Identification of an essential cysteine residue in human glutathione synthase

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Glutathione is essential for a variety of cellular functions, and is synthesized from γ-glutamylcysteine and glycine by the action of glutathione synthase (EC 6.3.2.3). Human glutathione synthase is a dimer of two identical subunits, each composed of 474 amino acids. Little is known about the structure–function relationships of mammalian glutathione synthases and, in order to gain a greater understanding of this critical enzyme, we have probed the role of cysteine residues by chemical modification and site-directed mutagenesis. Preincubation with thiol reagents such as *p*-chloromercuribenzoate, *N*-ethylmaleimide, iodoacetate and 5,5′-dithiobis-(2-nitrobenzoate) resulted in significant inhibition of recombinant human glutathione synthase. Each subunit contains cysteine residues at positions 294, 409 and 422, and we have prepared four different mutants by replacing individual cysteine residues, or all of the cysteine residues, with alanine. The C294A and C409A cysteine mutants retained significant residual activity, indicating that these two cysteine residues are not essential for activity. In contrast, substantial decreases in enzymic activity were detected with the C422A and cysteine-free mutants. This suggests that Cys-422 may play a significant structural or functional role in human glutathione synthase.

INTRODUCTION

Glutathione plays a significant role in a variety of intracellular processes [1–3]. The final step in the synthesis of glutathione is carried out by glutathione synthase (EC 6.3.2.3), which catalyses the addition of γ-glutamylcysteine to glycine in the presence of ATP [2]. Glutathione synthase has been studied from various sources in both prokaryotes [4,5] and eukaryotes [6–11]. Although cDNA clones/genes have been identified from *Escherichia coli* [12], *Schizosaccharomyces pombe* [13], rat kidney [14], *Xenopus laevis* [15], *Arabidopsis thaliana* [16] and human [17], the only recombinant enzyme to be structurally characterized in detail has been glutathione synthase from *E. coli* [18–22]. The *E. coli* enzyme, a tetramer of 306-residue subunits, appears to be structurally quite dissimilar to the mammalian glutathione synthases, which are dimers of 474-residue subunits [14,17,23]. In addition, there is little amino acid sequence identity between the human and *E. coli* enzymes. Thus previous studies of the *E. coli* enzyme shed little light on the relatively unstudied structure–function relationships of the mammalian glutathione synthases.

Previous studies have shown that rat glutathione synthase is inhibited by *p*-chloromercuribenzoate (pCMB), which suggests that cysteine residues may play a significant role in its structure and function [23,24]. There are three cysteine residues in rat and human glutathione synthases, and one of these appears to be conserved in the *X. laevis*, *Schiz. pombe* and *A. thaliana* sequences. Although it is structurally dissimilar, there is some evidence that cysteine residues may also contribute to the function of *E. coli* glutathione synthase [25,26].

Thiol reagents have been useful in studying the involvement of cysteine residues in catalytic reactions and the maintenance of enzyme structure. In addition, site-directed mutagenesis can be used to examine the role of particular residues. We have now used thiol reagents and site-directed mutagenesis to define the contribution of cysteine residues to the structure and function of human glutathione synthase.

EXPERIMENTAL

Materials

[α-^{33}P]dATP was purchased from Amersham. Cloning and expression vectors were obtained from Boehringer-Mannheim and Qiagen. Restriction endonucleases and buffers were purchased from Pharmacia, Boehringer-Mannheim and Promega Industries. Sequencing was carried out with a sequenase version 2.0 kit supplied by USB. Site-directed mutagenesis was carried out with a DNA Sculptor kit supplied by Amersham. Nickel-nitriloacetate (Ni-NTA)–agarose was purchased from Qiagen. Oligonucleotides used for mutagenesis were synthesized by the Australian National University Biomolecular Resource Facility (Canberra, Australia). γ-Glutamylcysteine was obtained from Sigma Chemical Co. All other chemicals used were of analytical grade.

Cloning of glutathione synthase cDNA into a pQE expression vector

A *Bam*HI–*Hin*dIII fragment encoding the full-length human glutathione synthase cDNA was excised from pGSH-S [17] and cloned into *Bam*HI–*Hin*dIII-digested pQE-31 (Qiagen). In order to position the glutathione synthase coding sequence as close as possible to the six histidine residues encoded in pQE-31, and also in the correct reading frame, the resulting plasmid (pQEGS) was digested with *Bam*HI and *Spe*I, blunt-ended with the Klenow fragment of DNA polymerase and re-ligated with T4 DNA ligase. This procedure deleted the sequence between the *Bam*HI and *Spe*I sites and generated the plasmid pQEGSS. This construct produced human glutathione synthase with the following additional N-terminal residues: MRGSHHHHHHTDPSVG.

