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D-Aspartate *N*-methyltransferase catalyzes biosynthesis of *N*-methyl-D-aspartate (NMDA), a well-known selective agonist of the NMDA receptor, in mice

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ABSTRACT

N-Methyl-D-aspartate (NMDA), which is a selective agonist for the NMDA receptor, has recently been shown to be present in various biological tissues. In mammals, the activity of D-aspartate *N*-methyltransferase (DDNMT), which produces NMDA from D-aspartate, has been detected only in homogenates prepared from rat tissues. Moreover, the enzymatic properties of DDNMT have been poorly studied and its molecular entity has not yet been identified. In this report, we show for the first time that the activity of DDNMT is present in mouse tissues and succeed in obtaining a partially purified enzyme preparation from a mouse tissue homogenate with a purification fold of 1900 or more, and have characterized the enzymatic activity of this preparation. The results indicate that DDNMT, which is highly specific for D-aspartate and is *S*-adenosyl-L-methionine-dependent, is a novel enzyme that clearly differs from the known methylamine-glutamate *N*-methyltransferase (EC 2.1.1.21) and glycine *N*-methyltransferase (EC 2.1.1.20).

1. Introduction

Ionotropic glutamate receptors that mediate much of the excitatory neurotransmission in mammalian brains are classified into three subfamilies: the α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) receptor, *N*-methyl-D-aspartate (NMDA) receptor and kainate receptor [1]. These subfamilies were categorized by differences in their affinity toward the synthetic agonists AMPA, NMDA and kainic acid [2]. The synthetic NMDA used for that classification is still used widely in neurological research as a selective agonist of the NMDA receptor. In addition, since the late 1900s synthetic NMDA has been known to induce the release of the luteinizing hormone, gonadtropin-releasing hormone, prolactin and growth hormone in mammals [3]. Although NMDA has long been considered an unnatural amino acid, it has recently been shown to be present in various biological tissues [4–8], including humans [9], rats [10,11] and mice [12–14]. Given the biological activity of synthetic NMDA, understanding the physiological mechanisms of endogenous NMDA is of significant interest and requires an explanation of NMDA biosynthesis. The activity of D-aspartate *N*-methyltransferase (DDNMT, NMDA synthase), which produces NMDA from D-aspartate (D-Asp), has been detected in tissue homogenates from rat hypothalamus, brain, adenohypophysis and liver [11]. However, its enzymatic properties have been poorly studied and its molecular entity has not been identified. Thus, characterization of DDNMT still requires much work. In addition, there is no report on DDNMT activity in mice, which are more commonly used as experimental animals in neuroscience research [15]. In this paper, we showed for the first time that DDNMT activity is present in mouse tissues and used various purification approaches in an effort to purify the enzyme. We succeeded in obtaining a partially purified enzyme preparation with a purification

Abbreviations: NMDA, *N*-methyl-D-aspartate; NMLA, *N*-methyl-L-aspartate; NMDG, *N*-methyl-D-glutamate; NMLG, *N*-methyl-L-glutamate; SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; D-Asp, D-aspartate; DDNMT, D-aspartate *N*-methyltransferase; Gly, glycine; NMGly, *N*-methylglycine; GNMT, glycine *N*-methyltransferase; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry; FDLA, N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide;; DDO, D-aspartate oxidase

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fold of 1900 or more and characterized this preparation.

2. Material and methods

2.1. Materials

Four week-old female Slc:ddY mice were purchased from Japan SLC (Shizuoka, Japan). Laparotomy was performed under anesthesia using 1.5% isoflurane and mice were euthanized by the cardiac blood collection method. Each tissue was quickly removed and frozen at −80 °C.

N^{α}-(5-Fluoro-2,4-dinitrophenyl)-L-leucinamide (FDLA) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The EDTA-free cOmplete™ protease inhibitors cocktail was purchased from Roche Diagnostics (Mannheim, Germany).

