The EMBO Journal vol.11 no.1 pp.57-62, 1992

Identification and characterization of an *Escherichia coli* gene required for the formation of correctly folded alkaline phosphatase, a periplasmic enzyme

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Communicated by W.Neupert

Tn*5* insertion mutations of *Escherichia coli* were isolated that impaired the formation of correctly folded alkaline phosphatase (PhoA) in the periplasm. The PhoA polypeptide synthesized in the mutants was translocated across the cytoplasmic membrane but not released into the periplasmic space. It was susceptible to degradation by proteases *in vivo* and *in vitro*. The wild-type counterpart of this gene (named *ppfA*) has been sequenced and shown to encode a periplasmic protein with a pair of potentially redox-active cysteine residues. PhoA synthesized in the mutants indeed lacked disulfide bridges. These results indicate that the folding of PhoA *in vivo* is not spontaneous but catalyzed at least at the disulfide bond formation step.

Key words: alkaline phosphatase/disulfide bond formation/periplasmic protein/protein disulfide isomerase/protein folding

Introduction

Translocation of newly synthesized secretory proteins across the membrane occurs without acquisition of the final tertiary and quaternary structures (Randall and Hardy, 1986), probably in an extended conformation (Rassow *et al.*, 1990). Recent studies indicate that the post-translocational protein folding that occurs within the mitochondrial matrix space is facilitated by molecular chaperones, the mitochondrial Hsp60 and Hsp70 (Ostermann *et al.*, 1989; Kang *et al.*, 1990). Chaperone-like factors in the *trans* side of the mitochondrial or endoplasmic reticulum membrane could facilitate not only the folding reaction but also the translocation reaction itself (Kang *et al.*, 1990; Vogel *et al.*, 1990). Although protein translocation across the bacterial cytoplasmic membrane has been studied extensively both genetically (for a review see Schatz and Beckwith, 1990) and biochemically (for a review see Wickner *et al.*, 1991), little is known about factors that act from the periplasmic side. Also, it is not known whether the folding-assembly of proteins in the periplasmic space is assisted by some catalyst.

Alkaline phosphatase (PhoA) of *Escherichia coli* is one of the most extensively studied periplasmic proteins (for a review see Coleman and Gettins, 1983). It is a zinc metalloprotein consisting of two identical subunits encoded by the *phoA* gene. Each subunit contains two intramolecular disulfide bridges. Biogenesis of this enzyme thus includes the synthesis of a precursor molecule with an N-terminal 22 residue signal sequence, translocation across the cytoplasmic membrane, removal of the signal sequence, folding of the polypeptide chain, disulfide bond formation, incorporation of Zn^{2+} and dimerization, not necessarily indicating the order. The final product of PhoA is resistant to proteolytic enzymes including trypsin (Roberts and Chlebowski, 1984). In contrast, PhoA molecules internalized in the cytoplasm due to a defect in the signal sequence or attachment to a cytoplasmic domain of other protein are enzymatically inactive (Boyd *et al.*, 1987) and susceptible to trypsin degradation (Akiyama and Ito, 1989). Apparently, export to the periplasm is necessary for this protein to be folded correctly, a feature that makes PhoA useful as a reporter of protein topogenesis (for a review see Manoil *et al.*, 1990).

In the work reported in this study, we attempted to isolate mutants in which the PhoA biogenesis is aborted at a post-translocational step. A gene termed *ppfA* has thus been identified, and shown to contain a potential redox-active site sequence, Cys-Pro-His-Cys. PhoA synthesized in the *ppfA* mutants was sensitive to proteases both *in vivo* and *in vitro* and lacking the disulfide bridges. The disulfide bond formation of PhoA and possibly other proteins in the bacterial periplasmic space appears to be catalyzed by the *ppfA* gene product.

