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Nerve Tissue-Specific Human Glutamate Dehydrogenase that Is Thermolabile and Highly Regulated by ADP

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Abstract: Glutamate dehydrogenase (GDH), an enzyme that is central to the metabolism of glutamate, is present at high levels in the mammalian brain. Studies on human leukocytes and rat brain suggested the presence of two GDH activities differing in thermal stability and allosteric regulation, but molecular biological investigations led to the cloning of two human GDH-specific genes encoding highly homologous polypeptides. The first gene, designated GLUD1, is expressed in all tissues (housekeeping GDH), whereas the second gene, designated GLUD2, is expressed specifically in neural and testicular tissues. In this study, we obtained both GDH isoenzymes in pure form by expressing a GLUD1 cDNA and a GLUD2 cDNA in Sf9 cells and studied their properties. The enzymes generated showed comparable catalytic properties when fully activated by 1 mM ADP. However, in the absence of ADP, the nerve tissue-specific GDH showed only 5% of its maximal activity, compared with ~40% showed by the housekeeping enzyme. Low physiological levels of ADP (0.05–0.25 mM) induced a concentration-dependent enhancement of enzyme activity that was proportionally greater for the nerve tissue GDH (by 550–1,300%) than of the housekeeping enzyme (by 120–150%). Magnesium chloride (1–2 mM) inhibited the nonactivated housekeeping GDH (by 45–64%); this inhibition was reversed almost completely by ADP. In contrast, Mg2+ did not affect the nonstimulated nerve tissue-specific GDH, although the cation prevented much of the allosteric activation of the enzyme at low ADP levels (0.05–0.25 mM). Heat-inactivation experiments revealed that the half-life of the housekeeping and nerve tissue-specific GDH was 3.5 and 0.5 h, respectively. Hence, the nerve tissue-specific GDH is relatively thermolabile and has evolved into a highly regulated enzyme. These allosteric properties may be of importance for regulating brain glutamate fluxes in vivo under changing energy demands. Key Words: Glutamate dehydrogenase—Human brain—Glutamate metabolism—ADP enzyme regulation—Enzyme thermolability. J. Neurochem. 68, 1804–1811 (1997).

Glutamate dehydrogenase (GDH), the enzyme that catalyzes the reversible oxidative deamination of glutamate to α-ketoglutarate, is expressed at high levels in mammalian brain (Smith et al., 1975). Although the exact function of GDH in brain is not fully understood, the enzyme is enriched in glutamatergic receptive regions (Aoki et al., 1987), where it is highly concentrated in astrocytes (10 mg of GDH protein/ml of mitochondrial matrix) (Rothe et al., 1994). These observations are consistent with a suggested role for this enzyme in the metabolism of synaptic glutamate (Plaitakis et al., 1984; Colon et al., 1986).

At present, the functional significance of GDH in nerve tissue remains uncertain. Although thermodynamically the GDH reaction favors glutamate synthesis, aspartate aminotransferase may be more important than GDH in forming glutamate from α-ketoglutarate in vivo (Palaiologos et al., 1988; Christensen et al., 1991). Cooper et al. (1979) previously showed that 13NH3 ammonia given systemically to rats failed to incorporate into brain glutamate, thus suggesting that GDH is not functioning toward the synthesis of this amino acid in nerve tissue. On the other hand, Yu et al. (1982) suggested that GDH may play a role in glutamate oxidation in astrocytes. Also, Erecinska and Nelson (1990) found that activation of GDH activity increased the rate of glutamate oxidation by rat brain synaptosomes. Other studies (Kuo et al., 1994) have also suggested that, in spite of the high GDH concentration in brain, the metabolic flux through this pathway is quite low in vivo, thus inferring that enzyme activity is regulated. Kuo et al. (1994) further suggested that, in rat brain, this is due to the inhibitory effect of magnesium and polyamines on GDH activity.

Previous studies have shown that GDH activity is reduced in leukocytes and fibroblasts of patients with...
multisystemic neurological disorders that are clinically and pathologically heterogeneous (Plaitakis et al., 1980, 1982; Duvoisin et al., 1983, 1988; Aubby et al., 1988; Abe et al., 1992). Our work with neurologic patients led to the finding that GDH is present in human tissues in “heat-labile” and “heat-stable” forms and that, in these patients, reduction in GDH activity was largely limited to the heat-labile component (Plaitakis et al., 1984). Similar results have been obtained by other (Konagaya et al., 1986; Kajiyama et al., 1988; Iwatsuji et al., 1989; Abe et al., 1992) but not all (Aubby et al., 1988; Duvoisin et al., 1988) investigators. Additional studies in our laboratory (Colon et al., 1986) showed the presence of two GDH activities in rat brain differing in their relative resistance to thermal inactivation, detergent extractability, and allosteric regulation characteristics. Further investigations at the protein level (Hussain et al., 1989) revealed that GDH purified from human brain consists of four electrophoretically distinct isoproteins. More recent studies have shown that two different GDH isoproteins are also present in bovine brain (Cho et al., 1995).

