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# Purification and Characterization of Glutamate Dehydrogenase as Another Isoprotein Binding to the Membrane of Rough Endoplasmic Reticulum

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**Abstract** Glutamate dehydrogenase (GDH) was purified from rough endoplasmic reticulum (RER) in rat liver using anion-exchange and affinity chromatography. As GDH has been known as an enzyme that exists mainly in the matrix of mitochondria, the properties of purified GDH were compared with those of mitochondrial GDH. The GDH activity in 0.1% Triton X-100-treated RER subcellular fraction was nearly the same as intact RER, whereas that of the mitochondrial fraction increased by 50% after the detergent treatment. In kinetic values, in addition, mitochondrial GDH had a higher $K_m$ value for $NADP^+$ than $NAD^+$, whereas the $K_m$ value for $NAD^+$ was higher than that for $NADP^+$ in the case of GDH of RER, which showed a difference in specificity to cofactors. Moreover, when two GDH isoproteins were incubated at 42°C or treated with trypsin, GDH from RER was more stable against heat inactivation and less susceptible to proteolysis than mitochondrial GDH in both cases. In addition, GDH of RER had at least five amino acids different from mitochondrial GDH when sequences of N-terminal and several internal peptide fragments were analyzed. These results showed that GDH of RER is another isoprotein of GDH, of whose properties are different from those of mitochondrial GDH. J. Cell. Biochem. 76:244–253, 1999. 

**Key words:** isozyme; proteolysis; heat stability; sequence; function

Glutamate dehydrogenase (EC 1.4.1.3, GDH) catalyzes the interconversion of α-ketoglutarate and glutamate, using $NAD^+$ or $NADP^+$ as a cofactor, and has been known as an enzyme existing exclusively in the matrix of mitochondria [Salganicoff et al., 1965]. While two structurally distinct forms of the enzyme showing specificity for either $NAD^+$ or $NADP^+$ are known to exist in lower organisms, all mammalian GDHs known are capable of using both cofactors [Smith et al., 1975]. Several recent studies have been conducted to show the existence of isoproteins of mammalian GDH. Four different forms of GDH isoproteins were purified from human cerebellum of normal subjects and patients with neurodegenerative disorders [Hussain et al., 1989]. However, the origin of this GDH polymorphism is unknown. These isoproteins could be the products of different genes or of different mRNA species generated by alternative splicing, or both. They could also arise by post-translational modifications, such as phosphorylation or proteolysis [Hussain et al., 1989; McCarthy et al., 1980]. Concurrent molecular biological studies showed the presence of four different-sized mRNA and multiple gene copies for GDH in humans [Mavrotalassitis et al., 1988; Amuro et al., 1988], suggesting a genetic basis for the multiplicity of this protein. To date, however, only one cDNA encoding human GDH is known [Mavrotalassitis et al., 1988; Banner et al., 1987; Nakatami et al., 1988]. In addition to these four isoproteins in human brain, two isoforms of GDH in bovine brain, GDH GDH II, and I were purified [Lee et al., 1995; Cho et al., 1995]. By examining the differences between the biochemical properties of GDH I and GDH II, such as N-terminal sequences of intact and tryptic-digested enzymes, kinetic parameters, optimal pH, and heat stability, two GDH isoproteins were revealed to be

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novel and distinct polypeptides [Cho et al., 1995].

In addition to these studies, showing the existence of isoproteins of GDH, reports have indicated that other subcellular fractions also contain GDH isoprotein different from mitochondrial GDH [Prisco et al., 1975; McDaniel 1995; Rajas et al., 1993; Colon et al., 1986]. The nuclear GDH from ox liver was different in primary structure and displayed different immunological properties from those of mitochondrial GDH [Prisco et al., 1975; McDaniel et al., 1995]. Furthermore, lysosomal GDH from pig liver and brain was purified and investigated to show that another GDH isoprotein, MP50, was closely associated with lysosomal membrane and extracted with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), a zwitterionic detergent [Rajas et al., 1993]. This study also demonstrated that GDH purified from lysosomal membrane fraction had the property of binding to a microtubule, one of cytoskeleton. Similarly, particulate GDH and soluble GDH were isolated from membrane-rich pellet fraction of rat brain and their properties were tested [Colon et al., 1986]. The particulate GDH had the same molecular mass, chromatographic behavior and enzyme activity as mitochondrial GDH. However, the differences between two GDH isoproteins were detected in their thermal stability and allosteric properties.

