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Brain L-glutamate decarboxylase: Purification and subunit structure

(γ-aminobutyric acid/neurotransmitter enzyme)

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ABSTRACT **Glutamate decarboxylase (GDCase; L-glutamate-1-carboxy-lyase, EC 4.1.1.15) was purified from whole rat brain ≈1300-fold to apparent homogeneity with a specific activity of 2.4 units per mg of protein by a combination of column chromatographies on DEAE-cellulose, hydroxylapatite, and gel filtration, and preparative nondenaturing polyacrylamide gel electrophoresis. The purified preparation contained a single protein band that comigrated with GDCase activity in three diverse analyses: nondenaturing regular (5%) and gradient (3.6–25%) polyacrylamide gel electrophoresis and isoelectric focusing at pH 4–7. The native molecular mass was calculated to be 120 ± 10 kDa from gradient polyacrylamide gel electrophoresis and 110 ± 10 kDa from gel filtration. Under the treatment with $NaDodSO_4$ and 2-mercaptoethanol, GDCase dissociated into two subunits of 40 ± 2 and 80 ± 4 kDa, as estimated from $NaDodSO_4$ gel electrophoresis. However, only a 40-kDa subunit was detected when GDCase was treated with 4 M urea plus $NaDodSO_4$ and 2-mercaptoethanol, suggesting that the 80-kDa subunit is the dimer of the 40-kDa subunit. In immunoblotting, polyclonal antibodies against GDCase reacted with both 40- and 80-kDa subunits, while monoclonal antibody reacted with only 80-kDa subunits. The isoelectric point of the native enzyme was 5.4. The K_m for glutamate was 1.59×10^{-3} M. In addition to L-glutamate, cysteine sulfinic acid was also decarboxylated at ≈10% of the rate of glutamate. The pH optimum was fairly broad, with a maximum at ≈7.3. The enzyme was strongly inhibited by carbonyl-trapping agents, sulfhydryl reagents, thiol compounds, and β-methylene-DL-aspartate.**

γ-Aminobutyric acid (GABA) has been established as a major neurotransmitter in the mammalian central nervous system from physiological, biochemical, pharmacological, and morphological studies (1–5). The reaction involved in GABA biosynthesis is the decarboxylation of glutamic acid by glutamate decarboxylase (GDCase; L-glutamate 1-carboxy-lyase, EC 4.1.1.15). Hence, GDCase has been used as a specific marker for GABAergic neurons and their processes. Since the purification of GDCase from mouse brain to homogeneity was achieved in this laboratory and specific antibodies against mouse brain GDCase became available, much progress has been made in the identification of GABAergic neurons and their synaptic connectivities by immunocytochemical techniques (for review, see ref. 5). Unfortunately, the supply of these well-characterized monospecific antibodies against mouse brain GDCase has been depleted. Although antibodies against rat brain GDCase have been prepared and used in immunocytochemical studies, these antibodies were not raised from the purified GDCase preparation and their specificity has not yet been fully characterized (6). Hence, we decided to undertake the purification of GDCase from rat brain to homogeneity to ensure a continuous supply of pure antigen for the production of specific polyclonal and monoclonal antibodies. In addition, we also would like to compare GDCase from mouse brain and rat brain in terms of molecular mass, subunit structure, kinetic properties, and possible isozymes. In this communication, we describe the purification of rat brain GDCase to homogeneity; the characterization of its physical, chemical, and kinetic properties; and its possible subunit structure. The agreements and discrepancies between the results of the present study and those in the literature are also discussed. Portions of the present communication have been presented in a preliminary report (7, 8).

MATERIALS AND METHODS

Enzyme Assay. GDCase was assayed by a radiometric method measuring the formation of $^{14}CO_2$ from L-[1-^{14}C]glutamic acid as described (9, 10).

Protein Assay. Protein was assayed by the protein-dye binding method as described (11). Bovine serum albumin was used as a standard.

Preparative Polyacrylamide Gel Electrophoresis. Preparative polyacrylamide gel electrophoresis was performed as described (10, 12) except that the size of gels was changed from 0.127 × 15 × 15 cm to 0.15 × 15 × 40 cm.

