Localization of Thioredoxin from *Escherichia coli* in an Osmotically Sensitive Compartment

(Received for publication, September 30, 1981)

Charles A. Lunn† and Vincent P. Pigiel§

From the McCollum-Pratt Institute and The Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

The low molecular weight dithiol protein thioredoxin was released from *Escherichia coli* strain W3110 grown to mid-log phase by several nondisruptive treatments. Rapid freezing of cells in media or in a Tris buffer (pH 7.4) containing 3 mM EDTA released greater than 90% of the thioredoxin. Thioredoxin was also quantitatively released by the osmotic shock procedures described by Nossal, N. G. and Heppel, L. A. (1966) *J. Biol. Chem* 241, 3055. These conditions release only 20% of the soluble nucleotide pool and less than 1% of the cytoplasmic glucose-6-phosphate dehydrogenase, inconsistent with extensive cytoplasmic leakage.

Release of thioredoxin by osmotic shock exhibited many characteristics in common with the osmotic release of the protein synthesis elongation factor Tu (EF-Tu), one of a group of cytoplasmic proteins associated with the inner surface of the inner membrane. As distinct from periplasmic proteins, both thioredoxin and EF-Tu were retained within spheroplasts prepared with lysozyme and EDTA. The release of both thioredoxin and EF-Tu was also uniquely sensitive to cations, with 1 mM MgCl₂ in the osmotic shocking buffer sufficient to block the release of both thioredoxin and EF-Tu. Release of the periplasmic protein alkaline phosphatase was unaffected by the presence of cations.

We conclude that thioredoxin belongs to a class of cytoplasmic proteins released by osmotic shock. The ability to isolate thioredoxin with a membrane fraction prepared under gentle lysis conditions supports a peripheral association with the cytoplasmic membrane. Cytosolic localization was confirmed by the inaccessibility of thioredoxin in spheroplasts to either membrane-impermeant reagents specific for the active site thiols or to thioredoxin-specific antibodies. We argue from these data that the release of thioredoxin and other cytoplasmic proteins by osmotic shock reflects localization at sites where both the inner and outer membranes are contiguous, first described as adhesion sites by Bayer, M. E. (1968) *J. Gen. Microbiol.* 53, 395.

Thioredoxin is a low molecular weight dithiol protein distributed ubiquitously in nature and present in large amounts

* This work was supported by Grant GM-20122 from the National Institutes of Health and Grant PCM-01397 from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by Graduate Trainee Grant 5T32 GM07231. This work represents partial fulfillment of the requirements for the degree of Doctor of Philosophy.

§ To whom all inquiries should be addressed.

MATERIALS AND METHODS

Materials

[8-¹⁴C]GDP (144 Ci/mmol) was purchased from Amersham. Egg white lysozyme (9 × crystallized) was purchased from Sigma. Iodoacetic acid (free acid) was also purchased from Sigma and recrystallized with either/benzene immediately before use. Monobromobimane (Thiolyte MB) and monobromotrinethylammoniobimane (Thiolyte MQ, Calbiochem) were used without further purification and prepared immediately before use. Gel electrophoresis grade agarose was purchased from Bethesda Research Laboratories, and Triton X-100 was purchased from the Packard Instrument Co.

Antibodies to thioredoxin were prepared in rabbits as described (21). For immunofluorescence experiments, both thioredoxin and preimmune antibody preparations were absorbed to formalin-fixed whole cells. For this procedure 25 µl of antibody (purified through

11424
Osmotic Shock Release of Thioredoxin

DEAE-blue cellulose and representing 5 μl of crude serum was added to 6 x 10^7 cells (fixed in 2.5% formaldehyde, 25 °C, 5 min) then diluted in 200 μl water, 1 x sucrose, pH 8. After incubation at 4 °C for 30 min, the cells were removed by centrifugation and the serum was used directly. Antibody titer to thioredoxin (1 nmol of thioredoxin/ml of serum) was unchanged by this preadsorption.

Cell Growth: E. coli

W3110 (thy') was grown at 37 °C in Tiss minimal media containing 0.05% casamino acids (Difco vitamin-free), 0.1% glucose, and 0.3 mM phosphate (22). Innovations (1 % v/v) into TCG media were made from cells actively growing in L broth. Cells for experimentation were harvested at density of 3 x 10^9 cells/ml by centrifugation at 4 °C.

