Expression, purification, and characterization of recombinant human glutamine synthetase

Chad D. LISTROM*, Hiroki MORIZONO*, Basavapatna S. RAJAGOPAL†, Mark T. McCANN†, Mendel TUCHMAN† and Norma M. ALLEWELL*†

*Department of Biochemistry, College of Biological Sciences, University of Minnesota, St. Paul, MN 55108, U.S.A., †Department of Pediatrics, and Laboratory Medicine and Pathology, University of Minnesota Medical School, Minneapolis, MN 55455, U.S.A.

A bacterial expression system has been engineered for human glutamine synthetase (EC 6.3.1.2) that produces approximately 60 mg of enzyme (20\% of the bacterial soluble protein) and yields approx. 8 mg of purified enzyme per litre of culture. The recombinant enzyme was purified 5-fold to apparent homogeneity and characterized. It has a subunit molecular mass of approx. 45000 Da. The $V_{max}$ value obtained using a radioactive assay with ammonia and $\text{L-[G-}^3\text{H]glutamic acid as substrates was 15.9 \mu mol/min per mg. 40}$\% higher than that obtained in the colorimetric assay (9.9 \mu mol/min per mg) with hydroxylamine replacing ammonia as a substrate. $K_m$ values for glutamate were 3.0 mM and 3.5 mM, and for ATP they were 2.0 mM and 2.9 mM for the radioactive and spectrophotometric assays respectively. The $K_m$ for ammonia in the radioactive assay was 0.15 mM. The midpoint of thermal inactivation was 49.7 °C. Hydroxylamine, Mg(II) and Mg(II)-ATP stabilized the enzyme against thermal inactivation, whereas ATP promoted inactivation. The pure enzyme is stable for several months in storage and provides a source for additional studies, including X-ray crystallography.

INTRODUCTION

Glutamine is the most abundant amino acid in mammalian blood, making up as much as 20\% of the total amino acid content [1]. In addition to its role as a constituent of proteins, it is required for the biosynthesis of several amino acids via transamination, and its amide nitrogen is utilized in many biosynthetic pathways, including the synthesis of purines, pyrimidines, glucosamine and carbamoyl phosphate [2]. Glutamine synthetase (GS) (EC 6.3.1.2) is an ATP-dependent enzyme that catalyses the formation of glutamine from glutamic acid and ammonia [1–5].

Liver GS is expressed exclusively in 1–3 cell layers surrounding the central vein of the liver lobule [6] and plays an important role in the detoxification of ammonia that ‘escapes’ the urea cycle. This role of GS has been demonstrated in experiments in which hyperammonaemia was induced by selectively eliminating hepatic perivenous cells [7]. The perivenous localization of GS and perportal location of ureogenesis were also confirmed by metabolic perfusion studies in rat liver [8]. In contrast with the urea cycle, which is a low-affinity, high-capacity system, the high affinity of GS for ammonia allows removal of low concentrations of this toxin (high-affinity, low-capacity system) [8,9]. Thus GS may play a modifying role in urea-cycle disorders that result in hyperammonaemia, although polymorphisms or inherited defects in human GS have not yet been identified. A second important site for ammonia detoxification by GS is muscle [10,11]. GS is also expressed in brain astrocytes [12]; however, the role of brain GS in preventing ammonia toxicity is still unclear. In addition, brain GS is thought to regulate levels of the neurotransmitter $\gamma$-aminobutyric acid (GABA) [13].

Mammalian GS has been isolated from rat liver [14–17], rat brain [18], sheep brain [19,20], sheep spleen [21], pig brain [22–24], Chinese hamster liver [25] and human brain [26,27], but a mammalian overexpression system has not yet been reported. We have developed such a system for human GS in Escherichia coli and have purified the enzyme to homogeneity. This overexpression system enables substantial amounts of pure enzyme to be obtained for biochemical studies, generation of antibodies and crystallization to determine the three-dimensional structure of the enzyme.

