Folding and Aggregation of \( \beta \)-Lactamase in the Periplasmic Space of \textit{Escherichia coli}\* \\

Gregory A. Bowden and George Georgiou‡ \\
From the Department of Chemical Engineering, University of Texas, Austin, Texas 78712 \\

High level expression of TEM \( \beta \)-lactamase results in the accumulation of precursor and mature protein in the insoluble fraction of \textit{Escherichia coli}. The mature polypeptide is sequestered in protein aggregates (inclusion bodies) located within the periplasmic space whereas the insoluble precursor is present in the cytoplasm. With the native \( \beta \)-lactamase, aggregation is observed when the rate of expression exceeds 2.5% of the total protein synthesis rate. Substitution of the native signal sequence with the outer membrane protein A (OmpA) leader peptide results in extensive aggregation of only the mature protein. Furthermore, for OmpA-\( \beta \)-lactamase, the accumulation of mature insoluble protein is independent of the rate of protein synthesis. These observations cannot be accounted by the kinetics of export of the OmpA-\( \beta \)-lactamase and the native precursor, therefore suggesting that the signal sequence affects the conformation of the newly secreted mature polypeptide and in turn, the folding pathway. Previously, we have shown that the aggregation of the mature protein secreted using its own signal sequence can be inhibited by growing the cells in the presence of non-metabolizable sugars such as sucrose (Bowden, G., and Georgiou, G. (1988) Biotechnol. Prog. 4, 97-101). We show here that this phenomenon is not related to osmotic effects, changes in \( \beta \)-lactamase translation or precursor processing. It follows that the addition of sugars exerts a direct effect on the \textit{in vitro} pathway of aggregation and folding, in analogy with the well characterized effect of sugars \textit{in vivo}.

Under certain conditions, newly synthesised proteins do not fold properly \textit{in vivo} and are sequestered in amorphous protein aggregates. Protein aggregation is observed in cells grown at elevated temperatures (1), during the synthesis of abnormal polypeptides (2) and in cells expressing cloned gene products (3-6). Both heterologous and homologous polypeptides can form intracellular protein aggregates which are called inclusion bodies. Aggregates resulting from the expression of recombinant proteins have been observed in a wide range of microorganisms such as \textit{Escherichia coli}, \textit{Bacillus subtilis}, \textit{Erwinia carotovora}, and \textit{Saccharomyces cerevisiae} (7). Despite the widespread occurrence and biotechnological significance of inclusion bodies, the mechanism responsible for their formation is not well understood. Solubilization of the aggregated proteins usually requires strong denaturing conditions indicating that the polypeptide chains are incorrectly folded. In that respect, inclusion bodies resemble the aggregates that form \textit{in vitro} during the refolding of proteins from denaturant solutions. Detailed studies have shown that \textit{in vitro} protein aggregation arises from the association of partially folded intermediates and depends on protein concentration (8, 9). In agreement with these results, Haase-Pettingel and King (10) have shown that the aggregation of P22 tailspike protein in \textit{Salmonella typhimurium} is caused by the association of polypeptide molecules that fail to enter the folding pathway. According to the proposed model for the folding of the P22 tailspike, the extent of \textit{in vivo} aggregation depends on the accumulation of a soluble, partially folded intermediate. This intermediate may either fold to the native conformation or participate in intermolecular association processes that give rise to protein aggregates. Folding and aggregation are parallel pathways in kinetic competition with each other. Thus, the ratio of native to aggregated products is determined by the rate of accumulation of the folding intermediate, which depends on the rate of translation, as well as the rates of polypeptide folding and aggregation.

The relation between intracellular conditions, such as ionic strength or interactions with cellular components, and protein aggregation (or folding) is not clear. The growth temperature has been shown to drastically affect aggregation of the P22 tailspike protein as well as the formation of inclusion bodies by a variety of polypeptides expressed in \textit{E. coli} (11-13). However, the interpretation of these results is complicated since temperature not only influences the folding kinetics but also exerts a pleiotropic effect on cell physiology. Many of the processes that are thought to play a role in intracellular protein aggregation, such as the rate of translation and the interactions of partially folded polypeptides with chaperonins (14), exhibit a strong temperature dependence. In general, there are very few experimental systems in which the folding pathway has been studied \textit{in vivo} under conditions that do not have a profound influence on cell physiology.

