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Molecular Gene Cloning, Expression, and Characterization of Bovine Brain Glutamate Dehydrogenase

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A cDNA of bovine brain glutamate dehydrogenase (GDH) was isolated from a cDNA library by recombinant PCR. The isolated cDNA has an open-reading frame of 1677 nucleotides, which codes for 559 amino acids. The expression of the recombinant bovine brain GDH enzyme was achieved in *E. coli*. BL21 (DE3) by using the pET-15b expression vector containing a T7 promoter. The recombinant GDH protein was also purified and characterized. The amino acid sequence was found 90% homologous to the human GDH. The molecular mass of the expressed GDH enzyme was estimated as 50 kDa by SDS-PAGE and Western blot using monoclonal antibodies against bovine brain GDH. The kinetic parameters of the expressed recombinant GDH enzymes were quite similar to those of the purified bovine brain GDH. The K_m and V_{max} values for NAD^+ were 0.1 mM and 1.08 μmol/min/mg, respectively. The catalytic activities of the recombinant GDH enzymes were inhibited by ATP in a concentration-dependent manner over the range of 10 - 100 μM, whereas, ADP increased the enzyme activity up to 2.3-fold. These results indicate that the recombinant-expressed bovine brain GDH that is produced has biochemical properties that are very similar to those of the purified GDH enzyme.

Keywords: Expression, Glutamate dehydrogenase, Purification, Sequencing

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Introduction

Glutamate is thought to have several important functions. It is a major excitatory neurotransmitter (Fonnum, 1984) and is also known as the immediate precursor in the biosynthesis of γ-aminobutyric acid (GABA), a widely distributed inhibitory neurotransmitter. Glutamate has a greater presence in the brain than the other organs do (Hussain *et al*., 1989). Due to its neurotoxic potential, glutamate may be involved in the pathogenesis of human degenerative disorders (Plaitakis *et al*., 1982; Duvoisin *et al*., 1983). Since glutamate does not readily cross the blood-brain barrier, most of these amino acids in the central nervous system are produced from precursors through the action of several enzymes. One enzyme that is central to the metabolism of glutamate is glutamate dehydrogenase (GDH). This enzyme links glutamate with the Krebs cycle and provides a major pathway for the inter conversion of α-amino acids and α-keto-acids. In non-neural tissues, the oxidation of glutamate by GDH is considered to be linked to the synthesis of ATP as an energy source (Smith, 1979).

GDH (EC 1. 4. 1. 2-4) is a family of enzymes that catalyze the reversible deamination of L-glutamate to 2-oxoglutarate using NAD^+, $NADP^+$, or both as co-enzymes (Fisher, 1985). The GDH enzyme is expressed at high levels in the brain, liver, pancreas, and kidney, but not in muscles. There are three types of GDH that vary according to the co-enzymes; NAD(H)-specific GDH, NADP(H)-specific GDH, and GDH with mixed specificity, as well as the structure of GDHs, according to the sources (Veronese *et al*., 1974; Smith *et al*., 1975; McPherson and Wootton, 1983; Rice *et al*., 1985; Cho *et al*., 1995). The largest difference between the mammalian and bacterial GDH is the long antenna domain in mammalian GDH that is formed by the 48-amino acid insertion, beginning

at residue 395 (Peterson and Smith, 1999; Smith *et al*., 2001). The allosteric control of mammalian GDH activity by positive (ADP) and negative effectors (GTP) has been studied (Colman, 1991; Hudson and Daniel, 1993). The mammalian GDH is strictly regulated by allosteric activators and inhibitors (McPherson and Wootton, 1983; Cho *et al*., 1995). GTP inhibits enzyme turnover over a wide range of conditions by increasing the affinity of the enzyme for the product, making product release rate-limiting under all conditions in the presence of ADP (Peterson and Smith, 1999; Smith *et al*., 2001). ADP is a potent activator that decreases product affinity (Bailey *et al*., 1982; Smith *et al*., 2001). In contrast to vertebrate, bacterial GDH is not regulated by the allosteric regulators (Veronese *et al*., 1974; Rice *et al*., 1985). Thus, it has been suggested that the antenna domain that is unique to mammalian GDH has important roles in allosteric regulation (Peterson and Smith, 1999; Smith *et al*., 2001). The importance of the physiological nature of GDH has attracted considerable interest. The physiological significance of this regulation is highlighted by the recent identification of infants and children with an unusual hyperinsulinism-hyperammonemia (HI/HA) syndrome. These observations demonstrate that the allosteric regulation of GDH plays a crucial role in the regulation of insulin secretion. The mutated residues that are responsible for this pathology were mainly around the GTP site (Stanley *et al*., 1998). Twenty-four different mutations of GDH in patients with the HI/HA syndrome have been identified (Yorifuji *et al*., 1999; Miki *et al*., 2000; Stanley *et al*., 2000; MacMullen *et al*., 2001). All of these mutations occurred at amino acid residues that appeared to be directly or indirectly involved. They were also based on the crystal structure of bovine GDH, which is 95% identical with the human GDH (Peterson and Smith, 1999). Recently, the crystallization of bovine liver GDH was reported for the first time from mammalian sources (Peterson *et al*., 1997). However, remarkably little is known about the detailed structure of mammalian GDH, especially the brain enzymes.

