Heterologous expression of rat liver microsomal glutathione transferase in simian COS cells and *Escherichia coli*

Rolf WEINANDER,* Erifili MOSIALOU,* Jeff DEJONG,‡|| Chen-Pei D. TU,‡ Jeanette DYPBUKT,* Tomas BERGMAN,† Henry J. BARNES,§ Jan-Olov HÖÖG† and Ralf MORGENSTERN*

*Institute of Environmental Medicine and †Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-17177 Stockholm, Sweden, ‡Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA 16802, U.S.A. and §Department of Medical Nutrition, Karolinska Institutet, Huddinge University Hospital, F-60 Novum, 14186 Huddinge, Sweden

The cDNA coding for rat liver microsomal glutathione transferase was subcloned into the mammalian expression vector pCMV-5 and the construct was transfected into, and transiently expressed in, simian COS cells. This resulted in high expression (0.7% of the microsomal protein). The activity towards 1-chloro-2,4-dinitrobenzene in microsomes was 15–30 nmol/min per mg, which increased upon *N*-ethylmaleimide treatment to 60–200 nmol/min per mg. Control and antisense-vector-treated cells displayed very low activity (3–6 nmol/min per mg). A DNA fragment coding for rat microsomal glutathione transferase was generated by PCR, cloned into the bacterial expression vector pSP19T7LT and transformed into *Escherichia coli* strain BL21 (DE3) (which contained the plasmid pLys SL). Isopropyl β-D-thiogalactopyranoside (IPTG; 1 mM) induced the expression of significant amounts of enzymically active protein (4 mg/l of culture as measured by Western blots). The recombinant protein was purified and characterized and found to be indistinguishable from the rat liver enzyme with regard to enzymic activity, molecular mass and N-terminal amino acid sequence. Human liver cDNA was used to obtain the coding region of human microsomal glutathione transferase by PCR. This PCR product was cloned into pSP19T7LT, which, upon induction with IPTG, yielded significant amounts (9 mg/l of culture) of active enzyme in BL21 (DE3) cells. Thus, for the first time, it is now possible to express both human and rat microsomal glutathione transferase in an enzymically active form in *Escherichia coli*.

INTRODUCTION

Microsomal glutathione transferase [1,2] is the membrane-bound member of the glutathione transferase group of enzymes [3,4] that catalyse the conjugation of glutathione to hydrophobic electrophiles [5], thus serving in detoxication. The glutathione peroxidase activity and membrane location of microsomal glutathione transferase has been suggested to be vital to the protective function of the enzyme towards lipid peroxidation [6,7] and particularly hydrophobic substrates [8]. Rat liver microsomal glutathione transferase has a molecular mass of 17.3 kDa, with an amino acid sequence analysed both at the protein and cDNA level [9,10] and appears to have no closely related isoenzyme(s). The enzyme is present predominantly in the liver (endoplasmic reticulum and mitochondrial outer membrane) and has been purified from rat [11,12], mouse [13] and humans [14,15]. These species express closely related enzymes.

No systems for the heterologous expression of any microsomal glutathione transferase have as yet been developed. Such systems are needed to study structure–activity relationships and the role of the enzyme in cell toxicology. Here we describe the construction of expression vectors based on pCMV-5 (a mammalian expression vector [16]) and pSP19T7LT (a bacterial expression vector), expression of the rat enzyme in mammalian cells, expression with good yields of functional rat and human enzyme in bacteria and isolation of pure rat enzyme expressed in bacteria.

MATERIALS AND METHODS

Rat liver microsomal glutathione transferase cDNA isolation was described previously [10]. The *Eco*RI fragment compromising 906 bp including the coding region was inserted into the corresponding site in the pCMV-5 mammalian expression vector [16]. Sense and anti-sense plasmids were isolated [17] and named pCGS and pCGAS respectively. The same *Eco*RI fragment was used as a template for PCR, utilizing primers that incorporated suitable restriction sites into the product (*Nde*I–*Hin*dIII):

