Roles of Disulfide Bonds in Bacterial Alkaline Phosphatase*

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Alkaline phosphatase of Escherichia coli (a homodimeric protein found in the periplasmic space) contains two intramolecular disulfide bonds (Cys-168-Cys-178 and Cys-286-Cys-336) that are formed after export to the periplasmic space. The location-specific folding character of this enzyme allowed its wide usage as a reporter of protein localization in prokaryotic cells. To study the roles of disulfide bonds in alkaline phosphatase, we eliminated each of them by Cys to Ser mutations. Intracellular stability of alkaline phosphatase decreased in the absence of either one or both of the disulfide bonds. The mutant proteins were stabilized in a DegP protease-deficient strain, allowing accumulation at significant levels and subsequent characterization. A mutant protein that lacked the N-terminally located disulfide bond (Cys-168-Cys-178) was found to have Cys-286 and Cys-336 residues disulfide-bonded, to have a dimeric structure, and to have almost full enzymatic activity. Nevertheless, the mutant protein lost the tryptic-resistant conformation that is characteristically observed for the wild-type enzyme. In contrast, mutants lacking Cys-286 and Cys-336 were monomeric and inactive. These results indicate that the Cys-286-Cys-336 disulfide bond is required and is sufficient for correctly positioning the active site region of this enzyme, but such an active conformation is still insufficient for the conformational stability of the enzyme. Thus, a fully active state of this enzyme can be formed without full protein stability, and the two disulfide bonds differentially contribute to these properties.

Alkaline phosphatase (PhoA) is a periplasmic enzyme of Escherichia coli. It is a zinc- and magnesium-containing enzyme consisting of two identical subunits encoded by the phoA gene (1). Each of the subunits contains two intramolecular disulfide bridges, Cys-168-Cys-178 and Cys-286-Cys-336 (2). PhoA is initially synthesized as a precursor with an N-terminal 20-residue signal sequence that is removed during translocation across the cytoplasmic membrane. The mature 450-residue (or 449 when the N-terminal arginine residue is removed; Ref. 2) part of PhoA is then oxidized for its cysteine residues to form the disulfide bridges and is folded into a partially protease-resistant conformation (3). The subunit folding process is then followed by dimerization into the active metalloenzyme. This pathway of enzyme formation is completed within 2–3 min at 15 °C in the living cell (3).

The processes of folding/assembly of PhoA occur only after export to the periplasmic space (4–6). This property of PhoA has enabled its wide usage as a reporter of protein localization and membrane protein topology in prokaryotic cells (7, 8). The formation of disulfide bonds is a crucial event that triggers the correct folding of PhoA. In the cytoplasm, thioredoxin reductase and other factors seem to prevent disulfide bond formation on proteins (9). In the periplasm, the Dsb system facilitates disulfide bond formation (10, 11). It has been established that a periplasmic factor (DsbA) directly catalyzes oxidation of cysteines (12, 13). The membrane-bound factor DsbB is believed to reoxidize the reduced form of DsbA to allow it to function catalytically (14–16). Although biochemical studies on denaturation-renaturation as well as subunit association processes of PhoA were conducted previously (3, 17–22), and the structure of the enzyme was determined at a resolution of 2.8 Å (23) or 2.0 Å (24), the roles of the two disulfide bonds have not been precisely defined. In view of the importance of the disulfide bond formation for the formation of active PhoA enzyme, it is important to assign roles to each disulfide bond in the activity and stability of the enzyme. Thus, we have undertaken site-directed mutagenesis of cysteine residues of PhoA and examined the properties of the resulting mutant forms of the enzyme. The results show that the two disulfide bonds have different roles. Cys-286-Cys-336 was found to be required and was sufficient for the formation of the enzymatically active conformation, but was insufficient for supporting the protease-resistant structure, which proved to require Cys-168-Cys-178 as well. Thus, a fully active state of this enzyme can be formed without having the full stability of the protein.