Whereashile other works failed to show the precise function of the GDH isozyme, Rajas et al. [1996] suggested that GDH, which was able to bind to lysosomal membrane and microtubule, had functions other than its original activity. GDH from lysosome was found to be involved in the microtubule-dependent perinuclear localization of lysosome. Furthermore, GDH from bovine liver homogenate was identified as a RNA-binding protein and competed with different types of nucleotides [Preiss et al., 1993]. Excesses of tRNA, salmon testis DNA, or each of the four homoribopolymers were unable to compete for the RNA binding site. Total cytosolic RNA, however, successfully prevented binding of radiolabeled RNA substrate [Preiss et al., 1993]. Mitochondrial GDH interacting with guide RNA was also reported [Bringaud et al., 1997]. In this study, the gene for a 110-kDa polypeptide (GDH) from the T-V complex of *L. tarentolae* was isolated and cloned, which was found to interact with labeled gRNA and [$\alpha$-$^{32}$P]UTP-labeled endogenous RNA.

As mentioned above, several isoproteins of GDH have been identified. However, to our knowledge there has not been any study pointing to the existence of another isoprotein of GDH in membrane-bound form in rough endoplasmic reticulum (RER) of rat liver. We report the purification and characterization of GDH from RER of rat liver and demonstrate that this is an isozyme of GDH associated with membrane of RER.

## MATERIALS AND METHODS

### Materials

Female Wistar rats were obtained from Seoul National University Breeding Laboratories (Seoul, Korea). Hepes, Tris, $NAD^+$, NADH, $NADP^+$, NADPH, glutamate, $\alpha$-ketoglutarate, DEAE-Sepharose CL-6B, ATP-agarose, glutamate dehydrogenase (GDH) in rat liver (G5636), trypsin, EDTA, and Wet blotter were purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex G-50 was purchased from Pharmacia (Sweden), Molecular-weight markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Hercules, CA). The PVDF membrane (Immobilon-P®) was purchased from Millipore (Bedford, MA).

### Methods

**Subcellular fractionation.** Liver subcellular organelles (mitochondria, Golgi apparatus, crude microsome, and rough endoplasmic reticulum) were fractionated from female Wistar rats, which had been starved for 16–20 h before sacrifice by anesthesia. All subcellular fractionation steps were carried out at 4°C.

**Purification of mitochondria.** Rat liver was homogenized by Dounce homogenizer in 4 vol of buffer A (0.25 M sucrose, 10 mM Hepes, pH 7.4). The homogenate was centrifuged at 960*g* for 10 min. After removal of the resulting pellet, the supernatant was centrifuged at 12,000*g* for 30 min, to yield a mitochondrial pellet and postmitochondrial supernatant. The pellet had two distinct layers. After careful removal of the upper portion (mostly heavy microsomes), the lower portion, enriched in mitochondria, was resuspended in buffer A and recentrifuged at 12,000*g* for 30 min. As this pellet also had a slight upper layer, further separation was achieved by removing the upper

layer, followed by resuspending and centrifuging the pellet one more time under the same conditions. The resulting mitochondria pellet was resuspended in buffer A to 5–10 mg/ml.

**Purification of microsome.** The postmitochondrial supernatant was centrifuged at 105,000*g* for 1 h in 42.1 Ti rotor (Beckman, USA) to yield a crude microsomal pellet. The pellet was resuspended in buffer A to 5–10 mg/ml.

**Purification of rough endoplasmic reticulum (RER).** The resuspended crude microsomal fraction that was added with $CsCl_2$ to 15 mM was layered over the cushion of buffer B (1.3 M sucrose, 10 mM Hepes, 15 mM $CsCl_2$, pH 7.4) and centrifuged at 110,000*g* for 120 min in 42.1 rotor. The volume ratio of the microsome to the cushion was 7:3. The resulting pellet, RER, was resuspended in buffer A to 5–10 mg/ml.

**Purification of Golgi apparatus.** The crude microsomal pellet was resuspended gently, using a loose-fitting Dounce homogenizer in 52% sucrose, 10 mM Hepes (pH 7.4) and its sucrose density was adjusted to 43%; 1 vol of this fraction was placed in a SW 28 rotor (Beckman, USA) centrifuge tube, overlayered sequentially with 1 vol of 38.7%, 29%, and 8.2% sucrose in 10 mM Hepes (pH 7.4) buffer, and then centrifuged at 26,000 rpm in a SW 28 rotor for 1 h. The Golgi apparatus appeared in the 29%/38.7% sucrose interface. This layer was collected carefully with a syringe and resuspended in buffer A to 5–10 mg/ml.

