IMMUNOELECTRON MICROSCOPIC LOCALIZATION OF GLUTAREDOXIN AND THIOREDOXIN IN *ESCHERICHIA COLI* CELLS

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1. Introduction

Thioredoxin is a small (M_r 12 000) protein containing an oxidation–reduction active cystine disulfide bridge [1], that has been ascribed several functions. The reduced form, thioredoxin-$(SH)_2$, generated by NADPH and thioredoxin reductase, was the first isolated, in vitro hydrogen donor of ribonucleotide reductase [2]. It is also a general protein disulfide reductase; furthermore thioredoxin is an essential subunit of phage T7 DNA polymerase [3,4].

Glutaredoxin is another small acidic (M_r 12 000) protein originally discovered in an *Escherichia coli* mutant [5] lacking thioredoxin [6] and purified to homogeneity from *E. coli* wild-type cells [7]. Glutaredoxin enables the monothiol glutathione to be hydrogen donor for ribonucleotide reductase [5] and is structurally different from thioredoxin [7]. The relative contributions of thioredoxin and glutaredoxin in ribonucleotide reduction by normal cells are not yet known.

Attempts have been made to elucidate the cellular localization of *E. coli* thioredoxin by biochemical methods, e.g., extractions [6,8], however, no conclusive data have been presented so far. The cellular localization of glutaredoxin is unknown.

Here, we have used specific antibodies to probe these two proteins in *E. coli* cells using immunohistochemical techniques. Thioredoxin shows a dual localization; either it is found in the periplasmic space surrounding the cell, or it is primarily located in the nucleoid region in the center of cells. Glutaredoxin shows a cell wall localization either in the periplasmic space at one pole of the cell or in mesosome-like structures close to the cell membrane.

2. Experimental

2.1. *Preparation of reagents*

Homogenous thioredoxin and glutaredoxin from *E. coli* B were prepared as in [7,9].

The preparation of rabbit antiserum against thioredoxin has been described [10]. The same technique was used to prepare antiserum against glutaredoxin. In each case there was no detectable cross-reactivity with the heterologous antigen as tested by inhibition of enzyme activity, by radioimmunoassay or by binding to immunadsorbent columns (A. H., M. Dannbeck, unpublished).

Peroxidase-labeled protein A from *Staphylococcus aureus* (HRP-PA) was used as 'second antibody' in the pre-embedding immunohistochemical procedure. The conjugation of peroxidase to protein A was performed in two steps using glutaraldehyde as cross-linking reagent as detailed in [11].

Colloidal gold-labeled protein A (CG-PA) was used as 'second antibody' in the post-embedding immunohistochemical procedure. Reduction of chloroauric acid to colloidal gold was performed with sodium citrate according to [12]. Adsorption of PA to CG was performed at pH 5.5 which is close to the isoelectric point of PA as recommended in [13]. The diameter of the CG-particles and the adsorption of PA to CG was evaluated by negative staining as in [12].

2.2. *Bacterial cells*

Escherichia coli cells (strain 06 K13) were kindly supplied by Dr Stefan Lange, Department of Bacteriology, University of Gothenburg. The bacteria were grown in brain–heart-infusion broth (BH1, Difco Lab., Detroit MI) and harvested by centrifugation at the early stationary phase of growth.

