Sequencing and expression of a cDNA for human glutathione synthetase

Rayappa R. GALI and Philip G. BOARD*

Molecular Genetics Group, The John Curtin School of Medical Research, The Australian National University, P.O. Box 334, Canberra ACT 2601, Australia

A human brain cDNA clone encoding glutathione synthetase (EC 6.3.2.3) has been sequenced and expressed in *Escherichia coli*. The protein is 474 amino acids in length with a subunit molecular mass of 52 352 Da. The recombinant protein exhibits glutathione synthetase activity and occurs as a homodimer. The recombinant glutathione synthetase was purified to homogeneity and had a specific activity of 1.73 $\mu\text{mol}/\text{min}$ per mg of protein, an

isoelectric point of 5.35 and a pH optimum between 7.0 and 7.5. Southern blots of human genomic DNA hybridized with the glutathione synthetase cDNA revealed a relatively simple pattern of strongly hybridizing fragments, indicating the absence of a large gene family and suggesting that there may be only one glutathione synthetase gene in the human genome.

INTRODUCTION

Glutathione, a ubiquitous low-molecular-mass thiol, is important for a variety of biological functions. Considerable interest has been focused on its proposed roles in redox reactions, the protection of cells against reactive electrophiles and membrane transport [1]. It is synthesized by the consecutive actions of γ -glutamylcysteine synthetase and glutathione synthetase. In the second step glutathione synthetase (EC 6.3.2.3) catalyses the

synthesis of glutathione from γ -glutamylcysteine and glycine in the presence of ATP [2].

The intracellular concentration of glutathione ranges from 2 to 10 mmol/l and this high concentration suggests that severe

Figure 1 Structure and organization of plasmids pRB269 and pRG1

(a) Plasmid pRB269. The ubiquitin coding region is shown as an open box pointing in the direction of transcription/translation. (b) The *tac* promoter/ubiquitin coding region from (a) is expanded to show the DNA sequence at each end of the ubiquitin coding unit. Restriction enzymes are indicated above their recognition sequence. Ubiquitin amino acids are numbered and non-ubiquitin amino acids are in lowercase letters. Dots indicate a gap in the sequence. (c) Ubiquitin–GSH-S fusion insert in pRG1. Ubiquitin and glutathione synthetase coding regions are shown as open boxes; other regions of the plasmid and designations are as in (a) and (b). The arrows indicate the position of primers used to add bases encoding the *Sac*I site and the C-terminal residues of ubiquitin to the cDNA encoding the N-terminal sequence of glutathione synthetase.

Abbreviation used: GSH, reduced glutathione.

* To whom correspondence should be addressed.

The nucleotide sequence shown in Figure 2 will appear in the EMBL, DDBJ and GenBank Nucleotide Sequence Databases under the accession number L42531.