EXPERIMENTAL PROCEDURES

Bacterial Strains—E. coli strains KS272 (ΔphoA) and KS474 (ΔphoA degP41::Tn5) were described by Strauch and Beckwith (25). Strains MS3 and MS5 were derivatives of KS272 and KS474, respectively, into which F’lacλ lacPL8 lacZ Y+ A+ pro+ plasmid had been introduced from strain K267 (26). Strains BW313 (27) and MV1184 (28) were used for site-directed mutagenesis.

Plasmids—pMS8002 was a derivative of pBluescript-SK(−) carrying the phoA gene under the control of the lac promoter. It was constructed as follows. A polymerase chain reaction product was donated by N. Kusukawa of Takara Shuzo in which the phoA region of the chromosome was amplified using primers 5′ GAAAACAAAAACACTATTGCG (starting at the third nucleotide of phoA) and 5′ GTGACTGGCAATATAGTC (starting at the 75th nucleotide after the end of the phoA reading frame). It was treated with the Klenow enzyme and cloned into the EcoR1 site of pBluescript-SK(−). To eliminate any possible mutations introduced by polymerase chain reaction, a 1.4-kilobase pair BclI-KpnI fragment (representing most of the mature PhoA and some of the vector sequence) was replaced with the corresponding BclI-KpnI fragment that was prepared from a plasmid carrying the phoA gene of the confirmed sequence3 in which the phoA mature sequence had been simi-

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1 N. Kusukawa, unpublished data.
larly cloned at the EcoRV site of pT7Blue-Vector (Novagen). The remaining 5′ part of the *phaA* sequence was confirmed by sequencing. In the resulting plasmid (pMS001), the initiation codon of *phaA* was recreated but was in the same frame as the lacZa sequence of the vector. Therefore, the *Phal* site at the upstream region was subjected to cleavage. PhoA was in the Klenow enzyme and religation. The final product (pMS002) encodes the wild-type PhoA precursor.

Cys to Ser substitution mutations of PhoA were introduced into plasmid pMS002 by the procedures described by Kunkel (27), using the Muta-Genie Phagemid *In Vitro* Mutagenesis Kit (Bio-Rad). Synthetic oligonucleotides used as mutagenic primers were as follows: 5′-GGCG-CCACCTGTTGCGG-3′ for Cys-168 → Ser mutation, 5′-GATCGGAAATTCTC-3′ for Cys-178 → Ser mutation, 5′-AGTCACTGCTACGCAAA-3′ for Cys-286 → Ser mutation, and 5′-GAATCTTTCTGGGGCAAA-3′ for Cys-336 → Ser mutation (codons for Ser are underlined). Coding for PhoA(SCCC), pMS003 was first constructed using the Cys-168 → Ser primer. pMS004 (for PhoA(SSCC)), pMS006 (for PhoA(SSSC)), and pMS013 (for PhoA(SSCS)) were then constructed successively using appropriate primers. Similarly, pMS012 (for PhoA(CCSS)), pMS014 (for PhoA(CSCS)), and pMS015 (for PhoA(CCSS)) were constructed individually or in sequence. pMS016 (for PhoA(SSSS)) was constructed by replacing the EcoT14I-KpnI segment of pMS004 with that of pMS015. All the mutations were confirmed by sequencing.

Pulse-Chase and Immunoprecipitation—Cells were grown at 37 °C in an exponential phase in M9 medium (29) supplemented with 0.4% glucose and 20 μg/ml each of amino acids, except methionine and cysteine. The phoA gene under the lac promoter control was induced with 1 mM isopropyl-β-D-thiogalactoside. After 15 min, cells were pulse-labeled with 50 μCi/ml [35S]methionine (1100 Ci/mmol, obtained from American Radiolabeled Chemicals) followed by chase with unlabelled L-methionine (200 μCi/ml) for indicated periods. A portion of the culture was treated with trichloroacetic acid, and protein precipitates were dissolved in SDS for subsequent immunoprecipitation as described previously (11). Anti-PhoA serum was purchased from 5 Prime, Inc., Boulder, CO. Radioactive proteins were visualized and quantitated using a Bioimaging Analyzer BAS2000 (Fuji Film) in combination with a Discovery Series Densitometer (PD-1). Trypsin Sensitivity Assay—Trypsin resistance of various PhoA constructions in crude cell lysates was assessed essentially as described previously (5, 31), except that iodoacetamide was included (30).