TOYOPEARL Phenyl-650 resin was from TOSOH (Tokyo, Japan). HiTrap Blue HP, HiTrap Q FF, RESOURCE Q and Superdex 200 HR columns were from GE Healthcare UK Ltd. (Buckinghamshire, UK). Amicon Ultra-0.5 mL centrifugation filters were procured from Merck (Darmstadt, Germany). NMDA, *N*-methyl-L-aspartate (NMLA), *N*-methyl-D,L-glutamate, *N*-methylglycine, sodium bicarbonate, LC-MS grade acetonitrile, and 0.1% formic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). LC-MS grade methanol, biochemical grade *S*-adenosyl-L-methionine (SAM) and all other special grade reagents were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan).

2.2. Standard enzyme assay for DDNMT activity

DDNMT activity was measured by our reported liquid chromatography-electrospray ionization-tandem mass spectrometry method (LC-ESI-MS/MS method) [16] with minor modifications such as analytical column size and addition of internal standards. Briefly, the standard assay mixture containing 333 mM D-Asp, 20 mM SAM, 20 mM Tris-HCl buffer (pH 8.0) and an enzyme sample was incubated for 20 min at 30 °C. After quenching the reaction by adding 1 mL of cold methanol, the mixture was centrifuged at 12,000 ×*g* for 20 min at 4 °C. The supernatant was concentrated and dried under vacuum at 40 °C. The residue was dissolved in 125 μL of 0.5 M aqueous sodium bicarbonate solution containing 25 μM theanine as an internal standard. The resulting solution was derivatized with FDLA and subjected to LC-ESI-MS/MS to quantify NMDA. Blanks were run in parallel with each series of assays, and prepared as described above, except that cold methanol was added to the assay mixture before adding the enzyme sample. DDNMT activity was calculated by determining the increase in the amount of NMDA from an enzyme reaction for 20 min when compared with that of the blank.

For LC-ESI-MS/MS analysis, an Ultra HT Hydrosphere C18 column (3.0 × 50 mm, 2 μm) (YMC Corp., Kyoto, Japan) was used. The mobile phase was acetonitrile-methanol-0.1% formic acid (37:5:58, v/v/v) and the flow rate was 0.3 mL/min. Other LC and MS conditions were the same as described previously [16]. The MS/MS chromatograms of FDLA-derivatized NMDA and theanine were obtained by monitoring fragment ions (theanine: *m/z* 405; NMDA: *m/z* 288) generated from protonated ions (theanine: *m/z* 469; NMDA: *m/z* 442). The calibration curve plotted the NMDA concentration on the horizontal axis and the area ratio (NMDA/theanine) of the MS/MS chromatogram on the vertical axis using the measurement results of 0.25–25 μM authentic NMDA solutions containing 25 μM theanine.

2.3. Measurement of the activity before and after ammonium sulfate fractionation of various tissues

Approximately 0.5 g of frozen mouse tissue was homogenized with 2 mL of 20 mM Tris-HCl buffer (pH 8.0) containing cOmplete™ protease inhibitors cocktail using a Potter-Elvehjem homogenizer equipped with a Teflon pestle under ice cooling. The homogenate was centrifuged at 14,000 ×*g* for 10 min at 4 °C. A portion of the supernatant was dialyzed against 100 volumes of the same buffer three times (1 h each time), and then subjected to measurement of DDNMT activity as a sample before ammonium sulfate fractionation.

Ammonium sulfate was added to the remaining portion of the supernatant at 4 °C, with gentle stirring, to 10% saturation. The precipitate was removed by centrifugation at 14,000 ×*g* for 10 min at 4 °C, and ammonium sulfate was added again to the resulting supernatant of 10% saturation to give 20% saturation. The precipitate obtained after centrifugation (14,000 ×*g*, 10 min, 4 °C) was collected. The precipitate was dissolved in 3 mL of 20 mM Tris-HCl Buffer (pH 8.0) containing cOmplete™ protease inhibitors cocktail and dialyzed three times against 100 volumes of the same buffer (1 h each time). The DDNMT activity of the dialyzed solution was measured as the sample after ammonium sulfate fractionation.