Site-directed mutagenesis and cloning of mutants into expression vectors

The *Eco*RI fragment encoding the full-length cDNA was isolated

Abbreviations used: pCMB, *p*-chloromercuribenzoate; DTNB, 5,5′-dithiobis-(2-nitrobenzoate); NEM, *N*-ethylmaleimide; Ni-NTA, nickel-nitriloacetate.

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from pGSH-S and cloned into M13mp19. The single-stranded DNA produced from this clone was used for oligonucleotide-directed mutagenesis of the three cysteine residues in the glutathione synthase sequence. The cysteine residues were converted into alanines using the following oligonucleotides: C294A, 5′-AATGTCTGGGGCCTTGGCAGC-3′; C409A, 5′-CCGTAG-CAGGGCATTCTCAAA-3′; C422A, 5′-CTCTGAAATGGC-CTGGACCAC-3′. The mutations were carried out independently to produce individual cysteine-to-alanine mutants, and the primers also were combined in a single reaction to generate a cysteine-free enzyme. The M13mp19 mutagenized clones containing the desired mutations were identified by sequencing. The fragments containing the mutations were excised on *Apa*I–*Kpn*I fragments and ligated into the *Apa*I–*Kpn*I-cut pQEGSS vector to yield pC294A, pC409A, pC422A and pCYS free. The presence of the mutations in the respective expressing clones was confirmed by recloning into M13 and sequencing.

Expression and purification of wild-type and mutant isoenzymes

All the mutant and the wild-type enzymes were overexpressed in *E. coli* under the conditions previously described by Board and Pierce [27]. *E. coli* expressing wild-type and mutant glutathione synthase isoenzymes were collected by centrifugation from 1 litre cultures and, after resuspension in 50 mM sodium phosphate buffer, pH 7.5, containing 300 mM NaCl, were lysed by passage through a Sorvall Ribi cell disrupter. The cell lysate was centrifuged for 30 min at 10000 ***g***, and all the purification procedures were carried out at 0–4 °C. The supernatant (50 ml) was mixed with 10 ml of Ni-NTA resin for about 6 h by gentle rotation. The resin was then packed into a column and washed with several column volumes of 50 mM sodium phosphate buffer, pH 7.5, containing 300 mM NaCl until the A_{280} of the eluate was below 0.01. Non-specific binding proteins were eluted with 50 mM imidazole/HCl, pH 7.5, at a flow rate of 0.5 ml/min and the eluate (2 ml/tube) was screened by SDS/12 %-PAGE by the method of Laemmli [28] using a PHAST system (Pharmacia). Once the non-specific proteins had been removed, the imidazole concentration was increased to 500 mM and glutathione synthase was eluted from the column. The eluate in the initial tubes contained a few extra proteins, but the protein in the later tubes was essentially pure.

Enzyme assays

In experiments to evaluate the effects of thiol reagents on glutathione synthase enzyme activity, the enzyme (25 μg) was preincubated with thiol reagent for 30 min at 37 °C in 100 mM Tris/HCl, pH 7.5, and 1 mM EDTA in a total volume of 40 μl. The residual activity was determined by a method modified from that of Oppenheimer et al. [23]. Enzyme samples (10 μl) were added to an assay mixture (100 μl) containing 10 mM Tris/HCl, pH 8.2, 50 mM KCl, 5 mM γ-glutamylcysteine, 10 mM ATP, 5 mM [^{14}C]glycine, 20 mM $MgCl_2$, 2 mM EDTA, 5 mM phosphocreatine and 1 unit of creatine kinase for 30 min at 37 °C. The reactions were stopped by adding 0.9 ml of 20 mM acetic acid followed by 100 μl of Dowex 1-acetate. The labelled glutathione was allowed to bind to Dowex 1-acetate for a few minutes under gentle agitation. After washing with 20 mM acetic acid, the labelled glutathione was eluted with 1.5 M ammonium acetate and the radioactivity present was determined by liquid scintillation counting. Specific activities of the purified mutant enzymes were determined spectrophotometrically by linking the reaction to the oxidation of NADH and recording the change in absorbance at 340 nm by the method of Board et al. [29]. All protein determinations were carried out by the method of Bradford [30].

