Alkaline phosphatase (AP) is a nonspecific phosphomonoesterase that is ordinarily found in the periplasm of *Escherichia coli* during conditions of phosphate starvation (44). If retained in the cytoplasm, AP is enzymatically inactive (19, 28). This property of AP forms the basis for a convenient in vivo strategy for monitoring protein translocation across the cytoplasmic membrane (26).

We recently established that the enzymatic inactivity of AP in the cytoplasm is due to the inability of the intramolecular disulfide bonds that are present in the native enzyme to be formed in the cytoplasm (14). The following series of observations led us to this conclusion. There are two intramolecular disulfide bonds in each monomer of the native AP homodimer (21). These disulfide bonds must be formed for AP to achieve its enzymatically active conformation (17). These disulfide bonds are not formed when AP is retained in the cytoplasm of a wild-type strain (13). Mutants can be selected in which a variant of AP without a signal sequence (APΔ2-22) does acquire enzymatic activity in the cytoplasm (14). The mutations responsible for the activation of APΔ2-22 in the cytoplasm arise in the *trxB* gene, which codes for thioredoxin reductase, an enzyme with sulfhydryl redox activity (29). We proposed that thioredoxin reductase is required to maintain the reduced state of proteins in the cytoplasm. In support of the proposition, we observed that in the cytoplasm of a strain that lacks thioredoxin reductase, most or all of the APΔ2-22 has disulfide bonds (14). Because eradication of an activity likely to be involved in the control of disulfide bond formation in the cytoplasm was sufficient to activate AP in the cytoplasm, we concluded that AP was enzymatically inactive if retained in the cytoplasm specifically because its disulfide bonds could not ordinarily be formed in this compartment (14).

These findings point to some basic observations about disulfide bond formation in *E. coli* as well as in eukaryotic cells. In general, disulfide bonds are absent from cytoplasmic proteins and are found only in exported proteins (38, 41). These disulfide bonds are formed during or following translocation of the protein across the cytoplasmic membrane or across the membrane of the endoplasmic reticulum (2, 3, 7, 13, 31, 34). In recent years, it has become clear that cells do not rely upon spontaneous oxidation to accomplish this process and that disulfide bond formation is in fact facilitated. In *E. coli*, the Dsb proteins promote disulfide bond formation in proteins of the cell envelope (1). In the absence of the Dsb proteins, disulfide bonds are formed with greatly diminished speed and efficiency.

Thus, for proteins of the *E. coli* cell envelope that have disulfide bonds in their native structures, two features of cell physiology make the formation of these disulfide bonds unlikely should the proteins be retained in the cytoplasm. Thioredoxin reductase is likely to be involved in a process that antagonizes the formation of disulfide bonds in the cytoplasm. And there are presumably no Dsb proteins or an equivalent system to facilitate the formation of disulfide bonds in the cytoplasm.

In the present study, we report that disulfide bonds can form slowly in AP that is retained in the cytoplasm of wild-type (*trxB*−) cells, provided that the cells are no longer growing. This process results in the accumulation of active AP in the cytoplasm and can therefore lead to spurious data in the context of AP fusion protein studies. We therefore offer a simple method of preventing activation. Study of this disulfide bond formation may give insight into the means by which it is prevented in growing cells. The phenomenon can also be exploited for the production in the *E. coli* cytoplasm of foreign proteins that have disulfide bonds in their native structures.
MATERIALS AND METHODS

Materials. The media used for growth and maintenance of E. coli strains, the reagents used for radiolabeling and immunoprecipitation, and the sources for biochemical reagents have been described previously (15). Iodoacetamide (I-6125) was obtained from Sigma Chemical Co. A 1 M stock solution was prepared in 10 mM Tris-HCl, pH 8.0, and maintained frozen in aliquots at −20°C. An aliquot was used and refrozen (at −20°C) a few times before being discarded.