Analytical Polyacrylamide Gel Electrophoresis. The purified GDCase extract from preparative polyacrylamide gel electrophoresis was concentrated and analyzed on 5% polyacrylamide slab gels. The conditions were the same as those used in the preparative gels except that the size of gel was reduced from 0.15 × 15 × 40 cm to 0.075 × 15 × 10 cm and the staining solution was silver nitrate (13) instead of Coomassie brilliant blue.

Analytical $NaDodSO_4$/PAGE. About 0.5 μg of the purified GDCase was treated with 1% $NaDodSO_4$ and 0.2% 2-mercaptoethanol at 100°C for 5 min or 4% $NaDodSO_4$ and 0.1% 2-mercaptoethanol for 3 min. In one experiment, GDCase was treated with 4 M urea plus 4% $NaDodSO_4$ and 0.1% 2-mercaptoethanol. The $NaDodSO_4$-treated sample was then applied to 10% polyacrylamide slab gels (0.75 mm × 15 cm × 10 cm) containing 0.1% $NaDodSO_4$. The electrophoretic conditions were the same as those described for the nondenaturing gels except that the running buffer contained 0.1% $NaDodSO_4$.

Immunoblotting Test. The immunoblotting test was conducted according to Towbin *et al.* (14) with modifications as described (15). Polyclonal and monoclonal antibodies used in this study have been extensively characterized, including

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Abbreviations: GABA, γ-aminobutyric acid; GDCase, glutamate decarboxylase.

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immunodiffusion, immunoelectrophoresis, immunoprecipitation, dot immunoassay, immunoblotting, and enzyme-linked immunoadsorbent assay as detailed elsewhere (15). For the control experiments, the same amount of IgG isolated from preimmune serum was used instead of anti-GDCase IgG.

Gradient Polyacrylamide Gel Electrophoresis. Gradient electrophoresis was performed as described (10) with minor modifications. Slab gels (0.75 mm thick) were made with a continuous linear gradient from 3.6% to 25% or 6% to 10% acrylamide and run at 7 V/cm constant voltage for 12–24 hr at 4°C. Adjacent parallel lanes were assayed for GDCase activity or stained for protein with $AgNO_3$. Molecular size standards included thyroglobulin (669 kDa), ferritin (440 kDa), catalase (247 kDa), alcohol dehydrogenase (140 kDa), transferrin (90 kDa), bovine serum albumin (67 kDa), and ovalbumin (43 kDa).

Isoelectric Focusing. Horizontal slab agarose gels were formed on 10 × 15 cm glass plates according to a protocol described by the manufacturer (Bio-Rad). An equal mixture of pH 4–6 and pH 5–7 ampholytes was used to give a proper pH gradient. Samples were applied in <5 μl and gels were focused for 2.5 hr at 0.5 W/cm constant power. Adjacent lanes were either cut in 0.3-cm lengths and assayed for GDCase activity or stained for protein. An additional lane not containing any sample was cut in 0.3-cm lengths, macerated in 2 ml of water, allowed to stand for 1–4 hr, and the pH was measured.

Kinetic Studies. The K_m for glutamate was determined by using six glutamate concentrations from 1 to 30 mM with the standard assay conditions. The specific radioactivity of glutamate was kept constant. Blanks lacking enzyme were run at each substrate concentration.

The substrate specificity was tested at a final concentration of 4 mM for each substrate using standard assay conditions. In addition to 20 naturally occurring L-amino acids, cysteine sulfinic acid, α-ketoglutarate, and taurine were tested. Blanks lacking enzyme were run for each substrate.

The sensitivity to various inhibitors (at 8–12 concentrations) was investigated using standard assay conditions. The carbonyl-trapping agent aminooxyacetic acid was tested at concentrations from 1 nM to 10 mM. The sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) was tested at concentrations from 3 nM to 3 μM. The thiol compound 3-mercaptopropionic acid was tested at concentrations from 0.1 μM to 10 mM. The β,γ unsaturated amino acids, β-methylene-DL-aspartate, β-ethylidene-DL-aspartate and β-methylene-DL-glutamate were tested at concentrations from 10 μM to 1 mM. The effects of α-ketoglutarate (0.1–15 mM), sodium chloride (5–140 mM), and zinc acetate (0.1 μM–10 mM) were also tested.

