Kinetic properties of missense mutations in patients with glutathione synthetase deficiency

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Patients with hereditary glutathione synthetase (GS) (EC 6.3.2.3) deficiency present with variable clinical pictures, presumably related to the nature of the mutations involved. In order to elucidate the relationship between genotype, enzyme function and clinical phenotype, we have characterized enzyme kinetic parameters of missense mutations R125C, R267W, R330C and G464V from patients with GS deficiency. One of the mutations predominantly affected the K_m value, with decreased affinity for glycine, two mutations influenced both K_m and V_{max} values, and

one mutation reduced the stability of the enzyme. This characterization agrees well with predictions based on the recently reported crystal structure of human GS. Thus our data indicate that different mutations can affect the catalytic capacity of GS by decreasing substrate affinity, maximal velocity or enzyme stability.

Key words: γ -glutamyl cycle, 5-oxoprolinuria, haemolytic anaemia.

INTRODUCTION

To date, more than 65 patients with inborn errors in the metabolism of GSH, in 56 families, have been reported [1]. Glutathione synthetase (GS) (EC 6.3.2.3) deficiency is the most common of these disorders and exhibits autosomal recessive inheritance. It is probable that homozygosity for GS alleles that results in a totally inactive enzyme or the absence of protein expression would be lethal. Clinically, two variants can be distinguished. The mild form is restricted to erythrocytes and is thought to be caused by mutations that reduce the stability of the enzyme. The patients have low GSH levels in erythrocytes, but not in nucleated cells, and often have mild haemolytic anaemia. The severe form is caused by mutations that impair the catalytic function of the enzyme. These patients are referred to as 'generalized GS deficient' and present with massive urinary excretion of 5-oxoproline, haemolytic anaemia and metabolic acidosis, often in association with progressive impairment of central nervous system function. It is unclear if there is extensive polymorphism arising from functionally normal GS sequence variants. Although the 5' and central regions of the GS cDNA are not well represented in the expressed sequence tag ('EST') database, a search [2] has identified a number of GS sequence variants. However, most variation appears in non-coding regions, and the frequency of these potential polymorphisms has yet to be determined (P. G. Board, unpublished work). GS catalyses the final step in the biosynthesis of GSH according to:

The enzyme has been purified to homogeneity from several

sources, e.g. rat kidney [3], *Escherichia coli* [4] and yeast [5], but until now not to homogeneity from any human tissue. The human enzyme functions as a homodimer, with subunits containing 474 amino acid residues [6], encoded by a gene located on chromosome 20q11.2 [7]. Eighteen different mutations from 16 patients with GS deficiency have been described [8–10]. Many of these mutations affect residues that are within the protein core and active-site regions [11].

The recently reported crystal structure of human GS allows some predictions of the effects of naturally occurring mutations [11]. However, characterization of the mutant proteins is required to understand the complex interactions between structure, function and clinical phenotype. Furthermore, since GSH can be critical to the disposition of a range of therapeutic agents and the development of drug resistance, knowledge gained from the analysis of natural mutants may provide avenues for therapeutic intervention. The patients are often compound heterozygotes, which complicates kinetic analyses of specific mutations of enzymes isolated from blood samples. In the present investigation kinetic parameters of four naturally occurring missense mutations from patients with GS deficiency were investigated *in vitro* and the findings were correlated with the tertiary structure of the enzyme. The positions of the mutations within the GS subunit are illustrated in Figure 1. The properties of these mutations have not previously been studied and it was possible to compare the real characteristics of the proteins with predictions derived from the structure. The recombinant enzymes were His-tagged to facilitate purification. To evaluate putative effects of the added histidine residues on enzyme activity, wild-type His-tagged enzyme was compared to wild-type enzyme isolated from erythrocytes. This is the first time the human erythrocyte enzyme has been purified to homogeneity.

Abbreviations used: GS, glutathione synthetase; BTP, Bis-Tris Propane; γ -gluABA, L- γ -glutamyl-L- α -aminobutyric acid.

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Figure 1 A ribbon representation of the human GS monomer

The GS deficiency mutations discussed in the text are shown in blue. The cofactors (ADP is green, the sulphate which mimics the γ -phosphate is red and the magnesium ions are shown as two mauve spheres) and product (GSH is yellow) are also shown. This Figure was drawn using MOLSCRIPT [15].