EXPERIMENTAL

Materials

DEAE-Sepharose was purchased from Pharmacia, 2-mercapto-ethanol from Eastman Kodak, isopropyl thio-$\beta$-d-galactoside (IPTG) from Fisher Scientific, Ultrapure (NH$_4$)$_2$SO$_4$ from ICN Biomedicals, hydroxyapatite and acrylamide were from Bio-Rad, SDS was from Gibco BRL, and L-[G-$^3$H]glutamic acid was from Amersham Life Science. All other chemicals were purchased from Sigma Chemical.

Engineering of expression vector

The GS cDNA cloned into ‘Blue Script’ (pBS) was obtained from Dr. W. H. Lamers (University of Amsterdam, Amsterdam, The Netherlands) [28]. An $NdeI–Clai/SacI$ fragment containing the translation start of GS (+1) through base +124 was amplified by PCR using a forward primer with an $NdeI$ site (5′-CCCGG-GCATATGACCCCTCAAGTTCCC-3′), a reverse primer with $Clai$ and $SacI$ sites (5′-CCCCGGAGCTCATGATCAGATATACATGGC-3′) and the plasmid pBSHSGS as the template. The resulting fragment was digested with $NdeI$ and SacI and cloned into the expression vector pET21a+ (Novagen) to obtain the plasmid pETHGS1. Subsequently, the remaining portion of the GS cDNA was inserted into pETHGS1 using the internal $Clai$–HindIII fragment to obtain pETHGS2. The sequence of both strands of the PCR fragments was confirmed by

Abbreviations used: GABA, $\gamma$-aminobutyric acid; GS, glutamine synthetase; IPTG, isopropyl thio-$\beta$-d-galactoside.

† To whom correspondence should be addressed.
the dideoxynucleotide chain-termination method [29] using the Sequenase DNA sequencing kit (USB) and [\(\beta^32\)P]dATP (NEN).  

**Enzyme assays**

GS activity was measured using two different methods, a colorimetric approach as described by Wellner and Meister [30], with slight modifications, and an HPLC/radioactive method derived from an assay for ornithine transcarbamoylase developed by Tuchman et al. [31].

The colorimetric reaction mixture consisted of 100 mM imidazole/HCl, 50 mM sodium t-glutamate, 25 mM 2-mercaptoethanol, 20 mM ATP, 20 mM MgCl\(_2\) and 125 mM of freshly titrated hydroxylamine, which replaces NH\(_4\)Cl in this assay. Stock solutions of imidazole, t-glutamate and ATP were titrated to pH 7.2, and all reagents were stored on ice before assaying. 500 \(\mu\)l of the reaction mixture was pre-incubated for 2 min at 37 °C, and the reaction was initiated by the addition of 4 \(\mu\)g of enzyme. The reaction was quenched after 15 min with 750 \(\mu\)l of 0.37 M FeCl\(_3\)/0.67 M HCl/0.2 M trichloroacetic acid, and the absorbance was read at 535 nm against a blank without enzyme. Controls carried out with purified GS and lacking ATP or t-glutamate had no absorbance. All absorbances were within the linear range of the \(\gamma\)-glutamylhydroxamate standard curve. One unit of enzyme forms 1 \(\mu\)mol of \(\gamma\)-glutamylhydroxamate per minute.

The reaction mixture for the radioactive assay was essentially the same as the colorimetric assay, with the following modifications: 40 mM NH\(_4\)Cl replaced hydroxylamine, and 50 mM t-glutamate was mixed with t-[G-\(^3\)H]glutamate (49 mCi/\(\mu\)mol). t-[G-\(^3\)H]Glutamate accounted for less than 0.004 % of the total glutamate in the reaction mixture. A 3 \(\mu\)g amount of enzyme was used in the assay, and reaction times were varied between 5 and 15 min to obtain t-[G-\(^3\)H]glutamate peaks with greater than 10000 counts. Linearity for the radioactive assay was established by monitoring enzyme activity with 2-8 \(\mu\)g of enzyme and reaction times from 2 to 15 min. No deviation from linearity was observed over these ranges. The reaction was quenched with 40 \(\mu\)l of 30 % trichloroacetic acid and the assay mixture was filtered through a 0.2 \(\mu\)m filter. A 25 \(\mu\)l aliquot was injected into a 150 mm \(\times\) 4.6 mm, 5 \(\mu\)m particle size, NH\(_4\)HPLC column (Econosphere; Alltech Associates), and a binary solvent system [31] was used to separate t-[G-\(^3\)H]glutamine from t-[G-\(^3\)H]glutamate with a 1 mL/min flow rate. The two peaks eluted approx. 7 min apart. The HPLC column was connected to a radioactivity flow monitor (Radiomatic Instruments), which quantified the two peaks using a 250 \(\mu\)L flow cell and scintillation fluid/eluate ratio of 4:1. Units of activity are the same as in the colorimetric assay.