1	GGAGAACCCT	TCGCGGAGGA	AAGCGAACT	AGTGTGGGA	TGGCCACCA	50
1					M	4
51	CTGGGGGAGC	CTCTTCGAGG	ATAAACGACA	GCTAGAGGAG	CTGGCCAGGC	100
5	W--G--S--	L--L--Q--D	--K--Q--Q--	--L--E--E--	L--A--R--Q	21
101	AGGCGGTGGA	CCGGGCGCTG	GCTGAGGGAG	TATTGCTGAG	GACCTCACAG	150
22	--A--V--D--	R--A--L--L	A--E--G--V	--L--L--R--	T--S--Q--	37
151	GAGCCCACTT	CCTCGGAGGT	GGTAGGCTAT	GCCCCATTCA	CGCTCTTCCC	200
38	E--P--T--S	--S--E--V--	--V--S--Y--	A--P--F--T	--L--F--P--	54
201	CTCACTGGTC	CCCAGTGCCG	TGCTGGAGCA	AGCCTATGCT	GTGCAGATGG	250
55	S--L--V--	P--S--A--L	--L--E--Q--	A--Y--A--	V--Q--M--D	71
251	ACTTCAACCT	GCTAGTGGAT	GCTGTGAGCC	AGAAGCTGTC	CTTCTGGGAG	300
72	--F--N--L--	L--V--D--	A--V--S--Q	--N--A--A--	F--L--E--	87
301	CAAACTCTTT	CCAGCACCAT	CAACAGGAT	GACTTTACCG	CTCTCTCTTT	350
88	Q--T--L--S	--S--T--I--	--K--Q--D--	D--F--T--A	--R--L--F--	104
351	TGACATCCAC	AAGCAAGTCC	TAAAGAGGG	CATTGCCAG	ACTGTGTTC	400
105	D--I--H--	K--Q--V--L	--K--E--G--	I--A--Q--	T--V--F--L	121
401	TGGGCTGAA	TCGCTCAGAC	TACATGTTC	AGCGCAGCGC	AGATGGCTCC	450
122	--G--L--N--	R--S--D--	--R--S--F--Q	--R--S--A--	--D--G--S--	137
451	CCAGCCCTGA	AACAGATCGA	AATCAACAC	ATCTCTGCCA	GCTTTGGGGG	500
138	P--A--L--K	--Q--I--E--	I--N--T--	I--S--A--S	--F--G--G--	154
501	CCTGGCCTCC	CGAGCCCCAG	CTGTGCACCG	ACATGTTCTC	AGTGTCTCTGA	550
155	L--A--S--	R--T--P--A	--V--H--R--	H--V--L--	S--V--L--S	171
551	GTAAGACCAA	AGAAGCTGGC	AAGATCCTCT	CTAATAATCC	CAGCAAGGGA	600
172	--K--T--K--	E--A--G--	K--I--L--S	--N--N--P--	S--K--G--	187
601	CTGGCCCTCG	GAATTGCGAA	AGCCTGGGAG	CTCTACGGCT	CACCCAATGC	650
188	L--A--L--G	--I--A--K--	A--W--E--	L--Y--G--S	--P--N--A--	204
651	TCTGGTGCTA	CTGATTGCTC	AAGAGAAGGA	AAGAAACATA	TTTGACCAAG	700
205	L--V--L--	L--I--A--Q	--E--K--E--	R--N--I--	F--D--Q--R	221
701	GTGCCATAGA	GAATGAGCTA	CTGGCCAGGA	ACATCCATGT	GATCCGACGA	750
222	--A--I--E--	N--E--L--	L--A--R--N	--I--H--V--	I--R--R--	237
751	ACATTTGAAG	ATATCTCTGA	AAAGGGGTCT	CTGGACCAAG	ACCGAAGGCT	800
238	T--F--E--D	--I--S--E--	K--G--S--	L--D--Q--D	--R--R--L--	254
801	GTTTGTGGAT	GGCCAGGAAA	TTGCTTGGT	TTACTTCCGG	GATGCTACA	850
255	F--V--D--	G--Q--E--I	--V--V--V--	Y--F--R--	D--G--Y--M	271
851	TGCCTCGTGA	GTACAGCTCTA	CAGAATTGGG	AAGCAGTCT	ACTGCTGGAG	900
272	--P--R--Q--	Y--S--L--	Q--N--W--E	--A--R--L--	L--L--E--	287
901	AGGTCAATG	CTGCCAAGT	CCAGACATTT	GCCACCCAGC	TGGCTGGGAG	950
288	R--S--H--A	--A--K--C--	P--D--I--	A--T--Q--L	--A--G--T--	1004
951	TAAGAAGGTT	CAGCAGGAGC	TAAGCAGGCC	GGGCATGCTG	GAGATGTTGC	300
305	--K--K--V--	Q--Q--E--L	--S--R--P--	G--M--L--	E--M--L--L	321
1001	TCCCTGGCCA	GCCTGAGGCT	GTGGCCCGCC	TCCGCGCCAC	CTTTGCTGGC	1050
322	--P--G--Q--	P--E--A--	V--A--R--L	--R--A--T--	F--A--G--	337
1051	CTCTACTCAC	TGGATGTGGG	TGAAGAAGGG	GACCAAGGCCA	TCGCGAGGCC	1100
338	L--Y--S--L	--D--V--G--	--E--E--G--	D--Q--A--I	--A--E--A--	354
1101	CCTTGCTGCC	CCTAGCCGGT	TTGTGCTAAA	GCCCCAGAGA	GAGGGTGGAG	1150
355	--L--A--A--	P--S--R--F	--V--L--K--	P--Q--R--	E--G--G--G	371
1151	GTAACAACCT	ATATGGGGAG	GAAATGGTAC	AGGCCCTGAA	ACAGCTGAAG	1200
372	--N--N--L--	Y--G--E--	E--M--V--Q	--A--L--K--	Q--L--K--	387
1201	GACAGTGAGG	AGAGGGCCTC	CTACATCCTC	ATGGAGAAGA	TCGAACCTGA	1250
388	D--S--E--E	--R--A--S--	Y--I--L--	M--E--K--I	--E--P--E--	404
1251	GCCTTTTGG	AATTGCTCTG	TACGGCCTGG	CAGCCCTGCC	CGAGTGGTCC	1300
405	--P--F--E--	N--C--L--L	--R--P--G--	S--P--A--	R--V--V--Q	421
1301	ATGCACTTTC	AGAGCTGGGC	ATCTTTGGGG	TCTATGTACG	CGAGGAAAAG	1350
422	--C--I--S--	E--L--G--	I--F--G--V	--Y--V--R--	Q--E--K--	437
1351	ACACTCGTGA	TGAACAAGCA	CGTGGGGCAT	CTACTTCGAA	CCAAAGCCAT	1400
438	T--L--V--M	--L--K--H--	--V--G--H--	L--L--R--T	--K--A--I--	454
1401	CGAGCATGCA	GATGGTGGTG	TGGCAGCGGG	AGTGGCAGTC	CTGGACAACG	1450
455	E--H--A--	D--G--G--V	--A--A--G--	V--A--V--	L--D--N--P	471
1451	CATACCCTGT	GTGAGGGCAC	AACCAGGCCA	CGGGACCTTC	TATCTCTGCT	1500
472	--Y--P--V					474
1501	ATTTGTGCTT	CCTCTCCTAG	CCCTCTGAG	GGGTATCCTC	CTAAGACCT	1550
1551	CCAAAGTTT	TATGGAAGGG	TAAATATCTG	TACCTTCCCC	CAGCTTTCCA	1600
1601	TCTGAGGACC	AGAAAAGTGT	TGCTCTCCTT	AGATGAGATC	TAGAGCCCC	1650
1651	CAAAATCCTG	AGATGTGGGT	ATAGCTCAGG	GTAAGCTGCT	AAACCATTTA	1700
1701	CCAAAATAAA	GTATAGGCCA	TAGAATTTGA	AACCTGGCGC	AATAGATATA	1750
1751	GTACCGCAAG	GGAAAAGATGA	AAAATTATAA	CCAAGCATAA	TATAGCAAGG	1800
1801	ACTAACCCCT	G				1811

