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Purification and Characterization of a Soluble and a Particulate Glutamate Dehydrogenase from Rat Brain

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Abstract: Glutamate dehydrogenase (GDH) activity was determined in high-speed fractions (100,000 g for 60 min) obtained from whole rat brain homogenates after removal of a low-speed pellet (480 g for 10 min). Approximately 60% of the high-speed GDH activity was particulate (associated with membrane) and the remaining was soluble (probably of mitochondrial matrix origin). Most of the particulate GDH activity resisted extraction by several commonly used detergents, high concentration of salt, and sonication; however, it was largely extractable with the cationic detergent cetyltrimethylammonium bromide (CTAB) in hypotonic buffer solution. The two GDH activities were purified using a combination of hydrophobic interaction, ion exchange, and hydroxyapatite chromatography. Throughout these purification steps the two activities showed similar behavior. Kinetic studies indicated similar $K_m$ values for the two GDH fractions for the substrates $\alpha$-ketoglutarate, ammonia, and glutamate; however, there were small but significant differences in $K_m$ values for NADH and NADPH. Although the allosteric stimulation by ADP and $L$-leucine and inhibition by diethylstilbestrol was comparable, the two GDH components differed significantly in their susceptibility to GTP inhibition in the presence of 1 mM ADP, with apparent $K_i$ values of 18.5 and 9.0 $\mu$M GTP for the soluble and particulate fractions, respectively. The Hill plot coefficient, binding constant, and cooperativity index for the GTP inhibition were also significantly different, indicating that the two GDH activities differ in their allosteric sites. In addition, enzyme activities of the two purified proteins exhibited a significant difference in thermal stability when inactivated at 45°C and pH 7.4 in 50 mM phosphate buffer. Key Words: Glutamate dehydrogenase—Particulate—Soluble—GTP—Allosteric regulation. Colon A. D. et al. Purification and characterization of a soluble and a particulate glutamate dehydrogenase from rat brain. J. Neurochem. 46, 1811-1819 (1986).

Substantial amounts of glutamate dehydrogenase (GDH) activity are known to be present in mammalian brain (Kuhlman and Lowry, 1956; Williamson et al., 1967; Leong and Clark, 1984). Glutamate, the substrate of the GDH-catalyzed reaction, is thought to serve as excitatory transmitter in the nervous system (Fonnum, 1984). Because of its known neurotoxic potentials (Olney et al., 1971), the compound may be involved in human neurodegenerative processes (McGeer and McGeer, 1976; Coyle, 1982; Plaitakis et al., 1982) or mediate neuronal damage in response to various insults (Simon et al., 1984; Rothman, 1984; Wieloch, 1985). The exact function of GDH in nervous tissue under physiological or pathological conditions to this date remains obscure. However, the enzyme may be of particular importance in understanding the pathogenesis of human degenerative disorders because it has been described to be deficient in leukocytes and fibroblasts from patients with multisystem degeneration of the olivopontocerebellar atrophy (OPCA) type (Plaitakis, et al., 1980, 1984; Yamaguchi, 1982; Duvoisin et al., 1983).

Although studies on the purification and properties of brain glutamate dehydrogenase have been recently reported (Chee et al., 1979; McCarthy et al., 1980), the brain enzyme has not been studied as extensively as the liver GDH. Previous studies have shown that in animal liver the enzyme is localized in the mitochondrial matrix (Hodgeboom...
and Schneider, 1953), and this has been supported by the ease with which the enzyme is released in soluble form by tissue homogenization (Schmidt, 1963; Fahien et al., 1969).

On the other hand, there are indications from recent studies that distinct readily solubilized and membrane-associated forms of GDH may exist in human leukocytes and cultured skin fibroblasts that can be separated by high-speed centrifugation (Plaitakis et al., 1984). Furthermore, in patients with OPCA associated with a partial deficiency of GDH, the defect has been shown to be limited to the membrane-associated form of the enzyme, suggesting that the two activities may be under different genetic control (Plaitakis et al., 1984).

