Purification and characterization of glutathione reductase encoded by a cloned and over-expressed gene in *Escherichia coli*

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An expression vector, pKGR, for the *gor* gene from *Escherichia coli* encoding glutathione reductase was constructed by subcloning of an *Ava*II fragment of the Clarke & Carbon bank plasmid pGR [Greer & Perham (1986) *Biochemistry* **25**, 2736–2742] into the plasmid pKK223-3. The expression of glutathione reductase from the plasmid pKGR was found to have been successfully placed under the control of the *tac* promoter. Transformation of *E. coli* cells with this plasmid resulted in 100–200-fold increase in glutathione reductase activity in cell-free extracts. A rapid purification procedure for the enzyme, based on affinity chromatography on Procion Red HE-7B–CL-Sepharose 4B, was developed. The purified enzyme was homogeneous as judged by SDS/polyacrylamide-gel electrophoresis, and all its properties were consistent with the DNA sequence of the gene [Greer & Perham (1986) *Biochemistry* **25**, 2736–2742] and with those previously reported for *E. coli* glutathione reductase [Mata, Pinto & Lopez-Barea (1984) *Z. Naturforsch. C. Biosci.* **39**, 908–915]. These experiments have enabled an investigation of the protein chemical and mechanistic properties of the enzyme by site-directed mutagenesis.

INTRODUCTION

Glutathione reductase (EC 1.6.4.2) catalyses the reduction of GSSG by NADPH:

It is a widespread member of an important family of flavoprotein oxidoreductases that includes dihydrolipoamide dehydrogenase (EC 1.6.4.3) (Reed, 1974; Packman & Perham, 1982), thioredoxin reductase (EC 1.6.4.5) (Holmgren, 1980) and mercuric reductase (Fox & Walsh, 1982). All these enzymes are dimers with an M_r of about 105000, and all possess a disulphide bridge in each subunit which is alternately oxidized and reduced as part of the catalytic mechanism (reviewed by Williams, 1976). Amino acid sequences around the disulphide bridges in dihydrolipoamide dehydrogenase, glutathione reductase and mercuric reductase are highly homologous, implying that they have arisen by divergent evolution from a common ancestor (Perham *et al.*, 1978; Williams *et al.*, 1982; Fox & Walsh, 1983). On the other hand, thioredoxin reductase is sufficiently different for it to be likely that this enzyme has arisen by convergent evolution towards a common mechanism (Perham *et al.*, 1978).

The *lpd* gene of *Escherichia coli*, encoding dihydrolipoamide dehydrogenase (Stephens *et al.*, 1983), the *merA* gene of transposon Tn501 from *Pseudomonas aeruginosa*, encoding mercuric reductase (Brown *et al.*, 1983), and the *gor* gene of *E. coli*, encoding glutathione reductase (Greer & Perham, 1986), have been cloned and their nucleotide sequences determined. This in turn has enabled their complete primary structures to be inferred and compared. The complete amino acid sequence of human glutathione reductase (Krauth-Siegel *et al.*, 1982) has also been determined, which has permitted considerable light to be thrown on the reaction mechanism

from X-ray-crystallographic analysis of the protein at 0.2 nm resolution (Thieme *et al.*, 1981; Pai & Schulz, 1983).

Human and *E. coli* glutathione reductases are highly homologous in their amino acid sequences (Greer & Perham, 1986), making it certain that their three-dimensional structures will also be similar. Thus it should now be possible to test some of the predictions made about the catalytic mechanism of glutathione reductase (Pai & Schulz, 1983) by site-directed mutagenesis of the *E. coli* protein. Complementary work is being undertaken with mercuric reductase (Schultz *et al.*, 1985). To facilitate such a study, it is essential to have a system for expression of the cloned gene, preferably in a controllable expression vector, and a simple method for purifying the enzyme from transformed cells. We describe here the cloning of the *E. coli gor* gene into a plasmid vector that raises the glutathione reductase activity some 200-fold above that found in untransformed wild-type *E. coli* (strain JM101) and a simple purification method that permits the preparation of large quantities of *E. coli* glutathione reductase indistinguishable from that described by Mata *et al.* (1984).

MATERIALS AND METHODS

Materials

Complex bacteriological media were from Difco Laboratories and all media were prepared as described in Maniatis *et al.* (1982). L-[³⁵S]Methionine (800 Ci/mmol; for coupled transcription–translation) and [α -³⁵S]-thio[dATP triethylammonium salt (> 400 Ci/mmol; for DNA sequencing) were from Amersham International. Ethidium bromide, isopropyl β -thiogalactoside, 5-bromo-4-chloroindol-3-yl β -galactoside, NADPH, GSSG and amino acids were from Sigma Chemical Co.

