J. Biochem. Mol. Biol. Vol. 30, No. 5, pp. 332~336 September 30, 1997

Regulation of Two Soluble Forms of Brain Glutamate Dehydrogenase Isoproteins by Leucine

Jongweon Lee†, Jong Eun Lee[1], Soo Young Choi[2], and Sung-Woo Cho*

Department of Biochemistry, College of Medicine, University of Ulsan, Seoul 138-736
[1]Department of Anatomy, Yonsei University College of Medicine, Seoul 120-752
[2]Department of Genetic Engineering, College of Natural Sciences, Hallym University, Chunchon 200-702, Korea
(Received July 16, 1997)

Abstract : The stimulatory effects of leucine on the activities of two soluble forms of brain glutamate dehydrogenase isoproteins (GDH I and GDH II) have been studied at various conditions. There were significant differences between GDH I and GDH II in their sensitivities to the action of leucine. When the effects of varied leucine concentrations on GDH activities were studied in the direction of reductive amination of 2-oxoglutarate with NADPH as a coenzyme, a marked activation was observed for both isoproteins at leucine concentrations up to 10 mM, whereas both isoproteins showed activation to a lesser extent with NADH as a coenzyme. The stimulatory effects of leucine on GDH activities in the direction of the oxidative deamination of glutamate were also observed, but to a much lesser extent. Leucine relieved the inhibition of GDH I by GTP and this resulted in an increase in the apparent activation by leucine in the presence of GTP. 2-Oxoglutarate was found to give rise to high substrate inhibition and leucine significantly reduced the substrate inhibition in the presence of 200 μM NADH. Thus, the effects of leucine might be composed of a direct effect on the enzyme together with a relief of high substrate inhibition.
Key words : glutamate, glutamate dehydrogenase isoproteins, leucine, neurodegenerative disorders

Glutamate is a major excitatory neurotransmitter (Fonnum, 1984) and is also known to be the immediate precursor in the biosynthesis of γ-aminobutyric acid, a widely distributed inhibitory neurotransmitter. Due to its neurotoxic potentials, glutamate may be involved in the pathogenesis of human degenerative disorders (McGeer and McGeer, 1976; Plaitakis *et al.*, 1982). One enzyme central to the metabolism of glutamate is glutamate dehydrogenase (GDH) (EC 1.4.1.3). GDHs are a family of enzymes which catalyze the reversible deamination of L-glutamate to 2-oxoglutarate using NAD^+, $NADP^+$ or both as coenzymes (Stillman *et al.*, 1993).

Since the pathology of the disorders associated with GDH defects is restricted to the brain, the enzyme may be of particular importance in the biology of the nervous system. The importance of the pathophysiological nature of the GDH-deficient neurological disorders has attracted considerable interest. Hussain *et al.* (1989) detected four different forms of GDH isoproteins from the human cerebellum of normal subjects and patients with neurodegenerative disorders. The isoproteins are differentially distributed in the two catalytically active isoforms of the enzyme (Plaitakis *et al.*, 1993). The enzyme isolated from one of the patients with a variant form of multisystem atrophy displayed a marked reduction of one of the GDH isoproteins (Hussain *et al.*, 1989). The origin of the GDH polymorphism is not known. It was reported that four differently sized mRNAs and multiple gene copies for GDH exist in the human brain (Mavrothalassitis *et al.*, 1988). A novel cDNA encoded by an X chromosome-linked intronless gene also has been isolated from human retina (Shashidharan *et al.*, 1994).

Although the three-dimensional structure of GDH from microorganisms is available (Baker *et al.*, 1992), no crystal structure has been reported for mammalian GDH and thus remarkably little is known about the structure of mammalian GDH, especially brain enzyme. Recently, we have isolated two soluble forms of glutamate dehydrogenase isoproteins (designated GDH I and GDH II) from bovine brain (Cho *et al.*, 1995) and the regulatory sites within the GDH isoproteins have been identified (Cho *et al.*, 1996; Cho and Lee, 1996; Kim *et al.*, 1997). The results from our recent studies demonstrate that the bovine brain GDH isoproteins are different gene

*To whom correspondence should be addressed.
Tel : 82-2-224-4278, Fax : 82-2-224-4278
E-mail : swcho@amc.ulsan.ac.kr
†Present address: Department of Parasitology and Institute of Tropical Medicine, Yonsei University College of Medicine, Seoul 120-752, Korea

products rather than the results of posttranslational modifications (Cho *et al.*, 1995; 1996a; Kim *et al.*, 1997).

