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Cooperative Binding of γ-Glutamyl Substrate to Human Glutathione Synthetase

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Human glutathione synthetase is responsible for catalyzing the final step in glutathione biosynthesis. It is a homodimer with a monomer subunit MW of 52 kDa. Kinetic analysis reveals a departure from linearity of the Lineweaver–Burk double reciprocal plot for the binding of γ-glutamyl substrate, indicating cooperative binding. The measured apparent K_m values for γ-glutamyl-α-aminobutyrate (an analog of γ-glutamyl-α-aminobutyrate) are 63 and 164 μM, respectively. Neither ATP (K_m of 248 μM) nor glycine (K_m of 452 μM) exhibits such cooperative binding behavior. Although ATP is proposed to play a key role in the sequential binding of γ-glutamyl substrate to the enzyme, the cooperative binding of the γ-glutamyl substrate is not affected by alterations of ATP concentration. Quantitative analysis of the kinetic results for γ-glutamyl substrate binding gives a Hill coefficient (*h*) of 0.75, indicating negative cooperativity. Our studies, for the first time, show that human glutathione synthetase is an allosteric enzyme with cooperative binding for γ-glutamyl substrate.

Key Words: **glutathione; glutathione synthetase; enzyme kinetics; cooperativity.**

Glutathione (GSH; L-γ-glutamyl-L-cysteinylglycine) is found in high concentrations in almost all prokaryotic and eukaryotic cells (1). It has a variety of important biochemical functions such as protection against oxidative damage, maintenance of thiol groups of both proteins and low molecular compounds, and participation in amino acid transport. GSH functions as a cofactor for several enzymes, and is associated with radiation and alkylating agent resistance of tumor cells (2–4). Low GSH levels have been associated with atherosclerosis, AIDS, hepatitis C, arthritis, stroke, acute respiratory distress syndrome, diabetes, cataracts and neurological disorders (1–3, 5).

Glutathione is synthesized by two sequential ATP-dependent reactions. In the first reaction (Eq. [1]), the rate-limiting step for GSH synthesis, γ-glutamylcysteine synthetase catalyzes the formation of γ-glutamylcysteine. The second reaction (Eq. [2]) is catalyzed by glutathione synthetase (EC 6.6.2.3)(GS) to form GSH.

L-Glutamate + L-cysteine + ATP ⇒ L-γ-glutamyl-L-cysteine + ADP + Pi [1]

L-γ-Glutamyl-L-cysteine + glycine + ATP ⇒ GSH + ADP + Pi [2]

Glutathione, the product of GS (5–7), normally feedback inhibits γ-glutamylcysteine synthetase, thus, regulating cellular GSH levels. In GS deficiency, γ-glutamylcysteine synthetase is no longer regulated due to GSH levels, thus causing excessive formation of γ-glutamylcysteine. The later is converted by γ-glutamyl cyclotransferase into 5-oxoproline and cysteine. Cysteine is recycled for GSH synthesis, fueling a futile cycle, while 5-oxoproline is hydrolyzed to glutamate by 5-oxoprolinase (8, 9). The amount of 5-oxoproline formed in GSH deficiency exceeds the capacity of 5-oxoprolinase, and it accumulates in the body fluids, causing metabolic acidosis and 5-oxoprolinuria (2, 8–10).

Three forms of GS deficiency are distinguished clinically (11): the mild form is restricted to erythrocytes; the moderate and severe forms are expressed in multiple tissues. Patients with the mild form of GS deficiency have low GSH levels in erythrocytes, and often have mild hemolytic anemia. Patients with moderate form of GS deficiency are characterized by massive urinary excretion of 5-oxoproline, hemolytic anemia,

Abbreviations used: GSH, glutathione; GS, glutathione synthetase; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; LB, Luria broth.