Primer 1 (upper 1): 5′-CG*CATATG*GCTGACCTCAAG *Nde*I site

Primer 2 (upper 2): 5′-CG*CATATG*GTTTTTGCCAACCCGG-AA *Nde*I site

Primer 3 (lower): 5′-CG*AAGCTT*ATTACAAGTACAGTCTG *Hin*dIII site

Primer 1 yields a full-length construct, whereas primer 2 yields a construct encoding a truncated form of the enzyme lacking the first 41 amino acids. The PCR products were isolated by low-melting-temperature-agarose electrophoresis and subsequently purified from the gel (Magic-PCR-prep; Promega) and cut with *Nde*I and *Hin*dIII. The resulting 470 bp and 344 bp products were ligated into the bacterial expression vector pSP19T7LT, which had been cut with the same restriction enzymes and gel-purified. The *Escherichia coli* expression plasmid, pSP19T7LT, is a high-copy-number plasmid derived from pSPORT-1 (GIBCO–BRL) in which a DNA fragment containing the translation-initiation region of the bacteriophage T7 gene 10 had been introduced downstream of the *lac* and synthetic T7 promoter sequences of pSPORT-1. A T7 transcription terminator sequence had been introduced immediately following the last restriction

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; NEM, *N*-ethylmaleimide; IPTG, isopropyl β-D-thiogalactopyranoside.

||Current address: Laboratory of Biochemistry and Molecular Biology, Rockefeller University, 1230 York Avenue, New York, NY 10021-6399, U.S.A.

site (i.e. *Hin*dIII) in the pSPORT-1 multiple cloning site. The coding sequence of microsomal glutathione transferase is introduced into the plasmid so as to create an expression unit consisting of tandem *lac*/T7/ promoters, the T7 gene 10 leader, microsomal glutathione transferase cDNA and a T7 transcription terminator sequence. All constructs were transformed into *E. coli* JM109 as described in [18], positive colonies were identified and the plasmids isolated followed by transformation into *E. coli* BL21 (DE3) (which harboured the plasmid pLys SL) using the same protocol. The plasmid pLYS SL is a derivative of pLys S [19] in which the phi 3.8 promoter has been removed to prevent 'around the plasmid transcription' of the T7 lysozyme gene [19]. Details of the construction of plasmids pSP19T7LT and pLys SL can be obtained from H.J.B. on request. Glycerol stocks were prepared and stored frozen at −70 °C for subsequent use as starting material for the expression experiments.

Human liver cDNA (generously provided by Inger Johansson, Department of Medical Biochemistry and Biophysics, Karolinska Institutet) was used as a template for human microsomal glutathione transferase-specific primers based on a previously published cDNA sequence [20] in order to isolate the coding sequence of this enzyme. The same flanking restriction sites were introduced, and the product was inserted into pSP19T7LT and transformed into *E. coli* JM109 and BL21 (DE3) as described for the rat enzyme.

Primer (upper): 5′-GCGA*CATATG*GTTGACCTCACCCAG *Nde*I site

Primer (lower): 5′-GAGC*AAGCTT*TTACAGGTACAATTT-ACTTTTC *Hin*dIII site

The sequences of all PCR-generated constructs were determined by the dideoxy-chain-termination method of Sanger et al. [21] using Sequenase I (U. S. Biochemical Corp.) to ensure the fidelity of *Taq* DNA polymerase extension of the primers. Two typographic errors in the human microsomal glutathione transferase sequence became evident when this PCR product was cloned and sequenced (the errors were clearly typographic, since the deduced amino acid sequence given was correct [10] and in accordance with our sequencing results). Changes comprise nucleotides 128 (G to T) and 266 (A to T) (numbered with the initiator codon *A*TG as 1).

Transient expression in mammalian cells was accomplished by transfection with DEAE-dextran and DNA (performed as described in [16]) with COS cells as recipients [COS-1; simian fibroblast-like (CRL-1650)]. Microsomes were isolated by standard procedures [22]. Measurement of microsomal glutathione transferase activity [11] was performed as described below, except that a lower concentration, 0.2 mM 1-chloro-2,4-dinitrobenzene (CDNB), was used as the second substrate to reduce background. Lipid peroxidation was determined as described previously [6]. Western blots were performed as described below.

The origin, species and organ, of cell types used to screen for microsomal glutathione transferase are as follows. Cell types commercially available from the American Type Culture Collection (Rockville, MD, U.S.A.) are indicated in parentheses with their order number. Hep G2, human liver carcinoma (HB 8065); BT 20, human breast carcinoma (HTB 19); MCF 7 S/R, human breast carcinoma, adriamycin-sensitive or -resistant (HTB 22); HOF, human oral fibroblasts; NF, human normal skin fibroblasts (CRL 1508); XP 12BE, human xeroderma-pigmentosum skin fibroblasts, complementation group A (CRL 1223); PC-12, rat adrenal phaeochromocytoma (CRL 1721); L6, rat skeletal-muscle myoblasts (CRL 1458); RINm5F, rat insulinoma; V79, Chinese-hamster lung (CCL 93); McCoy, mouse (CRL 1696); NB II A, mouse neuroblastoma (CCL 131); L1210, mouse lymphomas (CCL 219); 1c1c7, mouse hepatoma; F10, rat smooth muscle.

