

## *Escherichia coli* Alkaline Phosphatase Fails To Acquire Disulfide Bonds When Retained in the Cytoplasm

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Received 14 June 1991/Accepted 21 September 1991

**The cysteines of the *Escherichia coli* periplasmic enzyme alkaline phosphatase, which are involved in disulfide bonds in the native enzyme, were found to be fully reduced when the protein was retained in the cytoplasm. Under these circumstances the cysteines remained reduced for at least several minutes after the synthesis of the protein was completed. This contrasted with the normally exported protein, wherein disulfide bonds formed rapidly. Disulfide bond formation accompanied export and processing. The implications of these findings for the inactivity of the enzyme in the cytoplasm are discussed.**

The properties of the enzyme bacterial alkaline phosphatase (AP) have provided the basis for a convenient *in vivo* strategy for investigating protein localization and topology in *Escherichia coli* (for a review, see reference 18). This strategy has been developed around the observation that the normally periplasmic AP is enzymatically active only if exported from the cytoplasm (5). Retention in the cytoplasm by a variety of signal sequence mutations or deletions yields an inactive protein that is typically unstable (11, 17, 20). In cases where some fraction of wild-type export is preserved, the amount of activity correlates well with the amount of export (19). Thus the gene for AP, *phoA*, modified so as to no longer contain the information that codes for its signal sequence, can be fused to a gene of interest. Export of AP and enzymatic activity will thereby require that export information be present in the target protein.

The inactivity of AP in the cytoplasm of *E. coli* has not been explained. One possibility, that rapid degradation in the cytoplasm preempts proper folding, has been discounted (5). Rather, the reverse appears to be true—that the failure of the protein to assume a native, enzymatically active conformation in the cytoplasm results in its rapid degradation. Indeed, cytoplasmic AP is susceptible to proteolytic digestion at concentrations where the native AP is fully resistant, indicating that cytoplasmic AP is either misfolded or unfolded (1).

The native enzyme is well characterized both structurally and enzymologically (15). The crystal structure has been refined to a 2.0-Å (0.2-nm) resolution (14). The enzyme is a twofold symmetrical homodimer. There are no interchain covalent linkages, but each monomer contains two intrachain disulfide bonds, and these involve all of the cysteines in the protein. Each monomer contains an active site that includes the hydroxyl group of Ser-102 and three cation binding sites which bind two Zn<sup>2+</sup> ions and one Mg<sup>2+</sup> ion. The native enzyme functions as a nonspecific phosphomonoesterase.

One approach to investigating cytoplasmic inactivity is to determine the ways in which the cytoplasmic protein differs from its well-characterized periplasmic counterpart. The cytoplasmic protein, to begin with, has a signal sequence and the periplasmic protein does not. Cytoplasmic precursor has been purified and is able to fold into an active conformation

*in vitro* although with slow kinetics (12). The signal sequences of other *E. coli* periplasmic proteins have been shown to retard the folding of the mature portion in purified systems (16, 22). It is unlikely, however, that such a phenomenon could itself explain the cytoplasmic inactivity of AP since if the coding region for the signal sequence is eliminated from the *phoA* gene, the resulting protein is still unable to assume an active conformation in the cytoplasm (8). In addition, there are many enzymatically active periplasmic AP fusion proteins with long amino-terminal extensions derived from unrelated proteins. From the standpoint of both folding and activity, AP appears to be indifferent to material at its amino terminus.

This report describes experiments that demonstrate another distinction between the cytoplasmic protein and native AP. The cysteines of AP retained in the cytoplasm are reduced. Oxidation of the sulfhydryls to disulfides accompanies export of the protein from the cytoplasm. We speculate as to whether the absence of disulfides is sufficient to explain the enzymatic inactivity of the cytoplasmic protein.

**Assay for the redox state of cysteines in AP.** The reduced and oxidized forms of AP and of its derivatives such as PhoA61, the cytoplasmically localized product of a strong signal sequence mutation of *phoA* (20), can be distinguished by migration through sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. The oxidized form of each protein is the faster-migrating species. Whole-cell lysates were exposed to the sulfhydryl alkylating agent iodoacetamide (IA), and the electrophoretic migration of the resulting proteins was compared to markers for reduced and oxidized forms. This strategy was adopted from an earlier study in which the *in vivo* redox state of the cysteines in the TEM1  $\beta$ -lactamase was determined (24).

