The periplasmic fates of misfolded MalE31, a defective folding mutant of the maltose-binding protein, were determined by manipulating two cellular activities affecting the protein folding pathway in host cells: (i) the malEp promoter activity, which is controlled by the transcriptional activator MalT, and (ii) the DegP and Protease III periplasmic proteolytic activity. At a low level of expression, the degradation of misfolded MalE31 was partially impaired in cells lacking DegP or Protease III. At a high level of expression, misfolded MalE31 rapidly formed periplasmic inclusion bodies and thus escaped degradation. However, the manipulated host cell activities did not enhance the production of periplasmic, soluble MalE31. A kinetic competition between folding, aggregation, and degradation is proposed as a general model for the biogenesis of periplasmic proteins.

Among the functions of protein degradation in living cells is the elimination of misfolded proteins with abnormal conformations (1). Because misfolded proteins can induce cellular stress (2), proteolysis is a major component of the highly conserved regulatory heat shock response. Recently, a new heat shock sigma factor, σE (or σ24), involved in extra-cytoplasmic stresses has been discovered in Escherichia coli (4). In the periplasmic compartment, among many proteases, the DegP (also known as HtrA or Do) protease is the only protease, identified as a heat shock protease, involved in the degradation of misfolded proteins (3, 4). The degP gene, which is under the transcriptional regulation of sigma σE, is essential for the survival of E. coli above 42 °C (5).

Our aim is to understand the exact relationships between export and folding of bacterial periplasmic proteins. We are using a model system based on the maltose-binding protein (MalE or MBP) of E. coli. The MalE protein serves as the periplasmic receptor for the high affinity transport of maltose and maltodextrins (6). Because of its key role in maltose transport, correct export and folding of MalE into the periplasm is essential for cells to utilize maltose as a carbon source. This feature facilitated the use of genetic selections for analyzing MalE export (7) and folding (8). MalE is synthesized in the cytoplasm as a precursor protein, preMalE, with an amino-terminal signal sequence. This signal sequence serves to target the precursor protein to the periplasm. As the precursor enters the periplasm, the signal sequence is cleaved off by the signal peptidase. Upon removal of the signal peptide, the protein is released from the membrane into the periplasm and refolds to its native structure. The three-dimensional structure of MalE consists of two discontinuous domains constructed from secondary structural αβ units surrounding a cleft that forms the binding site for maltose and maltodextrins (9, 10).

We previously identified amino acid substitutions of MalE that are critical for the in vivo folding (11). Among these mutations, the most defective variant, MalE31, corresponds to the double substitution of G32D and I33P in the αβ loop connecting the helix I to strand B of the N-domain. In vivo, the MalE31 precursor was correctly exported, based on the kinetics of signal peptide processing, but the defective folding of the mature protein in the periplasm led to the formation of inclusion bodies (11). However, the modified loop, which is apart from the maltose-binding site, did not perturb the maltose-binding affinity of the purified protein, renatured from inclusion bodies. One major physiological consequence for the cells overproducing MalE31 was an increase in σE activity, and, hence, DegP synthesis (12). However, this increased level of DegP did not degrade a series of misfolded MalE31 variants (8). In this study, we have examined the production of MalE31 in several protease-deficient strains to clarify these contradictory observations and to specify precisely the different kinetic parameters between folding, aggregation, and degradation of proteins in the periplasm of E. coli.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The bacterial strains used in this study are listed in Table I. The degP and ptr mutants were constructed by standard P1 transduction (13) of HS2619 (14) using RS474 (6) and SF103 (15) as donors and selecting for kanamycin and chloramphenicol, respectively. The recA1 allele was introduced by cotransduction with sth::Tn10. All strains carried a non-polar deletion of the chromosomal malE gene (14), and pop6499 gave constitutive, high level expression of the maltase operons because it contained the malT allele (16). Plasmids pHCE31 and pHCE-33P are pBR322 derivatives that carry the malE alleles under the control of their own promoters; the MalT-dependent malEp promoter (11). Plasmid pOM82 (pSC101 derivative) and pMDX (pBR322 derivative) encoded the lacZ gene under the control of the malE promoter (17) and the tac promoter, respectively. Plasmid pT31H is a pBR322 derivative of pTH18 (18) expressing the malE31 gene under the control of the tac promoter. Plasmid p7059 is a pHCM derivative that carries a deletion of the ribosome binding site and the first 256 codons of the malE gene (19) and was used as a negative control.