In \textit{E. coli} and other Gram-negative bacteria, polypeptides that are secreted into the periplasmic space become exposed to an environment considerably different from the cytoplasm. Due to the permeability of the outer membrane, the redox potential, pH, and ionic composition in the periplasmic space vary depending on the extracellular environment. The conformation of newly secreted polypeptides can be characterized by their susceptibility to externally added proteases in cells that have been converted into spheroplasts. For example, by taking advantage of the trypsin accessibility of partially folded \( \beta \)-lactamase, Minsky et al. (15) established that an intermediate bound on the periplasmic side of the inner membrane is formed prior to the release of the protein in soluble form in the periplasmic space. The accessibility of the periplasmic space to externally added agents can provide a convenient experimental system for identifying the conditions that de-

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‡To whom correspondence should be addressed.
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TERMINE PROTEIN AGGREGATION IN VIVO

At relatively low or moderate expression levels, β-lactamase is soluble in the periplasmic space and fractionates almost quantitatively with the osmotic shock fraction (16). However, elevated rates of protein synthesis cause the formation of aggregates which are confined between the inner membrane and the cell wall (17). The formation of inclusion bodies in the periplasmic space of E. coli has also been observed with other proteins (18-20). To monitor the aggregation quantitatively in vivo, we chose E. coli strain RB791(pJG108) because this strain is more convenient to handle than RB791(pJG107), the parent strain, which grows poorly at high densities. The plasmid pJG108, a 9.6-kilobase vector provided by M. Inouye (24), has the pTacII (4.6 kilobase) promoter system described earlier (23). The plasmid pJG107, a 10.6-kilobase vector provided by the same investigator, has been described previously (28). Protein concentrations were determined by the Bradford method (29) using the Bio-Rad assay reagent with bovine serum albumin as the standard.

PULSE-CHASE EXPERIMENTS— Cultures were grown in minimal salts media supplemented with 0.2% fructose, 50 μg/ml ampicillin, and 19 mM potassium phosphate buffer, pH 6.5. IPTG (1 mM) was added at mid-exponential phase. Two milliliters of cultures were used for the pulse-chase experiments. [35S] methionine incorporation, 40-100 μl of culture were diluted into 95 ml of identical media at various times after the addition of IPTG. Following a 15-min incubation period, [35S]methionine was added to the culture at a final concentration of 4 μCi/ml. Samples (1 ml) were withdrawn at 20-s intervals for up to 2 min and mixed with 110 μl of 10% trichloroacetic acid at 0°C. Subsequently, samples were heated for 10 min at 90°C to hydrolyze [35S]-labeled tRNA and placed on ice for 30 min. Proteins were precipitated by centrifugation at 16,000 × g for 20 min, washed with cold 100% ethanol, and dissolved in 50 mM Tris-HCl, pH 8.0, containing 1% SDS. The rate of [35S]methionine incorporation into β-lactamase was measured after immunoprecipitation with an anti-β-lactamase antibody (15). The spheroplast suspension was divided in half, and one-half of the labeling was measured using the electrocellular particle sizing apparatus with a 30 μm aperture (Coulter Electronics Inc.) after appropriate dilution into particle-free buffer.

The rate of precursor processing was determined as follows. One h after the addition of 0.1 mM IPTG, 2 ml of culture were diluted into 20 ml of identical media. After 15 min incubation, [35S]methionine was added at a final concentration of 4 μCi/ml. The chase was initiated by adding unlabeled methionine prewarmed to 37°C at a final concentration of 10 mg/ml. One ml samples were withdrawn at 20-s intervals and mixed with 110 μl of 10% trichloroacetic acid at 0°C. β-Lactamase was immunoprecipitated and the immune complexes were resolved by SDS-PAGE. The β-lactamase bands were identified by autoradiography. A prototype Clayton densitometer (developed by L. Poulson at the University of Texas at Austin) was used to scan the developed x-ray film.