Figure 2 The nucleotide sequence of a glutathione synthetase cDNA and the deduced amino acid sequence

depletion could have major consequences. Patients with glutathione synthetase deficiency have lowered GSH levels and this can lead to a variety of disorders including metabolic acidosis, 5-oxoprolinuria, mental retardation and haemolytic anaemia [3–5].

Although glutathione synthetase has been purified from yeast [6], *E. coli* [7], rat kidney [8], human erythrocytes [9], bovine erythrocytes [10] and bovine lens [11], the nucleotide sequence is available only from *E. coli* [12], *Schizosaccharomyces pombe* [13], anaplasma centrale [14] and *Xenopus laevis* [15]. Until now *E. coli* and *Schizosaccharomyces pombe* are the only species from which cloned glutathione synthetase has been successfully expressed and well characterized [12,16–19]. Studies of glutathione synthetase from mammalian sources have suggested that the enzyme is a dimer ranging in size from 118 to 150 kDa. However,

it is not clear whether variation in the molecular mass is due to large species differences or the experimental conditions.

Since altered levels of human glutathione synthetase have implications for many diseases it was of interest to study glutathione synthetase in detail. In this study we have sequenced a cDNA encoding human glutathione synthetase and expressed the recombinant enzyme in *E. coli*. The human enzyme shows substantial similarity with the sequence reported for the large subunit of *X. laevis* glutathione synthetase [15]. The identification and expression of this clone clarifies the subunit structure of mammalian glutathione synthetases and will help define the role of glutathione synthetase in the regulation of intracellular glutathione concentrations. In addition, it will now be possible to study the mutations causing glutathione synthetase deficiency and 5-oxoprolinuria.

EXPERIMENTAL

Materials

The [γ - 32 P]dATP and [14 C]glycine were purchased from Brestec (Adelaide, Australia) and Amersham (U.K.) respectively. Cloning vectors were obtained from Boehringer-Mannheim and Promega (Australia). Restriction endonucleases and buffers were purchased from Pharmacia (Australia); Boehringer-Mannheim (Australia); and Promega Industries (Australia). The expression plasmid pRB269 was kindly provided by Dr. Rohan Baker of JCSMR, Australian National University. Sequencing was carried out with a Sequenase version 2.0 kit supplied by USB (U.S.A.). DE-52 cellulose was purchased from Whatman (U.K.). AffiGel Blue was obtained from Bio-Rad. Oligonucleotides were synthesized by the Australian National University Biomolecular Resource Facility (Canberra, Australia). All other chemicals used were of analytical grade.