Bacterial strains and plasmids. Strains are listed in Table 1. Most experiments were carried out with E. coli DBH4, a derivative of strain MC1000 (9), which contains the phoA ProH deletion (20). Strain ADS87 is a derivative of DBH4, contains a single chromosomal copy of the wild-type phoA gene under tac promoter control, and was constructed by integration of a derivative of phagemid pDHB5059 at the phoA locus (DphoA ProHH deletion) as described previously (15). The Δpho deletion in strain AD370 is derived from strain DF657 (39), the trxB::kan disruption in strain AD404 is derived from strain A304 (35), and the ΔtrxA307 deletion in strain AD500 is derived from strain A307 (36).

Plasmids and phagemids from which are expressed mutant versions of AP that are localized to the cytoplasm and the N-terminal sequences of these proteins are listed in Table 2. Strains carrying plasmid pAD135 also carried either plasmid pACYCClacPl (6) or plasmid pACYCClacPl-CAM (15).

Enzymatic assays of AP. Cultures were grown in minimal M63 medium containing 0.2% ribose and glycerol, 50 µg (each) of 18 amino acids (no cysteine or methionine) per ml, and appropriate antibiotics (15). Expression of proteins such as APΔ2-22 from the tac promoter was induced with 5 mM isopropyl-thio-β-D-thigalactopyranoside (IPTG) for 25 min. The final optical densities of the cultures at 600 nm were generally from 0.3 to 0.5. Cells were washed twice with a buffer consisting of 50 mM NaCl, 10 mM NH4Cl, 10 mM MgCl2, and 40 mM 3-(N-morpholino)propanesulfonic acid (MOPS) (K+ salt), pH 7.3, and then resuspended in this buffer. Washed cells were assayed at 28°C as described previously (8).

To prevent the spontaneous activation of AP that is localized to the cytoplasm, cells were added to prechilled microcentrifuge tubes containing iodoacetamide to yield a final concentration of 1 mM. The wash buffer described above was supplemented with 1 mM iodoacetamide as well.

Subcellular fractionation of AP activity. Cultures were grown in minimal M63 medium containing 0.2% ribose and glycerol, 50 µg (each) of 18 amino acids (no cysteine or methionine) per ml, and appropriate antibiotics (15). Expression of APΔ2-22, wild-type AP, or the MalF A fusion protein from the tac promoter was induced with 5 mM IPTG for 2 h. The final optical density at 600 nm was approximately 0.6. Three milliliters of the culture was removed to ice and then washed twice with wash buffer containing no iodoacetamide. The washed cells were concentrated twofold in wash buffer and maintained on ice for a total of 5 h, with occasional vortexing. The cells were then washed with spheroplast buffer supplemented with protease inhibitors (15) in order to remove the Mg2+. Subcellular fractionation was carried out as described previously (14), and the fractions and lysed cells were assayed for AP, β-galactosidase, and β-lactamase as described previously (15). In addition, AP assays were carried out on 1 ml of the culture that had been treated with iodoacetamide and washed as described above. These assays measured the small amount of AP activity that had accumulated in the periplasm of these strains during the course of the induction. The value was subtracted from the activity measured in the lysed cells and the spheroplast supernatant.

Radio labeling and immunoprecipitation. The procedures for the radiolabeling of cells and the immunoprecipitation of their contents have been described previously (15). Nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously (13).
was apparent or could be inferred from the correlation coefficients calculated from a least-squares fit of the data (Fig. 3).

An increase was also observed with strains DHBS043 and DHBS045, which express larger AP fusion protein derivatives of MalF that are inserted into the cytoplasmic membrane with their AP moieties localized to the cytoplasm (Fig. 2 and 3). In none of these cases was the increase comparable in magnitude.

