Site-directed mutagenesis establishes cysteine-110 as essential for enzyme activity in human γ -glutamyl hydrolase

Karen J. CHAVE, John GALIVAN and Thomas J. RYAN¹

Division of Molecular Medicine, Wadsworth Center, New York State Department of Health, Albany, NY 12201-0509, U.S.A.

γ -Glutamyl hydrolase (GH), which hydrolyses the γ -glutamyl conjugates of folic acid, is a key enzyme in the maintenance of cellular folylpolyglutamate concentrations. The catalytic mechanism of GH is not known. Consistent with earlier reports that GH is sulphhydryl-sensitive, we found that recombinant human GH is inhibited by iodoacetic acid, suggesting that at least one cysteine is important for activity [Rhee, Lindau-Shepard, Chave, Galivan and Ryan (1998) *Mol. Pharmacol.* **53**, 1040–1046]. Using site-directed mutagenesis, the cDNA for human GH was altered to encode four different proteins each with one of four cysteine residues changed to alanine. Three of the mutant proteins had activities similar to wild-type GH and were inhibited by

iodoacetic acid, whereas the C110A mutant had no activity. Cys-110 is conserved among the human, rat and mouse GH amino acid sequences. The wild-type protein and all four mutants had similar intrinsic fluorescence spectra, indicating no major structural changes had been introduced. These results indicate that Cys-110 is essential for enzyme activity and suggest that GH is a cysteine peptidase. These studies represent the first identification of the essential Cys residue in this enzyme and provide the beginning of a framework to determine the catalytic mechanism, important in defining GH as a therapeutic target.

Key words: antifolates, folate metabolism, folylpolyglutamates.

INTRODUCTION

Many enzymes involved in producing precursors for DNA synthesis require folate as a cofactor. Antifolate drugs such as methotrexate (MTX), which impair folate function, are the primary treatments for many cancers. The retention and efficacy of folates and antifolate drugs within the cell are dependent on the addition of a poly- γ -glutamate chain to the monoglutamate [1,2]. Folylpolyglutamate synthetase catalyses the sequential addition of glutamate [3,4] and γ -glutamyl hydrolase (GH, EC 3.4.19.9) catalyses the removal of glutamate from folyl- and antifolylpoly- γ -glutamates [5]. In conjunction with the folate transport systems, the balance between GH and folylpolyglutamate synthetase activity regulates the amount of glutamylation of folate and antifolate drugs in the cell.

GH activity in mammalian cells is found in the lysosomes [3,6–9]. However, in cell culture, the major part of the synthesized enzyme is secreted into the medium [10]. In humans, GH activity has been detected in plasma [11], bile [12], pancreatic juice [13] and jejunal mucosa [14]. The enzyme has been purified from a number of mammalian tissues [15–19] and cell lines [9,10,19,20]. Rat GH and human GH are the most extensively studied proteins and are glycoproteins [20,21]. GH from different species has a different specificity in the hydrolysis of the poly- γ -glutamyl tail. For example, the rat GH enzyme acts as an endopeptidase [19] hydrolysing the innermost γ -glutamyl bond and releasing the poly- γ -glutamate chain as a single unit. Conversely GH isolated from human sources (hGH) removes only the carboxyl terminal glutamate or di- γ -glutamate during the reaction [20].

The cDNAs encoding GH from rat and human sources have been isolated in this laboratory [21,22] and a mouse GH cDNA has recently been isolated [23]. The hGH cDNA has been expressed in both an insect expression system [20] and *Escherichia coli* [22]. The first 24 amino acids encoded by the hGH cDNA are a signal peptide, which is removed during processing [20].

Therefore, the N-terminal amino acid of the mature hGH enzyme is equivalent to Arg-25 in the published hGH sequence [22]. The amino acid numbering in the present study is based on the N-terminal arginine of the mature protein being number 1.

Early studies demonstrated that GH is sulphhydryl-sensitive and is inhibited by iodoacetic acid or *p*-hydroxymercuribenzoate (pHMB) [3,14,16]. Studies on GH in lysosomes isolated from murine S180 cells indicate that accumulation of reduced sulphhydryls in the lysosome activates GH [24]. All of this evidence suggested that at least one cysteine residue is catalytically important. However, until recently pure preparations of GH have not been available to determine if a single cysteine is critical. Recent studies with pure GH preparations from our laboratory verified the earlier findings of sulphhydryl sensitivity [10,20,21].