Molecular biological studies (Mavrothalassitis et al., 1988; Shashidharan et al., 1994) revealed that multiple GDH-specific genes are present in the human. At least two of these genes are functional. The first, designated GLUD1, is an intron-containing gene mapping to human chromosome 10; the mRNA of this gene is expressed in all tissues (housekeeping) (Hanauer et al., 1987; Mavrothalassitis et al., 1988; Anagnou et al., 1993; Michaelidis et al., 1993) and is the only GDH-specific message found in human liver (Shashidharan et al., 1994). The second, designated GLUD2, is encoded by an X-linked intronless gene, the mRNA of which is specifically expressed in neural and testicular tissues (Shashidharan et al., 1994).

The polypeptides predicted by the two genes share a 96% amino acid sequence homology (Shashidharan et al., 1994), with this similarity in structure probably accounting for the inability to separate them in a clean form from human tissues. The availability of recombinant DNA technology has permitted us now to obtain and study each of these human GDH isoenzymes in pure (unmixed), catalytically active forms by expressing the corresponding cDNAs in the insect Spodoptera frugiperda (Sf9) cells. Results revealed that the nerve tissue-specific isofrom has evolved into a highly regulated enzyme that is markedly activated by ADP at concentrations that can occur when cellular energy charge declines.

**MATERIALS AND METHODS**

**Materials**

Sf9 cells and the baculovirus expression vectors were obtained from Invitrogen (San Diego, CA, U.S.A.). Insect cell media and fetal calf serum were obtained from Life Technol-
each of these cDNAs was PCR-amplified (Shashidharan et al., 1994). The products obtained were subcloned, ligated in a sense specific (GLUD2-derived GDH; C) GDHs were obtained by expression of the corresponding cDNAs in Sf9 cells as described in Materials and Methods. The enzyme extracts were mixed with 1:1 (vol/vol) bovine serum albumin (8 mg/ml) in 100 mM (pH 7.4) phosphate buffer. The enzyme mixture was incubated at 45.5°C in Eppendorf tubes. Aliquots were removed at specified intervals and assayed at pH 8.0 in the presence of 1.0 mM ADP as described in Materials and Methods. Data are mean ± SEM (bars) values of three determinations. Baseline activity refers to enzyme activity at 0 min of incubation.

Expression of GLUD1 and GLUD2 cDNA in Sf9 Cells

A GLUD1 cDNA and a GLUD2 cDNA derived from human liver and retina mRNA, respectively, were expressed in the baculovirus expression system. The coding region of each of these cDNAs was PCR-amplified (Shashidharan et al., 1994). The products obtained were subcloned, ligated to the baculovirus transfer vector (pVL 1392; Invitrogen), and used to transform Escherichia coli (INVAlphaF'). The proper orientation of the insert was verified by DNA sequencing. Sf9 cells were cotransfected with the plasmid DNA and modified baculovirus DNA (BaculoGold; Pharmingen) and were incubated at 27°C for 4 days. The recombinant virus was amplified with two additional rounds of infection. Sf9 cells were harvested and homogenized in a buffer containing 0.05 M Tris-HCl (pH 7.4), 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5 M NaCl. The extracts obtained were used for enzyme assays, kinetic analyses, thermal inactivation, and immunoblotting, as described above. The protein concentrations were determined as previously described (Lowry et al., 1951).

Enzyme assay

GDH was assayed spectrophotometrically in the direction of reductive amination of α-ketoglutarate. For this, the reaction mixture of 1 ml contained 50 mM triethanolamine buffer (pH 8.0, except as indicated), 100 mM ammonium acetate, 150 μM NADPH, and 2.6 mM EDTA. ADP was also added to 1 mM unless otherwise specified. The reaction was started with addition of α-ketoglutarate to 8 mM (Colon et al., 1986).