Fractionation Procedures

Freeze-Then—Cell pellets were resuspended to approximately 7 x 10^7 cells/ml in 50 mM Tris, 3 mM EDTA, pH 7.4 (Tris-EDTA buffer), then quickly frozen in a dry ice/ethanol bath. The frozen suspensions were slowly thawed in an ice bucket, and the cells pelleted by centrifugation (4 °C); the soluble fraction was analyzed for thioredoxin protein using rocket immunoelectrophoresis (see below). The residual cell fraction was also analyzed for thioredoxin after resuspension in Tris-EDTA buffer followed by sonication at 4 °C (setting 5, 2 min, 50% duty cycle, using a Branson model 350 sonicator equipped with a 1/8" microtip).

Osmotic Shock—Osmotic shock was performed as described by Nossal and Heppel (23). A cell pellet from freshly grown cells was resuspended to a density of 7 x 10^7 cells/ml in a plasmolysis buffer containing 50 mM Tris, pH 7.4, 2.5 mM EDTA, and 20% (w/v) sucrose, and incubated at room temperature for 10 min. The resulting pellet was resuspended by vortexing in cold (2 °C) deionized water (to a cell concentration of 7 x 10^7 cells/ml), and then incubated at 4 °C for 10 min. The resulting supernatant obtained after centrifuging cells (i.e. the osmotic shockate) was then assayed for thioredoxin protein. Where designated, the osmotic shock step was carried out in the presence of either magnesium or calcium chloride. The residual cell pellet was resuspended in Tris-EDTA buffer and assayed as described above.

Determination of Trichloroacetic Acid-soluble Nucleotides Released on Osmotic Shock—Samples of plasmolysisates, osmotic shockates, and sonicated cell residue fractions were treated with cold 5% (w/v) trichloroacetic acid, and the resulting precipitates were removed after 60 min at 4 °C by centrifugation at 12,000 x g for 15 min. The absorbance at 260 nm was determined on the resulting supernatants. These supernatants all showed absorbance spectra characteristic of nucleotides (A260/A280 = 2.0) at 290 nm.

Lysozyme-EDTA Spheroplasts— The procedure of Kacab (24) was utilized, and the efficiency of spheroplast formation was determined by measuring the decrease in turbidity at 550 nm of the treated cells upon dilution in distilled water. With this assay the efficiency of making spheroplasts was greater than 90%.

Formaldehyde-fixed spheroplasts were prepared by incubating spheroplasts (1 x 10^9 cells) in 50 mM Tris, pH 7.4, 40% (w/v) sucrose) in 2.5% formaldehyde at room temperature for 5 min. The reaction was stopped by dilution in 1 volume of 1 M glycine in the above buffer.

Enzyme and Protein Assays

Thioredoxin—Thioredoxin protein was quantified using one-dimensional rocket immunoelectrophoresis (25) as described (21). The assay was sensitive to levels of thioredoxin to 0.5 pmol and was linear through 5 pmol. In modified forms of thioredoxin were determined by crossed rocket immunoelectrophoresis, as described. First dimension electrophoresis was performed at 6 °C in 1% (w/v) agarose gels containing 30 mM Tris, 100 mM glycine, 1% (v/v) Triton X-100, and 1 mM EDTA, pH 8.6. Following electrophoresis for 2 h at 5 V/cm, gel track segments were cut transversely, then fused with an antibody-containing plate. Second dimension electrophoresis, staining of gel, and quantitation of rocket areas were performed as described elsewhere (21).

Alkaline Phosphatase—Alkaline phosphatase activity was determined according to the method of Torrini (27).

Elongation Factor—Elongation factor Tu was quantified using the GDP exchange assay of Geiser and Gordon (28). Estimation of EF-

Tu protein levels was also determined by rocket immunoelectrophoresis (25) using a partially purified IgG fraction of rabbit anti-(EF-Tu) kindly supplied by Dr. D. L. Miller (Roche Institute, Nutley, NJ).

Glucose-6-phosphate Dehydrogenase—G-6-P dehydrogenase was assayed using the spectral assay described by Kornberg and Horecker (29).