EXPERIMENTAL

Materials

[1- 14 C]Glycine and the DNA Sculptor Kit were purchased from Amersham (Little Chalfont, Bucks., U.K.). DEAE-Sepharose CL-6B, thiopropyl-Sepharose 6B, Sephacryl S-300, Polybuffer (pH 6.5–4) and restriction endonucleases were obtained from Pharmacia Biotech. Bis-Tris Propane (BTP) was purchased from Sigma. Mercaptopyrindine-Sepharose 6B was produced as described previously [12]. Primers coding the missense mutations R125C, R267W, R330C, and G464V were synthesized at the Australian National University Biomolecular Resource Facility (Canberra, Australia). Sequence analysis was performed using a Sequenase version 2.0 kit supplied by USB (Cleveland, OH, U.S.A.). Protein assay reagent was purchased from Bio-Rad (Sundbyberg, Sweden). All chemicals were of analytical grade. Human erythrocyte concentrates were obtained from the Blood Bank, Akademiska Hospital, Uppsala, Sweden. This study was approved by the ethical committee of the Karolinska Institute.

Preparation of haemolysate

Erythrocytes were centrifuged at 5000 *g* for 20 min, before washing the cells twice with six vol. of PBS. One vol. of 5 mM Tris/HCl (pH 7.4)/1 mM EDTA was then added to the packed

erythrocytes, prior to sonication. Haemolysate was subsequently obtained, after centrifugation at 25000 *g* for 30 min.

Purification of GS

GS was purified from the haemolysate by ion-exchange chromatography, thiophilic adsorption chromatography, gel filtration, chromatofocusing and ammonium sulphate precipitation. The stepwise use of the different columns is described below. Fractions were collected throughout the purification for activity measurements. All procedures were performed at 5 °C. All column sizes are given as diameter $\times$ height.

DEAE-Sepharose

DEAE-Sepharose CL-6B (200 ml) was equilibrated with 20 mM BTP (pH 6.5), and suspended in 1000 ml of haemolysate (containing 138 g of protein). The mixture was agitated for approx. 2 h, collected in a Buchner funnel and washed with BTP buffer. The matrix was placed in a 3.2 cm $\times$ 25 cm column and washed with BTP buffer. At pH 6.5 haemoglobin did not bind to the matrix and was thus eluted. The adsorbed proteins were eluted stepwise with 0.05, 0.15 and 0.5 M KCl in BTP buffer. The enzyme activity was detected in the fractions eluted with BTP buffer containing 0.15 M KCl. These fractions were pooled (489 mg of protein), and K_2SO_4 was added to a final concentration of 0.5 M.

Table 1 Purification of GS from human erythrocytes

GS was purified from the haemolysate by ion-exchange chromatography, thiophilic adsorption chromatography, gel filtration, chromatofocusing and ammonium sulphate precipitation.

Fraction	Volume (ml)	Total protein (mg)	Purification factor	Yield of activity (%)
Haemolysate	1000	138 000	1	100
DEAE-Sepharose	670	490	160	56
Thiopropyl-Sepharose	102	42	1710	52
Mercaptopyrindine-Sepharose	43	5.2	10600	39
Sephacryl S-300	41	3.7	11 400	30

Thiopropyl-Sepharose

The sample was then applied to a 2 cm × 20 cm column of thiopropyl-Sepharose 6B equilibrated with a buffer containing 10 mM Tris/HCl (pH 7.8) and 0.5 M K₂SO₄. After washing with equilibration buffer, the enzyme was eluted with 10 mM Tris/HCl (pH 7.8)/1.5 M NaCl. Fractions with enzyme activity were pooled (42 mg of protein), and K₂SO₄ was added to a final concentration of 0.5 M.

Mercaptopyrindine-Sepharose

The sample was passed through a 2 cm × 16 cm column of mercaptopyrindine-Sepharose 6B equilibrated with a buffer containing 10 mM Tris/HCl (pH 7.8) and 0.5 M K₂SO₄. After washing with equilibration buffer, the enzyme was eluted with 10 mM Tris/HCl (pH 7.8)/1.5 M NaCl. The active fractions were pooled (5.2 mg of protein) and concentrated by ultrafiltration (Immersible CX-10; Millipore, Sundbyberg, Sweden).

Sephacryl

The concentrated sample (3.5 ml) was gel filtered through a 2 cm × 82 cm column of Sephacryl S-300 equilibrated with 20 mM BTP buffer. The fractions containing enzyme activity were pooled.

