5 mg/ml and 1 mg/ml were identical therefore seem to indicate that the recoveries achieved at both concentrations are the highest achievable in the conditions of the experiment. The low maximum recovery yield resulting from the solubilization-renaturation of inclusion body protein suggests that some of the contaminants integrated in the inclusion bodies enhance reaggregation of the protein during the refolding step.

To examine the effect of protein contaminants on the renaturation process, the reaggregated and the soluble fractions obtained from the refolding of 5 mg/ml of inclusion body protein from 5 M GuHCl were run on a SDS polyacrylamide gel (Figure 7). The soluble protein fraction obtained by refolding all three types of inclusion bodies was almost exclusively composed of mature β-lactamase. Essentially all the contaminating proteins initially present in the inclusion bodies were sequestered in the aggregates which were formed after the removal of the denaturant. This seems to indicate that the contaminating proteins tend to promote aggregation. Previous work on the refolding of tryptophanase (*9*) clearly showed that the interactions leading to aggregation of this protein are highly specific. Addition of other proteins or even crude cell lysate did not affect the recovery yields. On the other hand, the interactions leading to the aggregation of egg white lysozyme have been shown to be highly non-specific (*10*). The effect of protein contaminants on the refolding yield cannot be assessed at this point. Other types of contaminants such as phospholipids may also influence the

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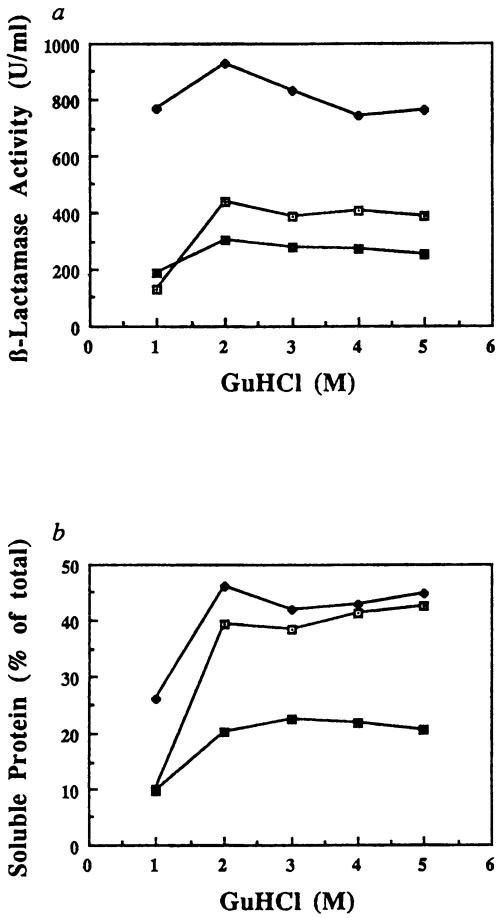

Figure 5. β-lactamase activity (a) and percent of soluble protein (b) obtained upon refolding of inclusion body proteins from RB791(pGB1) (⊡), RB791(pKN) (■) and RB791(pJG108) (◆). The inclusion bodies were first solubilized in 50 mM potassium phosphate buffer, pH 7.0 containing 5 mM DTT and various concentrations of GuHCl. The samples were then dialyzed against 50 mM potassium phosphate buffer, pH 6.0 for three hours at 23°C. The protein concentration was 1 mg/ml. The final GuHCl concentration was 0.015 M.

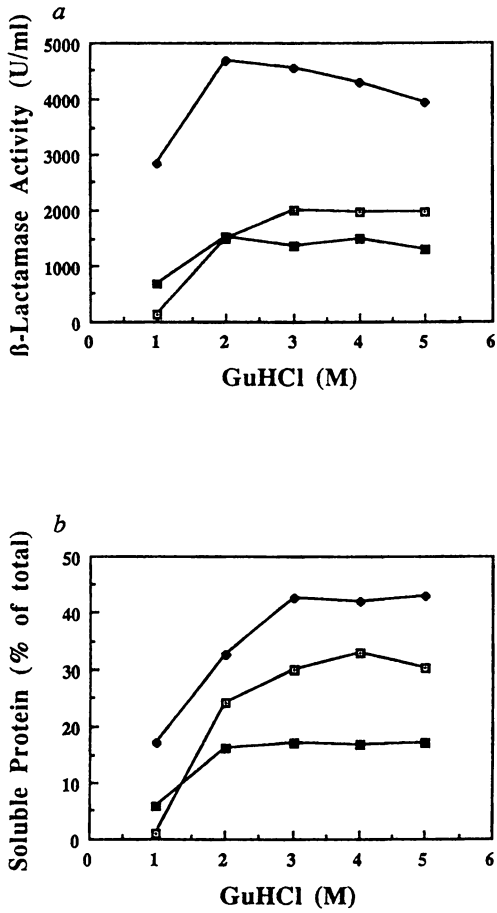

Figure 6. Same as Figure 5 except that the concentration of inclusion bodies was 5 mg/ml.

