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Glutamine Synthetase Isolated from Human Brain: Octameric Structure and Homology of Partial Primary Structure with Human Liver Glutamine Synthetase

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Abstract—Glutamine synthetase (GS) has been purified from the cytosolic fraction of non-frozen human brain tissue. The purified GS migrated as a main band around 44 kD on reducing SDS-PAGE. Two-dimensional electrophoresis revealed heterogeneity within subunits of GS. The masses of eight different peptides from a tryptic digest of GS as measured by high resolution MALDI-MS matched with the respective masses from an *in silico* tryptic fingerprint of the Swiss-Prot database entry of human liver GS, proving that at least 24% of the primary sequences of GS from brain and liver are identical. Sedimentation equilibrium profiles obtained from analytical ultracentrifugation experiments at 10°C showed that human brain GS is mainly octameric. The quaternary structure of human brain GS at 10 μM (subunit concentration) was not significantly affected by cations, such as magnesium (5 and 20 mM) or manganese (0.2 and 1 mM) within the range of pH 7.1-7.8.

Key words: glutamine synthetase, human brain, primary structure, octameric structure, analytical ultracentrifugation, quasi-elastic light scattering

Glutamine synthetase (GS, EC 6.3.1.2) is one of the key enzymes of glutamate metabolism. Because it is a key enzyme of the brain glutamine/glutamate cycle and of its regulatory role in the brain glutamate neurotransmitter pool [1], it is essential to understand the molecular structure of human GS (huGS) and factors that influence its activity. It seems that huGS is involved in Alzheimer's disease [2-4] and schizophrenia [5] as there are alterations in activity and level of brain huGS in these two pathologies of the human central nervous system.

In mammalian tissues, GS is not uniformly distributed. Most GS activity is found in liver, brain, and kidney, while minor activities are in muscle, spleen, testes, and heart [6, 7]. Liver GS was studied in relation to hepatic damage [8] and a human liver cDNA for GS was sequenced [9, 10]; the human GS gene was expressed in *Escherichia coli* and the active enzyme was purified in preparative amounts [11].

On the basis of their primary structures, bacterial and mammalian GSs belong to different classes of the large family of GS proteins. The quaternary structures of all known GSs are proposed to be homooligomeric with variable oligomerization (e.g., octamers, dodecamers) and stacking symmetry. Although the subunit stacking and folding in bacterial GS has been intensively investigated [12-14], little has been published on the mammalian GS in general and on huGS found in brain in particular.

For sheep brain GS, a concentration dependent equilibrium between several oligomers that is influenced by metal ions has been identified *in vitro*. A homooctameric quaternary structure was proposed for this enzyme *in vivo* [17]. The quaternary structure of huGS may be similar to that of sheep brain GS, although many biochemical properties of these two proteins are different [15, 18]. The data obtained from native gel electrophoresis suggested that huGS migrated as a tetramer [15] or as an oligomer with an estimated molecular mass of 360-400 kD [16].

We have reported a preparative method for the purification of huGS from frozen [18] and non-frozen [19] brain tissue with improved protein yield enabling the study of its quaternary structure. Our unpublished data on

Abbreviations: GS) glutamine synthetase; huGS) human glutamine synthetase; MALDI-MS) matrix-assisted laser desorption ionization mass spectrometry; FPLC) fast performance liquid chromatography.

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the formation of microtubule structures from tubulin suggested that enzymes purified from non-frozen brain tissue might be closer to their native structure than those purified from frozen tissue. Hence, in contrast with previous studies, our starting material was a cytosolic fraction of non-frozen human brain tissue.

MATERIALS AND METHODS

Human brain. The starting material for huGS purification (human brain hemisphere samples obtained at autopsy from two individuals with no history of mental or neurological disorders) was the same as previously described [19]. Brain samples (total weight 920 g) were placed on ice and processed no later than 6 h after death.

Purification of huGS from brain. The purification procedure differed from previously described [19] in minor modifications: tissue was minced with a scalpel, suspended in five volumes of ice-cold 10 mM HEPES, pH 7.4, containing 220 mM mannitol, 70 mM sucrose, 0.2 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (buffer proposed for isolation of mitochondria [20]) and then homogenized with an IKA-Werk Ultra-Turrax knife homogenizer (Germany). Insoluble material was removed by centrifugation in two steps, at 650*g* for 10 min and then at 27,000*g* for 30 min; a cut of 20-40% relative ammonium sulfate saturation was used for further GS purification as previously described [19]. The preparation obtained was further concentrated on a PM-10 membrane filter (Amicon, USA) and subjected to size exclusion chromatography in 50 mM Tris-HCl, pH 7.5, with 100 mM NaCl, 1.5 mM 2-mercaptoethanol using a 0.9 × 60 cm column packed with Sepharose CL-6B (gel and FPLC system; Pharmacia, Sweden). The purified human GS was supplemented with 40% glycerol and stored at −20°C. For structural investigations glycerol was removed by small scale size exclusion chromatography. A 1.5-ml aliquot of purified huGS (about 0.4 mg huGS in 1 ml) was concentrated to a final volume of 850 µl in a centrifugal filter device (Ultrafree 10 kD; Millipore, USA), 200 µl aliquots of the protein concentrate were fractionated using a Superdex 200 gel filtration column (PC 3.2/30, installed in a SMART system; Pharmacia), equilibrated either with 50 mM Tris-HCl, pH 7.5, or 50 mM MOPS, pH 7.1, both containing 200 mM NaCl and 1 mM 2-mercaptoethanol. Enzymatic huGS activity was evaluated during the enzyme purification by the Mn^{2+}-stimulated transferase assay system as described [18, 19].