Allosteric regulation of human recombinant GDHs

Stimulation of GDH activity by ADP was carried out by adding increasing amounts of ADP (final concentration, 0.05–1.0 mM) to the above assay mixture. Similar ADP stimulation studies were also carried out at different pH values using 0.1 M phosphate buffer (pH 6.5, 7.0, 7.5, or 8.0). The effects of spermine and Mg2+ on GDH activity were determined by adding either compound to the reaction mixture (final concentration, 1–2 mM) with or without ADP.

Heat inactivation of recombinant GDHs

Sf9 cell extracts containing expressed GLUD1- or GLUD2-derived protein were mixed (1:1) with 100 mM sodium phosphate buffer (pH 7.4) containing 8 mM bovine serum albumin. The samples were incubated at 45.0°C in a shaking water bath; aliquots were removed at specific intervals and assayed immediately as described above with the addition of 1.0 mM ADP (final concentration) to the reaction mixture.

Purification and N-terminal amino acid sequencing of recombinant GDHs

Both recombinant human GDHs were partially purified from Sf9 cell extracts according to a previously described method (Hussain et al., 1989), and the amino acid sequence of their N-terminus was determined. In brief, the purification method involves ammonium sulfate fractionation and hydrophobic interaction chromatography. A 30–65% ammonium sulfate cut of a high-speed (10,000 g for 1 h) supernatant of these extracts was obtained and dissolved in Tris-HCl buffer (0.05 M, pH 6.0) containing ammonium sulfate to 15% saturation. It was then loaded onto a column (1.5 × 30 cm) packed with phenyl-Sepharose-4B and equilibrated with the same buffer. The column was washed with 200 ml of equilibration buffer (to remove any nonspecifically bound proteins) and eluted with a total of 250 ml of a double linear gradient of 0–90% ethylene glycol and 15–0% ammonium sulfate. Fractions containing GDH activity were pooled, dialyzed against deionized distilled water, and loaded on a discontinuous 5–15% linear gradient polyacrylamide gel containing 0.1% sodium dodecyl sulfate as previously described (Hussain et al., 1989). The proteins were transferred to a polyvinylidene difluoride membrane according to the method of Matsudaira (1987) using 250 mM constant current for 4 h in a Bio-Rad electroblothing apparatus. The electrophoretically transferred polyvinylidene difluoride membrane was washed in deionized distilled water for 5 min, stained with 0.1% Coomassie Brilliant Blue R250 in 50% methanol for 30 s, and then destained for 3–5 min in 50% methanol/10% acetic acid. It was finally rinsed in deionized distilled water and air-dried. The GDH band was cut out and used for N-terminal amino acid sequencing (Matsudaira, 1987).
Statistical analysis
The significance of the effect of Mg\(^{2+}\) on recombinant human GDH activities was analyzed by an unpaired two-tailed \(t\) test. The statistical analysis was carried out using Microsoft Excel version 4.0 software. The Hill plot coefficient and the cooperativity index were calculated using Enzfit software (Elsevier-BIOSOFT, Cambridge, U.K.).

RESULTS

Production of recombinant human GDHs
The specific multiplication of recombinant baculovirus containing either the \(GLUD1\) or the \(GLUD2\) cDNA in the Sf9 cell cultures infected by the recombinant viruses was verified by \(SalI\) digestion of a PCR-amplified DNA segment containing a \(SalI\) restriction site unique to the \(GLUD2\) cDNA (Shashidharan et al., 1994). As shown in Fig. 1, this treatment resulted in complete cleavage of the \(GLUD2\) cDNA-derived PCR product, but it left uncut the \(GLUD1\) cDNA-derived DNA segment. Immunoblots of Sf9 cell extracts using anti-GDH antiserum (Shashidharan et al., 1994) revealed that cells infected with both recombinant baculoviruses showed GDH-positive immunoreactivity, the density of which increased between day 2 to 5 post-infection (data not shown).

Sequencing of the N-terminus of the recombinant proteins revealed that both the \(GLUD1\) - and \(GLUD2\)-generated polypeptides had been processed within the host cells in a manner similar to that occurring for the mature mammalian GDH, which involves removal of the leader peptide (Fig. 2). Moreover, these sequencing data confirmed the presence of leucine and alanine at position 3 for the nerve tissue-specific (\(GLUD2\)-derived) and the housekeeping (\(GLUD1\)-derived) GDH, respectively, as predicted by the corresponding cDNAs (Fig. 2), thus verifying the specific expression of the cDNAs in the corresponding cell lines.

