RseA Binding to the Periplasmic Domain of RseA Modulates the RseA:σE Interaction in the Cytoplasm and the Availability of σE-RNA Polymerase*

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The Escherichia coli σE regulon has evolved to sense the presence of misfolded proteins in the bacterial envelope. Expression of periplasmic chaperones and folding catalysts is under the control of σE RNA polymerase. The N-terminal domain of RseA sequesters σE in the cytoplasmic membrane, preventing its association with core RNA polymerase. The C-terminal domain of RseA interacts with RseB, a periplasmic protein. The relative concentration of σE:RseA:RseB is 2:5:1 and this ratio remains unaltered upon heat shock induction of the σE regulon. Purification from crude cellular extracts yields cytoplasmic, soluble σE RNA polymerase as well as membrane sequestered σE-RseA and σE-RseA:RseB. RseB binding to the C-terminal domain of RseA increases the affinity of RseA for σE by 2- to 3-fold (Kd = 50–100 nm). RseB binds also to the misfolded aggregates of MalE31, a variant of maltose binding protein that forms inclusion bodies in the periplasm. We discuss a model whereby the RseB-RseA interaction represents a measure for misfolded polypeptides in the bacterial envelope, modulating the assembly of σE RNA polymerase and the cellular heat shock response.

Folding and assembly of proteins in the bacterial envelope requires mechanisms and factors that are distinct from those of the cytoplasmic heat shock chaperones GroEL/GroES and DnaK/DnaJ/GrpE (1). Protein misfolding in the cytoplasm is sensed directly by the general heat shock transcription factor σ32 (RpoH) (2). In contrast, two independent pathways have evolved to sense the presence of misfolded polypeptides in the periplasm and in the outer membrane of Escherichia coli (3). The CpxRA system comprises a two-component signal transduction system. It appears that the histidine kinase CpxA senses the presence of misfolded polypeptides in the periplasm, resulting in autophosphorylation and phosphotransfer to the aspartyl of the cytoplasmic CpxR response regulator. CpxR then activates the transcription of various genes that act on the bacterial envelope (3).

The second pathway requires σE, a sigma factor that associates with core RNA polymerase to generate the transcriptionally active αββ′-σE(σE(σE)) complex (4–7). σE RNA polymerase recognizes specific promoters and under heat shock conditions causes a 2- to 3-fold induction of transcription (5, 6). Similar to σ32 regulation, this modest increase in transcriptional activation is presumably due to the high level of basal expression of σE-dependent promoters, because folding catalysts in the bacterial envelope are required at all times, even prior to the exposure to extreme stress. Consistent with this view is the observation that rpoE, the structural gene for σE, is essential for the growth of E. coli under all conditions (8). The activity of σE RNA polymerase is controlled by rseA and rseB (regulator of sigma E), two genes that are encoded within the σE operon. Deletion of rseA leads to a 9-fold increase in transcription of σE-dependent promoters, whereas deletion of rseB results in a 2-fold stimulation (9, 10).

RseA is inserted into the cytoplasmic membrane via a single transmembrane segment, positioning the N-terminal domain in the cytoplasm and the C-terminal domain in the bacterial periplasm. Co-immunoprecipitation and affinity chromatography experiments revealed the existence of interactions between σE and RseA and between RseA and RseB. The N-terminal cytoplasmic domain of RseA alone is sufficient to bind σE and to prevent transcription of σE RNA polymerase (9, 10), presumably by sequestering the sigma factor from RNA polymerase. Gross and co-workers (11) showed that, under conditions of extreme stress, i.e. when misfolded outer membrane proteins accumulate in the bacterial periplasm, RseA may be rapidly degraded, leading to transcriptional activation at σE-dependent promoters. Proteolysis of RseA appears to require DegS (11). DegS is a putative periplasmic protease anchored to the inner membrane (12). Although these results provide an explanation for the regulation of σE RNA polymerase by controlling the stability of the RseA anti-sigma factor, this model does not account for the formation of σE-RseA:RseB complexes.