### Purification of GDH From RER of Rat Liver

The RER proteins (80 mg) were centrifuged for 1 h at 105,000*g* in 42.1 rotor and resuspended in 25 ml of buffer C (0.6 M NaCl, 10 mM Hepes, pH 7.4). After incubation for 10 min on ice, the sample was centrifuged at 105,000*g* for 40 min. To desalt the supernatant extracted with 0.6 M NaCl, the Sephadex G-50 column (1.0 × 60 cm, 48 ml) equilibrated with buffer D (30 mM Tris, pH 7.8) was carried out at a flow rate of 0.5 ml/min in the isocratic condition. Then 10 ml of the desalted fraction was loaded onto DEAE-Sepharose column (1.5 × 17 cm, 30 ml) equilibrated with buffer D, followed by washing out the unbounded proteins with 40 ml of the buffer D and eluting with NaCl linear gradient (50–300 mM) in buffer D. The fractions containing GDH activity were pooled and applied to the affinity column chromatography of ATP-agarose (1 ml) equilibrated with buffer D at a flow rate of 0.5 ml/min. After washing with 5 ml of buffer D at the same flow rate, ATP-agarose column was eluted with buffer E (1 mM ATP, 30 mM Tris, pH 7.8) at a flow rate of 1 ml/min. The fractions containing high GDH activity were pooled and a further purification step was carried out using Resource Q (1 ml). At a flow rate of 1 ml/min, the column was eluted with NaCl gradient (80–300 mM) in buffer D after washing the column with 10 ml of buffer D. The active GDH fractions were combined and adjusted to 15% glycerol before storing the purified GDH at 4°C.

### Enzyme Assay of GDH

The determination of GDH activity was performed by the modified method of Cho et al. [1995]. Unless otherwise specified, the activity was measured by using highly purified GDH from RER subcellular fraction and commercial GDH (mitochondrial GDH) from rat liver. Thus mitochondrial fraction indicates mitochondrial subcellular fraction, whereas mitochondrial GDH means highly purified GDH purchased from Sigma. GDH activity was measured spectrophotometrically in the direction of oxidative deamination of glutamate by following the increase in absorbance at 340 nm. The reaction mixture in which GDH activity assay was performed contained 25 mM glutamate, 50 mM Tris (pH 9.5), 2.5 mM EDTA, 1.4 mM NAD, and 1 mM ADP. The reaction was started with the addition of enzyme into the reaction mixture at room temperature (RT). The initial rate of activity was measured for the first minute after the reaction started. One unit of enzyme was defined as the amount of enzyme required to reduce 1 µmol of $NAD^+$ at RT. A reaction in the direction of reductive amination of α-ketoglutarate was also performed in a reaction mixture containing 50 mM Tris (pH 8.0), 100 mM ammonium acetate, 2.5 mM EDTA, 1 mM ADP, 10 mM α-ketoglutarate, and 1 mM NADH at RT. This reaction was started by the same method as mentioned above, the definition of one unit also being the same except for in the measurement of the amount of oxidated NADH.

For kinetic studies, the reaction was carried out by varying the concentration of the substrate under investigation, while keeping other components at the concentration indicated above, including 1 mM ADP.

In measuring optimal pH, Tris was replaced by sodium phosphate (pH 6.0–8.0) and glycine

(pH 8.5–10.0) at the same concentration (50 mM), whereas other components in both directions were kept the same as for the standard reaction mixture.

**Heat stability test.** Two GDH isoproteins were incubated in 10 mM Hepes (pH 7.4) at 42°C. Aliquots were removed at specified intervals (2, 4, 6, 8, 10, 15, and 20 min) and assayed immediately in both directions (oxidative deamination of glutamate and reductive amination of α-ketoglutarate) by adding the reaction mixture while adjusting other components to the same concentration as indicated above.

**Limited proteolysis by trypsin.** Two GDH isoproteins were incubated with trypsin (1:100, w/w) at 37°C in 10 mM Hepes, pH 7.4. The remaining activity of GDH treated with trypsin was checked at various times (5, 10, 20, 30, 40, and 60 min). The aliquots were withdrawn and the activity assayed by the addition of the standard assay mixture adjusting to 50 mM Tris (pH 9.5), 25 mM glutamate, 2.5 mM EDTA, 1.4 mM NAD, and 1 mM ADP. The intactness of GDH isoproteins treated with trypsin was analyzed by 10% SDS-PAGE, which was stained by Coomassie blue.

### Marker Enzyme Assays

The marker enzymes used for checking the purity of the isolated subcellular fractions were glucose 6-phosphatase for RER, cytochrome-c oxidase for mitochondria, α-mannosidase II for Golgi apparatus, and alkaline phosphodiesterase I for plasma membrane, respectively. The activity of each enzyme was measured using the documented procedures [Storrie and Madden, 1990].

### Microsequencing

For N-terminal sequencing, approximately 20 µg of purified GDH was applied to SDS-PAGE (10%), followed by blotting to PVDF membrane (Wet blotters, Sigma). After staining and destaining the PVDF membrane, the GDH band obtained on the membrane was sent to Dr. Robert Scharkmann's laboratory in University of Utah for the sequence analysis. For internal sequencing, CNBr (100-fold treated the purified GDH by molar mass, 24 h, at RT) or trypsin (1:50 w/w, 2 h, at 37°C), followed by the separation of fragments by the SDS-PAGE. The steps that followed were the same as described above.