Cell Fractionation—Cells were grown in LB medium (13) supplemented with 0.1 mg/ml carbenicillin and 0.2% maltose, at 30 °C for 3 h (A600 ≈ 1). Cells from 10 ml of the culture (5 × 10⁸ cells/ml) were harvested by centrifugation and fractionated by spheroplast preparation as described previously (8). The periplasmic and membrane fractions were analyzed by SDS-polyacrylamide gel electrophoresis (12.5% acrylamide). Proteins were visualized by either Coomassie Blue or silver staining and scanned with a MasterScan I Interpretive densitomer.
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Table I

| Name          | Relevant genotype      | Origin                           |
|---------------|------------------------|----------------------------------|
| HS2019        | MC1400 mleE444         | Laboratory collection            |
| pop6499       | MC1400 malTΔmleE444 recA1 | Laboratory collection            |
| pop6590       | HS2019 recA1 srl::Tn10  | Laboratory collection            |
| KS474         | RS727 degP43Δ(leuR+Kan') | Ref. 4                           |
| SF103         | RS727 ptr-32::I Cm'     | Ref. 15                          |
| NSS2          | HS2019 degP43Δ(leuR+Kan') recA1 | This study                     |
| NSS5          | NSS ptr-32::I Cm'       | This study                       |
| NSS6          | HS2019 ptr-32::I Cm' recA1 srl::Tn10 | This study                     |

FIG. 1. Fractionation of soluble and insoluble MalE variants. Cells grown in rich medium containing 0.2% maltose at 30 °C for 3 h were fractionated from spheroplasts. Periplasmic (soluble) and membrane fractions (insoluble), corresponding to 10⁶ bacteria, were separated by SDS-polyacrylamide gel electrophoresis (12.5% acrylamide). The gels were stained with Coomassie Blue or silver-stained. A, strains harboring pHCE31 or pA709 plasmid. B, strains harboring pHCE31-33P or pA709 plasmid.

To determine the exact susceptibility of misfolded MalE31 to periplasmic degradation, we constructed three isogenic protease-deficient strains lacking either the DegP protease (NS2) or the Protease III (NS6) or both (NS5; Table I). Protease III (also known as P), the ptr gene product, is another periplasmic protease involved in the degradation of exported protein fusions (15). The steady-state level of MalE31 in these different protease-deficient strains is shown in parallel with the strain carrying the malT allele (Fig. 1A). The presence of the malT allele in the pop6499 strain ensured that the malE31 gene encoded by pHCE31 is expressed at a high and constitutive level, even in absence of maltose. Because no cytoplasmic accumulation and complete processing of the MalE31 precursor were previously reported in this genetic background (11), the preprotein translocase did not limit the periplasmic level of MalE31 under these conditions of high expression. Thus, intracellular distribution between soluble and insoluble fraction is given by cellular fractionation into periplasmic and membrane fractions, respectively. The MalE31 protein was found predominantly in the membrane fraction (Fig. 1A). The level of periplasmic MalE31 was very low, and the protein could be only detected after silver staining the gel. However, although the deficiency of protease and the promoter strength had no effect on the soluble protein, it had pronounced effects on the level of aggregated MalE31. From the membrane fractions, production of aggregated MalE31 ranged from a high level (in strain pop6499) to undetectable (in strain pop6590).