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Deoxy- and dideoxy-nucleoside 5'-triphosphates used in DNA sequencing were from P-L Biochemicals. Ultrapure agarose, dithiothreitol and CsCl were from Bethesda Research Laboratories. Procion Red HE-7B linked to CL-Sepharose 4B was a gift from Dr. C. R. Lowe (Biotechnology Centre, University of Cambridge). All other chemicals were of analytical-reagent grade wherever possible. Glass-distilled water was used throughout.

The restriction enzymes *Hind*III, *Ava*II, *Eco*RI and *Sma*I were purchased from New England Biolaboratories. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. T4 DNA polymerase was from Pharmacia. T4 DNA ligase and the Klenow fragment of *E. coli* DNA polymerase I were generously made available by Dr. R. T. Hunt (Department of Biochemistry, University of Cambridge). The expression vector pKK223-3 was from Pharmacia.

Plasmid construction and DNA sequencing

Plasmid or bacteriophage RF DNA were prepared on a 100 ml scale as described by Maniatis *et al.* (1982). For the purposes of screening, plasmids and RF DNA were prepared on a small scale by the rapid boiling technique of Holmes & Quigley (1981). Endonuclease digestion of DNA was carried out as recommended by the enzyme suppliers. For analytical digests 1 µg of DNA was overdigested ten times for 1 h in a total volume of 25 µl. For preparative digests, 100 µg of DNA was overdigested ten times in a total volume of 400 µl overnight. DNA fragments were purified by submarine agarose-gel electrophoresis. The gels were electrophoresed in TAE buffer (40 mM-Tris/20 mM-sodium acetate/10 mM-EDTA, pH 8.2) at 5V/cm. Nucleic acid was stained with ethidium bromide in running buffer (0.5 µg/ml) for 20 min, and DNA was detected by using a long-wavelength u.v. transilluminator. The appropriate gel band in the stained agarose gel was excised and placed in an ISCO gel electroelution cup and electroeluted in accordance with Allington *et al.* (1978). The eluted DNA was purified further by phenol, chloroform and diethyl ether extraction (Maniatis *et al.*, 1982).

Vector DNA was cut with the appropriate enzyme, treated with calf intestinal alkaline phosphatase if required and then ethanol-precipitated (Maniatis *et al.*, 1982). End-repair of DNA with T4 polymerase, ligation of DNA fragments and transformation of cells were also carried out as described by Maniatis *et al.* (1982).

Bacteriophage single-stranded DNA was prepared and sequenced by the chain-termination method (Sanger *et al.*, 1980; Biggin *et al.*, 1983).

Coupled transcription-translation of DNA *in vitro*

Coupled transcription-translation of DNA was carried out as described by Zubay (1974) with a cell-free system derived from *E. coli* strain PR7 (Howe *et al.*, 1982).

SDS/polyacrylamide-gel electrophoresis

Cell-free extracts were electrophoresed in 15% polyacrylamide slab gels in the presence of SDS (Laemmli, 1970). Gels were then either stained with Coomassie Brilliant Blue or silver-stained (Morrissey, 1981).

Growth of cells and preparation of cell-free extracts

All cells were grown with vigorous shaking in 2 × TY medium supplemented with the appropriate antibiotics

until the required cell density was reached. For small-scale preparation of extract, a 10 ml culture of cells was grown and then centrifuged at 300 *g* for 5 min. The medium was decanted and the pellet washed in 2 ml of sonication buffer (50 mM-potassium phosphate, pH 8.0). Cells were again collected by centrifugation (3000 *g* for 5 min) and resuspended in 1 ml of sonication buffer. The suspension was then sonicated in three pulses each of 8 s on power setting 2 on a Heat Systems cell disruptor. The cell extract was centrifuged (11500 *g* for 5 min) to yield a cell-free supernatant. Protein concentration was determined either by the method of Lowry *et al.* (1951) or by that of Bradford (1976); bovine serum albumin was used as standard.

Assay of glutathione reductase

Glutathione reductase activity was estimated at 30 °C by the GSSG-dependent oxidation of NADPH measured by the decrease in the absorbance at 340 nm (Mata *et al.*, 1984). Each assay mixture contained 10 µl of 10 mM-NADPH, 10 µl of 120 mM-GSSG, the sample to be assayed (10–100 µl) and 0.1 M-potassium phosphate buffer, pH 7.6, to a final volume of 1 ml.