## RESULTS

### Subcellular Fractionation and Isolation of RER

The subcellular fractionation (mitochondria, Golgi apparatus, microsome, and RER) from rat liver homogenate was carried out by the method of differential and gradient centrifugation [Packer et al., 1997]. The activity values (SA, RSA) of their marker enzymes (cytochrome-c oxidase, α-mannosidase II, alkaline phosphodiesterase I, and glucose 6-phosphatase) are shown in Table I. In isolated RER fraction the remaining activity of cytochrome c oxidase was very low (less than 10%), as compared with that of isolated mitochondria frac-

**TABLE I. Marker Enzyme Activity in Isolated Subcellular Fractions***

| Fraction | Homogenate | Mitochondria | Golgi apparatus | Microsome | RER |
|---|---|---|---|---|---|
| Cytochrome c oxidase | | | | | |
| SA | 0.66 | 1.32 | 0.66 | 0.55 | 0.10 |
| RSA | 1.00 | 2.00 | 1.00 | 0.83 | 0.15 |
| α-Mannosidase II | | | | | |
| SA | 0.11 | 0.17 | 0.62 | 0.13 | 0.06 |
| RSA | 1.00 | 1.54 | 5.63 | 1.18 | 0.54 |
| Alkaline phosphodiesterase | | | | | |
| SA | 0.43 | 0.59 | 1.81 | 1.35 | 0.05 |
| RSA | 1.00 | 1.37 | 4.21 | 3.14 | 0.12 |
| Glucose 6-phosphatase | | | | | |
| SA | 0.72 | 0.17 | 0.32 | 3.50 | 3.60 |
| RSA | 1.00 | 0.24 | 0.44 | 4.86 | 5.00 |

*Subcellular fractions (mitochondria, Golgi apparatus, crude microsome, and RER) were prepared as described under Methods. The enzymes indicated were assayed using each fraction, and the data are given as the specific activity (SA, units per milligram of protein) and the relative specific activity (RSA, $SA_{fraction}/SA_{homogenate}$). One unit of activity corresponds to 1 µmol of substrate changed/min.

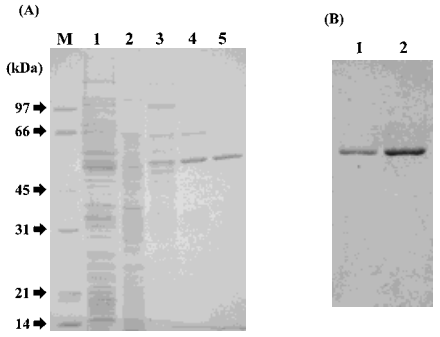
tion. Thus, the possibility of contamination by mitochondria in RER fraction was thought to be negligible. In our experiment, the mitochondrial fraction isolated by high-speed centrifugation (12,000*g*) also had high GDH activity (see Fig. 2). These results demonstrate that GDH of RER displayed the difference in binding specificity to the subcellular fraction from mitochondrial GDH.

## Purification of GDH

GDH in RER fraction was extracted with 0.6 M NaCl buffer from the membrane of RER without any detergent. It was a different characteristic from that of other membrane-bound forms of GDH from lysosome of pig liver and brain [Rajas et al., 1996], which could not be extracted with 0.6 M NaCl buffer but was solubilized in CHAPS, a zwitterionic detergent. However, GDH purified from RER was not thought to be a soluble protein in either the cytosol or lumen of RER, because the both supernatants obtained after ultracentrifugation (105,000*g*, and hypo-osmotic lysis did not contain GDH activity (data not shown). Thus, GDH of RER seems to be a peripheral membrane protein because the interaction of GDH with RER membrane was not strong. The amount of extracted protein was approximately 40% of RER fraction when the total amount of protein was investigated. By applying this extract to DEAE-Sepharose, ATP-agarose, and Resource Q in turn, highly purified GDH was obtained as shown in Figure 1A. In ATP-agarose column chromatography, GDH bound to ATP-agarose resin and was eluted by 1 mM ATP. This result showed that GDH of RER had a possibility of specific binding affinity to ATP. As indicated in Figure 1B, purification fold to RER fraction was 606, indicating high purification. The molecular weight of the purified GDH was estimated as 56 kD, which was the same as that of GDH in mitochondria.

## Properties of Two GDHs

Before comparing the properties of two purified GDH isoproteins, the activities of GDH in the intact membrane vesicles, mitochondria, and RER, were investigated. As shown in Figure 2, when the membrane vesicles were treated with 0.1% Triton X-100, mild nonionic detergent for 10 min, the activity of solubilized mitochondrial fraction was increased nearly 1.5 times relative to the activity in intact mitochondrial fraction, whereas the activity of GDH in RER fraction treated with Triton X-100 was nearly the same as that in intact RER fraction. This result showed that the binding region of GDH from RER was the outside of the RER membrane, unlike the mitochondrial GDH in the matrix of mitochondria.




