Enzymatic Properties of Glutamate Dehydrogenase in Rabbit Masseters

by

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Introduction

L-glutamate dehydrogenase (EC 1.4.1.3) catalyzes the following reaction:
 α -ketoglutarate + NH_4^+ + NAD(P)H^+ + H^+ — L-glutamate + NAD(P) + H_2O . Many papers have been published on the enzymatic properties of L-glutamate dehydrogenase (GDH) in various mammalian tissues [1–11], in bacteria [12–14], and in plants [15,16]. In oral tissue, KOHATA [6] reported on the subcellular distribution of GDH in rabbit dental pulp and MAKINO [14] reported on the enzymological properties of GDH in *Fusobacterium* from human dental plaque, such as molecular weight, coenzyme specificity and its kinetic mechanism. However, little has been reported on the enzymological properties of GDH in masseters and its enzymatic characterization is not well-known. In order to clarify the role of GDH associated with metabolic pathways on glucose and amino acids, we prepared a scheme for this study. In this paper GDH from rabbit masseters was partially purified by column chromatography on DEAE-cellulose and its enzymatic properties are described.

Materials and methods

Materials—Albino rabbits of both sexes, weighing about 2 kg, were used in this study. The heads were stored frozen until use and employed within 4 days. Masseter fragments were obtained from the jaws using a surgical knife. For homogenization, fragments were obtained and minced with a razor on a glass-plate set on ice.

Enzyme preparation—All procedures were carried out at 4°C. The masseters (wet weight: 100 mg) were homogenized in 2 ml of 0.01 M triethanolamine-HCl buffer (pH 8.2) in a glass homogenizer and then centrifuged at 10,000 × g for 15 minutes. A supernatant fraction was applied on the DEAE-cellulose column (1 × 15 cm) and equilibrated with 0.01 M triethanolamine-HCl buffer (pH 8.2). Elution was performed in steps with the addition of KCl at 0 M, 0.1 M, 0.2 M, and 0.5 M concentrations in the above buffer to give 5 ml for each fraction.

Enzyme assay—The reaction mixture was composed of triethanolamine-HCl buffer (pH 7.4) 20 μmol , α -ketoglutarate (α -KG) 2.0 μmol , NH_4Cl 120 μmol , NAD(P)H 0.08 μmol and the enzyme preparation in a final volume of 0.4 ml. The reaction was carried out for 30 minutes at 37°C and terminated by the addition of

0.4 ml of 10% perchloric acid. After 2.0 ml of 10 N NaOH containing 0.01% hydrogen peroxide was added, the solutions were reincubated for 1 hour at 37°C. The fluorescence originating from the NAD(P) that formed was measured with a fluorescence spectrophotometer^a at an excitation wavelength of 365 nm and an emission wavelength of 470 nm.

Molecular weight—The molecular weight was determined by the upward flow technique on a 2.6×100 cm column of Sephadex G-200 that was equilibrated with 0.01 M triethanolamine-HCl buffer (pH 8.2) and contained 0.2 M KCl. The elution of proteins was carried out at 4°C with the same buffer and a fraction of 2.0 ml each was collected and assayed for enzyme activity. The column was previously calibrated with the following proteins of known molecular weight: cytochrome C (13,500), hen egg albumin (45,000), aldolase (158,000) and ferritin (450,000).

Results

Partial purification by chromatography on DEAE-cellulose—The elution profile of enzyme activity is illustrated in Fig. 1. The major enzyme activity was

Fig. 1 DEAE-cellulose column chromatography of glutamate dehydrogenase. The elution of proteins from a DEAE-cellulose column (void volume: 8 ml) was performed with step additions of KCl at concentrations as indicated in triethanolamine-HCl buffer (pH 8.2). The effluent fraction of 5.0 ml each were collected and assayed for the enzyme activity.

a. Hitachi fluorescence spectrophotometer 650-10M, Japan.

eluted as a single peak with 0.2 M KCl in 0.01 M triethanolamine-HCl buffer (pH 8.2). The active fraction was tested for GDH activity under the standard assay conditions excluding α -KG, NH_4Cl and NADH. In either case, no NAD formation could be detected. The active fraction served as the enzyme preparation in this study.