We report here the intracellular concentration of σE-RseA:RseB, revealing a ratio of 2:5:1. When examined under heat shock, this ratio appeared largely unaltered, and we observed only a modest increase in RseA degradation. Affinity purification experiments revealed the presence of σE-RNA polymerase, σE-RseA, and σE-RseA:RseB complexes. RseA binding to RseB increased the affinity of the anti-sigma factor for σE. Furthermore, RseA-RseB binding sequesters σE in the membrane, preventing the association of the sigma factor with core RNA polymerase. We discuss a model whereby the binding of RseB with a six-histidine tag appended at the C terminus; RseAσE, a variant of σE with a six-histidine tag appended at the N terminus; MalE, maltose binding protein; MalE31, mutant allele of maltose binding protein; Ni-NTA; nickel-nitrioltriacetic acid; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.

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The abbreviations used are: E: αββ′, core polymerase; RpoE and σE, sigma E transcription factor; EσE and αββ′-σE, holoenzyme complexed to sigma E; Rse, regulator of σE; RseA_His, a variant of RseA with a six-histidine tag appended at the C terminus; RseAσE, a variant of σE with a six-histidine tag appended at the N terminus; MalE, maltose binding protein; MalE31, mutant allele of maltose binding protein; Ni-NTA; nickel-nitrioltriacetic acid; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.
to the C-terminal domain of RseA modulates its affinity for $\alpha^E$ and the activity of $\alpha^E$ RNA polymerase.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Strains and plasmids used are listed in Table I. Sequences of primers used in this study can be obtained from the authors upon request. Cells were grown in Luria broth (LB) medium (13) at 30 °C, using the appropriate antibiotic at the following concentrations: ampicillin (100 $\mu$g/ml), spectinomycin (50 $\mu$g/ml), tetracycline (15 $\mu$g/ml), and kanamycin (50 $\mu$g/ml). Induction of $\text{RseA}_{\text{His}6}$ and $\text{RseA}_{\text{His}6}$ was accomplished by addition of arabinose (0.2%) to cultures of $E. coli$ strains harboring PB3 or pDM2232. Mutations were transduced into various backgrounds using the P1 bacteriophage (13).

**Cloning Procedures and Gene Replacement**—Plasmid PB3 encodes $\text{RseA}_{\text{His}6}$, a variant of $\text{RseA}$ with a six-histidine tag appended at the C terminus. The $\text{RseA}$ gene was amplified from the chromosome using primers RseA-3 and RseA-6, introducing a 5'-NcoI site immediately preceding the start codon and a 3'-insertion of six histidine codons followed by a stop codon and a BamHI site. $\text{RseA}_{\text{His}6}$ was cloned into pBAD-A (Invitrogen) cut with NcoI and BamHI, yielding PB3. Plasmid pDM2232 encodes $\text{RseA}_{\text{His}6}$ with a six-histidine tag appended at the N terminus of RseB. RseB was polymerase chain reaction-amplified from the chromosome using primers RpoE-P3 and RseA-5, introducing a 5'-BamHI site and a 3'-EcoRI site, respectively. This polymerase chain reaction product was cloned in vector pBAD-B (Invitrogen) using the BamHI and EcoRI restriction sites. Purified $\text{RseA}_{\text{His}6}$ was cleaved with enterokinase to remove the N-terminal His tag.

**Intracellular Concentration and Fractionation of $\sigma^E$, RseA, and RseB**—$E. coli$ MC4100 or LM1910 were grown at 30 °C or 43 °C to an $A_	ext{opt}$ of 0.6. Culture aliquots were removed and plated on agar medium to count the number of colony forming units per milliliter. Cells of 200-ml culture were collected by centrifugation at 3000 × g for 10 min and washed twice with 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5. Cells were suspended in 20 ml of the same buffer and lysed in a French pressure cell at 14,000 p.s.i. Unbroken cells were removed by centrifugation at 3000 × g for 10 min, and 2% octylglucoside was added to the supernatant. The detergent extract was centrifuged at 100,000 × g for 30 min at 4 °C, and the supernatant was subjected to chromatography on 1 ml of Ni-NTA (Qiagen), pre-equilibrated with buffer A supplemented with 10 mM imidazole, and proteins were eluted with buffer A and 0.5 M imidazole. Eluted proteins were analyzed by SDS-PAGE and immunoblotting with antibodies against $\alpha^E$, RseA, or RseB. For fractionation experiments, cells of 50-ml culture were harvested as described above and suspended in 10 mM HEPES, 66 mM KCl, 10 mM MgOAc, pH 7.6. After lysis in a French pressure cell at 14,000 × g for 10 min, samples were centrifuged at 10,000 × g for 30 min at 4 °C. The supernatant (soluble protein) and sediment (membrane proteins) were separated and analyzed by 12% SDS-PAGE, followed by immunoblotting.