The pH optimum was determined using 2-(*N*-morpholino)-ethanesulfonic acid, sodium salt, potassium phosphate, and Tris buffers. The pH was varied from 5.6 to 9.1 with several points of overlap at ranges intermediate between the optimal buffering capacities of each buffer. Controls lacking enzyme were included for each pH.

Preparation of Starting Material. Rat brains were rapidly dissected, collected in ice-cold homogenization medium [1 mM 2-aminoethylisothiouronium bromide hydrobromide (AET)/0.2 mM pyridoxal-5′-phosphate (PLP)/1 mM EDTA/1 mM benzamidine hydrochloride/0.1 mM phenylmethylsulfonyl fluoride/soybean trypsin inhibitor at 20 μg/ml, pH 7.0], and used fresh. Unless stated otherwise, all procedures were performed at 4°C and all solutions contained 1 mM AET, 0.2 mM PLP, and 1 mM EDTA at pH 7.0. Ten percent homogenates were prepared with a motor-driven Teflon pestle in a glass homogenizer. The homogenates were centrifuged at 100,000 × g_{max} for 60 min. Supernatants were decanted and pellets thus obtained were extracted two additional times with the homogenization medium containing 25 mM potassium phosphate. The three supernatants were combined, concentrated in an Amicon DC2 Dialyzer/Concentrator with a 30-kDa cutoff hollow fiber cartridge, and the buffer was adjusted to 25 mM potassium phosphate.

DE-52 Chromatography. The concentrated high-speed supernatant was applied at 100 ml/hr to a 5 × 40 cm column of DE-52 that had been equilibrated in 25 mM potassium phosphate. The column was then washed with 2 bed vol of the equilibration buffer. A 2-bed vol of a linear gradient made of equal volumes of 25 mM potassium phosphate (pH 7.0) and 300 mM potassium phosphate (pH 6.4) was used to elute the column as described (16).

Hydroxylapatite Chromatography. Fractions from DE-52 column with a specific activity >11.6×10^{-3} unit/mg were loaded at 100 ml/hr on a 2.5 × 20 cm column of spheroidal hydroxylapatite equilibrated in 1 mM potassium phosphate. The column was washed with 1 bed vol of equilibration buffer followed by 5 bed vol of 10 mM potassium phosphate. The column was further eluted with 8 bed vol of a linear gradient made of equal volumes of 10 and 100 mM potassium phosphate as described (16).

Gel Filtration Chromatography. Fractions from the preceding step with a specific activity >104×10^{-3} unit/mg were concentrated and loaded on a 2.6 × 90 cm Ultrogel AcA 44 column equilibrated in 25 mM potassium phosphate. GDCase was eluted from the column with the same buffer at 30 ml/hr as described (16). Sample volume was <1% of the bed volume.

RESULTS

Purification. The purification of GDCase from 100 rat brains is summarized in Table 1. Six steps were used to purify GDCase to apparent homogeneity with an ≈1300-fold purification over the homogenate and a yield of 1.7% of the total activity.

DE-52 Chromatography. GDCase activity started to appear at ≈30 mM and peaked at ≈130 mM. Fractions with specific activities >50% that of the peak fraction were pooled and concentrated in the DC2 concentrator followed by dialysis against 1 mM potassium phosphate.

Hydroxylapatite Chromatography. The GDCase activity started to appear around 15 mM and activity peaked at ≈35 mM. Fractions with specific activities >60% that of the peak fraction were pooled and concentrated by ultrafiltration in an Amicon stirred cell with PM30 membranes, and the buffer was adjusted to 25 mM.

Gel Filtration Chromatography. A single symmetrical peak of GDCase activity was obtained. Fractions with specific activities >80% of the peak fraction were pooled and concentrated by ultrafiltration.

Preparative Polyacrylamide Gel Electrophoresis. Recovery of GDCase activity from preparative electrophoresis was ≈30%. The R_f value of GDCase was ≈0.51.

Criteria of Purity. The purity of the GDCase solution prepared from preparative gels was established by three distinct methods. A single protein band that comigrated with GDCase activity was obtained in three different systems—namely, nondenaturing 5% polyacrylamide gels (Fig. 1), nondenaturing polyacrylamide gradient gels of 3.6–25% (Fig. 2) or 6–10% (data not shown), and narrow range isoelectric focusing gels (Fig. 3).

