Folding and Assembly of Bacterial Alkaline Phosphatase in Vitro and in Vivo*

Yoshinori Akiyama and Koreaki Ito

From the Department of Cell Biology, Institute for Virus Research, Kyoto University, Kyoto 606-01, Japan

Alkaline phosphatase (PhoA), localized in the periplasmic space of Escherichia coli, is a homodimeric metalloprotein containing two intramolecular disulfide bonds. We attempted to clarify the folding-assembly pathways of this enzyme by allowing in vitro-synthesized PhoA polypeptide to fold into active enzyme and by examining the occurrence of similar pathways in vivo by pulse-chase experiments. PhoA (lacking the signal sequence) that was synthesized in a coupled transcription-translation system was effectively converted into active enzyme when incubated with either oxidized glutathione, periplasmic proteins, or purified DsbA protein in the presence of Zn\(^{2+}\). The first appreciable event in the activation of initially unfolded translation product (species I) was the disulfide bond formation, which was immediately followed by folding into a partially trypsin-resistant monomer (species II), and then by assembly into active dimer (species III). The species II PhoA molecules, but not the species III molecules, were found to be sensitized to trypsin in the presence of a reducing agent, dithiothreitol. Pulse-chase studies showed that PhoA acquires disulfide bonds immediately after the biosynthesis, whereas it acquires resistance to both dithiothreitol and trypsin in the presence of a reducing agent, dithiothreitol. PhoA molecules internalized in the cytoplasm due to a lack of functional signal sequence as well as those synthesized in the dsbA-deficient cell are reduced and protease-sensitive (Bardwell et al., 1991; Kamitani et al., 1992; Derman and Beckwith, 1991).

Although biochemical studies on denaturation-renaturation and subunit association processes of PhoA were conducted previously (Schlesinger and Barrett, 1965; Schlesinger, 1965; Reynolds and Schlesinger, 1967; Applebury and Coleman, 1969; Falk et al., 1982), it will be important to revisit these problems with emphasis placed on their relevance to the in vivo processes. We initiated the present investigation by re-examining the activation process of in vitro-translated PhoA molecules (Inouye et al., 1977) to define intermediates in the folding-assembly pathway. Our results show that the translation product of phoA that is reduced, unfolded, and sensitive to trypsin is triggered by disulfide bond formation for subsequent folding into the folded monomers. This is then followed by conversion into the native-like dimers. A peculiar feature found with the in vitro folded monomers, the reducing agent sensitivity, facilitated the identification of a similar intermediate in vivo.

MATERIALS AND METHODS

Bacterial Strains—The following E. coli K12 strains were used. SM138 (phoR) and KS474 (degP41(kan)) were described by Kami- tani et al. (1992) and Strauch and Beckwith (1988), respectively. AD269 and AD270 were constructed by introducing degP41(kan) (kan\(^{1}\) and ptr-32::Cmr) (Baneyx and Georgiou, 1991), respectively, into SM138 by P1 transduction.

Preparation of the Periplasmic Fraction—KS474 or SM138 cells were grown in L broth (typically 1 liter) to a late-log phase and subjected to cold osmotic shock (Neu and Heppel, 1965). Bacterial components released into ice-cold H\(_2\)O (90 ml) were concentrated 8-fold by dialysis against polyethylene glycol 20,000, followed by extensive dialysis against 10 mM Tris-HCl, pH 8.1. Protein concentration

1 The abbreviations used are: PhoA, alkaline phosphatase; PMSF, phenylmethylsulfonyl fluoride; TLCK, N\(^{-}\)p-tosyl-L-lysine chloromethyl ketone; DTSSP, dithiothreitol.
RESULTS