**Pulse-labeling Experiments**—$E. coli$ MC4100 was grown at 30 °C to $A_	ext{opt}$ of 0.7 in M9 minimal media lacking methionine and cysteine. Cells were pulse-labeled for 1 min with 50 $\mu$Ci of $[^{35}S]$methionine/cysteine (Express S35 protein labeling mix, PerkinElmer Life Sciences). Incorporation of radioactive amino acids was quenched by the addition of excess unlabeled amino acids (per milliliter of culture: 100 $\mu$l of 10% casamino acids, 2% methionine, and 2% cysteine). The stress response was initiated by shifting the temperature from 30 to 43 °C. Two kinds of experiments were conducted. Cultures were labeled at 30 °C for 1 min and quenched. Immediately after the addition of the quench (chase), cultures were divided into two equal aliquots. One sample was incubated at 30 °C, whereas the other was placed at 43 °C. During the other experiment, cultures were divided into two equal aliquots prior to labeling. One culture was incubated at 30 °C, labeled for 1 min, and quenched. Another culture aliquot was incubated at 43 °C, labeled for 1 min, and quenched. Labeled cultures were incubated for various amounts of time, and a 100-ml sample was withdrawn and precipitated with trichloroacetic acid. Precipitates were washed in acetone, suspended in 10 ml of 0.1 M Tris-HCl, 4% SDS, pH 7.0, by heating to 95 °C for 5 min. The samples were centrifuged at 13,000 × g for 10 min to remove any insoluble material. A 5-ml aliquot was removed and analyzed in a scintillation counter to account for the total amount of labeled protein. After adjusting for equal counts, aliquots were subjected to immunoprecipitation and samples analyzed by SDS-PAGE and phosphorimaging. Protein half-lives were determined by analyzing data with the exponential decay equation $l = l_0 \times e^{-k t}$, where $l_0$ and $l$ represent the intensity of the radioactive proteins at time 0 and time $t$ after beginning of chase. Experiments were conducted in triplicate each time. To determine whether temperature shift to 43 °C induced the $\sigma^E$ regulon, we examined the synthesis rate of $\beta$-galactosidase driven either by the rpoHPS or the htrL promoters (known $\sigma^E$- and $\sigma^D$-dependent promoters, respectively), as described by Nagai et al. (14) using the anti-$\beta$-galactosidase antibody from 5 Prime → 3 Prime, Inc. (Billerica, MA).

**Table I. Bacterial strains and plasmids**

| Strains        | Relevant characteristics | Source                  |
|----------------|--------------------------|-------------------------|
| MC4100         | $\text{araD139} \Delta(\text{argF-lac})$ U169 | Our collection           |
| LM1910         | $\text{lacZT4} \Delta(\text{galE thi rpsL phoA araD139})$ leu : $\text{Tn10}$ | Our collection           |
| pop6499        | $\text{malT1} \Delta(\text{malE nonpolar deletion})$ | (16)                     |
| BC1            | LM1914 $\Delta\text{rseA} \Delta\text{rseB} \Delta\text{rseC}^-; + \text{5} \text{pDM2232}$ | Our collection           |
| BC4            | Same as BC1 background MC4100 | (Our collection)         |
| BC38           | $\text{rpoE}$ $\Delta\text{rseA} \Delta\text{rseB} \Delta\text{rseC}^-; + \text{5} \text{pDM2232}$ | Our collection           |
| BC39           | $\text{rpoE}$ $\Delta\text{rseA} \Delta\text{rseB} \Delta\text{rseC}^-; + \text{5} \text{pDM2232}$ | Our collection           |
| SR1710         | $\text{RseA} \Delta\text{rseB} \Delta\text{rseC}^-; + \text{5} \text{pDM2232}$ | (5)                     |
| SR1588         | $\text{RseA} \Delta\text{rseB} \Delta\text{rseC}^-; + \text{5} \text{pDM2232}$ | (5)                     |

Plasmids

| pHCM8 | Encoding wild type malE | (16) |
|-------|-------------------------|------|
| pHCM631 | Encoding malE31 | (16) |
| pSR2695 | pOHK2 rseB $^+\text{Kan}^R$ | (10) |
| pBC3 | pBAD-A expressing RseA$m^d$ Amp$^R$ | This study |
| pDM2232 | pBAD-B expressing RseA$m^d$, Amp$^R$ | This study |
RESULTS