Physical Characterization. The native molecular mass of GDCase was determined by two methods. Nondenaturing polyacrylamide gradient gels were calibrated with seven molecular mass standards. Based on the logarithmic relationship between mobility and relative molecular mass (17), the calculated size was 120 ± 10 kDa. The plot of logarithm of molecular mass versus elution volume (18) from gel filtration

Table 1. Purification of GDCase from rat brain

Sample	Total activity, units*	Total protein, mg	Specific activity,† units/mg × 10^{-3}	Yield, %	Purification, -fold
1. Homogenate	41.8	23,200	1.8	100	—
2. Supernatant	29.2	12,700	2.3	70	1.3
3. DEAE-cellulose	14.0	1,210	11.6	33	6.4
4. Hydroxylapatite	4.90	47.2	104	12	58
5. Gel filtration	2.06	6.6	312	4.9	170
6. Gel electrophoresis	0.72	0.30	2400	1.7	1300

Purification of GDCase was from 100 brains.
*One unit = 1 μmol of product formed per min at 37°C under standard conditions.
†Specific activities of the peak fractions were as follows: sample 3, 15.1; sample 4, 128; sample 5, 349.

chromatography on AcA 44 based on five standards corresponded to a mass of 110 ± 10 kDa. The isoelectric point based on the location of both enzyme activity and protein staining in a pH 4–7 agarose gel was 5.4 ± 0.15 (Fig. 3).

Subunit Structure. When purified rat brain GDCase was treated with $NaDodSO_4$ and 2-mercaptoethanol under different conditions and analyzed on $NaDodSO_4$/PAGE, two protein bands of about equal intensity were obtained (Fig. 4*A*). However, when GDCase was treated with 4 M urea in addition to $NaDodSO_4$ and 2-mercaptoethanol, only one protein band corresponding to the lower molecular mass subunit was obtained (Fig. 4*B*). When two sets of standard proteins were run under the same conditions, a linear relationship was found between the logarithm of the molecular sizes and the corresponding relative mobilities (Fig. 4*C*). From this standard curve, the molecular masses of the two subunits observed in Fig. 4*A* were determined to be 40 ± 2 and 80 ± 4 kDa, respectively. The GDCase subunit observed in Fig. 4*B* corresponded to a molecular mass of 40 kDa.

In the immunoblotting test, polyclonal anti-GDCase crossreacted with both 80- and 40-kDa subunits, while monoclonal anti-GDCase (12-24) crossreacted with only the 80-kDa subunit (Fig. 5).

Kinetic Characterization. The K_m for glutamate, determined from a Lineweaver–Burk plot (19), was calculated to be 1.59 ± 0.14 × 10^{-3} M.

With a detection limit of ≈1% of the GDCase activity, with L-glutamate as substrate, none of the following compounds served as substrate: α-ketoglutarate, taurine, and 19 other naturally occurring L-amino acids. Cysteine sulfinic acid was decarboxylated at ≈10% the rate of glutamate. The pH profile was rather broad, with an optimum at pH ≈ 7.3.

The enzyme was found to be sensitive to inhibitors of mouse brain GDCase. Inhibition of 50% of the GDCase activity occurred at the following concentrations for each inhibitor: 5,5′-dithiobis(2-nitrobenzoic acid), 2.5 μM; aminooxyacetic acid, 1 μM; NaCl, 17.5 mM; α-ketoglutarate, 9 mM; 3-mercaptopropionic acid, 15 μM; zinc acetate, 25 μM. Among the β,γ unsaturated amino acids tested, β-methylene-DL-aspartate was the most potent inhibitor, inhibiting 50% of GDCase activity at 0.1 mM. β-Ethylidene-DL-aspartate and β-methylene-DL-glutamate had no effect on GDCase activity at 1 mM.

DISCUSSION

Although GDCase has been purified or highly purified from several species, including mouse, bovine, catfish, rat, and human brain (9, 10, 12, 16, 20–23), the purification procedures used in the present studies are different from those described previously in several aspects.