(C)

| Lane | Fraction | Specific Activity (units/mg) | Purification fold |
|---|---|---|---|
| 1 of (A) | RER subcellular fraction | 0.0489 | 1 |
| 2 of (A) | 0.6M NaCl washing | 0.222 | 4.54 |
| 3 of (A) | DEAE-Sepharose eluent | 1.26 | 25.8 |
| 4 of (A) | ATP-agarose eluent | 13.8 | 281 |
| 5 of (A) | Resource Q eluent | 29.7 | 606 |

**Fig. 1.** **A:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purification of glutamate dehydrogenase (GDH) from rat liver rough endoplasmic reticulum (RER). For each lane, 30–40 μg of proteins in fractions containing high GDH activity after each purification step were applied to 10% SDS-PAGE, followed by staining with Coomassie blue. The fractions were (1) RER subcellular fraction, (2) resulted supernatant after ultracentrifuging (105,000*g*, 1 h), resuspending with 0.6 M NaCl buffer and recentrifuging (105,000*g*, 40 min), (3) DEAE-Sepharose fractions eluted by NaCl linear gradient (50–300 mM), (4) ATP-agarose fractions eluted by 1 mM ATP, and (5) Resource Q fractions eluted by NaCl linear gradient (80–300 mM). **Lane M**, molecular-weight marker. **B:** SDS-PAGE of mitochondrial GDH and GDH of RER. (1) GDH purchased from Sigma, (2) GDH of RER. **C:** Specific activity and purification fold for each purification step. The values indicated are the specific activity of GDH and purification fold to RER fraction for each lane of SDS-PAGE (1–5). GDH activity was measured at RT in the direction of deamination of glutamate in the presence of 1 mM ADP.

## Kinetic Studies

The optimal pH for GDH activity in the direction of reductive amination of α-ketoglutarate was about 8.0 for both GDH isoproteins. In the direction of glutamate oxidation, however, both isoproteins displayed optimal GDH activity around pH 9.0–9.5. In most previous studies [Hussain et al., 1989; McCarthy et al., 1980],

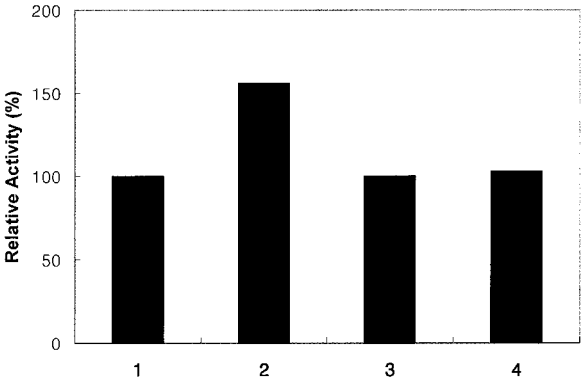



**Fig. 2.** Difference in glutamate dehydrogenase (GDH) activity for mitochondrial fraction and rough endoplasmic reticulum (RER) fraction after treating with 0.1% Triton X-100. The values of y-axis represent the relative activity of subcellular fraction to that of each fraction untreated with Triton X-100. **Lane 1**, GDH activity of intact mitochondrial fraction; **lane 2**, GDH activity of detergent-treated mitochondrial fraction; **lane 3**, GDH activity of intact RER fraction; **lane 4**, GDH activity of detergent-treated RER fraction

the GDH activity has been assayed within the pH range of 7.5–8.0 in the direction of glutamate oxidation. In our study, the GDH activities measured at pH 7.5–8.0 was only one-half that measured at pH 9.5 in the direction of glutamate oxidation (data not shown), the same result reported by Cho et al. [1995]. Therefore, all enzyme assays were performed at pH 9.5 in the direction of glutamate oxidation unless otherwise indicated.

The kinetic studies for glutamate, $NAD^+$, and $NADP^+$ were performed in the direction of deamination of glutamate to compare the $K_m$ values of the two GDH isoproteins. As shown in Table II, $K_m$ values for the glutamate showed little difference when the concentration of either cofactors was varied. However, small differences were detected in the $K_m$ values for the cofactors. In GDH of RER, the $K_m$ value for $NAD^+$ was more than twice that for $NADP^+$, whereas mitochondrial GDH had nearly twice the $K_m$ value for $NADP^+$ that it did for $NAD^+$. This result indicates the difference in specificity to the cofactors between the two GDH isoproteins. While mitochondrial GDH had higher affinity to $NAD^+$, GDH of RER to $NADP^+$.

## Heat Stability

As incubation time at 42°C was increased, the activity of mitochondrial GDH decreased rapidly in comparison with GDH of RER in both directions (Fig. 3). After 20 min, mitochondrial GDH was reduced to 20% of its initial activity, whereas that of GDH of RER retained 80%. It showed better thermal stability of GDH in RER. As there were similar differences in both directions in similar manners, the differences were not ascribed to the condition of the reaction mixture but to its original properties.