In view of these considerations, in the present study we determined GDH activity in high-speed fractions (100,000 g for 1 h) isolated from whole homogenates of thoroughly disrupted rat brain tissue and found most of the sedimented activity to be tightly bound to the high-speed pellet (HSP; membrane-associated). Readily solubilized (probably of mitochondrial matrix origin) and particulate brain enzyme activities were purified to homogeneity and their properties were studied. Results of this study have been presented in part (Colon et al., 1985).

MATERIALS AND METHODS

Preparation of crude soluble and particulate GDH

Adult male Sprague-Dawley rats (200–250 g weight) were killed by decapitation and the cerebri quickly removed, washed in 50 mM Tris-HCl, pH 7.4, and subjected to three or four freeze/thaw cycles on dry ice. A 20% (wt/vol) homogenate of the minced tissue in 50 mM Tris-HCl, pH 7.4, was prepared by glass-to-glass shearing (0.004–0.006 inch clearance) for 5 min at 0–5°C with a motor-driven pestle (300 rpm). For some experiments the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (0.1 mM) was included in the homogenization buffer. Centrifugation for 10 min at 480 g yielded a low-speed supernatant (LSS) which was further fractionated, at 100,000 g for 60 min, into a high-speed supernatant (HSS) containing the soluble GDH activity, and a high-speed, membrane-rich, pellet (HSP) containing the particulate GDH. For experiments using these crude GDH preparations, the HSP was resuspended in 50 mM Tris-HCl, pH 7.4 (volume equal to that of LSS), and assays were performed as described below, with the addition of Triton X-100 (0.05% final concentration) to the reaction mixture. To test the extractability of the particulate GDH activity, aliquots of HSP suspensions were treated with various detergents such as Triton X-100 (0.1–2%), digitonin (0.1%), deoxycholate (0.1%), cetyltrimethylammonium bromide (CTAB) (0.05–1%), or salt at high ionic strength, such as KCl (0.2–2 M) or NaCl (0.2–2 M) for 1 h at 5°C and then recentrifuged at 100,000 g for 60 min. The second HSP was again resuspended in 50 mM Tris-HCl, pH 7.40, buffer and GDH assays were performed as described below. In addition, aliquots of HSP suspensions (in 50 mM Tris-HCl) were subjected to sonication and then recentrifuged as described above.

Purification of soluble and particulate GDH

The HSS represented the starting material for the purification of the readily solubilized GDH activity. For the purification of the particulate GDH activity the HSP was gently rinsed with 50 mM Tris-HCl buffer, pH 7.40, then resuspended in the same buffer, now containing 0.05% CTAB, a cationic detergent that was found capable of extracting most of the particulate enzyme. The HSP-CTAB extract was then used as the starting material for the purification procedures described below. The procedures beyond this point were identical for both GDH fractions. All purification steps were done at 0–5°C.

Hydrophobic interaction chromatography. Following ammonium sulfate fractionation of the starting material, the 30–60% ammonium sulfate pellets were redissolved in a minimum volume of 50 mM Tris-HCl, pH 6.0, containing ammonium sulfate to 15% saturation, and loaded onto a column of phenyl-Sepharose (Pharmacia) 1.5 cm × 29 cm, equilibrated in the same buffer. The column was washed with two bed volumes (100 ml) of start (column equilibration) buffer to remove any unbound protein, followed by elution with a total of 250 ml of a double gradient of 0–90% ethylene glycol and 15–0% ammonium sulfate in 50 mM Tris-HCl at pH 6.0. Fractions containing the GDH activity were pooled and the ethylene glycol and residual ammonium sulfate removed by a 2-h dialysis (at 4°C) against several changes at 25 mM Tris-HCl, pH 7.5, containing 0.1 M KCl (to protect the enzyme activity) in preparation for ion-exchange chromatography. GDH has been found to be unstable in Tris buffer in the absence of salt ions (KCl or NaCl) or ethylene glycol.