Just before quasi-elastic light scattering and analytical ultracentrifugation experiments, an additional size exclusion chromatography purification step was performed with purified huGS, to remove the stabilizing reagent glycerol and any unspecific aggregates that might have formed during the storage period, as well as possibly further reducing the amount of contaminants.

The huGS eluted from the Superdex 200 PC 3.2/30 column as an almost symmetrical peak with the position of a globular protein of about 290 kD. Single fractions from the peak region were more homogeneous than the starting material when analyzed by SDS-PAGE and were pooled for quasi-elastic light scattering and analytical ultracentrifugation analyses. Apparently, this pool did not contain contaminants between 55 and 90 kD, whereas bands at 33 and around 100 kD were slightly reduced.

SDS-PAGE and two-dimensional PAGE. SDS-PAGE was carried out in 12 × 13.5 × 0.075-cm slab gels containing 10% acrylamide [21] in a model 220 chamber (Bio-Rad, Austria) or on ready made 4-20% pre-cast gels (Novex, USA) as indicated. Gels were stained with Coomassie Brilliant Blue R-250.

Two-dimensional electrophoresis in polyacrylamide gels was performed according to O'Farrell [22] with minor modifications as described previously [19] using a GE-2/4 LS gel electrophoresis apparatus (Hoefer-Pharmacia, Sweden) for separation in the first dimension. SDS-PAGE in the second dimension was performed using the model 220 device (Bio-Rad). Gels were stained with Coomassie Brilliant Blue R-250, scanned with a soft laser scanning densitometer (model Hi-speed 1D/2D Laser Scanner, Zeineh, USA), and images were processed using the Zeineh programs (Biomed software, Biomed Instruments, Inc., USA).

Tryptic protein digestion within cut out bands from polyacrylamide gels. Bands were excised and placed into Eppendorf tubes. Each band was de-stained with 100 µl of 30% acetonitrile in 50 mM ammonium bicarbonate and dried in a vacuum evaporator. Each dried gel piece was rehydrated with 4 µl of 3 mM Tris-HCl, pH 9.0, containing 50 ng trypsin (Promega, USA). After 16 h at room temperature, 7 µl of H_2O was added to each gel piece and the samples were shaken in a Titrimax 1000 shaker (Heidolph, Germany) at position 5 for about 10 min. Four microliters of 50% acetonitrile, containing 0.3% trifluoroacetic acid, the standard peptides des-Arg-bradykinin (Sigma, USA, 904.4681 daltons), and adrenocorticotropic hormone fragment 18-39 (Sigma, 2465.1989 daltons) in water, was added to each gel piece and shaken as above.

Amino acid sequencing. Amino acid sequencing of selected peptides was performed as described [23].

Matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS). MALDI-MS analysis was performed as described elsewhere [24] with some modifications. The application of the samples was performed with a SymBiot I sample processor (PE Biosystems, USA). The peptide mixture (1.5 µl) was simultaneously applied with 1 µl of matrix consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile containing 0.1% trifluoroacetic acid.

Samples were analyzed in a time-of-flight mass spectrometer (Reflex 3, Bruker Analytics, Germany). The accelerating voltage was 20 kV. Peptide matching and protein searches were performed automatically with the use of in-house developed software [25]. The peptide masses were compared with the theoretical peptide masses of all available proteins from all species. Monoisotopic masses were used and a mass tolerance of 0.0025% was allowed. The algorithm used for determining the probability of a false positive match with a given MS-spectrum is described elsewhere [25]. Four matching peptides were required for an identity assignment. Unmatched peptides or miscleavage sites were not considered.

Quasi-elastic light scattering. Quasi-elastic light scattering analysis was done using a MSTC instrument (Protein Solutions Ltd., UK) with a 200 mW solid state laser as light source (824 nm), a thermostatted sample cell and an avalanche photodiode as photon detector mounted at a fixed angle of 90°. Insoluble components and dust were removed from samples during filling of the sample cell by using a microfilter system (Protein Solutions Ltd., UK) and filtering through bovine serum albumin saturated 0.1 μm pore-sized filters (Anodisc 13; Whatman, UK). The removable sample cell required 12 μl for filling of the optical pathway but at least 20-30 μl of sample were required for the overall filling process including filtration. Measurements were done at 20°C.

Data were analyzed and the translational diffusion coefficient was determined using the software Dynamics, version 3.3 (Protein Solutions Ltd.). This software allows monomodal or bimodal analysis by fitting the auto correlation function obtained with either one or two exponentials, respectively. Furthermore, the software computes a polydispersity parameter that reflects the broadness of distribution of molecular species [26].

Analytical ultracentrifugation. Analytical ultracentrifugation measurements were performed with the analytical ultracentrifuge Optima XL-A (Beckman, USA) mainly following established protocols [27]. Sedimentation equilibrium runs were performed at 10 or 20°C, using a four hole rotor with six channel cells, that allowed us to run nine parallel samples. Samples were prepared by mixing huGS, either dissolved in 50 mM Tris-HCl buffer, pH 7.8, or in 50 mM MOPS buffer, pH 7.1, with tenfold stock salt solutions. The data were evaluated by fitting different models to the measured profiles using the software DISCREEQ (P. Schuck; NIH, USA) [28]. Baseline values were always experimentally determined by meniscus depletion for 2 h at 40,000 rpm at the end of the experiment.

