Human hereditary glutathione synthetase deficiency: kinetic properties of mutant enzymes

Runa NJÄLSSON^{*1}, Katarina CARLSSON^{*}, Vikas BHANSALI[†], Jia-Li LUO[‡], Lennart NILSSON[§], Rudolf LADENSTEIN[§], Mary ANDERSON^{||}, Agne LARSSON^{*} and Svante NORGREN^{*}

^{*}Department of Paediatrics, Karolinska Institutet, Karolinska University Hospital, 141 86 Stockholm, Sweden, [†]Department of Biology, University of North Texas, Denton, TX 76203, U.S.A., [‡]Hisun Group Corp. Ltd., 46 Waisha Road, Jiaojiao, Taizhou, Zhejiang, People's Republic of China, [§]Department of Biosciences at NOVUM, Centre for Structural Biochemistry, Karolinska Institutet, 141 57 Stockholm, Sweden, and ^{||}Department of Chemistry and Physics, Texas Woman's University, P.O. Box 425859, Denton, TX 76204-5859, U.S.A.

Patients with hereditary glutathione synthetase deficiency suffer from haemolytic anaemia, 5-oxoprolinuria, metabolic acidosis, recurrent bacterial infections and various degrees of central nervous system dysfunction. To investigate the molecular basis of the mutations associated with this disease, seven naturally occurring missense mutations [L188P (Leu¹⁸⁸ → Pro), D219A, D219G, Y270C, Y270H, R283C and P314L] were expressed using a His-tagged, *Escherichia coli*-based expression system. Effects of the mutations on kinetic properties, including negative co-operative binding of γ -glutamyl substrate, were evaluated. The mutation P314L did not have any major effect on these parameters and

was classified as a neutral mutation. The remaining mutations decreased $V_{\max}$ to 2–27 % of wild-type activity. Negative co-operativity for γ -gluABA (L- γ -glutamyl-L- α -aminobutyric acid) was abolished in five mutant recombinant enzymes, whereas for one mutant enzyme, this co-operativity changed from negative to positive. The structural consequences of the mutations were interpreted on the basis of the known structure of the wild-type enzyme.

Key words: co-operativity, glutathione synthetase, kinetic property, mutant enzyme, reaction velocity, substrate affinity.

INTRODUCTION

The tripeptide glutathione (L- γ -glutamyl-L-cysteinyl-glycine, GSH) is involved in several essential intracellular processes, and its importance for normal cellular function is well documented [1]. The synthesis and degradation of GSH occurs via the γ -glutamyl cycle. The final step in its synthesis is catalysed by the enzyme GS (glutathione synthetase; EC 6.3.2.3) as follows:

The rare autosomal recessive disorder, GS deficiency (MIM 266130), is associated with haemolytic anaemia, metabolic acidosis, 5-oxoprolinuria, central nervous system dysfunction and recurrent bacterial infections [1]. Genetic investigations have revealed disease-causing mutations in the *GSS* gene [2–6] and the clinical phenotypes can be classified according to the severity of the disease as mild, moderate and severe [7]. We postulate that the clinical heterogeneity in GS deficiency reflects variation in enzyme structure and function caused by the different mutations.

The human GS enzyme is a homodimer of subunits containing 474 amino acid residues, encoded by a single-copy gene located on chromosome 20q11.2 [8]. *In vitro* analysis of naturally occurring missense mutations showed that mutations could affect the stability, catalytic capacity and substrate affinities of the enzyme [9]. The three-dimensional structure of the wild-type enzyme is known and predictions were made about the consequences of some naturally occurring mutations in GS deficiency [10]. However, considering a theoretical approach alone may cause misinterpretations of the actual enzyme mutations, e.g. the R330C (Arg³³⁰ → Cys) mutant enzyme was initially suggested to be polymorphic with normal function [10], but was later shown, by *in vitro* analysis, to have a compromised enzyme stability [9]. In

the present study, we investigate the molecular characteristics of the missense mutations found in GS-deficient patients by analysing the enzyme kinetic parameters together with interpretations of the structural consequences on the basis of the wild-type structure. This combination provides a powerful tool for a thorough analysis of the mutations associated with GS deficiency.