Bacterial expression was performed as follows: a small aliquot (5–10 μl) of bacterial glycerol stock was grown in 1.5 ml of 2 × YT [17] overnight at 37 °C. The culture was diluted 1:100 in terrific broth [23] and grown until the A_{600} was 0.4–1.2. At this point expression was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), the temperature was switched to 30 °C and the culture allowed to grow for another 2–12 h. All steps were performed in the presence of ampicillin (75 μg/ml) and chloramphenicol (10 μg/ml) with shaking at 240 rev./min. Aliquots were taken to monitor the increase in enzyme activity [11] and immunoreactive protein (0–12 h). At the peak level cells were pelleted and resuspended in 15 mM Tris/HCl (pH 8.0)/0.25 M sucrose/0.1 mM EDTA/1 mM GSH. The cells were lysed by sonication using four 30 s pulses from an MSE Soniprep 150 sonifier at 40–60 % of maximum power. $MgCl_2$ was added to a final concentration of 6 mM, and DNA and RNA were hydrolysed by incubation with DNase I (4 μg/ml) and RNase A (4 μg/ml) for 30 min at 4 ° C with gentle stirring. Cell debris was removed by centrifugation at 5000 ***g*** for 10 min. The supernatant was then centrifuged at 250000 ***g*** for 60 min and the membrane pellets were suspended in 10 mM potassium phosphate (pH 7.0)/20 % glycerol/0.1 mM EDTA/1 mM GSH.

Purification of rat liver microsomal glutathione transferase from bacteria was performed as follows: membranes (isolated from 1 litre of culture) were solubilized by addition of an equal volume of 10 mM potassium phosphate (pH 7.0)/20 % glycerol/0.1 mM EDTA/1 mM GSH/6 % Triton X-100 and 15 min incubation on ice. Hydroxyapatite chromatography was performed by a batch procedure whereby solubilized membranes were adsorbed to 5 g of hydroxyapatite (Bio-Gel HTP; Bio-Rad) equilibrated with 10 mM potassium phosphate (pH 7.0)/20 % glycerol/0.1 mM EDTA/1 mM GSH/1 % Triton X-100 (hereafter referred to as 'buffer A') for 15 min. The hydroxyapatite was pelleted by a low-speed centrifugation pulse and washed with 2 vol. of buffer A, followed by 1 vol. of 50 mM potassium phosphate in buffer A. Microsomal glutathione transferase was eluted with 0.4 M potassium phosphate in buffer A and desalted by dialysis for 24 h against 50 vol. of buffer A. Further purification was performed by ion-exchange chromatography on a CM-Sepharose CL 6B (Pharmacia) column equilibrated with buffer A. The enzyme was eluted with 0.2 M KCl in buffer A, and 0.2 ml fractions were collected at a flow rate of 0.3 ml/min. Fractions containing the peak activity were pooled and subjected to SDS/PAGE, Western blotting and N-terminal amino-acid-sequence analysis as described below.

Determination of the N-terminal amino acid sequence of the expressed and purified rat enzyme was performed by automated Edman degradation using an Applied Biosystems 470A instrument. Amino acid phenylthiohydantoin derivatives were analysed by reverse-phase HPLC essentially as described in [24].

Enzyme-activity assays with different substrates were performed as described in [12,25], except that the Triton X-100 concentration was raised to 1 % with crude fractions in order to avoid an increase in turbidity.

SDS/PAGE was performed as described by Laemmli [26] in 15 % polyacrylamide gels, followed by Western blotting and immunodetection with specific antisera towards the rat liver enzyme [27]. Purified rat and human microsomal glutathione transferase were used as standards. Rainbow marker molecular-mass standards were from Amersham International.

Protein bands were revealed by staining with Coomassie Brilliant Blue R-250 and quantification was by the method of Peterson [28].

All chemicals were from common commercial sources and of the highest purity available.