Chromatography on DEAE-Sepharcel. The dialyzed pool of GDH-containing phenyl-Sepharose fractions was loaded onto a column of DEAE-Sepharcel, 1.5 cm × 29 cm, equilibrated in the same buffer used for dialysis. Unbound protein was washed out with two bed volumes of start buffer, followed by elution with a 200 ml gradient of 0.1–0.6 M KCl in 25 mM Tris-HCl, pH 7.5. Fractions containing the GDH activity were pooled.

Chromatography on hydroxyapatite. The pool of DEAE-Sepharcel GDH-containing fractions was loaded, without buffer exchange, on a small column of hydroxyapatite, 1 cm × 12 cm, equilibrated in 20 mM Na phosphate buffer, pH 7.5. Preliminary batch experiments demonstrated that this procedure was satisfactory and compatible with complete binding. The column was first washed with 60 ml equilibration buffer, then eluted with a 120-ml gradient of 20–200 mM sodium phosphate at pH 7.5.

Enzyme assays

GDH activity was assayed spectrophotometrically or fluorometrically in the direction of reductive amination of α-ketoglutarate in a medium containing 50 mM triethanolamine buffer (pH 8.0), 100 mM ammonium acetate, 90 μM NADH, 2.6 mM EDTA, and 1 mM ADP and the reaction started with the addition of α-ketoglutarate to 8 mM (Schmidt, 1963; Plaitakis et al., 1984). Initial rates were recorded at 25°C. GDH activity was also measured fluorometrically in the direction of glutamate oxidation, at 28°C, in a medium containing 20 mM K-phosphate, pH 7.7; 300 mM nicotinamide; 0.4 mM Na cyanide; 1.4 mM NAD; and 0.67 mM ADP. The reaction was started with the addition of l-glutamate to 12.9 mM.

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Thermal inactivation

The hydroxypatite-purified GDH activities, in approximately 100 mM sodium phosphate buffer, pH 7.5, were mixed 1:1 with bovine serum albumin, 8 mg/ml in water, to achieve a protein concentration of 4 mg/ml in 50 mM phosphate buffer, pH 7.4. The samples were incubated in a shaking water bath maintained at 45 ± 0.5°C, and aliquots removed at specified intervals and assayed immediately as described above.

Substrate kinetics and stimulation-inhibition studies

Unless otherwise specified, the purified GDH fraction was used for kinetic studies. The assays were performed by varying the substrate under investigation while keeping the other substrates and reagents at the optimal concentrations already indicated above. α-Ketoglutarate was varied between 0.8 and 8 mM, ammonium acetate between 10 and 100 mM, NADH between 16 and 100 μM, NADPH between 30 and 120 μM, and glutamate between 0.8 and 13 mM.

Stimulation-inhibition studies with ADP, l-leucine, GTP, and diethylstilbestrol (DES) were performed using purified GDH, as well as crude HSS and HSP prepared with and without the inclusion of PMSF. ADP was varied between 0.025 and 1 mM and l-leucine between 1 and 10 mM. The GTP concentration was varied from 5 to 40 μM and assayed in the presence of 1.0 mM ADP while all substrates and reagents were kept at the concentrations already specified. Finally, DES concentration was varied between 0.25 and 1 μM assayed in the presence of 1 mM ADP.

Sodium dodecyl sulfte-polycrylamide gel electrophoresis (SDS-PAGE) and protein determination

Electrophoretic separations and sample pretreatment were performed essentially as described by Laemmli (1970). Protein determination was by the method of Lowry et al. (1951).

Materials

ADP, GTP (lithium salt), NAD, NADH, NADPH, α-ketoglutaric acid, and bovine liver glutamate dehydrogenase were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A. CTAB (5% solution), DES, and l-glutamic acid (monosodium salt) were from Sigma Chemical, St. Louis, MO, U.S.A. l-Leucine was obtained from Cal Biochem. Phenyl-Sepharose and DEAE-Sephadex were purchased from Pharmacia, Uppsala, Sweden. Hydroxyapatite, Bio-gel HT was obtained from BioRad. NADH and NADPH solutions were made fresh on the day of the assays. α-Ketoglutaric acid was dissolved in distilled water and titrated to pH 6.7 with KOH. DES was dissolved in water with the addition of a few drops of 0.1 M KOH.