EXPERIMENTAL

Materials and patients

Primers were purchased from DNA Technology (Århus, Denmark). The *Escherichia coli* strain BL21(DE3)Star and *Pfu* DNA polymerase were purchased from Stratagene (Amsterdam, The Netherlands). Bacteria were lysed using a French pressure cell press obtained from American Instrument Co. (Silver Spring, MD, U.S.A.). Ni-NTA (Ni²⁺-nitrilotriacetate) resin was obtained from Qiagen (Crawley, West Sussex, U.K.), and the Centricon YM-50 spin columns were from Millipore (Bedford, MA, U.S.A.). ECL[®] (enhanced chemiluminescence) Western blotting reagents were purchased from Amersham Biosciences (Uppsala, Sweden) and reagents used for protein determination were from Bio-Rad Laboratories (Hercules, CA, U.S.A.).

γ -gluABA (L- γ -glutamyl-L- α -aminobutyric acid) was synthesized as described in [11]. Other biochemical reagents were obtained from Sigma. Enzyme kinetics was analysed using Sigma Plot software from SPSS Science (Chicago, IL, U.S.A.). The crystal structure of human wild-type GS (PDB code 2HGS; <http://www.rcsb.org/pdb>) was used for structural interpretations of the mutations.

Clinical data of the patients and the mutation analyses are described elsewhere [2,3,12].

Abbreviations used: γ -gluABA, L- γ -glutamyl-L- α -aminobutyric acid; GS, glutathione synthetase; Ni-NTA, Ni²⁺-nitrilotriacetate.

¹ To whom correspondence should be addressed (e-mail runa.njalsson@mednut.ki.se).

Construction and *in vitro* expression of mutant GS cDNA

An *Xho*I–*Bam*HI fragment encoding the full-length wild-type human GS cDNA was excised from the expression vector pQE-31 [13] and subcloned into the corresponding sites of the expression vector pET-15b. The missense mutations L188P, D219A, D219G, Y270C, Y270H, R283C and P314L were separately generated from this construct by site-directed PCR mutagenesis based on the overlap extension method [14] using *Pfu* DNA polymerase. The wild-type construct was used as a template, and all mutations were confirmed by sequencing. The GenBank® accession number NM_000178.2 was used for sequence referencing. Primer information is available on request.

Wild-type and mutant cDNAs were expressed in *E. coli* BL21(DE3)Star. Transformed cells were grown at 37 °C in LB (Luria–Bertani) medium containing 50 mg/l ampicillin to an absorbance A_{600} 0.6, before inducing expression of the constructs for 2 h by the addition of 0.25 mM isopropyl-1-thio- β -D-galactopyranoside. Cells from a 2 litre culture were harvested by centrifugation at 5000 *g* for 10 min. The pellets were washed once with 0.9% NaCl and then stored at –80 °C until use.

Bacterial pellets (approx. 5 g wet weight) were suspended in 20 ml of resuspension solution (50 mM NaH_2PO_4 /300 mM NaCl/10 mM imidazole, pH 8.0) and lysed by passaging twice at 6900 kPa through a French pressure cell press. After centrifugation at 14 000 *g* for 30 min, the supernatant was saved and the pellet was washed with an additional 10 ml of the resuspension solution. Ni-NTA (5 ml slurry) was added to the pooled supernatants, and the protein was allowed to bind overnight.

The Ni-NTA resin was centrifuged (1000 *g* for 3 min), added to a column (column diameter, 0.7 cm), and washed with 250 ml of 50 mM NaH_2PO_4 /300 mM NaCl/30 mM imidazole, pH 8.0 (1.5 ml/min). The enzyme was eluted using 50 mM NaH_2PO_4 /300 mM NaCl/250 mM imidazole (pH 8.0).

Purification was performed at 0–4 °C. The enzyme was stored at –80 °C in 50 mM NaH_2PO_4 /300 mM NaCl (pH 7.5) and 50% (v/v) glycerol subsequent to the removal of imidazole on a Centricon YM-50 column.

SDS/PAGE and Western blotting

The eluted enzyme was assessed for purity and degradation by denaturing PAGE and Western blotting with a polyclonal human GS-specific antiserum [6]. The blotted protein was visualized using ECL® Western blotting reagents.