Molecular weight—Molecular weight was determined by gel filtration chromatography on Sephadex G-200. As shown in Fig. 2, GDH activity was observed to be eluted at a position corresponding to a molecular weight of approximately 300,000.

Effects of pH and buffers—The pH of maximum enzyme activity was measured with the following buffers: 50 mM triethanolamine-HCl, 50 mM imidazole-HCl, 50 mM Tris-HCl and 50 mM phosphate. The optimum pH was observed to be nearly constant in the wide range of pH 6.2 to pH 8.6, as shown in Fig. 3. A pH of 7.4 in the triethanolamine-HCl buffer was used for the standard assay.

Coenzyme specificity—Coenzyme specificity was examined using NADPH in the reaction mixture under standard assay conditions instead of NADH (Table 1). GDH activity, using NADPH as a coenzyme, was 60% of that using NADH. The K_m values for NADH and NADPH were calculated to be 0.052 mM and 0.062 mM, respectively.

K_m values for α -KG and NH_4Cl —The K_m values for α -KG and NH_4Cl were examined using NADH and NADPH, respectively, in the reaction mixture under standard assay conditions (Table 2). The K_m values for α -KG and NH_4Cl using NADH were calculated to be 0.67 mM and 150 mM by means of double reciprocal plots [17]. The K_m values for α -KG and NH_4Cl using NADPH were calculated to be 0.63 mM and 150 mM by the above method.

Fig. 2 Molecular weight determination of glutamate dehydrogenase. A Sephadex G-200 column was equilibrated with 0.01 M triethanolamine-HCl buffer (pH 8.2), containing 0.2 M KCl, prior to gel filtration. The column was calibrated with the standard proteins which were monitored by absorbance at 280 nm. The effluent fractions of 2.0 ml each were collected and assayed for enzyme activity.

Fig. 3 Effects of pH and buffers on enzyme activity. Assays were performed as described under "Materials and methods," except that the pH and buffers were varied as indicated. ●, 50 mM triethanolamine-HCl buffer; ○, 50 mM imidazole-HCl buffer; ▲, 50 mM Tris-HCl buffer; △, 50 mM phosphate buffer.

Table 1 Coenzyme specificity for glutamate dehydrogenase

	NADH	NADPH
K _m (mM)	0.052	0.062
Relative activity (%)	100	60

Assays were performed in the presence of each compound as a coenzyme.

Table 2 K_m values for α-KG and NH₄Cl

	NADH	NADPH
α-KG	0.67	0.63
NH ₄ Cl	150	150

K_m (mM)

Assays were performed as described under "Materials and methods."

Effects of nucleotides—The effects of nucleotides on the catalytic function of enzymes were examined using NADH and NADPH in the reaction mixture under standard assay conditions (Table 3, Table 4). This enzyme was activated by AMP and ADP, but was inhibited by GTP, GDP, ITP, CTP and UTP. The extent of inhibition using NADH was larger than that using NADPH. In order to determine the activation type for AMP and the inhibition type for GTP, the activity and inhibition measurements were analyzed by means of double reciprocal plots [17]. As a result, the activation type for AMP in Fig. 4 was found to be parallel with respect

Table 3 Effects of nucleotides on enzyme activity using NADH as a coenzyme

Nucleotides	Concentrations (mM)				
	0.025	0.1	0.25	1.0	2.5
AMP	128	135	136	155	155
ADP	112	112	112	130	130
ATP	100	100	100	100	100
CMP	100	100	100	100	100
CDP	100	100	100	80	72
CTP	86	77	61	54	46
GMP	100	95	78	47	28
GDP	54	19	11	0	0
GTP	5	0	0	0	0
IMP	100	100	92	76	50
IDP	100	78	74	42	31
ITP	56	20	9	3	3
UMP	100	100	100	100	100
UDP	100	100	87	57	47
UTP	87	80	37	25	14

Relative activity (%)

Assays were performed in the presence of each nucleotide concentration as indicated.