Previous mutagenesis studies indicated that the extent of periplasmic inclusion body formation varied with the nature of amino acid residues at positions 32 and 33 of MalE (8). The MalE-133P variant that displayed less severe folding defects than MalE31 had a soluble/insoluble distribution of 40/60 (11). At the steady-state, the periplasmic levels of MalE-133P were similar whatever the producing strain (Fig. 1B). Once again, differences were observed only for the aggregated protein sedimenting with membrane fractions (Fig. 2). Although the basal level in pop6590 is higher than for MalE31, insoluble production of MalE-133P was similarly enhanced in the protease-deficient strains. Increased production in the three protease-deficient strains (NS2, NS5, and NS6) relative to the isogenic pop6590 strain suggests decreased degradation of misfolded MalE31 and MalE-133P. Although the effects of the degP and ptr mutations were approximately cumulative, DegP deficiency was more effective on the degradation of both misfolded proteins (Fig. 2). However, the greatest amount of insoluble MalE31 was found in the malT strain (pop6499), even though this strain is degP+, ptr+.

The production level of MalE31 in the pop6590 strain, assessed from both the periplasmic and membrane fractions shown in Fig. 1B, was very low compared with the pop6499 strain. We performed two sets of experiments to test whether the expression of the malE31 gene was poorly induced and/or whether the corresponding protein was correctly synthesized and exported but rapidly degraded.

First, the high level expression of malE31 obtained in the pop6499 strain could stem primarily from the activation of the MalT-dependent promoter. Thus, we quantified the malEp promoter activity in the malT (pop6590) and malT (pop6499) genetic backgrounds using two different transcriptional gene fusions: malEp-lacZ encoded by pOM82 and tac-lacZ encoded by pMDX (Fig. 3A). The synthesis of β-galactosidase directed from the malEp promoter was 5-fold higher in pop6499 than in pop6590. However, in both strains, transformed by pMDX, the same level of β-galactosidase activity was measured. This result suggested that translational levels in both strains are similar when a gene is transcribed from a MalT-independent promoter. Further comparisons of these strains were investi-
gated with pT31H, a plasmid expressing \textit{malE31} under the control of the \textit{tac} promoter. The membrane fractions, prepared as described above, showed a similar level of aggregated MalE31 in both strains (Fig. 3B) and confirmed the transcriptional fusion data obtained with pMDX.

Second, pulse-chase experiments were performed to measure the intracellular stability of MalE31 in both strains (Fig. 4). MalE31 appeared to be rapidly degraded in the \textit{malT} \textsuperscript{c} strain; in fact no protein was observed after a 1-min chase (half-life < 30 s). In contrast, the protein showed no degradation in pop6499 after 1 h. From Fig. 4, it is evident that MalE31 is considerably more stable in the \textit{malT} \textsuperscript{c} strain; this stability can be attributed to the insoluble misfolded protein. Conversely, intracellular degradation appeared to be a major cause of the low level production of MalE31 observed in pop6590 cells.

Previous transcriptional studies on \textit{degP} activity performed with a fusion of the \textit{degP} promoter to the \textit{lacZ} gene have demonstrated that \textit{degP} transcription is increased in cells overproducing MalE31 (12). However, this increased cellular level of DegP did not degrade the misfolded MalE31, overproduced in the pop6499 strain (8). To test, more directly, the steady-state level of DegP production on MalE31 overproduction in pop6499 and pop6590 cell extracts, we performed an immunoblot analysis with anti-DegP antibodies. Data on Fig. 5A confirmed those obtained previously with the \textit{degP-lacZ} fusion: the cellular level of DegP increased with the level of insoluble MalE31. However, when cellular fractionation was carried out, the periplasmic amount of DegP did not reflect its level in whole cell extracts. Indeed, DegP was found mainly associated with membrane fractions, and the level of DegP in these insoluble fractions increased with the extent of MalE31 aggregation (Fig. 5B). This result suggests that export of DegP could be blocked in the membrane (if the export pathway is overloaded or partially jammed by the MalE31 precursor) or, alternatively, that DegP aggregated with misfolded MalE31 in the periplasm. To determine whether the anomalous cellular localization of DegP provoked by the overproduction of MalE31 could be related to an inhibition of the export pathway, the signal sequence processing of DegP precursor was examined by pulse-chase experiments. There was no significant difference in DegP precursor processing observed in pop6499 cells carrying pHCM31 or pΔ709 (data not shown). Therefore, under over-

![Fig. 2. Insoluble production of MalE variants.](image)

Relative steady-state levels of insoluble MalE31 and MalE-I33P were determined from densitometric analysis of the gels shown in Fig. 1 and normalized to the level of the outer membrane protein OmpA. The amount of MalE31 or MalE-I33P in the pop6499 cells was taken as 100%.