In order to avoid excessive substrate depletion when determining the \(K_m\) for ammonia with the radioactive assay, reaction times were varied from 2 to 5 min and 2 \(\mu\)g of enzyme was used. The sensitivity of the radioactive assay was enhanced by directly collecting the glutamine peaks from the HPLC column and counting them for radioactivity in a Beckman LS 7500 scintillation counter using a scintillation fluid/eluate ratio of 5:1.

**Protein purification**

Transformed *E. coli* cells were grown overnight at 37 °C in 50 ml of Luria broth containing 100 \(\mu\)g/ml ampicillin. An aliquot of 10 ml of this culture was transferred to 1 litre of the same medium, and expression of GS was induced with 0.1 mM IPTG when the cell density reached \(A_{590}\) of 0.4–0.8. The cells were allowed to grow overnight at 25 °C and were harvested by centrifugation. All centrifugations were performed with a GSA rotor \((r_{max} \approx 14.57\) cm) at 4 °C and 4000 \(g\) for 15 min unless otherwise specified. The pelleted cells were resuspended in 10 mM potassium phosphate/10 mM 2-mercaptoethanol, pH 7.2, and centrifuged. Typical wet cell weights were 3 g of cells/litre of culture. The cells were resuspended in approx. 10 ml of the phosphate buffer per g of cells, sonicated on ice for 10 min using a 90 %, on/10 % off pulse, and centrifuged.

The remainder of the enzyme purification was essentially as described by Tate et al. [16]. The supernatant from the centrifugation step (35 ml) was loaded on to a 7.0 cm \(\times\) 3.0 cm hydroxypatite column that had been equilibrated in 10 mM potassium phosphate/10 mM 2-mercaptoethanol, pH 7.2, at room temperature and a flow rate of 1.2 ml/min. The column was washed first with 100 mM potassium phosphate/2 mM 2-mercaptoethanol, pH 7.2, until the absorbance approached the baseline and then with 160 ml of a linear 150–300 mM potassium phosphate/10 mM 2-mercaptoethanol, pH 7.2, gradient. GS eluted in the second half of the gradient as a small broad peak on the trailing shoulder of the gradient peak. Fractions of 5 ml each were collected; the active fractions were pooled, yielding a total volume of 50–100 ml, and were precipitated with 2 M (NH\(_4\))\(_2\)SO\(_4\) for 30 min on ice. The precipitated protein was collected by centrifugation in a SS-34 rotor \((r_{max} \approx 10.70\) cm) at 4 °C and 23500 \(g\) for 40 min, resuspended in 15 ml of 10 mM imidazole/HCl/2 mM EDTA/5 mM 2-mercaptoethanol, pH 7.9, and dialysed against this same buffer overnight. This dialysed solution (19 ml) was loaded on to a 1.5 cm \(\times\) 10 cm DEAE-Sepharose fast-flow column at room temperature and a flow rate of 4.8 ml/min. GS eluted as a sharp peak (15–20 ml) with 20 mM imidazole/HCl/2 mM EDTA/5 mM 2-mercaptoethanol, pH 6.2. A very small peak eluted just before GS but did not contain GS activity and was not collected with the GS peak.

Purity was assessed by SDS/PAGE as described by Sambrook et al. [32]. The purified protein was dialysed against 10 mM imidazole/HCl/2 mM EDTA/5 mM 2-mercaptoethanol, pH 7.2, and stored at 4 °C. The enzyme retained its activity under these storage conditions for several months. GS was stored as an (NH\(_4\))\(_2\)SO\(_4\) precipitate at 4 °C for longer periods. Protein concentration was determined with a dye-binding assay (BioRad) using BSA as a standard [33].