First of all, the starting material used for GDCase purification is different. Previously, we used the lysate of synaptosome containing crude mitochondria fraction as the starting material, which is only ≈25% of the total GDCase activity (9), whereas the present procedures use the high-speed supernatant of whole brain hypotonic homogenates, which contain at least 90% of the total GDCase activity as the starting material. Hence, the present procedures minimize the chance of selecting a specific population or pool of GDCase for purification. Second, in contrast to prior studies of human and rat brain GDCase (20, 22), we avoided the use of low pH and increased temperatures, which are known to alter protein conformation and their properties. Third, the use of Triton was avoided, since this detergent binds to protein molecules and forms a micelle, which alters the physical, chemical, and kinetic properties of the enzyme. Fourth, since numerous proteolytic enzymes have been described in mammalian systems (24), several different inhibitors for proteolytic enzymes were included in the homogenizing medium to

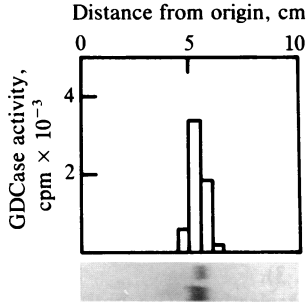

FIG. 1. Nondenaturing polyacrylamide gel electrophoresis of GDCase. Concentrated GDCase extracts from preparative gels were electrophoresed on a 5% analytical gel as described. Two lanes (*Lower*) were silver-stained for protein (upper band, 100 ng; lower band, 500 ng). A parallel lane was cut in 0.5-cm slices and assayed for GDCase activity (*Upper*).

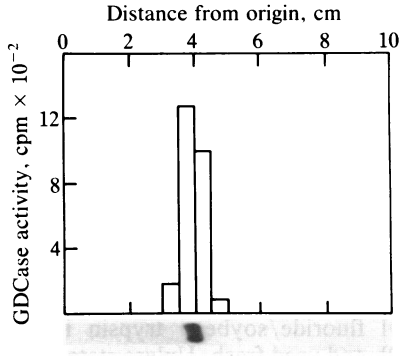

FIG. 2. Nondenaturing polyacrylamide gradient gel electrophoresis of GDCase. Concentrated GDCase extracts from preparative gels were electrophoresed on a linear 3.6–25% gradient gel as described. One lane containing 1 μg of pure GDCase was silver-stained for protein (*Lower*); a parallel lane was cut in 0.5-cm slices and assayed for enzyme activity (*Upper*).

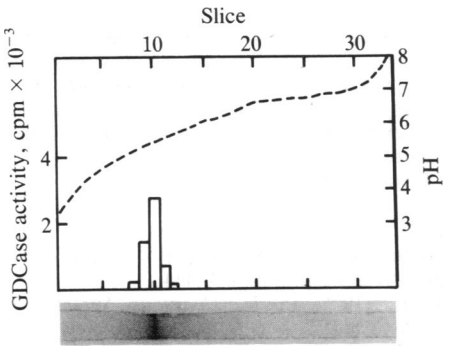

FIG. 3. Isoelectric focusing of GDCase. Concentrated GDCase extracts from preparative gels were focused in a pH 4–7 agarose gel. Parallel lanes were silver-stained (500 ng) for protein (*Lower*), cut in 0.3-cm slices, and assayed for GDCase activity (open bars), or cut in 0.3-cm slices and macerated in water for pH determination (----).

minimize proteolytic alterations that might mask the existence of, or artificially produce, different forms of the enzyme.

GDCase purified under nondenaturing conditions as described in this report appears to be homogeneous in terms of size and charge as judged from the results of regular and gradient polyacrylamide gel electrophoresis and isoelectric focusing, in which only one protein band with comigrating GDCase activity was obtained. The molecular size estimates from gradient gels and gel filtration are smaller than those described for rat and human GDCase (20, 22) but larger than estimates for the mouse enzyme (16) and rat enzyme (25). The isoelectric point, pH optimum, inhibitor sensitivities, and K_m for glutamate are similar to those found for mouse, bovine, rat and human GDCase (6, 16, 20, 22, 26). In contrast to the mouse enzyme, which can decarboxylate L-aspartate at ≈3% of the rate of L-glutamate, the GDCase preparation purified from rat brain does not decarboxylate aspartic acid, although it can also decarboxylate cysteine sulfinic acid (in addition to glutamate) as reported for the purified bovine enzyme (12) and partially purified rat enzyme (27). It is conceivable that under abnormal conditions, such as change in pH, substrate concentrations or inhibitors may make the biosynthetic capacity of taurine physiologically significant.