![Fig. 3. Promoter activity in malT\textsuperscript{c} and malT\textsuperscript{c} strains.](image)

A, activity of \textit{malEp} and \textit{tac} promoters was tested in the \textit{malT} \textsuperscript{c} (pop6590) and \textit{malT} \textsuperscript{c} (pop6499) strains, harboring either pOM82 (\textit{malEp-lacZ}) or pMDX (\textit{ptac-lacZ}) plasmids, respectively. \textit{β}-Galactosidase activity (Miller units) was measured in maltose-induced (pOM82) or isopropyl-1-thio-\textit{β}-D-galactopyranoside-induced (pMDX) strains using the average of four determinations with their standard deviation. B, expression of the \textit{malE31} gene under the control of the \textit{tac} promoter. pop6590 and pop6499 strains harboring pT31H were fractionated as described in the legend to Fig. 1. Membrane fractions were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. Lane 1, molecular weight standards. Lane 2, pT31H in pop6590. Lane 3, pT31H in pop6499. The bands corresponding to LamB, MalE31, and OmpA are indicated by arrows.
production of MalE31, DegP coaggregated with misfolded MalE31 in inclusion bodies.

Several studies carried out on various proteins produced in the cytoplasm of *E. coli* supported a correlation of thermodynamic stability with the extent of inclusion body formation (22–24). The results described here suggest that instead of thermodynamic stability, dynamic folding properties of the newly translocated protein would better reflect the final distribution among alternative pathways. We therefore determined whether the amino acid substitutions at positions 32 and 33 had altered the folding rate of MalE. The refolding process of the wild-type and variant proteins after denaturation in 4 M GdnHCl, as monitored by the fluorescence of tryptophan residues, appeared to be at least biphasic (Fig. 6). The first phase was too fast to be analyzed by manual mixing (dead time of 10 s). However, this phase was detected because its amplitude accounted for 40% of the total signal recorded between native and denatured proteins. The remainder of the change in fluorescence accompanying refolding (60%) was attributed to a slow phase with a rate constant that varies between the different MalE variants (Table II). The final renaturation yields of the MalE variants, reported in Table II, reflected well their intrinsic tendency to misfold. The MalE-I33P protein purified from the soluble periplasmic fraction (by osmotic shock) displayed similar refolding kinetic parameters to those of the protein purified from inclusion bodies (data not shown). It is interesting to note the correlation between the refolding rate and the extent of corresponding soluble MalE protein in the periplasmic fractions (Table II).

**DISCUSSION**

This work was initiated to delineate the determinants of the fate of misfolded proteins, dictated by the interplay between host cell characteristics and protein folding parameters. By modulating cellular factors involved in the folding pathway of misfolded variants of MalE, we showed that: (i) at low level promoter activity, the protein was rapidly degraded by at least two known periplasmic proteases, DegP and Protease III; (ii) at high level promoter activity, the protein aggregated and formed inclusion bodies; and (iii) the level of soluble protein depended neither on promoter activity nor on proteolytic activity but could be determined by the ratio of rate constants governing

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**FIG. 4. Degradation of MalE31 in the pop6590 strain.** pop6590 (malT<sup>+</sup>) and pop6499 (malT<sup>c</sup>) strains harboring pHCM31 were grown in minimal medium containing 0.4% glycerol and 0.4% maltose at 30 °C. Cells were labeled for 2 min with [35S]methionine and incubated for 0–60 min with an excess of unlabeled methionine. Lane U corresponds to the control plasmid pD709 in pop6499 strain.

**FIG. 5. Effect of overproduced MalE31 on DegP.** Fractions from cellular fractionation experiment shown in Fig. 4A were separated by SDS-polyacrylamide gel electrophoresis, and immunoblot analysis of the gel was performed by using a rabbit antibody directed against DegP. A, whole cell extracts. B, periplasmic and membrane fractions. Lanes 1, pHCM31 in pop6590. Lanes 2, pHCM31 in pop6499. Lanes 3, pHCM31 in NS2. Lanes 4, pD709 in pop6499.

