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Glycine-144 is required for efficient folding of outer membrane protein PhoE of *Escherichia coli* K12

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Short stretches of similar sequences have been detected in unrelated bacterial outer membrane proteins (Nikaido and Wu (1984) Proc. Natl. Acad. Sci. USA 81, 1048–1052). In the most pronounced similarity region, only a glycine residue is absolutely conserved. To investigate whether this glycine residue is essential for outer membrane incorporation, oligonucleotide-directed mutagenesis was applied to replace this residue, i.e. Gly-144, as well as two other Gly-residues in pore protein PhoE. Substitution of Gly-52 and Gly-258 by Ala and Val, respectively, did not influence outer membrane incorporation. However, the substitution of Gly-144 by Leu affected the efficiency of outer membrane incorporation. After in vitro synthesis this mutant protein was less efficiently precipitated with monoclonal antibodies that recognize conformational epitopes than wild-type PhoE, showing that the mutation interferes with correct folding of the protein.

Outer membrane protein; Protein sorting; Protein folding; Site-directed mutagenesis; PhoE protein; *Escherichia coli*

1. INTRODUCTION

Exported proteins in Gram-negative bacteria are synthesized with an N-terminal signal sequence. The signal sequence is essential for translocation across the cytoplasmic membrane but it does not contain sorting information required for the ultimate localization in the cell envelope [1]. The nature of sorting information in outer membrane proteins seems to reside in the overall conformation of the protein, rather than in a short stretch of amino acid residues [2,3]. Nevertheless, short stretches of similarity have been observed in the primary sequences of unrelated outer membrane proteins [4], suggesting that they may contain sorting information. Some evidence for the involvement of the most pronounced similarity region in the sorting process has been obtained in case of OmpA protein [5]. Mutant proteins in which segments including amino acids 160–172 were deleted, accumulated in the periplasm whereas deletions elsewhere in the protein prevented proper insertion in, but not association to the outer membrane. Sequence comparisons have recently been updated [6] and it appears that only a glycine residue, corresponding to Gly-144 in PhoE protein, is absolutely conserved in this segment.

PhoE protein of *Escherichia coli* is a pore-forming outer membrane protein. The native form of the protein is a trimer. The synthesis of the protein is induced when cells are grown under phosphate limitation [7]. According to a model proposed for the topology of PhoE, the protein traverses the outer membrane 16 times mostly as amphipathic β-sheets [8]. Gly-144 is located in this model at the periplasmic side of the membrane and might be involved in the formation of a reverse turn. In the present study, we have investigated the role of Gly-144 in the biogenesis of PhoE. This residue and two other glycine residues which may also be involved in the formation of reverse turns at the periplasmic side of the membrane were replaced by turn-blocking residues and the effect of these mutations on PhoE biogenesis was studied.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains, phages and growth conditions*

E. coli K-12 strains CE1224 [1] and CE1248 [9] are deleted for the *phoE* gene and do not produce OmpF and OmpC proteins due to *ompR* mutations. Strain CE1248 contains in addition a *phoR* mutation, resulting in constitutive expression of the *pho* regulon. Phage TC45*hr*N3 [9], which uses PhoE protein as receptor, is from our laboratory stock. Phage sensitivity of strains was determined by testing for plaque formation. Unless otherwise indicated, cells were grown under aeration at 30°C in yeast broth or in a synthetic medium in which the phosphate concentration can be varied [10]. When necessary, the media were supplemented with chloramphenicol (25 μg/ml).

2.2. *DNA techniques and plasmids*

Plasmid DNA was prepared as described by Birnboim and Doly [11], followed by CsCl/ethidium bromide isopycnic centrifugation. Recombinant DNA techniques were performed essentially as described by Maniatis et al. [12]. For site-directed mutagenesis, mutant identification and sequencing the same methods were used as previously [13]. The sequence analysis revealed no other mutations than the

desired substitutions, i.e. codon 52 from GGT to GCT, codon 144 from GGC to CTC and codon 258 from GGT to GTT (where codon 1 corresponds to the codon for the first amino acid of mature PhoE). The mutations resulted in the substitution of the Gly residues by Ala, Leu and Val residues, respectively. The mutations were introduced into the PhoE expression plasmid pJP29 [3] by exchanging the corresponding restriction fragments. The plasmids were designated as pJP29-A52, pJP29-L144 and pJP29-V258, respectively.

pJP370 was created by inserting a cartridge in pJP29 [14]. It encodes a PhoE protein in which the signal sequence is replaced by only two residues, Met and Ser. Similarly, this cartridge was inserted into pJP29-L144, yielding pJP371.