Cultures were grown in minimal M63 medium containing 0.2% each ribose and glycerol and supplemented with 19 amino acids (no methionine) to an optical density at 600 nm of approximately 0.3. Cultures were pulse labeled with [<sup>35</sup>S]methionine (1,000 Ci/mmol) at 15  $\mu$ Ci/ml for 1 min, at the end of which 0.1 volume of freshly prepared 1% cold methionine was added. Immediately thereafter, two 700- $\mu$ l samples were withdrawn from each culture and added to equal volumes of 10% trichloroacetic acid prechilled on ice. After the mixture was vortexed, 700  $\mu$ l was removed from each tube to a fresh tube, to give four samples per culture. Trichloroacetic acid precipitation of the labeled cells was allowed to proceed on ice for at least 1 h. The pelleted

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precipitates were washed twice with 90% acetone–0.1 N HCl and then dried in a desiccator.

The four pellets from each culture were treated as follows. Two were resuspended in 50  $\mu$ l of deaerated carboxymethylation buffer (1.5% SDS, 5 mM EDTA, 100 mM Tris-HCl [pH 9.0]), and to one of these was added dithiothreitol (DTT) to 35 mM. Both samples were boiled for 10 min. Each then received 50  $\mu$ l of a freshly prepared 100 mM solution of IA in carboxymethylation buffer. Carboxymethylation was carried out at room temperature for at least 1 h. These manipulations were performed under argon gas in order to prevent in vitro oxidation of sulfhydryls. The carboxymethylated samples were then diluted and immunoprecipitated with polyclonal sera raised against AP and against ribose-binding protein as described previously (13) except that the *Staphylococcus aureus* pellets were washed in a high-salt buffer (1 M NaCl, 1% Triton X-100, 1 mM EDTA, 50 mM Tris-HCl [pH 8.0]) and boiled in a Laemmli SDS-polyacrylamide gel electrophoresis (PAGE) sample preparation buffer containing no  $\beta$ -mercaptoethanol (2% SDS, 15% glycerol, bromphenol blue, 125 mM Tris-HCl [pH 6.8]). The protease inhibitor phenylmethylsulfonyl fluoride was present at 150  $\mu$ g/ml during carboxymethylation and immunoprecipitation.

The other two pellets were prepared as markers for the oxidized and reduced forms of the protein of interest. Both were boiled in carboxymethylation buffer and then directly diluted and immunoprecipitated as described above. Two SDS-PAGE sample preparation buffers were used; one contained  $\beta$ -mercaptoethanol (reduced form) and one did not (oxidized form). A 10% gel was used for SDS-PAGE.

**Cytoplasmic AP does not have disulfide bonds.** The procedure described above was carried out on strain Mph1061, which expresses the PhoA61 mutant cytoplasmic precursor protein, and strain Mph42, which expresses mature, wild-type AP (20). Both proteins are expressed constitutively. The result is shown in Fig. 1a. The mobility of the IA-treated mature AP corresponded to that of the oxidized marker, confirming the presence of disulfide bonds. By contrast, the mobility of the IA-treated PhoA61 protein corresponded to that of the reduced marker, indicating that these disulfide bonds are absent in PhoA61. The samples that were reduced with DTT prior to treatment with IA served as controls for the efficiency of carboxymethylation. As expected, their migration corresponded to that of the reduced markers.

In Fig. 1b is shown the same experiment for the MalF A fusion, a well-characterized cytoplasmic AP fusion protein (6). Synthesis of this protein was induced for 20 min prior to a pulse labelling and a 5-min chase. At both early and late chase points, migration of the carboxymethylated protein corresponded to that of its counterpart that was boiled in DTT prior to carboxymethylation. Likewise, the product of another mutant *phoA* gene, in which the material that codes for the signal sequence is deleted completely, was fully reduced after a pulse and remained fully reduced after a 7-min chase (data not shown).

**Disulfide bond formation accompanies export of AP from the cytoplasm.** Since the cysteines of AP are reduced in the cytoplasm and oxidized in the periplasm, it seemed likely that oxidation was associated with the export process. This association was examined with *phoA73*, a weak signal sequence mutant. Only 30% of the PhoA73 protein is exported, and this export occurs very slowly, over the course of about 1 h (19). The carboxymethylation procedure was performed on strain Mph56, in which the PhoA73 protein is expressed constitutively. The labelled culture was chased for 45 min