2.4. Purification of DDNMT from the pancreas of mouse

All procedures were carried out at 4 °C. Column chromatography was performed with a BIO-RAD NGC Chromatography System (Bio-Rad, Hercules, CA) and several appropriate columns.

The pooled pancreases from eighty mice were homogenized with five volumes of 20 mM Tris-HCl buffer (pH 8.0) containing cOmplete™ protease inhibitors cocktail using a Potter-Elvehjem homogenizer equipped with a Teflon pestle under ice cooling. The homogenate was centrifuged at 14,000 ×*g* for 10 min at 4 °C. The supernatant obtained was fractionated by ammonium sulfate precipitation (10–20% saturation). The resulting precipitate was dissolved in 10 mM potassium phosphate buffer (pH 7.0) containing 2 M NaCl and loaded at 1.0 mL/min onto a TOYOPEARL Phenyl-650 column (1.6 × 15 cm) equilibrated with the same buffer. After the column was washed with 10 mM potassium phosphate buffer (pH 7.0) containing 2 M NaCl, a stepwise elution was performed with decreasing concentrations of NaCl (1.2, 1.0 and 0 M) dissolved in 10 mM potassium phosphate buffer (pH 7.0) at a flow rate of 2.0 mL/min. The activity of each fraction was measured and fractions with DDNMT activity were pooled. The pooled fraction was dialyzed three times (1 h each time) against 100 volumes of 20 mM Tris-HCl buffer (pH 8.0) for buffer exchange and loaded at 0.5 mL/min onto a HiTrap Blue HP column (0.7 × 2.5 cm) equilibrated with the same buffer. After the column was washed with the same buffer, a stepwise elution was performed with increasing concentrations of NaCl (0.45, 0.60 and 1 M) dissolved in 20 mM Tris-HCl buffer (pH 8.0) at a flow rate of 0.5 mL/min. The activity of each fraction was measured and fractions with DDNMT activity were pooled. The pooled fraction was dialyzed three times (1 h each time) against a 100 volumes of 20 mM Tris-HCl buffer (pH 8.0) for buffer exchange. The dialyzed fraction was loaded at 0.2 mL/min onto a RESOURCE Q column (0.64 × 3.0 cm) equilibrated with the same buffer, and was then eluted by using a linear gradient (90 mL) from 0 to 0.6 M NaCl in 20 mM Tris-HCl buffer (pH 8.0) at a flow rate of 0.2 mL/min. The activity of each fraction was measured. Fractions with DDNMT activity were pooled, concentrated with Amicon Ultra-0.5 mL centrifugation filters and stored at 4 °C until use.

2.5. Expression and purification of the mouse glycine N-methyltransferase in Escherichia coli

Expression of mouse glycine *N*-methyltransferase (GNMT) in *Escherichia coli* (*E. coli*) was performed according to Luka et al. [17]. The *E. coli* cells were suspended in lysis buffer (100 mM Tris-HCl, 5 mM EDTA, 1 mM β-mercaptoethanol, pH 8.0) and lysed by sonication. The lysate was dialyzed against 100 volumes of 20 mM Tris-HCl buffer (pH 8.0) for 2 h and then applied to a HiTrap Q FF column (5 mL) equilibrated with the same buffer. After washing the column with the same buffer, recombinant GNMT was eluted by using a linear gradient (135 mL) from 0 to 0.3 M NaCl in 20 mM Tris-HCl buffer (pH 8.0) at a flow rate of 1.0 mL/min. Fractions containing the enzyme were

Table 1
Purification of DDNMT from the mouse pancreas.