In Vitro Protein Folding Experiments— RB791(pJG108) cultures were grown in conditions described above. The cells were centrifuged and lysed by French pressing in lysis buffer composed of 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM EDTA. The insoluble fraction was pelleted by centrifugation at 12,000 × g for 10 min and resuspended in the same buffer with the addition of 10 mM EDTA and 2% (v/v) Triton X-100. The inclusion bodies were incubated for 10 min at room temperature, washed twice with lyse buffer, and resuspended in 50 mM potassium phosphate, pH 6.5. The purity of the β-lactamase was determined by SDS-PAGE and found to be >95%. An aliquot of the inclusion body suspension (500 μl of culture) was solubilized by the addition of a guanidine hydrochloride solution (4 M final concentration). The refolding kinetics of the β-lactamase was determined by diluting the guanidine hydrochloride (0.2 M final concentration) with 50 mM potassium phosphate, pH 6.5, and measuring the enzymatic activity (as described above) at 5-min intervals. Similarly, the folding kinetics of β-lactamase from RB791(pJG107) cultures were determined with enzyme previously isolated and purified by P. Valax, University of Texas, Austin.

RESULTS

Formation of Aggregates—In E. coli, the overexpression of TEM β-lactamase using a tac promoter results in accumulation of large amounts of protein inclusion bodies which are confined between the inner membrane and the cell wall (17). The polypeptide chains are held together by noncovalent interactions and can be solubilized in 6 M guanidinium hydrochloride. Removal of the denaturant by dialysis results in quantitative recovery of the β-lactamase activity. The total level of β-lactamase accumulation depends on the concentration of the inducer IPTG and the time of addition to the culture. To determine the effect of inducer addition, different concentrations of IPTG were added to mid-exponential phase cells grown in minimal media. After overnight growth, 1 ml aliquots of the culture were lysed and separated into total soluble and insoluble fractions. The amount of β-lactamase...
The purified $\beta$-lactamase from the soluble fraction has a specific activity of 2.4% of the total protein synthesis rate. Since both the precursor and the mature form of $\beta$-lactamase are present in the insoluble fraction, the actual rate of accumulation of the mature polypeptide in the periplasmic space is greater than the total rate of $\beta$-lactamase synthesis. Pulse-chase experiments demonstrated that in cultures induced with 0.1 mM IPTG, 65% of the precursor is not processed to mature protein even after 5 min of chase (Fig. 2A). No processing was evident even after 2 h post-chase but the amount of precursor was reduced, most likely due to proteolysis. The cellular location of the pre-$\beta$-lactamase was investigated by its accessibility to trypsin after conversion of the cells into spheroplasts. For these experiments the cultures were grown in the presence of 0.3 mM sucrose to minimize the aggregation of the mature protein (21). From Fig. 3, it can be seen that the pre-$\beta$-lactamase was protected from proteolytic degradation in intact spheroplasts but was completely degraded in lysed cells after 15 min of incubation with trypsin. This result shows that under conditions of very high expression, the precursor accumulates in the cytoplasm, probably because a certain component of the export machinery becomes limiting. Since the precursor fractionates with the insoluble fraction, it must be present in a high molecular weight complex formed either by self-association or by binding to the cytoplasmic membrane. The conformation of the cytoplasmic precursor was not characterized further. Many proteins which are sequestered in inclusion bodies become highly resistant to degradation (6). This is not the case for the unprocessed pre-$\beta$-lactamase, suggesting that it is present