2.3. *Isolation and characterization of cell fractions*

Cell envelopes were isolated by differential centrifugation after ultrasonic disintegration of the cells [15]. The accessibility of proteins in cell envelope fractions for trypsin was tested as described previously [16]. The protein patterns were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE [15].

2.4. *Pulse-label and pulse-chase experiments*

Cells, grown under phosphate starvation, were labelled with [^{35}S]methionine for 30 s at 30°C and subsequently chased with an excess of non-radioactive methionine as described [3].

To measure the kinetics of assembly of PhoE into a trypsin-resistant form, cells were radiolabelled for 30 s at 25°C. After a chase period at the same temperature, samples were rapidly frozen in liquid nitrogen. The cells were pelleted by centrifugation and resuspended in 0.5 ml 100 mM Tris-HCl (pH 8.0), 5 mM EDTA, 2.5% Triton X-100 and 50 μg Trypsin (Serva). The suspension was kept on ice for 30 min. Subsequently, a 3-fold excess of Trypsin Inhibitor (Serva) was added. The pellet obtained after 10 min centrifugation in an Eppendorf centrifuge was resuspended in sample buffer and incubated for 30 min at 37°C. The samples were analysed by SDS-PAGE and autoradiography.

2.5. *In vitro synthesis, immunoprecipitation and trimerization*

Conditions for the in vitro synthesis of PhoE proteins, the outer membrane-induced trimerization and immunoprecipitation with monoclonal antibodies (mAbs) which recognize conformational epitopes [17], were as described [18].

3. RESULTS

3.1. *Expression of mutant proteins*

The plasmids pJP29-A52, pJP29-L144 and pJP29-V258, encoding mutant PhoE proteins with substitutions of the Gly residues in positions 52, 144 and 258 by Ala, Leu and Val, respectively, were constructed as described in section 2.2. The plasmids were introduced into *phoR* strain CE1248. Cell envelopes of cells grown overnight at 30°C in yeast broth were isolated and analysed by SDS-PAGE (Fig. 1). Whereas the amounts of the pJP29-A52 and pJP29-V258 encoded proteins are comparable to the wild type PhoE protein level, there was substantially less pJP29-L144 encoded PhoE protein present in the cell envelopes. This effect was even more pronounced when cells were grown at 42°C (Fig. 1).

To investigate whether the reduced amount of the L144-mutant protein was due to reduced synthesis, pulse-label and pulse-chase experiments were performed. CE1224 cells, carrying the pJP29 derivatives, were grown under phosphate limitation and radio-labelled

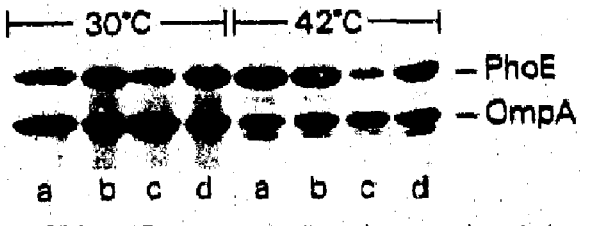

Fig. 1. SDS-PAGE patterns of cell envelope proteins of plasmid-containing derivatives of strain CE1248 after growth of the cells at 30°C or 42°C. Plasmids present were: (a) pJP29; (b) pJP29-A52; (c) pJP29-L144 and (d) pJP29-V258. The position of the wild-type PhoE protein is indicated.

for 30 s, followed by 0, 3, or 10 min chase period. The radiolabelled proteins were analysed by SDS-PAGE and autoradiography. No differences in the amounts of the mutant proteins and the wild-type PhoE protein were observed at any of these time points (not shown). Apparently, the mutations do not affect the synthesis of the proteins. The reduced amount of the pJP29-L144 encoded protein in the cell envelope fraction of an overnight culture (Fig. 1, lane c) must therefore be explained by slow partial proteolytic degradation in the cell. The processing kinetics of wild-type and mutant PhoE proteins were all similar (results not shown), suggesting normal export from the cytoplasm.