Purification step	Total activity[a] (nmol/min)	Yield (%)	Protein (μg)	Specific activity (μmol/min per mg)
1. Homogenate	nd	–	556000[b]	nd
2. Ammonium Sulfate Fractionation (10–20% saturation)	375	100	127000[b]	0.0030
3. Phenyl TOYPEARL	103	28	7470[c]	0.0138
4. HiTrap Blue	9.5	2.5	249[c]	0.0381
5. RESOURCE Q	4.8[d]	1.3	0.811[c]	5.86

[a] The activity that produced NMDA from D-Asp and SAM was measured using the standard enzyme assay with LC-ESI-MS/MS, as described in the Materials and methods section. nd, not detected.
[b] Proteins were quantified using the Bio-Rad Quick Start Bradford Protein Assay kit (Bio-Rad).
[c] Protein was quantitatively determined by the area normalization method, as described in the Materials and methods section.
[d] The volume of the final enzyme preparation was 1.0 mL, and the activity per mL of the enzyme preparation was 4.8 nmol/min per mL.

confirmed by SDS-PAGE, as described by Laemmli [18]. The enzyme was pooled, concentrated using Amicon Ultra-0.5 mL centrifugation filters and stored at 4 °C until use. The purity of the GNMT preparation estimated by SDS-PAGE was ~90%.

2.6. Protein determination

2.6.1. Bradford method

The Bradford method was used for protein quantification in the ammonium sulfate fractionation described in Sections 2.3 and 2.4 and the purification process described in Section 2.6. Proteins were quantified using the Quick Start Bradford Protein Assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Bovine serum albumin was used as the standard.

2.6.2. Area normalization method

This method was used for protein quantification of pooled active fractions from the column chromatography procedure described in Section 2.4. The peak area of the active fraction and the total peak area on the chromatogram obtained by monitoring the absorption at 280 nm were calculated by ChromLab software Ver. 4.0 (Bio-Rad, Hercules, CA) and the following formula was applied:

$$\begin{aligned}&(\text{Amount of protein in active fraction})\\&\quad = (\text{Amount of protein applied to column})\\&\qquad \times (\text{Peak area of active fraction})/(\text{Total peak area})\end{aligned} \quad (1)$$

2.7. Substrate specificity

For DDNMT, D-Asp, L-aspartate (L-Asp), D-glutamate (D-Glu), L-glutamate (L-Glu) and glycine (Gly) were tested as substrates. For the recombinant GNMT, Gly and D-Asp were tested. The activity was measured under standard enzyme assay conditions (Section 2.2), but the reaction mixture contained 333 mM D-Asp or another substrate. In addition, LC-MS/MS analysis of *N*-methyl amino acids produced by the enzymatic reaction was performed by monitoring the following fragment ion: *N*-methyl-D,L-aspartate, *m/z* 288 generated from the protonated ion at *m/z* 442; *N*-methyl-D,L-glutamate, *m/z* 280 generated from the protonated ion *m/z* 456; and *N*-methylglycine, *m/z* 277 generated from the protonated ion at *m/z* 384. For DDNMT, methylamine was also tested instead of SAM as the methyl donor.

The relative activity of DDNMT was determined with the activity using D-Asp and SAM as 100%. The relative activity of GNMT was determined with the activity using Gly and SAM as 100%.

2.8. Characterization of DDNMT

2.8.1. Molecular mass determination

The native molecular mass was determined by gel filtration on a Superdex 200 HR column (1.0 × 30 cm) with chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and aldolase (158 kDa) as the molecular mass marker proteins. The enzyme preparation with DDNMT activity obtained by RESOURCE Q column chromatography described in Section 2.4 was applied to the column and eluted with a 20 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl at a flow rate of 0.1 mL/min. Elution of the molecular mass marker proteins was monitored by absorption at 280 nm, and elution of DDNMT was monitored by activity.

2.8.2. Effect of pH

Enzyme activity was measured at various pH values under the same conditions as the standard enzyme assay, except that 20 mM potassium phosphate buffer (pH 5.0–7.0) or 20 mM Tris-HCl buffer (pH 7.0–10.0) was used as the reaction buffer.

2.8.3. Effect of temperature

Enzyme activity was measured at various temperatures (10, 20, 30, 37, 40 and 45 °C) using a standard enzyme assay.