Table 4 Effects of nucleotides on enzyme activity using NADPH as a coenzyme

Nucleotides	Concentrations (mM)				
	0.025	0.1	0.25	1.0	2.5
AMP	105	111	124	135	135
ADP	100	100	114	131	131
ATP	100	100	100	100	100
CMP	100	100	100	100	100
CDP	100	100	100	100	100
CTP	100	100	88	81	68
GMP	100	92	87	83	74
GDP	97	78	38	11	8
GTP	10	0	0	0	0
IMP	100	100	100	100	91
IDP	100	100	96	93	73
ITP	77	55	25	5	3
UMP	100	100	100	100	100
UDP	100	100	100	92	88
UTP	100	100	91	65	46

Relative activity (%)

Assays were performed in the presence of each nucleotide concentration as indicated.

to NADH and convergent with respect to α -KG and NH_4Cl . The inhibition type for GTP, in Fig. 5, was found to be uncompetitive with respect to NADH and non-competitive with respect to α -KG and NH_4Cl .

Effects of metal ions—The effects of various metal ions on enzyme activity were examined in the reaction mixture under standard assay conditions (Table 5). This enzyme was inhibited by each metal ion and it was strongly inhibited by Zn^{2+} , Ca^{2+} , Hg^{2+} and Cd^{2+} .

Fig. 4 Activation of AMP on enzyme activity. Assays were performed as described under "Materials and methods." The data are represented as double reciprocal plots of the initial velocities against NADH (in A), NH_4Cl (in B) or α -KG (in C).

Effects of metabolic intermediates in glycolysis—The effects of metabolic intermediates on the catalytic function of enzymes were examined. As seen in Table 6, this enzyme was inhibited by 2,3-bisphosphoglycerate, 2-phosphoglycerate, glucose 6-phosphate and phosphoenolpyruvate, whereas the other compounds had little effect on enzyme activity.

Effects of metabolic intermediates in the TCA cycle—The effects of metabolic intermediates on the catalytic function of enzymes were examined. As seen in Table 7, this enzyme was inhibited by malate, succinate and fumarate, and then was strongly inhibited in particular by malate. The other compounds had little effect on enzyme activity. In order to determine the inhibition type of malate, the activity

Fig. 5 Inhibition of GTP on enzyme activity. Assays were performed as described under "Materials and methods." The data are represented as double reciprocal plots of the initial velocities against NADH (in A), NH_4Cl (in B) or α -KG (in C).

measurements, as a function of substrate concentration, were analyzed by means of double reciprocal plots [17]. As a result, the inhibition type for malate, in Fig. 6, was found to be competitive with respect to α -KG and noncompetitive with respect to NH_4Cl and NADH.

Kinetic studies—The kinetic pattern on the reaction mechanism was determined by changing a substrate concentration at various fixed levels of the other substrates and high concentrations of one of the three substrates, NADH, α -KG and NH_4Cl , respectively (Fig. 7). As a result, the double reciprocal plots [17] of the reaction rate against the α -KG concentration at several fixed concentrations of NADH showed

Table 5 Effects of metal ions on enzyme activity

Metal ions	Concentrations (mM)				
	0.01	0.05	0.1	0.5	1
Mg ²⁺	95	70	54	29	25
Ni ²⁺	94	88	73	28	13
Ba ²⁺	100	100	100	94	83
Sr ²⁺	90	86	83	80	77
Cu ²⁺	57	37	15	0	0
Co ³⁺	73	62	60	41	30
Ca ²⁺	83	82	80	67	54
Cd ²⁺	71	52	31	8	0
Hg ²⁺	70	28	13	—	—
Zn ²⁺	9	0	0	0	0

Relative activity (%)

Assays were performed in the presence of each metal ion concentration as indicated.