RESULTS

Expression of rat liver microsomal glutathione transferase in mammalian cells

Transfection of COS cells with plasmid pCGS (0.05–0.18 μg of DNA/cm^2) increases the glutathione transferase activity towards CDNB 5–10-fold in microsomes (Table 1). The increase in microsomal glutathione transferase protein (as detected by Western blots) is from barely detectable in controls to 7 μg/mg of microsomal protein. This level is of the same order as that in rat liver microsomes (31 μg/mg of microsomal protein), underlining the efficiency of the procedure. The optimal amount of added DNA and time of incubation after transfection are 0.07 μg/cm^2 and 72 h respectively. Activation by *N*-ethylmaleimide (NEM) increases the CDNB activity up to 8-fold. The activity in control and pCGAS-transfected cells is very low, although it is interesting to note that activation does occur. This is a phenomenon not previously observed in material of non-hepatic origin [29], with the exception of nasal mucosa [30].

In order to identify suitable candidates for future transfection experiments, the content of microsomal glutathione transferase in several cell types was investigated by immunoblot analysis (Table 2). The reactivity of the rabbit anti-(rat enzyme) serum is expected to vary with cells from different species, resulting in an approximate indication of the relative content (this reasoning also applies to simian COS cells, where the control levels cannot be quantified exactly by immunochemistry). The enzyme could be detected in all cell lines investigated. It was found that Hep G2, BT 20, V79WT, NB II A, L1210 and RIN m5F cells had a high content of microsomal glutathione transferase, whereas XP 12BE, 1c1c7, MCF7 R, NF and HOF cells had very low amounts and could thus be useful for transfection experiments with the enzyme.

Bacterial expression of rat and human microsomal glutathione transferase

The expression vector pSP19T7LT containing the coding sequence of rat microsomal glutathione transferase was transformed into *E. coli* BL21 (DE3). Induction with 1 mM IPTG yielded comparatively high amounts of active protein (4 mg/l of culture) as determined by Western blots. The enzyme appears to be localized in the bacterial inner membrane, as 70–80 % of the protein was found in the membrane pellet after ultracentrifugation. The CDNB-conjugating activity of the recombinant enzyme is indistinguishable from the one obtained in rat liver microsomes (2.9 μmol/min per mg of immunoreactive protein). We also expressed a truncated form of the enzyme that lacks the first 41 amino acids, since it is known that this portion can be removed from the purified protein by proteolysis/ion-exchange chromatography [31]. The truncated form (residues 42–154) retains its activity in this case. Upon expressing this part of the protein in bacteria, however, no enzymic activity could be observed, even though expression of a polypeptide with the

Table 2 Content of microsomal glutathione transferase in different cell types as determined by immunoblot analysis of total protein from 0.5 × 10^6 cells

Cell type	Arbitrary content
MCF7 S	Medium
MCF7 R	Low
XP 12 BE	Low
NF	Low
Hep G2	High
HOF	Low
COS-1	Low
BT 20	High
L6	Medium
F10	Medium
PC-12	Medium
RIN m5F	High
1c1c7	Low
McCoy	Medium
NB II A	High
L1210	High
V79 WT	High

Table 1 Glutathione transferase activity towards CDNB in microsomes and cytosol from COS cells that were transfected with an expression vector for microsomal glutathione transferase

Student's *t* test of significance: (1) control and antisense-construct-treated cells before and after NEM activation, 4.4 ± 1.9 ($n = 8$) versus 10.2 ± 4.4 [$n = 7$ (additional data included)] ($P < 0.01$); (2) control and antisense-construct-treated cells, 4.4 ± 1.9 ($n = 8$) compared with all sense-construct-treated cells at 72 h, 22.8 ± 5.7 ($n = 4$) ($P < 0.01$); (3) all sense-construct-treated cells at 72 h before and after NEM activation, 22.8 ± 5.7 ($n = 4$) versus 143 ± 45 ($n = 4$) ($P < 0.001$).

			Specific activity (nmol of CDNB/min per mg)			
			pCGS (sense)		pCGAS (antisense)	
Subcellular fraction	DNA (μg/cm^2)	Time (h) post-transfection	Unactivated	NEM-treated	Unactivated	NEM-treated
Microsomes	0	48	3.3	5.2		
	0.05	48	17	90	2.6	
	0.09	48	20	94	3.1	
	0.18	48	15	62	2.5	
	0.05	72	16	93	4.2	
	0.07	72	28	200	5.6	15
	0.09	72	27	150	8.0	
	0.18	72	20	130	5.6	
Cytosol	0	48	56			
	0.05	48	53		43	