## Limited Proteolysis

To monitor whether there is a difference in the stability against protease attack, two GDH isoproteins were incubated with trypsin at 37°C. As shown in Figure 4A, GDH of RER was less susceptible to trypsin than GDH of mitochondria, and the difference was more conspicuous than in the case of heat stability. That was also confirmed by comparing the intensity of protein band in SDS-PAGE after treatment of trypsin (Fig. 4B). After 60 min, the intensity of the stained protein band of mitochondrial GDH (lane 1) was reduced to nearly zero. In the case of GDH of RER, however, the intensity of the protein band (lane 2) remained nearly intact. Moreover, different numbers of fragments and different fragmentation patterns were produced after tryptic treatment. In GDH of RER, only one fragment of similar molecular weight with intact GDH was produced by trypsin, whereas different-sized fragments were also produced in mitochondrial GDH. The middle-sized fragment appeared within a shorter incubation time, and the intensity of that fragment was stronger than that of the upper fragment, which was same size with that of GDH of RER.

## Microsequencing

Table III presents the sequences obtained with five fragments. The sequence of fragment 1 was an N-terminal sequence of GDH; the other four sequences were internal sequences of GDH obtained after treating with trypsin (fragments 1 and 3) and CNBr (fragments 2 and 4). Whereas two of the sequences (fragments 2 and 4) were exactly identical to those of GDH, known as mitochondrial enzyme of rat liver, others (fragments 1, 3, and 5) had different amino acids from mitochondrial GDH. Although an N-terminal sequence (fragment 1) of GDH from RER was consistent with another sequence reported as a different N-terminal sequence of GDH from rat liver [Amuro et al., 1989], two other different sequences (fragments 3 and 5) have not yet been reported. Considering that GDH has a very high conserved se-

**TABLE II. Kinetic Values of Two GDH Isoproteins***

| Varied substrate | Fixed substrate | $K_m$ (mM) Mitochondrial GDH | $K_m$ (mM) GDH of RER |
|---|---|---|---|
| Glutamate | $NAD^+$ | 4.61 ± 0.32 (4) | 5.93 ± 0.24 (4) |
| Glutamate | $NADP^+$ | 20.7 ± 2.6 (4) | 23.8 ± 1.7 (4) |
| $NAD^+$ | Glutamate | 0.364 ± 0.026 (4) | 0.924 ± 0.035 (4) |
| $NADP^+$ | Glutamate | 0.637 ± 0.025 (4) | 0.443 ± 0.012 (4) |

*The kinetic studies were done in 50 mM Tris (pH 9.5) by varying the concentration of one substrate under investigation, while keeping other components in the same conditions of standard reaction mixture, including 1 mM ADP. Values are means ± SD. Numbers in parentheses indicate number of experimental determinations.

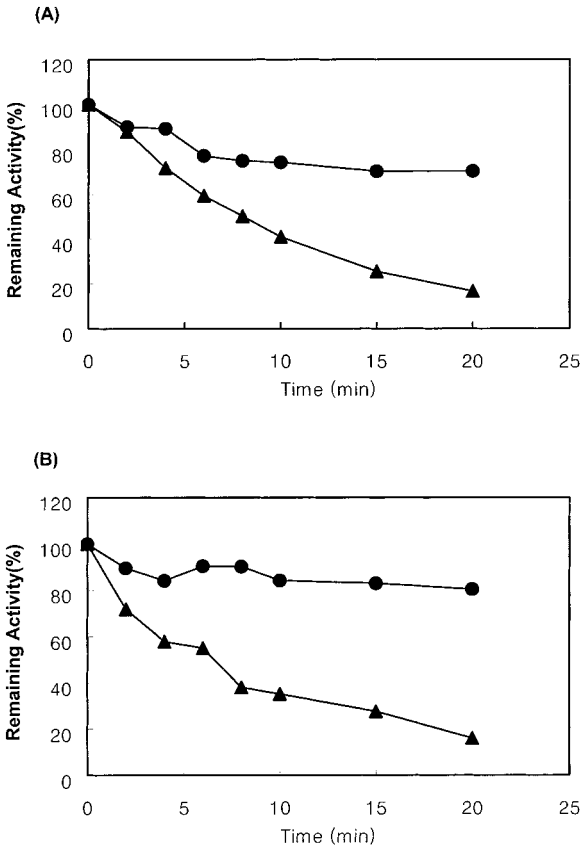



**Fig. 3.** Heat stability of glutamate dehydrogenase (GDH) isoproteins from rough endoplasmic reticulum (RER) and mitochondria. GDH isoproteins were incubated at 42°C in 10 mM HEPES, pH 7.4. At the designated time, aliquots were withdrawn and the activity was assayed in both directions. **A:** Oxidative deamination of glutamate. **B:** Reductive amination of α-ketoglutarate. Remaining activities are expressed relative to each control. ●, GDH of RER; ▲ GDH of mitochondria.

quence homology, these differences in sequence are somewhat meaningful.