RESULTS

Purification of wild-type and mutant enzymes

Although we have previously described the expression of human glutathione synthase in *E. coli*, the cloning of the glutathione synthase cDNA into a pQE vector that encodes six histidine residues at the N-terminus has enhanced the expression and simplified the purification procedure. The recombinant enzymes were purified to apparent homogeneity in a single step by Ni-NTA–agarose chromatography (Figure 1). The presence of extra amino acids at the N-terminus did not appear to affect the catalytic reaction, as the specific activity of the wild-type enzyme prepared in this manner (2.25 μmol/min per mg) is significantly higher than that obtained by us after a multi-step purification procedure (1.73 μmol/min per mg) [15].

Effects of thiol reagents on glutathione synthase activity

The thiol reagents pCMB, iodoacetate, *N*-ethylmaleimide (NEM) and 5,5′-dithiobis-(2-nitrobenzoate) (DTNB) all inactivated glutathione synthase in a non-linear dose-dependent manner (Figure 2). Because of its low solubility, DTNB was only evaluated at concentrations up to 5 mM. It is evident that approx. 50 % of activity was inhibited by low concentrations of reagents; however, 100 % inhibition was never achieved, even at high thiol reagent concentrations. This suggests that the initial reaction of a thiol reagent with an essential cysteine residue may decrease the possibility of a further reaction with either another essential cysteine residue or the equivalent cysteine residue in the other subunit of the dimeric enzyme.

Site-directed mutagenesis of cysteine residues

Since the action of different thiol reagents clearly indicated a significant role for cysteine residues in glutathione synthase, each cysteine residue was mutated to alanine in order to identify the particular cysteine residue(s) responsible for the inhibition. The

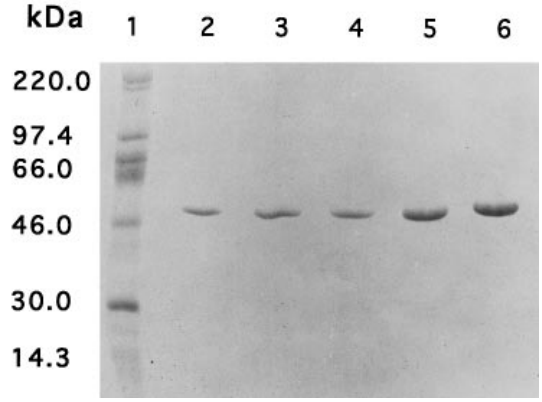

Figure 1 SDS/PAGE of purified recombinant glutathione synthase and mutant isoenzymes

Wild-type and mutant recombinant glutathione synthase proteins were analysed in an SDS/12.5%-PAGE gel on a Pharmacia Phast Gel System. Lane 1, molecular mass markers; lanes 2–5, purified C294A, C409A, C422A and cysteine-free mutants respectively; lane 6, purified wild-type human glutathione synthase.

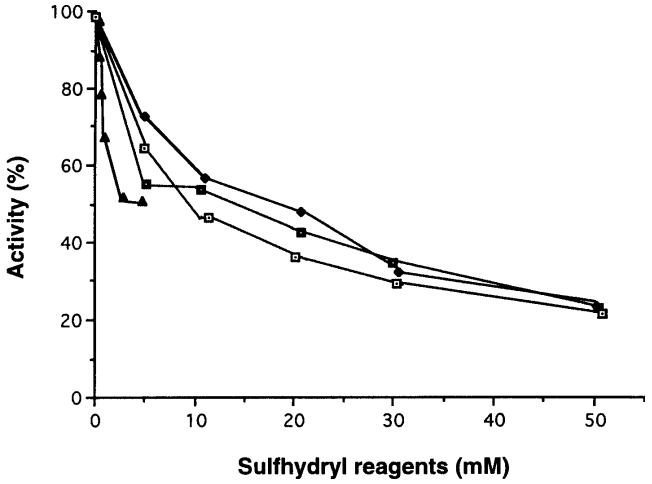

Figure 2 Inactivation of wild-type recombinant glutathione synthase by thiol reagents

Purified wild-type glutathione synthase was incubated with the thiol ('sulfhydryl') reagents DTNB (▲), pCMB (◆), NEM (□) and iodoacetate (■) in 100 mM Tris/HCl, pH 7.5, and 1 mM EDTA. Glutathione synthase activity was determined as described in the Experimental section, and is expressed as a percentage of the activity in the absence of thiol reagents. Results shown are means of four experiments.