Chromatofocusing

The gel filtration peak containing GS was concentrated (3.7 mg of protein) and run on an FPLC Mono P HR 5/20 column (Pharmacia Biotech). Polybuffer, with pH range 6.5–4.0, was then used to elute the enzyme. The active fractions were pooled, and solid ammonium sulphate was supplemented to a concentration of 0.4 g/ml. The mixture was stirred for 30 min, before the precipitate was collected by centrifugation and dissolved in PBS. The final sample contained 0.5 mg of enzyme in 1.3 ml of buffer. The purity of the enzyme was assessed by a Pharmacia Phast System, with Phast Gel Gradient 8–25 and Phast Gel SDS Buffer Strips.

Construction and expression of mutant cDNAs

The wild-type GS cDNA was inserted into the His-tag vector pQE-31 (Qiagen, Clifton Hill, Australia), resulting in the addition of 6 histidine residues at the N-terminus of the protein [13]. Mutated cDNAs were constructed by PCR, employing mutant specific primers and the DNA Sculptor Kit. Constructs were confirmed by DNA sequencing using a Sequenase version 2.0 kit. Mutant cDNAs were expressed in *E. coli* TG1 and isolated as

described previously [8]. The purity of the recombinant enzymes was determined as described above.

Enzyme assay

The enzyme activity was determined as described previously [14], with the following modifications: the total incubation volume was 100 µl, containing 12 mM L-γ-glutamyl-L-α-aminobutyric acid (γ-gluABA), 16 mM [1-¹⁴C]glycine (specific activity 9.3 × 10⁹ Bq/mol), 8000 units/l pyruvate kinase, 4 mM phosphoenolpyruvate, 4 mM ATP, 100 mM Tris/HCl (pH 8.6), 25 mM KCl, 6 mM MgCl₂ and 1 g/l BSA. Following incubation at 37 °C for 60 min the reaction was stopped by the addition of 10 µl of 20 % (v/v) perchloric acid. Denatured protein was removed by centrifugation at 17000 g for 3 min, and the supernatant was applied to a 0.5 cm × 4 cm Dowex acetate column. Remaining [1-¹⁴C]glycine was eluted with 6 ml of 20 mM acetic acid. The absorbed product, ¹⁴C-γ-glutamyl-aminobutyril-glycine (ophthalmic acid), was eluted with 6 ml of 1.5 M ammonium acetate. Radioactivity was analysed by liquid scintillation counting. The kinetic constants K_m and V_{max} were determined by varying the concentrations of γ-gluABA, glycine and Mg²⁺/ATP (concentration ratio 3:2) respectively. Enzyme activity is expressed as µmol [1-¹⁴C]glycine incorporated/min per mg of protein.

K_m and V_{max} values were determined from the enzyme activity data by non-linear regression analysis, using GraFit Version 4 (Erithacus Software, Horley, Surrey, U.K.). For each substrate and enzyme, the concentration curve experiments were repeated twice. Results for V_{max} are consequently means ± S.D. of six determinations.

RESULTS

A protocol for the purification of wild-type GS from human control erythrocytes is outlined in Table 1. From 138 g of haemolysate proteins, approx. 0.5 mg of purified enzyme was obtained. The four different missense mutants R125C, R267W, R330C and G464V, as well as the wild-type enzyme, were expressed as His-tagged proteins in *E. coli*. One litre of each *E. coli* culture yielded 10–100 mg of recombinant enzyme. The purity of all isolated enzyme preparations was verified by SDS gel electrophoresis. Each preparation resulted in a single band (results not shown). The K_m and V_{max} data are shown in Table 2. Due to the high K_m for glycine in the G464V mutant, the highest velocity which could be reached in the γ-gluABA and ATP/Mg²⁺ concentration curves was only 10 % of V_{max} . The V_{max} for mutation G464V was therefore derived from the glycine con-

Table 2 The kinetic properties, $V_{\max}$ and K_m , of erythrocyte and His-tagged recombinant GS

Kinetic parameters were determined using increasing concentrations of each substrate ranging from 0.1–18 times the K_m in the presence of 12–18 times the determined K_m of the other substrates. Activity data are given as means $\pm$ S.D. The $V_{\max}$ for mutant R330C decreased successively. Therefore the range of $V_{\max}$ is given rather than the mean.

