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SurA Assists the Folding of *Escherichia coli* Outer Membrane Proteins

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Many proteins require enzymatic assistance in order to achieve a functional conformation. One rate-limiting step in protein folding is the *cis-trans* isomerization of prolyl residues, a reaction catalyzed by prolyl isomerases. SurA, a periplasmic protein of *Escherichia coli*, has sequence similarity with the prolyl isomerase parvulin. We tested whether SurA was involved in folding periplasmic and outer membrane proteins by using trypsin sensitivity as an assay for protein conformation. We determined that the efficient folding of three outer membrane proteins (OmpA, OmpF, and LamB) requires SurA in vivo, while the folding of four periplasmic proteins was independent of SurA. We conclude that SurA assists in the folding of certain secreted proteins.

The *surA* gene was discovered in a screen designed to identify genes required for survival of *Escherichia coli* during stationary phase (35). Tormo et al. showed that strains lacking *surA* lost plating ability during stationary phase, but they did not address the question of the biochemical function of SurA (35). We have now proceeded to characterize the phenotypes of *surA* mutants in more detail. Although we do not yet understand how the lack of SurA leads to loss of viability during stationary phase, here we present results demonstrating that SurA is involved in folding extracytoplasmic proteins.

As an initial approach to understanding the function of SurA, we began a battery of simple phenotypic tests. The colony morphology of *surA* mutants grown in rich medium was slightly mucoid. The *surA* mutants also exhibited sensitivity to bacitracin, vancomycin, and bile salts. Since *E. coli* is normally resistant to these agents because of the barrier afforded by the outer membrane, these results suggested that SurA functioned in maintaining outer membrane integrity. The *surA* mutants also became misshapen and easily lysed during stationary phase, again consistent with envelope defects.

Concurrently with the initial characterization of the mutant phenotypes, we determined the nucleotide sequence of *surA*. The nucleotide sequence determined was identical to that deposited in GenBank by Yura et al. as part of the *E. coli* genome sequencing effort (accession number D10483) (38). The predicted amino acid sequence of SurA revealed that the first 20 residues constitute a typical secretion signal sequence with a consensus leader peptidase cleavage site (36), suggesting that SurA is a secreted protein. Consistent with this, we found that SurA was released from the cell after osmotic shock (20), indicative of periplasmic localization. We next wanted to sequence the amino-terminal end of SurA to see if the leader sequence was cleaved. To this end, *E. coli* ZK126 (W3110 *tna-2* Δ*lacU169*) carrying pSurA-Eco (a pUC19 derivative with the 5.2-kb *Eco*RI chromosomal fragment carrying *surA*) was osmotically shocked to release periplasmic proteins (20). Ammonium sulfate was added to the extract to 60% saturation. Precipitated proteins were resuspended in and dialyzed against 20 mM Tris, pH 8. The dialyzed proteins were subjected to fast protein liquid chromatography (FPLC) with a MonoQ column (Pharmacia/LBK) and eluted with a 0 to 0.2 M NaCl gradient in 20 mM Tris, pH 8. SurA was eluted at 50 mM NaCl. After desalting with a Pharmacia PD-10 column, the fractions containing the major peak were subjected to the same FPLC protocol. The major peak was then subjected to 10 cycles of Edman degradation. The determined amino acid sequence, Ala-Pro-Gln-Val-Val-Asp-Lys-Val-Ala-Ala, was identical to residues 21 to 30 of the predicted SurA protein sequence. These results demonstrate that mature SurA is a periplasmic protein with a processed N-terminal signal sequence.

An important clue regarding the biochemical function of SurA was suggested by amino acid sequence comparisons (28). SurA and several other proteins were shown to have sequence similarities with parvulin, a newly discovered cytoplasmic peptidyl prolyl isomerase from *E. coli* (23). The periplasmic localization of SurA and the pleiotropic envelope defects of *surA* mutants, coupled with similarity to a peptidyl prolyl isomerase, prompted us to hypothesize that SurA could be involved in folding periplasmic and outer membrane proteins in *E. coli*. Before testing this hypothesis, we constructed a *surA* deletion to avoid the appearance of revertants of the *surA1* allele (a mini-Tn*10* insertion) (35). A 1,496-bp DNA fragment containing most of *surA* and part of the adjacent *pdxA* gene (*Nru*I to *Eco*RV) was deleted from pSurA-Eco, and a kanamycin resistance gene cassette was cloned into the deletion. To introduce the deletion into the chromosome, the resulting plasmid was linearized and electroporated into *E. coli* V355 (*lac gal rpsL recD1014*) (30). Kanamycin-resistant, ampicillin-sensitive transformants were obtained, and the mutant allele was then transduced into ZK126 by using P1*vir*, with selection for kanamycin resistance. The deletion allele was designated Δ*surA3*.