Localization of Thioredoxin Using Indirect Immunofluorescence Microscopy—Formaldehyde-fixed spheroplasts were resuspended to 1 x 10^11 cells/ml in 50 μl of phosphate-buffered saline, pH 7.4, containing 20% (w/v) sucrose. Aliquots were permeabilized by incubation with 0.5% Triton X-100 at 4 °C for 10 min. Antibodies were added and incubation continued at 4 °C for 15 min. The incubation was then diluted in 10 volumes of wash buffer containing 5% (v/v) newborn calf serum in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, Hank's salts (purchased as a 1 x stock from Gibco), 20% sucrose with 2.5 mM dithiothreitol. Spheroplasts were pelleted (100 x g, 10 min) and then resuspended in 10 μl of DEAE-purified goat anti-rabbit IgG antibodies (2 mg/ml, conjugated with 1.1 fluorescein groups/globin) by the procedure of Cebra and Goldstein (26). After incubation at 4 °C for 10 min, cells were diluted in 10 volumes of wash buffer without dithiothreitol, pelleted, then resuspended, and viewed in a Leitz epifluorescence microscope equipped with a 50-watt mercury lamp.

Modification of Thioredoxin in Vivo Using Membrane-permeant and -impermeant Bimanuals—Freshly harvested cells or spheroplasts were resuspended to 1 x 10^9 cells/ml in an argon-flushed buffer containing 50 mM Tris, pH 7.4, 40% (w/v) sucrose, 1 mM EDTA. This modified form of thioredoxin was prepared from thioredoxin by reduction in 1.1 M dithiothreitol. Freshly prepared monobromobimane or mono-N-trimethylammoniobimane was added to the incubation mixture, and reactions were carried out under anaerobic conditions at 4 °C. Antibodies were added by the addition of 2 mM iodoacetate, reacted for 15 min at 4 °C, and then finally quenched with 0.1 M acetate buffer, pH 4.1. The ability of the biamines to block reaction with iodoacetate was then determined by two-dimensional crossed rocket immunoelectrophoresis.

RESULTS

Osmotic Sensitivity of Thioredoxin Association with Bacterial Cells—Selective release of thioredoxin from E. coli W3110 was first observed upon rapid freezing of cultures grown in Tiss medium and harvested in log phase (3 x 10^9 cells/ml). Upon thawing, 60 to 80% of the thioredoxin was released directly into the culture medium. Quantitative release of thioredoxin required removal of divalent metal ions from the cell suspensions with either EDTA or EGTA. For example, when freshly harvested cells were resuspended in 50 mM Tris, 3 mM EDTA (pH 7.4), frozen rapidly in a dry ice/ethanol bath, and then thawed, release of thioredoxin was greater than 90% (Table I). Freezing cells in the presence of an osmotic stabilizer such as sucrose (up to 40% w/v) had no effect on this release. The efficiency of this release was reduced in stationary phase cells.

The conclusion that this freeze-thaw procedure selectively released bacterial proteins was based upon the amount of protein released (approximately 4% of the cell's total protein, as determined by protein assay) and upon the spectrum of proteins released, as assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Both high and low molecular weight polypeptides were released by the freeze-thaw procedures, and several polypeptides with molecular weights less than that of thioredoxin were retained by the residual cell fraction (data not shown). In general, unique classes of proteins showed distinct retention characteristics (Table I). Glucose-6-phosphate dehydrogenase, a 56 x 10^6 cytoplasmic protein, was not released at all, and alkaline phosphatase, a dimer of 80 x 10^6 located in the periplasm, was only partially retained (46% released). The protein synthesis EF-Tu, a 44 x 10^6 protein associated with the inner surface of the cytoplasmic membrane (30), was only partially released (24% released).