### RESULTS

Cells expressing certain variants of AP that are localized to the cytoplasm gradually acquire enzymatic activity when they are no longer growing. As part of our effort to understand the basis for the enzymatic activity of AP in the cytoplasm, we had constructed phagemid pAID135, from which are expressed AP without its signal sequence, i.e., APΔ2-22 (15). Strains such as AD135 and WP12, which carry this phagemid, gave rise to pale blue colonies on agar plates containing the chromogenic indicator for AP, 5-bromo-4-chloro-3-indolyl phosphate (XP) as was expected for strains that export little AP to the periplasm. When cultures were assayed for AP activity, however, the values obtained were variable and often unexpectedly high. Manoil's group encountered similar anomalies in its work with strains expressing AP fusion proteins that would be expected to be localized to the cytoplasm. The group observed that activity increased with the length of time that the cells were allowed to sit in buffer at ambient temperature (24). We found that the same was true for strains expressing APΔ2-22. Over a period of 5 h, during which time cells of strain AD135 were held at ambient temperature after having been washed at ambient temperature, the AP activity increased 10-fold (Fig. 1a). A 40-fold increase was observed when the cells were washed at 4°C and held on ice (Fig. 1a). This increase represents activation of at least 30% of APΔ2-22 (15). A similar pattern was observed with a strain expressing the MalF fusion protein A (MalF A). The AP moiety of this protein fusion to the integral cytoplasmic membrane protein MalF is localized to the cytoplasm (Fig. 2). AP activity increased nearly 20-fold when cells of strain DHBS046 were washed and held at ambient temperature for 5 h and 45-fold when these procedures were performed in the cold (Fig. 1b).

A linear time-dependent increase in activity was observed with strains expressing either a mutant AP precursor protein with a strong signal sequence mutation (phaA61) or other AP fusion proteins (Table 1). The amino-terminal sequences of these fusion proteins lack the features of a signal sequence and would not be expected to promote export (43). For all but one of these strains, a time-dependent linear increase in AP activity

### TABLE 1. Subcellular fractionation of AP activity after activation

<table>
<thead>
<tr>
<th>Enzyme Fraction</th>
<th>AD370/WP12 (APΔ2-22)</th>
<th>AD387 (AP)</th>
<th>DHBS046 (MalF A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysed Cells</td>
<td>857</td>
<td>2,660</td>
<td>478</td>
</tr>
<tr>
<td>Spheroplasts</td>
<td>686</td>
<td>52</td>
<td>428</td>
</tr>
<tr>
<td>Supernatant</td>
<td>162</td>
<td>2,584</td>
<td>32</td>
</tr>
<tr>
<td><strong>Lactamase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysed Cells</td>
<td>1,416</td>
<td>ND</td>
<td>888</td>
</tr>
<tr>
<td>Spheroplasts</td>
<td>47</td>
<td>ND</td>
<td>28</td>
</tr>
<tr>
<td>Supernatant</td>
<td>1,452</td>
<td>ND</td>
<td>914</td>
</tr>
<tr>
<td><strong>β-Galactosidase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysed Cells</td>
<td>284</td>
<td>363</td>
<td>394</td>
</tr>
<tr>
<td>Spheroplasts</td>
<td>254</td>
<td>354</td>
<td>346</td>
</tr>
<tr>
<td>Supernatant</td>
<td>32</td>
<td>22</td>
<td>57</td>
</tr>
</tbody>
</table>

* The experiment was carried out twice with slightly different induction times, and essentially the same results were obtained. The data presented are from one trial. Values for strains AD370 and WP12 were averaged. Standard assays of AP were carried out prior to the incubation of the cells on ice in order to determine the activity accumulated in the growing cells during the induction (strain AD370, 50 U; strain WP12, 54 U; strain AD387, 2,769 U; and strain DHBS046, 2 U). In an exercise described in Materials and Methods, these values were subtracted from what was measured in the lysed cells and the supernatant fractions in order to generate the entries in the table for strains AD370/WP12 and DHBS046.