We wanted to determine if SurA assists the folding of outer membrane or periplasmic proteins. To this end we utilized a trypsin sensitivity assay, since unfolded proteins are more sensitive to proteolytic degradation than folded proteins (2). Several periplasmic or outer membrane proteins were individually tested for folding kinetics in both wild-type strains and strains lacking SurA. Bacterial cultures were grown to mid-exponential phase at 37°C (optical density at 600 nm = 0.3) in M63 minimal medium (18) containing 0.2% maltose. Cells were pulse-labeled with 20 μCi of [^{35}S]methionine per ml for 30 s and then chased with an equal volume of medium containing 0.8% unlabeled methionine and 200 μg of chloramphenicol per ml. At specified time points, 1-ml aliquots were transferred to tubes containing 0.1 ml of a prechilled solution containing

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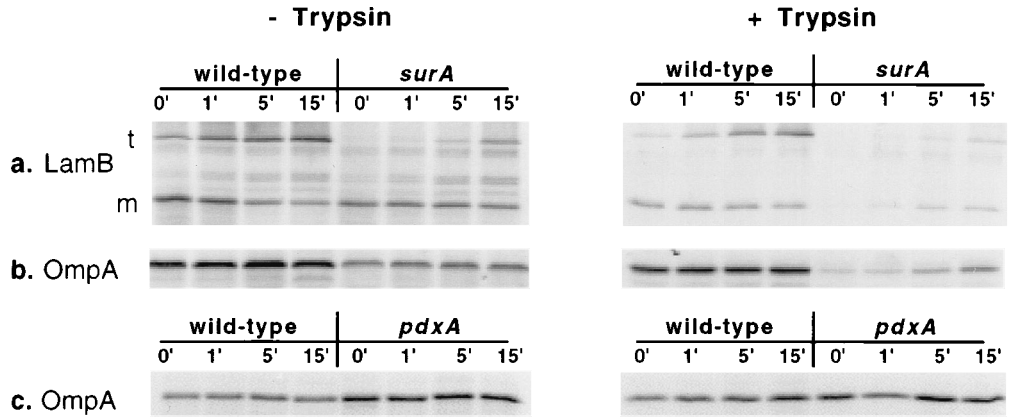

FIG. 1. Trypsin sensitivity of outer membrane proteins. Outer membrane proteins were tested for sensitivity to trypsin. Cultures were grown, labeled, and lysed as described in the text. Specific antisera were then used to immunoprecipitate proteins. (a) LamB immunoprecipitated from ZK126 and ZK126 Δ*surA3* (m, monomer; t, trimer); (b) OmpA immunoprecipitated from ZK126 and ZK126 Δ*surA3*; (c) OmpA immunoprecipitated from ZK126 and ZK126 *pdxA*::Km. Times in minutes are indicated above the lanes.

2% sodium azide and 2 mg of chloramphenicol per ml and then were immediately frozen on dry ice. Cells were permeabilized with 20% sucrose in 15 mM Tris (pH 8)–7 mM EDTA–2.5 μg of lysozyme per ml–0.5% Triton X-100. One-half of each sample was treated with 10 μg of trypsin per ml for 20 min at 4°C, and 1.25 mg of trypsin inhibitor per ml and 0.35 mg of phenylmethylsulfonyl fluoride per ml were then added to all samples. Specific antisera against OmpA (gift of J. Beckwith), OmpF (gift of R. Misra), LamB (gift of T. Silhavy), maltose-binding protein (MBP) (purchased from New England Biolabs), and soluble lytic transglycosylase and the 30- and 49-kDa endopeptidases (gifts of J. Höltje) were then used to immunoprecipitate these periplasmic and outer membrane proteins from the samples (8). After addition of Laemmli loading buffer (13), the samples were boiled for 3 min (except 10 min for OmpA and 10 min at 70°C for LamB) and electrophoresed in sodium dodecyl sulfate-polyacrylamide gels. The gels were dried, and labeled proteins were detected by autoradiography (8).