RESULTS

Purification of huGS and characterization of its subunits by electrophoresis in polyacrylamide gel. For structural investigations, huGS was purified from non-frozen brain tissue supernatants as recently published [19]. SDS-PAGE of purified human brain GS using a 4-20% polyacrylamide gel gradient gel showed a prominent band at about 44 kD that agreed well with the computed molecular mass of a human liver GS monomer primary sequence (42.1 kD, Swiss-Prot database entry P15104). Some faint protein bands, mainly at about 33, 55-90, and 100 kD, were also observed (Fig. 1, lanes *1* and *2*).

Two-dimensional electrophoresis in polyacrylamide gel was carried out to compare subunit properties of purified GS from non-frozen and frozen human brain tissue [15, 18]. The patterns for GS purified from non-frozen and frozen tissues are shown in Fig. 2. The huGS purified from both non-frozen and frozen tissues gave several spots (at least five and six respectively) on two-dimensional electrophoresis in polyacrylamide gel, all located in the p*I* interval from 6.7 to 6.4 and corresponded to a molecular mass around 44 kD. The density distribution between the spots in huGS purified from non-frozen tissue differed from that of huGS isolated from frozen tissue. Relative spot densities (in percentages, from basic to acid p*I*) for huGS in the first case were estimated as 7 : 53 : 22 : 14 : 4, whereas the spot densities for huGS from frozen tissue were estimated as 23 : 26 : 23 : 17 : 8 : 3.

Characterization of purified huGS by quasi-elastic light scattering. The obtained correlation function fitted

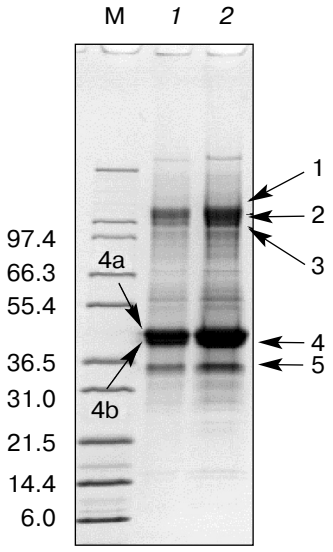

Fig. 1. SDS-PAGE pattern of huGS (two different concentrations, lanes *1* and *2*) purified on Superdex 200 column. Protein bands No. 1-5 were analyzed by mass spectrometry. Lane M, mixture of standard proteins (bovine lung aprotinin, chicken egg white lysozyme, soybean trypsin inhibitor, carbonic anhydrase, porcine lactate dehydrogenase, bovine liver glutamate dehydrogenase, bovine serum albumin, rabbit muscle phosphorylase *b*, *E. coli* β-galactosidase, rabbit muscle myosin); their molecular masses are given on the left in kD.

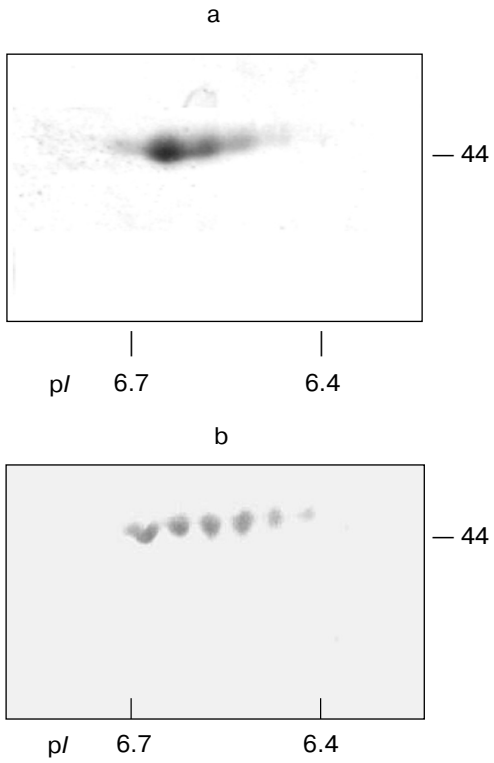

Fig. 2. Polyacrylamide gels stained with Coomassie R-250 after two-dimensional electrophoresis of purified huGS from non-frozen (a) and frozen (b) brain tissue. The benchmark for molecular mass is given on the right (in kD), and the p*I* scale is given at the bottom of each panel.

well with a single exponential function, returning a translational diffusion coefficient of $D_{w,20} = (3.2 \pm 0.1) \cdot 10^{-7}$ cm^2·sec^{-1}. The size distribution of scattering particles appeared to be narrow. Assuming globular molecular shape, a molecular mass of about 270 kD was calculated from this diffusion coefficient using the Stokes–Einstein equation.

Characterization of purified huGS by analytical ultracentrifugation. Samples for sedimentation equilibrium analysis with a huGS concentration of 0.4 mg/ml (10 μM subunit concentration) were prepared in two different buffers and supplemented with magnesium and manganese cations at different concentrations as described in Table 1.

Wavelength scans between 200 and 400 nm were taken of all samples immediately after reaching a rotor speed of 3,000 rpm. With the exception of sample No. 8, where no absorption was seen because 1 mM $ZnCl_2$ precipitated the huGS protein which had pelleted, all other samples appeared to be within a suitable concentration range for equilibrium sedimentation analysis. Comparison of radial scans at 5,000 rpm and 10°C obtained after 16, 23, and 40 h proved that equilibrium was essentially reached by 40 h.