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and metabolic acidosis. The severely affected patients present with impairments of CNS function on top of this. The mild form of the deficiency is thought to be due to mutations that affect the stability of the enzyme, while the moderate and severe forms are generally caused by mutations that impair the catalytic function of the enzyme. Kinetic studies of human recombinant mutant GS have revealed that mutations might affect both the stability of the enzyme and its ability to bind substrates, as well as to catalyze the reaction after substrate binding, i.e., K_m and V_{max} (12).

The most highly purified and most active mammalian GS has been purified from rat kidney (13). Extensive studies on the kinetic properties of this enzyme have been conducted on both native and recombinant rat GS (13–15). Previously (14), it was reported that native rat GS exhibited an unusual property for the binding of γ-glutamyl substrate. For example, the K_m values for γ-glutamyl-α-aminobutyrate (a non-thiol analog of γ-glutamylcysteine) could not be determined by normal kinetic models because of the departure from linearity of the double reciprocal plots. The apparent K_m values for γ-glutamyl-α-aminobutyrate were only estimated by extrapolation of the curved plot to be between 20 and 200 μM. This unusual kinetic behavior of recombinant rat GS was shown to be caused by negative cooperativity in the binding of the γ-glutamyl substrate (13). This finding provides an explanation for the departure from linearity on the Lineweaver–Burk plot of γ-glutamyl substrate. However, the structural mechanism for this kinetic phenomenon is not yet clear.

Since human GS is reported to share a high amino acid sequence identity (89%) with rat GS (15–17), it is likely to share the mechanism proposed for the rat enzyme (6). Sequence analysis identified several residues near the ATP binding domain of human GS as being different from those of rat enzyme. Since there is neither a crystal structure for the rat enzyme, nor a detailed kinetic study on the human enzyme, our studies focused on investigating whether human GS, like the rat enzyme, exhibits negative cooperativity in binding γ-glutamyl substrate.

MATERIALS AND METHODS

Materials. γ-Glutamyl-L-α-aminobutyrate (an analog of γ-glutamylcysteine) was synthesized as described (14). DNA modifying enzymes were obtained from New England Biolabs, Inc. (Beverly, MA). All reagents were obtained from Sigma (St. Louis, MO) unless indicated, and were of the highest purity possible.

Expression in E. coli and purification. The wild-type human GS was subcloned into pT7-7 plasmid for expression. The enzyme was expressed and purified using BL21/DE3 cells. Freshly transformed colonies were used to inoculate 2 liters of LB containing ampicillin (200 μg/ml), which was incubated at 37°C. When the cells reached 0.8 OD_{550}, protein expression was induced by the addition of isopropyl-β-D-galactopyranoside (0.8 mM). Cells were harvested by centrifugation 6–12 h after induction and were washed with 0.85% NaCl solution.

The recombinant enzyme was purified as described (13) with minor modifications. All steps were carried out at 4°C. Cells (~6 g, wet weight) were resuspended in Buffer A (50 mM imidazole hydrochloride, pH 7.4), sonicated, and the cellular debris removed by centrifugation (5000*g*, 10 min). The resulting supernatant was applied to a DE-52 column (Whatman, Maidstone England) (2.4 × 10 cm) previously equilibrated with Buffer A. The column was washed with 50 ml of Buffer A, and the enzyme was eluted (50 ml/h) with a linear gradient established between 400 ml of Buffer A and 400 ml of Buffer A containing 400 mM NaCl. Fractions containing enzyme activity were pooled and ammonium sulfate was added to 60% saturation. After stirring for 30 min at 4°C, the solution was centrifuged (12,000*g*, 30 min). The pellet was resuspended with 1 ml of Buffer A and applied to a Sephadex G150 column (Sigma, St. Louis, MO) (1.2 × 100 cm, 6 ml/h) preequilibrated with Buffer A. Fractions containing enzyme activity were pooled and ammonium sulfate was added to a final concentration of 1 M. After stirring, the solution was centrifuged (3000*g*, 10 min).