Results

Tn5 insertion mutations that lower PhoA activity

To isolate mutants in which the PhoA biogenesis is impaired due to a defect at a post-synthesis step, we first mutagenized a *phoA* deletion strain (KS272) by random transposition of Tn*5*. A mixture of mutagenized cells were then transformed with plasmid pKY192 carrying a *bla* (for β-lactamase)-*phoA* gene fusion. Transformant colonies on a selective agar medium containing a chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (XP) were screened for those with reduced PhoA activities, by looking for pale blue or white colony color. We used the fusion gene which was under the control of the constitutive *bla* promoter to minimize the possibility of obtaining mutants affected at the transcription level. After elimination of mutants with alterations in the plasmid or with too subtle reduction in the enzyme activity, we obtained two mutants whose PhoA enzyme activities were ~30% of the wild-type value. The Tn*5* insertions in these mutants were then transduced into the parental strain carrying pKY192 as well as into a *phoA*$^+$ *phoR*$^-$ strain (SM138) with no plasmid. In both cases, transductants that received the Tn*5* were shown to have PhoA enzyme activities reduced again by ~70%. Genetic crosses using Hfr strains suggested that both Tn*5* insertions were located at 80-90 min on the chromosome. These results established that the Tn*5* insertions [termed *ppfA-33*::Tn*5* and *ppfA-101*::Tn*5*; *ppf* for *p*eriplasmic *p*hosphatase (or *p*rotein) *f*ormation] were responsible for the reduced PhoA activities. The mutational

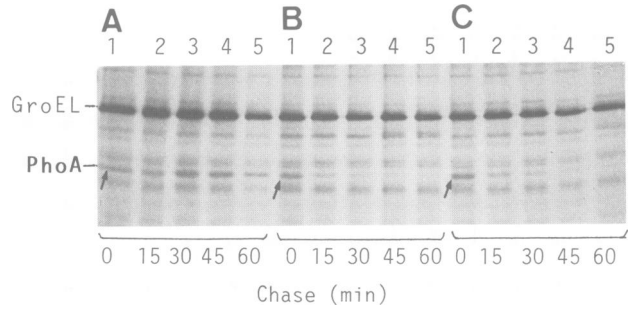

Fig. 1. Synthesis and *in vivo* stability of PhoA. Cells of SM138 (**A**, *ppfA*$^+$), SK33 (**B**, *ppfA-33*::Tn*5*) and SK101 (**C**, *ppfA-101*:Tn*5*) were pulse labeled with [^{35}S]methionine for 2 min followed by chase with unlabeled methionine for the indicated period (min). Samples were immediately treated with trichloroacetic acid and subjected to immunoprecipitation with a combination of antisera against the GroEL protein and PhoA. Shown are autoradiograms after SDS−PAGE.

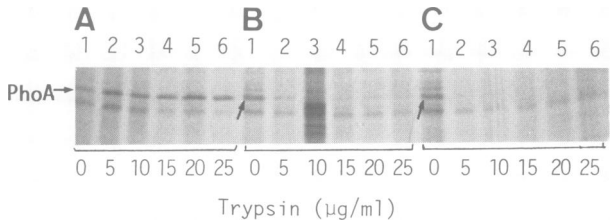

Fig. 2. Trypsin sensitivity of pulse labeled PhoA. Cells of SM138 (**A**), SK33 (**B**) and SK101 (**C**) were pulse labeled with [^{35}S]methionine for 2 min and subjected to freezing and thawing in the presence of lysozyme. Cell lysates were then treated with trypsin of indicated concentrations, and immunoprecipitated with anti-PhoA serum.

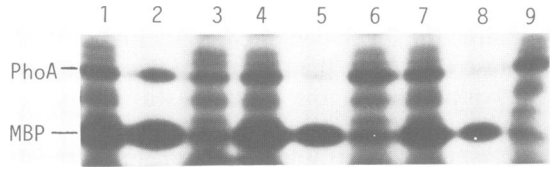

Fig. 3. Localization of pulse-labeled PhoA and maltose binding protein. Cells of SM138 (lanes 1−3), SK33 (lanes 4−6) and SK101 (lanes 7−9) were grown with glycerol and maltose as carbon sources, pulse labeled with [^{35}S]methionine for 2 min, and fractionated into spheroplast (lanes 3, 6 and 9) and periplasm (lanes 2, 5 and 8) fractions following lysozyme−sucrose−EDTA treatment. Equivalent amounts of unfractionated samples were directly treated with trichloroacetic acid (lanes 1, 4 and 7). Each fraction, after trichloroacetic acid precipitation, was immunoprecipitated with antisera against PhoA and maltose binding protein.

effects were independent of whether the promoter, the translation initiation site, or the signal sequence was derived from *bla* or *phoA*. In all the subsequent experiments, the *phoA*$^+$ *phoR*$^-$ *ppfA*::Tn*5* transductants were used as mutant strains.