In this study, we report the cloning and overexpression of bovine brain GDH in *E. coli* by using the pET-15b expression vector containing a T7 promoter, purification, and characterization of recombinant enzyme. In addition, the purified recombinant GDH has very similar properties to the purified bovine brain GDH.

Materials and Methods

Materials NADH, 2-oxoglutarate, glutamate, and ADP were purchased from Sigma (St. Louis, USA). Bovine brains were obtained from Majang Slaughterhouse (Seoul, Korea). The glutamate dehydrogenase proteins were purified from bovine brain by the method developed at our laboratory (Cho *et al*., 1995). Ni^{2+}-nitrilotriacetic acid Sepharose superflow was purchased from Qiagen (Hilden, Germany). Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Duchefa Co. (Haarlem, Netherlands). Goat anti-mouse IgG, conjugated with horseradish peroxidase (HRP), was purchased from Sigma. Fetal bovine serum (FBS), basal medium Eagle, Dulbecco's modified Eagles' medium (DME), and penicillin-streptomycin antibiotics were purchased from Gibco BRL (Grand Island, USA). All of the other chemicals and reagents were the highest analytical grade available.

Construction of recombinant bovine brain GDH fusion gene (pET-bGDH) A bovine GDH cDNA was isolated using the polymerase chain reaction (PCR) technique from the bovine brain cDNA library. On the basis of the cDNA sequence of bovine GDH, two oligonucleotides were synthesized. The forward primer contained the *Xho*I restriction site and the reverse primer contained the *Bam*HI restriction site. The purified PCR products were ligated into a TA cloning vector (Promega, Madison, USA) and then transformed into a DH5α competent cell. Plasmids of the selected colonies were purified by using the alkaline lysis method (Sambrook *et al*., 1989). The purified TA vector containing bovine GDH cDNA was digested with *Xho*I and *Bam*HI. The purified insert was ligated into a pET-15b expression vector (Novagen, Madison, USA), which had been digested with the same restriction enzymes. The cells were plated on LB agar containing ampicillin (100 μg/ml), and the plasmid DNA was purified and examined by DNA sequencing. General methods for cloning were as described previously (Sambrook *et al*., 1989).

Purification of pET-bGDH fusion protein The host *E. coli* BL21 (DE3) (Novagen) was transformed with pET-bGDH and then the transformants were selected on a LB plate containing ampicillin. The selected colonies were cultured in a LB medium (Sparks, USA) containing ampicillin. IPTG was added to the final concentration of 1 mM, and the incubation was continued for 3 h. The cells were harvested and a 5 ml binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) was added for 10 min and sonicated. After centrifugation (15,000 × *g*, 30 min), the supernatants containing pET-bGDH were immediately loaded onto a 2.5 ml Ni^{2+}-nitrilotriacetic acid sepharose column. After the column was washed with 10 volumes of a binding buffer and six volumes of a washing buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), the proteins were eluted with an elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Purified proteins were combined and the salts were removed using PD-10 column (Pharmacia, Uppsala, Sweden) chromatography (Koh *et al*., 2001). The protein concentration was estimated by the Bradford procedure using bovine serum albumin as the standard (Bradford, 1976). The purified GDH proteins were analyzed by SDS-PAGE and recognized by Western blot using monoclonal antibodies that were previously produced in our laboratory against the bovine brain GDH (Choi *et al*., 1999).