Activation of in Vitro-synthesized PhoA—Inouye et al. (1977) showed previously that PhoA protein synthesized in the 52-coupled transcription-translation system could be converted into an enzymatically active form after prolonged incubation in the presence of Zn++. We re-examined this reaction using plasmid pB7A4 (Akiyama et al., 1992) as a template. The bla (β-lactamase) transcription and translation initiation regions on pB7A4 directed the synthesis of PhoA without the signal sequence. Postsynthetic incubation with Zn++ resulted in the appearance of low and variable levels of active enzyme. Addition of GSSG, E. coli periplasmic fraction, or purified DsbA protein (data not shown) markedly accelerated the enzyme formation (Fig. 1). GSSG and DsbA should catalyze rapid formation of them under optimal redox conditions (Akiyama et al., 1992); whereas GSSG stimulates linear and gradual formation of the disulfide bonds, DsbA catalyzes rapid formation of them under optimal redox conditions (Akiyama et al., 1992). We have not sorted out the periplasmic components that were responsible for the stimulation of enzyme formation, but DsbA should have contributed to the reaction.

Folding Intermediates in Vitro—During GSSG or periplasmic protein-assisted activation of in vitro-synthesized PhoA, samples were removed periodically and treated with trypsin before electrophoretic analysis. Initially, the PhoA molecules were subjected to lysozyme treatment in 20% sucrose and then treated with 0 or 50 μg/ml trypsin for 30 min at 0°C as described previously (Akiyama and Ito, 1989). DTT (10 mM) was present throughout the manipulations for the last set of samples. The trypsin reaction was terminated by the addition of 1 mM PMSF and 1 mM TLCK (final concentrations, 1 mM) and proteins were precipitated with an equal volume of ice-cold 10% trichloroacetic acid. Proteins were collected by centrifugation, washed with acetone, and dissolved in SDS sample buffer (Laemmli, 1970) containing 1 mM PMSF and 1 mM TLCK.

For assessing disulfide bond formation, 5-μl portions of samples were withdrawn and immediately mixed with 200 μl of 5% trichloroacetic acid. After washing with acetone, protein precipitates were dissolved in SDS sample buffer containing 0.1 M iodoacetamide but no β-mercaptoethanol for SDS-gel electrophoresis (Akiyama et al., 1992).

For assessing PhoA enzymatic activity, a 29-μl portion of the sample was mixed with 100 μl of 1 M Tris-HCl, pH 8.1, 12.5 μl of 1 M NaF, and 12.5 μl of 0.4% Sigma 104, and incubated at 37°C for 1 h. Absorbance at 420 nm was measured as described (Manoil and Beckwith, 1985).

Detection of a DTT-sensitive Folding Intermediate in Vivo—Cells were grown at 15°C to midlogarithmic phase in M9 medium supplemented with thiamin, 18 amino acids other than Met and Cys, and 0.4% glucose, pulse-labeled with about 3.7 MBq/ml of [35S]methionine for 30 s, and chased with 200 μg/ml unlabeled methionine. At indicated time points, three 100-μl portions were withdrawn. The first set of samples was mixed with 100 μl of 10% trichloroacetic acid. The second set of samples was rapidly mixed with 4 μl of 2% NaN3, 16 pl of 0.4% Sigma 104, and incubated at 37°C for 1 h. Absorbance at 450 nm was measured as described (Manoil and Beckwith, 1985).

Sucrose Gradient Centrifugation of in Vitro-synthesized PhoA—A sample (either in vitro-synthesized phoA with or without activation or periplasmic fraction containing normal PhoA molecules) made up to 348 μl was layered on the top of 4.8 ml of 5-20% sucrose, centrifuged (348 μl) containing 1 mM PMSF and 1 mM TLCK. For assessing trypsin resistance, 5-μl portions were removed during incubation, diluted into 47.5 μl of 10 mM Tris-HCl, pH 8.1, and mixed with 2.5 μl of 1 mg/ml trypsin solution in 10 mM Tris-HCl, pH 8.1. After incubation for 30 min in an ice bath, the reaction was terminated by the addition of PMSF and TLCK (final concentrations, 1 mM), and proteins were precipitated with an equal volume of ice-cold 10% trichloroacetic acid. Proteins were collected by centrifugation, washed with acetone, and dissolved in SDS sample buffer (Laemmli, 1970) containing 1 mM PMSF and 1 mM TLCK. For assessing disulfide bond formation, 5-μl portions of samples were withdrawn and immediately mixed with 200 μl of 5% trichloroacetic acid. After washing with acetone, protein precipitates were dissolved in SDS sample buffer containing 0.1 M iodoacetamide but no β-mercaptoethanol for SDS-gel electrophoresis (Akiyama et al., 1992).