2.8.4. Determination of the Michaelis–Menten constant (K_m)

The enzyme preparation used for determining the K_m of SAM was obtained by RESOURCE Q chromatography given in Table 1. The enzyme preparation used to determine the K_m for D-Asp was newly purified through all steps given in Table 1. Enzyme activity was measured under the same conditions as the standard enzyme assays, except that the concentration of D-Asp or SAM was varied. For determining the K_m toward D-Asp, enzyme activity was measured using seven concentrations of D-Asp over the range of 1.5–94 mM and 30 mM SAM. For determining the K_m toward SAM, enzyme activity was measured using 333 mM D-Asp and five concentrations of SAM over the range of 2.5–40 mM.

Computer programs using the methods of Wilkinson [19] were used to obtain statistical estimations of enzyme kinetics, and the values obtained are given as the mean ± standard error.

2.9. Assay for D-aspartate oxidase

D-Aspartate oxidase activity was measured as reported previously [20].

3. Results and discussion

3.1. Tissue distribution of DDNMT activity

The measurement of D-aspartate *N*-methyltransferase activity was performed with minor modifications to our previous method [16]. Although the C18 column length was shortened to reduce the analysis time, good separation between NMDA, NMLA and theanine (internal standard) was achieved (Fig. S1), and the use of an internal standard achieved the same linearity ($r^2 > 0.999$) for the calibration curve of NMDA created using the same concentration range (0.25–25 μM)

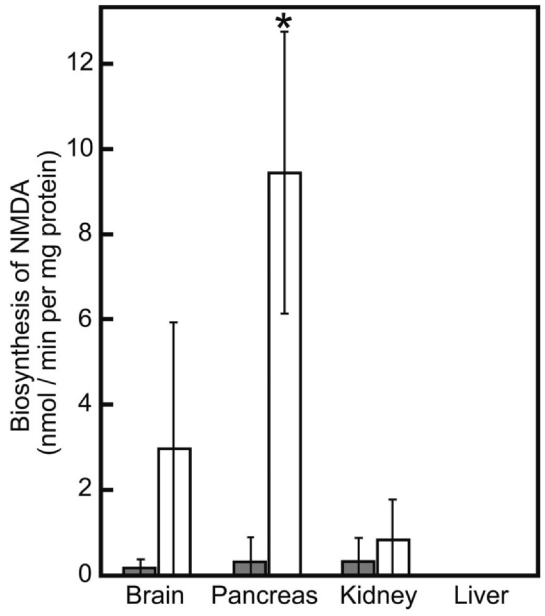

Fig. 1. Specific activity before and after ammonium sulfate fractionation of mouse tissue homogenates. The activities were measured before (black bar) and after (white bar) ammonium sulfate fractionation (10–20% saturation) of a homogenate prepared from the tissues of a 4-week-old female mouse using the standard enzyme assay. The specific activities in each tissue are shown as the mean ± standard deviation ($n = 3$). The asterisk indicates a significant difference ($p < 0.05$) before and after ammonium sulfate fractionation using the paired-sample *t*-test.

reported previously.

The DDNMT activity from various tissues of female mice before and after ammonium sulfate fractionation was determined (Fig. 1). In the tissue homogenate before the ammonium sulfate fraction prepared from each of the three individuals, enzyme activity was detected in all brain samples and not detected in any liver samples. In the kidney and pancreas, there were individuals in which activity was detected and individuals where activity was not detected. After ammonium sulfate fractionation (10–20% saturation) of the tissue homogenate, activity was detected in the pancreas of all individuals, and the specific activity increased about 30-fold ($p < 0.05$). In brain samples, the specific activity increased 16-fold. Effects from the ammonium sulfate fractionation on specific activity were not observed for kidney samples, and no activity was detected in all liver samples even after ammonium sulfate fractionation.