Aberrant forms of PhoA are produced in the ppfA mutants

To examine the synthesis and translocation of PhoA, the wild-type and the *ppfA* mutant cells were pulse labeled with [^{35}S]methionine for 1 or 2 min, directly treated with trichloroacetic acid followed by solubilization with SDS and immunoprecipitation. Both of the *ppfA* mutants gave almost exclusively the mature form of PhoA whose differential synthesis rates did not significantly differ from that in the wild-type cells (Figure 1, compare lanes A1, B1 and C1). Thus, PhoA biogenesis was taking place normally up to the stage of translocation of the signal sequence cleavage site.

Upon chase with unlabeled methionine, PhoA molecules labeled in the mutants were degraded with half lives ~10 min (Figure 1, lanes B2−B5 and C2−C5), whereas that labeled in the wild-type cells was stable (Figure 1, lanes A2−A5). To examine whether PhoA polypeptides produced in the mutants were folded into the protease-resistant conformation, lysates of the pulse-labeled cells were treated with increasing concentrations of trypsin at 0°C (Figure 2). Unlike the normal PhoA, which was resistant to trypsin (Figure 2A), the same polypeptides synthesized in the *ppfA-33*::Tn*5* and the *ppfA-101*:Tn*5* mutants were degraded by trypsin at concentrations as low as 5−10 μg/ml (Figure 2B and C). Occasionally, we observed a poor recovery of the pulse labeled PhoA when the mutant cell lysates were left at low temperature but not when the cultures were directly treated with trichloroacetic acid. These results indicate that the PhoA molecules synthesized in the *ppfA* mutant cells did not attain the correctly folded structure, and were subject to degradation by some endogenous protease *in vivo* (and occasionally *in vitro*) or by trypsin *in vitro*. In contrast to PhoA, maltose binding protein, another periplasmic protein lacking disulfide bonds, was stable and trypsin resistant even when produced in the *ppfA* mutant cells (data not shown).

When cells were fractionated into the spheroplast and periplasmic fractions, PhoA molecules labeled in the *ppfA* mutants were recovered from the former (Figure 3, lanes 6 and 9), whereas maltose binding protein in the same cells was largely found in the periplasmic fraction (lanes 5 and 8). The spheroplast-associated PhoA molecules were accessible to digestion by externally added trypsin (Figure 4B). Thus, the aberrant forms of PhoA in the *ppfA* mutants should have translocated the cytoplasmic membrane to the periplasmic side, but have not been released into the periplasmic fraction. Possibly, the lack of proper folding makes the polypeptide stick to the outer surface of the cytoplasmic membrane. Maltose binding protein was fractionated normally in the mutant cells.

Characterization of the ppfA gene

Chromosomal DNAs from the *ppfA-33*::Tn*5* and the *ppfA-101*::Tn*5* mutants were digested with *Bam*HI, electrophoresed through agarose, and probed with a DNA fragment derived from the Tn*5* sequence. Both gave two signals at ~19 kb and 4.3 kb with slight strain-dependent mobility differences (data not shown). We were then able to clone the 4.3 kb fragments by virtue of the kanamycin resistant marker carried on them, yielding plasmids pSK33 and pSK101, respectively, for the *ppfA-33*::Tn*5* and *ppfA-101*::Tn*5* mutants (Figure 5A). The cloned *Bam*HI fragments were then tested for hybridization with the filter-immobilized *E.coli* genomic DNA library of Kohara *et al.* (1987). Hybridization in both cases was observed specifically with clones 9B11, 10H11 and 8D12, locating the Tn*5* insertions at the coordinate 3660 kb or 87 min of the *E.coli* chromosome.