Our work led to the finding that GDH is present in bovine brain in "heat-labile (GDH I)" and "heat-stable (GDH II)" forms (Cho *et al.*, 1995). Similar results were reported by other investigators showing that reduction in GDH activity in patients with neurodegenerative disorders was largely limited to the heat-labile form (Plaitakis *et al.*, 1984; Abe *et al.*, 1992). Very recently, it has been reported that nerve tissue-specific human GDH is thermolabile and highly regulated by ADP (Shashidharan *et al.*, 1997). It has been reported that the presence of two GDH activities in rat brain differ in their relative resistance to thermal inactivation, detergent extractability, and allosteric regulation characteristics (Colon *et al.*, 1986). It also has been reported that GDH is allosterically activated by certain neutral amino acids including leucine and its analogues (McGivan *et al.*, 1973; Couee and Tipton, 1989). To our knowledge, comparison of the detailed structure and functions of any GDH isoproteins rarely has been reported. It is, therefore, essential to have a detailed structural and functional description of the various types of brain GDH to elucidate the pathophysiological nature of the GDH-deficient neurological disorders.

In the present work, we have comparatively examined the effects of leucine on two different types of GDH isoproteins (GDH I and GDH II) homogeneously purified from bovine brain in view of the central role of GDH in cerebral metabolism and in GDH-deficient neurodegenerative disorders.

Materials and Methods

Materials

NADH, NADPH, 2-oxoglutarate, glutamate, ADP, GTP, and L-Leu were purchased from Sigma Chemical Co. The GDH isoproteins were purified from bovine brain by the method developed in our laboratory (Cho *et al.*, 1995) and were homogeneous as judged by Coomassie-stained gradient SDS-polyacrylamide gel electrophoresis. All other chemicals and solvents were reagent grade or better.

Enzyme assay

GDH activity was measured spectrophotometrically in the direction of reductive amination of 2-oxoglutarate by following the decrease in absorbance at 340 nm as described before (Cho *et al.*, 1995). All assays were performed in duplicate, and initial velocity data were correlated with a standard assay mixture containing 50 mM triethanolamine, pH 8.0, 100 mM ammonium acetate, 0.1 mM NADH, and 2.6 mM EDTA at 25°C. GDH concentrations were adjusted to give a measured rate of less than 0.04 absorbance units per min. The reaction started with the addition of 2-oxoglutarate to a 10 mM final concentration. One unit of enzyme was defined as the amount of enzyme required to oxidize 1 mmol of NADH per min at 25°C. GDH activity was also measured in the direction of glutamate oxidation in 50 mM Tris/HCl, pH 9.5 containing 1.4 mM NAD, and 2.6 mM EDTA at 25°C. The reaction was started by the addition of glutamate to a 25 mM final concentration.

Activation of GDH isoproteins by leucine

Unless otherwise specified, the highly purified GDH fractions were used for activation studies. Stimulation studies with leucine were performed at various concentrations in assay buffer at 25°C as described in figure legends. Initial velocities were determined as described above and the data were fitted by a least-squares method. For the determination of the effects of substrate concentration on the activation by leucine, the assays were carried out by varying the substrate under investigation while keeping the other substrate and reagents at the optimal concentration indicated above. In some experiments, the effects of GTP on activation by leucine were tested by including it in the incubation as described in the figure legends. Each experimental point represents the mean of triplicate determinations.