As an example, the sedimentation equilibrium profile of huGS at pH 7.8 in the absence of added metal ions (sample No. 1), measured at 275 nm after 40 h at 5,000 rpm, is shown in Fig. 3. The profile fits well with a single-component model, returning a molecular mass of 335.1 kD which is close to the theoretical mass of octameric huGS (336.5 kD). The octamer model is well confirmed by the

Table 1. Molecular masses as obtained from best fits of the one component model to profiles at 280 and 275 nm (averages) as obtained after 40 h at 5000 rpm and 10°C

Sample No.	Conditions	Molecular mass, kD	Quality***	X_r^2 (280/275 nm)
1*	no added salt, Tris-HCl	339 ± 5	++	0.57/0.55
2	no added salt, MOPS	357 ± 3	++	1.85/1.64
3	+ 5 mM $MgCl_2$, Tris-HCl	350 ± 3	++	1.98/1.57
4	+ 20 mM $MgCl_2$, MOPS	360 ± 1	++	0.89/1.81
5**	+ 1 mM $MnCl_2$, Tris-HCl	348 ± 5	+	1.05/1.49
6	+ 200 μM $MnCl_2$, Tris-HCl	346 ± 2	++	0.72/0.89
7	+ 1 mM $MnCl_2$, MOPS	354 ± 3	+/++	1.26/1.42
8****	+ 1 mM $ZnCl_2$, Tris-HCl			

* Figure 3 shows the radial distribution of this sample at 275 nm.
** Figure 4 shows the radial distribution of this sample at 275 nm.
*** Quality is a subjective judgment of fit residuals (magnitude and regularity of distribution): ++, high quality; +, poor but acceptable quality.
**** No data were obtained from sample 8 because of protein precipitation.

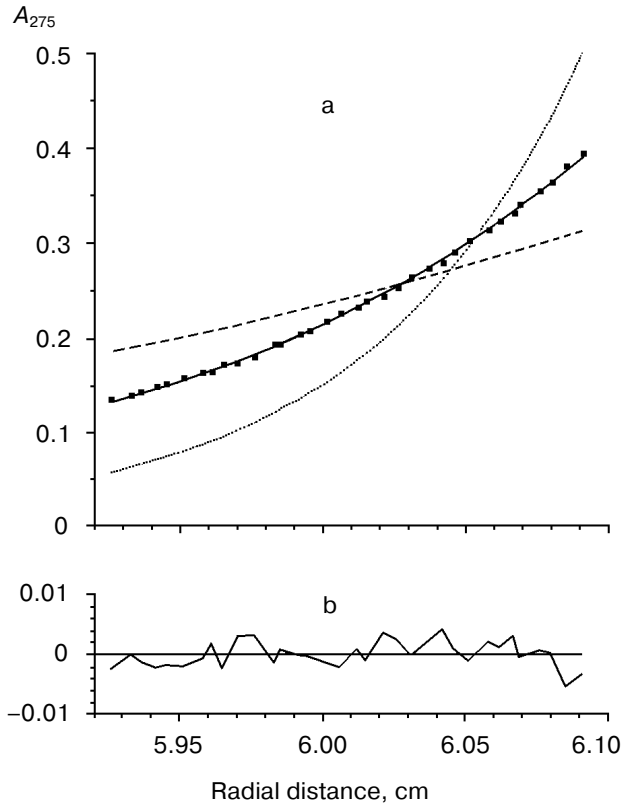

Fig. 3. Sedimentation equilibrium profile of sample No. 1 recorded after 40 h at 5,000 rpm and 10°C. a) Radial absorbency distribution measured at 275 nm (squares) was fitted with the single-component model (solid line), returning a molecular mass of 335.1 kD. For better orientation, "best fits" based on a tetramer (dotted line) or a 16-mer (dashed line) model are also included; b) radial dependent residuals representing deviations of the experimental data from those generated by the octamer model.

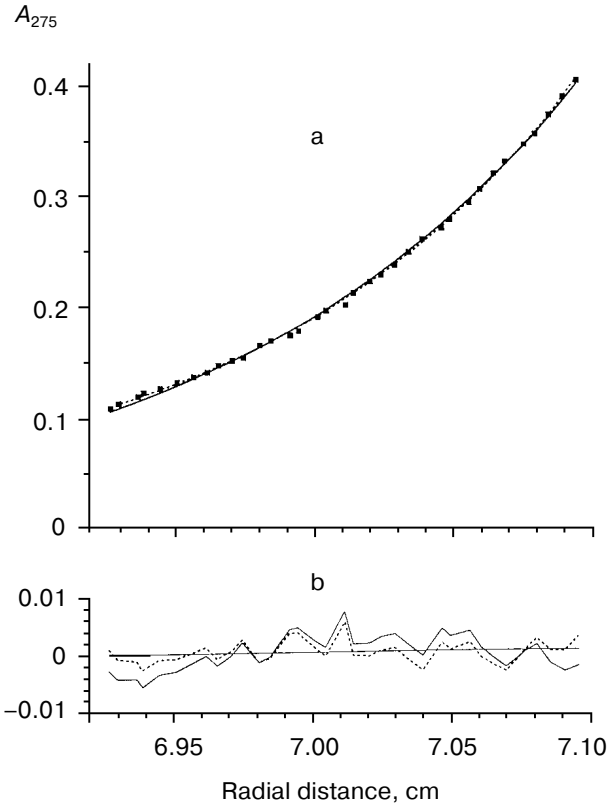

Fig. 4. Sedimentation equilibrium profile of sample No. 4 recorded after 40 h at 5,000 rpm and 10°C. a) Radial absorbency distribution measured at 275 nm (squares) was fitted with the single-component model (solid line), returning a molecular mass of 344.3 kD or with a three component model (dotted line), returning 19% tetramers, 69% octamers, and 12% 16-mers; b) radial dependent residuals representing deviations of the experimental data from those generated by the single component model (solid line) or the three component model (dotted line).

magnitude and regularity of distribution of the fit residuals.