Purification of PhoA and PhoA(SSCC)—To overproduce these proteins, the *phaA* genes were cloned under the control of the *are* promoter. pMS002 and pMS004 were linearized with KpnI and then partially digested with EcoRI. An approximately 5-kilobase fragment containing the phoA open reading frame was recovered and cloned into pBAD22 (36) that had been digested with EcoRI and KpnI. The resulting plasmids (named pMS019 and pMS021, respectively, for PhoA and PhoA(SSCC)) were introduced into strain MS5. Cells were cultivated in 4 liters of M9 amino acid medium described above (except containing 0.4% arabinose), and periplasmic fractions were prepared and analyzed as described previously (32). PhoA proteins were purified according to the procedure of Chaidaroglou et al. (37). Approximately 10 and 0.5 mg of proteins of at least 90% purity (as judged from the SDS-PAGE profiles) were obtained for PhoA and PhoA(SSCC), respectively.

RESULTS AND DISCUSSION

Construction and Intracellular Stability of Cys to Ser Substitution Mutants of PhoA—The *phaA* gene, cloned under the control of the *lac* operator/promoter on a plasmid vector, was subjected to site-directed mutagenesis to construct a series of 8 mutants in which a Cys codon was converted into a Ser codon singly or in combination. For the sake of convenience, these mutant forms of PhoA were designated by PAGE, polyacrylamide gel electrophoresis.

The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.

\[ ^2 \text{The four-letter notations, with C for cysteine and S for serine, indicate the aspartic acid residues, 168, 178, 286, and 336, in that order.} \]

\[ ^3 \text{The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.} \]
strains (Fig. 1), it was possible to accumulate mutant forms of PhoA using this periplasmic protease-deficient strain as a host.

The Cys-286-Cys-336 Disulfide Bond Is Essential, whereas the Cys-168-Cys-178 Disulfide Bond Is Dispensable for the PhoA Activity—The wild-type and mutant forms of PhoA were allowed to accumulate in the degP mutant cells, and their relative amounts were determined by subjecting whole cell extracts to SDS-PAGE and visualizing PhoA by immunoblotting. PhoA activities of the same cultures were assayed, and relative specific activities were determined by dividing the enzymatic activities by relative intensities of PhoA in the immunoblotting experiments (Table I). Only PhoA(SSCC) and PhoA(SCCC) were found to have significant enzyme activity (Table I). Since PhoA(CCSS), PhoA(CCCS), PhoA(CCSSC), PhoA(SSSS), and PhoA(SSCS) were all inactive (see Table I for (CCS); data not shown for the other mutants), the presence of both Cys-286 and Cys-336 was required for the activity. In contrast, Cys-168 and Cys-178 were dispensable for the enzymatic activity.

PhoA(SSCC) was as active as the wild-type enzyme (Table I), suggesting that Cys-286-Cys-336 is the sole activity-supporting disulfide bond in PhoA. To confirm this notion, PhoA and PhoA(SSCC) were overproduced and purified. Specific activities of these preparations were about the same (Table I). Interestingly, PhoA(SSCC), which contains an additional cysteine, was less active than PhoA(SSCC). We have evidence indicating that PhoA(SSCC) tends to form an aberrant disulfide bond.