Results and Discussion

The existence of brain GDH isoproteins has been only recently recognized (Hussain *et al.*, 1989; Shashidharan *et al.*, 1994). The studies of the brain GDH isoproteins are far less encompassing in protein function and structure of these isotypes. It is only recent that detailed information about the three-dimensional structure of any GDH has become available (Teller *et al.*, 1995; Baker *et al.*, 1991). No crystal structure is available for mammalian GDH and thus remarkably little is known about the chemistry of the active site of GDH, especially brain enzyme. It is, therefore, essential to have a detailed structural description of different types of GDH. Recently, we have isolated two soluble forms of glutamate dehydrogenase isoproteins, GDH I and GDH II, from bovine brain (Cho *et al.*, 1995). Unlike most of the previous reports which presented a soluble and a particulate form of GDH (Rajas and Rouset, 1993; Plaitakis *et al.*, 1993), both GDH I and GDH II were readily solublized and no detergents were required for the initial extraction step (Cho *et al.*, 1995). In the present work, we comparatively examined the effects of leucine on two different types of GDH isoproteins (GDH I and GDH II).

The effects of leucine on GDH activities have been

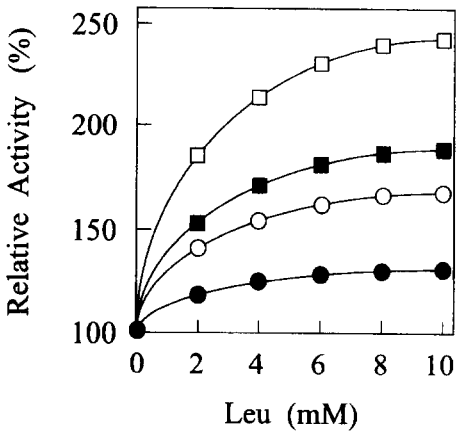

Fig. 1. Activation of GDH isoproteins by leucine in the direction of reductive amination. GDH isoproteins were assayed in the direction of reductive amination as described in the Materials and Methods with 0.1 mM NADH or 0.1 mM NADPH. The relative activities are expressed as percentage of each control. Closed circle (GDH I with NADH); open circle (GDH I with NADPH), closed square (GDH II with NADH); open square (GDH II with NADPH).

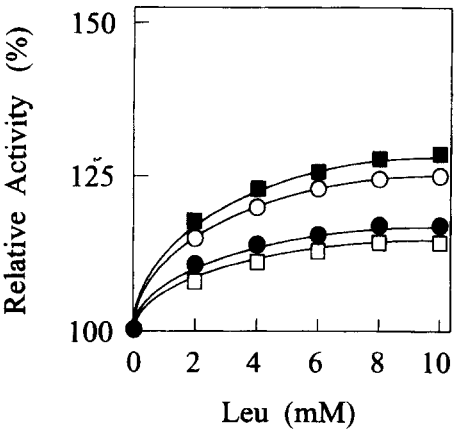

Fig. 2. Activation of GDH isoproteins by leucine in the direction of glutamate oxidation. GDH isoproteins were assayed in the direction of glutamate oxidation with 1.4 mM NAD^+ or 1.4 mM $NADP^+$. The relative activities are expressed as percentage of each control. Closed circle (GDH I with NADH); open circle (GDH I with NADPH), closed square (GDH II with NADH); open square (GDH II with NADPH).

studied at various conditions for both GDH I and GDH II. There were significant differences between GDH I and GDH II in their sensitivities to the action of leucine. When the effects of varied leucine concentrations on GDH activities were studied in the direction of reductive amination of 2-oxoglutarate with NADPH as a coenzyme, a marked activation was observed for both isoproteins at leucine concentrations up to 10 mM, whereas both isoproteins showed activation to a lesser extent with NADH as a coenzyme as shown in Fig. 1. The largest activation was observed with GDH II when NADPH was used as a coenzyme (Fig. 1).

The stimulatory effects of leucine on GDH activities in the direction of the oxidative deamination of glutamate were also observed, but to a much lesser extent as shown in Fig. 2. A slightly higher activation in a dose-dependent manner was observed for GDH I when $NADP^+$ was used as a coenzyme (Fig. 2). The effects, however, were reversed in GDH II, showing a higher activation when assayed with NAD^+ (Fig. 2).