Sedimentation equilibrium profiles of the other samples (No. 2-7) after 40 h at 5000 rpm were also well fitted with a model involving only one component. Averaged molecular masses as returned from the best fits of the 280 and 275 nm profiles are given in Table 1. Assuming that the minor differences between the samples were not significant, the overall average molecular mass was 351 ± 8 kD.

The qualities of the fits of all samples increased slightly when, besides octamers, tetramers, and 16-mers (dimers of octamers), were involved in the model. As an example, the sedimentation equilibrium profile of huGS, supplemented with 1 mM $MnCl_2$ at pH 7.8 (sample No. 5) and measured at 275 nm, is shown in Fig. 4. It fits well with a single-component model, in this case returning a molecular mass of 344.3 kD which is about 2% higher than the theoretical mass of a huGS octamer. The best fit based on a three component model (19% tetramers, 69% octamers, and 12% 16-mers, dashed line) can hardly be distinguished from the single component model. The slightly higher quality of the three component model was indicated by the higher regularity of distribution of the corresponding residuals. With all other samples the improvement of fit quality with the three component model relative to the single component model was smaller.

The weight percentages for the single molecular species as returned from the four component model for all measured samples were as follows: octamers, 73-92%, tetramers, 4-16%, 16-mers, 2-11%, and higher order oligomers, less than 2%. Note that values of the statistical parameter X_r^2 (reduced X^2) are in all cases somewhat lower when compared with those listed in Table 1, indicating better fits.

After centrifugation at 5,000 rpm for 40 h, the speed was increased to 7,000 rpm for 28 h and then to 10,000 rpm for another 20 h. No sedimentation equilibrium profile obtained at 7,000 or 10,000 rpm could be fitted with acceptable quality to a single component model (data not shown). Apparently, sample heterogeneity had increased after incubation of the enzyme for more than two days in the absence of glycerol at 10°C.

In another sedimentation equilibrium experiment we investigated huGS at different concentrations: 0.4, 0.1, and 0.04 mg/ml. This experiment was performed with 6,000 rpm at 20°C. Because of the lower buffer viscosity at the higher temperature of 20°C, equilibrium was already reached after about 20 h. However, samples appeared to be less homogeneous in this than in the 10°C experiment, as the best fits of the obtained profiles with the one component model were of poor quality. Average molecular masses as returned by fitting of six different profiles per concentration (after 21, 31, and 35 h at 6,000 rpm; two wavelengths each) to the one component model despite the quality issue were 339 ± 13, 277 ± 6, and 254 ± 11 kD, for 0.4, 0.1 and 0.04 mg/ml, respectively, indicating a weak dissociation equilibrium.

Identification of proteins that co-purified with huGS. Some proteins apparently co-purified with huGS (Fig. 1). Since these proteins may undergo specific interactions with GS, we initially determined their identity by MALDI-MS or coupled ion-spray MS/MS.

Various amounts of huGS were applied to SDS-PAGE (Fig. 1, lanes *1* and *2*). Bands 1, 2, 3, 4a, 4b, and 5 were cut out, cleaved by tryptic digestion, and then analyzed by MALDI-MS. Results are compiled in Table 2. Bands No. 1-3 clustered around 110 kD, and 32 of 54 determined peptide masses matched with theoretical masses of tryptic peptides computed from the sequence of human contactin precursor protein (Swiss-Prot database entry No. Q12860), which has a theoretical molecular mass of 113.3 kD.

Band No. 4 may be a doublet. We therefore analyzed bands 4a and 4b separately and obtained similar results from both analyses: six and five, respectively, peptide masses precisely matched with tryptic sequences of the human liver GS gene product (Swiss-Prot entry P15104). Overall, the masses of the identified GS peptides constituted 24% of the primary sequence of the hypothetical cDNA translate.

One selected peptide obtained from band No. 5 (around 33 kD) was fragmented into amino acids by coupled MS/MS. All (eight out of eight) obtained masses from this MS/MS spectrum fitted the amino acid sequence of the 2150.03 dalton peptide LTGFHETSNINDFSAGVANR, position 300 to 319 of human liver GS. The same peptide as judged from the mass entity was also detected by MALDI-MS of peptides derived from bands No. 4a and 4b (Table 2).

DISCUSSION

The present study is the first report of structural investigations of native huGS from brain and was made possible by our recent report of a purification scheme for this cytosolic enzyme from non-frozen brain tissue in sufficient amounts to enable such studies [19].

Different heterogeneity of huGS purified from non-frozen or frozen tissues. Previous investigations of GS from human brain have been mainly done with enzyme purified from frozen tissue [15, 16, 18, 19]. The distribution of huGS in subcellular fractions obtained during purification of this enzyme from non-frozen human brain [19] as well as from the brains of other mammalian species [29, 30] suggests that the GS pool contains compartmentalized GS, associated with a crude mitochondrial or particulate fraction, and completely soluble cytosolic GS, associated with the fraction that is free from membrane or subcellular components. Nothing is known about the identity extent between the compartmentalized and cytosolic GS forms in mammals, particularly in human brain. The huGS purified from non-frozen brain tissue in the present work contains mostly cytosolic GS, whereas huGS purified from frozen tissue contained compartmentalized as well as cytosolic GS.

