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Demonstration of a Folded Monomeric Form of Porin PhoE of *Escherichia coli* In Vivo

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The porins in the outer membranes of gram-negative bacteria are trimeric proteins. A folded monomeric form of the *Escherichia coli* porin PhoE, with a higher electrophoretic mobility than that of the denatured protein, has recently been detected in in vitro folding studies. To investigate the possible biological significance of the folded monomer, we attempted to detect this form in vivo. After pulse-labeling, folded monomers could be detected by immunoprecipitation. Furthermore, folded monomers were detected in a preparation of mutant PhoE porins, in which the subunit interactions were weakened by a E-66→R substitution. Together, these results show that the folded monomer is not an in vitro folding artifact but an integral part of the native trimer.

The outer membrane of *Escherichia coli* contains several proteins that form pores through which nutrients can pass (12). The functional form of these porins is a trimer. The synthesis of one of the porins, PhoE, is induced under phosphate limitation. Outer membrane proteins are synthesized as precursors with an amino-terminal signal sequence, which is removed during or after translocation through the inner membrane (15). Little is known about the subsequent folding and assembly of these proteins and their insertion into the outer membrane.

In our laboratory, the folding of PhoE protein has been studied after its translation in vitro (6, 22). Besides PhoE trimers, a folded monomeric form of PhoE which could be precipitated with PhoE-specific monoclonal antibodies (MAbs) that recognize conformational epitopes in the native protein was detected. When samples were not heated and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at 4°C and low amperage, this folded monomer migrated faster than did denatured monomers. This aberrant electrophoretic behavior resembles the well-documented heat modifiability of monomeric outer membrane proteins, such as OmpA (7). Heating of the samples at 56°C resulted in denaturation of the folded monomeric form of PhoE, while trimers were resistant to this temperature (6). Heating for 10 min at 100°C resulted in denaturation of all folded forms of PhoE protein. The folded monomeric PhoE form was suggested to represent an assembly intermediate (6). Alternatively, this form could represent an in vitro off-pathway folding artifact (16). In this study, we investigated the possible biological significance of the folded monomer by the detection of this PhoE form in vivo.

Detection of folded PhoE monomers in vivo in pulse-chase experiments. To investigate the possibility that the folded PhoE monomer is an in vivo assembly intermediate of PhoE, pulse-chase experiments were performed. CE1224 cells (19) carrying *phoE* plasmid pJP29 (2) were pulse-labeled, and after various chase periods, aliquots were frozen in liquid nitrogen. Subsequently, immunoprecipitations were performed with the conformation-dependent PhoE-specific MAb PP4-1 (a generous gift of M. Kleerebezem) to detect folded PhoE molecules (6). The use of detergents containing a phenyl group (e.g., Triton X-100) to prevent unspecific precipitation during the procedure was avoided, since they could possibly induce PhoE folding in vitro after the breaking of cells (22). Instead, the detergent Tween 20, which does not induce the folding of PhoE protein in vitro (22), was included in the buffer. After pulse-labeling and immunoprecipitation, folded monomers of PhoE were detected (Fig. 1A). These folded forms had the same electrophoretic mobility as did the folded monomers previously detected in vitro. Furthermore, they showed a similar temperature sensitivity in that they were stable in sample buffer at room temperature (Fig. 1A, lanes a) and denatured at 56°C (Fig. 1A, lanes b). No proteins migrating to the position of PhoE trimers were detected in the samples that had not been heated before electrophoresis. These trimers are probably present in aggregates that are visible near the top of the gel and that fall apart in individual trimers after heating the samples at 56°C. The amount of folded PhoE monomers decreased during the longer chase periods (Fig. 1A), suggesting that they represent assembly intermediates. However, it cannot be excluded that the folded monomers observed after the incubation of samples at room temperature prior to SDS-PAGE were actually generated by dissociation from metastable trimers, as previously described for OmpF and LamB (9, 11). Thus, the observed chase of the folded PhoE monomers may not be due to a trimerization reaction but to stabilization of the trimers. This possibility might explain the observation that the folded monomers were chased away with very slow kinetics. Scanning the autoradiogram revealed that the amount of folded monomers decreased from 32% of the total amount of PhoE detected directly after the pulse to 6% after a 30-min chase (Fig. 1B). Nevertheless, the pulse-chase experiments strongly suggest that the folded monomeric form of PhoE that was earlier detected in in vitro folding experiments (6) does exist in vivo, either as an assembly intermediate on its own or as part of a metastable trimer.