It has been well documented that the allosteric effects of nucleotides on GDH are complex depending on the pH, substrate concentration and coenzyme used (Cho *et al.*, 1995; Bailey *et al.*, 1982). ADP and ATP have been found to be activators of GDH. ADP also has been known to reduce high substrate inhibition by NADH (Bailey *et al.*, 1982). In the present work, the activities of the GDH isoproteins obtained at saturating concentrations (10 mM) of leucine were not significantly increased in the presence of ADP or ATP and the apparent activation by leucine was decreased in the presence of ADP or ATP (data not shown).

GTP has been known as an inhibitor of GDH (Shoemaker and Haley, 1993; Pal and Colman, 1979; McCarthy and Tipton, 1984) and our previous work also showed that the bovine brain GDH isoproteins were inhibited by GTP at different extents (Cho *et al.*, 1996). The effects of GTP on activation by leucine was studied for both GDH I and GDH II. As shown in Fig. 3, leucine relieved the inhibition of GDH I by GTP and this resulted in an increase in the apparent activation by leucine in the presence of GTP. Similar effects, but to different extents, were observed with GDH II as shown in Fig. 4. Therefore, qualitatively, GTP showed similar ef-

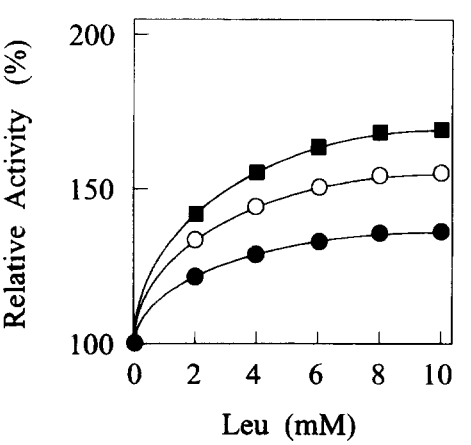

Fig. 3. Effect of GTP on the activation of GDH I by leucine. GDH I was assayed in the direction of reductive amination with 0.1 mM NADH at various concentrations of GTP. The relative activities are expressed as percentage of each control. The GTP concentrations were 0 mM (closed square), 0.2 mM (open circle), and 0.5 mM (closed circle).

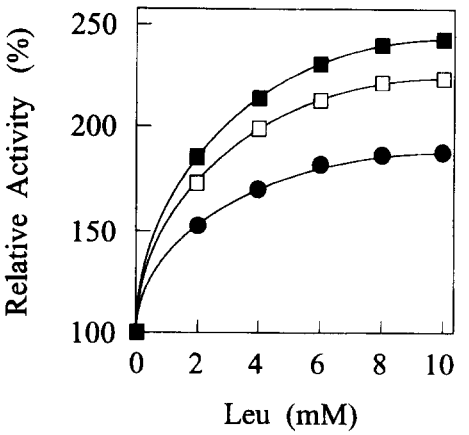

Fig. 4. Effect of GTP on the activation of GDH II by leucine. Details were as described in Fig. 3 except that GDH II was used.

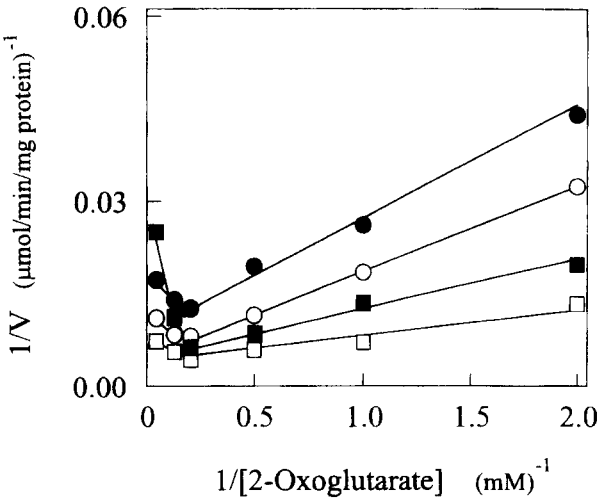

Fig. 5. Effect of substrate concentration on the activation of GDH isoproteins by leucine. GDH I was assayed in the direction of reductive amination at various concentrations of 2-oxoglutarate with 100 mM NADH or 200 mM NADH. Closed circle (100 mM NADH without leucine); open circle (100 mM NADH with 10 mM leucine). closed square (200 mM NADH without leucine); open square (200 mM NADH with 10 mM leucine).

fects on the activation of both GDH I and GDH II by leucine, however quantitatively, there were differences as shown in Fig. 3 and Fig. 4. The *in vivo* roles of GTP on the activation of GDH by leucine, however, still remains to be answered, since the magnesium complexes of this compound, which may be the predominant form, have little effect on the enzyme (McCarthy and Tipton, 1984).