We initially compared huGS samples purified from frozen and non-frozen brain tissues by two-dimensional electrophoresis in polyacrylamide gel, wherein both preparations yielded typical spot patterns reflecting microheterogeneity as reported for huGS purified from frozen brain [16, 18]. The differences in the spot intensities in our two-dimensional gels for these preparations may reflect different ratios of huGS subunits derived from cytosolic and compartmentalized GS released by freezing-thawing.

Different huGS subunits may represent individual gene products (in particular, two different genes are known for mitochondrial and cytosolic GS isoforms in *Drosophila melanogaster* [31]). Several GS-like sequences have been identified in the human genome but while some are presumably genes others may be pseudogenes [32]. Furthermore, huGS may be an isozyme. Isozymes of huGS could arise from post-translation modification that probably occurs to a different extent within the cytosolic and the cellular compartments. The release of huGS from subcellular organelles is certainly influenced by the freezing process. Our MS studies identified a tryptic huGS peptide VQAMYIWIDGTGEGLR unmodified and as a derivative with oxidized methionine. Methionine residues are susceptible to oxidation in bacterial GS [33], but methionine oxidation is an often observed artifact caused during peptide preparation for MS and the physiological significance of this observation is unclear. Finally, the isozyme distribution of huGS may also depend on the individual brain. Elucidation of this point requires population analysis.

Table 2. Peptides derived from bands No. 1-4b (Fig. 1) identified by MALDI-MS analysis and compared with the Swiss-Prot database

Band No.	Mass, daltons	Swiss-Prot entry No.	Start	End	Peptide sequence	Δ, %
	1007.56		836	843	IVESYQIR	0.000
	1047.59		960	968	HSIEVPIPR	0.000
	1065.51		969	977	DGEYVVEVR	–0.001
	1091.56		617	626	ATSVALTWSR	–0.002
	1194.73	Q12860:	226	235	FIPLIPIPER	0.001
1	1267.63	matches:	857	867	VQVTSQEYSAR	0.000
	1286.72	13/18	722	732	ELTITWAPLSR	0.001
	1384.73		942	953	VLYRPDGQHDGK	0.002
	1521.78		559	571	NFMLDSNGELLIR	0.000
	1537.75		559	571	NFMLDSNGELLIR (mod.)	–0.001
	1606.87		236	249	TTKPYPADIVVQFK	–0.001
	1668.81		668	680	AVDLIPWMEYEFR	0.000
	1979.01		819	835	VLSSSEISVHWEHVLEK	–0.001
	2637.33		125	147	STEATLSFGYLDPFPPEERPEVR	0.002
	1091.57	Q12860:	617	626	ATSVALTWSR	–0.001
	1194.71	matches:	226	235	FIPLIPIPER	–0.001
	1267.62	9/18	857	867	VQVTSQEYSAR	–0.001
2	1286.70		722	732	ELTITWAPLSR	–0.001
	1384.71		942	953	VLYRPDGQHDGK	0.001
	1521.74		559	571	NFMLDSNGELLIR	–0.002
	1668.76		668	680	AVDLIPWMEYEFR	–0.003
	1978.98		819	835	VLSSSEISVHWEHVLEK	–0.002
	2637.33		125	147	STEATLSFGYLDPFPPEERPEVR	0.002
	956.49		690	698	GEPSIPSNR	0.001
	977.51		643	650	TILSDDWK	0.002
	1091.56	Q12860:	617	626	ATSVALTWSR	–0.002
3	1194.71	matches:	226	235	FIPLIPIPER	–0.001
	1267.59	10/18	857	867	VQVTSQEYSAR	–0.003
	1286.69		722	732	ELTITWAPLSR	–0.001
	1521.70		559	571	NFMLDSNGELLIR	–0.005
	1607.81		408	421	ILALAPTFEMNPMK (mod.)	–0.001
	1668.79		668	680	AVDLIPWMEYEFR	–0.001
	2637.32		125	147	STEATLSFGYLDPFPPEERPEVR	0.001
	956.51		107	114	RPAETNLR	–0.001
	994.50	P15104:	269	276	YIEEAIEK	–0.001
	1002.47	matches:	174	181	DIVEAHYR	–0.003
4a	1808.88	6/18	26	41	VQAMYIWIDGTGEGLR	–0.001
	1824.85		26	41	VQAMYIWIDGTGEGLR (mod.)	–0.002
	1875.88		341	357	RPSANCDPFSVTEALIR	–0.003
	2150.00		300	319	LTGFHETSNINDFSAGVANR	–0.001
	956.53		107	114	RPAETNLR	0.000
	1002.51	P15104:	174	181	DIVEAHYR	0.001
	1279.62	matches:	15	25	QVYMSLPQGEK	–0.001
4b	1808.85	5/18	26	41	VQAMYIWIDGTGEGLR	–0.003
	1824.83		26	41	VQAMYIWIDGTGEGLR (mod.)	–0.004
	2149.98		300	319	LTGFHETSNINDFSAGVANR	–0.002

Notes: Mass, as analyzed by MS; Δ, percentage of deviation from theoretical molecular mass; (mod.), oxidized methionine. Matches: *m*/*n*, from *n* obtained masses *m* matched well with theoretical masses of peptides from Swiss-Prot database entry Q12860 or P15104, according to our criteria [24].