The catalytic mechanism of GH has yet to be defined. A better understanding of the mechanism could lead to the specific inhibition of this enzyme and increased efficacy of antifolate drugs. Human GH has four cysteine residues. Comparison of the amino acid sequences derived from the available three cDNAs indicate that two of these residues (Cys-19 and Cys-110) are conserved in the human, rat and mouse GH proteins. In the present study we have used the opportunity afforded by an expression system for hGH to determine which cysteine(s) is critical for activity by using site-directed mutagenesis of the cDNA encoding the mature hGH protein to replace systematically each of the four cysteine residues in hGH protein with alanine.

EXPERIMENTAL

Construction of C19A, C110A, C124A and C290A mutants of hGH

A cDNA encoding the 294 amino acid mature form of hGH protein with an N-terminal methionine preceding the first ar-

Abbreviations used: GH, γ -glutamyl hydrolase; hGH, human γ -glutamyl hydrolase; MTX, methotrexate (4-NH₂-10-CH₃PteGlu); OBG octyl β -D-glucoside; pHMB, *p*-hydroxymercuribenzoate.

¹ To whom correspondence should be addressed (email: thomas.ryan@wadsworth.org).

Table 1 Sequences of the primer pairs used for mutagenesis of the hGH sequence

The codon changed is underlined and mismatched basepairs are in bold. The sense oligonucleotides are indicated by + and the antisense oligonucleotides by −.

Mutant	Oligonucleotide sequence
C19A +	5'-GCC-CAT-CAT-CGG-AAT-ATT-AAT-GCA-AAA- AGC -CCG-TAA-TAA-AGT-C-3'
C19A −	5'-GAC-TTT-ATT-ACG- GCG -TTT-TTG-CAT-TAA-TAT-TCC-GAT-GAT-GGG-C-3'
C110A +	5'-CCT-GTG-TGG-GGC-ACA- GCG -CTT-GGA-TTT-GAA-GAG-C-3'
C110A −	5'-GCT-CTT-CAA-ATC-CAA- GCG -CTG-TGC-CCC-ACA-CAG-G-3'
C124A +	5'-GCT-GAT-TAG-TGG-AGA- GCG -CTT-ATT-AAC-TGC-CAC-AG-3'
C124A −	5'-CTG-TGG-CAG-TTA-ATA- AGG -CCT-CTC-CAC-TAA-TCA-GC-3'
C290A +	5'-CTT-CAT-TTC-AGC- AAG -CTT-ACA-TAT-TTG-ATT-GAA-AGT-C-3'
C290A −	5'-GAC-TTT-CAA-TCA-AAT-ATG-TAA- GCT -TGC-TGA-AAT-GAA-G-3'

ginine residue had previously been subcloned into pET-24a (pET-hGH, [22]) (Novagen, Madison, WI, U.S.A.). Site-directed mutagenesis was performed with the QuickChange kit (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's protocol using 125 ng of each primer for the C110A mutation and 150 ng for the other mutations, and annealing temperatures of 55 °C for C110A and 53 °C for the other mutations. This method requires the use of two oligonucleotides containing the desired mutation and complementary to opposite strands of the vector. The sequences of the pairs of oligonucleotides synthesized for the mutagenesis are presented in Table 1.

Plasmid DNA was purified using the Plasmid Midi kit (Qiagen, Santa Clarita, CA, U.S.A.). The sequences of all mutant clones were confirmed using automated DNA sequencing on either an Applied Biosystems 373A or 377A DNA sequencer (Perkin-Elmer, Branchburg, NJ, U.S.A.).