Previously, we suggested that mouse brain GDCase may be a hexamer consisting of 15-kDa subunits (16, 28). In the present study, we have shown that rat brain GDCase dissociated into two subunits of 40 and 80 kDa, respectively, by the treatment with $NaDodSO_4$ and 2-mercaptoethanol. It seems rather unlikely that the appearance of the two protein bands on $NaDodSO_4$/PAGE can be explained on the basis of impurities in the enzyme preparations, since only one protein band—namely, 40-kDa subunit—was observed when GDCase was treated with a combination of urea and $NaDodSO_4$ plus 2-mercaptoethanol. Furthermore, the purified GDCase preparations have been shown to be homogenous under three diverse systems—e.g., nonnaturing 5% polyacrylamide gels, nondenaturing polyacrylamide gradient gels of 3.6–25% or 6–10%, and in narrow-range isoelectric focusing gels. In all three analyses, the position of the protein band is coincident with GDCase activity. Since the native enzyme has a molecular mass of 120 kDa, it is reasonable to conclude that rat brain GDCase consists of two subunits of 80 and 40 kDa, respectively, and the 80-kDa subunit is a dimer of the 40-kDa subunit.

Maitre *et al.* (22) reported that rat brain GDCase had a molecular mass of 140 kDa and consisted of two identical 67-kDa subunits. Spink *et al.* (25) reported that all three forms of rat brain GDCase (α, β, and γ) had a similar molecular mass of 100 kDa. The discrepancy between our results and

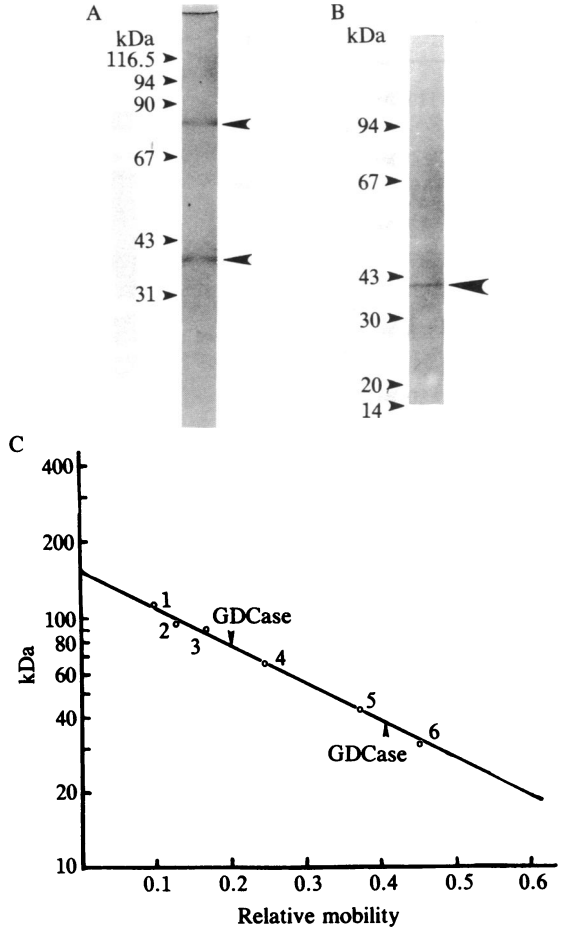

FIG. 4. $NaDodSO_4$/PAGE. (*A*) About 0.5 μg of the purified rat brain GDCase was treated with 4% $NaDodSO_4$ and 0.1% 2-mercaptoethanol at 100°C for 3 min and then applied to 10% polyacrylamide gel as described. The gel was stained with silver-staining solution. Arrows indicate the position of the standard protein markers: 1, β-galactosidase, 116 kDa; 2, phosphorylase b, 94 kDa; 3, transferrin, 90 kDa; 4, bovine serum albumin, 67 kDa; 5, ovalbumin, 43 kDa; 6, carbonic anhydrase, 31 kDa. (*B*) The conditions were the same as those described in *A* except the amount of purified GDCase used was 0.3 μg instead of 0.5 μg and the sample buffer contained 4 M urea in addition to 4% $NaDodSO_4$ and 0.1% 2-mercaptoethanol. (*C*) Molecular mass estimation for GDCase in $NaDodSO_4$/PAGE. Arrows indicate the position of the two GDCase subunits in the gel (*A*).