Table 6 Effects of metabolic intermediates in glycolysis on enzyme activity

Compounds (mM)	Concentrations (mM)				
	0.025	0.1	0.25	1.0	2.5
glucose 6-phosphate	100	84	80	78	46
fructose 6-phosphate	100	100	100	92	72
fructose 1,6-bisphosphate	100	100	100	100	100
dihydroxyacetone phosphate	100	100	100	100	100
glyceraldehyde-phosphate	100	100	100	100	100
3-phosphoglycerate	100	100	100	100	100
2,3-bisphosphoglycerate	95	74	67	55	34
2-phosphoglycerate	100	98	80	60	50
phosphoenolpyruvate	100	100	90	64	48
lactate	100	100	100	100	100
glucose 1-phosphate	100	100	100	100	90

Relative activity (%)

Assays were performed in the presence of each metabolic intermediate concentration as indicated.

Table 7 Effects of metabolic intermediates in the TCA cycle on enzyme activity

Compounds (mM)	Concentrations (mM)				
	0.025	0.1	0.25	1.0	2.5
citrate	100	100	100	100	100
isocitrate	100	100	100	100	100
succinate	100	100	100	53	31
fumarate	90	88	76	61	54
malate	90	83	52	15	0

Relative activity (%)

Assays were performed in the presence of each metabolic intermediate concentration as indicated.

Fig. 6 Inhibition of malate on enzyme activity. Assays were performed as described under "Materials and methods." The data are represented as double reciprocal plots of initial velocities against NADH (in A), NH_4Cl (in B) or α -KG (in C).

parallel straight lines at a high concentration of NH_4Cl . And then, the double reciprocal plots [17] of the reaction rate against α -KG or NADH concentrations at several fixed concentrations of NH_4Cl showed convergent straight lines at a high concentration of NADH or α -KG.

Effects of L-glutamate—The effects of L-glutamate on the catalytic function of enzymes were examined. As a result, this enzyme was inhibited by L-glutamate. In order to determine the inhibition type of L-glutamate, the activity measurements, as a function of substrate concentration, were analyzed by means of double recip-

Fig. 7 Kinetics of glutamate dehydrogenase. The data are represented as double reciprocal plots of the initial velocities against α -KG or NADH, according to LINEWEAVER and BURK [17]. In A, α -KG was varied as indicated, at fixed NH_4Cl concentrations of 38 mM, 50 mM, and 75 mM. In B, α -KG was varied as indicated, at fixed NADH concentrations of 38 mM, 50 mM, 63 mM, 75 mM and 200 mM. In C, NADH was varied as indicated, at fixed NH_4Cl concentrations of 25 mM, 38 mM, 63 mM and 75 mM.

rocal plots [17]. As seen from Fig. 8, the inhibition type for L-glutamate was found to be noncompetitive with respect to α -KG and noncompetitive with respect to NADH and NH_4Cl .

Discussion and conclusions

GDH from rabbit masseters was isolated by chromatography on DEAE-cellulose as a single peak with elution of GDH activity using KCl at an 0.2 M concen-

Fig. 8 Inhibition of L-glutamate on enzyme activity. Assays were performed as described under "Materials and methods." The data are represented as double reciprocal plots of the initial velocities against NADH (in A), NH_4Cl (in B) or α -KG (in C).

tration in triethanolamine-HCl buffer (pH 8.2). This peak, as the sample, was subsequently used to study its enzymatic properties.

The molecular weight of this enzyme was determined at approximately 300,000 by filtration chromatography, like that of rat liver [8].

The optimum pH of GDH from rabbit masseters was studied over a wide range, pH 6.2 to pH 8.6. The distribution of the active level was rather broad, but was slightly higher between the pHs of 7.2–7.6, like that of rabbit dental pulp [6], human liver [7], chicken liver [2] and rat kidneys [5].