The abbreviations used are: EF-Tu, elongation factor Tu; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid.
within the periplasmic space. However, this is not necessarily the case, as several examples of cytoplasmic proteins released by osmotic shock are known (31). Indeed a member of this so-
called group D class of cytoplasmic proteins (31), the elongation factor Tu, was co-released with thioredoxin (Table I) under a variety of conditions involving manipulation of the osmotic shock procedure. To differentiate between periplasmic and cytosolic localization, the release of thioredoxin was examined after the formation of lysosome-EDTA spheroplasts, a less ambiguous diagnostic test for defining strict periplasmic localization (31). For this purpose cells suspended in a hypertonic media (30 mM Tris, 20% (w/v) sucrose, pH 7.4) were treated with EDTA (1 mM) and egg white lysozyme (10 mg/ml) at 4°C for 30 min. These conditions disrupt the cell's outer envelope, thus exposing the periplasmic space to the external environment without the physical manipulations required for osmotic shock. Greater than 95% of the cells were converted to spheroplasts by this treatment. Under these conditions neither thioredoxin or EF-Tu were released from spheroplasts while there was nearly quantitative release of alkaline phosphatase (Table I). No glucose-6-phosphate dehydrogenase activity was released upon spheroplast formation.

To determine whether retention of thioredoxin with spheroplasts was due to binding to the outer surface of the inner membrane, we applied the technique of indirect immunofluorescence. Formaldehyde-fixed spheroplasts were incubated with rabbit anti-thioredoxin at 4°C with fluorescein-conjugated goat anti-rabbit immunoglobulin. As shown in Fig. 2, no antibody staining was observed using preimmune antiserum either with or without detergent permeabilization, or with whole spheroplasts incubated with anti-thioredoxin serum. Labeling was observed only with Triton-permeabilized spheroplasts incubated with thioredoxin antibody. Consistent with this observation was the inability to reduce the antibody

Table I

| Treatment | Thioredoxin Released % | Alkaline Phosphatase Released % | EF-Tu Released % |
|-----------|------------------------|--------------------------------|-----------------|
| Sonication| 100                    | 100                            | 100             |
| Freeze-thaw| 92                    | 46                             | 24.1            |
| Plasmolysis| 9                    | 0                              | 0.7             |
| Osmotic shock| 100                  | 94                             | 60-80           |
| Lysozyme-EDTA spheroplasts| 0                 | 96                             | 0.05            |

*Cells were grown to mid-log phase, harvested by centrifugation (4°C), resuspended in the appropriate buffer, and subjected to the various gentle release procedures, as described under "Materials and Methods." The percent release of each protein is compared to the total detected upon sonication. In each experiment the total protein (or activity) detected in individual fractions always equaled the amount detected in sonication. The total amounts detected/10^9 cells were: 23.4 pmol for thioredoxin, 0.9 nmol of p-nitrophenyl phosphate hydrolyzed/min for alkaline phosphatase, 29.7 GDP-bound pmol for EF-Tu, and 30 nmol of NADPH formed/min for glucose-6-phosphate dehydrogenase.

Thioredoxin Is Not a Periplasmic Protein.—The quantitative release of both thioredoxin and alkaline phosphatase by osmotic shock suggested that both proteins might reside within the periplasmic space. However, this is not necessarily the case, as several examples of cytoplasmic proteins released by osmotic shock are known (31). Indeed a member of this so-

FIG. 1. Release of thioredoxin and trichloroacetic acid (TCA)-soluble nucleotides by osmotic shock. The selectivity of thioredoxin release by osmotic shock was addressed by measuring the concomitant release of low molecular weight nucleotides. After plasmolysis with different sucrose concentrations, cells were osmotically shocked by dilution according to Nossal and Heppel (23). Samples were then assayed for thioredoxin release (○—○) and for the release of trichloroacetic acid-soluble nucleotides (△—△) as described under "Materials and Methods." The acid-soluble material released at the plasmolysis stage was less than 1% of the total released upon osmotic shock.
Osmotic Shock Release of Thioredoxin

Tu reflects a common localization derives from the hypersensitivity of osmotic shock release to cations in the shocking buffer. These conditions are known to block release of EF-Tu, but not of alkaline phosphatase (30). Fig. 3 shows that the increasing concentrations of magnesium chloride in the osmotic shockate selectively blocked the release of thioredoxin and EF-Tu while having little effect on alkaline phosphatase release. Calcium chloride was equally effective in blocking thioredoxin release (Fig. 4). This sensitivity to added divalent cations was also observed in cells plasmolyzed in EGTA (data not shown) suggesting that release was probably mediated by breaking a calcium-stabilized interaction.

Membrane Association of Thioredoxin—It has been suggested that the release of cytoplasmic proteins by osmotic shock requires a peripheral membrane association with the inner surface of the inner membrane (31). Such an association was observed for EF-Tu when cells were lysed under gentle conditions (30). To test this model for thioredoxin, membranes were prepared using the gentle lysis technique of Kaback (24).