### Table 1

| Plasmid     | [IPTC] | Induction time | Total $\beta$-Lactamase | % $\beta$-Lactamase synthesis |
|-------------|--------|----------------|-------------------------|-------------------------------|
| pTacl\(1\)  | 0.1    | 1              | 246                     | 34.0                          | 10.7                          |
| pTacl\(1\)  | 0.1    | 2              | 147                     | 25.2                          | 13.3                          |
| pTacl\(1\)  | 0.02   | 1              | 239                     | 7.3                           | 2.4                           |
| pJG108      | 0      |                | 357                     | 3.5                           | 0.8                           |
| pJG108      | 0.1    | 1              | 281                     | 6.8                           | 1.9                           |
| pJG108      | 0.1    | 2              | 304                     | 16.0                          | 4.1                           |
| pJG108      | 0.1    | 3              | 310                     | 7.5                           | 5.3                           |

*The rate for the uninduced RB791(pJG108) culture was measured 1 h after the time it would have been induced.*

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2 P. Valax, unpublished observations.
space of cells induced with 0.1 mM IPTG becomes equal to 3.8% of the total protein synthesis rate.

Effect of the Signal Sequence on Aggregation—Plasmid pJG108 contains the gene for the mature TEM \( \beta \)-lactamase fused to the OmpA signal sequence and expressed from the \( lpp \) promoter. Upon secretion, cleavage of the signal sequence gives rise to the mature \( \beta \)-lactamase in which the N-terminal amino acid is identical to the native sequence (24). Ghreyeb et al. (24) have reported that in induced cultures the mature protein is aggregated due to the high level of expression. It was of interest to compare the effect of the OmpA signal sequence on the aggregation of the mature \( \beta \)-lactamase. From Fig. 4 it can be seen that extensive aggregation occurs at all concentrations of IPTG as well as in uninduced cultures. The production of a large amount of \( \beta \)-lactamase in uninduced cultures indicates that the \( lpp \) promoter is leaky in strain RB791. In contrast to the results obtained with RB791(pTac11), the predominant species in the insoluble fraction corresponds to the mature protein. A very small amount of the precursor was detectable in immunoblots of the insoluble fraction (data not shown).

The relationship between the rate of accumulation of the mature polypeptide in the periplasmic space and protein aggregation was investigated. The OmpA-\( \beta \)-lactamase precursor was processed at a rate identical to that of the native protein even in cultures induced with 0.1 mM IPTG (Fig. 2). The half-life of the precursor, about 45 s, is consistent with observations by Bolla et al. (36). Since the ompA-\( \beta \)-lactamase precursor does not accumulate in the cytoplasm, the formation of mature \( \beta \)-lactamase is determined solely by the rate of protein synthesis. The specific rate of incorporation of \(^{35}\)S methionine into the trichloroacetic acid precipitate was higher in RB791(pJG108) compared with RB791(pTac11), reflecting slight differences in the growth rate (Table I). For cultures induced with 0.1 mM IPTG, a higher rate of \( \beta \)-lactamase synthesis was obtained from the tac promoter in cells containing plasmid pTac11. Under these conditions, only 35% of the pre-\( \beta \)-lactamase was processed into the mature protein (Fig. 2A). When the fraction of processed protein is taken into account, then the maximum rates of mature \( \beta \)-lactamase

![Diagram](https://image-url.com)

**Fig. 2.** Kinetics of \( \beta \)-lactamase processing. Pulse-chase experiment for \( E. \) coli RB791 containing either pJG108 or pTac11 for different growth conditions. A, fraction of the \( \beta \)-lactamase precursor remaining as a function of time following the addition of chase. Q, pTac11 (0.1 mM IPTG); ♦, pTac11, supplemented with 0.15 M raffinose (0.1 mM IPTG); ■, pTac11 (0.02 mM IPTG); ♣, pJG108 (0.1 mM IPTG). B, semilog plot of the change in unprocessed precursor as a function of time. In this plot the radioactivity of the unprocessed precursor remaining after 5 min was subtracted from each time point.