Enzyme assays and kinetic analysis

All kinetic analyses were performed on purified recombinant His-tagged human GS. Enzyme activity was measured by coupling ATP hydrolysis with the oxidation of NADH via reactions catalysed by pyruvate kinase and lactate dehydrogenase. In this system, the generation of NAD^+ is directly proportional to the rate of formation of ADP by GS. γ -GluABA, a non-thiol γ -glutamylcysteine analogue in which the thiol has been replaced by a methyl group, was used in place of γ -glutamylcysteine to avoid oxidation of the thiol group. Specific activity was measured by the addition of purified GS to 0.2 ml of a preincubated (37 °C) reaction mixture containing 100 mM Tris/HCl (pH 8.2), 50 mM KCl, 20 mM MgCl_2 , 5 mM sodium phospho(enol)-pyruvate, 10 mM ATP, 10 mM glycine, 10 units/ml lactic acid dehydrogenase (Type II rabbit muscle), 10 units/ml pyruvate kinase (Type III rabbit muscle), 0.2 mM NADH and 20 mM γ -gluABA. To determine the K_m (app) values, all substrate concentrations, except one, were held constant at saturation levels,

and the remaining substrate concentration varied at least 10-fold around the K_m value. The rate of the reaction was monitored continuously in a spectrophotometer at 340 nm. Control reactions were performed in the absence of γ -gluABA. Enzyme activity was expressed in terms of μmol of product formed per min per mg of protein at 37 °C. Protein concentrations were determined by the Bradford method [15]. Activities were measured on a single preparation of each enzyme, and samples were run in duplicate or triplicate. The coefficients of variation were less than 10% for all concentrations of the substrates used in the affinity experiments, except for the lowest γ -gluABA concentration in the analysis of D219A (21%).

Using non-linear regression and the Michaelis–Menten equation, K_m (app) values for ATP and glycine and $V_{\max}$ values were deduced. Non-hyperbolic curves were replotted as Eadie–Hofstee plots (v versus $v/[S]$) to demonstrate deviations from Michaelis–Menten kinetics. The data were further analysed by inserting $V_{\max}$, $[S]$ and v into the Adair equation:

$$v = \frac{V_{\max} \left(\frac{[S]}{K_m} + \frac{[S]^2}{\alpha K_m^2} \right)}{1 + \frac{[S]}{K_m} + \frac{[S]^2}{\alpha K_m^2}} \quad (1)$$

This yielded the K_m (app) value for the binding of γ -gluABA to its first site (K_m^1) and the interaction factor α . By multiplying these two values, the K_m value for the binding of the second molecule of γ -gluABA was estimated (K_m^2). The Adair equation (eqn 1) describes the Koshland model used to explain negative co-operativity [16]. Here, $[S]$ represents substrate concentration, $V_{\max}$ the maximum velocity [$\mu\text{mol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$], v the initial velocity, K_m the substrate concentration at $V_{\max}/2$ and α the interaction factor. An interaction factor $\alpha > 1$ indicates negative co-operativity. By plotting $\log[v/(V_{\max} - v)]$ against $\log[S]$ (the Hill plot), the Hill coefficient h was obtained (slope of the curve). For negative co-operativity, h is < 1 . Possible structural effects of the mutations were based on visual inspection of the wild-type GS structure (PDB code 2HGS) in the vicinity of the mutated sites.

RESULTS

Seven naturally occurring GS missense mutations and the GS wild-type were expressed in an *E. coli* system, isolated and investigated *in vitro*. Figure 1 illustrates the GS subunit with the positions of the mutated side chains. From 2 litres of culture solution, 0.7–7.4 mg of GS enzyme protein was obtained. Recombinant GS proteins were isolated to at least 95% purity as evaluated by SDS/PAGE. The enzyme kinetic results of purified His-tagged wild-type and mutated GS enzymes are summarized in Table 1. The mutated recombinant enzymes are referred to by the mutation they contain, and the construct containing the unmutated sequence referred to as the wild-type.

The $V_{\max}$ values for six of the seven mutant GS enzymes were substantially decreased (2–27%) when compared with the wild-type enzyme and the seventh mutant enzyme (P314L) had normal activity.

All recombinant enzymes displayed normal Michaelis–Menten kinetics for glycine and ATP. The L188P and D219A mutant enzymes displayed the largest decreases in ATP affinity, with the K_m values being 10 and 15 times that of the wild-type respectively. There were no major effects on the affinity for glycine, where K_m values ranged from 58% (R283C) to 130% (L188P) of the wild-type K_m value.

