FEBS Letters 354 (1994) 191–194

FEBS 14728

Immunogold localization of glutathione transferase B1-1 in *Proteus mirabilis*

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Received 30 August 1994; revised version received 22 September 1994

Abstract By using the immunolabelling technique, the cellular localization of glutathione transferase in *Proteus mirabilis* was investigated. Evidence was obtained indicating a significant higher content of glutathione transferase in the periplasmic than cytoplasmic space. This result further support the idea that bacterial glutathione transferase is involved in xenobiotic detoxication.

Key words: Proteus mirabilis; Glutathione transferase; Immunogold labelling; Western blotting

1. Introduction

Glutathione transferases (GST; EC 2.5.1.18) are a family of multifunctional dimeric proteins that catalyse the conjugation of reduced glutathione to a large variety of exogenous and endogenous hydrophobic electrophiles [1–4]. GST are also involved in intracellular binding and transport of hydrophobic compounds, including heme, bile acids, bilirubin and polycyclic hydrocarbons, participate in the synthesis of prostaglandins and leukotrienes, and may play a key role in the elimination of toxic organic hydroperoxides [1–4]. The structure and function of cytosolic mammalian GST species have been the subject of numerous investigations and the various forms so far characterized can be grouped into at least four distinct classes: alpha, mu, pi and theta [5,6].

GST have also been characterized from several bacteria [7–11]. We have previously purified and characterized a GST isoenzyme from *Proteus mirabilis*, designated GSTB1-1 (previous name Pm-GST-6.0) having structural, kinetic and antigenic properties different from the GST characterized from mammalian sources [8]. A very low identity (less than 20%) with the primary structures of alpha, mu and pi classes GST resulted, indicating that it represents the prototype of a new GST family (bacterial class) [12]. Several studies indicate that in addition to detoxication reactions GST may be involved in a variety of other distinct roles in bacteria [13] including the degradation of lignin [14] and the reductive dechlorination of pentachlorophenol [15]. To date, no information is available on the enzyme location in the bacterial cell. To better understand this aspect of bacterial GST, we have investigated the cellular localization of GSTB1-1 in *Proteus mirabilis* by using the immunogold labelling and Western blotting techniques.

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2. Materials and methods

2.1. Purification of GSTB1-1 and antibody preparation

Glutathione transferase from *Proteus mirabilis* (GSTB1-1) was purified according to Di Ilio et al. [8].

Antiserum against GSTB1-1 was raised in rabbit via two injections of 100 μg of protein in Freund's complete adjuvant [8].

2.2. Preparation of cells

Proteus mirabilis was grown aerobically at 37°C in 5 litres of Tryptone Soya Broth (Unipath), harvested by centrifugation, and washed twice in 10 mM-potassium phosphate buffer, pH 7.0. Separate pellets were prepared from the broth culture to determine the tests.

2.3. Preparation of periplasmic, cytoplasmic and total cellular extracts

The first part of the pellet was subjected to osmotic shock according to Neu and Heppel [16]. The cells were removed by centrifugation, the supernatant was collected and referred to as the periplasmic fraction. The cell pellet remaining after osmotic shock was disrupted by cold sonication (five bursts of 3 min each, at 300 W) with a Labsonic 1510 (Braun) sonicator. The particulate material was removed by centrifugation at 105,000 × *g* for 1 h and the supernatant was designated as cytoplasmic fraction.

The second part of the pellet was cold sonicated (five bursts of 3 min each, at 300 W) and centrifuged to obtain the total cellular extract.

All fractions were concentrated and dialysed by ultrafiltration and used for further studies.

2.4. SDS-PAGE and Western blotting analysis

SDS-PAGE in discontinuous slab gels was done by the method of Laemmli [17]. The SDS concentration was 0.1% (w/v), and the spacer and the separating gels contained 3% and 12.5% (w/v) acrylamide, respectively. Phosphorylase b (M_r 94,000), bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,100), and α-lactalbumin (M_r 14,200) were used as standards (Pharmacia).

Proteins were electrophoretically transferred from polyacrylamide gel onto nitrocellulose membrane (Bio-Rad Transblot System) according to the method of Towbin et al. [18], with sligth modification previously described [19].