Expression and purification of wild-type and mutant hGH

Mutant plasmids were used to transform *E. coli* strain BL21(DE3)pLysS to kanamycin resistance. Cultures (20 ml) were grown for 16 h at 37 °C, 225 r.p.m. in tryptone-phosphate broth [25], containing 30 µg/ml kanamycin and 34 µl chloramphenicol. An aliquot (6 ml) of this culture was used to inoculate 500 ml of the same medium. Cultures were incubated at 27 °C, 225 r.p.m. until the OD_{600nm} was 0.5–0.6. Isopropyl β-D-thiogalactoside was added to a final concentration of 1 mM and cultures were incubated for a further 3 h. Cells were harvested by centrifugation and stored at −80 °C. Cells from 1 litre of broth were resuspended in 50 ml of 50 mM Tris/HCl pH 7.5 buffer containing 500 mM NaCl and Complete protease inhibitor cocktail (Boehringer-Mannheim, Indianapolis, IN, U.S.A.). The mixture was sonicated and clarified by centrifugation at 20000 g for 30 min at 4 °C. A 20–60% ammonium sulphate precipitate was resuspended in 50 mM sodium acetate, pH 5.8, 1 mM octyl β-D-glucoside and 5% v/v glycerol (buffer A). After dialysis at 4 °C against buffer A and clarification by centrifugation at 30000 g, 4 °C for 120 min, the dialysate was applied in two aliquots to a Protein Pak SP column (1.0 cm × 10 cm, Waters Division of Millipore Corp., Milford, MA, U.S.A.) equilibrated in buffer A. The protein was eluted (1 ml/min) with a linear gradient in buffer A of 0 to 1 M NaCl over 60 min. Fractions were assayed for hGH activity or for hGH protein by Western blot. Fractions containing hGH were pooled and frozen at −80 °C. Wild-type hGH and the C19A, C110A and C290A mutants eluted as two peaks, whereas

the C124A mutant eluted as a single peak coincident with the first peak for the other hGH proteins. Western blotting analysis indicated that the hGH in both peaks was of the same M_r . There was 8–10 times more hGH protein in peak 1 than in peak 2. A statistically significant amount (40–82%) of enzyme activity of proteins purified on the SP column was lost when stored either at 4 °C or at −80 °C. For further purification, thawed aliquots of peak 1 were incubated for 2 h at 4 °C with 2 ml of pHMB agarose (0.8 µmol/ml agarose) (Sigma Chemical Co., St Louis, MO, U.S.A.) equilibrated in 50 mM sodium acetate, pH 5.8, 20 mM octyl β-D-glucoside, 500 mM NaCl (buffer B). The matrix was poured into a column (0.75 cm × 4.5 cm) and washed with buffer B. hGH protein was eluted with buffer B containing additionally 10 mM dithiothreitol and 50 mM β-mercaptoethanol. EDTA (1 mM final) was added to each fraction to inhibit remaining proteases. Fractions containing hGH were pooled and stored at −80 °C.

Protein concentration assay

Protein concentrations were determined using the Coomassie Plus protein assay reagent (Pierce, Rockford, IL, U.S.A.) with bovine serum albumin as the standard. The concentrations of purified wild-type and mutant hGH proteins were normalized according to the intensity of their intrinsic fluorescence spectra relative to the spectra of C110A (the most pure protein).

Activity assays

hGH activity in chromatography fractions was measured with 4-NH₂-10-CH₃PteGlu₅ (Schirk Labs, Jona, Switzerland) as previously described [20]. The specific activities and reaction kinetics of hGH were determined using varying concentrations of 4-NH₂-10-CH₃PteGlu₂ as substrate (Schirk Labs). The kinetic constants K_m and V_{max} were calculated using a VMS Fortran-program for a Digital Equipment Corp. VAX station 4000 computer, which fits experimental data to the Michaelis-Menten equation by the method of Cornish-Bowden and Eisenthal [26] using weighted linear regression. Iodoacetate inhibition was performed as previously described using 0.5 mM iodoacetate for 60 min [20].

SDS/PAGE

Proteins were separated using 12% (w/v) SDS/PAGE gels. Protein bands were visualized by silver staining (Bio-Rad, Hercules, CA, U.S.A.), following the manufacturer's protocol.

Western blotting

hGH protein was determined by Western blotting as previously described [20].

Measurement of intrinsic fluorescence

Intrinsic fluorescence of wild-type and mutant hGH proteins was measured on a Perkin-Elmer LS-50B fluorescence spectrometer, at a concentration of 0.58 µg/ml in 50 mM sodium acetate, pH 5.5, 50 mM β-mercaptoethanol, 1 mM octyl β-glucoside. Excitation at 280 nm produced emission spectra with a maximum at 337–338 nm that were identical in shape and λ_{max} for all the purified GH proteins.