Although leucine is known as an allosteric activator, the leucine binding site within any GDH has not been identified. GDH from *Neurospora crassa* is not regulated by GTP or ADP. This enzyme contains 48 less residues than mammalian GDH, and there is little identity between the 100 residues in the C-terminus (Wootton *et al.*, 1974). Studies have shown that chemical probes can at least partially desensitize bovine liver GDH to GTP inhibition while not affecting catalytic activity. The amino acids modified by these chemical probes were shown to be a residue in the C-terminus (Coffee *et al.*, 1971; Piszkiewicz *et al.*, 1971). It seems likely that the regulatory ADP and GTP binding domains are located in this region. Although a crystal structure of any mammalian GDH is not available yet, the regulatory purine nucleotide binding sites are thought to reside in the C-terminal half of GDH (Smith *et al.*, 1975). An analysis of the three-dimensional structure of the mammalian enzyme should supplement the understanding of the action of the allosteric regulators.

Fig. 5 shows the effects of substrate concentration on the activation of GDH I by leucine. The results of varying the concentration of 2-oxoglutarate at two different fixed concentrations of NADH (100 μM and 200 μM) in the presence or absence of 10 mM leucine are shown. The higher concentration of 200 μM NADH used was in the range giving inhibition by this coenzyme. 2-Oxoglutarate was found to give rise to high substrate inhibition both in the absence and presence of 10 mM leucine (Fig. 5). Leucine, however, significantly reduced the substrate inhibition in the presence of 200 μM NADH (Fig. 5). The effects of substrate concentration on the activation of GDH II by leucine were similar to those obtained with GDH I (data not shown). Thus, the effects of leucine might be composed of a direct effect on the enzyme together with a relief of high substrate inhibition.

A final comment concerns the role of leucine as a possible endogenous activator of GDH in the CNS *in vivo*. The half-maximal concentrations of leucine for activating GDH isoproteins were approximately 0.5~1.0 mM. The concentration of leucine in rat brain is 0.1~0.2 mM (Erecinska *et al.*, 1984), but it rises in such pathological states as ischemia (Erecinska *et al.*, 1984) and diabetes (Brosnan *et al.*, 1984). It is tempting, therefore, to speculate that leucine may activate GDH in pathological conditions where leucine concentrations are elevated.

As many proteins have functions distinct from those for which they were originally identified, it will also be of interest to assess the other roles of GDH isoproteins. Recently, Preiss et al. (1993; 1995) have reported that the bovine liver GDH contains a binding site for heteropolymeric RNA with the highest affinity for an as yet undefined nucleotide consensus sequence or structure. It also has been reported that GDH from pig liver and brain possesses an ATP-dependent high-affinity microtubule-binding activity (Rajas and Rouset, 1996; 1993). The potential physiological relevance of the brain GDH

isoproteins remains to be answered.

Acknowledgement

This work was supported by a Non-Directed Research Fund from the Korea Research Foundation (1995) S.-W. Cho.

References

Abe, T., Ishiguro, S. I., Saito, H., Kiyosawa, M. and Tamai, M. (1992) *Invest. Ophthalmol. Vis. Sci.* **33**, 447.

Bailey, J., Bell, E. T. and Bell, J. E. (1982) *J. Biol. Chem.* **257**, 5579.

Baker, P. J., Britton, K. L., Engel, P. C., Farrants, G. W., Lilley, K. S., Rice, D. W. and Stillman, T. J. (1991) *Proteins Struct. Funct. Genet.* **12**, 75.

Baker, P. J., Britton, K. L., Rice, D. W., Rob, A. and Stillman, T. J. (1992) *J. Mol. Biol.* **228**, 662.

Brosnan, J. T., Forsey, R. G. P. and Brosnan, M. E. (1984) *Am. J. Physiol.* **247**, C450.