![Diagram](https://image-url.com)

**Fig. 3.** Trypsin accessibility of the precursor in spheroplasts of induced RB791(pTac11) cells. SDS-PAGE of the insoluble protein fraction of spheroplasts treated with trypsin for various times. Lane 1, molecular weight standards; Lanes 2–5, intact spheroplasts exposed to 0.1 mg/ml trypsin for 0, 5, 10, and 15 min, respectively. Lanes 6–9, lysed spheroplasts treated with trypsin, for the same times, respectively.

in a protease accessible conformation. On the other hand, the precursor did not migrate with the membrane fraction when a modified sucrose gradient centrifugation procedure (31) was used to separate aggregated proteins from membranes (data not shown).

The kinetics of formation of mature protein are not affected by the accumulation of pre-\( \beta \)-lactamase in the cytoplasm. As can be seen from Fig. 2B when the fraction of the precursor which cannot be translocated is subtracted from the total initial amount present at the beginning of the chase, then the processing rate is the same regardless of the concentration of inducer. The half-life of the pre-\( \beta \)-lactamase which is competent for processing was approximately 45 s, in close agreement with previous results (35). When the fraction of processed \( \beta \)-lactamase is taken into account, the adjusted relative rate of accumulation of mature protein in the periplasmic

![Diagram](https://image-url.com)

**Fig. 4.** Distribution of \( \beta \)-lactamase in the soluble and insoluble fractions of RB791(pJG108) cultures. Samples were prepared as in Fig. 1 except that the equivalent of 50 \( \mu \)l of culture were loaded in each lane, and after SDS-PAGE the gel was stained. Lanes 1–5, insoluble fractions from cultures grown with 0, 0.0001, 0.005, 0.05, 0.1, and 1.0 mM IPTG, respectively. Lane 7: molecular weight standards. The specific activities of the \( \beta \)-lactamase in the soluble protein fractions corresponding to each lane are also shown.
accumulation are very similar: 11.9 cpm/10^6 cells/s for cells harboring pTac11 and 16 cpm/10^6 cells/s for RB791(pJG108), 1 and 2 h after induction, respectively. On the other hand, the amount of soluble β-lactamase, as measured by the specific activity, was 3-fold higher in RB791(pTac11) compared with RB791(pJG108) (compare Figs. 1 and 4). Since the rate of accumulation of newly secreted β-lactamase in the periplasmic space is approximately the same in both cases, the difference in the extent of aggregation is not caused by an increase in the concentration of partially folded mature polypeptides. Similarly, in uninduced cultures of RB791(pJG108) where aggregation is extensive (Fig. 4), the β-lactamase synthesis rate is less than half of pTac11 harboring cells induced with 0.02 mM IPTG (Table I) although in the latter all the protein is soluble. Consequently, the difference in the extent of aggregation is not due to the protein synthesis rate.

Since a number of molecular manipulations have occurred in the construction of pJG108, a silent missense mutation created in the primary sequence could affect the folding kinetics of the protein. The in vitro folding kinetics of β-lactamase purified from inclusion bodies isolated from RB791(pJG108) were compared with β-lactamase purified from RB791(pTac11) (see “Experimental Procedures”) and were found to be identical (data not shown). Since the folding kinetics of the mature protein as well as the signal sequence processing kinetics are identical for both OmpA-β-lactamase and the native protein, these observations suggest that the effect of the signal sequence on the aggregation of the mature protein is exerted at a step preceding the cleavage of the signal sequence by signal peptidase I.

Inhibition of Aggregation by the Addition of Sugars—The ratio of soluble to aggregated β-lactamase in RB791(pTac11) can be increased by growing the cells in the presence of certain sugars (21). The inhibition of aggregation depends on the concentration of the sugar in the growth medium. For the same concentration, the extent of the inhibition decreases in the order: raffinose (M, 595) > sucrose (M, 342) > sorbose (M, 180). The molecular weight of these compounds is small enough so that they equilibrate within the periplasmic space of E. coli (37) but are not transported into the cytoplasm and therefore are not metabolized. However, the addition of sugars changes the osmotic pressure of the growth medium. For example, 0.3 M sucrose increase the osmotic pressure of the growth medium from 220 to 480 mOs/kg H_2O. Changes in osmotic pressure can affect the growth rate, cell dimensions, and the expression of certain genes (38, 39). To determine the effect of the osmotic pressure on protein aggregation, RB791(pTac11) cells were grown in media with 0.3 M sucrose for the osmotically equivalent concentrations of NaCl (0.185 M). The addition of NaCl did not affect protein aggregation or the specific activity of soluble β-lactamase (Fig. 5).