**Thermal inactivation**

A sample of 50 \(\mu\)l of GS (0.4 mg/ml) in 10 mM imidazole/HCl/2 mM EDTA/5 mM 2-mercaptoethanol, pH 7.9, was heated for 10 min at the desired temperature \(\pm 1\) °C and cooled on ice for 5 min, and 10 \(\mu\)l was added to the colorimetric assay mixture. The activity assay was performed as described earlier except that the reaction was initiated with hydroxylamine. Enzyme activity was measured as a percentage of a control sample that was not heated. To determine whether substrates prevented or promoted thermal inactivation, GS was incubated with various substrates or KCl at concentrations of 30 mM for 10 min at 51 °C and then assayed as described above.

**RESULTS**

Human GS was cloned into an inducible PET 21a(+) expression vector and purified to homogeneity. A representative purification is shown in Table 1. The enzyme constituted approx. 20 % of the total soluble protein of the bacterial cell. Bacterial GS levels were determined by measuring GS activity in *E. coli* cells containing
Recombinant human glutamine synthetase

Table 1 Purification of recombinant human GS

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysate</td>
<td>35.0</td>
<td>262.2</td>
<td>511.8</td>
<td>2.0</td>
<td>1.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Cell supernatant</td>
<td>35.0</td>
<td>181.9</td>
<td>307.5</td>
<td>1.7</td>
<td>0.9</td>
<td>60.1</td>
</tr>
<tr>
<td>Hydroxyapatite fraction</td>
<td>96.0</td>
<td>22.3</td>
<td>86.4</td>
<td>3.9</td>
<td>2.0</td>
<td>16.9</td>
</tr>
<tr>
<td>DEAE fraction</td>
<td>17.0</td>
<td>7.1</td>
<td>67.3</td>
<td>9.5</td>
<td>4.9</td>
<td>13.2</td>
</tr>
</tbody>
</table>

Figure 1 SDS/PAGE of human GS at various stages in the purification

Lane 1, cell lysate; lane 2, hydroxyapatite fraction after precipitation with 2 M (NH₄)₂SO₄ to concentrate the fraction and dialysis to remove excessive salt; lane 3, purified GS after fast-flow DEAE chromatography.

the pETOTCII plasmid bearing the human ornithine trans-carbamoylase gene but not the human GS gene. Simply measuring GS activity in uninduced cells that contain the human GS gene was not adequate, as there is some ‘leakage’ of the pETHGS2 plasmid. Controls omitting glutamate and ATP were also incorporated into all measurements of GS activity in crude extracts, as Ehrenfeld et al. [34] have reported that glutaminases can produce artificially high activities in crude extracts by catalysing ATP-independent formation of γ-glutamylhydroxamate. Less than 1% of GS activity in crude extracts was found to be contributed by E. coli GS.

The enzyme lost all activity when cells were sonicated in an imidazole buffer, but dialysis in imidazole later in the purification did not reduce activity. About 30–40% of the enzyme aggregated into inclusion bodies, and approx. 75% of the enzyme loaded on to the hydroxypatite column was lost in the wash fractions. Reloading the wash fractions on to the hydroxypatite column did not substantially increase the yield. The specific activity of the purified enzyme was 9.0 ± 1.0 µmol/min per mg. Yields from several preparations ranged from 10 to 20%, with 1 litre of culture typically yielding 8 mg of pure enzyme.

SDS/PAGE showed only a single band, indicating that the enzyme is composed of subunits with identical mass. Figure 1 illustrates the purity of GS after the DEAE column. Calibration with proteins of known molecular mass indicated a monomer mass of 45000 Da, in agreement with previous studies. Yamamoto et al. [26] and Tumani et al. [27] report a value of 44000 Da for the monomer of human brain GS, which is believed to assemble as an octamer. Reported monomer molecular masses from other mammalian species range from 42000 Da in Chinese hamster liver [25] to 49000 Da in sheep brain [16]. A Western blot showed that purified human GS was immunoreactive against polyclonal antibodies produced in rabbit (results not shown).