Figure 1 SDS, 12.5% (w/v) polyacrylamide gel electrophoresis of purified wild-type and mutant hGH proteins (0.58 μ g per well)

(A) Silver-stained gel, (B) Western blot using a polyclonal antibody (1:10000 dilution) against hGH expressed in insect cells. 1, wild-type; 2, C19A; 3, C110A; 4, C124A; 5, C290A.

proteins were expressed in *Escherichia coli* using the pET expression system [27]. Wild-type hGH and the C19A, C110A, C124A and C290A mutants were initially purified on a SP cation-exchange column. In the case of the C110A mutant no fractions contained hGH activity.

The SP purified proteins were further purified on a column of pHMB linked to agarose [28]. Using this procedure wild-type hGH was isolated with an overall yield of 11%. Analysis by SDS/PAGE and Western blotting of pooled fractions indicated hGH was purified to near homogeneity (Figure 1). The molecular mass of the major band at 35 kDa is in agreement with the theoretical mass of the cDNA encoded protein [22]. In most preparations there was a minor non-immunoreactive protein that copurified with hGH and had a fractionally lower molecular mass.

Activity of wild-type and mutant hGH proteins

The specific activities of the purified hGH proteins are summarized in Table 2. Wild-type, C19A, C124A and C290A hGH proteins had activity and all produced a similar product distribution of methotrexate polyglutamates when 4-NH₂-10-CH₃PteGlu₅ was used as a substrate (results not shown). The specific activities of C19A and C124A were significantly lower than for the wild-type protein but these proteins had a higher amount of contaminating protein (Figure 1). The activities of wild-type, C19A, C124A and C290A proteins were reduced 70–94% by incubation with 0.5 mM iodoacetate for 60 min.

The K_m and V_{max} for the active hGH proteins with 4-NH₂-10-CH₃PteGlu₂ were determined (Table 2). The V_{max} values for the wild-type and active mutants were not significantly different, indicating that the differences in specific activities of the purified proteins (Table 2) were due to an increased amount of contaminant protein. The K_m values for 4-NH₂-10-CH₃PteGlu₂ with the C19A, C124A and C290A mutant hGH proteins were not significantly different, indicating a similar affinity for this substrate. The C110A mutant had no activity (less than 0.02% of wild-type enzyme) when assayed with either 4-NH₂-10-CH₃PteGlu₂ or the better substrate, 4-NH₂-10-CH₃PteGlu₅.

RESULTS

Expression and purification of wild-type and mutant hGH proteins

Site-directed mutagenesis was used to generate C19A, C110A, C124A and C290A variants of hGH. Wild-type and mutant

DISCUSSION

The cDNA encoding the mature human GH has been expressed in *E. coli* and the expressed protein purified to near homogeneity. Four mutant hGH cDNAs, each having one cysteine codon

Table 2 K_m , V_{max} and specific activities purified wild-type hGH and mutant hGH proteins

K_m and V_{max} activities were measured using variable 4-NH₂-10-CH₃PteGlu₂ substrate concentrations. Specific activities were measured using 200 μ M 4-NH₂-10-CH₃PteGlu₂ for 5 min.

Protein	K_m (μ M)	V_{max} (nmol 4-NH ₂ -10-CH ₃ PteGlu ₂ hydrolysed min ⁻¹ · μ g ⁻¹ protein)	Specific activity (nmol 4-NH ₂ -10-CH ₃ PteGlu ₂ hydrolysed min ⁻¹ · mg ⁻¹ protein)
Wild-type	49.0 ± 14.8	2.6 ± 1.0	1913 ± 32
C19A	22.2 ± 5.1	1.1 ± 0.4	1000 ± 72*
C124A	30.3 ± 6.4	1.0 ± 0.1	725 ± 79*
C290A	23.1 ± 3.1	2.0 ± 0.4	1406 ± 463
C110A	—	—	No activity†

Results are mean ± S.D. of three experiments.

* Compared with wild-type $P < 0.01$.