The same probes were also used to detect plasmid clones carrying the wild-type counterpart of this region among a collection of pACYC184-based random clones of *E.coli*

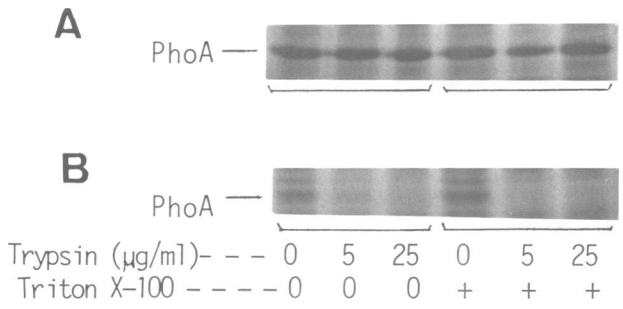

Fig. 4. Accessibility of PhoA to external trypsin. Cells of SM138 (**A**) and SK101 (**B**) were converted to spheroplasts and treated with indicated concentrations of trypsin in the presence or absence of 1% Triton X-100 as indicated. Samples were trichloroacetic acid precipitated and immunoprecipitated with anti-PhoA serum.

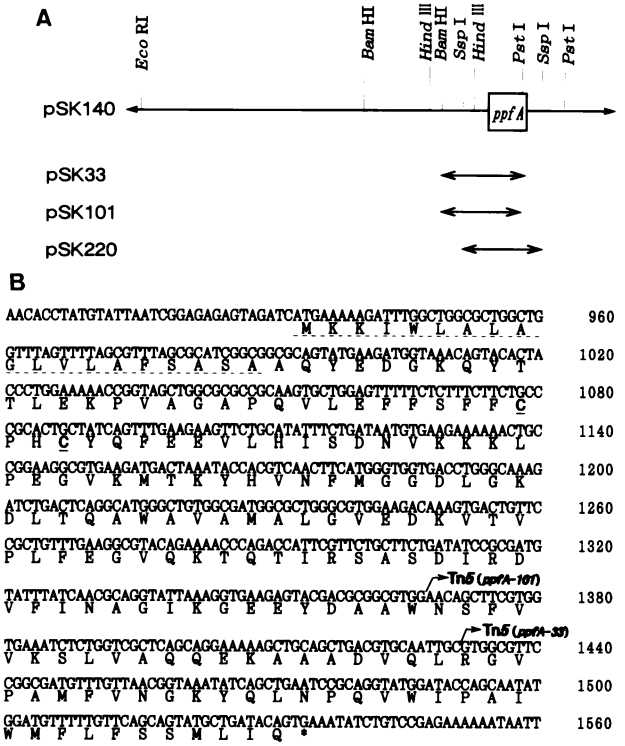

Fig. 5. Chromosomal region around *ppfA* and nucleotide and deduced amino acid sequences of *ppfA*. (**A**) The 7.4 kb fragment cloned in pSK140 are indicated with some restriction enzyme cleavage sites as well as the *ppfA* open reading frame. Arrows represent chromosomal regions carried in plasmids indicated. (**B**) The sequences of the *ppfA* gene and its product are shown. The putative signal sequence is underlined with dotted line and the putative redox active cysteines are shown by underlined boldface. Shown also are the sites of Tn*5* insertions. The *Bam*HI site preceding *ppfA* (see A) was numbered 1.

chromosomal fragments. The plasmid thus obtained, pSK140, carried ~7.4 kb chromosomal fragment (Figure 5A) and fully complemented the decreased PhoA activity of the *ppfA* mutants (data not shown). DNA sequencing was first carried out for the chromosomal part of pSK33 and pSK101 using a primer corresponding to a Tn*5* sequence. Making use of the above sequence information, we then sequenced the pSK140 and its derivatives for 2192 bases surrounding the sites at which the Tn*5* insertions had taken place in the mutants. Within the sequenced region, we could identify only one ATG-initiated open reading frame