* ND, not determined.
to that observed with strains AD135 and DHB5046. No increase was observed with the following strains: (i) Mph42, which expresses wild-type AP; (ii) DHB519, which expresses an AP fusion protein derivative of MalF, MalF B, whose AP moiety is localized to the periplasm (Fig. 2), and (iii) Mph29, which expresses no AP at all (data not shown).

Activation corresponds to the folding of AP into its native conformation. The disulfide bonds of AP must be formed for AP to acquire its native, enzymatically active conformation (17), and these disulfide bonds are absent from APΔ2-22 in growing cells (13). The increase in activity of APΔ2-22 must therefore be accompanied by the acquisition of disulfide bonds in the protein. In parallel with the increase in activity, the reduced APΔ2-22 was indeed converted into oxidized protein whose migration on nonreducing SDS-PAGE matched that of the native enzyme (Fig. 4). If disulfide bond formation was inhibited, as by treatment of cultures with the sulfhydryl-alkylating agent iodoacetamide, the increase in activity was likewise abolished (Fig. 1).

Activation was also inhibited if cells were washed and held at 37°C (Fig. 1). Cells that were grown at ambient temperature had little AP activity immediately after growth, but activity accumulated in a manner resembling that of cells grown at 37°C once growth was suspended (data not shown). Therefore, cell lysis did not take place in growing cells even if they were growing at lower temperatures. Instead, activation was initiated once the cells ceased growing, with the rate and extent of activation determined by the temperature at which the cells were held following growth. We verified that indeed no growth had occurred during these incubations. The cells were washed and held in a buffer deficient in nutrients, and no increase in optical density or in viable counts was observed. Activation was not prevented by 50 mM NaN₃, which is presumably sufficient to kill the cells. The buffer in which the cells were washed and held had only small effects on the rate and extent of activation (data not shown). The effects of pH and ionic strength were not investigated.

Activation occurs in the cytoplasm. AP is unable to fold in the cytoplasm of growing cells because the formation of disulfide bonds is prevented in this compartment (14). A trivial explanation for the activation we observed, then, was that prolonged incubation of cells in buffer resulted in their lysis and the release of the AP to the medium. Liberated from the cytoplasm, the AP could then fold. We found no evidence, however, in support of this explanation. The integrity of the cells was unaffected by prolonged incubation in cold buffer. As mentioned above, optical density and viable counts were unchanged at the end of the incubations. When a culture of strain AD135 was lysed with lysozyme, only 30 U of AP activity was measured. When this lysate was then maintained on ice for 5 h, AP activity increased only slightly, to 50 U. Therefore, cell lysis cannot and does not account for the activation. On the contrary, cellular integrity is actually required for the activation.

The observation that cellular integrity was preserved raised the question of where within the cell the activation was occurring.

FIG. 4. Disulfide bond formation in APΔ2-22 during activation. Strain AD135 was grown in 10 ml of M63 minimal medium containing 0.2% (each) ribose and glycerol and supplemented with 50 μg (each) of 18 amino acids (no cysteine or methionine) per ml, 10 μg of tetracycline per ml, and 100 μg of ampicillin per ml. Expression of APΔ2-22 was induced with 5 mM IPTG for 25 min, after which the culture was pulse radiolabeled with 300 μCi of [35S]methionine for 1 min and chased for 30 s. The culture was then chilled on ice after part was treated with iodoacetamide. Cells were then pelleted and washed with chilled buffer with or without iodoacetamide. After the washes, part of the culture that had been treated with iodoacetamide was precipitated with trichloroacetic acid in preparation for immunoprecipitation and part was used for enzymatic assays of AP. The same was done for the culture that had not been treated with iodoacetamide, both immediately after the washes and at the indicated times thereafter. Approximately 35 min elapsed between the end of the radiolabeling and the beginning of the first set of assays (incubation time 0 min). The immunoprecipitations were carried out with antibodies to AP and to ribose-binding protein, and immunoprecipitates were fractionated on nonreducing SDS-PAGE. Markers for the migration of reduced (red.) and oxidized (oxi.) AP were derived from a previous radiolabeling of the phoA+ strain AD158, β-Mercaptotetanol was present in the lane containing the marker for reduced AP. iodo, iodoacetamide; AP red., reduced AP; AP oxi., oxidized AP. Enzymatic activities were iodoacetamide (iodo), 8 U; 0 min, 30 U; 15 min, 60 U; 30 min, 79 U; 1 h, 126 U; 2 h, 172 U; 3 h, 222 U; 4 h, 281 U; and 5 h, 303 U.