Identification of co-purified proteins with huGS. A 33-kD polypeptide that co-purified with huGS is a fragment of huGS. Probably this fragment was able to form hetero oligomers with full-length huGS subunits and therefore was not separated from the full-length species during the purification process. It is unlikely that this 33-kD polypeptide represents another *in vivo* form of huGS as we never observed 33-kD bands in immunoblots of human brain extracts that had been stained with polyclonal anti-GS antibodies and we conclude that the 33-kD fragment is an artifact. Another ~113-kD protein that co-purified with huGS was identified by mass spectrometry as brain contactin precursor. Co-purification of this protein may indicate a specific affinity for huGS that could be of physiological relevance, but further analysis of the possibility was beyond the scope of the present work.

Primary structure homology of huGS from liver and brain. The N-terminus of huGS is blocked and thus direct N-terminal sequencing is complicated [34]. Since this observation was obtained from huGS isolated from frozen brain tissue, N-termini are probably blocked in both the compartmentalized and the cytosolic forms of huGS, as was found for the mitochondrial GS precursor in the liver of spiny dogfish [35]. Because of the lack of comparison between the N-termini of compartmentalized and cytosolic GS, the mechanism of GS translocation into subcellular membrane structures (including mitochondria) remains unknown.

The partial amino acid sequences of three internal tryptic peptides of huGS indicate homology between GS from human brain and liver [34]. In the present work additional information regarding the primary structure of huGS in brain enabled a more extended comparison with the liver huGS sequence (Swiss-Prot database access No. P15104).

MALDI-MS analysis of eight tryptic peptides obtained from huGS bands of a SDS-PAGE gel revealed molecular masses that fitted well with those of the corresponding peptides from liver huGS. When the complete data base was searched with the experimentally obtained masses, the huGS entry was the only one identified, confirming that our fitting criteria had been set sufficiently high. Furthermore, we sequenced one of these peptides using the coupled MS/MS technology. The obtained sequence was in complete agreement with that expected from the liver sequence. Overall, the identified peptides cover about 24% of the complete liver huGS primary sequence. On the other hand, several peptides of the GS sequence were not identified by MALDI-MS. These missing peptides were not detected either for technical reasons (they may be unsuitable for MALDI-MS analysis because of their physical properties), or because they carry post-translation modifications or because their sequences differ from those of the liver form of huGS as deposited in the Swiss-Prot database. We conclude from our data that at least 24% of the huGS primary sequence is identical in liver and brain and that this part of the primary sequence has no post-translation modifications.

Quaternary structure of huGS from brain. The study of huGS quaternary structure and subunit association is very important for understanding of enzymatic reaction mechanism and also for the development of quantitative methods of huGS level determination. We investigated the quaternary structure of huGS isolated from non-frozen brain by several techniques. In size exclusion chromatography huGS eluted relatively early, indicating an oligomer with an estimated molecular mass of about 290 kD. However, the determination of molecular mass by size exclusion chromatography gives wrong results when the analyzed protein has a non-globular shape or when nonspecific interactions with the gel matrix occur [27]. Quasi-elastic light scattering measurements are done in free solution and are therefore not influenced by any matrix. Using this technique we measured a diffusion coefficient that can be translated to a molecular mass of about 270 kD if one assumes globular molecular shape. This mass is in good agreement with the result from size exclusion chromatography. However, as the molecular shape of huGS is unknown, its correct mass remains unresolved [26]. The huGS preparation behaved monodisperse in quasi-elastic light scattering analysis, indicating that there was a narrow distribution of molecular species.

Unlike size exclusion chromatography and quasi-elastic light scattering, the sedimentation equilibrium technique of analytical ultracentrifugation provides accurate molecular masses of proteins in solution without knowledge of their actual molecular shape [27]. In our sedimentation equilibrium experiments with purified cytosolic huGS from non-frozen brain, the radial distribution profiles obtained after 40 h were well fitted by an octameric one component model. All fits improved slightly when further oligomers (tetramers and dimers of octamers) were involved in the model and the fitting algorithm always returned the presence of at least 80% of the octameric form at a protein concentration of 0.4 mg/ml. Addition of 0.2-1.0 mM Mn^{2+} or 5-20 mM Mg^{2+} or variation of the pH (7.8 and 7.1) had no significant effect on the results. After 40-h centrifugation, sample heterogeneity increased, probably because of protein instability.

Dissociation and association of huGS subunits may be dependent on protein concentration. Average molecular masses as computed from sedimentation equilibrium profiles that were obtained at different protein concentrations were found to decrease with decreasing protein concentration. However, the quality of the data that we obtained from these experiments did not justify a more quantitative description of this hypothetical huGS oligomerization process.

This is the first analysis of the quaternary structure of huGS by analytical ultracentrifugation. An octameric structure was reported for GS obtained from liver mito-

chondria of dogfish [35]. A dissociation equilibrium has been described for ovine brain GS [17]. At a high enzyme concentration (~3.8 mg/ml) and in the presence of manganese the enzyme was exclusively octameric, whereas lower oligomeric forms were only apparent at a lower concentration of 0.13 mg/ml and in the absence of manganese [17].