Statistical analysis

The $K_m$ values ± SD were obtained by the method of Wilkinson (1961). The significance of the differences in $K_m$ were estimated by the use of the Student’s $t$ test. The Hill plot coefficient, binding constant, and the cooperativity index for the GTP inhibition were evaluated using the equation originally described by Hill according to the method discussed by Cornish-Bowden (1979).

RESULTS

Approximately 60% of the GDH activity present in the LSS of whole brain homogenate was found to be associated with the HSP (particulate) and the remaining was soluble (Table 1). Most of the particulate GDH activity was not extractable with any of the nonionic detergents used, whereas high-molarity salt solutions solubilized about 25% of this activity (Table 2). In contrast, the cationic detergent CTAB was found capable of extracting about 60% of the particulate GDH activity (Table 2); repeated extractions with 0.05% of CTAB (in 50 mM Tris-HCl, pH 7.40) resulted in the removal of most of the remaining GDH activity from the HSP (Table 3). The use of SDS and urea destroyed the enzymatic activity whereas sonication of the HSP did not result in solubilization of GDH activity.

The chromatographic separations of the particulate GDH is shown in Fig. 1; Recoveries and enrichments of protein and enzymatic activity during the various purification steps are shown in Table 3. Similar results were also obtained during purification of the readily solubilized enzyme activity.

In general, throughout the chromatographic separations both activities behaved similarly. Peak activities were eluted from the phenyl-Sepharose column at viscosities corresponding to an apparent ethylene glycol concentration of 54%. Both fractions were eluted from the DEAE column at approximately 0.155 M KCl, and from the hydroxyapatite column at approximately 100 mM phosphate at peak activity. Approximately 0.04% of the protein and 14% of the activity were recovered after hydroxyapatite chromatography (Table 3; Fig. 1).

| TABLE 1. Distribution of GDH activity in rat forebrain fractions |
|-----------------|-----------------|-----------------|
| Fraction | Specific activity (μmol NADH oxidized/mg protein/h) | Percent total activity | Total protein (g) |
| WH | 22.9 ± 2.4 | 100 | 5.2 |
| LSP | 20.3 ± 4.2 | 48.4 ± 9.9 | 3.0 |
| LSS | 26.9 ± 4.3 | 50.3 ± 6.7 | 2.2 |
| HSP | 37.4 ± 6.9 | 30.8 ± 2.5 | 1.0 |
| HSS | 18.6 ± 3.7 | 19.6 ± 9.2 | 1.2 |

The GDH specific activity is expressed in μmol NADH oxidized/mg protein/h. Values are means ± SEM from four different experiments. GDH activity was measured in the direction of reductive amination of α-ketoglutaric acid as described in Materials and Methods. The GDH activity was not extractable with any of the nonionic detergents used, whereas high-molarity salt solutions solubilized about 25% of this activity. If the detergent and the ADP were not added to the reaction mixture at the time of assay, the activity of the pellets was markedly underestimated. The fractions were prepared as described in Materials and Methods. HSP and HSS were prepared from the LSS. The low-speed pellet (LSP) and HSP were resuspended in 50 mM Tris-HCl, pH 7.40, buffer in volume equal to that of the whole homogenate (WH) and LSS, respectively. The total protein values are from a representative experiment in which the forebrains of 50 adult rats (weight 200–250 g) were used for the preparation of the fractions yielding 290 ml of total homogenate.
TABLE 2. Extraction of HSP GDH activity by detergents and salt solutions