2.5. Immunogold staining

Cells of *Proteus mirabilis* were fixed according to Francesconi et al. [20] with modifications as follows. Cells were centrifuged for 5 min and were fixed in (v/v) 2% paraformaldehyde/0.1% glutaraldehyde in 100 mM cacodylate buffer, pH 7.2, for 4 h at 4°C. The cell pellet was dehydrated sequentially in 50, 75, 90% dimethylformamide and embedded in Lowicryl K4M followed by UV polymerization [21]. Thin

SSDI 0014-5793(94)01115-X

sections were placed on 200-mesh nickel grids. For immunostaining the grids were incubated for 10 min at room temperature in blocking buffer (0.05 M Tris-HCl, 1% sodium azide and 0.1% bovine serum albumin), pH 7.6. The grids were treated with normal goat serum (1:20 in blocking buffer) for 30 min at room temperature, and incubated with anti-GSTB1-1 diluted up to 1:240 in blocking buffer over night. After several washes in diluted buffer (0.2 M Tris-HCl, 0.9% NaCl and 0.1% bovine serum albumin), pH 8.2, reaction with secondary antibody (10 nm gold goat anti-rabbit IgG; British BioCell) was performed for 1 h diluted 1:6 in diluted buffer. After washing, sections were stained in uranyl acetate and lead citrate. Sections not incubated with primary antibody were used as control. Observations were carried out with Zeiss EM 109.

2.6. Statistical analysis

The micrographs were printed to count, at a final magnification of 64,000 ×. A square network corresponding to 38.5 lines per μm on the specimen was placed over the prints, resulting in 1,482 squares per μm^2 and a resolution of 26 nm. Each of the single squares was judged to belong to one of the following classes: (i) gold particles on periplasmic area; (ii) periplasmic area without gold; (iii) gold particles on cytoplasmic area; (iv) cytoplasmic area without gold. Gold spheres were determined to be in a square only if the center of the particle laid within it.

A number of twenty-four bacteria were evaluated in this manner and average values ± the standard deviation (S.D.) of the gold spheres counted on squares delimiting the two compartments were calculated.

To evaluate the incidence of the background on the total count, an equal number of squares in cell free-areas corresponding to the squares of each cell studied was counted. The total gold particles counted in the squares present on cells were compared with the gold particles counted in an equal number of squares outside the cells.

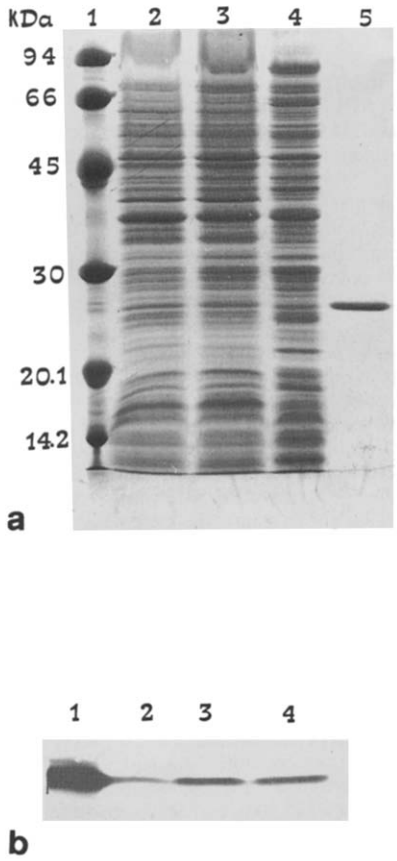

Fig. 1. (a) SDS-PAGE analysis of total, cytoplasmic and periplasmic proteins of *Proteus mirabilis* cells. Lane 1, standards and their M_r values, from top to bottom are: phosphorylase b (M_r 94,000), bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,100), and α-lactalbumin (M_r 14,200); lane 2, whole-cell proteins; lane 3, cytoplasmic fraction; lane 4, periplasmic fraction; lane 5, GSTB1-1 purified from *Proteus mirabilis* (M_r 22,500). (b) Western blot analysis of total, cytoplasmic and periplasmic proteins of *Proteus mirabilis* cells using antibodies raised against GSTB1-1. Lane 1, GSTB1-1 purified from *Proteus mirabilis*; lane 2, periplasmic fraction; lane 3, cytoplasmic fraction; lane 4, whole-cell proteins.

3. Results and Discussion

In Fig. 1 is reported the immunoblotting analysis of total, periplasmic and cytoplasmic extracts of *Proteus mirabilis* using anti-GSTB1-1 serum. A positive cross-reaction with both cytoplasmic and periplasmic extracts was seen.