The observation that the activity cannot be detected with the homogenate, but the activity can be detected in the fraction partially purified by ammonium sulfate fractionation, suggests the presence of a DDNMT inhibitor and/or an enzyme that reduces NMDA produced by DDNMT in mouse tissues. D-aspartate oxidase (DDO, EC 1.4.3.1), which catalyzes the oxidative deamination of acidic D-amino acids, has long been reported to exist in various biological tissues including mice [20]. Interestingly, mammalian DDO showed similar activity toward NMDA as D-Asp, a physiological substrate [21–25]. Additionally, DDO-deficient mice showed a marked increase in NMDA levels compared with wild-type mice [12]. When DDO activity was measured in the ammonium sulfate fractionation step of the mouse pancreas in the present study, about 20% of the DDO activity was present in the fraction (10–20% saturation) containing DDNMT, but about 80% of the DDO activity was present in a different fraction (Fig. S2). Therefore, separation of DDNMT activity and DDO activity by ammonium sulfate fractionation may be one of the reasons why DDNMT activity can be detected in many individuals. However, the reason that no effect of ammonium sulfate fractionation on specific activity was observed in the kidney samples is probably that there was 100-fold more DDO activity in the kidney than in the pancreas, the DDO continued to affect DDNMT activity after ammonium sulfate fractionation (10%–20% saturation).

The DDO activity in the mouse liver in this study was less than half that of the kidney (data not shown). The absence of DDNMT activity after ammonium sulfate fractionation (10%–20% saturation) in the liver cannot be explained by DDO activity alone, but may be because of inhibition by enzymes other than DDO and/or lower expression levels of DDNMT in the liver.

D'aniello et al. reported that DDNMT activity in rat tissues showed similar values in total brain and liver and was not detected in kidney [10,11]. Differences in the distribution of DDNMT activity in rats and mice may also be affected by differences in DDO activity in both animals.

No significant gender difference was observed for DDNMT activity after ammonium sulfate fractionation of pancreas and brain samples (data not shown).

3.2. Purification of DDNMT from the pancreas of mice

In the brain and pancreas, the specific activity increased more than 10-fold after ammonium sulfate fractionation. These tissues are suitable raw sources for enzyme purification. In the present study, we attempted to purify the enzyme from the pancreas because samples taken from this tissue following ammonium sulfate fractionation showed the highest specific activity.

Using 80 mice pancreases as starting material, purification was performed using ammonium sulfate fractionation and column chromatography with phenyl TOYPEARL, Blue Sepharose and RESOURCE Q (Table 1). The homogenate showed no DDNMT activity but the fraction obtained by ammonium sulfate fractionation (10–20% saturation) showed activity. The enzyme was expected to have high hydrophobicity because of the observed precipitation at 20% ammonium sulfate. Thus, hydrophobic interaction chromatography using Phenyl TOYPEARL was employed. DDNMT activity was detected in fractions eluted at 1.0 M NaCl.

The adenosyl part of SAM, which acts as the methyl donor of DDNMT, is similar to the adenine part of the nicotinamide cofactor. Therefore, adsorption of DDNMT to Blue Sepharose (GE Healthcare) was expected. In affinity chromatography using a HiTrap Blue column packed with Blue Sepharose, DDNMT activity was detected in the adsorbed fraction.

The specific activity of the final active preparation obtained by RESOURCE Q chromatography increased by ~1900-fold when compared with that of the enzyme preparation after ammonium sulfate fractionation. Gel filtration chromatography loaded with the final active preparation showed a single peak of activity (Fig. S3); however, homogeneity using SDS-PAGE could not be confirmed by the small amount of protein purified in the present study.

The lack of confirmation of homogeneity of the purified enzyme did not completely eliminate the possibility that NMDA production from D-Asp involves several enzymes rather than a single one. However, the observation that the final preparation had high specific activity following purification via multiple steps and that a single sharp activity peak was obtained by gel filtration chromatography indicates that NMDA production from D-Asp was catalyzed by a single enzyme.