In our experiments with huGS the content of octamers was high when no metal ions were added to the preparation. To preserve maximal enzymatic activity and native quaternary structure, we had avoided metal depletion during purification of huGS (in a control experiment huGS was incubated with 2 mM EDTA which was subsequently removed by dialysis, about 30% of the initial activity was lost; data not shown). The huGS used in the present studies had high enzymatic activity and presumably contained endogenous metal ions. These metal ions were apparently sufficient to stabilize the octameric structure.

Future experiments will address the potential heterogeneity of quaternary structures of compartmentalized and cytosolic huGS from brain and other tissues and the functional basis for the octameric structure of brain huGS.

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REFERENCES

1. Hertz, L., Yu, A. C. H., Kala, G., and Schousboe, A. (2000) *Neurochem. Int.*, **37**, 83-102.
2. Gunnersen, D., and Haley, B. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 11949-11953.
3. Le Prince, Gh., Delaere, P., Fages, Ch., Lefrancois, Th., Touret, M., Salanon, M., and Tardy, M. (1995) *Neurochem. Res.*, **20**, 859-862.
4. Robinson, S. R. (2000) *Neurochem. Int.*, **36**, 471-482.
5. Tereshkina, E. B., Boksha, I. S., Savushkina, O. K., and Burbaeva, G. Sh. (2000) *Zh. Nevropatol. Psikhiat. im. S. S. Korsakova*, **6**, 51-53.
6. DeMarco, V., McCain, M. D., Strauss, D., Chakrabarti, R., and Neu, J. (1997) *Placenta*, **18**, 541-545.
7. Labow, B. I., Souba, W. W., and Abcouwer, S. F. (1999) *Am. J. Physiol.*, **276**, E1136-E1145.
8. Gebhardt, R., Schmid, H., and Fitzke, H. (1989) *Experientia*, **45**, 137-139.
9. Gibbs, C. S., Campbell, K. E., and Wilson, R. H. (1987) *Nucleic Acids Res.*, **15**, 6293.
10. Christa, L., Simon, M. T., Flinois, J. P., Gebhardt, R., Brechot, C., and Lasserre, C. (1994) *Gastroenterology*, **106**, 1312-1320.
11. Listrom, C. D., Morizono, H., Rajagopal, B. S., McCann, M. T., Tuchman, M., and Allewell, N. M. (1997) *Biochem. J.*, **328**, 159-163.
12. Atkins, W. M., Cader, B. M., Hemmingsen, J., and Villafranca, J. J. (1993) *Protein Sci.*, **2**, 800-813.
13. Dabrowski, M. J., Yanchunas, J., Jr., Villafranca, B. C., Dietze, E. C., Schurke, P., and Atkins, W. M. (1994) *Biochemistry*, **33**, 14957-14964.
14. Yanchunas, J., Dabrowski, M. J., Schurke, P., and Atkins, M. (1994) *Biochemistry*, **33**, 14949-14953.
15. Yamamoto, H., Konno, H., Yamamoto, T., Ito, K., Mizugai, M., and Iwasaki, Y. (1987) *J. Neurochem.*, **49**, 603-608.
16. Tumani, H., Shen, G. Q., and Peter, J. B. (1995) *J. Immunol. Meth.*, **188**, 155-163.
17. Denman, R. B., and Wedler, F. C. (1984) *Arch. Biochem. Biophys.*, **232**, 427-440.
18. Boksha, I. S., Tereshkina, E. B., and Burbaeva, G. Sh. (1995) *Biochemistry (Moscow)*, **60**, 1299-1303.
19. Boksha, I. S., Tereshkina, E. B., and Burbaeva, G. Sh. (2000) *J. Neurochem.*, **75**, 2574-2582.
20. Wyss, M., Schlegel, J., James, P., Eppenberger, H. M., and Walliman, T. (1990) *J. Biol. Chem.*, **265**, 15900-15908.
21. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
22. O'Farrell, P. H. (1975) *J. Biol. Chem.*, **250**, 4007-4021.
23. Lahm, H., and Langen, H. (2000) *Electrophoresis*, **21**, 2105-2114.
24. Fountoulakis, M., and Langen, H. (1997) *Analyt. Biochem.*, **250**, 153-156.
25. Berndt, P., Hobohm, U., and Langen, H. (1999) *Electrophoresis*, **20**, 3521-3526.
26. Schönfeld, H.-J., Pöschl, B., and Müller, F. (1998) *Biochem. Soc. Trans.*, **26**, 753-758.
27. Schönfeld, H.-J., and Behlke, J. (1998) *Meth. Enzymol.*, **290**, 269-296.
28. Schuck, P. (1994) *Progr. Colloid Polym. Sci.*, **94**, 1-13.
29. Herzfeld, A., and Estes, N. A. (1973) *Biochem. J.*, **133**, 59-66.
30. Ward, H. K., and Bradford, H. F. (1979) *J. Neurochem.*, **33**, 339-342.
31. Caizzi, R., Bozzetti, M. P., and Caggese, C. (1990) *J. Mol. Biol.*, **212**, 17-26.
32. Wang, Y., Kudoh, J., Kubota, R., Asakawa, S., Minoshima, S., and Shimizu, N. (1996) *Genomics*, **37**, 195-199.
33. Berlett, B. S., Levine, R. L., and Stadtman, E. R. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 2784-2789.
34. Burbaeva, G. Sh., Boksha, I. S., and Tereshkina, E. B. (1998) *J. Neurochem.*, **71**S1, S84A.
35. Laud, P. R., and Campbell, J. W. (1994) *J. Mol. Evol.*, **39**, 93-100.