We first tested the trypsin sensitivity of the outer membrane protein LamB, which is both the receptor for phage λ and the maltodextran porin (25, 33). LamB monomers are assembled into metastable trimers which are stabilized after insertion into the outer membrane (19). In the wild-type strain, total LamB concentrations remain constant during the 15-min chase and are also resistant to trypsin treatment (Fig. 1a). In contrast, significantly fewer LamB trimers were detected in the Δ*surA3* strain even in the absence of trypsin. This suggested that the monomers are not in the proper state to be assembled into trimers. In the presence of trypsin, the amount of detectable monomer is greatly reduced in the Δ*surA3* strain, and stable trimers are detectable only after a 5-min chase. The slow accumulation of folded monomers seen in the trypsin-treated samples of the Δ*surA3* strain is probably due to slow spontaneous prolyl isomerization. Prolyl isomerization is known to occur spontaneously, although the reaction proceeds at a much lower rate than when catalyzed by an isomerase (29).

In order to test if the folding of another outer membrane protein was also affected by the absence of SurA, we assayed the trypsin sensitivity of OmpA (21). Similar to LamB, OmpA is resistant to trypsin in the wild-type strain and sensitive to trypsin in a Δ*surA3* mutant (Fig. 1b). We also tested a third outer membrane protein (OmpF) and obtained similar results (not shown). Because the Δ*surA3* allele is known to be polar on downstream genes, it was necessary to determine if the trypsin sensitivity was due to the polar nature of the mutation. To this end, we tested the trypsin sensitivity of OmpA in a strain (ZK126 *pdxA*::Km) carrying an insertion in the gene immediately downstream of *surA*, *pdxA* (26). No differences between the wild-type strain and ZK126 *pdxA*::Km were detected (Fig. 1c). Thus, the effects on trypsin sensitivity are the result of the lack of SurA and are not due to polar effects of the Δ*surA3* mutation.

Are all secreted proteins improperly folded in a *surA* mutant? This is not the case. Four additional proteins were tested and showed no difference in trypsin sensitivity in the Δ*surA3* mutant (Fig. 2). MBP (Fig. 2c) (11), which is a periplasmic protein required for maltodextran uptake, and the peptidoglycan-hydrolyzing enzymes soluble lytic transglycosylase (Fig. 2b) and the 30- and 49-kDa endopeptidases (Fig. 2a) (9) were all equally stable in the wild-type and Δ*surA3* strains, even after trypsin treatment. Although not all proteins require prolyl isomerization to achieve a mature conformation, studies of the folding kinetics of MBP indicate that two folding intermediates differ in the isomerization state of prolyl residues (3). Thus, the SurA independence of MBP folding is not due to a lack of prolyl residues to isomerize. The three outer membrane proteins tested require the activity of SurA to achieve a fully folded, trypsin-resistant state. In contrast, none of the four periplasmic proteins are affected in the absence of SurA. Determination of whether this is indicative of a dedicated role of SurA in the folding of outer membrane proteins will require a larger sample size.

The selective degradation of unfolded proteins is important for proper functioning of the cell (17). In the periplasm, one enzyme that appears to degrade unfolded proteins is DegP (14, 31, 32). Overproduction of outer membrane proteins leads to the induction of DegP (16). We hypothesized that an increase in unfolded outer membrane proteins might also lead to an increase in DegP. To test this hypothesis, we immunoprecipitated labeled proteins with antisera raised against MBP and DegP (31). The results shown in Fig. 3 indicate that the amount of DegP is increased in the Δ*surA3* mutant. Quantitation of radioactivity in the DegP band (relative to that in the internal MBP control) revealed a sixfold increase in the amount of DegP. This finding suggests that an increase in unfolded proteins in the periplasm induces the expression of *degP*. Transcription of *degP* is controlled by the alternative