Fig. 2. Indirect immunofluorescence microscopy of spheroplasts using thioredoxin antibodies. The topological orientation of thioredoxin protein was determined by indirect immunofluorescence microscopy. Formaldehyde-fixed spheroplasts were incubated with purified rabbit thioredoxin antibody in the presence (A and B) or absence (C and D) of 0.5% (v/v) Triton X-100. Cells were washed, treated with fluorescein-conjugated goat anti-rabbit antibody, washed, and then scored by fluorescence microscopy. Studies using preimmune antisera, or thioredoxin antisera preincubated with excess pure thioredoxin, gave no labeling. Shown are Triton-permeabilized spheroplast aggregates photographed under bright field (A) and fluorescent (B) illumination. Sealed spheroplasts are shown under bright field (C) and fluorescent (D) illumination. Magnification, 420 X.

Fig. 3. Effect of MgCl2 on release of thioredoxin and EF-Tu after EDTA plasmolysis. Cells were plasmolyzed in 20% (w/v) sucrose in 50 mM Tris, pH 7.4, 2.5 mM EDTA and then diluted into a shocking solution containing magnesium chloride in the concentrations indicated (23, 30). Similar data were observed using cells plasmolyzed in 2.5 mM EGTA. Release of thioredoxin (●●●●●●), EF-Tu (■■■■■■), and alkaline phosphatase (▲▲▲▲▲▲) were determined as described for Table I and under “Materials and Methods.” Percentage of EF-Tu release was determined relative to the protein’s osmotic release of the protein in the absence of magnesium chloride.

Titer to thioredoxin following serum incubation of whole spheroplasts (data not shown).

Thioredoxin thus fulfills all the criteria established for group D proteins (31), generally defined as cytoplasmic proteins released by osmotic shock but retained by spheroplasts. Further evidence that the co-release of thioredoxin and EF-

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**Table II**

| Treatment  | Thioredoxin | EF-Tu | G-6-P dehydrogenase |
|------------|-------------|-------|---------------------|
| Dilution + Mg²⁺ | 20–24 | None detected | 4.3 |
| Dilution   | 9 | None detected | 3.2 |

"Cells grown to mid-log phase were treated with lysozyme and EDTA to prepare spheroplasts according to the method of Kaback (24) and were lysed by dilution either in the presence or absence of MgCl2 (1 mM). Membrane fraction was then prepared as described under "Materials and Methods" according to the method of Kaback (24). The resulting membrane pellet was solubilized by sonication in 1% (w/v) Triton X-100 and then assayed for thioredoxin, elongation factor Tu, and for contaminating glucose-6-phosphate dehydrogenase.

The percentage recovered in the membrane fraction was computed as the total relative to that detected on sonication of intact cells (see Table I)."
As shown in Table II, 20% of the thioredoxin was recovered in the membrane pellet, compared with the membrane association of less than 5% of the glucose-6-phosphate dehydrogenase. Thioredoxin was enriched in the membrane fraction by 8-fold. This association was sensitive to the presence of divalent cations during breakage, as only 9% of the thioredoxin was present in the membrane pellet if no magnesium chloride was present during cell breakage.

These results were comparable to those reported previously (16) which showed that approximately 30% of the thioredoxin was associated with the membrane fraction. In this study an alternative method of gentle cell lysis (32) was used in which spheroplasts were suspended in sodium chloride (0.1 M), dithiothreitol (1 mM) and subjected to a heat shock (1 min, 37 °C). Immediately upon breakage, magnesium chloride was added to 10 mM. Chromatography of the resuspended membrane fraction on Bio-gel A-50 showed association of thioredoxin with the several high molecular weight species also containing ribonucleotide diphosphate reductase. Some thioredoxin appeared to chromatograph free (i.e. as an uncomplexed species) and this proportion increased with storage time, indicating a weak, labile association.