Thermal stability characteristics of \(GLUD1\) - and \(GLUD2\)-generated GDH
Heat-inactivation studies revealed that the two recombinant proteins differed in their relative resistance to thermal denaturation. At 45°C (pH 7.4), heat inactivation proceeded faster for the nerve tissue-specific GDH (half-life = 0.5 h) than for the housekeeping enzyme (half-life = 3.5 h), as shown in Fig. 3.
Regulation of recombinant GDH activities by ADP

Enzyme assays of Sf9 cell extracts revealed that expression of the GLUD1 or GLUD2 in these cells generated proteins capable of catalyzing the reversible interconversion of glutamate to $\alpha$-ketoglutarate in the presence of either NAD(H) or NADP(H). Because the insect GDH shows an absolute specificity for NAD(H) (Shashidharan et al., 1994) and the human GDH is capable of using both cofactors (Plaitakis et al., 1980), measurement of enzyme activity in the presence of NADP(H) provided a simple way of eliminating all background activity.

The specific activities of the two recombinant human GDHs were similar when analyzed in the presence of 1 mM ADP. However, the two enzymes differed markedly in their ability to interconvert glutamate to $\alpha$-ketoglutarate when assayed in the absence of allosteric effectors; the nerve tissue-specific GDH was largely inactive, whereas the housekeeping enzyme showed about half of its maximal specific activity (Fig. 4). ADP at 0.05–0.25 mM induced a proportionally greater degree of stimulation for the nerve tissue-specific than for the housekeeping enzyme. This differential effect increased at higher nucleotide concentrations (0.5–1.0 mM), with 1.0 mM ADP stimulating the nerve tissue-specific GDH to ~2,000% of baseline activity while inducing only a 2.5-fold activation of the housekeeping enzyme (Fig. 4).

Effect of pH on ADP activation of recombinant human GDHs

Enzyme assays, performed at various pHs, revealed that the nonstimulated activity (baseline) of the GLUD1-derived GDH (housekeeping) increased by about fourfold as the pH of the reaction buffer increased from 6.5 to 8.0 (Fig. 5); however, the ADP activation of the GLUD1 isoenzyme remained proportionally the same (200–250% of baseline activity; Fig. 5). In contrast, whereas the baseline activity of the GLUD2-derived GDH (nerve tissue-specific) was unaffected by varying the pH of the reaction buffer, the ADP activation of this isoenzyme was enhanced with increasing pH; addition of 0.5 mM ADP (final concentration) at pH 6.5, 7.0, 7.5, and 8.0 increased the activity of the GLUD2-derived GDH to 600, 700, 1,238, and 2,100% of baseline activity, respectively (Fig. 5). Hence, increasing the pH of the reaction buffer between 6.5 and 8.0 enhances the nonstimulated (baseline) activity of the housekeeping GDH, whereas it renders the nerve tissue-specific isoenzyme increasingly sensitive to allosteric activation by ADP.
Effect of magnesium chloride on recombinant human GDHs

In the absence of ADP, magnesium chloride (1.0 and 2.0 mM) inhibited the housekeeping GDH (by 45 and 64%, respectively), but it had no effect on the nonstimulated nerve tissue-specific enzyme (Fig. 6). In the presence of low physiological concentrations of ADP (0.05–0.25 mM), however, this inhibitory effect of magnesium chloride was completely abolished (Fig. 7). Magnesium had little effect on the baseline activity of the nerve tissue-specific GDH, but it modified its allosteric regulation by preventing most of the activation of this enzyme at 0.05–0.25 but not at 1.0 mM concentrations of ADP. As a result, the hyperbolic ADP activation curve became sigmoidal in the presence of magnesium chloride (Fig. 6). This change was significant by Hill plot analysis, which showed that the Hill plot coefficient for ADP (0.05–0.25 mM), but it had no effect on the housekeeping GDH (by 45 and 64%, respectively), but this inhibition was significant by Hill plot analysis, which showed that the Hill plot coefficient for ADP stimulation was 1.24 ± 0.02 in the absence of magnesium chloride and 1.81 ± 0.04 (p < 0.001) in the presence of the cation. Spermine, shown to be inhibitory to rat brain GDH (Kuo et al., 1994), had no effect on either human GDH isoenzyme (data not shown).