The resulting supernatant was applied to a Phenyl Sepharose (Pharmacia) column (0.6 × 5 cm) preequilibrated with Buffer A containing 0.5 M ammonium sulfate. The enzyme was eluted with a linear gradient established between 50 ml of the starting buffer and 50 ml of Buffer A. The fractions containing enzyme activity were pooled and concentrated (~2 ml) (Amicon). SDS/PAGE showed that the protein was homogenous with greater than 99% purity.

Enzyme assays and kinetic analysis. All kinetic analyses were done using purified recombinant GS. The enzyme activity was measured at 37°C using a spectrophotometeric assay, which couples ADP production to NADH oxidation (15). Buffer (100 mM Tris–HCl, pH 8.2, 50 mM KCl, 20 mM $MgCl_2$, 5 mM sodium phosphoenolpyruvate, 0.2 mM NADH) was mixed with 10 units of pyruvate kinase (Type III rabbit muscle) and 10 units of lactic acid dehydrogenase (type II rabbit muscle), as well as GS substrates to a final reaction volume of 1.0 ml. In the standard assay, the substrate, γ-glutamyl-α-aminobutyrate was used in place of γ-glutamylcysteine to avoid the complication of thiol oxidation. The assay was initiated by the addition of purified human GS. For specific activity measurements, the concentration of ATP, γ-glutamyl-α-aminobutyrate, and glycine were 1, 5, and 10 mM, respectively. Kinetic parameters were determined using the standard assay described above with various substrate concentrations. For the determination of K_m values, two substrates were held at saturating levels, while the third was varied by at least 10-fold around the putative K_m. Control reactions, in the absence of γ-glutamyl-α-aminobutyrate, were conducted to confirm that the measured ATP hydrolysis was substrate specific rather than a nonspecific ATPase. The rate of decrease in the absorbance at 340 nm was monitored at 37°C. A unit of enzyme activity is defined as the amount that catalyzes 1 μmole of product per minute. Protein concentration was determined by the Lowry method (18) using bovine serum albumin as the standard. Kinetic data were plotted and nonlinear regression analysis was performed using SigmaPlot software (SPSS Science Inc.).

RESULTS AND DISCUSSION

Recombinant human GS was purified to apparent homogenity using the efficient expression and purification procedure described in Materials and Methods, which allows accurate kinetic studies of non-tagged wild type enzyme. The apparent kinetic parameters of human GS determined in this study are listed in Table 1. The V_{max} of the purified recombinant human enzyme is 5.6 μmol/min/mg, which is similar to that previously reported for recombinant His-tagged human GS (12). The apparent K_m values for glycine (452 μM) and ATP (248 μM) were obtained from linear double reciprocal plots of 1/*v* versus

TABLE 1

Apparent Kinetic Parameters of the Recombinant Human Glutathione Synthetase

	V_{max} (μmol/mg/min)	K_m^{app} (μM) [γ-glu-α-AB]	[ATP]	[Gly]	*h* coefficient
Recombinant human GS	5.6	63/164[a]	248	452	0.75
Recombinant rat GS[b]	11.3	42/302[a]	37	913	0.55

Note. The enzymatic assays were carried out as described under Materials and Methods, and values are average of four separate purifications in duplicate measurements.

[a] The second K_m values were calculated as described under Results and Discussion.

[b] Data were from reference [12].

1/[S]. In contrast, similar attempts to determine the K_m values for γ-glutamyl-α-aminobutyrate in the presence of saturating concentrations of glycine and ATP gave distinctly nonlinear double reciprocal plots (Fig. 1A). The pattern of the curved double reciprocal plots with γ-glutamyl-α-aminobutyrate suggested that the human GS might be an allosteric enzyme. For an allosteric enzyme, the binding of one substrate molecule induces structural or electronic changes that alter the affinity for the other vacant site(s), and then the velocity curve no longer follows Michaelis–Menten kinetics. The departure from linearity of the double reciprocal plot, together with the finding of two active sites on the homodimer (15) suggest that there may be cooperative binding of the γ-glutamyl substrate for human GS.