Substrate titration curves were fit as described by Morizono et al. [35] using the non-linear least-squares regression program NONLIN [36] to determine $K_m$ and $V_{max}$ values (Figure 2). Table 2 shows the $K_m$ and $V_{max}$ values for L-glutamate and ATP obtained from the two assay methods and for ammonia as determined by the radioactive assay. While the $K_m$ values varied only slightly between the two assay methods, the $V_{max}$ values were substantially higher in the radioactive assay, which uses the natural substrate, ammonia, rather than the artificial substrate,
Table 2  Comparison of $V_{\text{max}}$ and $K_m$ for radioactive and colorimetric assays

<table>
<thead>
<tr>
<th></th>
<th>L-Glutamate</th>
<th>ATP</th>
<th>Ammonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioactive assay</td>
<td>$V_{\text{max}}$, µmol/min per mg</td>
<td>$K_m$, mM</td>
<td>$V_{\text{max}}$, µmol/min per mg</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>15.9 ± 1.6</td>
<td>13.8 ± 1.8</td>
<td>15.4 ± 2.7</td>
</tr>
<tr>
<td>$K_m$</td>
<td>3.0 ± 1.2</td>
<td>1.8 ± 1.0</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>Colorimetric assay</td>
<td>$V_{\text{max}}$, µmol/min per mg</td>
<td>$K_m$, mM</td>
<td>$V_{\text{max}}$, µmol/min per mg</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>9.9 ± 0.6</td>
<td>9.4 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>$K_m$</td>
<td>3.5 ± 0.7</td>
<td>2.8 ± 1.1</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3  Thermal inactivation of human GS in 10 mM imidazole/HCl/2 mM EDTA/5 mM 2-mercaptoethanol, pH 7.9

The curve was generated with enzyme from two independent preparations and was fit assuming a two-state transition. The calculated error in the temperature at the midpoint of the transition corresponds to the 99% confidence interval. Mean percentages and S.D. values were obtained from four values in the fully active and fully inactive phases and six values in the transition region.

Figure 4  Substrate stabilization/inactivation of human GS

The enzyme was incubated for 10 min at 51 °C, cooled on ice and assayed. The concentration of all substrates and added KCl was 30 mM. Mean percentages and S.D. values were obtained from four values except in the absence of substrate, where eighteen values were available.

hydroxylamine. In the ATP titrations, ATP became inhibitory when its concentration exceeded the Mg(II) concentration. This effect is consistent with the results of Iqbal and Wu [15] for rat liver GS and of Tiemeier and Milman [25] for Chinese hamster liver GS. At a Mg(II)/ATP ratio of 0.6, we observed a reduction in GS activity of approx. 43%.

The midpoint of thermal inactivation for GS was 49.7 ± 2.0 °C (Figure 3). Figure 4 shows the percent of GS activity remaining after incubating near its midpoint of inactivation in the presence of substrates or KCl. Incubation with hydroxylamine, Mg(II) or Mg(II)-ATP during the heating cycle stabilized GS against inactivation, while incubation with ATP alone promoted almost complete inactivation. Glutamate and glutamine, the natural product of catalysis, had no effect on thermal stability. Although KCl is not a substrate, it was included to determine whether the stabilization effects observed with substrates resulted from ionic strength protection. GS activity was neither enhanced nor inhibited in control experiments in which the enzyme and substrate incubations were not heated.