Derivatives of strain CE1248 carrying pJP29, pJP29-A52, pJP29-L144 and pJP29-V258 were all sensitive to the PhoE-specific phage TC45*hr*N3. However, cells expressing the L144-mutant protein gave turbid plaques. This is in agreement with the reduced amount of this mutant protein, detected in the cell envelope preparations (Fig. 1). Nevertheless, these experiments show that the mutant proteins are correctly incorporated into the outer membrane. This result was confirmed in ELISA experiments [19], which showed that the binding of three mAbs, PP1-4, PP2-1 and PP3-4 [17,19] which recognize conformational epitopes on the cell-surface exposed part of PhoE protein, to intact cells was not affected by the mutations.

3.2. *Kinetics of outer membrane assembly*

Wild-type PhoE protein, imbedded as a trimer in the outer membrane, is protected against trypsin activity [20]. Trypsin treatment of the cell envelopes, shown in Fig. 1, demonstrated that the mutant proteins are also resistant to the protease in this conformation (not shown). Thus, possible effects of the glycine replacements on the kinetics of outer membrane incorporation can be studied by testing the trypsinsensitivity of the mutant proteins in pulse-chase experiments. CE1224 cells carrying pJP29, pJP29-A52, pJP29-L144 and pJP29-V258 were radio-labelled for 30 s at 25°C. This low temperature was chosen to slow down the very rapid assembly process. After different chase periods with non-radioactive methionine, the cells were lysed and incubated with trypsin as described in section 2.

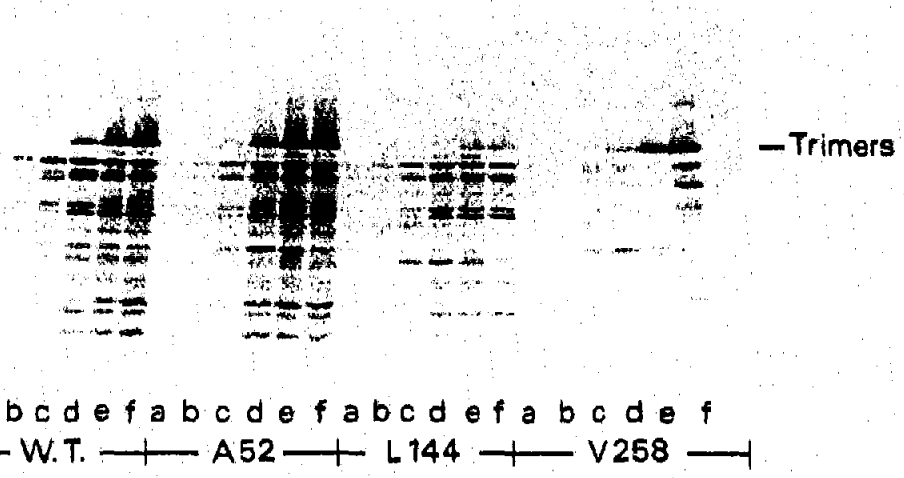

Fig. 2. Autoradiogram of [^{35}S]methionine labelled proteins from cells of strain CE1224 separated by SDS-PAGE. Plasmids present were pJP29, pJP29-A52, pJP29-L144 and pJP29-V258. The cells were starved for phosphate and pulse-labelled with [^{35}S]methionine for 30 s. The pulse was followed by a chase period of 0 s, 15 s, 45 s, 2 min, 8 min and 30 min in lanes a, b, c, d, e and f, respectively. Subsequently the cells were lysed and incubated with trypsin. The samples were solubilized in sample buffer for 30 min at 37°C. The position of the PhoE trimers is indicated.