Dissociation of recombinant PhoE trimers into folded monomers. The three-dimensional structure of the *E. coli* porins OmpF and PhoE has been solved (5). Each monomer of these porins consists of a β-barrel structure composed of 16 β-strands, which span the outer membrane. Short turns align the channel at the periplasmic side, and large loops are exposed to the exterior of the cell. The third loop, L3, is buried into the pore, forming a constriction zone at half the height of

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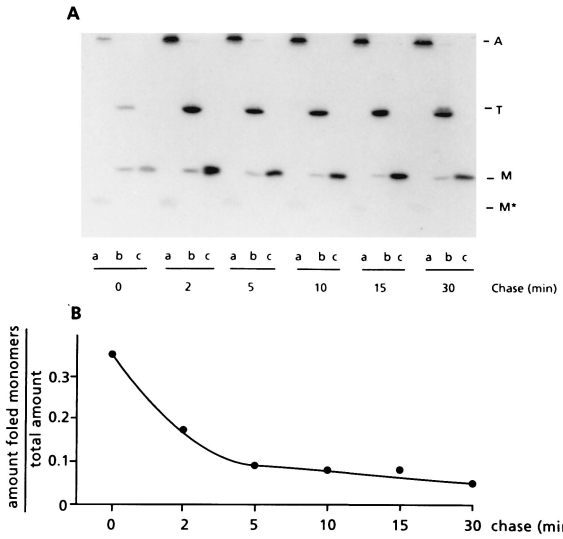

FIG. 1. (A) Pulse-chase experiment. *E. coli* CE1224 cells containing pJP29 were induced under phosphate limitation in a synthetic medium (18) for 5 h at 37°C. After pulse-labeling with [^{35}S]methionine for 30 s, the culture was chased with an overload of nonradioactive methionine (2). At the indicated time points, samples of 600 μl were immediately frozen in liquid nitrogen. Upon defrosting, 600 μl of a twofold-concentrated immunoprecipitation buffer described previously (6), except that Triton X-100 was replaced by Tween 20, was added together with 2 mg of protein A-Sepharose CL-4B and 1 μl of ascites fluid containing the PhoE-specific MAb PP4-1. After 1 h of incubation at room temperature, pellets obtained after 20 s of centrifugation in a microcentrifuge were washed twice with the immunoprecipitation buffer containing Tween 20 and once with a buffer containing sodium deoxycholate (6). Pellets were incubated for 4 min in 0.2 M glycine-HCl (pH 4) to release the PhoE molecules from the antibodies. Samples were divided in three parts and incubated for 10 min at room temperature (lanes a), 56°C (lanes b), and 100°C (lanes c) prior to SDS-PAGE at 4°C and 20 mA. The positions marked A, T, M, and M* indicate aggregates, trimers, monomers, and folded monomers, respectively. (B) Quantification of the results. The bands of the denatured monomers (panel A, lanes c), representing the total amount of PhoE synthesized, and of the folded monomers (panel A, lanes a) were scanned with an LKB 2222-010 ultrascan-XL laser densitometer. The percentage of PhoE present in the folded monomeric form at each time point indicated is plotted.