Enzyme	Mutation	$V_{\max}$ (μ mol/min per mg of protein)	K_m γ -gluABA (mM)	K_m glycine (mM)	K_m ATP (mM)
Erythrocyte	—	5.676 $\pm$ 2.053	0.99 $\pm$ 0.07	1.37 $\pm$ 0.01	0.23 $\pm$ 0.03
Wild type*	—	6.014 $\pm$ 1.402	0.65 $\pm$ 0.22	1.34 $\pm$ 0.34	0.22 $\pm$ 0.03
R125C*	373C $\rightarrow$ T	0.017 $\pm$ 0.004	1.79 $\pm$ 0.45	12.39 $\pm$ 0.84	0.06 $\pm$ 0.01
R267W*	799C $\rightarrow$ T	0.008 $\pm$ 0.002	10.12 $\pm$ 2.35	8.58 $\pm$ 3.24	0.44 $\pm$ 0.30
R330C*	988C $\rightarrow$ T	4.401 $\pm$ 0.300	0.72 $\pm$ 0.36	1.51 $\pm$ 0.08	0.29 $\pm$ 0.01
G464V*	1391G $\rightarrow$ T	1.608 $\pm$ 0.169	0.22 $\pm$ 0.03	130.40 $\pm$ 12.16	0.11 $\pm$ 0.06

* Recombinant His-tagged enzyme.

centration curves. The $V_{\max}$ of the mutant R330C decreased markedly with repeated freezing and thawing (the range is shown in Table 2). The R125C and R267W mutants exhibited markedly reduced but consistent $V_{\max}$ values, as well as increased K_m values for all of the substrates.

DISCUSSION

The genotype–phenotype relationship in human GS deficiency was addressed by expression of four mutants and kinetic analysis of the purified recombinant mutant enzymes. The mutations and the heterogeneous clinical symptoms of the patients studied have been described previously [8–10]. The four patients are compound heterozygotes for missense mutations affecting amino acid residues that are conserved between rats and humans.

Although kinetic properties of His-tagged recombinant enzymes have been studied previously [13], it was essential to confirm that the His-tag did not influence the catalytic properties of the enzymes. Thus comparison between wild-type His-tagged enzyme and wild-type enzyme isolated from erythrocytes demonstrated that the addition of the His-residues had minimal effects on enzyme kinetics. The experimental finding agrees with predictions from the crystal structure of His-tagged wild-type human enzyme. The His-tag is disordered in the crystal structure, as are the first two residues of the mature protein [11]. The third N-terminal amino acid residue is located on the surface of the protein and is approx. 28 Å (1 Å = 0.1 nm) from the active-site.

Hence the His-tag, which is joined to the N-terminus, is not expected to influence activity. Although GS has been purified from several tissues in other species, this is the first protocol for purification of human GS to homogeneity. The results indicate that GS constitutes at least 0.01 % of the soluble protein in human erythrocytes.

As illustrated in Table 2, the R125C and R267W mutations reduced the $V_{\max}$ to 0.2 % and 0.1 % of the wild-type activity, respectively. This suggests that these residues are of critical importance for formation of the active-site and catalysis. Examination of the crystal structure (Figures 1 and 2, left panel) shows that the guanidine group of Arg¹²⁵ forms a number of important contacts in the active-site including a salt-link with Asp¹²⁷ and hydrogen bonds to a sulphate ion, which mimics the position of the cleaved γ -phosphate, to the main-chain carbonyl of Ser¹⁵¹ and to the carbonyl of the cysteine moiety of the substrate. It has been proposed that Arg¹²⁵ neutralizes the negative charge of the γ -phosphate group of ATP during the reaction and that the main-chain amide of Ser¹⁵¹ stabilizes a reaction intermediate [11]. Asp¹²⁷ is engaged in a complicated hydrogen-bonding network with active-site residues. In accord with these observations, the kinetic data demonstrate that the substitution of Arg¹²⁵ with cysteine appears to effect both the reaction mechanism and substrate binding. Although there is one direct contact between the arginine and substrate/product, it is only one of 95 interactions [11] and so the loss of one hydrogen-bonding interaction is likely to have only a limited effect on substrate binding. The

Figure 2 Stereo pictures of the micro environments around the site of two of the missense mutations

Left panel: Arg¹²⁵. The two magnesium ions are shown as open circles. Right panel: Arg²⁶⁷. The polar interactions between the side-chains of these residues and surrounding atoms are shown by the dashed lines and the distance between polar atoms is measured in Ångströms. These Figures were drawn using MOLSCRIPT [15].