FIG. 3. Activation of other cytoplasmic variants of AP. Cultures were grown and induced at 37°C for enzymatic assays of AP. At the end of the induction period, part of each culture was pelleted in a microcentrifuge tube that had been precooled on ice. Cells were washed at 4°C with chilled washed buffer and then held on ice. At the times indicated, assays were begun by the addition of washed cells to prewarmed assay mixtures containing permeabilizing agents and p-nitrophenyl phosphate. Approximately 25 min elapsed between when growth was suspended and when the first set of assays (incubation time 0 min) were begun. The strains are PS30 (U3), PS77 (U6), PS113 (SecE-115), Mph1061 (PhoA61), SEC13 (SEC/Fusion D13), DHB5043 (MalF/Fusion C), and DHB5045 (MalF/Fusion D). PS30, PS77, PS113, and Mph1061 were assayed in one experiment, and the remaining strains were assayed in a second experiment. For each strain, correlation coefficients for a least-squares fit of the data to a straight line are given in parentheses.

FIG. 2. Positions of AP fusions to MalF (adapted from an illustration in reference 6).
We have described a peculiar process in which a protein that is enzymatically inactive in the cytoplasm of growing cells slowly acquires enzymatic activity in the cytoplasm once the growth of the cells is arrested. We emphasize that the cells must not be growing in order for the activation to occur. AP is not enzymatically active in the cytoplasm of growing E. coli. Strains that express AP\(\Delta 2-22\) do not satisfy selections for active cytoplasmic AP (14). Export of a small amount of AP\(\Delta 2-22\) to the periplasm accounts for the activity that is present in these strains and for the pale blue color of the colonies they form on agar plates containing XP (15). For AP\(\Delta 2-22\) to become enzymatically active in the cytoplasm of growing cells, mutations in cellular factors such as thioredoxin reductase are required (14).

Iodoacetamide should be present during the preparation of cells for AP assays so that spurious results are avoided. The observation that AP is only enzymatically active if exported to the periplasm forms the basis for its use as a reporter for protein export (26). The present study was prompted by a finding that appeared to be inconsistent with that observation. These experiments demonstrate that there really is no inconsistency, that the activation of AP is not due to its illegitimate export to the periplasm or to any process that occurs during growth of the cells. Nevertheless, the ready activation of AP in the cytoplasm of nongrowing cells can lead to spurious AP activity measurements and therefore to erroneous conclusions about protein export and topology. We therefore recommend that iodoacetamide be present during the preparation of cells for enzymatic assays of AP (see Materials and Methods). We found that 1 mM iodoacetamide inhibits activation almost completely in our assays, but higher concentrations may be necessary for strains that produce a great deal of fusion protein or for cultures assayed at high densities. Iodoacetamide should be used routinely in assays of cellular AP performed in conjunction with AP fusion protein studies. We note that iodoacetamide is not an effective substitute for iodoacetamide (data not shown), presumably because the charged species is less capable of traversing the cytoplasmic membrane.