## DISCUSSION

In this report, we demonstrate that GDH of RER has some properties that are distinguishable from those of mitochondrial GDH. First, unlike mitochondrial GDH, which is known as a soluble protein, GDH of RER seems to be a peripheral membrane protein since GDH could be extracted with 0.6 M NaCl buffer from the membrane of RER without detergent. The cytosolic fraction obtained after ultracentrifugation (105,000*g*) and the intravesicular fraction obtained by hypo-osmotic pressure-dependent lysis had no GDH activity (data not shown). In RER fraction treated with 150 mM NaCl buffer (10 mM Hepes, pH 7.4) including or not including 0.25 M sucrose, the extracted supernatant in both cases has very slight amount of GDH activity (<1%, data not shown). This finding indicated that the RER form of GDH cannot be extracted from membrane at physiological ionic strength (~150 mM NaCl), and GDH of RER was not the same protein with mitochondrial GDH that was bound to microsomal membrane before or during homogenization and cell fractionation. In addition, GDH of RER is thought to associate with the outside of the RER membrane. This could be deduced from the fact that GDH activity did not increase after the RER membrane was treated with Triton X-100 (mild nonionic detergent) and that the intravesicular fraction did not contain any GDH activity. Two other groups also purified another membrane-bound form of GDH from pig liver and brain [Rajas et al., 1993] or rat brain [Colon et al., 1986]. However, their purification scheme did not contain any steps to remove the mitochondrial fraction, which had high GDH activity. In addition, GDH from pig liver and brain could not be extracted by 0.6 M NaCl buffer, while GDH from rat brain was not treated by high salt extraction. The above facts made it clear that GDH purified by our group is not the same protein as that purified by other groups.

Second, GDH of RER displayed higher stability against heat inactivation or tryptic proteolysis than mitochondrial GDH. The membrane-

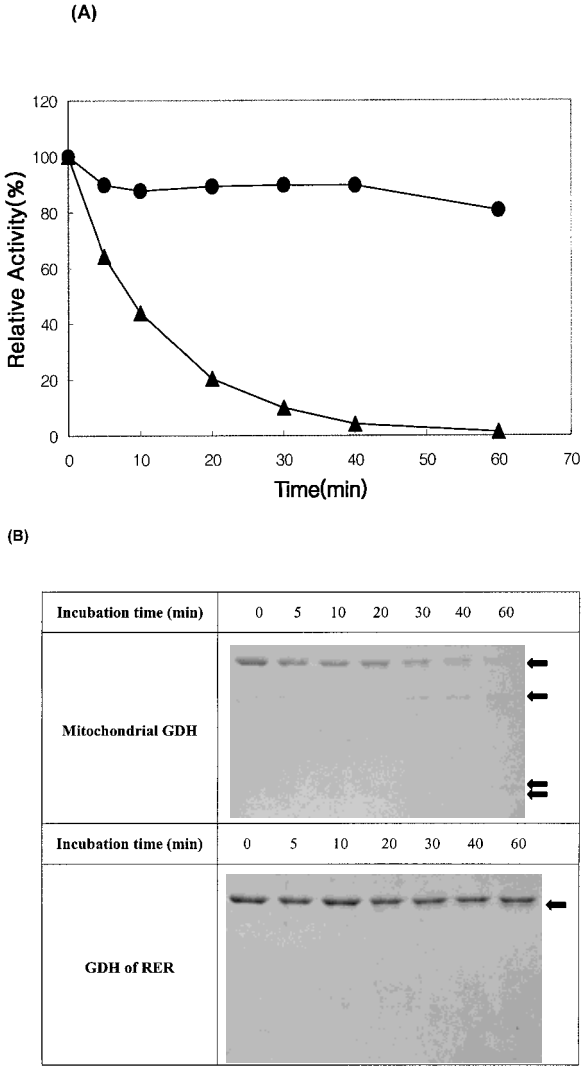



**Fig. 4.** Limited proteolysis (1:100, w/w) of glutamate dehydrogenase (GDH) isoproteins by trypsin. Two GDH were incubated with trypsin (1:100, by mass) at 37°C. **A:** At the designated time, aliquots were withdrawn and the activity assayed by the addition of the standard assay mixture. ● GDH of rough endoplasmic reticulum (RER); ▲ GDH of mitochondria. Remaining activities are expressed relative to each control. **B:** The intactness of GDH isoproteins against trypsin was analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie blue. The numbers above the stained band indicate the incubation time with trypsin under the same conditions as in **A**.