† Using an extended assay (16 h) and a 10-fold increase in protein concentration, an activity of 0.35 nmol · mg⁻¹ · min⁻¹ could have been detected.

Table 3 Alignment of known GH amino acid sequences between amino acids 16–21 and 103–118

Amino acids highlighted in bold are conserved in all three GH sequences, underlined are Cys-19 and Cys-110.

Species	Amino acids	
	16–21	103–118
<i>Homo sapiens</i> *	MQKCRN	YFPVWGTCLGFEELS L
<i>Mus musculus</i> †	MQECFG	HFPVWGTCLGFEELS V
<i>Rattus norvegicus</i> ‡	MQECYG	HFPVWGTCLGFEELS V

* Reference 22.

† Reference 23.

‡ Reference 21.

replaced by an alanine codon, were created by site-directed mutagenesis. The mutant cDNAs were expressed in *E. coli* and the expressed proteins were purified to near homogeneity in an identical manner. All four cysteine mutant proteins bound to pHMB coupled agarose, indicating that there was more than one cysteine residue in hGH, which bound this matrix.

Activity assays of the wild-type and mutant proteins indicated that the C110A mutant had no hGH activity, whereas the $V_{\max}$ activities of C19A, C124A and C290A were not significantly different from wild-type activity. The C19A, C124A and C290A mutant proteins were inhibited by iodoacetate, suggesting that all these mutant proteins still contained a catalytically essential cysteine residue, namely Cys-110. An amino acid sequence alignment of the human [22], rat [21] and mouse [23] GH proteins indicated that Cys-19 and Cys-110 were invariant in all three sequences (Table 3). Since the C19A mutant had a $V_{\max}$ similar to the wild-type hGH, Cys-19 cannot be catalytically essential. There are no equivalents of Cys-124 or Cys-290 in rat or mouse GH proteins. Therefore, it is unlikely that either is involved in catalysis.

The C19A, C124A and C290A proteins all had similar K_m values with the substrate 4-NH₂-10-CH₃PteGlu₂. Although hGH cleaves both at the carboxyl terminal and penultimate γ -glutamyl bond, the penultimate bond is favoured [20]. It has also been demonstrated that 4-NH₂-10-CH₃PteGlu₂ is a poor substrate for hGH relative to longer chain polyglutamates [20,29], presumably because hydrolysis can occur only at the terminal γ -glutamyl bond. Given the complex nature of cleavage of substrates with longer chain lengths, it is currently impossible to obtain detailed kinetic data for these more favoured substrates. The similar product distribution shown when the active mutants were assayed with 4-NH₂-10-CH₃PteGlu₅, favouring cleavage at the penultimate γ linkage [20] suggests, that these mutations had no effect on the specificity of the location of the γ -bond cleavage.

The loss of activity when Cys-110 is mutated to alanine and the fact that Cys-110 is conserved in the amino acid sequences of human, rat and mouse GH indicates that this is the essential cysteine that has been suggested by chemical modification but not previously established. The presence of an essential cysteine suggests the hypothesis that GH is a cysteine peptidase. A catalytically essential cysteine is invariably accompanied by an essential histidine as a proton donor [30,31]. A search of the GH sequence alignments identified five totally conserved histidine residues. Any of these five residues could represent the catalytically essential histidine.

Cysteine-histidine peptidases can contain a catalytic dyad (e.g. caspases [31]) or contain an additional catalytic residue which stabilizes the thiolate-imidazolium pair and correctly orientates the histidine. Examples of a third catalytic residue are: asparagine in papain [31], aspartate in ubiquitin C-terminal hydrolase L3 [32] or glutamate in rhinovirus 3C protease [33]. Site-directed mutagenesis and molecular modelling studies are currently proceeding to establish the hypothesis of GH being a cysteine peptidase and to identify other essential catalytic residues.

The present study establishes that Cys-110 is required for the activity of GH. The hypothesis that GH is a cysteine peptidase contrasts with the characterization of glutamate carboxypeptidase II, a membrane-bound zinc metallopeptidase that also cleaves folyl polyglutamates [34,35]. The differing catalytic mechanisms may allow the design of inhibitors specific for the two enzymes in order to elucidate the role that these individual enzymes play in folate metabolism and in antifolate activity in neoplastic tissue.

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