COLDIN and FRIEDEN [9] reported that GDH from bovine liver had NAD- and NADP-dependent. MAKINO [14] reported that GDH from *Fusobacterium* had NAD-dependent. We also studied GDH from rabbit masseters using NADH and NADPH as coenzymes. The activity of this enzyme using NADPH was 60% of that using NADH. However, the K_m values for NADH were similar to that for NADPH.

In the oxidation of NADH, the K_m value for α -KG was calculated to be 0.6 mM by means of double reciprocal plots [17], like that of bovine liver [13] and *Fusobacterium* [14]. The K_m value for NH_4Cl was calculated to be 150 mM by the above method, like that of rat liver and kidneys [10].

FRIEDEN [5] reported that the GDH from various mammalian tissues was inhibited by Zn^{2+} . GARLAND [16] reported that the GDH from *Pisum Sativum* was activated by low concentrations of Mn^{2+} , Ca^{2+} and Zn^{2+} , but it was inhibited by high concentrations of Zn^{2+} . The activity of GDH from rabbit masseters was also studied over a wide range, 0.01 mM to 1 mM concentrations. This enzyme was inhibited by each metal ion and was significantly inhibited by Zn^{2+} , Cu^{2+} , Hg^{2+} and Cd^{2+} , like the report of FRIEDEN [5].

FRIEDEN [5] reported that GDH from various mammalian tissues was activated by ADP. On the other hand, LEHMANN and PELEIDERER [7] reported that GDH from human liver was inhibited by GTP. Therefore, we examined the effects of various nucleotides on GDH from rabbit masseters using NADH or NADPH as coenzymes. As a result, enzyme activity was activated by AMP and ADP, and those were inhibited by GTP, GDP, ITP, CTP and UTP. The extent of inhibition showed that enzyme activity using NADH had larger values than those using NADPH as a coenzyme, like the report on bovine liver [5]. In order to clarify the effects of AMP and GTP on this enzyme the activation mechanism of AMP and the inhibition mechanism of GTP were examined by means of double reciprocal plots [17]. As a result, in the case of AMP, it was found to be parallel with respect to NADH and convergent with respect to α -KG and NH_4Cl . In the case of GTP, it was found to be uncompetitive with respect to NADH and noncompetitive with respect to α -KG and NH_4Cl . These results may suggest, like the report of FRIEDEN [4], that GDH from beef liver has a binding site which is highly specific for purine nucleotides, other than a binding site for NADH.

LEJOHN and STEVENSON [15] reported that GDH from taxonomically related fungi was inhibited by phosphoenolpyruvate, citrate and isocitrate. Therefore, we examined the effects of metabolic intermediates on the catalytic function of this enzyme. As a result, this enzyme was inhibited by 2,3-bisphosphoglycerate, glucose 6-phosphate, 2-phosphoglycerate, succinate and fumarate, and then it was significantly inhibited by low concentrations of malate by 50% at 0.25 mM. In order to clarify the effect of malate against the catalytic function of this enzyme, the inhibition mechanism of malate was examined by means of double reciprocal plots [17]. As a result, it was found to be competitive with respect to α -KG and noncompetitive with respect to NH_4Cl and NADH. The results may suggest the occurrence of an apparent decrease in the activity of this enzyme through a binding of malate to the binding site of α -KG because α -KG and malate are dicarboxylates.

The kinetic pattern on the reaction mechanism of this enzyme was determined by changing a substrate concentration at various fixed levels of the co-substrate. As a result, it was found to be the "Ordard" mechanism proposed by CLELAND [19]. In order to determine the reaction mechanism of this enzyme in detail, the inhibition mechanism of L-glutamate was examined. As a result, it was found to be non-competitive with respect to α -KG and uncompetitive with respect to NADH and NH_4Cl . The results may suggest that the reaction mechanism of this enzyme is an "Ordered Ter Bi" mechanism, like that of bovine liver [3] and *Fusobacterium* [14]. This mechanism consists of a sequential order of addition of substrates with pyrimidine nucleotides added first. In the oxidation of NADH ammonium ions add to the enzyme before α -KG.

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