Intracellular Concentration of \( \sigma^E \), RseA, and RseB—To measure the cellular concentration of \( \sigma^E \), RseA, and RseB, \( E. coli \) was grown under non-heat shock as well as heat shock conditions. Cells were lyzed and extracted with 2% octylglucoside, and insoluble material was removed by centrifugation. Soluble proteins in the sample were separated on SDS-PAGE and analyzed by immunoblotting using specific antisera. Immunoreactive signals of cell extracts were compared with signals generated by a dilution series of purified protein with known concentration. We measured concentrations of 21 (\( \pm 5 \)) fmoles of RseA, and 10 (\( \pm 3 \)) fmoles of RseB per mg of cellular proteins (numbers in parenthesis represent standard deviations obtained in three independent experiments). Similar ratios (2.5:1) between \( \sigma^E \)-RseA-RseB were measured with \( E. coli \) strains MC4100 and LMG194. Approximately 80 \( \sigma^E \), 200 RseA, and 40 RseB molecules reside in an \( E. coli \) LMG194 cell. Others had recently measured the concentration of \( \sigma^E \) and obtained a similar number (approximately 10 molecules of \( \sigma^E \) per cell) (15). Under heat shock conditions, the ratio of \( \sigma^E \), RseA, and RseB remained unaltered even though the cellular concentration of these polypeptides was increased by 1.5- to 2-fold.

Stability of RseA under Heat Shock Conditions—Previous work reported degradation of pulse-labeled RseA under stress conditions (11). We examined the stability of RseA by growing \( E. coli \) cells (MC4100) in minimal medium, pulse-labeling newly synthesized protein with \( ^{35} \)S-methionine/cysteine, and inducing stress via temperature shift to 43 °C. At timed intervals before and after the heat shock signal, culture aliquots were precipitated with trichloroacetic acid and proteins were solubilized in hot SDS. B, synthesis rate of beta-galactosidase driven from the rpoH3 E-\( \sigma^E \)-dependent promoter (filled squares) or the htrL E-\( \sigma^E \)-dependent promoter (open circles). Cells were grown in minimal medium at 43 °C, and proteins were labeled with \( ^{35} \)S-methionine/cysteine for 1 min and chased with excess unlabeled methionine/cysteine (200 \( \mu \)g/ml) for 1 min prior to trichloroacetic acid precipitation. Immunoprecipitated RseA and beta-galactosidase were separated on SDS-PAGE, and radioactive signals were quantified using a PhosphorImager (Molecular Dynamics). Experiments were performed in triplicate, and results were averaged. A and B show data that are representative of five independent experiments.

Fig. 1. Stability of RseA under heat shock conditions. A, stability of RseA under heat shock (open squares) and non-heat shock (filled circles) conditions. E. coli cells (MC4100) were grown in minimal medium at 30 °C, and newly synthesized proteins were pulse-labeled with \( ^{35} \)S-methionine/cysteine. Culture aliquots were either incubated at 30 °C (non-heat shock) or 43 °C (heat shock). At timed intervals before and after the heat shock signal, culture aliquots were precipitated with trichloroacetic acid and proteins were solubilized in hot SDS. B, synthesis rate of beta-galactosidase driven from the rpoH3 E-\( \sigma^E \)-dependent promoter (filled squares) or the htrL E-\( \sigma^E \)-dependent promoter (open circles). Cells were grown in minimal medium at 43 °C, and proteins were labeled with \( ^{35} \)S-methionine/cysteine for 1 min and chased with excess unlabeled methionine/cysteine (200 \( \mu \)g/ml) for 1 min prior to trichloroacetic acid precipitation. Immunoprecipitated RseA and beta-galactosidase were separated on SDS-PAGE, and radioactive signals were quantified using a PhosphorImager (Molecular Dynamics). Experiments were performed in triplicate, and results were averaged. A and B show data that are representative of five independent experiments.