DNA sequencing Plasmid DNA was purified for sequencing using the alkaline lysis method (Sambrook *et al*., 1989) from 3 ml of a LB medium containing ampicillin that was incubated for 16 h at 37°C. The cloned plasmid DNA sequence was confirmed with a fluorescence-based automated sequencer (Model 373A; Applied Biosystems Inc., Foster City, USA).

Enzyme assay GDH activity was measured spectrophotometrically in the direction of the reductive amination of 2-

oxoglutarate by measuring the decrease in absorbance at 340 nm, as described previously (Ahn *et al*., 1999b). All of the assays were performed in triplicate and the initial velocity data was 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. Because *E. coli* only has NADP(H)-dependent GDH (Hanahan, 1983; McPherson and Wootton, 1983), the enzyme assay was performed with NADH as a co-enzyme, as described elsewhere (Cho *et al*., 1995). GDH concentrations were adjusted to give a measured rate of about 0.04 absorbance units per min. One unit of enzyme was defined as the amount of enzyme required to oxidize 1 μmol of NADH per min at 25°C. GDH activity was also measured in the direction of oxidative deamination of glutamate by following the increase in absorbance at 340 nm, as described previously (Ahn *et al*., 1999a).

Results and Discussion

Glutamate metabolism is linked with the aminotransferase reaction, ureagenesis, the tricaboxylic acid (TCA) cycle, GABA and glutathione synthesis. Glutamate completes amino acid degradation through its oxidative deamination by glutamate dehydrogenase (GDH) to α-ketoglutarate and free ammonia. Recently, the clinical importance of glutamate metabolism has been highlighted by the identification of individuals with an unusual hyperinsulinism/ hyperammonemia (HI/HA) syndrome (Brusilow and Horwich, 1995; Weinzimer *et al*., 1997; Stanley *et al*., 1998; 2000). However, the precise roles of GDH in the central nervous system and the predominant direction of the reaction it catalyzes remain unclear. GDH may play a role in a number of human neurodegenerations. Therefore, the regulation of GDH, an important regulator of glutamate, might be worth examining clinically.

To produce recombinant bovine brain GDH fusion proteins in *E. coli*, the GDH gene was amplified from a bovine brain cDNA library by PCR. We isolated three cDNA clones by screening a bovine brain cDNA library. We ligated the three clones for 3 h. Restriction enzyme digestion analyses then revealed that the cloned bovine brain GDH contained full-length bovine brain cDNA (Fig. 1). To confirm the nucleotide sequence of the bovine GDH encoding regions, the complete nucleotide sequence was elucidated, as described in Materials and Methods. The nucleotide and deduced amino acid sequences of the bovine brain GDH that was cloned in this experiment are deposited at GenBank™ AY256856. The open-reading frame was found to consist of 1677 bp, encoding a protein of 559 amino acids. The nucleotide sequence of the cloned cDNA from the bovine brain cDNA library showed a high homology (90% identity) with human brain GDH. We previously reported the identification and characterization of bovine and human GDH (Cho *et al*., 1995; Lee e*t al*., 2001a; 2001b; Yoon *et al*., 2002). GDH is a ubiquitous enzyme in most organisms and plays an essential role in cellular nitrogen and carbon metabolism. GDH is one of the enzymes whose sequence is highly conserved in a wide range of organisms. In addition, GDH is highly expressed in the brain, liver, kidney, and pancreas.

To develop an expression system that overexpresses bovine brain GDH protein for purification, we constructed the recombinant bovine brain GDH expression vector, pET-bGDH. The GDH expression vector contained a cDNA sequence encoding bovine GDH and six histidine residues at the amino-terminus (Fig. 2). Bacterial cells were induced with IPTG and lysed at 4°C in phosphate buffered saline (PBS). The cells were then disrupted by sonication and centrifuged. Crude extracts that were obtained from the supernatant were electrophoresed in 10% SDS-polyacrylamide slab gels. Figure 3A shows the protein bands that are visualized by Coomassie brilliant blue staining. The recombinant GDH proteins, marked by arrows in the lane 1 (expressed bGDH) and lane 2 (purified bGDH), indicate that the protein was expressed at a very high level as a major component of the total soluble cellular protein. Recombinant bovine brain GDH proteins were purified homogeneously from cell lysates by Ni^{2+}-nitrilotriacetic acid Sepharose affinity chromatography (Fig. 3A). The yields of recombinant protein that were obtained were approximately 30 mg/l culture. The recombinant bovine brain GDH proteins have an estimated molecular mass of approximately 50 kDa by SDS-PAGE. The purified products were Western blotted using a monoclonal antibody to a bovine brain GDH. The monoclonal antibodies against human and bovine brain GDH were produced by fusion experiments in our laboratory (Choi *et al*., 1999). The expressed and purified recombinant bovine brain GDH enzyme was detected as corresponding bands in Fig. 3B.