Sequencing and expression

A cDNA clone with sequence similarity to the first 300 bp of a putative glutathione synthetase cDNA from *X. laevis* was identified during random sequencing of cDNA clones from a human brain cDNA library [20]. This clone, which we have termed pGSH-S, was digested with *Eco*R1 and the resulting 1.8 kb cDNA fragment was inserted into the cloning site of M13 phage mp19 to generate single-stranded DNA templates. The resulting recombinant phage DNA was sequentially deleted from one end of the insert by the 3' to 5' exonuclease activity of T4 DNA polymerase [21]. Deletions of various sizes were generated and the size of each deletion was estimated by agarose gel electrophoresis of the subcloned phage DNA. Deleted clones were sequenced by the dideoxy termination method [22].

The expression plasmid pRB269 [23] in which a yeast ubiquitin gene is inserted in front of a *Tac* promoter was used to co-express human glutathione synthetase as a ubiquitin fusion. To position the glutathione synthetase cDNA downstream of the ubiquitin gene and in the same reading frame, primers were designed for PCR amplification of a 150 bp fragment from the N-terminal sequence of the glutathione synthetase cDNA. The forward primer (5'-GTGCCGCGGTGGTATGGCCACCAACTGG) includes a *Sac*II site from the C-terminus of the ubiquitin gene and the reverse primer (5'-GGCTCCTGTGAGGTCTCTC) was positioned 3' of an *Ap*I site in the glutathione synthetase cDNA. The amplification was carried out in a 100 μ l reaction containing 200 μ M dNTPs, 10 pmol of each primer, 1.5 units of *Taq* polymerase (Promega) in 1 $\times$ *Taq* polymerase buffer (Promega) and 10 ng of pGSH-S. The amplification was carried out for 35 cycles (96 $^{\circ}$ C for 1 min, 52 $^{\circ}$ C for 1 min, 72 $^{\circ}$ C for 1 min). The

Human:	1	MATNWGSLLDKQKQLEELARQAVDRALAEGLVLLRTSQEPTSSEVVSYPFTLFPSPVPSA	60
Xenopus	1	MADLWDDIYNDTKLLEELAPIAIDAALLQGVLMTKESPNSSDVVSFAPFALLPSPVPSA	60
Human:	61	LLEQAYAVQMDFNLLVDAVSQNAAFLEQTLSSSTIKQDDFTARLFDIHKQVLKEGIAQTTF	120
Xenopus:	61	LFEQAKCVQEDFNTLVDRISQDTSFLEQVLSSTIKVDDFIRRLFAIHKQVQVEDCTQEVF	120
Human:	121	LGLNRSDYMFQRSADGSPALKQIEINTISASFGGLASRTPAVHRHVSLSKTEAGKIL	180
Xenopus:	121	LGINRSDYMFDCRDDGTPALKQIEINTIAASFGGLASRTPAVHQHVLKFLRKSEBSSSIL	180
Human:	181	SNNPSKGLALGIAKAWELYGSPNALVLLIAQEKERNIFDQRAIENELLARNIHVIRRTFE	240
Xenopus:	181	TNDAVEGIGWGIAHAWALYGSVDATVMFLVENEQRNILDQRFIEAELCKRNVRVIRRRLA	240
Human:	241	DISEKGSLLDQDRRLFVDGQEIADVYFRDGYMPRQYSLQNWEARLLERSHAAKCPDIATQ	300
Xenopus:	241	DVFERGTLDEERHLFIDGYEVAVAYFRTGYVPQDYTEQDWEARLMLERSRAVKCPDVPTQ	300
Human:	301	LAGTKKVQQLSRPGMLEMLLPQPEAVARLRATFAGLYSLDVGEEGDQAI AEALAAPSR	360
Xenopus:	301	LVGTKKVQQLSRPQILEKFLDPKPEAVARIRETFTGLYSLDIGEEGDEAVRVALANPDQ	360
Human:	361	FVLKPQREGGNNLYGEEMVQALKQLKDSEERASYILMEKIEPEPFENCLLRPGSPARVV	420
Xenopus:	361	FVLKPQREGGNNLYGEELKEKLQECKDSEERTSYILMDKINPKPLKNCLLRAGGRVQIS	420
Human:	421	QCISELGIFGVYVRQEKTLVMNKHVGHLLRTKAIEHADGGVAAGVAVLDNPYPV	474
Xenopus:	421	ECISELGMFGVYVRHRDQMIYYDQVGHLLRTKAIEHSDGGVAAGVAVLDNPYLV	474

