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Purification and study of a bacterial glutathione S-transferase

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A glutathione S-transferase from *Escherichia coli* has been purified approximately 800-fold with an 11% activity yield by passage through DEAE Sephacel and glutathione-agarose affinity columns. Its functional form is a homodimer of two 24 000 Da polypeptides that catalyzes the binding of glutathione and 1-chloro-2,4-dinitrobenzene with K_m values of 0.25 and 1.5 mM, respectively. Optima of pH and temperature were 7.5 and 35°C. The activity was stimulated (30%) by ethylenediaminetetraacetic acid. The N-terminal amino acid sequence was: Met-Leu-Leu-Phe-Ile-Leu-Pro-Gly-Ala.

Glutathione transferase; Enzyme purification; N-terminal sequence; Western blotting; *(Escherichia coli)*

1. INTRODUCTION

Glutathione S-transferases from eucaryotic organisms have been the subject of intensive research because of their capacity to catalyze the binding between glutathione and a large range of biologically active molecules carrying an eletrophilic center, which probably gives them a key role in detoxification. Glutathione S-transferases have been encountered in all kinds of eucaryotes from yeasts to man, being present in a diversity of tissues and organs, where they appear as different isoenzymes even in a single individual (for examples of recent reviews on glutathione S-transferases see [1–3].

In bacteria, the subject has been neglected until very recently. Shishido [4] showed the existence of an activity compatible with the presence of a glutathione S-transferase in extracts of *Escherichia coli*. Later on, a report on purification of 3 forms of glutathione S-transferases from *Proteus mirabilis* [8] appeared and was followed by two recent papers, which tend to demonstrate a wide distribution of the enzyme in procaryotic organisms [9,10]. Furthermore, it turned out that dichloromethane utilization by *Hyphomicrobium* sp. and *Methylobacterium* sp. was dependent of a dehalogenase that catalyzed the binding of glutathione to the haloalkane [11,12].

Our interest in these enzymes is a consequence of the finding that fosfomycin (an antibiotic which inhibits peptidoglycan formation) is enzymatically modified in vivo by the binding of a molecule of glutathione [5]. Attempts to purify this enzyme by standard methods used in glutathione S-transferase work [6,7] rendered a molecule able to catalyze the formation of *S*-(2,4-dinitrophenyl)glutathione from glutathione and 1-chloro-2,4-dinitrobenzene but uncapable of modifying fosfomycin. This paper deals with the isolation and partial characterization of that glutathione S-transferase from two of the most used strains of *E. coli* in cloning studies and compares this enzyme with those previously found and also with the 'atypical' glutathione S-transferase responsible for fosfomycin modification in bacteria [13].

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2. MATERIALS AND METHODS

2.1. *Purification and structural determinations*

E. coli HB101 or *E. coli* K12 JM83 were used as sources of the enzyme. Stationary cultures grown at 37°C under aeration overnight were cooled on ice, centrifuged (15300×*g* for 10 min) and washed twice with 20 mM Tris-HCl buffer (pH 8) containing 1 mM EDTA and 0.1 mM *p*-phenyl methylsulphonyl fluoride (buffer A) and then resuspended in the same buffer to 400 mg (wet weight) per ml. The cells were broken by passage through a French Press, the extract was ultracentrifuged (100 000×*g* for 1 h), and the supernatant was applied to a DEAE-Sephacel (Pharmacia) column (32 ml bed vol.) equilibrated with buffer A. After washing with the same buffer, elution was performed with a linear gradient of 0–250 mM NaCl in 300 ml of buffer A. Active fractions were pooled, dialyzed in buffer A and applied to a glutathione agarose affinity column (Sigma) (10 ml bed vol.) equilibrated with the same buffer. After washing, the bound enzyme was released by elution with 20 mM glutathione in buffer A and the active fractions dialyzed and stored at either -20°C or lyophilized. The protein concentration was determined by the method of Lowry et al. [14]. Analysis of the purity of the protein as well as molecular weight determinations were done by SDS-polyacrilamide gel electrophoresis as described by Laemli [15] and size exclusion HPLC [13] using as molecular weight standards those contained in the LMW kit of Pharmacia. Amino acid sequence determinations were done by automated Edman degradation on an Applied Biosystems 477A protein gas phase sequencer (Applied Biosystems). Protein immunoblotting was performed by the method of Towbin et al. [16] followed by detection of the antigen-antibody complexes by

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the alkaline phosphatase conjugated anti-antibody system of Blake et al. [17].

2.2. *Enzymatic assays*

Glutathione S-transferase activity was assayed by the spectrophotometric method of Habig et al. using 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene as substrates [18]. A unit of enzyme activity was defined as the amount that catalyzes the formation of 1 μmol of *S*-(2,4-dinitrophenyl)glutathione per min at 30°C (A_{340} = 10 $mM^{-1}cm^{-1}$) [19].