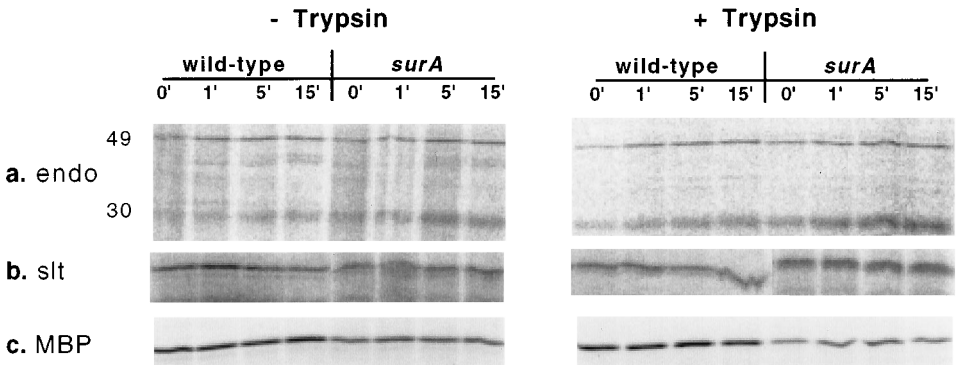

FIG. 2. Trypsin sensitivity of periplasmic proteins. Four periplasmic proteins were tested for sensitivity to trypsin. endo, 30- and 49-kDa endopeptidases; slt, soluble lytic transglycosylase. Times in minutes are shown above the lanes; numbers on the left are molecular masses in kilodaltons.

sigma factor σ^E (6), and Rouviere and Gross indeed found mutations in *surA* in a search for mutations that increased σ^E activity (27). The Cpx signal transduction pathway is known to induce *degP* transcription in response to overproduction of the NlpE lipoprotein but not in response to overproduction of outer membrane proteins (4). It will be interesting to determine if the SurA-dependent induction of *degP* utilizes the Cpx pathway.

We have shown that SurA assists the folding of outer membrane proteins. Protein folding proceeds through a series of intermediates. Some steps between intermediates are spontaneous and rapid, while others are slower and may require the action of folding catalysts (5). Periplasmic and outer membrane proteins translocate across the cytoplasmic membrane in a mostly unfolded state (24). After translocation, these proteins fold, often with enzymatic assistance. An example of posttranslocation folding is the formation of disulfide bonds, which in *E. coli* is catalyzed by DsbA (2). Another slow step in protein folding is prolyl isomerization; therefore, secreted proteins may require the action of periplasmic prolyl isomerases for proper folding (15). SurA may be such an enzyme, given that it has sequence similarity with parvulin, the cytoplasmic prolyl isomerase from *E. coli* (22, 23, 28). SurA is involved in protein folding in vivo, suggesting that it may be a prolyl isomerase. However, we have not been able to demonstrate such an activity in vitro. In addition to SurA, several other proteins have sequence similarity with parvulin (28); among these are NifM, PrsA, and PrtM (10, 12, 37). While none of these proteins have been tested for prolyl isomerase activity in vitro, the phenotypes of cells lacking them suggest that they may play a role in protein folding. The loss of NifM results in the production of inactive nitrogenase in *Klebsiella pneumoniae* (10), PrsA is involved in exoprotein secretion in *Bacillus subtilis* (12), and PrtM is required for proteinase maturation in *Lactobacillus lactis* (37).

The results presented here constitute the first indication of in vivo folding substrates for a member of the parvulin-like family of proteins. The enzymatic activity of prolyl isomerases has been extensively characterized in vitro (29). However, the role that these enzymes play in protein folding in vivo remains poorly understood. The recent demonstrations that human immunodeficiency virus virion particles contain a prolyl isomerase associated with the major viral capsid protein Gag and that the NinaA prolyl isomerase found in the *Drosophila* photoreceptor associates with rhodopsin provided the first indications of possible in vivo substrates for prolyl isomerases (1, 7, 34). Our studies show for the first time that some proteins remain unfolded in the absence of a putative prolyl isomerase. Knowledge of in vivo substrates of prolyl isomerases should provide the foundation for more detailed biochemical analyses of this important class of proteins.

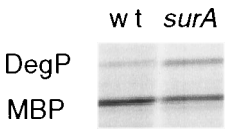

FIG. 3. Periplasmic protease DegP is induced in a *surA* mutant. Cultures of ZK126 and ZK126 Δ*surA3* were grown, labeled, and lysed as described in the text. Labeled proteins were immunoprecipitated with antisera raised against MBP and DegP. wt, wild type.

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