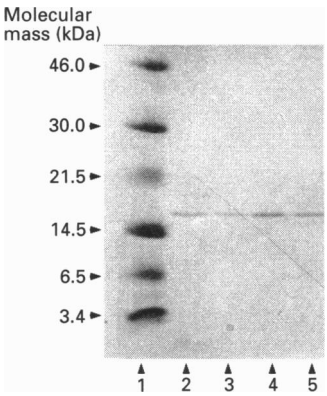

Figure 1 SDS/PAGE of microsomal glutathione transferase purified from *E. coli*

Lane 1, Rainbow molecular-mass markers; lane 2, purified microsomal glutathione transferase from rat liver (1 μg); lanes 3–5, purified recombinant microsomal glutathione transferase from *E. coli* membranes (1, 1.7 and 1.4 μg). Protein bands were revealed by staining with Coomassie Brilliant Blue R-250. For experimental details, see the Materials and methods section.

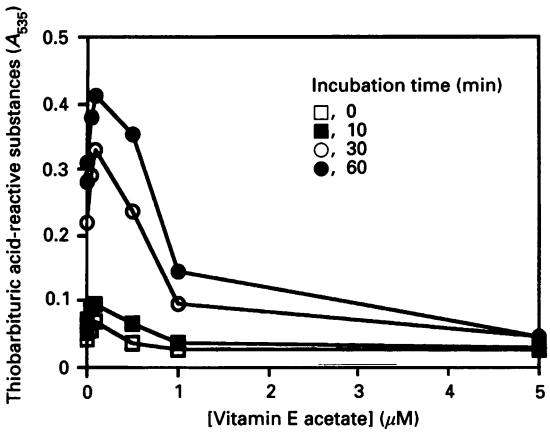

Figure 2 Lipid peroxidation in microsomes isolated from COS cells

The effect of vitamin E acetate addition 72 h prior to isolation is shown. Details are given in the Materials and methods section.

appropriate molecular mass did occur (as determined by Western blots). Furthermore, only 20% of the protein was recovered in the membrane fraction, whereas 80% was found in the 5000 *g* pellet, indicating the formation of inclusion bodies. It thus appears that the first 41-amino-acid stretch, which contains a membrane spanning region, is important for the correct folding and localization of the enzyme, although it does not contribute to the active site.

Essentially the same purification method that was devised for rat liver microsomes was used for purification of recombinant rat microsomal glutathione transferase. This method yields a purified protein that has the expected enzymic properties, electrophoretic mobility, immunoreactivity, N-terminal amino-acid sequence (Figure 1 and Table 3). Thus the first heterologous expression–purification method for microsomal glutathione transferase has been established.

Human microsomal glutathione transferase could also be expressed successfully in bacteria by the methods described here. The yield (9 mg/l) and specific activity [4.4 μmol/min per mg of immunoreactive protein (in a partially purified fraction)] is comparable with that obtained for the rat enzyme. Purification of the human enzyme expressed in bacteria is currently being developed.

We have examined lipid peroxidation in microsomes isolated from COS cells. It was realized that α-tocopherol acetate (a vitamin E derivative) had to be added to the media to mimic the situation when microsomes are isolated from rat liver. When α-tocopherol acetate is not added to the cells, microsomes are very vulnerable to lipid peroxidation induced by ADP-Fe and ascorbate. No protection by added GSH could be observed. When a range of α-tocopherol acetate concentrations were added to the cells (last 72 h) before isolation of microsomes, lipid peroxidation was completely inhibited by high concentrations ($\geqslant 5$ μM) (Figure 2). Lower concentrations ($\leqslant 0.5$ μM) that did not protect by themselves restored GSH-dependent protection. In this case microsomes from both sense- and antisense-transfected cells displayed protection. A slightly better GSH-dependent protection was apparent with the sense construct [64 ± 19% and 42 ± 12% ($n = 4$) GSH-dependent reduction in malonaldehyde formation between 30 and 60 min for the sense and antisense constructs respectively (the difference is not significant)]. Since there is some variability in expression and, especially, the protection by addition of α-tocopherol acetate, it has been difficult to ascertain the potential protective role of

Table 3 Specific activities of recombinant rat microsomal glutathione transferase with different substrates

Enzyme was expressed and purified from *E. coli* BL 21 (DE3) as described in the Materials and methods section. Values are means $\pm$ S.D. of at least triplicate determinations.