In the present study, the pattern of the curved double reciprocal plot indicates a negative cooperative binding with respect to the γ-glutamyl substrate. According to the negative cooperative model, shown in Scheme 1, upon the binding of the first substrate molecule, the enzyme presumably undergoes a conformational change that alters the initial dissociation constant, K_s, for the second substrate by a factor α. In general terms, K_s gives an indication of the affinity of the enzyme for the substrate. Under the steady state conditions used in these experiments, the Michaelis–Menten equilibrium assumptions are valid and thus the Michaelis constant is approximately the same as the dissociation constant ($K_m \cong K_s$). Based on Scheme 1, the velocity equation for two substrate binding sites is thus modified as (19)

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

where [S] is the substrate concentration, V_{max} is the maximum velocity, v is the initial velocity, K_m is the substrate concentration at $\frac{1}{2}V_{max}$, and α is the interaction factor.

For the calculation of kinetic results, the velocity data for γ-glutamyl-α-aminobutyrate were fitted by nonlinear least-squares regression analysis with the equation above. The calculated K_m value for γ-glutamyl-α-aminobutyrate is 63 μM, and the calculated apparent factor α value is 2.6. Based on the model for cooperative substrate binding, when α is greater than 1, there is negative cooperativity where the binding of the first substrate molecule decreases the affinity of the enzyme for the second substrate molecule. The K_m value for the second site of γ-glutamyl-α-aminobutyrate binding is obtained by multiplying the

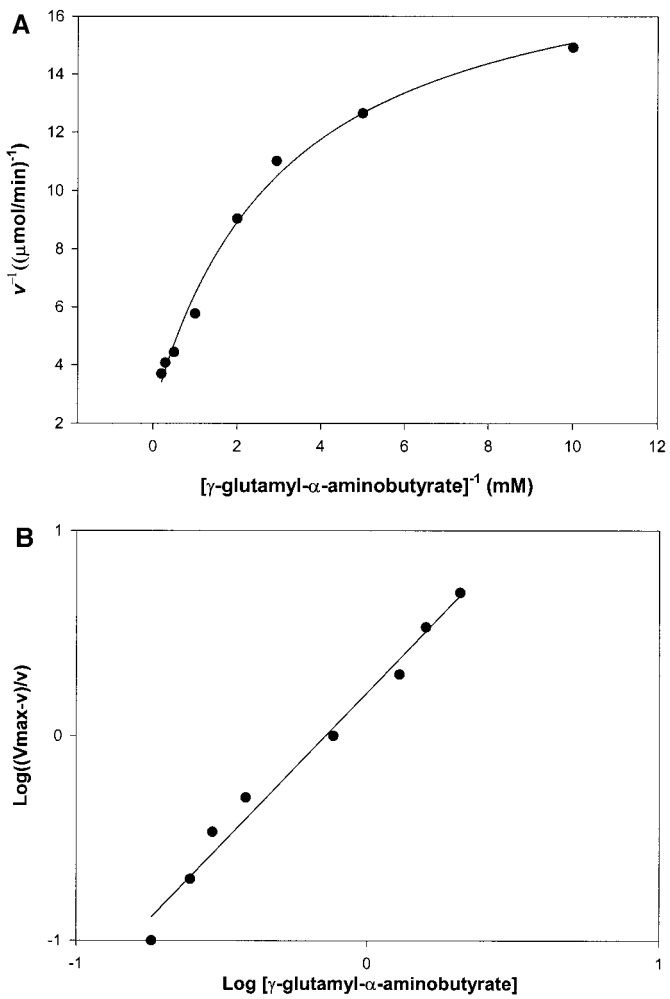
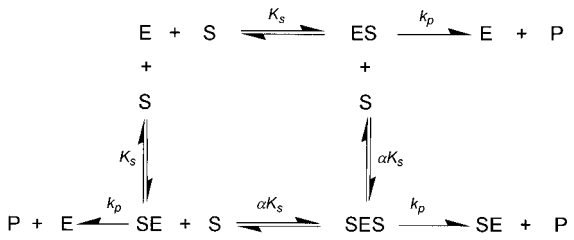