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Folding Intermediates in Vitro—During GSSG or periplasmic protein-assisted activation of in vitro-synthesized PhoA, samples were removed periodically and treated with trypsin before electrophoretic analysis. Initially, the PhoA molecules were almost entirely sensitive to trypsin (Fig. 2, lanes 1 and 2). This state of PhoA is designated as species I. After incubation for about 0.5 h, they attained a partial resistance to trypsin, producing a fragment (PhoA') that was about 2 kDa smaller than the intact PhoA polypeptide (Fig. 2, lanes 3 and 4). This partially trypsin-resistant PhoA is designated as species II. At this time point, conversion from species I to species II was almost quantitative. Upon further incubation, most of PhoA became resistant to trypsin, its electrophoretic mobility being unchanged by trypsin treatment (Fig. 2, lanes 11 and 12). This form of PhoA was designated as species III. Under the conditions used, about 70% of the PhoA molecules were eventually converted into species III. Qualitatively similar results were obtained when either periplasmic fraction or GSSG was used as a stimulating agent, with somewhat higher yield of species III with the former. The results with oxidized glutathione indicate that the oxidative conditions are crucial for the initiation and continuation of the folding reactions.

The reaction mixture at 4 h that contained species II and III, as well as that incubated without added periplasm in which PhoA remained in species I, were fractionated by sucrose gradient centrifugation (Fig. 3A). The peak fractions were examined for trypsin resistance. The PhoA species I sedimented as a single peak at fractions 8–10. It was trypsin-sensitive as expected (Fig. 3, A and B, No Peri). The activated PhoA preparation contained materials that sedimented faster.
than species I, forming two overlapping peaks (Fig. 3A, +Peri). The species III PhoA molecules (those unaffected by trypsin) peaked at fractions 5 and 6 (see Fig. 3B, fraction 6), whereas the species II PhoA molecules (those producing PhoA' upon trypsin treatment) peaked at fractions 8 and 9 (see Fig. 3B, +Peri Fraction 9). Sedimentation profile of the enzymatic activity of the activated preparation was similar to that of species III (Fig. 3C, circles). The native PhoA in the periplasmic fraction of a phoR phoA' strain was also centrifuged in a parallel gradient and found to sediment at the same position (fraction 6) as the species III molecules (Fig. 3C, squares). Thus, the PhoA species III should represent enzymatically active dimer. The species I and II sedimented at similar positions, the former being slightly slower than the latter. It is likely that the species I is in unfolded monomer and the species II is in folded monomer structures. The above interpretation was supported by the observation that omission of Zn²⁺ and addition of EDTA during the activation reaction resulted in the accumulation of species II. Zn²⁺ and Mg²⁺ are known to be required for in vitro dimerization of PhoA (H. Schlesinger and Barrett, 1965).

The species II of PhoA has an additional peculiarity that differentiates it from the native-like species III. The trypsin resistance of the native PhoA, as well as of species III, was maintained even when they were treated with 10 mM DTT (Fig. 4A, lane 3, and B, lane 4). In contrast, most of the species II PhoA became trypsin-sensitive after treatment with DTT (Fig. 4B, compare lanes 2 and 4). This sensitivity to DTT suggests that species II contains disulfide bonds that are somehow accessible to the reducing agent in the aqueous environment. Reduction of the disulfide bonds in species II will then unfold the protein.