Our very limited survey of AP fusion proteins gives us little sense of how often one should expect to encounter activation to the extent we have seen with AP\(\Delta 2-22\) and the MalF A fusion protein. For many of the proteins listed in Table 2, the activation was inconsequential and measured AP activity would not depart significantly from the correct in vivo value if cultures were assayed within 1 h of the suspension of growth (Fig. 3). It is probably of some significance here that two laboratories in which much effort has been directed towards the use and refinement of the AP fusion system stumped upon this phenomenon only some 5 years after the strategy was developed. Nevertheless, substantial activation is not unique to AP\(\Delta 2-22\) and MalF A, and has also been observed from fusion proteins to β-galactosidase and to chloramphenicol acetyltransferase, which contain only a few amino acids upstream of the AP moiety (24). It has also been observed from an AP fusion protein to the beginning of the cytoplasmic membrane protein MalG (42). We have been unable to discern any motifs or patterns common to the amino acid sequences preceding the AP moiety of those proteins from which the greatest activation is observed (Table 1). However, the length of these sequences is likely to be important. The proteins from which the greatest activation is observed have fewer than 40 amino acids preceding the AP moiety. The longer sequences may be more likely to antagonize the folding of the AP moiety or its dimerization, particularly if they can adopt secondary structure. The signal sequences of several proteins have been proposed to retard the folding of the purified precursor in just this way (23, 30). The extent of activation is most likely not a simple function of the amount of the protein present in the cytoplasm. Although the steady-state levels of the proteins surveyed for Fig. 3 were not determined quantitatively, we estimate the steady-state level of PhoA61 to be roughly the same as that of AP\(\Delta 2-22\) (12).

Wild-type cells that are not growing may simulate a thioredoxin reductase mutant. Activation results from the folding of AP into its native conformation in the cytoplasm. This folding is prevented in growing cells at the level of disulfide bond formation. It was possible that AP\(\Delta 2-22\) was folding in the cytoplasm, where it cannot fold in growing cells, or that AP\(\Delta 2-22\) was passing into the periplasm and folding there. Subcellular fractionation was performed on cells that were expressing AP\(\Delta 2-22\) and that had been held on ice for 5 h. The AP that accumulated during that period appeared in the cytoplasmic fraction (Table 2).

In order to determine whether activation required particular cytoplasmic factors, the activation profile was examined in strains carrying null mutations in those genes whose products have been shown to influence the ability of disulfide bonds to be formed in the cytoplasm. Elimination of thioredoxin reductase, for example, results in disulfide bond formation in AP\(\Delta 2-22\) in the cytoplasm and consequently in the activation of the protein in growing cells (14). Elimination of thioredoxin itself results in the in vivo activation of AP\(\Delta 2-22\) as well, but only at lower temperatures (12). Only a small amount of activation beyond that which had already occurred in vivo for a strain carrying a disruption of the thioredoxin reductase gene was observed during 5 h of incubation on ice (Fig. 5). But when cells carrying a deletion of the thioredoxin gene were incubated on ice for 5 h, both the rate of activation and the total accumulated AP activity were diminished considerably with respect to those of a wild-type strain (Fig. 5).

DISCUSSION

FIG. 5. Activation of AP\(\Delta 2-22\) in the absence of thioredoxin and thioredoxin reductase. Cultures were grown, induced, and assayed as described in the legend to Fig. 3, except that incubation time 0 min indicates the time at which cell growth was suspended and the cultures were transferred to ice. The strains are Trx− AD370 (two trials) and WP12 (one trial), trxB:kan AD404 (two trials with one isolate and one trial with another independent isolate), and ΔtrxA AD500 (one trial each with five independent isolates).
formation (14). It would seem, then, that whatever mechanism prevents disulfide bonds from forming in the cytoplasm of growing cells is gradually dissipated or abruptly suspended when growth is suspended. Because the formation of disulfide bonds does occur in the cytoplasm of growing cells that lack thioredoxin reductase (14), it is conceivable that the cytoplasm of wild-type cells comes to resemble the cytoplasm of a cell that lacks thioredoxin reductase when growth is suspended. Indeed, when the growth of a strain that lacks thioredoxin reductase was suspended, little activation occurred beyond that which had already occurred during growth of the cells. Thioredoxin reductase reduces disulfide bonds in proteins such as ribonucleotide reductase by transferring electrons from NADPH to the disulfide via a flavin cofactor and thioredoxin (29). If NADPH is dissipated in cells once their growth is stopped, thioredoxin reductase would be disabled. In addition, thioredoxin reductase may function ineffectively or not at all at the low temperatures at which activation is observed.