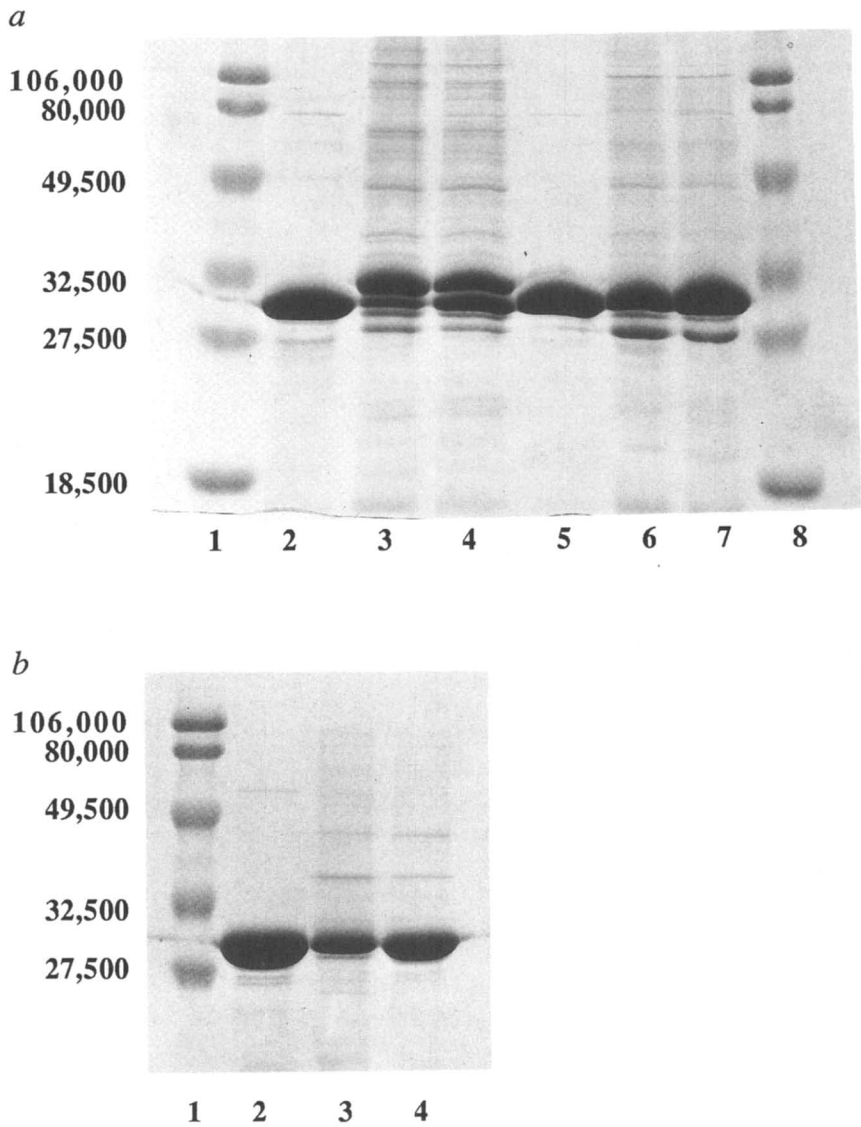

Figure 7. SDS-PAGE analysis of the renaturation of inclusion body protein. The samples correspond to the renaturation of 5 mg/ml of protein from potassium phosphate buffer pH 7.0 containing 5 M GuHCl, 5 mM DTT into the same buffer, pH 6.0. a. Lanes 2-4: RB791(pKN); Lanes 5-7: RB791(pGB1); Lanes 1 and 8 are molecular weight standards. b. Lanes 2-4: RB791(pJG108); Lane 1 is the molecular weight standard. Lanes 2a, 5 and 2b correspond to the soluble protein obtained after renaturation. Lanes 3a, 6 and 3b correspond to the reaggregated protein. Lanes 4a, 7 and 4b show the composition of the purified inclusion bodies.

recovery of active protein. The fact that the β-lactamase precursor of the RB791(pKN) inclusion bodies was found exclusively in the aggregated fraction is not surprising since the leader sequence of β-lactamase is very hydrophobic and has been shown to slow the folding kinetics (*39*), factors which favor aggregation.

In this report, we have shown that the structure of inclusion bodies and the conformation of the polypeptide chains within the inclusion bodies depend on the cellular compartment where aggregation occurs. These results suggest that the association mechanisms leading to the different types of inclusion bodies are different. Furthermore, the renaturation studies indicate that some cellular components present in the inclusion bodies may affect the recovery of active protein upon refolding.

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