Amino acid composition and sequence analysis

Amino acid analysis was carried out on an LKB 4400 amino acid analyser. The samples were hydrolysed for 24, 48 and 96 h in 6 M-HCl, as described by Perham (1978). *N*-Terminal sequence analysis was kindly carried out by Applied Biosystems on a gas-phase sequencer.

Determination of native M_r value

The M_r of *E. coli* glutathione reductase was determined by gel-filtration fast protein liquid chromatography on a Pharmacia Superose 12 column in 50 mM-potassium phosphate buffer, pH 7.5. The following proteins were used as standards (numbers in parentheses indicate the assumed M_r values): ferritin (480000), rabbit fructose-bisphosphate aldolase (160000), calf alkaline phosphatase (140000), bovine serum albumin (67500), chicken ovalbumin (44000), carbonic anhydrase (32000), α -chymotrypsinogen A (25000), horse heart myoglobin (18800), hen egg-white lysozyme (14388) and ox insulin (11466).

RESULTS AND DISCUSSION

Construction of an expression vector for glutathione reductase

Insertion of the *gor* gene into a suitable M13 bacteriophage would not only provide a convenient source of single-stranded DNA for site-directed mutagenesis experiments, but the possibility existed that the high copy number of the recombinant bacteriophage DNA within the host cell might cause a desirable over-expression of the inserted *gor* gene. To test this, the *gor* gene was excised as part of a 3.3 kbp *Ava*II fragment from the plasmid pGR described by Greer & Perham (1986) and inserted into the *Sma*I site of RF DNA of bacteriophage M13mp8. Examples of *gor*-gene insertion in both orientations were obtained, as demonstrated by DNA sequence analysis. The glutathione reductase activity in cell-free extracts of *E. coli* JM101 cells infected with the bacteriophage constructs carrying the *gor* gene was determined after prior induction of the cells with isopropyl β -thiogalactoside. No increase in glutathione

Fig. 1. Expression of the *gor* gene in *E. coli* strain SG5

Samples of cell extracts of *E. coli* strain SG5 were prepared and analysed by means of SDS/polyacrylamide-gel electrophoresis, as described in the Materials and methods section. Protein bands were stained with Coomassie Brilliant Blue. Track A, purified *E. coli* glutathione reductase; track B, cell extract of *E. coli* SG5 transformed with plasmid pKGR; track C, cell extract of *E. coli* SG5 transformed with plasmid pKK223-3; track D, cell extract of *E. coli* strain SG5.

reductase activity in bacteriophage-infected cells, compared with non-infected *E. coli* JM101 cells, was detected. Furthermore, SDS/polyacrylamide-gel electrophoresis of cell-free extracts demonstrated that large amounts of an inactive glutathione reductase were not being produced; and coupled transcription-translation of the bacteriophage RF DNA revealed that very little, if any, expression of the *gor* gene occurred *in vitro*. A more powerful expression system was therefore sought. This was provided by the plasmid pKK223-3, in which the inserted gene should come under the control of the strong *tac* promoter.

Samples of recombinant bacteriophage RF DNA carrying the correct and the reverse-orientation *gor* gene were digested with the enzymes *Eco*RI and *Hind*III to produce 3.3 kbp fragments incorporating the *gor* gene. These were isolated and directionally cloned into *Eco*RI-*Hind*III-digested plasmid pKK223-3. The constructs were transformed into *E. coli* JM101 and selected

for by growth on 2×TY/ampicillin plates. The orientation of the inserts was of course known and the presence of the *gor* gene was confirmed by restriction analysis.

Expression of the *gor* gene from the recombinant plasmid (designated pKGR) carrying the *gor* gene in the correct orientation, i.e. under the control of the *tac* promoter, was investigated in both *lac* i^q (JM101) and non-*lac* i^q (SG5) strains of *E. coli*. Strain SG5 (Greer & Perham, 1986) has the added advantage of lacking the *gor* gene, so that no background of glutathione reductase activity is present in these cells (Fig. 1). With plasmid-transformed *E. coli* JM101 before isopropyl β-thiogalactoside induction, glutathione reductase activity was found to be twice that of non-transformed cells. After the induction with isopropyl β-thiogalactoside (commenced at *A*₆₀₀ equal to 0.5), glutathione reductase activity was found to increase over a period of 90 min, at which point maximum expression was reached. Maximum expression was 100-fold greater than in non-transformed *E. coli* JM101. Similar studies in *E. coli* strain SG5 indicated that expression was 200-fold greater than non-transformed strain JM101 and was independent of isopropyl β-thiogalactoside. The identity of the glutathione reductase band was verified by immunoblotting the gel with antibody raised against purified *E. coli* glutathione reductase.