3. RESULTS

3.1. *Purification and structural properties*

Typical purification parameters of glutathione S-transferase from *E. coli* are summarized in table 1. Extraction from both *E. coli* HB101 and *E. coli* JM83 gave similar results. A single peak of glutathione S-transferase activity was eluted between 160 and 175 mM NaCl from the DEAE-Sephacel column with an increase in specific activity of around 6.5 times. After passage through the glutathione-affinity column, the specific activity further increased to around 800 times, with an overall recovery of around 11% of the initial activity. SDS-polyacrilamide gel electrophoresis of the final preparation showed a single band of around 24 000 Da (fig.1). However, runs of the purified protein in an HPLC molecular size exclusion column together with molecular weight markers, indicated that the activity eluted at a point corresponding to a protein of 48 000 Da. It is then concluded that the active form of the enzyme is a homodimer of two equal polypeptides of 24 000 Da. The amino terminal sequence of the protein was determined to be Met-Leu-Leu-Phe-Ile-Leu-Pro-Gly-Ala.

3.2. *Kinetic properties*

The initial velocity of *S*-(2,4-dinitrophenyl)-glutathione formation was dependent on the amount of enzyme added, confirming thus its nature ([5], data not shown). The enzymatic activity proceeded optimally at pH 7 and 35°C and was not dependent on any of the following cations (1 mM) Na^+, K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+}. Addition of Fe^{2+} or Pb^{2+} to the reaction vessels resulted in formation of precipitates, while Hg^{2+} completely inhibited the reaction. On the contrary, addition of up to 10 mM EDTA (tetrasodium salt) increased the rate of the reaction up to 30%, remaining constant afterwards. The enzyme was stable both after lyophilization and when stored at −20°C but not at 4°C; the activity was gradually lost after incubations over 45°C for 60 min. The apparent K_m values for glutathione and CDNB were 0.25 and 1.5 mM, respectively. Substitution of glutathione by other thiol-containing compounds such as cysteine, *N*-acetylcysteine, 2-β-mercaptoethanol resulted in at least a 10 times decrease of enzymatic efficiency. Finally, fosfomycin was not recognized as a substrate in spite of the fact that its biological inactivation is achieved by enzymatic addition of glutathione [5]. Furthermore, antibodies raised against the fosfomycin-modifying enzyme did not react with the glutathione S-transferase described here.

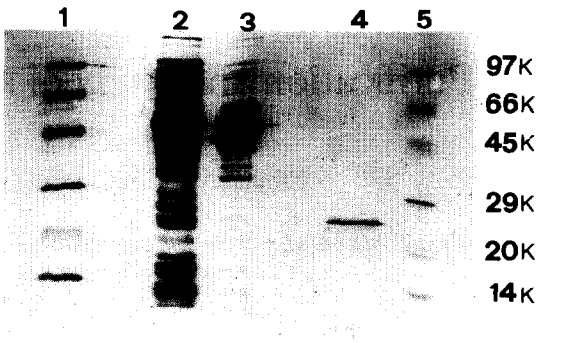

Fig.1. SDS-polyacrilamide gel electrophoresis of glutathione S-transferase isolated from *E. coli*. (1,5). Size standards: (from top to bottom) phosphorylase B (M_r 97 400), bovine albumin (M_r 66 000), egg albumin (M_r 45 000), carbonic anhidrase (M_r 29 000), trypsin inhibitor (M_r 20 000), lysozyme (M_r 14 000). (2) Extract after ultracentrifugation. (3) Active fractions from the DEAE-Sephacel column. (4) Purified enzyme after passage through the glutathione affinity column.

4. DISCUSSION

During our studies on plasmid-mediated fosfomycin resistance (Fo^r), we found that it was exerted through conjugation of the antibiotic with a molecule of glutathione which resulted in the opening of the epoxide group essential for its antimicrobial activity [5]. *E. coli* Fo^r cells produced a 16 000 Da polypeptide upon expression of the corresponding resistance determinant [20] and their extracts were able to support the so-called

Table 1

Purification of a glutathione S-transferase from *E. coli* JM83

	Enzyme activity		Protein (mg/ml)	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
	(units/ml)	(total units)				
100 000×*g* supernatant	0.116	3.487	36.5	0.0032	100	1
DEAE-Sephacel chromatography	0.067	0.98	3.275	0.0204	28	6.5
GSH-agarose affinity chromatography	0.058	0.385	0.15	2.55	11.05	801.9

Experimental details are given in the text. One unit of enzyme produces 1 μmol of *S*-(2,4-dinitrophenyl)glutathione/min at 30°C.