Figure 3 The deduced amino acid sequences of human and *X. laevis* glutathione synthetases

Vertical lines show the positions of identical residues.

amplified fragment was digested with *Sac*II and *Apa*I and, after isolation by agarose gel electrophoresis, the 111 bp fragment was ligated with *Sac*II- and *Hind*III-digested pRB269 and a 1689 bp *Apa*I–*Hind*III-cleaved fragment containing the remainder of the glutathione synthetase coding region. This three-way ligation generated the expression plasmid pRG1 (Figure 1). The cDNA that was amplified by the PCR reaction was subsequently resequenced to confirm that no errors had been introduced.

The plasmid pRG1 was transfected into TG1 cells containing the plasmid pRB173 that expresses the ubiquitin-specific protease Ubp2. This protease efficiently cleaves the ubiquitin fusion proteins expressed by pRB269 to yield the recombinant protein free of ubiquitin at the N-terminus [23]. Cultures of *E. coli* containing pRG1 and pRB173 were grown under the conditions previously described [24] except for the addition of glucose (6.3 g/l) to the medium. The addition of glucose was made to suppress the expression of *E. coli* tryptophanase which has a similar isoelectric point and subunit molecular mass to that of human glutathione synthetase and interfered with the purification.

Enzyme purification

E. coli expressing recombinant glutathione synthetase from pRG1 were collected by centrifugation from a 5 l culture and after resuspension in 50 mM imidazole/HCl, 10 mM MgCl₂, pH 7.4 (buffer A), were lysed by passage through a Sorvall Ribi cell disrupter. The cell lysate was centrifuged for 60 min at

20000 *g* and all purification procedures were carried out at 0–4 °C.

The supernatant was applied to a 2.6 cm × 27 cm DEAE cellulose column which was previously equilibrated with buffer A. After washing with several column volumes of the same buffer, the column was eluted with a linear gradient of buffer A and buffer A containing 0.5 M NaCl. The active fractions were concentrated on an Amicon concentrator using PM10 DIAFLO ultrafiltration membranes and applied to a 2.6 cm × 60 cm Sephacryl 200 (Pharmacia) column equilibrated with buffer A. The enzyme was eluted with a buffer flow rate of 0.5 ml/min. The active fractions were pooled and subjected to chromatography on a 2 cm × 6 cm column of AffiGel Blue (Bio-Rad). The column was washed with buffer A and the protein was eluted with a gradient from 0 to 0.5 M NaCl in buffer A. The active fractions were again pooled and after dilution in 1.5 vol. of buffer A were subjected to chromatofocusing on a Mono P column (Pharmacia) over the pH range 6.7 to 5. The peak of activity emerged at pH 5.35 and was essentially pure. Some fractions on the shoulders of the peak contained trace contamination that could be detected on Coomassie Blue-stained SDS/PAGE gels.

Molecular mass determination

Molecular mass determination was carried out by gel filtration and SDS/PAGE. Purified glutathione synthetase was passed through an FPLC Separose-12 column (Pharmacia) and elution

was carried out in a buffer consisting of 50 Tris/HCl, pH 7.4, in comparison with protein standards of known size (β -amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; BSA, 66 kDa; carbonic anhydrase, 29 kDa). SDS/PAGE was carried out on 12 % vertical gels in a Mini-Protein II cell (Bio-Rad) [25].

Enzyme assays

Glutathione synthetase activity was measured at 37 °C by a previously described method [26] except that γ -glutamylcysteine was used as a substrate. Di- γ -glutamylcysteine was prepared from oxidized glutathione by the action of carboxypeptidase B [27] and was reduced with an excess of 2-mercaptoethanol. Enzyme activity is expressed as μ mol of [14 C]glycine incorporated/min per mg of protein. All protein determinations were carried out by the method of Peterson [28].

Southern blot analysis

Human genomic DNA samples prepared from peripheral blood leucocytes were digested with *Pst*I, *Hind*III, *Bam*H1 or *Eco*R1. Digested DNAs were separated by agarose gel electrophoresis and blotted to Hybond N⁺ nylon membrane [29]. DNA was fixed on the membrane under long-wave UV light (365 nm) for 5 min and dried at 55 °C. The 1.8 kb cDNA fragment which contains the entire glutathione synthetase open reading frame was labelled with [α - 32 P]dATP by random priming and used as a hybridization probe. Hybridization was carried out in Nasmyth's solution [30] at 65 °C for 18 h. Filters were washed in 2 $\times$ SSC/0.1 % SDS (SSC: 0.15 M NaCl/0.015 M sodium citrate) at 65 °C and exposed to Fuji film at -70 °C.