Table 1 Specific activities of wild-type and mutant glutathione synthase isoenzymes

Results are means ± S.D. of four experiments.

Enzyme	Specific activity (μmol/min per mg)
Wild-type	2.25 ± 0.04
C294A	2.43 ± 0.16
C409A	1.61 ± 0.08
C422A	0.015 ± 0.003
Cysteine-free	0.006 ± 0.001

specific activities of the wild-type and mutant enzymes are shown in Table 1. The C294A mutant retained catalytic activity equivalent to that of the wild-type enzyme, while the C409A mutation resulted in a decrease in specific activity of 28 %. In contrast, the C422A mutant and the mutant enzyme that was devoid of cysteine residues lost 95–98 % of the wild-type activity.

Table 2 Effects of thiol reagents on wild-type and mutant glutathione synthase isoenzymes

Purified wild-type and mutant glutathione synthase enzymes were preincubated with various thiol reagents for 30 min at 37 °C. Glutathione synthase activity was determined as described in the Experimental section, and is expressed as a percentage of the activity obtained in the absence of thiol reagent. Activities are the means of four experiments. ND, not detectable.

	Activity (% of control)			
Enzyme	NEM (20 mM)	pCMB (20 mM)	Iodoacetate (20 mM)	DTNB (3 mM)
Wild-type	43.0	48.0	33.0	46.8
C294A	46.3	59.5	27	47.3
C409A	76.0	45.8	16.3	46.5
C422A	ND	ND	ND	ND
Cysteine-free	ND	ND	ND	ND

These data indicated that Cys-422 is the most important of the three cysteine residues in human glutathione synthase, and suggested that the inhibition by thiol reagents was probably due to reaction with Cys-422. Treatment of the mutant enzymes with thiol reagents confirmed this suggestion. As shown in Table 2, mutants C294A and C409A were inhibited by thiol reagents to about the same degree as the wild-type enzyme. Both of these mutants have a normal cysteine residue at position 422, and this residue clearly remains susceptible to modification by thiol reagents.

DISCUSSION

The use of the pQE expression vector described in this study has tremendously shortened the time required for purification of glutathione synthase. Despite the addition of extra N-terminal residues, the specific activity of the recombinant wild-type enzyme purified by nickel chelate chromatography is substantially higher than that of the enzyme purified by ion-exchange chromatography and gel filtration [17]. This elevation in specific activity presumably results from the speed of purification and diminished degradation of the enzyme, although a positive effect of the additional N-terminal residues cannot be excluded. This method of expression and purification also allows the preparation of mutant enzymes that have little detectable enzymic activity. It would be extremely difficult to purify inactive mutants by the previously described series of ion-exchange and gel-filtration steps [17].

Although previous studies of rat glutathione synthase demonstrated inhibition by pCMB, other thiol reagents such as NEM, iodoacetate and DTNB were apparently without significant effect. Our present results with recombinant human glutathione synthase show significant inhibition not only with pCMB but also with NEM, iodoacetate and DTNB. This comprehensive inhibition with a range of thiol reagents of different size and complexity argues strongly in favour of a significant structural or functional role for cysteine residues in human glutathione synthase.

To focus on the role of individual cysteine residues we used site-directed mutagenesis to convert each cysteine residue to alanine. Mutation to alanine was chosen as this is the most conservative change in terms of size and charge, and any resulting functional change can be largely attributed to the loss of the thiol group. Although the C294A and C409A mutants were unaffected or retained substantial activity, the C422A mutation resulted in an almost complete inactivation of the enzyme. As expected, a similar result was obtained with the cysteine-free enzyme. This result strongly suggests that Cys-422 plays a significant role in enzyme function, although it does not give any indication as to whether this effect is mediated through structural changes or via a specific role in catalysis.

An alignment of the amino acid sequences of glutathione synthases from human, rat, *Schizosaccharomyces pombe*, *Xenopus laevis* and *Arabidopsis thaliana* (not shown) reveals that three cysteines found in the human sequence are conserved in the rat and *Xenopus laevis* sequences. However, Cys-294 appears to be also conserved in *Schizosaccharomyces pombe* and *Arabidopsis thaliana*. The conservation of Cys-294 initially suggested that this residue may be the most significant; however, this conclusion is clearly not supported by our experimental results. As the three-dimensional structure of the eukaryotic enzyme is not known, our sequence alignment was not based on any structural in-

formation, and it is probable that we do not yet have sufficient information to reliably align the sequences from such evolutionarily divergent species.

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