In a previous study, we reported the purification and characterization of a soluble form of GDH from bovine brain (Cho *et al*., 1995; Lee *et al*., 1995). Pure bovine brain GDH was obtained by using a combination of Q-Sepharose, Hydroxyapatite, Heparin-Sepharose, and Phenyl-superose chromatography. The purified enzymes molecular mass was approximately 57.5 kDa by SDS-PAGE using a gradient gel. Four mg of the purified GDH protein was obtained with a final yield of 5%. However, another group reported the purification of a GDH from bovine brain using a combination of ammonium sulfate fractionation and chromatography on DEAE-cellulose and GTP-Sepharose (McCarthy *et al*., 1980). A similar result was observed by Hussain *et al*., who purified GDH from the cerebellum of normal subjects and patients with degenerative neurological disorders. This group purified human GDH by ammonium sulfate fractionation and chromatography on phenyl-Sepharose and GTP-Sepharose (Hussain *et al*., 1989).

Our first goal was to determine the best system for producing the most stable and soluble protein that could be easily purified in large quantities. Our results indicated that using our experimental system, the bovine brain GDH enzyme can be easily purified. In addition, the purified GDH enzyme was similar to authentic bovine brain GDH.

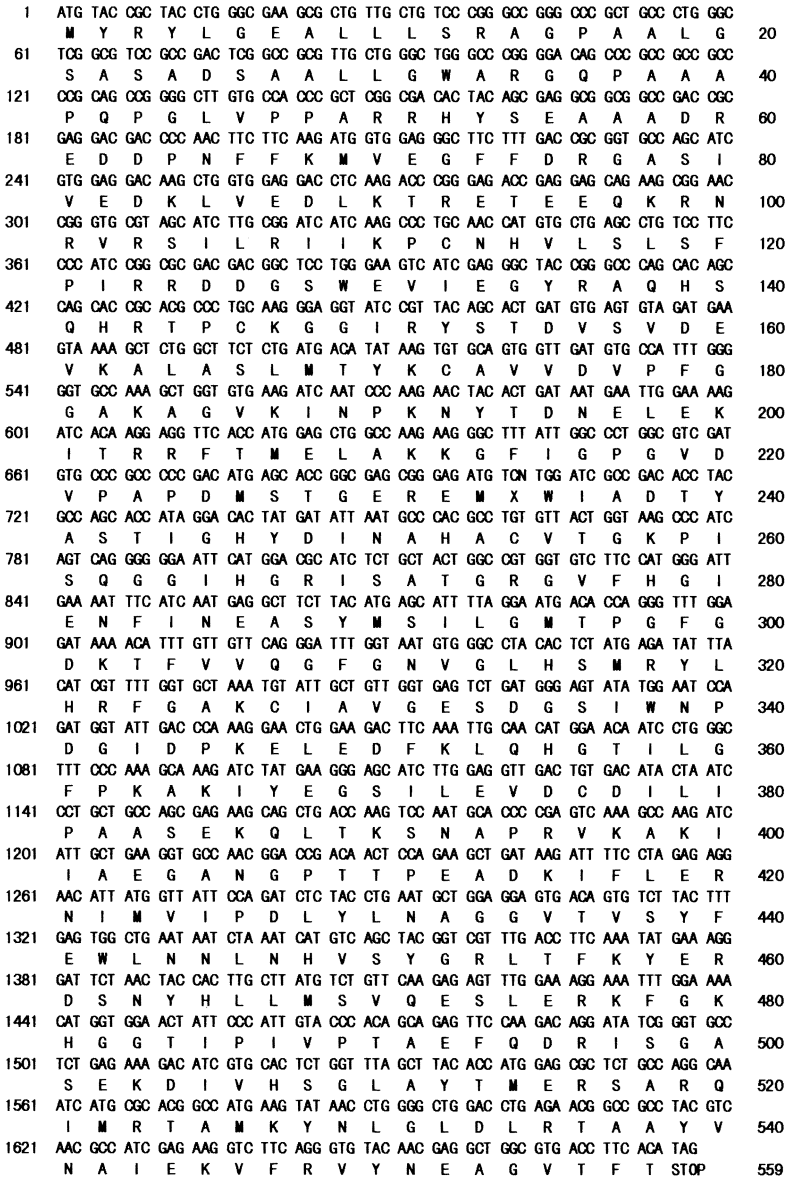

Fig. 1. Nucleotide and deduced amino acid sequence of recombinant bovine brain GDH. The nucleotides are numbered (left margin), beginning with the first base of the ATG initiator codon for GDH; the deduced amino acid residues are also numbered (right margin).