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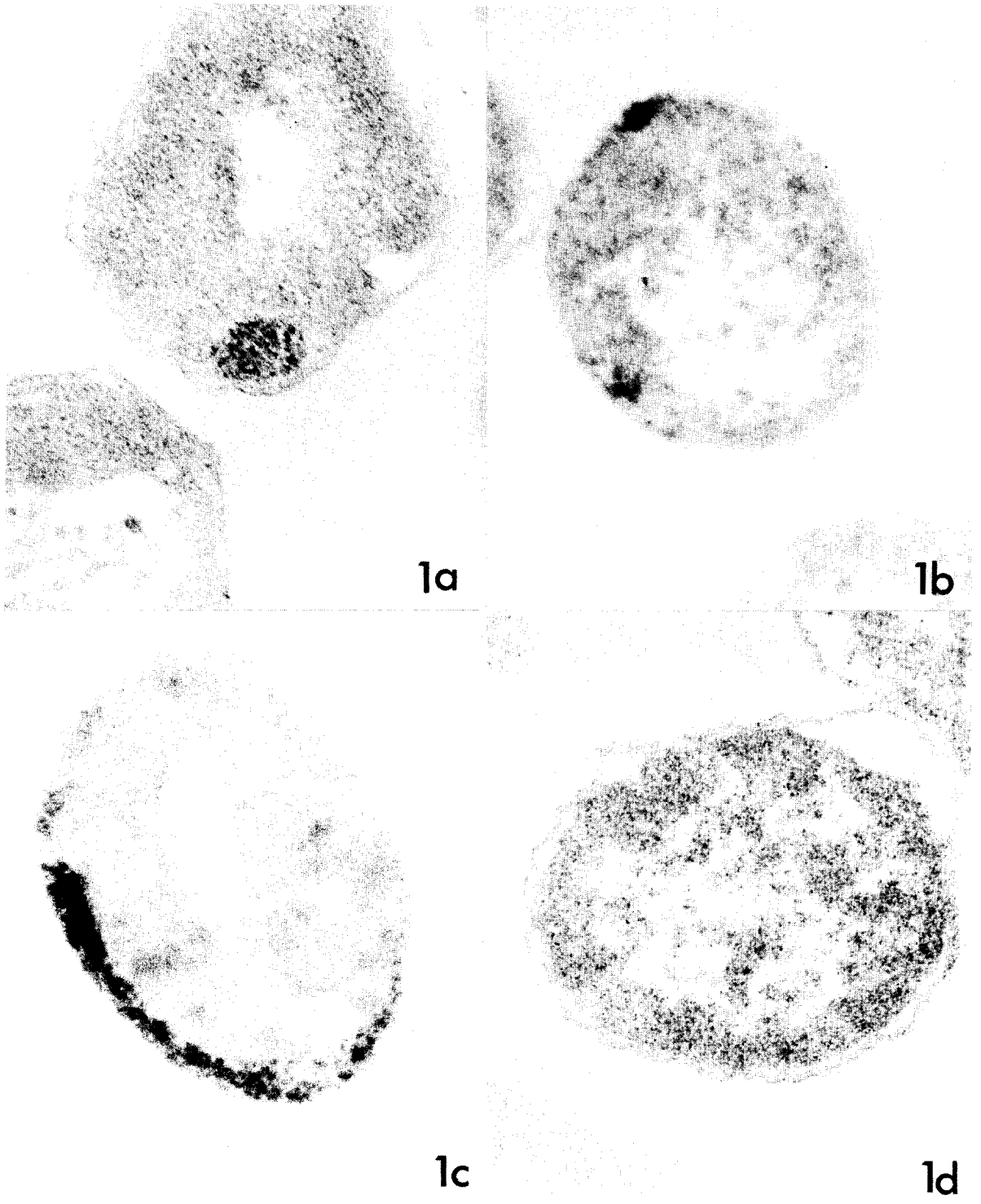

Fig.1. *Escherichia coli* cells incubated in antiserum directed against glutaredoxin according to the pre-embedding procedure: (1a) cell treated with EDTA and lysozyme prior to fixation with formaldehyde (×55 000); (1b) cell fixed with glutaraldehyde (×55 000); (1c) cell heated as in (1a) (×55 000); (1d) control incubated in normal rabbit serum after fixation with glutaraldehyde (×55 000).

2.3. *Pre-embedding immunohistochemical procedure*

The cells were divided into 6 groups. Three groups were treated with ethylenediamine tetraacetic acid (EDTA) and lysozyme [14] to remove the cell wall before fixation and 3 groups were directly transferred to fixation in formaldehyde (4% in 0.15 M cacodylate buffer (pH 7.2), containing 0 01% sialic acid) or glutaraldehyde (3% in 0.15 M cacodylate buffer (pH 7.2). After fixation (2 h at 4°C), the cells were rinsed in cacodylate buffer, and excess of aldehyde was inactivated by addition of lysine after fixation (0.02 M) for 2 h. In some experiments, the cells were treated with EDTA + lysozyme after fixation. The fixed and rinsed cells were incubated in dilutions of antiserum for 16 h followed by thorough rinsing in the cacodylate buffer 4 times for 30 min each. Incubation with HRP-PA was then performed for 2–4 h with subsequent rinses before the final transfer of the cells to 0.15 M cacodylate buffer (pH 7.2) containing 0.5 mg 3-3-diaminobenzidine/ml and 0.01% hydrogen peroxide [15]. Post-fixation was performed for 2 h in 2% osmium tetroxide dissolved in 0.15 M cacodylate buffer (pH 7.2) dehydration in graded series of ethanol and embedding in EPON was performed according to routine procedures [16].