RESULTS

The glutathione synthetase cDNA clone

The cDNA cloned in pGSH-S was completely sequenced on both strands and is 1811 bp in length. The nucleotide sequence and deduced amino acid sequence are shown in Figure 2. The 5' non-coding region is 39 bp; the open reading frame covers 1422 bp and the 3' non-coding region extends for 350 bp. There was no obvious poly(A) addition signal or poly(A) tract at the 3' end of this clone. The open reading frame encodes a peptide of 474 residues with a deduced molecular mass of 52352 Da. The deduced amino acid sequence reported for the *X. laevis* glutathione synthetase is compared with the deduced human sequence in Figure 3 and reveals 64 % identity. There is approximately 40 % identity between the human glutathione synthetase amino acid sequence and the yeast sequence (results not shown).

Southern blot analysis

To gain an estimate of the number of glutathione synthetase sequences in the human genome, Southern blots of human leucocyte DNA digested with *Pst*I, *Hind*III, *Bam*H1 or *Eco*R1 were probed with 32 P-labelled 1.8 kb cDNA insert from pGSH-S. As shown in Figure 4, the cDNA hybridized very strongly to a single fragment in a *Pst*I digest and only weakly to two smaller fragments. The probe hybridized strongly to a limited number of fragments in each of the other restriction enzyme digests. This result suggests that there is not a large family of glutathione synthetase genes in the human genome and there may only be one active gene.

Expression and purification of recombinant glutathione synthetase

Attempts to express recombinant glutathione synthetase under the direct control of a *tac* promoter were not successful. The use

Figure 4 Southern blot of human DNA hybridized with a glutathione synthetase cDNA

Lanes 1 to 4 contained DNA digested with *Pst*I, *Hind*III, *Bam*H1 or *Eco*R1 respectively. The position of size standards from *Hind*III-digested phage DNA are indicated.

Table 1 Purification of recombinant glutathione synthetase

Step	Volume (ml)	Total protein (mg)	Activity		
			Total (units)	Specific (units/mg)	Yield (%)
<i>E. coli</i> extract	45	83	578	0.11	100
DEAE cellulose	110	141	979	0.12	38
Sephacryl 200	41	47	740	0.26	29
AffiGel Blue	42	4.6	434	1.57	17
Mono P	17	1.2	124	1.73	5

of a ubiquitin fusion expression system [23] with the co-translational removal of the N-terminal ubiquitin moiety generated active enzyme in *E. coli* lysates.

The recombinant enzyme was purified to apparent homogeneity by ion-exchange chromatography, gel filtration, dye ligand affinity chromatography and chromatofocusing (Table 1). In these experiments, the AffiGel Blue dye ligand chromatography step gave a substantial increase in purity during the purification process. SDS/PAGE of the enzyme from the Mono P chromatofocusing step showed the enzyme was essentially purified to homogeneity (Figure 5). The size of the recombinant peptide evident from SDS/PAGE compares well with the 52352 Da deduced from the cDNA sequence. The specific activity of the purified recombinant human enzyme was 1.73 μ mol/min per mg which is over twice the value reported for the human enzyme purified from erythrocytes [9]. In contrast a considerably

Figure 5 SDS/PAGE of purified recombinant human glutathione synthetase

Lane 1, molecular mass markers; lane 2, purified recombinant human glutathione synthetase expressed in *E. coli*; lane 3, total soluble protein extract of TG1 cells containing pRG1 and pRB173 and expressing human glutathione synthetase.

Figure 6 Effect of pH on activity of recombinant glutathione synthetase

All assays were carried out in duplicate at 37 °C using the buffer system of Beutler et al. [31].

higher specific activity of 13.8 $\mu\text{mol}/\text{min}$ per mg has been reported for rat kidney glutathione synthetase [26].

The N-terminal amino acid sequence of the purified recombinant enzyme started with alanine and was found to be identical with the sequence deduced from the cDNA over 15 residues. Although the initiating methionine residue was encoded in the construct, it appears to have been removed after cleavage of the ubiquitin fusion protein. Gel filtration experiments on an FPLC Superose 12 column gave an estimated molecular mass of 100 kDa (results not shown) which, when compared with the size of the encoded peptide, suggests that human glutathione synthetase is composed of two identical subunits. The pH optimum of glutathione synthetase was determined using the buffer system of Beutler et al. [31]. Maximum activity was obtained in the range pH 7 to pH 7.5 (Figure 6). The isoelectric point of human glutathione synthetase was found to be 5.35 by chromatofocusing (results not shown) and by gel isoelectric focusing on a PHAST apparatus (Pharmacia) (Figure 7).