<table>
<thead>
<tr>
<th>Protein / recovery</th>
<th>Activity / recovery</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent of total</td>
<td>µmol/hr (%)</td>
<td>µmol/mg protein/h</td>
</tr>
<tr>
<td>HSP activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer wash-solubilized GDH</td>
<td>1.2 ± 0.2</td>
<td>2.0 ± 0.8</td>
</tr>
<tr>
<td>Triton (1%) -solubilized GDH</td>
<td>8.0 ± 2.1</td>
<td>4.8 ± 0.7</td>
</tr>
<tr>
<td>Digitonin (0.1%) -solubilized GDH</td>
<td>1.4 ± 0.9</td>
<td>4.7 ± 2.8</td>
</tr>
<tr>
<td>Deoxycholate (0.1%) -solubilized GDH</td>
<td>9.9 ± 0.4</td>
<td>19.5 ± 3.5</td>
</tr>
<tr>
<td>NaCl (0.5 M)-solubilized GDH</td>
<td>27.1 ± 3.1</td>
<td>65.3 ± 7.2</td>
</tr>
<tr>
<td>KCl (0.5 M)-solubilized GDH</td>
<td>26.6 ± 3.5</td>
<td>64.4 ± 10.1</td>
</tr>
<tr>
<td>CTAB (0.05%)-solubilized GDH</td>
<td>60.3 ± 4.6</td>
<td>160.0 ± 25.6</td>
</tr>
</tbody>
</table>

The HSP was prepared from whole homogenates of rat forebrain as described in Materials and Methods. It was resuspended in 50 mM Tris-HCl, pH 7.40 buffer (3.5 mg protein/ml) in the absence or presence of detergents or salts at the final concentration indicated. The suspensions were allowed to stay at 0-5°C for 1 h. The resulting second HSP (resuspended in the same buffer) and HSS were tested for GDH activity measured in the direction of reductive amination of α-ketoglutarate as described in Materials and Methods. Triton X-100 (0.05%) and 1 mM ADP (final concentration) were added to the reaction mixture at the time of assay. Specific activity: µmol NADH oxidized/mg protein/h. Values are means ± SEM. Numbers in parentheses indicate the number of experiments. If the suspensions were allowed to stay at 0-5°C for up to 24 h, little additional GDH activity could be extracted. However, repeated extractions (2-4) with 0.05% CTAB resulted in solubilization of 80-90% of the total GDH activity present in the HSP. Thorough homogenization (for up to 10 min) of the HSP in the presence of 1.6% Triton X-100, as well as repeated extractions with this detergent, did not result in further solubilization of GDH activity.

Examination of the hydroxyapatite-purified GDH activities by SDS-PAGE revealed a single band, with similar electrophoretic mobilities for both GDH forms. These lagged slightly behind the activities by the membrane-rich high-speed pellets. When purified brain mitochondria were similarly treated approximately 80% of GDH activity than for the membrane-associated form, with half-lives at 45°C of 5.8 min and 12.3 min, respectively (Fig. 3; Table 4). GTP inhibited the particulate activity significantly more than the soluble in the presence of 1 mM ADP, with apparent K values of 18.5 and 9.0 µM GTP for the soluble and membrane-associated activities, respectively (Fig. 4; Table 4). The same patterns of relative sensitivities to GTP inhibition and effect of ADP were also found with crude HSS and HSP regardless of whether they had been prepared in the presence or absence of the protease inhibitor PMSF (10⁻⁴ M). These crude preparations were analyzed in the presence of Triton X-100 (0.05%) which allows an estimation of the particulate GDH activity without removing the enzyme from the membranes as described above. The Hill plot coefficient, the cooperativity index, and the binding constant, obtained from the data, also showed significant differences between the two activities (Table 4). The soluble enzyme displayed a more sigmoidal curve of GTP inhibition than did the membrane-associated form in the presence of 1 mM ADP (Fig. 4).

Stimulation of the two GDH activities by ADP and L-leucine or inhibition by DES were comparable (data not shown).