For affinity measurements, a 50% slurry of Ni-NTA resin was prepared by incubating RseA-GST with a 1 ml suspension of either 50 \( \mu \)l of RseA, 50 \( \mu \)l of RseA-Sepharose or 50 \( \mu \)l of RseA-RseB resin. Samples were incubated for 2 h at 20 °C. Beads were washed exhaustively with 20 column volumes of buffer B to remove unbound material. The concentration of bound RseB was found to be equal to the amount of RseA-GST present on the beads. Increasing concentrations of RseB were added to a 1 ml suspension of either 50 \( \mu \)l of RseA, 50 \( \mu \)l of RseA-Sepharose. Samples were incubated for 2 h at 20 °C and centrifuged at 15,000 × g for 5 min. The supernatant (850 \( \mu \)l) was removed, and proteins were precipitated with trichloroacetic acid. Sediments were washed with acetone, solubilized in 50 \( \mu \)l of 0.1 M Tris-HCl, 4% SDS, pH 7.0, and heated at 95 °C for 5 min. Samples were separated on 12% SDS-PAGE, electrotransferred to a polyvinylidene difluoride membrane, and immunoblotted with alpha-RseA or alpha-RseB antibodies. Immune complexes were detected with 12 nm (wt) protein A, and radioactive signals were compared with a dilution series of purified protein with known concentration. MalE31 Aggregates—\( E. coli \) pol6499 (i.e. strain MC4100 malT1) with a nonpolar deletion of malE, was used as a host for plasmids pHCM1 and pHCM5, encoding wild-type MalE and MalE31, respectively (16). E. coli pol6499 (pHCME1) and E. coli pol6499 (pHCME31) were grown in LB medium supplemented with 0.1 mg/ml ampicillin at 30 °C for 3 h. Cells were harvested by centrifugation, and suspensions were normalized to yield similar A595 nm. The cells were converted to spheroplasts by suspension in 10 mM Tris-HCl, 0.7 M sucrose, 1 mM methylmethanolsulfon fluoride (PMFSF), 5 mM EDTA, and incubation with lysozyme (0.2 mg/ml) for 10 min on ice. Samples were centrifuged, and the supernatant (periplasmic fraction) was separated from the sediment. The spheroplast sediment was washed and lysed by repeated freeze-thawing under hypo-osmotic conditions. Samples were centrifuged, and the supernatant (cytoplasm) was separated from the sediment. Pellets were washed twice using 10 mM Tris-HCl buffer, pH 7.5, and suspended in 0.1 M Tris-HCl, 2% Triton X-100, 1 mM PMFSF, pH 7.5. Samples were incubated at 20 °C for 30 min prior to centrifugation. Supernatants were removed and the pellets (insoluble fraction containing the MalE31 aggregates) were suspended in 10 mM Tris-HCl, pH 7.5. All samples were heated at 95 °C for 10 min and separated on 12% SDS-PAGE. Proteins were electrotransferred to polyvinylidene difluoride membrane and immunoblotted with alpha-MalE (New England BioLabs), alpha-RseA, alpha-RseB, and alpha-DecB antibodies.

Stability of RseA under heat shock conditions. A, stability of RseA under heat shock (open squares) and non-heat shock (filled circles) conditions. E. coli cells (MC4100) were grown in minimal medium at 30 °C, and newly synthesized proteins were pulse-labeled with \( ^{35} \)S-methionine/cysteine. Culture aliquots were either incubated at 30 °C (non-heat shock) or 43 °C (heat shock). At timed intervals before and after the heat shock signal, culture aliquots were precipitated with trichloroacetic acid and proteins were solubilized in hot SDS. B, synthesis rate of beta-galactosidase driven from the rpoH3 E-\( \sigma^E \)-dependent promoter (filled squares) or the htrL E-\( \sigma^E \)-dependent promoter (open circles). Cells were grown in minimal medium at 43 °C, and proteins were labeled with \( ^{35} \)S-methionine/cysteine for 1 min and chased with excess unlabeled methionine/cysteine (200 \( \mu \)g/ml) for 1 min prior to trichloroacetic acid precipitation. Immunoprecipitated RseA and beta-galactosidase were separated on SDS-PAGE, and radioactive signals were quantified using a PhosphorImager (Molecular Dynamics). Experiments were performed in triplicate, and results were averaged. A and B show data that are representative of five independent experiments.
of outer membrane protein. This fulminating degradation of RseA appears to be catalyzed by the periplasmic protease DegS and represents a fascinating mechanism of regulation. However, such dramatic changes in RseA concentration are clearly not required for the induction of the σE regulon under heat shock conditions.