In the DNA sequence of plasmid pKGR, there is a 700 bp stretch of DNA between the promoter and the ribosome-binding site of the *gor* gene (S. Greer, unpublished work). Moreover, within this stretch there is a poly(T) region of about 30 bases, originating from the Clarke & Carbon bank plasmid, which might reduce the efficiency of transcription of the *gor* gene by acting as a terminator structure. Various methods were used therefore in a systematic attempt to remove this intervening sequence.

These methods included (a) cloning of a *gor*-gene fragment produced by digestion with restriction endonucleases that cut closer to the ribosome-binding site, (b) *Bal*31 exonuclease digestion of an *Eco*RI-*Hind*III fragment from the M13 clone, and (c) attempts at a splint deletion (Waye *et al.*, 1983). None of these methods met with success. Many such experiments were carried out and either no recombinants were detected or unexpected constructs were formed that appeared to arise by a still unexplained process of DNA deletion. Although this evidence is indirect, a tentative conclusion must be that deletion of the intervening sequence upstream of the *gor* gene is in some way detrimental to the cell.

Purification of glutathione reductase

A purification method for *E. coli* glutathione reductase exists (Mata *et al.*, 1984), but it was considered necessary to develop an easy and rapid method for the purification of the enzyme that could be used for both large-scale and small-scale preparations. For this purpose, plasmid-transformed *E. coli* strain SG5 was used, since this is a strain in which the chromosomal *gor* gene is deleted. Moreover, expression of glutathione reductase activity was higher than in strain JM101, and it was independent of isopropyl β-thiogalactoside induction. Several procedures were tested and the one described below was found to be the most effective.

For the large-scale preparation of enzyme, a 10-litre culture of *E. coli* strain SG5 transformed with plasmid

Table 1. Purification of glutathione reductase from *E. coli* strain SG5 transformed with plasmid pKGR

For experimental details see the text.

Sample	Volume (ml)	Glutathione reductase activity		Protein		Specific activity (units/mg)	Yield (%)	Purification (fold)
		(units/ml)	(units)	(mg/ml)	(mg)			
Initial extract	114	45.8	5223	8	912	5.73	100	—
40–80%-satn.- (NH ₄) ₂ SO ₄ fraction	75	72.4	5426	7.7	578	9.39	104	1.6
Procion Red HE7B-CL- Sephacrose 4B eluate	120	42.7	5119	0.14	16.6	308	98	53.8
Superose 12 eluate	6.4	603.9	3865	1.81	11.6	334	74	58.3

Fig. 2. Purification of glutathione reductase from *E. coli* strain SG5 transformed with plasmid pKGR

Samples of protein at various stages in the purification of glutathione reductase were analysed by means of SDS/polyacrylamide-gel electrophoresis, as described in the Materials and methods section. Protein bands were stained with Coomassie Brilliant Blue. Track A, initial cell extract; track B, 40–80%-satn.-(NH₄)₂SO₄ fraction; track C, enzyme eluted from Procion Red HE-7B-CL-Sepharose 4B; track D, enzyme eluted from Superose 12.

pKGR was harvested by centrifugation (3000 *g* for 5 min), and the cells were washed in 100 ml of buffer A (20 mM-potassium phosphate buffer, pH 7.5, containing 1 mM-EDTA, 1 mM-dithiothreitol and 10 μ M-FAD) and re-collected by centrifugation. The cells were then suspended in 100 ml of buffer A and disrupted in a French press at 4 °C and cell pressure of 140 MPa (20000 lbf/in²). The extract was clarified by centrifugation at 15000 *g* for 50 min and fractionated with solid (NH₄)₂SO₄.