	Specific activity (μmol/min per mg)			
	Recombinant enzyme		Liver enzyme	
Substrate	Untreated	NEM-treated	Untreated	NEM-treated
CDNB	2.9 ± 0.02	35 ± 4	2.6 [25]	38.0 [25]
N-Acetylcysteine + CDNB	1.6 ± 0.08	0.91 ± 0.02	4.5	1.42
4-Chloro-3-nitrobenzamide	0.63 ± 0.03	0.83 ± 0.07	1.9[25]	1.9 [25]
Cumene hydroperoxide*	0.17 ± 0.05	n.d.†	0.08 [12]	0.8 [12]

*Glutathione peroxidase activity.
†n.d., not determined.

microsomal glutathione transferase. It will therefore be necessary to study a cell line devoid of the enzyme. We are currently trying to identify such cell lines as well as creating one by use of the antisense construct.

DISCUSSION

In order to study the role of a specific enzyme in the cell, inhibitors have been widely used. Owing to the inherent problems such as lack of selectivity, toxicity and inability to suppress the activity in question, alternate methods have been developed. Efficient synthesis of enzymes in cellular systems by means of transfection with expression vectors is a very attractive method with which to study the role of a protein in endogenous processes and in the metabolism of exogenous compounds. In cellular toxicology it is becoming apparent that the interplay of many enzyme activities involving different key steps for different chemicals are responsible for the varying toxicological responses. It is therefore of great value to be able to study defined reactions in cell types harbouring the enzymes of interest compared with identical controls lacking the activity instead of having to resort to many different animal models that, in addition, are less well defined. We describe here, for the first time, the development of an efficient system for the expression of rat liver microsomal glutathione transferase in mammalian cells.

A role has been suggested for microsomal glutathione transferase in protection against oxidative stress. Certainly the enzyme has the capacity to reduce phospholipid hydroperoxides and to conjugate hydroxyalkenals [6]. Whether this capacity will be reflected in transfected as opposed to control cells is an interesting objective for future studies.

An antisense vector is a good control in the sense that it is expected to be an equivalent of the sense vector regarding replication and mRNA production without producing protein. In our case the antisense-vector-treated cells are indistinguishable from cells not receiving DNA, indicative of its value as a control. The perfect control, however, would involve expression of the same amount of protein with the same basic structure but no activity. We are presently constructing inactive microsomal glutathione transferase by *in vitro* mutagenesis in order to be able to determine the role of the enzyme in cellular protection against toxicity.

In studies of structure–activity relationships it is of great value to be able to produce the enzyme of interest in large amounts harbouring defined mutations introduced by *in vitro* methodology. Mammalian membrane proteins have been particularly difficult to overproduce in *E. coli* in enzymically active form and, so far, only a few examples have been described (in [32]). Bacterial expression of both human and rat microsomal glutathione transferase was successfully accomplished by use of a system that was developed for the expression of cytochrome *P*-450 [33]. In comparison, both proteins are expressed optimally at 25–30 °C, whereas cytochrome *P*-450 often requires much longer expression periods for comparable yields. Paramount to the high-level expression was the use of an *E. coli* strain containing a stable genomic copy of T7 RNA polymerase under the control of the *lac* promoter and an expression construct with the T7 promoter and leader sequence driving the expression. In addition the bacteria used contained the plasmid pLysSL, which produces T7 lysozyme, which inhibits low-level uninduced expression by inhibiting T7 RNA polymerase [19].

It is now possible to perform experiments involving *in-vitro*-mutagenesis methods for examining structure–function relationships which would be impractical utilizing cell-tissue-culture expression. Targets for *in vitro* mutagenesis are amino acids previously identified by chemical modification or implicated in catalysis by analogy to cytosolic glutathione transferases [34] and possible determinants of membrane topology. Western blots from proteinase-digestion experiments with spheroplasts made from bacteria that express the enzyme show that the protein is localized in the periplasmic membrane in an orientation where the N-terminus faces the outside (H. Raza, R. Weinander and R. Morgenstern, unpublished work). Since the N-terminus is oriented inside the lumen of the endoplasmic reticulum [31], we have excellent complementary models for studying membrane topology.

Although we have obtained reasonable yields of expressed protein, optimization of the expression construct (preferred codon usage and minimization of mRNA secondary structure around the starting codon) should yield even higher amounts of microsomal glutathione transferase. Since we are trying to crystallize the enzyme, large amounts of protein that can be modified by *in vitro* mutagenesis could greatly improve the chance of success.

In conclusion, COS-cell transfection experiments offers an efficient means for obtaining cells with comparatively high levels of microsomal glutathione transferase. The role of the enzyme in protection against oxidative stress will be examined in this system. Bacterial expression and purification of microsomal glutathione transferase opens new possibilities for advanced structure–function-relationship as well as membrane-topology studies.

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