those reported by Maitre *et al.* (22) and Spink *et al.* (25) could be due to the difference in the purification procedures. For instance, Maitre *et al.* (22) and Spink *et al.* (25) used low pH (pH 5.4) and high temperature (48°C–51°C) treatment in their preparation of the starting material, while we used neutral pH 7.0 and low temperature (4°C) in our preparation. It is conceivable that the harsh conditions used by Maitre *et al.* (22) might have inactivated the major species of GDCase. This view is supported by the fact that they could only recover 30% of GDCase activity in the supernatant, while our recovery was >90%. On the other hand, we could recover only ≈30% of GDCase activity from preparative gel electrophoresis, suggesting that we may have selectively purified one form of GDCase. The identity or nonidentity of GD purified by Maitre *et al.* (22) and us can be determined conclusively when their complete amino acid sequences become available.

In view of the reports of multiple forms of GDCase (25, 29–32), it is somewhat surprising that we are unable to obtain distinct separation of different forms of GDCase by the purification procedures reported here. It is possible that the

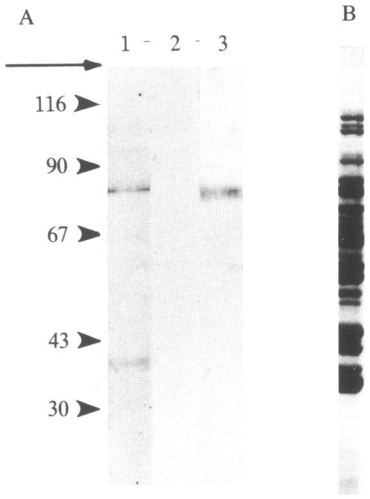

FIG. 5. Immunoblotting tests with polyclonal and monoclonal GDCase antibodies. About 20 μg of partially purified GDCase preparation (10% pure) was applied to 10% $NaDodSO_4$ slab gel and electrophoretically transferred to the nitrocellulose sheet. (*A*) Lane 1, stained with polyclonal anti-GDCase IgG, showing two protein bands corresponding to the position of GDCase subunits—namely, 80 and 40 kDa. Lane 3, stained with monoclonal anti-GDCase IgG (12-24), showing protein band corresponding to the position of GDCase α subunit (80-kDa subunits). Lane 2, stained with preimmune rabbit IgG, showing no reaction product. Arrow indicates starting position of the separating gel. Numbers on left represent the positions of standard molecular size markers in kDa. (*B*) Protein pattern of partially purified GDCase sample used in immunoblotting tests in *A*.

conditions used in the present study favor one form of GDCase, resulting in the loss of other forms of GDCase during the course of purification. This is consistent with the fact that, although the starting material—namely, the hypotonic high-speed supernatant—contains >90% of the total GDCase activity in the brain, the overall yield of GDCase activity as the purified preparation is only 1.7%. Since the recovery of GDCase activity from preparative polyacrylamide gel electrophoresis is only ≈30%, it is possible that multiple forms of GDCase do exist and some are rather unstable and inactivated under the present electrophoretic condition.

Another possible explanation is that the discrepancy is due to the difference in experimental conditions. For instance, we have shown previously that if GDCase extracted from hypotonic homogenates was fractionated with ammonium sulfate, we could obtain two distinct forms of GDCase—namely, high molecular weight and low molecular weight forms (12, 32). In our present study, we could also reproduce the same result if ammonium sulfate fractionation was used in the purification protocol (data not shown). However, if the ammonium sulfate step was omitted, only the smaller species of GDCase was obtained (data not shown). Hence, it is concluded that the high molecular weight form of GDCase is artificially produced by the treatment with ammonium sulfate. Since GDCase prepared from the crude mitochondrial fraction is insensitive to ammonium sulfate treatment (16), it is conceivable that GDCase is present *in vivo* as multiple forms that are affected differently by ammonium sulfate. If this is the case, it will be important to elucidate the role of various forms of GDCase in the function of GABA as a neurotransmitter.

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