**RseA Promotes Membrane Sequestration of σE**—RseA binding to σE prevents RNA polymerase function in vitro, and deletion of rseA leads to a constitutive activation of the σE-dependent response in vivo (9, 10). We wondered how many σE and RseB molecules were associated with RseA under normal growth conditions. E. coli cells (MC4100) were lysed in a French pressure cell at 14,000 p.s.i., and unbroken cells were removed by slow speed centrifugation. The supernatant was precipitated with trichloroacetic acid and analyzed by immunoblotting (Fig. 2). RseA sedimented with both fractions were precipitated with trichloroacetic acid and analyzed by immunoblotting were precipitated with trichloroacetic acid and analyzed by immunoblotting. In wild-type cells, 27% of RseB and 64% of σE were sequestered in the membrane envelope. As a control, all RseA (cytoplasmic membrane) sedimented during centrifugation (using an Ultracentrifuge), whereas all DsbC (periplasm) remained soluble. Neither RseB nor σE were sequestered within the membrane envelope of rseA mutant E. coli cells. rseB mutants (BC39) contained less sequestered σE (36%) than wild-type cells (64%).

FIG. 2. RseA-mediated sequestration of RseB and σE in the bacterial membrane envelope. E. coli cells (MC4100) were lysed in a French pressure cell, and lysates were centrifuged at 100,000 × g to separate soluble proteins in the cytoplasm and periplasm (S, supernatant) from insoluble proteins in the membrane envelope (P, pellet). Samples were precipitated with trichloroacetic acid and analyzed by immunoblotting with α-RseA, α-RseB, α-σE, and α-DsbC. In wild-type cells, 27% of RseB and 64% of σE were sequestered in the membrane envelope. As a control, all RseA (cytoplasmic membrane) sedimented during centrifugation (using an Ultracentrifuge), whereas all DsbC (periplasm) remained soluble. Neither RseB nor σE were sequestered within the membrane envelope of rseA mutant E. coli cells. rseB mutants (BC39) contained less sequestered σE (36%) than wild-type cells (64%).
wondered whether RseA and RseA-RseB displayed different affinities for σE and asked whether the anti-sigma activity of RseA is modulated by the binding to RseB. RseA His was purified from the crude cellular extracts of E. coli BC1 (rseArseB), and σE was removed by washing the resin with two column volumes of urea-containing buffer, yielding a 95% pure preparation of RseA His chelated to Ni-NTA beads. A 50% slurry of resin was dispensed into buffer containing purified σE, and crude cell extracts were subjected to affinity purification on Ni-NTA resin. Affinity chromatography of extracts from wild-type cells led to the co-purification of RseAHis with σE and RseB but not of RpoA (RNA polymerase α subunit). Deletion of wild-type rseA or rseB did not affect the co-purification of RseAHis with σE. B. E. coli cells (LMG194) were transformed with plasmid pBC1 encoding σE, and crude cell extracts were subjected to affinity purification on Ni-NTA resin. Affinity chromatography of extracts from wild-type cells led to the co-purification of σE with RseA, RseB, and RpoA. Deletion of rseA (BC5) abolished the co-purification of σE with RseB but did not affect the interaction between σE and RpoA.

### Purification of σE-RNA polymerase, σE-RseA, and σE-RseA-RseB complexes

#### A

|          | Wild-type | rseA− | Δ(rseA rseB) |
|----------|-----------|-------|-------------|
|          | L F W E   |       | L F W E     |
| α-RseA   |           |       | α-RseA      |
| α-RseB   |           |       | α-RseB      |
| α-σE     |           |       | α-σE        |
| α-RpoA   |           |       | α-RpoA      |

#### B

|          | Wild-type | rseA− | Δ(rseA rseB) |
|----------|-----------|-------|-------------|
|          | L F W E   |       | L F W E     |
| α-RseA   |           |       | α-RseA      |
| α-RseB   |           |       | α-RseB      |
| α-σE     |           |       | α-σE        |
| α-RpoA   |           |       | α-RpoA      |