Cho, S.-W., Lee, J. and Choi, S. Y. (1995) *Eur. J. Biochem.* **233**, 340.

Cho, S.-W., Ahn, J.-Y., Lee, J. and Choi, S. Y. (1996) *Biochemistry (US)* **35**, 13907.

Cho, S.-W. and Lee, J. E. (1996) *Biochimie* **78**, 817.

Coffee, C., Bradshaw, R., Goldin, B. and Frieden, C. (1971) *Biochemistry* **10**, 3516.

Colon, A. D., Plaitakis, A., Perakis, A. and Clarke, D. (1986) *J. Neurochem.* **46**, 1811.

Couee, I. and Tipton, K. F. (1989) *Biochem. J.* **261**, 921.

Erecinska, M., Nelson, D., Wilson, D. F. and Silver, I. A. (1984) *Brain Res.* **304**, 9.

Fonnum, F. (1984) *J. Neurochem.* **42**, 1.

Kim, S. W., Lee, J. Song, M.-S., Choi, S. Y. and Cho, S.-W. (1997) *J. Neurochem.* **69**, 418.

Hussain, M.H., Zannis, V.I. and Plaitakis, A. (1989) *J. Biol. Chem.* **264**, 20730.

Mavrothalassitis, G., Tzimagiorgis, G., Mitsialis, A., Zannis, V. I., Plaitakis, A., Papamatheakis, J. and Moschonas, N. K. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3494.

McCarthy, A. and Tipton, K. (1984) *Biochem. J.* **220**, 853.

McGeer, E. G. and McGeer, P. L. (1976) *Nature* **263**, 517.

McGivan, J. D., Bradford, N. M., Crompton, M. and Chappel, J. B. (1973) *Biochem. J.* **134**, 209.

Pal, P. and Colman, R. (1979) *Biochemistry* **18**, 838.

Piszkiewicz, B., Landon, M. and Smith, E. (1971) *J. Biol. Chem.* **246**, 1324.

Plaitakis, A., Berl, S. and Yahr, M. D. (1982) *Science* **216**, 193.

Plaitakis, A., Berl, S. and Yahr, M. D. (1984) *Ann. Neurol.* **15**, 144.

Plaitakis, A., Flessas, P., Natsiou, A. B. and Shashidharan, P. (1993) *Can. J.Neurol. Sci. Suppl.* **3**, S109.

Preiss, T., Hall, A. G. and Lightowlers, R. N. (1993) *J. Biol. Chem.* **268**, 24523.

Preiss, T., Sang, A., Chrzanowska-Lightowlers, Z. M. A. and Lightowlers, R. N. (1995) *FEBS Lett.* **367**, 291.

Rajas, F. Gire, V. and Rousset, B. (1996) *J. Biol. Chem.* **271**, 29882.

Rajas, F. and Rousset, B. (1993) *Biochem. J.* **295**, 447.

Shashidharan, P., Clarke, D. D., Ahmed, N., Moschonas, N. and Plaitakis, A. (1997) *J. Neurochem.* **68**, 1804.

Shashidharan, P., Michaelidis, T. M., Robakis, N. K., Kresovali, A., Papamatheakis, J. and Plaitakis, A. (1994) *J. Biol. Chem.* **269**, 16971.

Shoemaker, M. T. and Haley, B. E. (1993) *Bioconjugate Chem.* **32**, 1883.

Smith, E. L., Austen, B. M., Blumenthal, K. M. and Nyc, J. F. (1975) in The Enzymes (Boyer, P. D., ed.) Vol. XI, pp 293-367. Academic Press, New York.

Stillman, T. J., Baker, P. J., Britton, K. L. and Rice, D. W. (1993) *J. Mol. Biol.* **234**, 1131.

Teller, J K., Baker, P. J., Britton, K. L., Engel, P. C., Rice, D. W. and Stillman, T. J. (1995) *Biochim. Biophys. Acta* **1247**, 231.

Wootton, J., Chambers, G., Holder, A., Baron, A., Taylor, J., Fincham, J., Blumenthal, K., Moon, K. and Smith, E. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4361.