3.3. Substrate specificity

The substrate specificity of mouse DDNMT was examined using the final active preparation obtained by RESOURCE Q chromatography (Table 2). Mouse DDNMT catalyzes conversion of D-Asp to NMDA with high activity. *N*-methyl-D-glutamate (NMDG) was also produced when D-Glu was used instead of D-Asp as the substrate. DDNMT did not act on the L form of Asp or Glu, demonstrating that DDNMT has enantioselectivity for D-amino acids. NMDA was not produced from D-Asp when

Table 2
Substrate specificity of DDNMT from mouse and recombinant mouse GNMT.

	Substrate		Product	Relative activity (%)
Enzyme	Amino acid	Methyl group donor		
DDNMT[a]	D-Asp	SAM	NMDA	100
	L-Asp	SAM	NMLA	nd
	D-Glu	SAM	NMDG	31
	L-Glu	SAM	NMLG	nd
	Gly	SAM	NMGly	50
	D-Asp	Methylamine	NMDA	nd
	L-Asp	Methylamine	NMLA	nd
GNMT[b]	D-Asp	SAM	NMDA	nd
	Gly	SAM	NMGly	100

[a] The activity values of mouse DDNMT are shown as relative activities with 100% of the activity (2.8 μmol/min per mg) to produce NMDA from the substrates D-Asp and SAM. nd, not detected, i.e., below detection limits (5.4×10^{-4} μmol/min per mg for specific activity, 0.02% for relative activity).
[b] The activity values of recombinant mouse GNMT are shown as relative activities with 100% of the activity (6.2 μmol/min per mg) to produce NMGly from the substrates Gly and SAM. nd, not detected, i.e., below detection limits (3.9×10^{-4} μmol/min per mg for specific activity, 0.006% for relative activity).

methylamine was used instead of SAM as the methyl donor, showing that DDNMT is SAM-dependent. Methylamine-glutamate *N*-methyltransferase (EC 2.1.1.21, *N*-methyl-L-glutamate synthase) using methylamine as a methyl group donor and L-Glu as a substrate has been observed in bacteria [26]. DDNMT, which has enantioselectivity and SAM dependence, is clearly distinguishable from this bacterial enzyme.

The final purified DDNMT preparation also catalyzed Gly conversion to *N*-methylglycine (NMGly). GNMT (EC 2.1.1.20), which catalyzes the reaction to form NMGly and *S*-adenosylhomocysteine (SAH) from Gly and SAM, is present in various tissues including mammalian pancreas [27]. GNMT may catalyze the *N*-methylation of D-Asp but this has not been investigated. Therefore, we examined the substrate specificity of recombinant mouse GNMT prepared according to a previous report [17]. No catalytic conversion of D-Asp to NMDA was observed by recombinant GNMT (Table 2).

3.4. Properties of DDNMT

Other properties of DDNMT, which showed different substrate specificity when compared with that of GNMT, were investigated using the partially purified enzyme preparation. The native molecular mass of DDNMT was estimated to be approximately 78 kDa using Superdex 200 HR column chromatography (Fig. S3). In contrast, the molecular mass of mouse GNMT is reported to be 130 kDa, consisting of four subunits with subunit molecular masses of 33 kDa [17]. These differences in molecular mass seem to suggest that the molecular entities of DDNMT and GNMT are different.

The effect of pH and temperature on DDNMT activity was determined using the partially purified enzyme preparation. The optimal pH for activity was around pH 8 and the optimal temperature was ~35 °C (Fig. S4). The optimal pH of mammalian GNMT was 9 [27] and no optimal temperature was reported. Thus, the enzymes are distinguishable by their pH optima.