**Inaccessibility of Thioredoxin Active Site Thiol to Membrane-impermeant Reagents**—To determine whether the active site of thioredoxin was exposed to the extracellular space (i.e. protruding on the outer face of the inner membrane), the reactivity of the sulfhydryl groups of thioredoxin to several membrane-permeant and -impermeant reagents was investigated. Cells or lysates in an argon-flushed buffer containing 50 mM Tris, pH 7.4, 40% (w/v) sucrose, 0.1 mM dithiothreitol, and 1 mM EDTA were reacted with either the membrane-permeant monobromobimane or the membrane-impermeant monobromotrimethylammoniobimane (33) at 4 °C for up to 30 min. The reactions were stopped by adding an 8-fold excess of iodoacetic acid. The ability to block iodoacetic acid modification of thioredoxin by these reagents was then determined by two-dimensional rocket immunoelectrophoresis. Fig. 5 shows that the membrane permeant monobromobimane reacted efficiently with thioredoxin in either cells or lysates with a first order rate constant consistent with observed rates with hemoglobin (k = 0.061 min⁻¹) (34). The membrane-impermeant monobromotrimethylammoniobimane reacted rapidly with lysate thioredoxin (k = 0.5 min⁻¹), but was inhibited from reacting with thioredoxin in intact cells. Upon further incubation, modification did occur, but at a rate of 0.04 min⁻¹, 13 times slower than the reaction in lysates. This latter reaction may have resulted from the slow penetration of reagent or may have been due to cell breakdown in Tris-EDTA buffer. Similar results were observed using spheroplasts.

**DISCUSSION**

Several observations presented here are consistent with the peripheral association of thioredoxin with the inner surface of the inner (i.e. cytoplasmic) membrane. First, thioredoxin was quantitatively released from log phase cells by the osmotic shock techniques of Nossal and Heppel (23). Although this procedure is highly selective for the release of periplasmic proteins, a few examples exist for the release of cytoplasmic proteins as well. These group D proteins (31) include elongation factor Tu (30), the nucleotide catabolism enzymes thymidine phosphorylase, deoxyribozyme, deoxyribonuclease phosphorylase, deoxyribozyme, uridine phosphorylase, cytidine deaminase, and purine phosphoribosyltransferase (35-39). This osmotic shock procedure releases approximately 4% of the total cell protein and does not release recognized cytoplasmic marker proteins such as glucose-6-phosphate dehydrogenase (Table I). Second, unlike the efficient release of periplasmic proteins from spheroplasts prepared with lysyzyme and EDTA, thioredoxin was retained quantitatively. This behavior is consistent with the behavior of the class D proteins described above. Thioredoxin retained by these spheroplasts was inaccessible to chemical modification by the membrane-impermeant monobromotrimethylammoniobimane (Fig. 5) or to antibodies specific for thioredoxin (Fig. 2). These latter observations imply that the protein is not exposed to the outer surface of the inner membrane. Third, a significant fraction (20%) of the thioredoxin could be isolated within a membrane fraction. This association was dependent upon the presence of magnesium ions during cell disruption and membrane preparation and was observed only when cells were broken under gentle conditions, such as heat shocking of spheroplasts (16) or by dilution of spheroplasts (Table I). Cell breakage involving shear, sonication, or use of the French pressure cell produced lysates lacking detectable membrane association.

Although the effects of osmotic shock on bacterial cells has been extensively investigated, the mechanism by which this treatment causes release of cytoplasmic proteins is not clear. The resuspension of Gram-negative bacterial cells in hypotonic buffers results in an explosive expansion of the inner membrane against the outer envelope due to turgor generated by water influx into the cytoplasmic space. Such stress against weak points in the outer envelope generates finger-like extrusions projecting away from the cell surface (40). These extrusions are bounded either by the outer membrane only (thus
containing periplasmic materials) or by both the inner and outer membranes (containing cytoplasmic materials and regions of the inner membrane). As suggested (45), disruption of these latter structures would result in cytoplasmic protein release, with preferential release of those proteins contained within the extrusions. The generation of these extrusions by osmotic shock is insufficient to cause the release of cellular macromolecules, however. Such treatment can release only small molecular weight nucleotides, without releasing any detectable macromolecules (41). Cytoplasmic macromolecule release through these extrusions requires pretreatment with EDTA, a reagent which affects both membrane structure (42) and cell permeability to a variety of molecules (43) by removal of regions of the cell’s lipopolysaccharide coat (44).

