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

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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 Zn²⁺ ions and one Mg²⁺ ion. The native enzyme functions as a nonspecific phosphohydroxysterase.

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 sulphydryls 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 sulphydryl 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 β-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 labelled with [³⁵S]methionine (1,000 Ci/mmol) at 15 μCi/ml for 1 min, at the end of which 0.1 volume of freshly prepared 1% cold methionine was added. Immediately thereafter, two 700-μl samples were withdrawn from each culture and added to equal volumes of 10% trichloroacetic acid prechilled on ice. After the mixture was vortexed, 700 μl was removed from each tube to a fresh tube, to give four samples per culture. Trichloroacetic acid precipitation of the labelled cells was allowed to proceed on ice for at least 1 h. The pelletted
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 μl of deaerated carboxymethylating 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 μl of a freshly prepared 100 mM solution of IA in carboxymethylating 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 β-mercaptoethanol (2% SDS, 15% glycerol, bromphenol blue, 125 mM Tris-HCl [pH 6.8]). The protease inhibitor phenylmethylsulfonyl fluoride was present at 150 μg/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 carboxymethylating buffer and then directly diluted and immunoprecipitated as described above. Two SDS-PAGE sample preparation buffers were used; one contained β-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 on 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 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
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 β-lactamase is retained in the cytoplasm, its cysteines are in the form of reduced sulphydryls 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 sulphydryls 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 β-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 sulphydryl 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 Zn2+ and Mg2+ 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 Kd of AP for Zn2+ 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.

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