The activity of the GDH recombinant protein is of key importance. Thus, we determined the activities of GDH using the purified enzyme. As shown in Table 1, the specific activity of the recombinant bovine brain GDH was compared with that of the authentic bovine brain GDH enzymes. The purified recombinant bovine brain GDH that is produced in bacteria showed a similarity to that of bovine brain GDH. These results indicate that recombinant GDH has the full activity and function of bovine brain GDH. Additional catalytic activities of the recombinant enzyme are shown in Table 2. The recombinant bovine brain GDH had kinetic properties similar to those of bovine brain GDH in terms of its specific activities, K_m and V_{max}. We also investigated the effects of ATP and ADP on recombinant GDH enzyme activity. Recently, atomic structure of bovine GDH has suggested that ATP, another well known allosteric inhibitor, may bind to the same site as GTP (Stanley *et al*., 1998; Smith *et al*., 2001). Figure 4 shows that both recombinant GDH and bovine brain GDH enzymes were dose-dependently inhibited by ATP. Unlike the effects of ATP, ADP activated the recombinant GDH and bovine brain GDH up to 2.3-fold. ADP and ATP have just the opposite effect on GDH activity. ADP increases the reductive amination reaction velocity, whereas ATP inhibited the reaction at pH 8.0 and at a high concentration of NADH (Bailey *et al*., 1982). Because the atomic structure of bovine GDH suggests that the γ-phosphate of GTP dominates the GTP/GDH interactions (Smith *et al*., 2001), it is possible that ATP can bind to the GTP site (Stanley *et al*., 1998; Smith *et al*., 2001). The recent

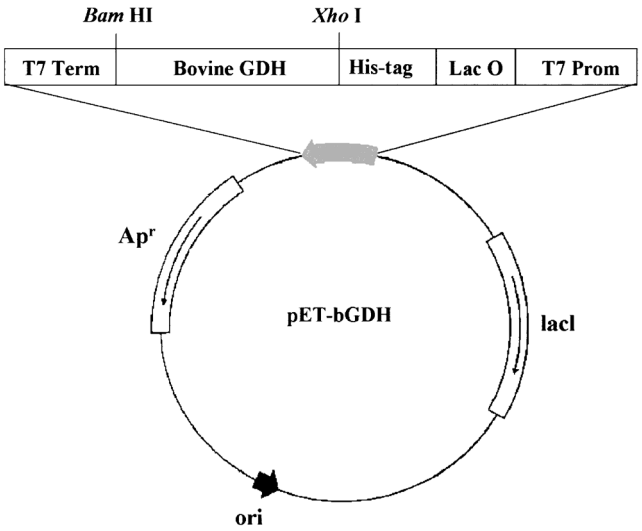

Fig. 2. Construction of the recombinant bovine brain GDH expression vector, pET-bGDH.

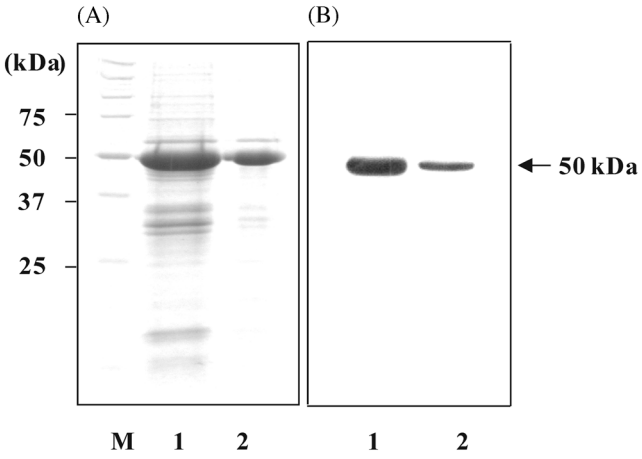

Fig. 3. Expression and purification of recombinant bovine brain GDH in *E. coli*. The protein extracts of the recombinant cells and purified proteins were analyzed by SDS-PAGE on a 10% gel and visualized with Coomassie brilliant blue (A). They were then subjected to an immunoblot analysis with monoclonal antibodies to bovine GDH (B). Lanes in A and B are as follows: lane 1, expressed pET-bGDH; lane 2, purified pET-bGDH.