During additions of (NH₄)₂SO₄ the pH was maintained at 7–7.5 by adding 2.5 M-K₂HPO₄ as necessary. The 40–80%-saturation precipitate, which contained the enzyme activity, was dissolved in about 40 ml of buffer B (5 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-EDTA and 1 mM-2-mercaptoethanol) and was dialysed against three changes of buffer B (2 litres each). The dialysed enzyme was then applied to a column (2.5 cm $\times$ 6 cm) of Procion Red HE-7B linked to CL-Sepharose 4B by the method described in Lowe *et al.* (1980). The column was washed with 0.1 M-KCl in buffer B, and the enzyme was then eluted by developing the column with 0.2 M-KCl in buffer B. Fractions (4 ml) were collected, and those containing glutathione reductase activity were pooled and concentrated to 2 ml by ultrafiltration through an Amicon PM10 filter. The concentrated enzyme was then injected in four portions into a Pharmacia fast-protein-liquid-chromatography apparatus fitted with a Superose 12 column equilibrated in 50 mM-potassium phosphate buffer, pH 7.5. The active fractions were again pooled. The purified enzyme had a specific activity of 334 units/mg, which compares well with that of 361 units/mg previously reported for the *E. coli* enzyme (Mata *et al.*, 1984). A typical purification procedure is summarized in Table 1, and analysis of the enzyme by SDS/polyacrylamide-gel electrophoresis at various stages in the purification is shown in Fig. 2.

Protein and enzymological characterization

The *N*-terminal amino acid sequence of the purified *E. coli* glutathione reductase was determined in a gas-phase sequencer. The sequence of the first 27 residues was

Table 2. Amino acid composition of *E. coli* glutathione reductase

Amino acid analysis was carried out on samples of glutathione reductase hydrolysed for 24, 48 and 96 h in 6 M-HCl containing 7 mM-2-mercaptoethanol, as described by Perham (1978). The values for valine, leucine and isoleucine are those taken from the samples hydrolysed for 96 h. Abbreviation: N.D., not determined.

Amino acid	Composition (residues/polypeptide chain)	
	Found	Calc. from DNA sequence
Asx	49	47
Thr	32	32
Ser	18	16
Glx	45	39
Pro	19	20
Gly	42	44
Ala	46	44
Cys	N.D.	6
Val	41	40
Met	10	12
Ile	30	33
Leu	28	27
Tyr	17	16
Phe	15	15
His	14	14
Trp	N.D.	3
Lys	24	24
Arg	17	18

found to match exactly that predicted from the DNA sequence of the *gor* gene (Greer & Perham, 1986) except that the *N*-terminal methionine was absent. The absence of this residue would be expected from the criteria of Flinta *et al.* (1986). An amino acid analysis of the purified enzyme was also carried out, and the results are compared with those expected from the DNA sequence in Table 2. The agreement is excellent apart from the values for glutamic acid.

The native and subunit M_r values of *E. coli* glutathione reductase were determined by means of gel filtration on Superose 12 and SDS/polyacrylamide-gel electrophoresis respectively. The subunit M_r was determined as 49000, again fitting in well with the value of 48717 predicted from the DNA sequence; and the native M_r was determined as 94000, indicating that, like the glutathione reductases from human and yeast (Williams, 1976), the *E. coli* enzyme exists as a dimer of identical subunits.

In the human enzyme the two subunits are linked by a disulphide bridge between Cys-90 and Cys-90' (Thieme *et al.*, 1981). In the *E. coli* enzyme no cysteine residue exists at or near this position (Greer & Perham, 1986) and no such inter-chain disulphide bridge is possible. As expected from this, SDS/polyacrylamide-gel electrophoresis of purified *E. coli* glutathione reductase generated a protein band with an estimated M_r of 49000, whether or not treatment with 2-mercaptoethanol was included in the preparation of the sample.

Steady-state kinetic analysis of the purified enzyme revealed that the K_m values for GSSG and NADPH were 70 μ M and 25 μ M respectively. These values compared

well with those of 66 μ M and 16 μ M respectively reported for the *E. coli* enzyme by Mata *et al.* (1984).

All in all, these results show that we have constructed an expression vector for *E. coli* glutathione reductase that produces the enzyme in the form expected and having properties consistent with those previously reported. The 200-fold amplification of glutathione reductase activity in the plasmid-transformed cells has permitted a simple purification procedure for the enzyme to be devised. Together with cloning and sequence analysis of the *gor* gene (Greer & Perham, 1986), this work can now form the basis of a study of the enzyme by X-ray crystallography and protein engineering.

We thank Applied Biosystems for determining the *N*-terminal sequence of the purified glutathione reductase and Dr. C. R. Lowe for a gift of Procion Red HE-7B linked to CL-Sepharose 4B. We are grateful to the Science and Engineering Research Council for financial support (Grant GR/D 05790) and a Research Studentship (to N. S. S.) and to St. John's College, Cambridge, for the award of a Benefactors' Studentship (to N. S. S.)

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Received 28 November 1986/17 February 1987; accepted 28 April 1987