DISCUSSION

GDH in mammalian brain has been reported to be associated with mitochondria (Salganicoff and DeRobertis, 1965: Van den Berg, 1974). Within these organelles, the enzyme is thought to be localized in the matrix, being largely solubilized with Triton X-100 (Salganicoff and DeRobertis, 1965). The present study used homogenization procedures of whole tissue that disrupt mitochondria and showed that, in addition to this readily solubilized brain GDH (probably originating from the mitochondrial matrix), there is in brain a substantial amount of enzyme activity that is associated with the membrane-rich high-speed (100,000 g for 1 h) pellet. When purified brain mitochondria were similarly treated approximately 80% of GDH activity

TABLE 3. Purification of particulate GDH from rat brain

<table>
<thead>
<tr>
<th>Protein / recovery</th>
<th>Activity / recovery</th>
<th>Specific activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg ( % )</td>
<td>µmol/hr (%)</td>
<td>µmol/mg protein/h</td>
<td>-fold</td>
</tr>
<tr>
<td>HSP—crude</td>
<td>1.250 (100)</td>
<td>31,750 (100)</td>
<td>25.4</td>
</tr>
<tr>
<td>HSP—CTAB (first extract)</td>
<td>132.4 (10.6)</td>
<td>20,654 (65.1)</td>
<td>156.0</td>
</tr>
<tr>
<td>HSP—CTAB (second extract)</td>
<td>117.2 (9.4)</td>
<td>8,767 (27.6)</td>
<td>74.8</td>
</tr>
<tr>
<td>30-60% (NH₄)₂SO₄ precipitate</td>
<td>110.6 (8.8)</td>
<td>25,792 (81.2)</td>
<td>233.2</td>
</tr>
<tr>
<td>Phenyl-Sepharose eluate</td>
<td>16.6 (1.3)</td>
<td>13,650 (43.0)</td>
<td>822.3</td>
</tr>
<tr>
<td>DEAE-Sepharose eluate</td>
<td>3.2 (0.3)</td>
<td>9,338 (29.4)</td>
<td>2,918</td>
</tr>
<tr>
<td>Hydroxyapatite eluate</td>
<td>0.5 (0.04)</td>
<td>4,444 (14)</td>
<td>10,100</td>
</tr>
</tbody>
</table>

The forebrains from 50 adult rats were used for the preparation of the crude HSP (HSP—crude) as described in Materials and Methods. It was extracted twice with 0.05% CTAB in 50 mM Tris HCl, pH 7.4 (HSP—CTAB). The two extracts were then pooled for ammonium sulfate precipitation and chromatography as described in Materials and Methods and the legend to Fig. 1.
was solubilized and the addition of Triton X-100 (1%) raised this value to about 90% of the total activity (Plaitakis et al., 1986). The activity found in the HSP was not extractable by a variety of methods that are ordinarily expected to extract proteins loosely bound to brain membranes (including Triton X-100). These results suggest that a considerable fraction of GDH is tightly bound to rat...
brain membranes, from which it may be largely removed by the cationic detergent CTAB. In this regard, it is important to note that the particulate GDH activity is markedly underestimated, especially in crude membrane-containing samples, if both a detergent (Triton X-100) and an activator (ADP or leucine) are not added to the reaction mixture during assay (Plaitakis et al., 1984).

Previous studies (Fahien et al., 1969) had indicated that low concentrations of CTAB efficiently solubilize GDH activity from liver homogenates, yielding an enzyme preparation of relatively high specific activity; this procedure has been often used as the initial step for the purification of the enzyme from various sources (Smith et al., 1975), including rat brain (Chee et al., 1979).

For the purification of the two GDH activities we used a combination of hydrophobic interaction, ion exchange (DEAE-Sephacel), and hydroxyapatite chromatography and obtained homogeneous proteins. Chee et al. (1979) have also used DEAE-Sephadex and hydroxyapatite columns for purification of rat brain GDH whereas others (McCarthy et al., 1980) have relied on a combination of DEAE-cellulose and a GTP-Sepharose affinity column. We have also been able to obtain homogeneous rat brain GDH activity with the use of a combination of a hydrophobic interaction and affinity GTP-Sepharose column chromatography (data not shown).