atomic structure of bovine liver GDH suggests that the allosteric regulation and negative cooperativity that was observed in mammalian GDH may be facilitated by the subunit interactions within the antenna regulation. It was suggested that these allosteric regulations are performed by changing the energy that is required to open and close the catalytic cleft during enzymatic turnover (Peterson and Smith, 1999; Smith *et al*., 2001).

Mammalian GDH is strictly regulated by several allosteric effectors. GTP inhibits the enzyme turnover over a wide range of conditions by increasing the affinity of the enzyme for the product, making the product release rate-limiting under all

Table 1. Activity of purified GDH enzymes from recombinant and bovine brain GDH

GDH	Specific activity (unit/mg)
Recombinant GDH	175
Bovine GDH	180

Table 2. Kinetic parameters of purified GDH enzymes from recombinant and bovine brain GDH

GDH	V_{max} (μmol/min/mg)	K_m (mM)
		NADH
Recombinant GDH	1.08 ± 0.03	0.10
Bovine GDH	1.12 ± 0.02	0.11

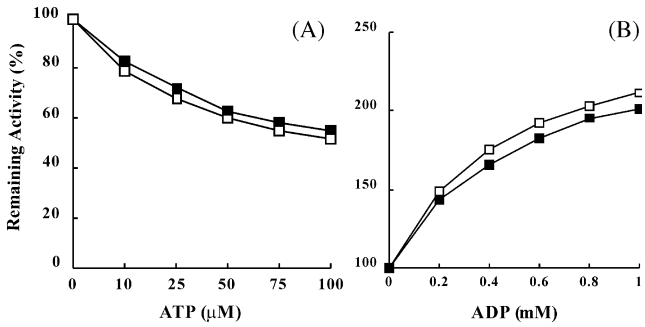

Fig. 4. The effects of ATP and ADP on the activities of the recombinant bovine brain GDH. The enzymes were preincubated with various concentrations of ATP or ADP in an assay buffer. The activities were assayed by the addition of the standard assay mixture with NADH as a co-enzyme. The remaining activities were expressed relative to each control. *Open circles*, recombinant GDH; *closed circles*, bovine brain GDH.

conditions in the presence of GTP (Peterson and Smith, 1999; Smith *et al*., 2001). In contrast, ADP is a potent activator by decreasing product affinity (Plaitakis *et al*., 2000).

It was recently reported that the HI/HA syndrome is caused by the mutation of the GDH gene, which affects GDH enzyme sensitivity to GTP-induced inhibition (Stanley *et al*., 1998). Therefore, the physiological significance of GDH in this regulation is significant. The structure of bovine GDH further supports the consideration that these mutations have indirect effects, either on the GTP binding or on the allosteric effects that are caused by GTP (Peterson and Smith, 1999; Smith *et al*., 2001). In either case, these results demonstrate the importance of GTP and the regulation of GDH activity in the mammalian system. For the last 20 years, the sequence identities and kinetic properties of mammalian GDHs from various sources (including human liver, human brain, rat brain, mouse brain, chicken liver, bovine liver, and bovine brain) have been reported by many researchers. It is very interesting that no other mammalian GDHs, except bovine liver GDH, have been known to show the second co-enzyme site, although the sequence identities between mammalian

GDHs are extremely high.

A recent study on this topic showed that GTP bound to GDH from *E. coli*, an allosteric site, regulatory mutations, structural mechanism, and also reverses the destabilizing effects of the GDH co-enzyme (Fang *et al*., 2002; Maurizi and Rasulova, 2002). This result strongly suggests that the unique 48-amino acid antenna region in mammalian GDH may not be totally responsible for the regulation of ADP and GTP.

The construction of a synthetic gene that encodes GDH would enable the generation of a large number of wild and site-directed mutations at several positions in the coding region. The high level GDH expression as a soluble protein in *E. coli* would allow the purification of large quantities of wild and mutant proteins for biochemical and structural studies. Our results, therefore, suggest that this combination of genetic and biochemical techniques could be used to address a broad range of questions related to the structure and function of GDH.

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