2.4. *Post-embedding immunohistochemical technique*

Bacterial cells were fixed overnight in 4% formaldehyde and 0.01% picric acid in 0.15 M cacodylate buffer (pH 7.2). The excess of aldehyde was removed by rinsing 4 times, 30 min each, followed by dehydration in ethanol and embedding in methacrylate:stryrene resin as in [16]. Thin sections were cut on a LKB Ultratome III ultramicrotome and collected on formvar-coated nickel-grids. The plastic resin was removed by incubation in xylene [17]. In some experiments the sections were rehydrated in graded series of ethanol, whereas in others, they were taken directly to incubation with normal rat serum for 1 h. Incubation of the grids with specific antiserum was performed for 2 h, normal rabbit serum was used as control. After a 3 × 5 min rinse in saline, the grids were placed on a drop of CG-PA solution for 2 h, rinsed in saline and post-stained with saturated uranyl acetate in water.

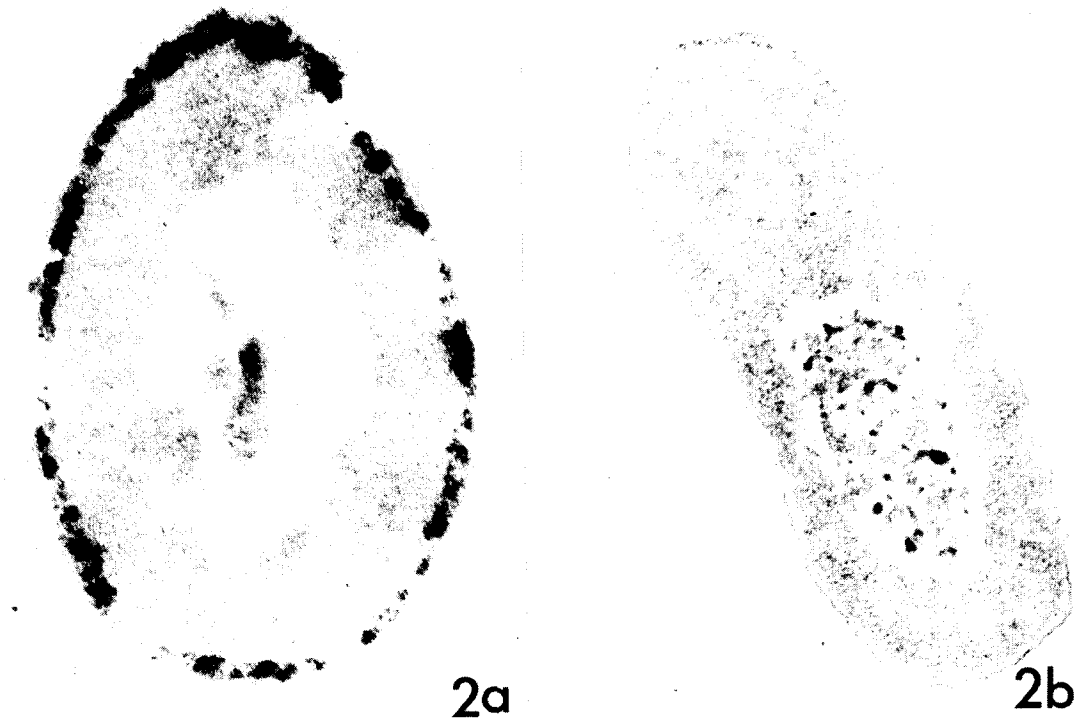

Fig.2. *Escherichia coli* cells incubated in antiserum against thioredoxin according to the pre-embedding procedure: (2a) cell fixed with glutaraldehyde (×55 000); (2b) cell heated with EDTA and lysozyme before fixation with formaldehyde (×35 000).