The enhancement of activation by low temperatures is most likely due to other factors as well. AP appears to fold more readily in the cytoplasm of growing cells at lower temperatures. The cytoplasmic chaperonin SecB, which antagonizes the folding of proteins to be exported and thereby maintains them competent for export (11), is required for the export of wild-type AP at 30°C but not at 37°C (22). Selections for the activation of AP in the cytoplasm (of growing cells) are less stringent at lower temperatures (12). The stability of AP is likely to be yet another factor. Because AP does not acquire its native conformation in the cytoplasm of growing cells, it is a ready substrate for cytoplasmic proteases (5). The proteolytic activity of the cytoplasm, at least with respect to AP, appears to be diminished at lower temperatures (40), leading to an increase in the amount of intact protein that can fold into active enzyme. These effects of low temperature may also account for our previous observation that the serine protease domain of murine urokinase is activated in the cytoplasm of a thioredoxin reductase mutant only once the growth of cells is suspended and the cells are chilled (14).

Cellular integrity was required for activation, suggesting that certain components of the cytoplasm participate in the process. If, as we propose, thioredoxin reductase does not function in cells that have ceased growing, a mechanism for activation is suggested. The thioredoxin that accumulates in the oxidized form would in turn oxidize sulfhydryls in the cytoplasm instead of maintaining their reduced state. Our results show that thioredoxin is indeed required for the efficient oxidation of AP at low temperature. We note, however, that we have ruled out precisely this mechanism as a possible explanation for the disulfide bond formation that occurs in the cytoplasm of (growing) thioredoxin reductase mutants (14). In that case, thioredoxin was found to be unnecessary for the observed disulfide bond formation. Those experiments were conducted at 37°C. It may be that the oxidizing ability of thioredoxin or the propensity of this activity is increased at lower temperatures.

The activation that we have described in this report could be exploited for the production of foreign proteins in E. coli. Many proteins of commercial value are exported from the cells in which they are produced and also have disulfide bonds in their native structures. Disulfide bond formation in E. coli ordinarily takes place in the periplasm, and so it is necessary either to ensure the export of these proteins to the periplasm or to express them in the cytoplasm in reduced form and then to oxidize them in vitro. Both of these approaches are fraught with difficulty and often result in a low yield of native protein (18).

If other proteins behave as AP does, an alternative strategy would be simply to express the foreign protein intracytoplasmically and to incubate the intact cells in cold buffer. On the basis of previous calculations, we estimate that approximately 30% of APA2-22 acquires activity (15). This may, however, be an underestimate, because all of the protein appears to be oxidized at the end of the incubation (Fig. 4). It is possible, though, that some of this oxidized protein has nonnative disulfide bonds. If the strategy does turn out to be efficient and applicable to a wide variety of proteins, it will afford a convenient means of producing proteins that are toxic to E. coli because the proteins do not attain their native conformations while the cells are growing.

The present study extends the observation we made in our previous study that the ability of E. coli to maintain the proteins of its cytoplasm in a reduced state represents an active process that draws upon the energy of the cell (14). It is therefore, perhaps, to have been anticipated that this ability would degenerate when the growth of cells was suspended. Oddly enough, and for reasons that continue to interest us, the cytoplasm of E. coli under these circumstances converts into an environment that is conducive to disulfide bond formation and in which the process may in fact be promoted. The cytoplasm of E. coli is well-equipped with factors to assist in the folding of proteins that are supposed to fold in this compartment, factors that have proven to be generally useful (32). It is therefore not surprising that the cytoplasm of E. coli cells whose growth has been suspended may make a very effective folding vessel for proteins that contain disulfide bonds.

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