The Fully Active PhoA(SSCC) Forms a Dimer but Does Not Form the Protease-resistant Conformation—Normal PhoA sediments at about 6 S in sucrose gradient centrifugation, whereas an unfolded monomer subunit sediments at around 2 S (3, 19). Periplasmic fractions were fractionated by sucrose gradient centrifugation and examined for sedimentation behaviors of PhoA proteins (Fig. 2). It was found that enzymatically active PhoA(SSCC) sedimented to similar fractions as the wild-type enzyme (Fig. 2, compare A and B). In contrast, PhoA(CCSS) (which was inactive) sedimented much more slowly (Fig. 2C) to the position similar to that of PhoA(SSSS) (data not shown) or that of reduced and denatured PhoA (3). These results indicate that PhoA(SSCC), but not PhoA(CCSS), is competent for the dimer formation.

The PhoA variants were also examined by PAGE at pH 8.8 under non-denaturing conditions. PhoA(SSCC) migrated to a similar position as the wild-type PhoA (Fig. 3, compare lanes 1 and 2). PhoA(CCSS) migrated much slower (Fig. 3, lane 3). PhoA(SSSS) also migrated slowly under the conditions used (data not shown). The basis for the slow migration of PhoA(CCSS) and PhoA(SSSS) is not known. It may be due to the lack of a compactly folded structure and/or different exposure of charged residues. In any case, the results of native PAGE are consistent with the notion that the overall structure of PhoA(SSCC) is similar to that of the wild-type enzyme.

To know whether the remaining cysteines in the mutant proteins are disulfide-bonded, we examined profiles of PhoA proteins in SDS-PAGE under non-reducing conditions. Normal PhoA with two disulfide bonds migrated faster than its reduced form (Fig. 4, compare lanes 1 and 2). Electrophoretic mobility of PhoA(SSCC) was indistinguishable from that of the oxidized wild-type enzyme (Fig. 4, compare lanes 3 and 4). After reduction with β-mercaptoethanol, all the PhoA molecules shown in Fig. 4 migrated identically at the position of the reduced wild-type PhoA protein (data not shown). Thus, Cys-286 and Cys-336 that remain in PhoA(SSCC) form an intramolecular disulfide bond.

Electrophoretic mobility of PhoA(CCSS) (Fig. 4, lane 5) was indistinguishable from that of PhoA(SSSS) (Fig. 4, lane 6) or of reduced PhoA. Either Cys-168 and Cys-178 in this mutant remain reduced or the Cys-168-Cys-178 disulfide bond affects

**TABLE I**

| PhoA variant | Cellular accumulation (relative value) | Activity | Relative specific activity | Specific activity of purified protein |
|--------------|----------------------------------------|----------|---------------------------|-------------------------------------|
| (CCCC) (wild-type) | 100 | 64 | 100 | 92 |
| (SCCC) | 58 | 8.4 | 23 | ND |
| (SSCC) | 86 | 54 | 98 | 94 |
| (CCSS) | 79 | 0 | 0 | ND |
| (SSSS) | 79 | 0 | 0 | ND |

a Determined by immunoblotting and quantitation of the stains by a Discovery Series Densitometer (PDI).
b The PhoA activity unit (7) was divided by the relative accumulation of the PhoA protein, and the value obtained was normalized by the wild-type value.
c Specific activity was indicated by the amount of p-nitrophenyl phosphate hydrolyzed (in μmol) per min/mg of protein at 25 °C and at pH 8.1.

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the electrophoretic mobility only negligibly. The close proximity of these residues in the primary sequence may suggest the latter possibility.

It was already shown in Fig. 1 that PhoA(SSCC) was unstable in vivo. Protease susceptibility of the mutant forms of PhoA was studied in vitro by trypsin digestion of cell extracts. Wild-type PhoA was resistant to up to 50 μg/ml trypsin examined (Fig. 5A, showing data for up to 10 μg/ml trypsin). PhoA(SSCC) was not resistant to even 2.5 μg/ml trypsin (Fig. 5B, lane 2). PhoA(CSSS) was not resistant to 5 μg/ml trypsin (Fig. 5C, lanes 2–5). These results show that the fully active and dimeric PhoA(SSCC) is still in loosely folded conformation.