Wild-type, P314L and D219A did not display normal Michaelis–Menten kinetics for γ -gluABA, but followed a distinct

Figure 1 Molecular model of a human GS monomer

Side chains of the mutated amino acid residues are shown in blue and all substrate molecules are shown in red. The Figure was drawn using the program Ribbons [20].

non-hyperbolic plot for the double-reciprocal plot. The corresponding Eadie–Hofstee plots for the wild-type (concave curve, Figure 2) and P314L enzymes indicated negative co-operativity, which is also supported by the results of interaction factor $\alpha > 1$ and Hill coefficient $h < 1$. D219A mutant GS showed positive co-operativity as indicated by $\alpha < 1$, $h > 1$ and a convex Eadie–Hofstee plot. The remaining mutant enzymes no longer showed negative co-operativity, as indicated by both α and h values being close to 1 and by linear Eadie–Hofstee plots for mutants Y270H, R283C and D219G (Figure 2). The K_m (app) value for the first binding site of γ -gluABA (K_m^1 in Table 1) was increased in D219A by approx. 9-fold compared with the wild-type K_m^1 value. The remaining mutant enzymes had K_m^1 values comparable with that of wild-type GS. For L188P mutant GS, binding of the second molecule γ -gluABA (K_m^2 in Table 1) was about twice that of the wild-type, but was similar or less than the wild-type in the remaining mutant GS enzymes.

Interpretations of the structural consequences of the mutations were based on a combination of the kinetic results and the published three-dimensional structure of the wild-type enzyme [10].

DISCUSSION

Seven naturally occurring missense mutations were expressed *in vivo*; the effects on the kinetic parameters of the corresponding mutant GS enzymes were analysed and compared with those of the wild-type enzyme. We have demonstrated previously that the His-tag has no effect on the reaction velocity and substrate affinities in the wild-type [9].

The V_{max} value obtained for the wild-type enzyme was $3.7 \mu\text{mol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ (Table 1). This value is consistent with that of previous reports [3,17], although it is slightly lower than that obtained previously by our group [9]. All the mutations studied in this report, except P314L, involve amino acid residues that are conserved for both humans and rats. With the exception of P314L, all the mutations caused decreased V_{max} values, ranging from 2 to 27 % of the wild-type value.

Residue 314 is on the surface of the GS subunit [10]. Exchanging Pro³¹⁴ with leucine did not cause major changes in the kinetic properties or co-operativity of γ -gluABA. In addition to being the only mutant with preserved negative co-operativity, this mutant also complements the GS-deficient yeast strain [2]. We conclude that this mutation is a neutral mutation without detrimental consequences to the enzyme function. The patient with this mutation had two additional mutations, one in *cis* with P314L, and cultured fibroblasts from this patient had only 5–10 % activity [2].

The V_{max} of L188P was only 9 % of the wild-type. Furthermore, the affinity for ATP in this mutant was severely decreased; the K_m (app) value was ten times higher than that of the wild-type enzyme. Leu¹⁸⁸ is situated in an α -helix, and introducing a proline residue into this secondary-structure element usually forces a 'kink' in the helix and probably disrupts it. Although Leu¹⁸⁸ is not in direct contact with the active site of the enzyme, the 'kink' will necessitate adaptive changes in the structure of its surrounding microenvironment. This α -helix is packed against an anti-parallel β -sheet involved in ATP binding [10], and the decreased affinity for ATP in this mutant probably reflects adaptive changes in this β -sheet.

In D219A, the V_{max} value was reduced to only 4 % of wild-type value. The apparent affinity for the binding of γ -gluABA to the first site was severely decreased, as was the binding of ATP. This mutation was the only one situated close to the subunit interaction surface and, interestingly, it was the only one where a change from negative to positive co-operative binding of γ -gluABA was observed. The change to a glycine instead of an alanine residue in this position apparently did not distort the residue interactions of the active site to the same extent, since binding of all the substrates was facilitated in the D219G mutant. D219G is associated with the mild clinical form of GS deficiency [2]. This mutant showed no

Table 1 Kinetic properties of wild-type and mutant GS

Enzyme (His-tagged)	V_{max} (%) [*]	K_m (app) (mM)					Hill coefficient (h)
		Glycine	ATP	K_m^1 (γ -gluABA)	K_m^2 (γ -gluABA)		
Wild-type	3.7 (100)	1.75	0.07	0.66	1.50		0.80
Y270C	0.06 (2)	1.43	0.05	0.94	1.31		0.90
D219A	0.14 (4)	2.07	1.05	5.73	2.71		1.55
Y270H	0.22 (6)	1.24	0.02	0.39	0.36		0.98
L188P	0.34 (9)	2.25	0.73	0.60	0.88		0.91
R283C	0.49 (13)	1.02	0.09	0.38	0.53		0.93
D219G	1.00 (27)	1.51	0.04	0.26	0.30		0.99
P314L	4.07 (110)	1.64	0.05	0.33	1.48		0.70

^{*} V_{max} values are given in $\mu\text{mol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$; mutant activities are expressed as a percentage of the wild-type value.