**FIG. 6. In vitro refolding kinetics of MalE variants.** Refolding kinetics were monitored by increased tryptophan fluorescence, excitation at 290 nm, and emission at 345 nm. Refolding was initiated by mixing proteins (10 μM) denaturated in 4.2 M GdnHCl, 25 mM Tris-HCl, pH 7.5, with 100 volumes of 25 mM Tris-HCl at 25 °C. Fluorescence intensities of the unfolding proteins in 4.2 M GdnHCl and native proteins were taken as 0 and 100%, respectively. The solid lines are fits of the data to a single exponential term.

**TABLE II**

| Protein       | Periplasmic yield<sup>a</sup> | Refolding rate<sup>b</sup> | Refolding yield<sup>c</sup> |
|---------------|-------------------------------|-----------------------------|-----------------------------|
| MalE-wt       | 23 ± 4                        | 6.2 ± 0.3                   | 85 ± 2                      |
| MalE-I33P     | 6 ± 1                         | 0.47 ± 0.01                 | 75 ± 1                      |
| MalE31        | 0.2 ± 0.1                     | 0.25 ± 0.01                 | 60 ± 1                      |

<sup>a</sup> Amount of MalE in soluble periplasmic fraction normalized to cellular protein content taken from Ref. 11.

<sup>b</sup> First order rate constant and normalized amplitude of the slow refolding phase followed by fluorescence at 25 °C.
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the competition between misfolding and folding.

In regards to periplasmic degradation, the high production level of MalE31 in pop6499 had two important consequences. First, increased amounts of aggregating species increases the formation of inclusion bodies, the compact nature of which renders them resistant to proteases. Such a protective effect is well documented in the literature (25). Second, although over-production of misfolded MalE31 increased the rate of DegP synthesis via the $\sigma^B$ response, the majority of that increased amount of proteas was localized in the insoluble fraction. Because DegP is intimately involved in the degradation of misfolded proteins in the periplasm (3), it is conceivable that DegP co-aggregated with misfolded MalE31 during the formation of inclusion bodies.

A working model for the periplasmic fates of the newly translocated protein is presented in Fig. 7 and used for simulations. Although some aspects are hypothetical, the main features of the model are as follows: (i) The single folding step involved in the formation of the native structure is competing with the initial misfolding step leading to aggregation or degradation. (ii) The misfolding species would originate from the formation of a wrong chain conformation (as an incorrect isomeric state of a peptidyl-prolyl peptide, see below). This isomerization step is followed either by further association steps involved in the formation of inclusion bodies (high order reaction) or by a degradation step of this partially structured and misfolded intermediate. (iii) Although the misfolded protein is assumed to be the only conformation of the protein that is a substrate for proteolytic degradation, correct simulations were obtained with the newly translocated protein as alternative protease substrate.

When $k_{\text{mis}}$ exceeds $k_{\text{fold}}$ (Fig. 7A), either the degradation flux (at low $k_{\text{export}}$) or the aggregation flux (at high $k_{\text{export}}$) is larger than the flux in the folding pathway. On the contrary, when the stationary concentrations $[\text{NewP}]_0$ and $[\text{MisP}]_0$ of the newly translocated protein and misfolded protein correspond, respectively, to the conditions $d[\text{NewP}]/dt = 0$ and $d[\text{MisP}]/dt = 0$. Thus, the steady-state of the system is given by the following equations:

$$[\text{NewP}]_0 = \frac{h_{\text{export}}}{k_{\text{mis}} + h_{\text{fold}}}$$

(Eq. 3)

$$[\text{MisP}]_0 = \frac{h_{\text{deg}} + \sqrt{\delta}}{2k_{\text{agg}}}$$

(Eq. 4)

where $\delta = k_{\text{deg}}^2 + (4k_{\text{agg}}h_{\text{mis}})h_{\text{export}}(h_{\text{fold}} + h_{\text{deg}})$. Finally, the stationary fluxes of the folding, aggregation, and degradation processes are as follows, respectively.