'general reaction' of glutathione S-transferases, in which a molecule of 1-chloro-2,4-dinitrobenzene is bound to a molecule of glutathione to give *S*-(2,4-dinitrophenyl)glutathione, whose formation can be followed spectrophotometrically [18]. This result was taken as an indication that fosfomycin was being modified by a bacterial glutathione S-transferase and its purification, based on well-established protocols for eucaryotic transferases [6,7] was undertaken. To our surprise we isolated a protein of 24 000 Da which although capable of supporting the formation of *S*-(2,4-dinitrophenyl)glutathione, did not catalyze fosfomycin modification, indicating that in those cells there were at least two distinct activities which used glutathione as a substrate in conjugation reactions, as was demonstrated later on ([13], this paper). This 24 000 Da polypeptide turned out to be one member of a family of bacterial glutathione S-transferases, which are currently being discovered and characterized, and thus its functional form is a homodimer similar in its kinetic properties to the enzyme isolated by Iizuka et al. [9], with minor differences regarding optimum temperature or the effect of different cations on its activity, and structurally related to the *Proteus mirabilis* Pm-GST-6.0, with which it shows 4 out of 9 identical amino acids in its amino terminus [21]. This enzymatic activity has been found in all strains tested in a study [10] which comprised several Enterobacteria as well as *Pseudomonas aeruginosa*, which could be taken as an indication that this is a general property of bacteria (at least of gram negatives capable of aerobic growth) and that it possibly plays an important role in the metabolism of these organisms.

In spite of the identity of some of the reactions catalyzed, no structural relationships were found between pro- and eucaryotic glutathione S-transferases, at least at the level of the N-amino acid terminus or immunological cross binding ([8,21], this paper). However, the fact that a dichloromethane dehalogenase from *Methylobacterium* sp. showed 3 zones of homology with the enzymes from eucaryotes [22] possibly indicates that the cloning and exact amino acid sequence of the procaryotic enzymes have to be known before a definite relationship can be ruled out. The absence of any relationship with the enzyme that mediates fosfomycin glutathionization, as judged by their lack of antibody cross reactivity, their different sizes (16 000 vs 24 000 Da), range of substrates other than glutathione and kinetic properties [13], is also of interest. Again the definite lack of relationship between these two enzymes cannot be ensured until the complete amino acid sequences can be compared. In order to do so we have started experiments to clone the 24 000 Da polypeptide genetic determinant.

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REFERENCES

[1] Mannervik, B. (1985) Adv. Enzymol. Relat. Areas Mol. Biol. 57, 357–417.
[2] Mannervik, B. and Danielson, U.H. (1988) CRC Rev. Biochem. 23, 283–337.
[3] Pickett, C.B. and Lu, A.Y.H. (1989) Annu. Rev. Biochem. 58, 743–764.
[4] Shishido, T. (1981) Agric. Biol. Chem. 45, 2951–2953.
[5] Arca, P., Rico, M., Braña, A.J., Villar, C.J., Hardisson, C. and Suárez, J.E. (1988) Antimicrob. Agents Chemother. 32, 1552–1556.
[6] Habig, W.H. and Jakoby, W.B. (1981) Methods Enzymol. 77, 218–231.
[7] Simons, P.C. and Vander Jagt, D. (1987) Methods Enzymol. 77, 235–237.
[8] Di Ilio, C., Aceto, A., Piccolomini, R., Allocati, N., Faraone, A., Cellini, L., Ravagnan, G. and Federici, G. (1988) Biochem. J. 255, 971–975.
[9] Iizuka, M., Inoue, Y., Murata, K. and Kimura, A. (1989) J. Bacteriol. 171, 6039–6042.
[10] Piccolomini, R., Di Ilio, C., Aceto, A., Allocati, N., Faraone, A., Cellini, L., Ravagnan, G. and Federici, G. (1989) J. Gen. Microbiol. 135, 3119–2125.
[11] Kohler-Staub, D. and Leisinger, T. (1985) J. Bacteriol. 162, 676–681.
[12] Scholtz, R., Wackett, L.P., Egli, C., Cook, A.M. and Leisinger, T. (1988) J. Bacteriol. 170, 5698–5704.
[13] Arca, P., Hardisson, C. and Suárez, J.E. (1990). Antimicrob. Agents Chemother. 34, in press.
[14] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[15] Laemli, U.K. (1970) Nature (Lond.) 227, 680–685.
[16] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[17] Blake, M.S., Johnston, K.H., Russell-Jones, G.J. and Gotschlich, E.C. (1984) Anal. Biochem. 136, 175–179.
[18] Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974) J. Biol. Chem. 249, 7130–7139.
[19] Mannervik, B. and Guthenberg, C. (1981) Methods Enzymol. 77, 231–235.
[20] Villar, C.J., Hardisson, C. and Suárez, J.E. (1986) Antimicrob. Agents Chemother. 29, 309–314.
[21] Di Ilio, C., Aceto, A., Piccolomini, R., Allocati, N., Caccuri, A.M., Barra, D. and Federici, G. (1989) FEBS Lett. 250, 57–59.
[22] La Roche, S. and Leisinger, T. (1990) J. Bacteriol. 172, 164–171.