No effect on the growth rate of induced cultures of RB791(pTac11) was observed in the presence of 0.15 M raffinose and a slight change (5%) was measured in cultures supplemented with 0.3 M sucrose. Higher concentrations of either sugar caused a more significant reduction in growth rate. The kinetics of protein synthesis and precursor processing were measured in order to determine if the inhibition of aggregation is caused by a reduction in the rate of accumulation of mature β-lactamase in the periplasmic space. Cultures were supplemented with 0.15 M raffinose (to maintain the same growth rate) and induced with 0.1 mM IPTG. The rate of incorporation of the label into the trichloroacetic acid precipitate and into β-lactamase was 290 and 37 cpm/10^6 cells/s, respectively. β-Lactamase corresponded to 10% of the total protein synthesis (corrected for methionine content), a value very similar to that obtained in cultures grown without raffinose. Similarly, no effect was observed on the kinetics of precursor processing (Fig. 2). Therefore, the rate of accumulation of mature β-lactamase is the same as in the absence of sugar. The osmotic pressure in the medium in the presence of 0.15 M raffinose is 385 mOs/kg H_2O. At this osmotic pressure there is little change in the volume of the periplasmic space (40). Since neither the periplasmic volume nor the rate of mature protein accumulation are altered by the presence of sugar, the concentration of newly secreted polypeptides must be approximately the same.

Fig. 6 shows the effect of increasing concentration of sucrose on the aggregation of β-lactamase in RB791(pJG108) cells induced with 0.1 mM IPTG. Since aggregation is more pronounced in RB791(pJG108), the increase in soluble protein is also more striking. An approximately 10-fold increase in specific activity occurs in cells grown in 0.4 M sucrose. The dependence of aggregation on sucrose concentration is similar to that reported earlier for cells overexpressing the native β-lactamase (pTac11).
At present there is relatively little information regarding the folding and assembly of secreted proteins in procaroytes (41, 42). In the cytoplasm, the precursor form of secreted polypeptides must be maintained in a conformation competent with export (43). At least two elements of the secretory process serve to modulate the tertiary structure of the precursor: (i) the presence of the signal sequence which, in addition to its other roles, also serves to retard folding (44), and (ii) interactions with chaperonins such as GroEL, GroES, trigger factor, and SecB (45). The folding events which follow the translocation through the membrane are not well defined. Under certain conditions a fraction of the mature polypeptides is unable to fold properly and aggregates forming inclusion bodies in the periplasmic space (7).

Elevated levels of expression of the native TEM β-lactamase result in the accumulation of both mature and precursor polypeptides in the insoluble fraction. The insoluble precursor is not accessible to trypsin in spheroplasted cells which is consistent with its expected cytoplasmic location. On the other hand, only the mature protein is observed in the insoluble fraction of cultures expressing a fusion between β-lactamase and the OmpA signal sequence, indicating that the aggregation of the precursor and the mature protein are not necessarily coupled. As can be deduced from the data in Fig. 1 and Table I, the accumulation of insoluble precursor is observed only when a certain threshold level of protein synthesis has been exceeded. In vivo and in vitro experiments have established that the chaperonins GroEL and GroES serve to maintain the pre-β-lactamase in a export competent conformation in the cytoplasm (46, 47). When the rate of protein synthesis is very high, the capacity of the chaperonin system may be exceeded. Any excess precursor chains should fold into an export incompetent conformation and eventually associate with each other (or with membrane components) forming a precipitable complex.