Figure 2 and Table 1 show the results obtained by immunolabelling thin sections of *Proteus mirabilis* cells with antibodies specific for GSTB1-1. After treatment with uracyl acetate and lead citrate, the grids were examined under the electron microscope and the representative fields observed were photographed. Twenty-four entire bacterial cells were analysed. Immunolabelling of these sections with specific antibodies and gold spheres localized and statistically verified the preponderant presence of GSTB1-1 in the periplasmic space (Fig. 2a,b). The count was made laying a grid of a net of squares, over a micrograph, as shown in Fig. 2c. Table 1 shows the number of the gold spheres counted in the different spaces examined. For each cell the squares were classified as containing periplasmic area with gold particles, periplasmic area without gold particles, cytoplasmic area with gold particles or cytoplasmic area without gold particles and were counted. Furthermore, the cells were checked to evaluate a possible clustering of gold spheres

Table 1
Gold spheres counts of GSTB1-1-immunolabelled *Proteus mirabilis* cells

Cell No.	No. of squares (percentage on the total corresponding area)			
	Gold on periplasm	Periplasm without gold	Gold on cytoplasm	Cytoplasm without gold
1	20 (8.2)	225	12 (1.6)	717
2	13 (6.7)	185	7 (1.2)	578
3	14 (6.9)	190	8 (1.4)	570
4	14 (7.7)	167	6 (1.2)	509
5	12 (6.8)	164	5 (1.1)	462
6	15 (7.7)	180	7 (1.1)	602
7	18 (7.9)	210	6 (1.0)	580
8	16 (7.5)	198	6 (1.0)	572
9	13 (6.4)	191	7 (1.1)	646
10	14 (5.7)	231	7 (0.9)	733
11	23 (8.1)	261	8 (1.4)	581
12	19 (7.4)	237	9 (1.5)	576
13	13 (6.1)	201	7 (1.2)	580
14	14 (7.6)	170	6 (1.1)	520
15	14 (7.0)	188	7 (1.1)	605
16	13 (6.4)	190	6 (1.0)	570
17	13 (7.6)	157	7 (1.3)	541
18	12 (7.0)	160	6 (1.1)	540
19	12 (6.0)	188	6 (1.5)	403
20	17 (7.2)	218	8 (1.4)	570
21	22 (8.1)	248	15 (2.2)	680
22	20 (7.7)	241	11 (1.6)	662
23	15 (5.5)	257	8 (1.1)	699
24	16 (6.8)	220	9 (1.6)	556
Average ± S.D.	7.1 ± 0.7		1.3 ± 0.3	