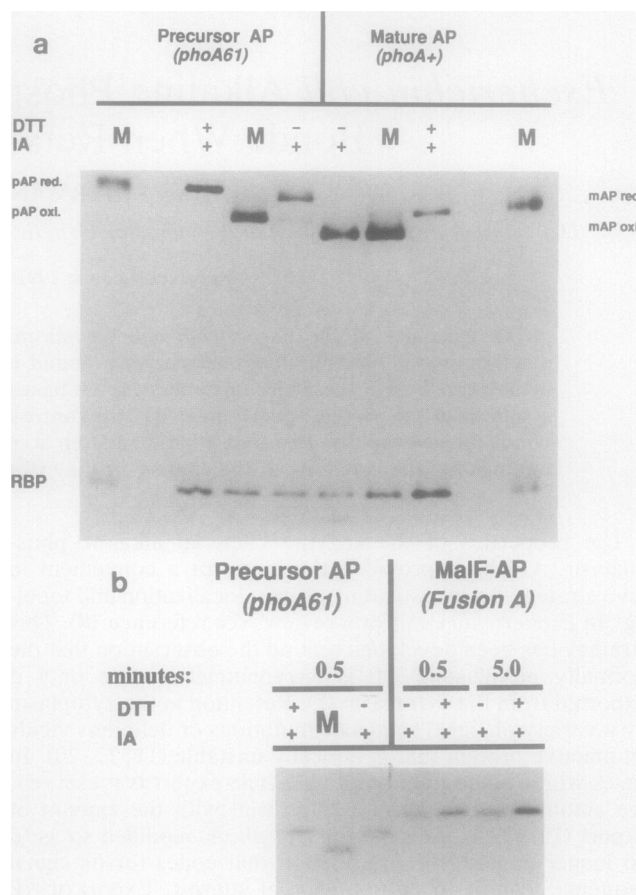


FIG. 1. Redox state of the cysteines in AP that is retained in the *E. coli* cytoplasm. Cultures were grown, pulse labelled, chased (in panel B only), lysed, treated with iodoacetamide, and immunoprecipitated, and the immunoprecipitates were subjected to electrophoresis as described in the text. Markers for reduced and oxidized forms of these proteins appear in lanes designated M. (A) PhoA61 mutant precursor protein. pAP, pre-AP; mAP, mature AP; red., reduced; oxi., oxidized; RBP, ribose-binding protein. (B) MalF A cytoplasmic AP fusion protein. M, oxidized form of the AP precursor; minutes, minutes into the chase. Ribose-binding protein bands are not shown in panel B.

with cold methionine, and aliquots were added to trichloroacetic acid at intervals.

The samples that were reduced prior to carboxymethylation (Fig. 2, left side) illustrate the slow export kinetics of this mutant. There was little mature protein visible before 5 min into the chase. However, by 45 min, nearly all of the precursor had been either degraded or exported and processed to the mature form. The corresponding samples that were carboxymethylated directly without prior reduction (right side) revealed that AP in the early stages of export is a reduced precursor that chases in the later stages of export into an oxidized mature form.

Disulfide bond formation in this system appeared to involve some discrete intermediates. For example, some fully reduced mature protein was visible at the intermediate time points, most conspicuously at 5 min. However, a few features of this system make it difficult to sort out precisely the relative kinetics of export and disulfide bond formation. First, the signal sequence mutation may retard processing of the precursor. The oxidized precursor that appeared at the

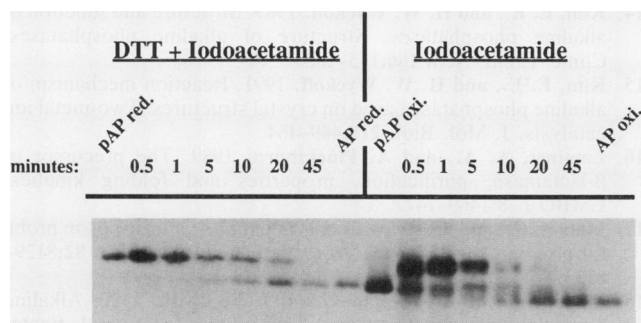


FIG. 2. Kinetics of disulfide bond formation in slowly exported AP. See the legend to Fig. 1 and the text for experimental procedure. Markers for precursor and mature AP were derived from pulse labelling of strains Mph1061 and Mph42. Markers for the reduced forms were generated by DTT treatment and carboxymethylation; markers for the oxidized forms were generated by air oxidation. Ribose-binding protein bands are not shown.

earliest time point may represent a portion of the total protein that is exported with wild-type or near wild-type kinetics and is only slowly processed. Second, that two disulfide bonds must form, not necessarily at equal rates, complicates the analysis. Markers for the expected intermediates are not easily generated. So while it cannot be said that export is required for disulfide bond formation, the profile of PhoA73 does illustrate that export and disulfide bond formation are associated processes.