The aggregation of native β-lactamase is a function of the protein synthesis rate. The increased rate of protein synthesis most likely leads to the accumulation of a putative folding intermediate which is prone to intramolecular association. In a similar manner, the aggregation of the β-lactamase from Staphylococcus aureus during refolding in vitro follows second order or higher kinetics and is therefore observed at higher protein concentrations (48). Biochemical and genetic studies have established that a partially folded intermediate is transiently bound on the external side of the cytoplasmic membrane during secretion (15, 49). While it is tempting to speculate that the membrane-bound polypeptide is the putative intermediate which gives rise to aggregate formation, no definite experimental proof is available at the moment.

We observed that substitution of the native signal sequence with that from OmpA causes a significant increase in the extent of aggregation of the mature protein. Considering that (i) the amino-terminal sequence of the mature protein is identical in both cases, (ii) the kinetics of precursor transport are the same, and (iii) the increase in the extent of aggregation is not caused by differences in the expression level, it follows that the signal sequence must be influencing the formation of an intermediate which is prone to aggregation. The effect of the signal sequence on the folding pathway of the mature protein may be explained by two different mechanisms. First, depending on the leader peptide, the precursor may be exported through separate pathways involving different components of the secretory system. There is at least one step in secretion in which precursors can interact with different components of the export machinery. Following translation, the precursor of β-lactamase interacts with the chaperonins GroEL and GroES, whereas pre-OmpA is bound by the trigger factor (50). If the transport of the native β-lactamase and the OmpA-β-lactamase takes place via separate pathways, then the conformation of the newly secreted mature protein may be different, affecting the folding pathway. However, export through different pathways is not very likely since the processing kinetics for the pre-OmpA-β-lactamase and the native precursor are identical. Alternatively, a second possibility is that the secretion pathway for the two precursors is the same and the signal sequence is influencing the folding of the mature protein. For example, the signal sequence may be responsible for the formation of certain elements of secondary structure which are retained during the membrane translocation process, or it may be affecting an early step in the folding of the secreted protein, prior to the processing event. In either case, the signal sequence must be influencing the conformation of an early intermediate in the folding of the mature protein which, in turn, leads to aggregation. In accord with this interpretation, mutations in the signal sequence have been shown to affect the release of β-lactamase from the outer surface of the cytoplasmic membrane (51).

For both the native protein and OmpA-β-lactamase, aggregation can be reduced by growing the cells in the presence of certain non-metabolizable sugars. The increase in correctly folded protein is not caused by changes in the rates of protein synthesis and is not related to the elevated osmotic pressure of the growth medium in the presence of sugars. The simplest explanation for this phenomenon is that the sugars exert a direct effect on polypeptide folding and aggregation in vivo. The stabilizing effect of sugars and polyalcohols on the native conformation of proteins in vitro is well known. According to the widely accepted model of Timasheff and co-workers (52) the stabilization of the native conformation arises from the preferential hydration of the polypeptide. One consequence of this effect is the inhibition of aggregation in vitro (53). Our results demonstrate that sugars can also inhibit protein aggregation in a physiological environment and thus establish a direct analogy with the in vitro studies. The inhibition of β-lactamase aggregation is observed with both the native protein and OmpA-β-lactamase and exhibits approximately the same dependence on the sugar concentration. Therefore, it is likely that in both cases the sugars act on the same step of the aggregation pathway.

The production of correctly folded recombinant polypeptides is of great interest for numerous fundamental studies and biotechnology applications. The above studies shed light on some of the factors that determine protein aggregation in vivo in E. coli. In agreement with previous studies (5, 10), we have observed that aggregation is determined by the folding pathway and the rate of polypeptide synthesis. For secreted proteins at least, the growth environment, and in particular the presence of non-metabolizable sugars, can exert a direct effect on the formation of inclusion bodies. There is also some evidence that other environmental factors such as ionic composition (54) and redox potential may also be critical for the correct folding of certain proteins. Thus, different genetic or physiological approaches may be taken in order to maximize the production of correctly folded proteins.

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G A Bowden and G Georgiou

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