To examine the temporal relationship between the disulfide bond formation and the acquisition of the partial trypsin resistance in species II, we examined their time courses using DsbA that can facilitate the former reaction rapidly. As reported previously, DsbA-dependent oxidation of thiols takes place after a lag period. Under the conditions used for the experiment reported in Fig. 5, the disulfide bond formation, as assessed by a shift in gel electrophoretic mobility (Derman and Beckwith, 1991; Kamitani et al., 1992; Akiyama et al., 2003).
were withdrawn at the indicated time points. One set of samples were directly subjected to SDS-polyacrylamide gel electrophoresis under nonreducing conditions. The ratios of the oxidized to total PhoA molecules were plotted (○). Another set of samples was treated with 50 μg/ml trypsin before gel electrophoresis. Amounts of the trypsin-resistant fragment (PhoA') relative to that of the 111-min sample were shown (△).

FIG. 5. Time courses of DsbA-induced disulfide bond formation and folding into PhoA species II. In vitro-synthesized PhoA was incubated with 0.55 mg/ml DsbA, under the redox conditions specified under "Materials and Methods." Duplicate samples were withdrawn at the indicated time points. One set of samples were directly subjected to SDS-polyacrylamide gel electrophoresis under nonreducing conditions. The ratios of the oxidized to total PhoA molecules were plotted (○). Another set of samples was treated with 50 μg/ml trypsin before gel electrophoresis. Amounts of the trypsin-resistant fragment (PhoA') relative to that of the 111-min sample were shown (△).

90 100 110 120
Relative amount
Incubation time (min)

were shown (△).

20 1992), occurred within a time window of 95–105 min after the addition of DsbA. The same samples were treated with trypsin to examine whether they were completely degraded or converted to the PhoA' molecules. These analyses indicated that the conversion from species I to species II occurred at 100 to 110 min, about 5 min later than the thiol oxidation. Thus, the disulfide bond formation precedes and probably triggers the rapid folding of species I molecule into the species II molecule.

In Vivo Pathway of PhoA Folding—The above conclusion that the disulfide bond formation is a prerequisite for the folding of PhoA holds also in vivo, since no trypsin-resistant PhoA species can be produced in the mutant cell in which dsbA has been disrupted by a transposon insertion (Kamitani et al., 1992; Bardwell et al., 1991). In contrast, the disulfide bond formation in wild type cells occurs very rapidly such that the reduced form of PhoA can never be detected even by a short pulse-labeling (such as for 30 s). Completion of the protease resistance delays somewhat, since substantial fractions of PhoA molecules appear to remain protease-sensitive immediately after pulse-labeling (Fig. 6B, 0 min; Bardwell et al., 1991).

To demonstrate more clearly the in vivo existence of a premature folding intermediate, we examined how rapidly PhoA acquires the resistance to reduction by DTT. Pulse-chased cultures were divided into three portions. The first portions were directly and immediately treated with trichloroacetic acid which should prevent subsequent enzymatic modifications of the polypeptides. The second portions were treated with lysozyme in the presence of 20% sucrose, whereas the last portions were immediately mixed with 10 mM DTT before the sucrose-lysozyme treatment. The latter two samples were left at 0 °C for 30 min with or without added trypsin and then treated with trichloroacetic acid. PhoA polypeptides were immunoprecipitated, and equivalent amounts were electrophoresed. As shown in Fig. 6, intensities of PhoA after a chase for 4 min or longer were unaffected by the different treatments described above. In contrast, PhoA molecules at shorter chase points disappeared when left in the presence of DTT (Fig. 6, compare A, B, and C for 0–2 min). Significant fractions of pulse-labeled PhoA were trypsin-sensitive (Fig. 6B, 0–2 min). Molecules that survived the DTT treatment were not further digested by trypsin (Fig. 6C). The degradation in the presence of DTT was due to some endogenous protease(s), as it was prevented by the inclusion of inhibitors of serine proteases (Fig. 7, lanes 3 and 4). It occurred in the degP (lane 5) and the ptr (lane 7) mutants, indicating that the responsible protease is different from DegP (Strauch and Beckwith, 1988) and protease III (Baneyx and Georgiou, 1991).