Proteins were analysed by SDS-PAGE and autoradiography (Fig. 2). After a 2 min chase period, small amounts of the proteins encoded by pJP29, pJP29-A52 and pJP29-V258 were protected against trypsin activity and present in a trimeric conformation. The amount of protected trimers increased during the 8 and 30 min chase periods. The assembly of the pJP29-L144 encoded protein appears to be retarded. Only after an 8 min chase period a small amount of this protein was found to be protected against the protease activity. This amount increased slightly during a 30 min chase period.

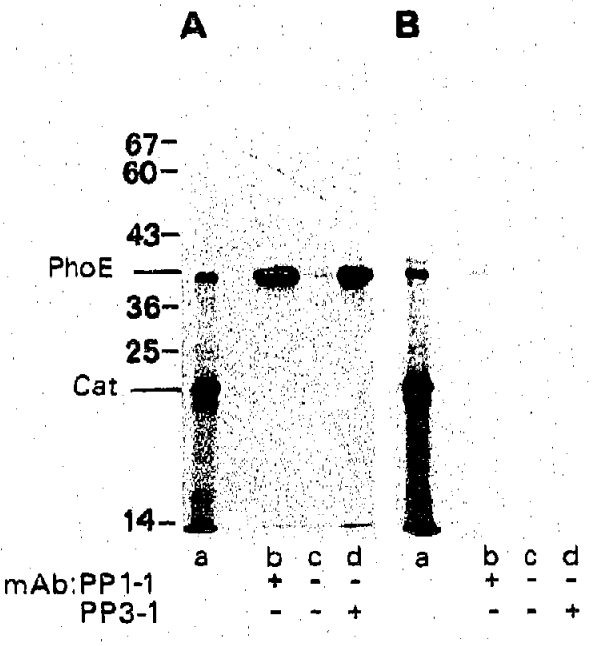

Fig. 3. Immunoprecipitations of wild-type (Panel A) and L144-mutant PhoE protein (Panel B). Plasmids pJP370 and pJP371 were used to direct the synthesis of quasi-mature PhoE proteins. Samples of 1 μl are shown in lanes a. Samples of 40 μl were used for immunoprecipitations with mAbs PP_{1-1} (lanes b) or PP_{3-1} (lanes d) or in the absence of PhoE-specific antibodies (lanes c). At the left, the positions of molecular mass standard proteins (in kDa) and of PhoE and chloramphenicol transacetylase (Cat) are indicated.

3.3. *In vitro folding of L144-mutant protein*

Recently, we have developed a system to study the folding and trimerization of PhoE protein in vitro [14,18]. In this system, the folding of in vitro synthesized PhoE protein is probed with mAbs which recognize conformational epitopes. The immuno-precipitated PhoE represents a folded monomer. The addition of outer membranes to the in vitro system is required to induce trimerization. This in vitro system was applied to study the nature of the assembly defect of the L144-mutant protein. Since the signal sequence interferes with the efficiency of folding [18], plasmids pJP370 and pJP371 were constructed (see section 2.2.) and used to direct the synthesis of quasi-mature forms of wild-type and L144-mutant PhoE in vitro (Figs 3A and B, lane a). The mutant protein was immunoprecipitated with the two different mAbs tested, but the efficiency was much reduced (7%) as compared to the wild type protein (Figs 3A and B, lanes b and d). This shows that the Gly-144→Leu substitution interferes with the folding of the monomer. Outer membrane-induced trimerization was apparently not affected by the mutation, since the amounts of wild-type and mutant trimers were proportional to the amount of folded monomers detected (result not shown).

4. DISCUSSION

We have shown that Gly-144 of PhoE protein, which is highly conserved in a short stretch of sequence

similarity between different outer membrane proteins [6], is essential for efficient outer membrane incorporation. However, it seems unlikely that this residue is part of a sorting signal in the primary sequence. Rather, it appears that the Gly-144→Leu substitution interferes with the folding of the protein, and thus prevents efficient outer membrane incorporation. The turn-promoting capability of glycine residues may be important in this respect. However, it should be noted that the substitution of two other glycine residues, which are also supposed to be involved in the formation of reverse turns at the periplasmic side of the membrane, did not affect outer membrane incorporation significantly. Therefore, Gly-144 seems to be of special importance for PhoE-folding.

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