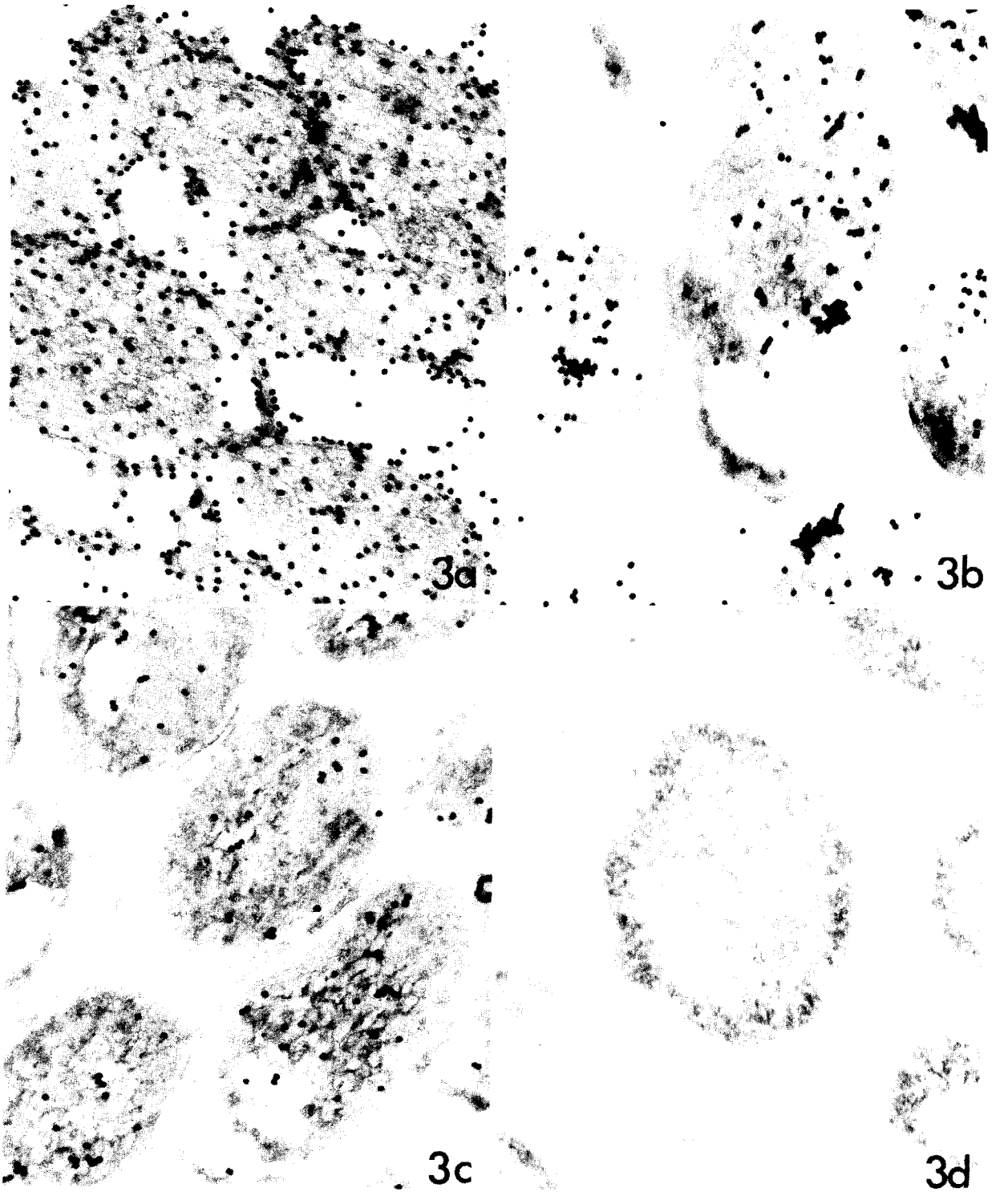

Fig.3. Post-embedding staining of *E. coli* cells: (3a) section incubated with antiserum against thioredoxin (×35 000); (3b) section incubated with antiserum against glutaredoxin (×35 000); (3c) section incubated in normal rabbit serum (×35 000); (3d) section incubated in saline and gold-labelled protein A (×35 000).