These results indicate that acquisition of the DTT resistance, a feature found in the native PhoA enzyme, requires on average about 2 min after the completion of the polypeptide.

DISCUSSION

Refolding of a purified protein in vitro does not necessarily represent the in vivo process of protein folding. For instance, whether the productive refolding depends on the chaperonin
function varies with temperature, ionic strength, protein concentration, and inclusion of a detergent (Mendoza et al. 1991). Although the in vitro system we used here could be taken as being closer to the in vivo situation than the conventional unfolding-refolding systems, in that it does not involve strongly denaturing agents, it is still far from mimicking the in vivo process. Most importantly, the step of membrane translocation is ignored and the folding is forced in the presence of the cytoplasmic components which PhoA should not meet in vivo while folding in the periplasm. Despite these shortcomings, we used the PhoA molecules synthesized in the coupled transcription-translation system for the following reasons. At present, it is not feasible to prepare inverted membrane vesicles in which periplasmic components are entrapped at significant concentrations. We succeeded to translocate a Bla-PhoA fusion protein into conventionally prepared inverted membrane vesicles and to get its signal sequence (derived from Bla) cleaved. However, PhoA molecules remained in a trypsin-sensitive conformation under such conditions. Thus, we pursued reactions without involving the membranes, as an initial attempt to study in vitro folding pathways of PhoA. The signal sequence encoding region was removed from the template, and the folding mixture was supplemented with the periplasmic fraction, an oxidative agent (GSSG), or the DsbA protein. It was observed that PhoA protein with attached Bla signal sequence was much less competent for the in vitro activation than the mature PhoA adopted in this study. This agrees with a proposed role of signal peptide in retarding folding reactions (Park et al., 1988; Liu et al., 1989).

Although a number of extracytoplasmic proteins contain disulfide bonds, they appear to fall into two classes with respect to their essentiality in establishing stable higher order structures. PhoA is one example in which disulfide bond formation is a prerequisite for a stable folding. In contrast, β-lactamase can be folded independently of the disulfide bond (Schultz et al., 1987; Laminet and Pluckthun, 1989). In our in vitro reaction, disulfide bond formation is immediately followed by folding into the PhoA species II (folded monomer with partial trypsin resistance). This is then followed more slowly by the formation of species III molecules (completely trypsin-resistant dimer) and of appearance of the enzymatic activity. Thus, a rate-limiting step in vitro may be the conversion of the species II to species III. Probably, a crucial reaction in this step is the dimerization of the PhoA subunits coupled with incorporation of zinc.

Our experiments did not address whether the in vitro folding of PhoA species I into species II as well as its dimerization into species III depended on other protein factors such as chaperones. However, the fact that even purified and denatured PhoA could be activated by purified DsbA indicate that the folding and assembly could proceed without chaperones,

at least in vitro. Importantly, our pulse-chase experiments demonstrated that a sequence of events similar to that revealed in vitro might take place in the cell. While disulfide bond formation in vivo is very rapid, the disulfide-bonded molecules initially remain sensitive to a reducing agent (DTT) which made them susceptible to some endogenous protease. On the basis of this sensitivity to DTT, we assume that this state of PhoA corresponds to the species II detected in vitro. A few more minutes later, PhoA molecules became resistant to DTT. Probably, dimerization of the species II-like molecules takes place during this period. Thus, the dimerization of the PhoA subunit might be rate-limiting in vivo as well.

The most interesting and important question here will be whether any factors other than DsbA participate in the folding and dimerization reactions in the cell. If they indeed do, mutations affecting their functions will define more clearly the different substeps of biogenesis of PhoA and other periplasmic proteins.

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