DISCUSSION

In this study, we evaluated the thermal stability and allosteric regulation characteristics of recombinant human GDH isoproteins generated by expression of GLUD1 (housekeeping) and GLUD2 (nerve tissue-specific) cDNAs in SF9 cells. Results revealed that the housekeeping GDH is relatively heat-stable, whereas the nerve tissue-specific enzyme is relatively heat-labile. Because activity of the heat-labile GDH is selectively reduced in some patients with multisystem degeneration, these results suggest a selective defect in the nerve tissue-specific GDH in these patients. This remains to be tested in future studies.

Evaluation of the regulatory properties of the two expressed GDH isoproteins revealed that the nerve tissue-specific GDH has evolved into a highly regulated enzyme showing little catalytic activity when assayed in the absence of allosteric effectors but being markedly activated (up to 1,300%) by relatively low physiological concentrations of ADP (0.02–0.25 mM). In contrast, the housekeeping GDH depends much less on ADP for its activation, maintaining ~50% of its maximal activity in the absence of this nucleotide.

Varying the pH of the reaction buffer had a differential effect on the allosteric regulation of the two isoenzymes by ADP. When the pH increased between 6.5 and 8.0, the nonstimulated (baseline) activity of the housekeeping GDH was enhanced, although the proportional activation of this isoenzyme by ADP was not altered. In contrast, increasing the pH between 6.5 and 8.0 markedly enhanced the ADP activation of the nerve tissue-specific GDH without affecting its baseline specific activity. Bailey et al. (1982) previously reported that ADP is inhibitory to bovine liver GDH when the reaction is carried at pH 6.5 and below. In the present studies, we found that ADP had no inhibitory effect on either human GDH isoenzyme at pH 6.5. Similar results have been reported by Cho et al. (1995) using bovine brain GDHs.

In addition, regulation of enzyme activity by magnesium was distinct for the two isoenzymes. The housekeeping GDH was inhibited substantially by 1.0 or 2.0 mM magnesium chloride when assayed in the absence of allosteric effectors, but this inhibition was
largely reversed by low physiological concentrations (0.05–0.25 mM) of ADP. Whereas magnesium had little effect on the baseline activity of the nerve tissue-specific GDH, it modified its allosteric regulation by preventing most of the activation of this enzyme at known to be in the range of 0.05–0.25 mM (depending on the rate of oxidative phosphorylation) (Gabriel et al., 1986). Hence, results of this study indicate that physiological levels of ADP and magnesium are capable of allosterically regulating human nerve tissue-specific GDH. In particular, magnesium is capable of shifting the relatively hyperbolic ADP stimulation curve to one that is clearly sigmoidal. As such, magnesium may play a role in keeping the nerve tissue-specific GDH idle at low physiological concentrations of ADP (high rate of phosphorylation), while permitting activation of this enzyme by higher concentrations of this nucleotide (low rate of phosphorylation).

When fully activated by ADP, the specific activity of GDH purified from human brain is ~160 μmol of glutamate/mg of protein/min (Hussain et al., 1989). Because the level of GDH in cerebellum is ~10 mg/ml of mitochondrial matrix (Rothe et al., 1994), this represents a capacity for interconverting 1,600 μmol of glutamate/min/ml of mitochondria matrix or 160 μmol of glutamate/min/ml of astrocytic volume (mitochondria account for ~10% of a cellular mass). When intracellular glutamate levels are low, unregulated high GDH function could deplete α-ketoglutarate with resultant impairment of the tricarboxylic acid cycle and other reactions that depend on this ketoacid. It is possible that the ability of the nerve tissue-specific GDH to remain relatively inactive at low ADP levels may represent an adaptation that permits the enzyme to attain high levels in astrocytes without interfering with metabolic homeostasis of these cells.

In light of the present findings, the variable GDH flux rates found in cultured astrocytes by several investigators (Yu et al., 1984; Erecińska and Nelson, 1990; Yudkoff et al., 1991; Farinelli and Nicklas, 1992) may relate to the state of GDH activation. Also, the present data suggest that under conditions of increased hydrolysis of ATP to ADP, GDH function may be enhanced, leading to increased oxidation or synthesis of glutamate by glial cells. With respect to the latter, the role of GDH in brain glutamate formation remains controversial, particularly given the fact that the \( K_m \) for ammonia is relatively high for this enzyme (~12–20 mM) (Dennis et al., 1977; Colon et al., 1986). It remains to be further studied whether these enzyme properties are essential for the regulation of glutamate metabolism under changing energy demands and whether this enzyme plays a role in protecting neurons against excitotoxicity under conditions of enhanced glutamate release and/or energy failure.

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REFERENCES