Both the readily solubilized and particulate rat brain GDH fractions behaved similarly throughout the chromatographic separations, and both also displayed a similar molecular mass on SDS-PAGE, apparently somewhat larger than the commercially obtained bovine liver GDH used as a marker. Also, the two enzymatic activities could not be readily distinguished by their kinetic constants for the various substrates of GDH, although small, but statistically significant, differences were found in $K_m$ values for NADH and NADPH. Our results on the $K_m$ for glutamate were similar to those reported by Chee et al. (1979); however, the $K_m$ values we obtained for $\alpha$-ketoglutarate (1.4 and 1.7 mM for the soluble and particulate GDH activities, respectively) were substantially different from those reported by Chee et al. (0.2 mM) but closer to those (1.0–1.5 mM) found by Dennis et al. (1977). This probably is due to the differences in the composition and the pH of the buffer used for assaying GDH and the use of the activator ADP by us and by Dennis et al. (1977) but not by Chee et al. (1979), since allosteric activators and buffer conditions have been reported to affect the enzyme kinetics (Bailey et al., 1982).

The two GDH activities were found to differ substantially in their allosteric inhibition by GTP and relative resistance to heat inactivation. Thus, GTP inhibited the particulate enzyme more effectively.

### TABLE 4. Comparison of rat brain soluble and particulate GDH

<table>
<thead>
<tr>
<th></th>
<th>Soluble</th>
<th>Particulate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kinetics:</strong></td>
<td>$K_m$: $\alpha$-ketoglutarate</td>
<td>$1.4 \pm 0.3$ mM (10)</td>
</tr>
<tr>
<td></td>
<td>$K_m$: NH$_4$</td>
<td>$18.3 \pm 1.9$ mM (9)</td>
</tr>
<tr>
<td></td>
<td>$K_m$: NADH</td>
<td>$79.7 \pm 9.9$ $\mu$M (8)</td>
</tr>
<tr>
<td></td>
<td>$K_m$: NADPH</td>
<td>$108.1 \pm 19.6$ $\mu$M (7)</td>
</tr>
<tr>
<td></td>
<td>$K_m$: Glutamate</td>
<td>$3.6 \pm 0.13$ mM (14)</td>
</tr>
<tr>
<td><strong>pH optima</strong></td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td><strong>Half-life at 45°C</strong></td>
<td>5.8 min</td>
<td>12.3 min</td>
</tr>
<tr>
<td><strong>GTP inhibition:</strong></td>
<td>Apparent $K_i$ at 1 mM</td>
<td>$18.5$ $\mu$M</td>
</tr>
<tr>
<td></td>
<td>Hill plot coefficient</td>
<td>$2.1 \pm 0.1$ (20)</td>
</tr>
<tr>
<td></td>
<td>Binding constant</td>
<td>$0.0022 \pm 0.0005$ (20)</td>
</tr>
<tr>
<td></td>
<td>Cooperativity index</td>
<td>7.8</td>
</tr>
</tbody>
</table>

The kinetic studies were done using purified soluble and particulate fractions as described in Materials and Methods. The Hill plot coefficient, binding constant, and cooperativity index were calculated by fitting the data to the Hill equation so that the sum of the squares of the errors is a minimum (Cornish-Bowden, 1979). Values are means $\pm$ SD. Numbers in parentheses indicate number of experimental determinations.
than the readily solubilized activity in the presence of the enzyme activator ADP in both crude preparations and the purified GDH fractions. The inhibition curve was sigmoidal for the soluble enzyme and more nearly hyperbolic for the particulate activity, and this difference was significant by Hill plot analysis. Denaturation by heat inactivation (at 45°C) of the enzyme activator ADP was determined at 45°C and pH 7.4. Samples of hydroxyapatite-purified GDH fractions with peak specific activities were mixed 1:1 (vol/vol) with bovine serum albumin solution containing 8 mg/ml in water and incubated at 45°C ± 0.5°C in small, capped, conical plastic tubes. The samples contained approximately 50 mM sodium phosphate buffer at pH 7.4. Aliquots were removed at 0, 5, 10, 15, 20, 30, and 60 min of incubation and assayed at pH 8.0 as described in Materials and Methods; ADP (1 mM final concentration) was added to the reaction mixture at the time of assay. The presence of ADP (1 mM), L-leucine (5–10 mM), or KCl (0.154–0.2 M) or ethylene glycol (50%) in the incubation medium was found to protect both GDH fractions from heat denaturation.