Several years ago it was shown by Pollitt and Zalkin that if the periplasmic enzyme TEM1  $\beta$ -lactamase is retained in the cytoplasm, its cysteines are in the form of reduced sulfhydryls and that oxidation to the disulfide occurs concomitantly with export from the cytoplasm (24). Here we have adopted essentially the same strategy and have shown that the same is true for AP. We are unaware of any other prokaryotic proteins for which the process of disulfide bond formation has been examined *in vivo*, but the few studies with eukaryotic proteins are consistent with these findings. Pulse-labelled cells exposed to IA yield either nascent chains or full-length proteins that contain native disulfides as well as sulfhydryls not yet oxidized to native disulfides. Taken together, these studies suggest that disulfides form during or following passage of the polypeptide chain through the cytoplasmic membrane of bacteria or rough endoplasmic reticulum membrane of eukaryotic cells (3, 4, 23).

The work with  $\beta$ -lactamase and the present study further suggest that disulfide bonds are unable to form in the *E. coli* cytoplasm. Both proteins may be retained in the cytoplasm for several minutes with no evidence of sulfhydryl oxidation. By contrast, oxidation occurs so rapidly upon export that it is unclear whether processing or disulfide bond formation occurs first (24 and Fig. 1a). The reducing potential of the cytoplasm, if not incompatible with the formation and maintenance of stable disulfide bonds in this compartment, is certain to antagonize the process (10, 27). Disulfide bond formation would be expected to occur then only with passage of the protein into the oxidizing environment of the periplasm. That very few cytoplasmic proteins have been reported to have stable disulfide bonds is in keeping with this hypothesis (25), and the few exceptions underscore the general principle. There is evidence, for example, that the disulfide bonds of bovine pancreatic trypsin inhibitor (BPTI) can form in the cytoplasm of *E. coli*. But BPTI is unusual in

that its native disulfide bonds can form efficiently in a highly reducing environment (21).

A mutant that is kinetically defective for disulfide bond formation in the periplasm of *E. coli* has recently been isolated and characterized (2). Although AP is exported to the periplasm in this mutant, IA trapping of pulse-labelled cells reveals that the mature form of AP is fully reduced. Most of this protein is degraded over the course of 15 min. About half of the small amount of AP that does persist is found to have oxidized during that time. This finding is consistent with those presented here, in that it suggests that AP emerges from a cytoplasmic environment in which it is fully reduced.

The present study was undertaken as part of an effort to understand why AP is enzymatically inactive when retained in the cytoplasm. The disulfide bonds of AP have been shown to be important for structure and activity. Two mutants of AP in which a cysteine is exchanged for another amino acid have been constructed (9). The cysteines changed in these two mutants participate in different disulfide bonds. Since the enzymatic activity of the resulting APs is either greatly diminished or eliminated altogether, disulfide bond formation is probably required for assembly of AP into a stable conformation.

Is the inability to form disulfide bonds the primary reason for the failure of AP to assume its native conformation in the cytoplasm? Or do disulfide bonds fail to form because some prior requirement for proper assembly cannot be satisfied? Although these questions cannot be answered until the *in vivo* folding pathway of the protein is elucidated, we believe that the inability to form disulfide bonds is a good candidate for the primary block. Both  $Zn^{2+}$  and  $Mg^{2+}$  are present in the cytoplasm, and many cytoplasmic metalloenzymes make use of these cations. It is unlikely that folding is inhibited by cation sequestration, particularly since the  $K_d$  of AP for  $Zn^{2+}$  is very low (7). The oligomeric state of AP that is retained in the cytoplasm has not been investigated. While there are many oligomeric enzymes in the cytoplasm, it is unlikely that dimerization of AP can proceed to completion between monomers that are themselves not properly folded.

It is to the benefit of the cell that AP cannot assume its native conformation in the cytoplasm. As a nonspecific phosphomonoesterase, a cytoplasmically active AP could be expected to seriously disrupt intracellular metabolism. Many periplasmic enzymes have degradative activities that could not be tolerated intracellularly, and many of these enzymes are likely to have disulfide bonds (26). If disulfide bond formation were in general prohibited in the cytoplasm, these proteins would be less competent for assuming their enzymatically active conformations in a compartment where they could be harmful than they would be for export to the compartment where they are of service.

We thank the laboratory of John Collier for generously supplying equipment and technical advice. We are especially grateful to Karl Reich, Cameron Douglas, and Brenda Wilson. We thank Catherine Lee and Dana Boyd for helpful advice and support.

This material is based upon work supported under a National Science Foundation Graduate Fellowship to A.D. and under grants from the American Cancer Society to J.B. J.B. is an American Cancer Society Research Professor.

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