Figure 2 Eadie-Hofstee plot for recombinant GS enzymes

Eadie-Hofstee plots were obtained for the binding of γ -gluABA by plotting initial velocity v against $v/[S]$, as shown for (A) wild-type, (B) Y270C, (C) D219A, (D) Y270H, (E) L188P, (F) R283C, (G) D219G and (H) P314L.

co-operativity; even though it was capable of binding its substrates with affinities similar to that of the wild-type, the enzyme had only 27% of the wild-type $V_{\max}$ value. The carboxylate group of the Asp²¹⁹ side chain interacts closely with one of the nitrogen molecules of the Arg²⁶⁷ side chain, namely Arg²⁶⁷-N η , and with the backbone amides of Phe¹⁵² and Gly¹⁵³ (Figure 3). Arg²⁶⁷-N η ,

in turn, forms two strong hydrogen bonds with GSH, as does the backbone amide of Ser¹⁵¹. If the Asp²¹⁹ side chain is replaced with alanine or glycine, these hydrogen bonds will be lost, possibly resulting in a destabilization of the γ -glutamyl substrate-binding site. There are, however, no direct interactions between Asp²¹⁹ and the ATP.

Figure 3 The microenvironment around Asp²¹⁹

Asp²¹⁹ in the wild-type structure of GS interacts with several active-site residues, as indicated by the broken lines. The distances between interacting molecules are expressed in Å. Glutathione is shown in cyan, its interacting oxygen in red, the side chain of Asp²¹⁹ in yellow, nitrogen atoms in purple and backbone carboxylate oxygen atoms in red. The Figure was generated with the program RasMol [21].

A major impact of the R283C mutation was the reduction in $V_{\max}$ value to 13 % of the wild-type. It has been proposed that introducing a cysteine residue at this position might lead to the formation of a disulphide bond with the nearby Cys²⁹⁴. Since the distance between these residues in the native form of the protein is approx. 12 Å (1 Å = 0.1 nm), any interaction probably occurs during protein folding [10] or could influence the equilibrium between folded and partially unfolded states of the protein.

Y270C probably causes inactivation of the enzyme by mixed disulphide formation [10]. To avoid autooxidation, however, the γ -glutamyl substrate used in our activity assays did not contain any thiol group. Despite this, the $V_{\max}$ value of this mutant enzyme was only 2 % of the wild-type value, suggesting an alternative unfavourable mechanism behind the decreased activity. Mutating the same residue to histidine caused a similar decrease in $V_{\max}$ value. The histidine residue introduced at position 270 could interact with the γ -phosphate of the ATP, probably also causing a decreased $V_{\max}$ value either by locking the γ -phosphate or inducing a less favourable conformation of the ATP. Substitution of one large aromatic side chain for another may facilitate substrate binding, as illustrated by the increased affinities of Y270H for all substrates and the absence of negative co-operativity. Even so, Y270H had a drastically reduced $V_{\max}$ value, only 6 % of the wild-type.

Missense mutations situated in regions of the enzyme that are not part of the active site or subunit interaction area of an enzyme might still cause decreased enzyme activity *in vivo* by compromising the stability of the enzyme through defective protein folding and increased interactions with proteases or chaperones. This is seen with missense mutations in phenylketonuria [18]. Disturbances of the folding process may also cause

mutant proteins to aggregate, as for example in Alzheimer's disease and Creutzfeldt–Jakob disease (see [19]). Structural predictions of mutant enzymes based on a three-dimensional structure of the wild-type protein do not take into account these factors. Still, in combination with site-directed mutagenesis, it provides a powerful tool to understand the molecular basis of diseases.

Earlier studies of naturally occurring GS mutations showed that missense mutations associated with GS deficiency could affect the stability and catalytic capacity of the enzyme and the affinity with which it binds its substrates [9]. Results of the present study demonstrate that mutations can also affect the cooperativity of the enzyme, and introducing different amino acids at the same position can have widely differing effects on the kinetic parameters. Kinetic analysis of naturally occurring mutations with GS deficiency is an important tool to gain knowledge about the mechanism behind this disease. So far, the only therapy offered to patients with GS deficiency is correction of the metabolic acidosis using bicarbonate and boosting of scavenger levels by administration of α -tocopherol and ascorbic acid [7]. Substrate affinity data of different mutations associated with this disease would make it possible to personalize future drug administration in this group of patients if a good delivery system for GSH precursors is developed.

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