Figure 7 Isoelectric point determination of human glutathione synthetase

Proteins were subjected to isoelectric focusing over the pH range 4 to 6.5 on a PHAST gel. Lane 1, isoelectric point standards; lane 2, purified recombinant human glutathione synthetase.

DISCUSSION

The human glutathione synthetase cDNA clone sequenced here was identified by its homology to a putative *X. laevis* glutathione synthetase cDNA clone that had originally been identified as a glutathione synthetase cDNA by cross-hybridization with an oligonucleotide derived from the gene encoding the large subunit of glutathione synthetase from *Schizosaccharomyces pombe*. The *X. laevis* amino acid sequence has only 39% identity with the yeast sequence [15]. Thus it was not absolutely certain that the *X. laevis* cDNA and pGSH-S encoded authentic glutathione synthetase enzymes. The expression of recombinant protein with glutathione synthetase activity has now confirmed the identity of pGSH-S and the *X. laevis* cDNA as glutathione synthetase clones.

Although *E. coli* glutathione synthetase is thought to be a tetramer and *Schizosaccharomyces pombe* glutathione synthetase is reported to be a heterodimer [13], the available evidence suggests that the equivalent mammalian enzymes are homodimers with subunits ranging in size from 50 to 65 kDa [10,11,26]. Although glutathione synthetase was previously purified from human erythrocytes [9] the subunit composition was not studied. The subunit size of human glutathione synthetase deduced from the nucleotide sequence in the present study falls clearly within the lower end of the range reported for other mammalian glutathione synthetases. The data obtained for the recombinant enzyme confirm the suggested homodimeric structure of the mammalian glutathione synthetases and clearly demonstrate that only one subunit type is essential for activity. Since the human and frog enzymes appear to be of identical length, it is highly likely that other mammalian glutathione synthetases will also be of the same size. Thus the range in molecular size reported for the rat, bovine and human enzymes may represent experimental variation.

While the specific activity of the purified recombinant human glutathione synthetase was slightly higher than the specific activity of the enzyme purified from human erythrocytes [9], it was markedly less than the level reported for the rat kidney enzyme [26]. It is not clear at this stage if this discrepancy represents a large degree of inter-species variation, or if the recombinant enzyme was partially degraded during purification.

Although the structure of the *E. coli* glutathione synthetase has been determined, the differences in sequence and subunit composition suggest that it is unlikely that the active site and residues required for substrate binding will be well conserved

between *E. coli* and humans. However, close consideration of highly conserved regions found in the frog, human and yeast enzymes may provide valuable areas in which to search for residues involved in function or the maintenance of essential structural features. One 16-residue segment (361–376) is almost perfectly conserved (15/16 residues) between human, frog and yeast; however, it is not clear what role this region plays. Since *p*-chloromercuribenzoate is reported to inhibit rat glutathione synthetase [26] it is possible that a cysteine residue may play a significant functional role. There are three cysteine residues in the human sequence and they are all conserved in the frog sequence. Only Cys-294 is conserved in the human, frog and yeast sequences, suggesting that this may be the most functionally significant cysteine. Many ATP- and GTP-binding proteins contain a phosphate-binding loop structure that is characterized by a glycine-rich sequence followed by a conserved lysine, and a serine or threonine residue (GXXXGKS/T) [32]. This motif does not occur in the human glutathione synthetase sequence, suggesting that the ATP-binding function of glutathione synthetase has evolved along a separate lineage.

The positive identification of a human glutathione synthetase cDNA has now opened the way for studies of the role of glutathione synthetase in regulating intracellular GSH concentrations. In addition a knowledge of the cDNA sequence will allow the identification of mutations causing glutathione synthetase deficiency and 5-oxoprolinuria. Such knowledge may be of use in prenatal diagnosis and genetic counselling.

Following the submission of this manuscript, the sequence of rat kidney glutathione synthetase was published [33]. The deduced amino acid sequence of the rat enzyme is identical in length and has 88% identity with the human sequence.

We thank Drs. Taka Suzuki and Rohan Baker for useful suggestions and Marjorie Coggan for excellent technical assistance. We are grateful to Dr. Dennis Shaw for carrying out the N-terminal sequence analysis.