RseB Binds the Periplasmic Aggregates of Misfolded MalE31—MalE31 is a mutant maltose binding protein that exhibits the remarkable property of aggregating in the periplasmic space of E. coli, after the translocation of MalE31 across the cytoplasmic membrane and signal peptide processing of the precursor. Expression of MalE31, but not of wild-type MalE, leads to the induction of σE RNA polymerase activity at the rpoH3 promoter, suggesting that the RseAB signaling device may sense the presence of misfolded (aggregated) MalE31 in the periplasmic space (7, 16, 17). To examine whether RseB binds to misfolded polypeptide, we asked whether RseB is sequestered within macromolecular aggregates that formed during overproduction of MalE31. E. coli carrying a knockout mutation of the malE gene was transformed with plasmids that allowed overproduction of either wild-type malE or malE31, respectively. After EDTA- and lysozyme-mediated spheroplasting of E. coli cells, samples were centrifuged, and the periplasmic contents in the supernatant (lane labeled Per, Fig. 5) were separated from the spheroplast sediment. Spheroplasts were lysed by multiple freeze thawing, and soluble proteins were separated by centrifugation. The remaining pellets were analyzed by immunoblotting (lane labeled Mb, Fig. 5), and their membrane protein contents were solubilized by extraction with Triton X-100 and separated from insoluble aggregates by a final centrifugation step (lanes labeled Sol and Ins, respectively, Fig. 5). Wild-type MalE was found soluble in the periplasmic space of fractionated E. coli cells (Fig. 5). In contrast, the periplasmic aggregates of MalE31 remained insol-
RseB Modulates RseA:σE Interactions

FIG. 4. Affinity of RseA<sub>his</sub> and RseA<sub>his</sub>-RseB for σ<sup>E</sup>. Ni-NTA resin precharged with either RseA<sub>his</sub> (0.1 mmol/ml resin, closed triangles) or RseA<sub>his</sub>-RseB (both at 0.1 mmol/ml resin, closed circles) were incubated with purified σ<sup>E</sup>. The binding of σ<sup>E</sup> to RseA<sub>his</sub> or RseA<sub>his</sub>-RseB was assessed by co-sedimentation of σ<sup>E</sup> with Ni-NTA-Sepharose beads during slow speed centrifugation. The concentration of σ<sup>E</sup> in the supernatant was measured by immunoblotting using [125I]protein A and PhosphorImager analysis. Increasing amounts of σ<sup>E</sup> were added to the beads and plotted against the concentration of σ<sup>E</sup> that co-sedimented with Ni-NTA. The inset shows a Scatchard plot analysis of the data, revealing a 1:1 association between σ<sup>E</sup> and RseA<sub>his</sub> both in the presence or absence of RseB. Dissociation constants K<sub>d</sub> 50 nM (σ<sup>E</sup>) RseA<sub>his</sub>-RseB and K<sub>d</sub> 100 nM (σ<sup>E</sup>)RseA<sub>his</sub> were calculated.

FIG. 5. RseB is captured within aggregates of misfolded MalE31. E. coli cells carrying a deletion of malE (popp499) were transformed with plasmids encoding wild-type MalE or MalE31. Celluar fractions were analyzed by immunoblotting with α-RseA, α-RseB, α-MalE, and α-DsbC. During fractionation, E. coli cells were treated with EDTA/lysozyme and centrifuged to separate proteins soluble within the periplasm (Per) from the spheroplast sediment. Spheroplasts were lysed and pellets (Mb) further extracted with Triton X-100 and centrifuged to separate soluble membrane proteins in the supernatant (Sol) from insoluble aggregates in the sediment (Ins). Wild-type MalE and DsbC were found in the periplasmic space, whereas MalE31 formed Triton X-100-insoluble aggregates. RseA sedimenated into the membrane fraction, but could be solubilized by Triton X-100 extraction. RseB was located within the insoluble aggregates of MalE31-producing cells. However, in the absence of MalE31, RseB was located predominantly in the soluble periplasmic- or Triton X-100-extractable fractions.