FIG. 1. (A) A representation of the Lineweaver–Burk double reciprocal plot for γ-glutamyl-α-aminobutyrate of purified wild-type human GS. The enzymatic assay was carried out by varying the concentration of γ-glutamyl-α-aminobutyrate while the concentrations of ATP and glycine were maintained at saturating concentrations. The data were a representation of four separate purified wild-type human GS and fitted by nonlinear least-squares regression analysis, and the line of best fit is shown. (B) A presentation of the Hill plot analysis of γ-glutamyl-α-aminobutyrate binding on the purified wild-type human GS. The binding of γ-glutamyl-α-aminobutyrate gives a Hill coefficient of 0.75.



SCHEME 1

first K_m values with the α factor. This calculation gives the second K_m value of 164 μM, indicating a change in affinity of enzyme–substrate interaction for the second substrate site by the factor α. This characteristic of cooperative binding is also reflected by the curved reciprocal plot of $1/v$ versus 1/[S] (Fig. 1A), in which the region with a high 1/[S] value corresponds to the normal hyperbolic saturation of the first (normal affinity) site at low substrate concentration. As the substrate concentration increases, the second site fills with lower affinity (higher K_m).

The observation of a nonlinear double reciprocal plot (Fig. 1A) and the calculated K_m values for the binding for γ-glutamyl-α-aminobutyrate, respectively, are consistent with the quantitative analysis of the cooperativity where the Hill plot is employed to describe the degree of cooperativity by the Hill coefficient (***h***). For the binding of γ-glutamyl-α-aminobutyrate, the ***h*** value was determined to be 0.75, indicating a negative cooperativity (Fig. 1B). Similar data analyses performed for both ATP and glycine give a Hill coefficient of 1 (data not shown), indicating that these two substrates do not display cooperative binding. The observed cooperative behavior of human GS appears to be localized to the γ-glutamyl substrate binding site.

Human GS has recently been identified as a member of ATP-grasp enzyme superfamily (20). Enzymes in this family catalyze a reaction that involves an ATP-dependent ligation of a carboxyl group carbon of one substrate with one amino group nitrogen of the second one to form an acylphosphate intermediate. In the case of GS, ATP reacts with γ-glutamylcysteine to form a γ-glutamylcysteinyl phosphate intermediate, which is consequently attacked by glycine to form GSH (6). Such a reaction mechanism indicates a close catalytic dependency between ATP and γ-glutamyl substrate, perhaps there might be a dependency of K_m for γ-glutamyl-α-aminobutyrate and on ATP concentrations. To examine this close relationship, various concentrations of ATP were used (between 1 and 10 mM) and the effects on γ-glutamyl-α-aminobutyrate kinetics determined. We found that even with these changes in ATP concentration, curved double reciprocal plots were obtained for γ-glutamyl-α-aminobutyrate (Fig. 2). The Hill coefficients (***h***) for each curve are 0.70, 0.74 and 0.75, indicating that the negative cooperative binding of the γ-glutamyl substrate does not change with changing ATP concentrations. Additionally, the binding of ATP is not cooperative for either rat (13) or human GS. These findings support the conclusion that the negative cooperativity is limited to the γ-glutamyl substrate-binding site.

In previous studies on rat GS (13), both thiol and non-thiol γ-glutamyl substrates (γ-glutamylcysteine and γ-glutamyl-α-aminobutyrate) displayed nonlinear double reciprocal plots. Both recombinant (non-glycosylated) (13) and isolated native rat kidney GS which contains carbohydrates (14), exhibit negative cooperative binding with respect to γ-glutamyl-α-aminobutyrate. These findings implicate that the thiol moiety of the γ-glutamyl substrate is not involved in this kinetic phenomenon, and glycosylation has no effect on the negative cooperative binding of γ-glutamyl substrate to GS.