REFERENCES

- 1 Meister, A. and Anderson, M. A. (1983) *Annu. Rev. Biochem.* **52**, 711–760
- 2 Meister, A. (1974) in *The Enzymes* (Boyer, P. D., ed.), 3rd edn., vol. 10, pp. 671–697, Academic Press, NY
- 3 Wellner, V. P., Sekura, R., Meister, A. and Larsson, A. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2505–2509
- 4 Prachal, J. T., Crist, W. M., Roper, M. and Wellner, V. P. (1983) *Blood* **62**, 754–757
- 5 Beutler, E., Gelbart, T. and Pegelow, C. (1986) *J. Clin. Invest.* **77**, 38–41
- 6 Mooz, E. D. and Meister, A. (1967) *Biochemistry* **6**, 1722–1734
- 7 Gushima, H., Miya, T., Murata, K. and Kimura, A. (1983) *J. Appl. Biochem.* **5**, 210–218
- 8 Meister, A. (1985) *Methods Enzymol.* **113**, 393–399
- 9 Majerus, P. W., Brauner, M. J., Smith, M. B. and Minnich, V. J. (1971) *J. Clin. Invest.* **50**, 1637–1643
- 10 Wendel, A., Schaich, E., Weber, L. and Flohe, L. (1972) *Hoppe-Zeyler's Physiol. Chem.* **353**, S523–S530
- 11 Rathbun, W. B., Sethna, S. S. and Buskirk, G. E. V. (1977) *Exp. Eye Res.* **24**, 145–158
- 12 Gushima, H., Yasuda, S., Soeda, E., Yokota, M. and Kimura, A. (1984) *Nucleic Acids Res.* **12**, 9299–9307
- 13 Mutoh, N., Nakagawa, C. W., Ando, S., Tanabe, K. and Hayashi, Y. (1991) *Biochem. Biophys. Res. Commun.* **181**, 430–436
- 14 Peters, J. M., Dalrymple, B. P. and Jorgensen, W. K. (1992) *Biochem. Biophys. Res. Commun.* **182**, 1040–1046
- 15 Habenicht, A., Hille, S. and Knochel, W. (1993) *Biochim. Biophys. Acta* **1174**, 295–298
- 16 Kato, H., Kobayashi, M., Murata, K., Nishioka, T. and Oda, J. (1989) *Agric. Biol. Chem.* **53**, 3071–3073
- 17 Tanaka, T., Kato, H., Nishioka, T. and Oda, J. (1992) *Biochemistry* **31**, 2259–2265
- 18 Hibi, T., Kato, H., Nishioka, T., Oda, J., Yamaguchi, H., Katsube, Y., Tanizawa, K. and Fukui, T. (1993) *Biochemistry* **32**, 1548–1554
- 19 Nakagawa, C. W., Mutoh, N. and Hayashi, Y. (1993) *Biochem. Cell. Biol.* **71**, 447–453
- 20 Adams, M. D., Kerlavage, A. R., Fields, C. and Venter, J. C. (1993) *Nature Genetics* **4**, 256–267
- 21 Dale, R. M. K., McClure, B. A. and Houchins, Y. P. (1985) *Plasmid* **13**, 31–40
- 22 Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H. and Roe, B. A. (1980) *J. Mol. Biol.* **143**, 161–178
- 23 Baker, R. T., Smith, S. A., Marano, R., McKee, J. and Board, P. G. (1994) *J. Biol. Chem.* **269**, 25381–25386
- 24 Board, P. G. and Pierce, K. (1987) *Biochem. J.* **248**, 937–941
- 25 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 26 Oppenheimer, L., Wellner, V. P., Griffith, O. W. and Meister, A. (1979) *J. Biol. Chem.* **254**, 5184–5190
- 27 Strumeyer, D. and Bloch, K. (1962) *Biochemistry* **9**, 52–55
- 28 Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- 29 Reed, K. C. and Mann, D. A. (1985) *Nucleic Acids Res.* **13**, 7207–7221
- 30 Nasmyth, K. A. (1982) *Cell* **30**, 567–578
- 31 Beutler, E., Mathai, C. K. and Smith, J. E. (1968) *Blood* **31**, 131–150
- 32 Saraste, M., Sibbald, P. R. and Wittinghofer, A. (1990) *Trends Biochem. Sci.* **15**, 430–434
- 33 Huang, C., He, W., Meister, A. and Anderson, M. E. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1232–1236