There are recent reports indicating that GDH is synthesized as a proenzyme of higher molecular weight which then is converted to the active enzyme by proteolytic cleavage (Mihara et al., 1982). McCarthy et al. (1980) have presented evidence that the N-terminus of bovine GDH can be modified when isolation is protracted (commercial bovine liver GDH) and such modified enzyme shows a slightly lower molecular weight than the nonproteolyzed enzyme. To prevent proteolysis they used the protease inhibitor PMSF in their isolation media. However, these authors found it necessary to keep the enzyme preparation at room temperature for 2 weeks to obtain the proteolytic changes present in the commercial bovine liver GDH. When their isolation method was rapid, the presence or absence of PMSF in the isolation media did not appear to affect the final results (McCarthy et al., 1980). These authors reported, in accord with previous observations (Talal and Tompkins, 1964), that the properties of the purified bovine liver and brain GDH were not distinguishable. A comparison of bovine brain GDH with the commercial bovine liver enzyme showed the kinetic properties to be the same except for the allosteric regulation (GTP inhibition) which was shown to be different, with the brain enzyme being more sensitive; this was attributed to proteolysis of the commercial preparation. On the other hand, Erwin (1969) and Chee et al. (1979) have shown that in rat the brain enzyme differs significantly from that isolated from liver or kidney in both kinetic properties and allosteric regulation.

In this study, isolation and purification of rat
brain GDH activities were carried out rapidly at 0.5°C. The results obtained were not affected by the presence or absence of PMSF in the isolation media and both soluble and particulate GDH fractions showed the same molecular weight on SDS-PAGE. The differences between the two GDH activities were found not only with the use of highly purified material but also with crude soluble and particulate brain preparations assayed under conditions that do not solubilize the latter activity. Therefore, it does not appear that these differences can be ascribed to artifacts of isolation, including the method used to remove the particulate enzyme from the membrane-rich fraction of brain homogenates. Whether or not the two GDH activities are different gene products or the results of posttranslational modifications of the GDH molecule can be firmly established only by sequencing.

Regardless of whether we are dealing with two structurally distinct GDH forms in brain, the tight association of one of the two GDH fractions with the membrane-rich particulate portion of brain homogenates suggests important implications for the function of the enzyme in brain. Studies on patients with OPCAs, associated with deficiency of particulate leukocyte GDH activity, showed a systemic defect in the catabolism of glutamate in these patients (Plaitakis et al., 1982). Since glutamate is known to be neurotoxic, the hypothesis has been suggested that the compound may accumulate in toxic amounts in the brain of the patients and cause neuronal degeneration. It has been argued that in the brain GDH may be primarily involved in the synthesis of glutamate, a contention supported by experimental evidence indicating that isolated brain mitochondria (unlike liver mitochondria) do not readily oxidize glutamate in vitro (Dennis and Clark, 1977, 1978). However, more recent studies by Yu et al. (1982), utilizing astrocytic cultures, have shown that most glutamate taken up by these cells is oxidized via GDH. Shank and Campbell (1983), in addition, have presented evidence indicating that glial cells produce α-ketoglutarate as a precursor of the transmitter glutamate pool and this formation may involve the GDH pathway. These data taken together suggest that brain GDH may have important functions both in the synthesis and oxidation of glutamate. The metabolism of glutamate has been shown to be compartmented in the brain (Berl and Clarke, 1983) but the distribution of GDH is not known. The present data showing that GDH exists in soluble and in particulate (most likely membrane-associated) forms in the brain raise the possibility that the differential distribution may serve different metabolic requirements in this organ. Thus, a membrane localization of the enzyme may expose it to a particular metabolic compartment of nerve or glial cells that may be distinct from that associated with the readily solubilized GDH. Such paradigms have already been shown with other enzymes related to the metabolism of putative neurotransmitters (Fonnum, 1968; Atterwill and Prince, 1978).

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REFERENCES
GLUTAMATE DEHYDROGENASE PURIFICATION AND ACTIVITY