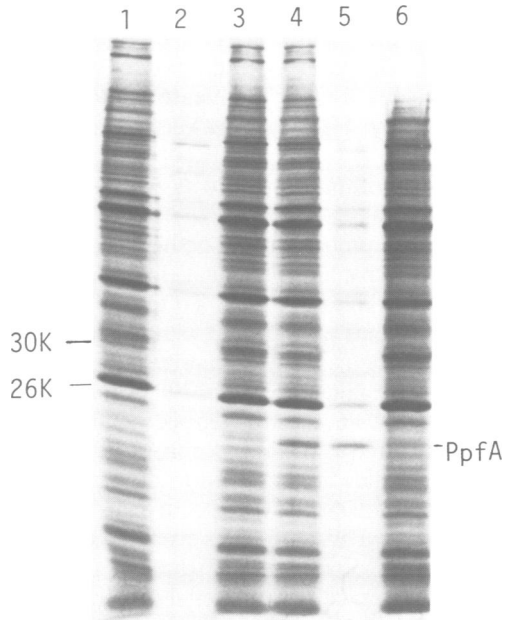

Fig. 6. Overproduction and localization of the *ppfA* gene product. MC4100 cells carrying pACYC184 (vector, lanes 1–3) or pSK140 (lanes 4–6) were pulse labeled with [^{35}S]methionine for 2 min, treated with trichloroacetic acid directly (lanes 1 and 4), or after fractionation into periplasmic (lanes 2 and 5) and spheroplast (lanes 3 and 6) fractions. Equivalent amounts were electrophoresed and autoradiographed. Positions of [^{14}C]Carbonic anhydrase (30 kDa) and chloramphenicol acetyltransferase (26 kDa) are shown on the left.

(ORF) of sufficient length, within which the *ppfA-33*::Tn*5* and *ppfA-101*::Tn*5* insertion sites were specified (Figure 5B). The ORF is preceded by typical ribosome binding sequences, although promoter sequences were not unequivocally identified. Introduction of a plasmid derivative, pSK220, that contained only this ORF and some surrounding sequences (Figure 5A), restored the PhoA enzyme activity of the *ppfA-33*::Tn*5* mutant (data not shown; for some unknown reason complementation was barely observed for the *ppfA-101*::Tn*5* mutant). From these and the following results, we conclude that this ORF represents the gene *ppfA* whose function is required for the full execution of the structure and function of the PhoA polypeptide.

Of the 200 amino acid long translated sequence of *ppfA*, the amino-terminal 19 residues (Figure 5B) have a feature typically expected for a translocation signal sequence, since it contains two lysines followed by some 12 hydrophobic amino acids and a region (Ala-Ser-Ala-Ala) expected for the leader peptidase cleavage site (von Heijne, 1986). Cells carrying pSK140 overproduced a periplasmic protein of ~20 kDa (Figure 6, lane 5), which was missing in those carrying the vector (Figure 6, lanes 1–3), pSK33 or pSK101 (data not shown). These results indicate that the *ppfA* gene product is a periplasmic protein.

The nucleotide and the deduced amino acid sequences of *ppfA* were examined for the existence of homologous sequences among the database entries, yielding no highly homologous ones. It is noted, however, that PpfA contains a sequence, Cys-Pro-His-Cys, near its amino-terminus (Figure 5B). The sequence motif Cys-X-X-Cys is known to be included in the active site of a class of proteins that catalyze thiol–disulfide exchange of proteins (for reviews, Freedman, 1989; Holmgren, 1989; Gilbert, 1990). Of 10

PpfA amino acid residues (Glu Phe Phe Ser Phe Phe Cys Pro His Cys) in the putative active site region, six (those underlined) are identical to one of the two active site regions of the yeast protein disulfide isomerase (Glu Phe Phe Ala Pro Trp Cys Gly His Cys; LaMantia *et al.*, 1991).

Disulfide bond formation of PhoA is aborted in the ppfA mutants

The presence of the probable redox-active cysteines in PpfA raised a possibility that it may facilitate the formation of the correct disulfide bridges in PhoA. To test this possibility, pulse–chased proteins were treated with iodoacetoamide to modify reduced cysteine residues to protect them from non-specific oxidation (Pollitt and Zalkin, 1983). PhoA immunoprecipitates were then electrophoresed with or without dithiothreitol treatment. Oxidized forms of a disulfide-containing polypeptide are expected to migrate faster than the reduced form in SDS–PAGE (Pollitt and Zalkin, 1983). In wild-type cells, PhoA migrates as the oxidized form immediately after pulse labeling, since its mobility was affected by treatment with dithiothreitol (Figure 7, compare A and B for lanes 1 and 2). Thus, the disulfide bond formation *in vivo* is a very rapid process. In contrast, PhoA molecules pulse labeled in the *ppfA* mutant cells were mostly in the reduced form which remained so after a chase for 9 min (Figure 7B, lanes 3–6). Recently, J.Beckwith and coworkers isolated similar mutants of a gene *dsbA* (presumably the same as *ppfA*) by a totally different approach, and showed that disulfide bond formation of several envelope proteins is defective (personal communication). The results that disulfide bond formation does not measurably occur in these mutants clearly indicate that it is a step catalyzed by PpfA/DsbA.