One of the objectives of this study was to determine whether human GS exhibits similar kinetic parameters as the rat enzyme. Our results show that the human GS, like the rat enzyme, has negative cooperativity associated with γ-glutamyl-α-aminobutyrate binding for the human enzyme. Human GS has a slightly higher K_m value for γ-glutamyl-α-aminobutyrate substrate to bind the first binding site, but has a substantially lower K_m for the binding of the secondary site as compared to those of the rat enzyme. This suggests that the first active site of the human enzyme binds the γ-glutamyl substrate less effectively than the rat enzyme, but the second active site binds the γ-glutamyl substrate more easily than the rat en-

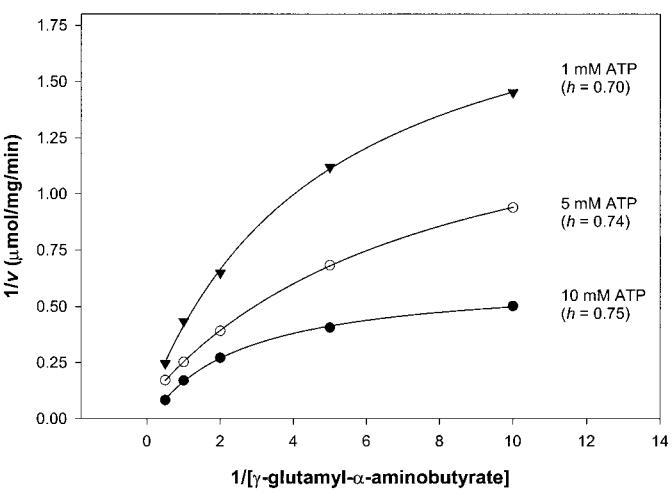


FIG. 2. A representation of the Lineweaver–Burk double reciprocal plot the purified wild-type human GS for γ-glutamyl-α-aminobutyrate at various ATP concentrations. The enzymatic assay was carried out at four separate purifications of human GS by varying the concentration of γ-glutamyl-α-aminobutyrate; the concentrations of ATP were at 1, 5, and 10 mM while glycine was maintained constant. The lines represent the simultaneous fit of all displayed date.

zyme does. It is also notable that the cooperativity of human enzyme is less than that of rat enzyme as reflected by the higher h coefficient value for human ($h = 0.75$) and lower values for rat enzymes ($h = 0.55$). Without the knowledge of the structure of the rat enzyme, it is not possible to explain exactly why human and rat enzyme behaves differently. However, it is plausible to suggest that non-conserved or semi-conserved residues involved in substrate binding and residues in regions for subunit dimerization, may play important roles in the difference of the kinetic effect.

The K_m values for ATP binding differ significantly between human and rat GS. The human enzyme has a much higher K_m value (248 μM) for ATP than that of rat enzyme (37 μM). Despite the high identity of the amino acid residues between the two enzymes, some variations in the local structure of the ATP binding site, as well as the non-conserved residues in the binding pocket may presumably account for the difference between the ATP K_m values of the two enzymes. In the case of glycine, the effective binding of this substrate is dependent to its proper alignment with γ-glutamyl phosphate intermediate. The lower K_m value for the binding of glycine in human enzyme might be an effect of a more effective binding of γ-glutamyl-α-aminobutyrate to the second binding site of the enzyme, by which the conformation change of the binding domain may facilitate a more effective binding of glycine.

In summary, our kinetic study of human GS shows that human GS is an allosteric enzyme. The finding of negative cooperativity for the binding of γ-glutamyl substrate explains the long-standing phenomenon of the non-linear Lineweaver–Burk double reciprocal plot for γ-glutamyl substrate (14) and supports the proposed acyl-phosphate intermediate formation mechanism for GS (6, 21). Negative cooperativity of mammalian enzyme is an unusual kinetic behavior and the structural basis for this effect requires further study.

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