R267W mutation caused a 15-fold increase in K_m for γ -gluABA and a 6-fold increase in the K_m for glycine. Arg²⁶⁷ is located in the substrate binding loop and the effect of the mutation on substrate binding is not surprising, since the bulky tryptophan substitution appears to be too large for the active-site defined in the crystal structure (Figures 1 and 2, right panel) [11]. Furthermore, the guanidine group of Arg²⁶⁷ is involved in numerous interactions such as salt-links to the γ -glutamyl moiety of the substrate and to Asp²¹⁹, as well as hydrogen bonds to the hydroxy groups of Ser¹⁵¹ and Tyr²⁶⁵. These contacts would be lost in the mutants. As previously mentioned, the main-chain amide of Ser¹⁵¹ is thought to stabilize a reaction intermediate. Asp²¹⁹ is a site of mutation for the mild form of GS deficiency and we have previously proposed that this mutation would disrupt a complicated network of hydrogen bonds in the active-site [11]. Tyr²⁶⁵ is located immediately preceding the substrate-binding loop. Hence mutation of Arg²⁶⁷ is predicted to affect both substrate binding and catalysis; this is borne out by the kinetic data. The R125C and R267W mutations can thus be classified as combined K_m and V_{max} mutations.

The kinetic data on the R330C mutant provide a new insight into the disease, because it was previously thought that it might represent a polymorphism with normal function (since the mutation occurred in a patient who also had Asp²¹⁹ and Leu²⁸⁶ mutations). Arg³³⁰ is located on the surface of the protein (on helix 16, Figure 1), forming a salt-bridge with Asp⁷¹, and is 22 Å distant from the active-site and hence not in a position to impact on substrate binding or catalysis. The results of the present study are in agreement with these structural observations as the R330C mutation caused insignificant changes in the K_m values for the substrates. However, as opposed to the other mutant enzymes, the V_{max} of this mutant decreased with repeated freezing and thawing. This indicates that the mutation affects the stability of the enzyme, rendering it more susceptible to oxidation or denaturation. After only one round of freezing and thawing, the V_{max} was higher than 70% of wild-type activity, indicating that the activity of this mutant *in vivo* may be very close to or just as high as the wild-type activity. After repeated rounds of freezing and thawing, the V_{max} decreased successively to only 5% of wild-type activity. The poor stability is probably related to the loss of the salt-bridge and the introduction of an accessible, reactive, cysteine residue. Since GS is a dimer, heterozygotes will form heterodimers between normal and mutant subunits. In examples such as the R330C mutant, it is possible that the inactivation of one subunit in a heterodimer could inactivate the complete dimer. This could lead to the loss of up to 75% of activity in such heterozygotes. The residual 25% of activity would result from the probability that 25% of the enzyme in a heterozygote will be wild-type homodimer. However, it is likely that, under normal conditions, 25% of normal activity might be sufficient to avoid the characteristic symptoms of GS deficiency. In addition to this, the Arg³³⁰ residue is located at the opposite end of the molecule to the dimer interface [11] so it would not be expected to destabilize the wild-type monomer in a heterodimer.

The G464V mutation clearly affected the affinity for glycine, causing an almost 100-fold increase in the K_m . The K_m values for the two other substrates were only slightly decreased. The glycine residue, shown in Figure 1, adopts main-chain ϕ and ψ angles (96° and 7°, respectively) that are outside the allowed values for non-glycine residues, and hence replacement by any other residue is likely to impair the conformation and flexibility of the alanine-rich loop, which is involved in binding of the glycine substrate

[11]. Thus the high K_m for glycine is in excellent agreement with predictions from the crystal structure. The V_{max} for this mutant was low, but might theoretically be able to reach normal levels if substrate concentrations are increased sufficiently. Thus this mutation is classified as a K_m mutation.

The present study demonstrates that specific missense mutations of GS cause distinct kinetic impairment of enzyme function, such as decreased substrate affinity, decreased maximal activity or decreased stability. We speculate that the variation in the clinical picture of patients with GS deficiency is correlated with the mutations involved. However, as many patients are compound heterozygotes with two different mutations a direct correlation between genotype and clinical phenotype may not always be evident. Further studies of natural and directed mutations of GS are required to obtain a greater understanding of the catalytic mechanism and the function of this important enzyme.

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