DISCUSSION

We have examined the role of RseA-RseB in signaling the presence of misfolded polypeptides in the periplasm of E. coli. Our current model predicts that RseA functions as an anti-sigma factor, sequestering σ<sup>BB</sup> from core RNA polymerase (α<sub>BB</sub>β′) (Fig. 6). Calculating the amounts of free and sequestered σ<sup>BB</sup> from the dissociation constant and cellular concentration of σ<sup>BB</sup> and RseA, 99% of the sigma factor should be tethered to RseA. This is clearly not the case as 36% of σ<sup>BB</sup> in wild-type and 64% in rseB mutant cells remained soluble within the bacterial cytoplasm. These soluble σ<sup>BB</sup> molecules are shown to be associated with RNA polymerase (α<sub>BB</sub>β′-σ<sup>BB</sup>) (Fig. 2), and we presume that very little sigma factor exists in an unbound (free) state. Thus, the concentration of soluble sigma factor must be accounted for by the affinity of σ<sup>B</sup> for RseA, RseA-RseB, as well as RNA polymerase core enzyme (α<sub>BB</sub>β′). We assume that σ<sup>B</sup> displays greater affinity for RNA polymerase (α<sub>BB</sub>β′) than for RseA. Overproduction of RseA should shift the physiological balance toward the sequestration of σ<sup>B</sup>, preventing the formation of α<sub>BB</sub>β′-σ<sup>BB</sup> RNA polymerase. This prediction is fulfilled as increased expression of RseA, similar to a

observations on the stability of these factors under non-heat shock and heat shock conditions (Fig. 2; and data not shown). As a control, periplasmic DsbC was not captured within the aggregates of MalE31.
reduction of $\sigma^E$ expression, is toxic for \textit{E. coli} cells, causing the rapid accumulation of suppressor mutations.

To alter the availability of $\sigma^E$ for core RNA polymerase under stress conditions, cells could either modify the affinity of the RseA-sigma factor interaction or reduce the relative concentration of the anti-sigma factor without altering that of $\sigma^E$ or RNA polymerase. In fact, both mechanisms appear to exist. Ades \textit{et al.} (11) reported specific, rapid degradation of all RseA molecules by the DegS protease in response to overproduction of outer membrane proteins. Due to its location in the periplasm, the DegS protease likely cuts the C-terminal portion of RseA, without affecting the N-terminal domain. DegS-initiated degradation is presumably completed by other proteases that cleave the N-terminal sigma factor binding domain of RseA. It appears that the massive degradation of RseA represents a state of extreme stress, because we did not observe massive RseA degradation when \textit{E. coli} cells were exposed to heat shock or overproduction of MalE31. Under these conditions, the overall concentration of $\sigma^E$, RseA, and RseB was unaltered even though transcription from $\sigma^E$-dependent promoters was increased by about 3- to 4-fold, respectively. Our data suggest that the association and disassociation of RseB with the C-terminal domain of RseA modulates the affinity of the anti-sigma factor complex for $\sigma^E$ (Fig. 6). We propose further that RseB binding to misfolded polypeptide affects its ability to interact with the C-terminal domain of RseA, thereby reducing the affinity of the anti-sigma factor and allowing for the association of $\sigma^E$ with core RNA polymerase. If so, one could view RseA-RseB complexes as representing a switch with an affinity for $\sigma^E$ that is equal or slightly above that of the sigma factor for RNA polymerase. Dissociation of RseB from the ternary $\sigma^E$-RseA-RseB complex may flip the switch and promote release of the sigma factor from RseA. In this manner, the $\sigma^E$-RseA-RseB complex may respond rapidly and reversibly to changes in the cellular environment that affect the folding of polypeptides in the bacterial envelope.

Regulatory mechanisms that provide for a cellular response to environmental stress are conserved in other microorganisms. $\sigma^E$ belongs to a family of sigma factors, members of which regulate extra-cytoplasmic functions and are referred to as extra-cytoplasmic factors (18, 19). Most Gram-negative organisms contain one or more \textit{rpoE}RseABC-like operon (20). Extra-cytoplasmic factors are also present in Gram-positive bacteria along with a cognate anti-sigma factor (20–22). \textit{Pseudomonas aeruginosa} regulates the expression of alginate, an extracellular polysaccharide virulence factor, via AlgU/AlgT (RpoE) RNA polymerase (23, 24). AlgU is the first gene of an operon, including \textit{algUnmucABC}, \textit{mucABC} represent homologs of \textit{rseABC} (25, 26). Deletion of \textit{mucA} or \textit{mucB} causes an increase in transcription of AlgU-dependent promoters, resulting in massive expression and secretion of alginate and a hypermucoid colony phenotype (25). Alginate production is not accompanied by a degradation of the MucA anti-sigma factor (27). Future work will need to examine the interaction of $\sigma^E$ with RNA polymerase to reveal the molecular mechanisms underlying the release of $\sigma^E$ from RseA-RseB complexes in the presence of misfolded polypeptide.

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\textit{RseB Modulates RseA: $\sigma^E$ Interactions}