Discussion

The recessive insertional inactivation of *ppfA* results in the deficiency of proper folding of a periplasmic protein PhoA, leading to the accumulation of reduced and unfolded polypeptide. This clearly indicates that protein folding in the bacterial periplasmic space is not always a spontaneous process. Formation of active alkaline phosphatase in the periplasm depends on at least one factor, PpfA, that is required for the formation of disulfide bonds. It is remarkable that a simple reaction like the oxidation of protein thiols is still to be catalyzed in the cell. Other reactions in the folding–assembly pathway of PhoA may well be catalyzed by as yet unidentified cellular factors, and the simple screening system we adopted may be useful for identification of such factors.

In wild-type cells, the formation of disulfide bonds in PhoA occurs soon after the synthesis of the polypeptide. The correctly folded active PhoA dimer is resistant to proteases and this conformation is not affected by treatment with disulfide reducing agents such as dithiothreitol (Y.Akiyama and K.Ito, unpublished results). However, we were able to detect an *in vivo* folding intermediate which is digested by trypsin or endogenous protease in the presence of dithiothreitol (Y.Akiyama and K.Ito, unpublished results). Similar intermediates could also be detected when *in vitro* translated PhoA was subjected to cell-free folding reactions (Y.Akiyama and K.Ito, unpublished results). On the other hand, PhoA in the wild-type cells is in the oxidized form

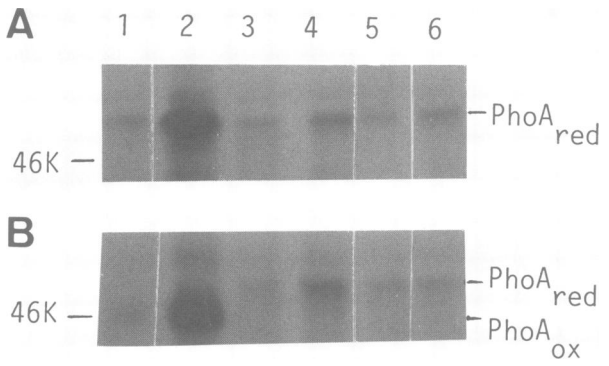

Fig. 7. Separation of disulfide-bonded and reduced forms of pulse–chased PhoA. Cells of SM138 (lanes 1 and 2), SK33 (lanes 3 and 4) and SK101 (lanes 5 and 6) were grown at 30°C and labeled with [^{35}S]methionine for 0.5 min, and chased for 0 (lanes 1, 3 and 5) or 9 (lanes 2, 4 and 6) min. Proteins were trichloroacetic acid precipitated, washed with acetone and dissolved in SDS solution containing iodoacetoamide. PhoA immunoprecipitates were electrophoresed with (**A**) or without (**B**) treatment by dithiothreitol. $PhoA_{red}$ and $PhoA_{ox}$ show positions of reduced and oxidized PhoA polypeptide, respectively. Position of [^{14}C]ovalbumin (46 kDa) is shown on the left.

immediately after synthesis (Figure 7). Thus, disulfide bond formation should be followed by folding and/or assembly into the final structure of the enzyme. During this process, adjustment of the disulfide bridges to the correct ones may occur. In the *in vitro* refolding pathway of some proteins, a series of non-native disulfide bonds are formed transiently (Creighton, 1986). It is striking that the PpfA-deficient conditions did not permit the effective formation of both the correctly and incorrectly oxidized PhoA species. Thus, PpfA acts at least in the oxidation of cysteine thiols. It should be addressed whether PpfA is also able to catalyze exchange of disulfide bonds or there are additional factors for the exchange.