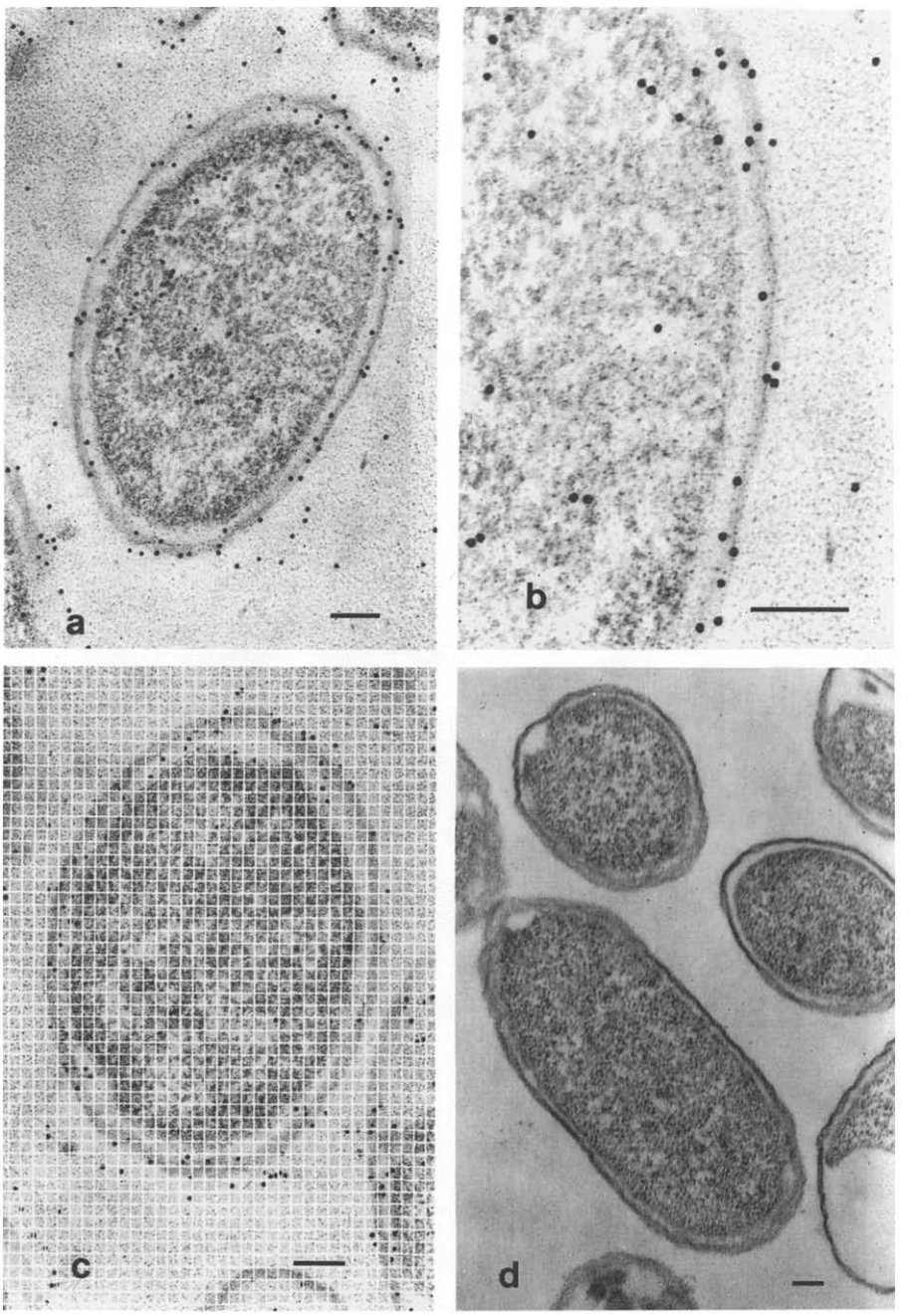

Fig. 2. Electron micrographs of immunogold-labelled *Proteus mirabilis* cells. (a) Cells treated with a primary antibody and gold-conjugated second antibody (magnification 64,000 ×) and (b) Its particular (magnification 2 × 64,000), (c) Network of 38.5 lines per μm over print for statistical analysis (magnification 64,000 ×) and (d) Control incubated without a primary antibody (magnification 40,000 ×). Bars = 0.1 μm.

in specific areas. No evidence of cluster of gold spheres was observed in both spaces.

The number of the gold spheres counted in squares on periplasmic compartment resulted higher than the number of the gold spheres included in squares on cytoplasmic compartment. The percentages of the number of the squares containing gold spheres on the total number of squares were 7.1 ± 0.7 and 1.3 ± 0.3 for periplasmic and cytoplasmic areas respectively ($P < 0.001$). Control sections incubated without primary antibody were devoid of label (Fig. 2d). The ratio between the gold particles counted in all squares of cell areas and the gold particles counted in an equal number of cell-free squares was found to be 4.7, confirming the low background and the validity of the results.

Thus, immunogold labelling technique clearly indicate a significant higher presence of GST in the periplasmic space as compared to the cytoplasmic area of *Proteus mirabilis*.

Three forms, indicated as Pm-GST-6.0 (GSTB1-1), Pm-GST-6.4 and Pm-GST-7.3, having the same antigenic determinant, were purified from *Proteus mirabilis* extract [8]. Thus whether

or not these three isoforms are located in different cell compartiments could not be established.

Owing to the putative role of GST in detoxification it is reasonable to suppose that this preponderant periplasmic GST location represent a boundary against the toxic effect of xenobiotics, including antibiotics. It has to be noted that previous experiments have demonstrated that GSTB1-1 is able to reduce the antimicrobial activity of β-lactam drugs [22].

Thus, our results strongly support the hypothesis of the inactivating role of this enzymatic system against several classes of antibiotics [19, 23] which represent a novel aspect of the general detoxification role of GST.

Experiments to point out a direct interaction between antibiotics and this enzyme are currently in progress.

Acknowledgements: We thank Prof. Benedetto Dainelli for helpful discussions and Michele Masulli and Renato Barbacane for their technical assistance. This work was supported by the Consiglio Nazionale delle Ricerche (CNR) Grant No. 92.02563.CT04 and Progetto Finalizzato 'Prevenzione e Controllo dei Fattori di Malattia' (FATMA), Sottoprogetto 'Qualità dell'Ambiente e Salute' No. 91.00146.PF41.

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