2.5. *Electron microscopy*

Thin sections of EPON-embedded cells treated according to the pre-embedding procedure and sections of methacrylate-embedded cells treated according to the post-embedding procedure were examined in a JEOL-100CX electron microscope with an accelerating voltage of 60 kV.

3. Results

Localization of glutaredoxin by the pre-embedding procedure resulted in an intense deposition of reaction products at two different locations. In some cells, the glutaredoxin was localized in well-demarcated cell membrane-associated spots (fig.1a,b), whereas in others the deposition of reaction product appeared in the periplasmic space between the cell membrane and the cell wall (fig.1c). This dual localization did not appear simultaneously in one single cell.

Localization of thioredoxin by the pre-embedding procedure also showed an intense specific reaction at two different locations. In some cells, thioredoxin was localized mainly to the periplasmic space (fig.2a) whereas in others, the main location was in connection with the nucleoid (fig.2b). The dual localization of thioredoxin sometimes appeared in one single cell (fig.2b), however, one location was always strongly predominant over the other. Incubation of the cells with normal rabbit serum did not give rise to any deposition of reaction products (fig.1d).

When using the post-embedding techniques, normal rabbit serum gave rise to a considerable background of deposited colloidal gold granules over the cytoplasm (fig.3c). This was not seen after incubation with CG-PA only (fig.3d). Compared with control sections (fig.3c) sections incubated with antiserum against thioredoxin showed a definite localization of gold granules to the cell wall and the periplasmic space (fig.3a). Incubation of thin sections with antiserum against glutaredoxin revealed a well-defined clustering of CG-granules close to the cell membrane at one pole of the cell (fig.3b).

4. Discussion

The results obtained by using immunoelectron microscopy for the localization of thioredoxin and glutaredoxin in *E. coli* cells demonstrate distinct different cellular distributions of these 2 proteins. Both the pre-embedding and post-embedding immunohistochemical techniques that were used have certain pitfalls, which may lead to false conclusions as to the localization of the antigens. In pre-embedding techniques, antibodies may fail to penetrate into the cells and antigen may diffuse away from its original location during the long preparation times. The penetration of antibodies was enhanced here by splitting the the cell wall with EDTA and lysozyme before or after fixation and attempts were made to minimize diffusion artefacts by a rapid fixation. It was thus possible to evaluate the contribution of such artefacts by comparison of the results obtained by the different procedures. To further elucidate the role of diffusion artefacts and poor penetration of the probe into the cells, a post-embedding technique was used. Using this technique with colloidal gold as label, the main pitfalls are the inactivation of antigen by the rough treatment during preparation of the specimen and the problems with background staining. Here, the results obtained by using pre-embedding and post-embedding techniques were fully consistent. This fact strongly suggests that we are observing the true localization of glutaredoxin and thioredoxin in living cells.

Glutaredoxin is only observed in a position close to the cell membrane; either within the periplasmic space at one part of the cell or highly concentrated to spots probably representing mesosomes. These structures, observed in electron micrographs of bacterial cells [19] are organelles of intracellular membranes suggested to be involved in a variety of processes including energy production, DNA replication and segregation and cell division [19,20].

The general periplasmic position of thioredoxin is consistent with functions in redox reactions and regulator reactions [1]. The presence of thioredoxin in the nucleoid region may represent a function linked to DNA metabolism either as hydrogen donor for ribonucleotide reductase producing deoxyribonucleotides for repair or recombination or as a structure component of a DNA–protein complex. In this context the function of thioredoxin, as an essential subunit of phage T7 DNA polymerase should be recalled [1,3,4].

The redistribution of glutaredoxin from a mesosomal and thioredoxin from the nucleoid to a distribution within the periplasmic space seen in some cells, implies a change from an intracellular strongly-bound pool of these proteins to an easier extractable pool.

Results of extractions show release of thioredoxin and elongation factor Tu from plasmolysed cells suggesting a localization of both proteins to an outer–inner membrane junction [8]. Several bacterial enzymes have been localized to the periplasmic space and variations in the extractability of bacterial enzymes have been shown to be related to the phase of growth of the cells [21,22]. There is thus reason to believe that the localization of glutaredoxin and thioredoxin varies with the cell cycle. Further studies on the localization of these proteins in different phases of growth using immunoelectron microscopy should give valuable information about the functional roles of glutaredoxin and thioredoxin.

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