Protein disulfide isomerase is widely distributed in the lumen of the endoplasmic reticulum of eurkaryotic cells and is thought to catalyze correct formation of disulfide bonds by oxidizing, reducing and exchanging them (Freedman, 1989). Although *in vitro* work (Bulleid and Freedman, 1988) supports the proposed function of protein disulfide isomerase in the biogenesis of secreted proteins, little information is available about whether this enzyme is essential for the biogenesis of disulfide-bonded proteins *in vivo*. Recent identification of the yeast gene for protein disulfide isomerase and its manipulations demonstrated that it is essential for viability of the yeast cell (LaMantia *et al.*, 1991). Although the existence of protein disulfide isomerase-like activity in the *E.coli* periplasm was noted (Barth *et al.*, 1988), no full account of this work has been published. We found that periplasmic fraction from wild-type cells markedly stimulated the execution of the enzymatic activity of *in vitro* translated PhoA polypeptide (without its signal sequence; Y.Akiyama and K.Ito, unpublished results). Relationships between this activity and PpfA are currently being investigated.

The Tn*5* insertions in *ppfA* did not crucially affect the cell viability, although they caused slightly slow growth and abnormal colony morphology in the presence of high concentrations of glucose under certain genetic backgrounds. At least three possibilities may account for these observations: (i) there is no disulfide-bonded periplasmic protein

that is growth essential; (ii) the Tn*5* insertions do not completely abolish the activity of PpfA; and (iii) background formation of disulfide bridges, either due to air oxidation or existence of additional PpfA-like factors in the cell, is sufficient for supporting cell growth. We are currently examining the effects of the *ppfA* mutations on the biogenesis of other cell envelope proteins, either containing or not containing disulfide bridges.

Although protein disulfide isomerase has two pairs of active site cysteines, PpfA contains only one. It should be addressed whether PpfA functions as a multimer in the cell. Protein disulfide isomerase and PpfA are not significantly homologous in the overall amino acid sequences, except for the 10 residues including the active site cysteines. In both cases the amino acid preceding the carboxy terminal cysteine is histidine, whereas that in thioredoxin is proline. A site directed replacement of the proline residue of *E.coli* thioredoxin by histidine makes the thioredoxin activities more like the isomerase activities (Krause *et al.*, 1991). Systematic comparison of active site sequences of the redox active proteins as well as their systematic alterations will give important clues on the structure−function relationships of this family of proteins. Finally, Ramseier *et al.* (1991) reported that a gene cluster in *Bradyrhizobium japonicum* with proposed functions in the biogenesis of *c*-type cytochromes contains an open reading frame with a thioredoxin-like motif.

Materials and methods

Strains

Escherichia coli K-12 strains KS272 (Δ*phoA* Δ*lacx74 galE galK thi rpsL*) and SM138, a *phoR* derivative of MC4100 (Casadaban, 1976), were provided by the J.Beckwith laboratory. Strains SK33 and SK101 were transductants of SM138 that received *ppfA-33*::Tn*5* and *ppfA-101*::Tn*5*, respectively.

Plasmids

The transposon Tn*phoA* was transposed onto the *bla* gene of pBR322 using published procedures (Manoil and Beckwith, 1985). In one of isolates, pBA71, the Tn*phoA* insertion occurred in-frame at the fifth codon of the *bla* mature sequence. pKY192 was a derivative of pBA71 from which a 6.4 kb *Pst*I fragment that included the kanamycin resistance gene of Tn*phoA* and a portion of *bla* had been removed.

pSK33 and pSK101 carried the kanamycin resistance gene of Tn*5* together with an adjacent chromosomal fragment of strains SK33 and SK101, respectively; chromosomal DNAs were digested with *Bam*HI and fragments of ~4.3 kb were recovered from agarose gels and cloned into the *Bam*HI site of pHSG399 (Takeshita *et al.*, 1987). pSK140 carried the wild-type *ppfA* region. Chromosomal DNA of a wild-type strain (W3110) was partially digested with *Sau*3AI and 5−15 kb fragments were cloned into the *Bam*HI site of pACYC184. About 5600 transformants were screened by colony hybridization using the ^{32}P-labeled *Bam*HI fragment of pSK33 as a probe, yielding one colony of increased signal. Plasmid from this colony (pSK140) carried an ~7.4 kb chromosomal fragment derived from the *ppfA* region. pSK220 was constructed by isolating an ~1.2 kb *Ssp*I fragment of pSK140 containing the *ppfA* ORF, and cloning it into the *Sma*I site of pHSG399.