The effect of D-Asp and SAM concentration on mouse DDNMT was investigated. DDNMT appeared to exhibit a Michaelis-Menten behavior over the concentration range of 1.5–94 mM for D-Asp and 2.5–40 mM for SAM (Figs. S5 and S6). The K_m values for D-Asp and SAM were 33.3 ± 2.6 mM and 35.0 ± 8.5 mM, respectively. In crude extracts prepared from starfish nerve, DDNMT activity shows a similar K_m for D-Asp (75 ± 28 mM) [8]. In contrast, rat GNMT shows Michaelis-Menten-type kinetics for Gly and S-shaped kinetics for SAM [28]. In mammalian GNMTs, the K_m for Gly and SAM are reported to be 2–20 mM and 0.03–0.2 mM, respectively [27]. The K_m for SAM of DDNMT was 100–1000 times greater than that of GNMT. The role of GNMT is to regulate the ratio of SAM/SAH in tissues, rather than the production of NMGly, which has no known essential metabolic functions [27]. Thus, GNMT activity with a lower K_m for SAM will be more sensitive to changes in SAM concentration than DDNMT. The larger K_m of DDNMT for SAM indicates that the role of DDNMT does not involve regulation of the SAM concentration.

Enhancement of postnatal DDO activity and D-Asp concentration decreases have been observed in the brains of humans [3,23], rats [3,23] and mice [29]. The D-Asp concentration in the brain is about 1/100 of the K_m value of mouse DDNMT, and at such a D-Asp concentration, DDNMT activity will be proportional to the D-Asp concentration. Therefore, the approximately 10-fold higher embryonic and perinatal D-Asp concentrations, compared with the concentrations in adults [3], would be expected to affect NMDA biosynthesis via DDNMT. There are no reports on the temporal profile of the levels of NMDA or DDNMT activity in different sexes and organs, or on any correlation between the concentration and activity, so similar analyses to previous studies [23,29–31] of the D-Asp levels and DDO activity will be required in the future.

4. Conclusions

The present study revealed the substrate specificity and other properties of DDNMT, which was highly purified from the mouse pancreas through various purification steps. The results suggested that DDNMT is a novel enzyme with SAM dependency and specificity toward D-Asp, which differs from known methylamine-glutamate *N*-methyltransferases (EC 2.1.1.21) and GNMT (EC 2.1.1.20).

In the pancreas, D-Asp is present in all islet cells, suggesting that it is involved in the intercellular or intracellular glutamate-signaling pathway in islets [32,33]. In recent years, it has been reported that the NMDA receptor is also present in the pancreas and is involved in regulating insulin secretion from pancreatic β-cells [34]. NMDA receptors have been suggested to play an important role as a target for treating diabetes [34]. The effects of selective synthetic agonists of the NMDA receptor on insulin secretion from islets of Langerhans and β-cells have also been investigated, and synthetic NMDA was found to increase insulin secretion [35]. However, the role and function of the NMDA receptor has not yet been elucidated in the pancreas, which contrasts the vast body of research examining the brain. The present study demonstrated for the first time the presence of mouse DDNMT, a pancreatic enzyme that can specifically act on D-Asp to produce NMDA, a potent agonist of the NMDA receptor. The pancreas also contains DDO, which can degrade NMDA [12]. In the future, it will be necessary to elucidate the physiological mechanism of endogenous NMDA, including the metabolic pathway of NMDA.

In mammals, the presence of DDNMT has also been reported in crude extracts from rat tissues [10,11]. However, the substrate specificity of rat DDNMT has not been investigated because the DDNMT activity assay used in previous studies cannot detect *N*-methyl amino acids other than NMDA. In the present study, an activity measurement method based on LC-ESI-MS/MS that can separate various *N*-methyl amino acids was used to reveal the substrate specificity of mouse DDNMT. Furthermore, unprecedented attempts to purify DDNMT from mice in the present study have clearly shown the presence of a molecular entity with NMDA-producing activity and a molecular mass of approximately 78 kDa. The acquisition of the DDNMT gene offers a variety of approaches to elucidate the detailed properties of DDNMT and the metabolism and physiological roles of NMDA. Thus, attempts to obtain the amino acid sequence of DDNMT from mammalian tissues through large-scale purification, and to search for the gene of DDNMT in mouse genome information will be performed. The results of the present study will underpin such attempts.

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Declaration of Competing Interest

The authors have no potential conflict of interest.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbapap.2020.140527.

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