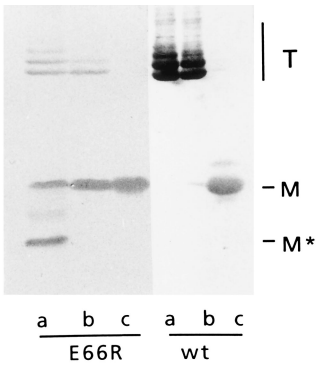

FIG. 2. Immunoblotting of mutant and native trimers isolated from the cell envelope. After overnight growth of CE1265 cells (8) containing plasmid pVG28 (encoding E66R mutant PhoE) or pJP29 (encoding wild-type [wt] PhoE) in L broth (19) at 37°C, cell envelopes were isolated. Peptidoglycan-associated proteins were extracted from cell envelopes and incubated for 10 min at room temperature (lanes a), 56°C (lanes b), and 100°C (lanes c) prior to SDS-PAGE at 4°C and 20 mA. After electrophoresis, proteins were electroblotted (20) and detected simultaneously with the PhoE-specific, conformation-dependent MAb PP4-1 and with MAb Me2-1 (generous gift of M. Kleerebezem), which recognizes denatured PhoE protein. The positions marked T, M, and M* indicate trimers, monomers, and folded monomers, respectively.

the channel, while the loop L2 is bended over toward a neighboring subunit, causing interactions with loop L3 and basic residues in the channel wall. One of these intersubunit interactions is a salt bridge between glutamate 66 in loop L2 and arginine 93 in the barrel wall. Because of numerous hydrophobic and hydrophilic subunit interactions, the PhoE trimers are very stable and harsh conditions (e.g., >75°C in 2% SDS) are required to dissociate them. Such conditions would also denature the folded monomeric form. Consequently, folded monomers have never been detected in preparations of wild-type PhoE. To verify whether the folded monomeric form detected on a gel is an integral part of the trimers, we attempted to weaken the subunit interactions. By site-directed mutagenesis, the Glu-66 residue was replaced by an arginine (E-66→R [E66R]), thereby disrupting the interaction with residue 93 of the neighboring monomer. First, an *Eco*RV fragment of plasmid pJP29 (2) was cloned (14) into *Sma*I-digested vector pTZ19U$_{rrh}$ (13), resulting in plasmid pVG26. Single-stranded DNA from pVG26 was used as a template in site-directed mutagenesis (13) with a mutagenic oligonucleotide and an origin repair oligonucleotide. The resulting mutant plasmid, pVG28, codes for the PhoE protein with the E66R substitution. This mutant PhoE pore protein was still assembled in the outer membrane since it could be detected on intact cells in an enzyme-linked immunosorbent assay (21) with the conformation-dependent MAb PP4-1 (results not shown). Cells expressing the mutant protein were resistant to the PhoE-specific phage TC45 (4), which confirms the supposition that loop L2 is part of the phage receptor site (3). Trimers were isolated from the outer membrane as previously described (1). Briefly, after ultrasonic disintegration of cells, membranes were collected by centrifugation, dissolved in a buffer containing 2% SDS, and incubated for 45 min at 40°C. Peptidoglycan-associated outer membrane protein complexes were collected by centrifugation, and PhoE was extracted by incubation in the same buffer, supplemented with 0.6 M NaCl, for 45 min at 40°C. Extracted proteins were concentrated by precipitation in ethanol and subsequently analyzed by SDS-PAGE and immunoblotting. When the preparation of the E66R mutant protein was incubated at room temperature prior to electrophoresis, folded monomers besides trimers could be detected (Fig. 2, lane a). An additional, faint band visible between the denatured and folded monomers (Fig. 2, lane a) could represent another folding intermediate or a folded monomer in association with lipopolysaccharide. No folded monomers were detected in the wild-type PhoE samples at room temperature. Apparently, the mutation indeed affected subunit interactions important for trimer integrity. These results show that the mutant PhoE trimers, extracted from cell envelopes, partially dissociate into monomers that retain tertiary structure. Apparently, the folded monomer previously detected in vitro is an integral part of the native trimer.

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