Isolation of the ppf::Tn5 mutants

Strain KS272 was mutagenized by random transposition of Tn*5* from λTn*5* as described by Kleckner *et al.* (1978). Kanamycin-resistant colonies were pooled and transformed with plasmid pKY192, using L-agar (Miller, 1972) plates containing kanamycin (25 μg/ml), tetracycline (25 μg/ml) and XP (20 μg/ml). Initially, some 20 colonies with pale blue or white colony color were obtained from a total of ~10^4 colonies. They were examined for the intactness of the plasmid by re-extracting and re-introducing it into the non-mutagenized parental cells, as well as for plasmid copy numbers by the method of Projan and Novick (1984). Those with normal plasmid were further examined by measurement of the PhoA enzymatic activities.

PhoA enzyme assay

Cells were grown in P-broth (Ito *et al.*, 1983) to mid-log phase, and the PhoA activity was measured essentially according to Manoil and Beckwith (1985).

DNA manipulations

Construction, purification and analysis of plasmid DNA, DNA hybridizations and other manipulations of DNA were carried out by the standard procedures (Sambrook *et al.*, 1989). Membrane filters with Kohara DNA Library (Kohara *et al.*, 1987) were provided by the National Institute of Genetics (Mishima, Japan). DNA sequencing was by the chain termination method using double-stranded templates and Sequenase version II (United State Biochemicals), according to the manufacturer's instruction. Primers were those commercially available as well as those synthesized by Applied Biosystem DNA synthesizer. Systematic introduction of deletions by exonuclease III digestion (Sambrook *et al.*, 1989) was also used to facilitate the sequencing.

Labeling, trypsin digestion, fractionation and immunoprecipitation

For pulse-labeling proteins, cells were grown on synthetic medium M9 (Miller, 1972) supplemented with 20 μg/ml each of amino acids (except methionine and cysteine) and 0.4% glucose (or other sugars as specified), usually at 37°C. A log phase culture was labeled with 50−200 μCi/ml of [^{35}S]methionine (American Radiolabeled Chemicals) for indicated period. Chase with unlabeled methionine was initiated by addition of 200 μg/ml L-methionine. Trypsin resistance of PhoA was examined as described previously (Akiyama and Ito, 1989) except that cells were disrupted by lysozyme−freezing−thawing treatment as described (Ito and Akiyama, 1991). Fractionation of cells into periplasmic and spheroplast fractions were done as described (Ueguchi and Ito, 1990). Disulfide bond formation in PhoA was assessed by the methods described by Pollit and Zalkin (1983). For immunoprecipitation, proteins were first precipitated and concentrated by trichloroacetic acid, either directly from the culture or after the above mentioned manipulations of the samples (Akiyama and Ito, 1989). Anti-PhoA serum was purchased from 5 Prime-3 Prime Inc., whereas anti-maltose binding protein serum was prepared in this laboratory.

Acknowledgements

We thank J.Beckwith for bacterial strains, S.Kishigami for preparing *E.coli* genomic clones on pACYC184, T.Taura for suggestions, A.Ishihama for Kohara phage DNA filters, H.Mori for conducting homology searches, K.Mochizuki for laboratory supplies, and J.Kataoka for preparation of the manuscript. A part of this work as well as the work by the Beckwith group were presented at a meeting *Maltose System: Molecular Genetics and Cell Biology* (September 1−6, 1991); I thank J.Beckwith for exchange of unpublished results. This work was supported by grants from the Ministry of Education, Science and Culture, from the Naito Foundation, and from the Nagase Science and Technology Foundation.

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Received on September 30, 1991

Note added in proof

The work of the Beckwith group has been published [Bardwell,J.C.A., McGovern,K. and Beckwith,J. (1991) *Cell*, **67**, 581–589]. Comparison of our sequence with that of Bardwell *et al.* (1991) and subsequent confirmatory experiments, revealed that the sequence reported in Figure 5 lacked one G at position 1483. Thus, the PpfA precursor should contain 208 (rather than 200) residues and the C-terminal 25 residues are: GMDTSNMDVFVQQYADTVKYLSEKK, in agreement with Bardwell *et al.* (1991) (however, note that Figure 2 in Bardwell *et al.* (1991) is incorrectly numbered for amino acids). The accession number for the sequence reported in this paper is X63186.