$$\phi_{\text{folding}} = \frac{d[\text{FoldP}]}{dt} = h_{\text{fold}}[\text{NewP}]_0$$

(Eq. 5)

$$\phi_{\text{aggregation}} = \frac{d[\text{AggP}]}{dt} = h_{\text{agg}}[\text{MisP}]_0^2$$

(Eq. 6)

$$\phi_{\text{degradation}} = \frac{d[\text{ProtP}]}{dt} = h_{\text{deg}}[\text{MisP}]_0$$

(Eq. 7)

Numerical simulations of Equations 5, 6, and 7 show the parametric dependence of the partition between folding, aggregation, and degradation. Curves were calculated for two distinct sets of $k_{\text{hid}}$ and $k_{\text{agg}}$ values. A, $k_{\text{hid}} = 0.1 \text{ s}^{-1}$ and $k_{\text{agg}} = 10 \text{ s}^{-1}$; B, $k_{\text{hid}} = 10 \text{ s}^{-1}$ and $k_{\text{agg}} = 100 \text{ s}^{-1}$, using the same values for other rate constants ($h_{\text{fold}} = 1 \text{ s}^{-1}$ and $h_{\text{deg}} = 100 \text{ s}^{-1}$). Combination of Equations 4, 6, and 7 shows that the $k_{\text{export}}$ value for which the relative predominance between aggregation and degradation fluxes becomes inverted is as follows.

$$k_{\text{export}} = \frac{3k_{\text{deg}}^2(h_{\text{mis}} + h_{\text{fold}})}{k_{\text{agg}}h_{\text{mis}}}$$

(Eq. 8)
$k_{\text{fold}}$ is larger than $k_{\text{mis}}$ (Fig. 7B), the flux through the folding pathway always exceeds the rate of formation of misfolded proteins. Hence, the aggregation and degradation fluxes are both lower than the folding flux, whatever the rate of protein translocation. The relative predominance of the degradation flux over the aggregation flux depends on all the rate constant values. The degradation flux predominates at low $k_{\text{export}}$ values. However, when the translocation rate increases, aggregation flux becomes more and more preferential.

This model is very similar to the general model presented by Kiefhaber et al. (26) for the kinetic competition between folding and aggregation, except for the export and degradation steps. Indeed, this outcome was incorporated into the model to support the results described here. Because kinetics of signal sequence processing of both MalE31 (11) and DegP precursors (data not shown) were unaffected by overproduction of misfolded MalE31, export to the periplasm does not constitute a critical step for the periplasmic folding pathway of bacterial proteins. The extent of aggregation is determined by both the rate of gene expression, which is related for MalE to the rate of export, and protein folding or misfolding rates. At high rates of expression, second order aggregation dominates over first order folding. Yet aggregation depends obviously on the existence of cellular proteases. At the misfolding stage a second kinetic competition between degradation and aggregation might occur, and a further increase in the rate of expression (as in the malT background) will favor the aggregation reaction. Obviously, in pop6499 cells the degradation rate of misfolded MalE was found to be much slower than the formation of aggregated bodies. An alternative possibility to balance the relative rates of species (Fig. 1). Under these conditions, the misfolded protein found to be much slower than the formation of aggregated pop6499 cells the degradation rate of misfolded MalE was $k_{\text{mis}}$ values. The degradation flux predominates at low $k_{\text{export}}$ values, whatever the rate of protein translocation. The relative predominance of the degradation pathway always exceeds the rate of formation of misfolded proteins, the bacterial periplasmic protein disulfide isomerases (31). Recently, by using the cellular effect of misfolded MalE31 on the $\omega^P$ response, Missiakas et al. (12) cloned two genes, surA and $\beta$pA, coding for two different families of peptidyl prolyl cis-trans isomerases. We may speculate that these catalysts act by accelerating a rate-limiting folding step on the productive pathway shown in Fig. 7. This possibility, which was not directly addressed in the present studies, is currently under investigation.

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