The above argument suggests two distinct pathways are available for protein release by osmotic shock, corresponding to the two classes of extrusions seen by electron microscopy. Periplasmic proteins pass through breaches of the E. coli envelope, while lipopolysaccharides by EDTA treatment. Cytoplasmic proteins like thioredoxin and EF-Tu pass through mixed inner and outer membrane extrusions. The blockage of thioredoxin and elongation factor Tu release by millimolar concentrations of cations, with no similar effect on periplasmic alkaline phosphatase release (Figs. 2 and 3), supports the physical distinction between these two pathways, and also specifies the membrane region involved in cytoplasmic protein release. Sensitivity of membrane penetration to cations suggests interaction with lipopolysaccharides (45) that have remained associated with the cell envelope after the initial EDTA extraction. Newly synthesized lipopolysaccharides, inserted into the outer membrane through the approximately 200 adhesion sites between the inner and outer membrane (19), are uniquely insensitive to extractions by EDTA (42). As lateral mobility of lipopolysaccharides is slow (46), these molecules would be expected to remain at these insertion sites as membrane-bound patches, insufficient to completely block macromolecule release, but sufficient to produce cation-dependent permeability changes as are seen in whole cells (47). As a result, the movement of proteins across these lipopolysaccharide insertion sites would be blocked by those factors affecting the aggregation state of the newly synthesized lipopolysaccharides.

Our present data do not allow us to state what the role of thioredoxin is at these membrane sites. As argued above, the cytoplasmic proteins releasable under osmotic stress are most likely localized in the membrane adhesion zones. These adhesion zones have been implicated in a variety of functions (see Ref. 31 for a review) including attachment of the lipopolysaccharide-specific phages, attachment of the bacterial chromosomes, export of F plasmids, and anchoring of flagella. Because the adhesion zones fuse the inner and outer membranes, they offer the possibility for sites of processing and sorting out of proteins for inclusion into the outer membrane or export to the periplasm (18, 48). The adhesion zones have also been implicated as the sites for lipopolysaccharide biosynthesis and/or insertion into the envelope (19). Possible roles for thioredoxin and/or EF-Tu with these various processes will have to await more detailed studies.

Our present studies have shown that thioredoxin is efficiently released from log phase cells by osmotic shock even though only 20 to 30% (16) of the thioredoxin was isolated in the membrane fraction. This apparent disparity may reflect some heterogeneity in membrane interaction due to a limitation in interaction sites or due to some difference in the two classes of thioredoxins. Efficient release, thus, only may reflect a physical localization, not necessarily a tight membrane association. Studies with cells grown under different physiological conditions may aid in sorting out this distinction. A membrane-associated function is suggested by the relatively tight membrane association of thioredoxin isolated from calf liver (49). Also, in plants, thioredoxin is reduced by a membrane bound ferredoxin-dependent thioredoxin reductase (6, 7).

It may be important that thioredoxin and EF-Tu share a number of molecular properties. Both proteins are made in an apparent excess of the amounts thought to be required for their defined roles (1 to 2 x 10^6 copies/cell for thioredoxin (1) and 7 x 10^6 copies/cell for EF-Tu (50)). Both proteins are required subunits for the replication of phage-specific nucleic acid, T3 phage requiring thioredoxin (12) and Q6 phage requiring EF-Tu (51). Both proteins share a common peptide of five amino acids (32). Although these similarities may all be coincidental, thioredoxin has been implicated directly in protein synthesis (8) possibly by a thiol-disulfide activation analogous to that shown for thioredoxin activation of the fructose biophosphatase in plants (6). Given the localization of these two proteins at the adhesion sites and the pleiotropic roles for each protein, it would not be surprising if these proteins shared some metabolic functions.

Acknowledgments—We would like to express our appreciation to E. Barry Skolnick for his helpful criticisms in designing the experiments on chemical modification of thioredoxin, to Dr. Michael Edidin and Kathleen S. Morgas, for their expert help with the indirect immunofluorescence microscopy, and to Dr. D. L. Miller for providing us with anti-(EF-Tu) sera.

Note Added in Proof—Localization of thioredoxin at the cell periphery has been demonstrated using immunoelectron microscopy by Nygren, H., Rozell, B., Holmgren, A., and Hansson, H.-A. (1981) FEBS Lett. 135, 145.

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Osmotic Shock Release of Thioredoxin

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