Roles of Disulfide Bonds in Activity and Stability of Alkaline Phosphatase—The results presented above indicate that of the two disulfide bonds in PhoA, the one between Cys-286 and Cys-336 is sufficient for the enzymatic activity. However, it is not sufficient to confer the protease-resistant conformation normally observed for this enzyme. The other disulfide bond (between Cys-168 and Cys-178) is important for stabilization of the protein.

PhoA is one of the enzymes that exhibit a high degree of thermal stability and protease resistance (38). Intramolecular disulfide bond formation is a prerequisite for its folding into an active dimer (3). PhoA has been widely used as a reporter of protein export from the cytosol (7, 8). The reducing environment of the cytosol prevents folding and activation of PhoA, whereas the periplasmic space contains DsbA, which facilitates oxidative folding of newly synthesized proteins (10, 11). PhoA molecules that are internalized because of a defect in the periplasmic space (degP) are not sufficient to confer the protease-resistant conformation normally observed for this enzyme. PhoA(SSCC) is still in loosely folded conformation.

The active site of this enzyme contains two atoms of zinc and one of magnesium and involves at least the following amino acid residues: Asp-327, Lys-329, His-331, His-372, and His-412 (25, 24, 39). The sequence (286–Cys–336) is essential for enzymatically active conformation of PhoA.

The active site residues are arranged in proper geometric configuration (24). In contrast, the conformational constraint imposed by the Cys-168-Cys-178 disulfide bond is limited within a single loop. Such a restriction may be trivial with respect to the formation of an active site and dimerization, but should be important for establishing a tight overall or local folding state that is crucial for the resistance of this enzyme to the action of proteases.

The finding that the Cys-168-Cys-178 disulfide bond is required for the tight folding without a major contribution to the active site formation (Table I) may corroborate the proposal (40) that the acquisition of a catalytic ability of a protein is often made possible at the expense of stability of the protein. By making a disulfide bridge, the Cys-168 and Cys-178 residues may have evolved to overcome such an intrinsic incompatibility between activity and stability of the enzyme. Subramaniam et al. (22) reported that during renaturation of guanidine hydrochloride-denatured PhoA, full enzymatic activity is achieved well before the establishment of the final conformation of the native enzyme. The PhoA(SSCC) molecule deserves structural studies.

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Differential in vivo roles played by DsbA and DsbC in the formation of protein disulfide bonds.

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Plasmid pMS002 and its derivatives used in the above two publications proved to contain an additional mutation for a Ser-401 → Cys substitution within PhoA. We traced this mutation back to the phoA plasmid (provided by others) that was used to substitute the amplified segment, as described in the first publication (page 6174, “Experimental Procedures”). Given this fact, we eliminated this unwanted mutation from most of the plasmid constructions and repeated key experiments presented in both publications. Mutant forms of PhoA are shown by the abbreviations described in both articles; however, the previous constructions are indicated by C* attached to the end, and its absence indicates new constructions without the Ser-401 → Cys mutation. Relative specific activity of PhoA[CCCC]* was about 80% of that of PhoA[CCCC]. PhoA[SSCC] was as active as PhoA[CCCC], whereas PhoA[SCCC] was about 50% as active as PhoA[CCCC]. We also observed a lowered cellular accumulation of PhoA[SSCC], consistent with its instability. Thus, the essential conclusions of the first publication hold. Although PhoA[SCCC] has a lower than normal enzyme activity, the abnormally migrating protein species reported in the second publication was not observed for PhoA[SCCC]. Thus, although the essential conclusion that DsbA introduces a disulfide bond of an aberrant combination (possibly involving residue 401 in PhoA[SCCC]) and that DsbC isomerizes that bond holds, the interpretation that the abnormal disulfide bond observed was between intrinsic PhoA residues was incorrect. We regret that we overlooked the mutation that existed in one of the starting materials. We thank Dr. George Georgiou for communicating their sequencing results of pMS003 and Yuki Takahashi for eliminating the mutation and characterizing the new PhoA derivatives.

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