**DISCUSSION**

Large amounts of recombinant human GS were produced by the inducible expression system described here. Pure human GS protein was obtained with only a 5-fold purification, although a large portion of the protein was lost to inclusion bodies and on the hydroxyapatite column. Further efforts to reconstitute the inclusion bodies into soluble enzyme and reduce the amount of enzyme lost on the hydroxyapatite column could increase the yield significantly. Chromatography at 4 °C may help improve the yield from the hydroxyapatite column. The enzyme was stable for several months in an imidazole buffer without preservatives, but 2-mercaptoethanol must be added to the storage buffer, as GS can be catalytically inactivated by oxidation [37]. Recombinant human GS is similar to other GSs in terms of monomer molecular mass and kinetic constants. GS was completely inactivated at 60 °C and had a midpoint of inactivation at 49.7 °C when incubated in 10 mM imidazole/HCl/2 mM EDTA/5 mM 2-mercaptoethanol, pH 7.9. Since GS is a cytosolic enzyme, it may be more stable if incubated at a pH closer to 7.0. Stahl and Jaenicke [22] reported a rapid loss of pig brain GS activity between 60 and 61 °C when incubated for 15 min in 50 mM imidazole/1 mM EDTA/10 mM thiglycol, pH 7.0. Substrates can stabilize the enzyme against thermal inactivation by binding to the enzyme and preventing the global and/or localized changes that cause inactivation. Hydroxylamine, Mg(II) and Mg(II)-ATP reduced thermal inactivation when incubated with GS at 51 °C for 10 min.

Surprisingly, ATP by itself promoted almost complete inactivation of the enzyme when incubated at elevated temperatures. ATP may disrupt interactions crucial to maintaining the structure of the enzyme or irreversibly bind to the enzyme so the Mg(II)-ATP complex, the required substrate for catalysis, cannot bind to the active site. The ATP inactivation of GS was temperature dependent, since enzyme and ATP that were incubated without heating did not show a reduction in enzyme activity. Hydrolysis of ATP cannot account for the reduced activity, since ATP heated by itself and then used in the assay resulted in normal activities. Glutamate and glutamine had no effect on the thermal stability of GS. KCl provided some non-specific stabilization, but it was not as effective as the substrates.

The $V_{\text{max}}$ values obtained in the radioactive assay with ammonia as substrate were approx. 40 %, higher than those obtained in the more widely used colorimetric method with hydroxylamine as substrate. There are at least two possible sources of the discrepancy between the $V_{\text{max}}$ values obtained with the two assay procedures. The colorimetric assay uses an artificial substrate,
whereas the radioactive assay uses the natural substrate. $V_{\text{max}}$ values for these two substrates may differ. Alternatively, a component of the colorimetric assay mixture may interfere with the assay. Wu [21] observed that increasing ATP concentrations interfere linearly with colour formation in the colorimetric assay and give reduced $V_{\text{max}}$ values. However, we were unable to identify any component of the colorimetric assay that would account for the $V_{\text{max}}$ difference between the two assays. Since the radioactive assay makes use of natural substrates, allows glutamine formation to be monitored directly and is less subject to interference, it is clearly the method of choice for studies of enzyme kinetics.

The $K_m$ value obtained for ammonia indicates that GS has a high affinity for ammonia, so that GS may act as a scavenger for ammonia. Since the affinity for ammonia is high, the involvement of GS in various tissues in ammonia detoxification may be limited by the amount of enzyme present. Cooper et al. [38] and Girard and co-workers [11,39] concluded that brain GS works at capacity, and there is little possibility that it can reduce high ammonia levels; however, Hod et al. [10] reported increased ammonia uptake and GS activity in skeletal muscle of hy- perammonaemic rats. The possibility that GS in extrahepatic tissues acts to trap excess ammonia and has a protective function in individuals with mutations in urea-cycle enzymes or other diseases that cause hyperammonaemia warrants further investigation.

Development of this expression system will enable regions of the enzyme that are critical to function to be identified. Naturally occurring mutations have not been identified yet, probably because ‘severe’ mutations in GS are lethal to the developing organism; for example, mutations in Drosophila melanogaster result in embryo lethal female sterility [40]. Human GS has a high degree of homology to GS from other species, with several stretches of amino acids that are strongly conserved. Kumada et al. [41] identified four regions that are functionally similar between prokaryotes and eukaryotes and suggested that the GS gene may be one of the oldest genes in existence. Hence, it will be feasible to use site-directed mutagenesis to determine whether highly conserved regions with known functions in prokaryotes have the same functions in the human enzyme.

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REFERENCES


13 Johnson, J. L. (1972) Brain Res. 37, 1–19


19 Pamiljans, V., Krishnaswamy, P. R., Dumville, G. and Meister, A. (1962) Biochemistry 1, 155–158