**TABLE III. Sequence Comparison Between Two GDH Isoproteins***

| Fragment no. | | |
|---|---|---|
| | | 1 10 |
| 1 | GDH of rat liver | SE[GP]TDREDD |
| | GDH of RER | SE[AA]TDREDD |
| | | 17 29 |
| 2 | GDH of rat liver | VEGFFDRGASIVE |
| | GDH of RER | VEGFFDRGASIVE |
| | | 41 49 |
| 3 | GDH of rat liver | NEE[QK]RNRV |
| | GDH of RER | NEE[AA]?N?V |
| | | 116 122 |
| 4 | GDH of rat liver | TYKCAVV |
| | GDH of RER | TYKCAVV |
| | | 347 356 |
| 5 | GDH of rat liver | IIAEGANGP[T] |
| | GDH of RER | IIAEGANGP[G] |

*Comparison of N-terminal (no. 1) and internal sequences (nos. 2–5) of GDH purified from RER with that of GDH of rat liver obtained from protein sequence database of Entrez at National Center for Biotechnology Information (NCBI). The amino acid is denoted by the single-letter code. The amino acid numbering is given above the sequences. Different amino acids appear in frame.

associated GDH mentioned above was also compared with soluble GDH for heat inactivation [Colon et al., 1986], and their result was consistent with ours. The particulate GDH was more stable than the soluble GDH. The values of the half-life at 45°C of particulate GDH and soluble GDH were 12.3 and 5.8 min, respectively. Thus, the higher thermal stability might be a unique feature of the membrane-bound form of GDH. By contrast, Cho et al. [1995] carried out heat stability and proteolysis tests with two soluble GDH isoproteins, GDH I and GDH II from bovine brain. These investigators found that GDH II, which was more stable in the heat stability test, was more susceptible to trypsin than was I. Thus, GDH of RER seems to be a different kind of protein from the soluble GDH isoproteins purified by Cho et al. [1995]. The cause of the stability is not fully understood, but it is likely that this stability can be ascribed to structural difference. No crystal structure has been reported for mammalian GDH, although the three-dimensional structure of GDH from microorganism is available [Baker et al., 1992]. This suggestion remains to be investigated.

Third, GDH of RER has different amino acid sequences from mitochondrial GDH. Except for the N-terminal sequence [Amuro et al., 1989], different amino acids of fragments 3 and 5 had not yet been reported. Therefore, this result also suggests a structural difference and the existence of another gene of GDH. That is, it is unlikely that GDH of RER is one of the isopro-

teins of GDH produced by post-translational modification. This postulation of the existence of another GDH gene is under debate [Nakatami et al., 1987; Amuro et al., 1990; Das et al., 1993].

Finally, GDH from RER seems to have a high affinity to ATP. While it is well documented that mitochondrial GDH binds to ADP and GTP specifically, it is not clear whether GDH has a binding site to ATP. As ATP did not inhibit nor activate the GDH activity, ATP appears to be involved in other function of GDH, if GDH of RER displays any other activity. Another group also purified GDH from pig thyroid lysosome using ATP-agarose column chromatography and showed that ATP inhibited GDH from binding to microtubule specifically [Rajas et al., 1993]. Thus, the membrane-bound form of GDH is thought to have a specific binding site to the ATP, as GDH mentioned above was also extracted from membrane fraction of lysosome.

This report cannot provide direct molecular biological evidence supporting the localization of GDH isoprotein to RER membrane, and there is another gene product of GDH. Rather, the study about other function of this protein will give another useful help to understand the reason and the mechanism of localization of GDH to RER membrane.

Whether GDH of RER plays a different role in regulating glutamate and/or $NH_3$ or has any other activity remains unknown. In human tissue and rat brain, the enzyme is shown to exist in two catalytically active forms, designated as soluble and particulate GDH. In some patients with multiple system atrophy, the decrease in enzyme activity is limited to the particulate form [Colon et al., 1986; Abe et al., 1992]. It is proposed that the particulate GDH from brain tissue did not display any other activity, but rather that it played a different role in the regulation of glutamate, as glutamate is a major excitatory neurotransmitter. In addition to playing a different role in regulation, GDH isoprotein displaying other activity was also studied. GDH from lysosomal membrane fraction was shown to be involved in microtubule-dependent perinuclear localization of lysosomes [Rajas et al., 1996]. On the other hand, GDH binding to mRNA or gRNA was reported [Preiss et al., 1993; Bringaud et al., 1997]. However, GDH of RER is not thought to display the activity of binding to mRNA or gRNA, as the GDH with which they showed the binding activity was mitochondrial enzyme. Thus, if GDH of RER displays any other activity, its role would appear to be involved in binding to microtubule. Work investigating this possibility is in progress.

In recent years, there have been a number of reports describing new function(s) for known proteins [Tong et al., 1988; Miles et al., 1991; Piatigorsky et al., 1989]. GDH might also be classified in the group of multifunctional proteins. This report, which identified and characterized the isoprotein of GDH in RER, provides further evidence for this postulate.

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