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D-Serine Dehydratase from Chicken Kidney: A Vertebral Homologue of the Cryptic Enzyme from *Burkholderia cepacia*

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D-Serine dehydratase (DSD) catalyses the conversion of D-serine to pyruvate and ammonia. D-Serine is a physiological modulator of glutamate neurotransmission in vertebrate brains. In mammals D-serine is degraded by D-amino-acid oxidase, whereas in chicken brain it is degraded by DSD, as we have recently demonstrated [Tanaka *et al.* (2007) *Anal. Biochem.* 362, 83–88]. To clarify the roles of DSD in avian species, we purified DSD from chicken kidney. The purified enzyme was a heterodimer consisting of subunits separable by SDS–PAGE but with identical N-terminal amino acid sequences. The prominent absorption at 416 nm and the inhibition of the enzyme both by hydroxylamine and by aminooxyacetate suggested that the enzyme contains pyridoxal 5′-phosphate as a cofactor. The enzyme showed the highest specificity to D-serine: the k_{cat}/K_m values of DSD for D-serine, D-threonine and L-serine were 6.19×10^3, 164 and 16 $M^{-1}s^{-1}$, respectively. DSD was found immunohistochemically in the proximal tubules of the chicken kidney. Judging from the amino acid sequence deduced from the cDNA, chicken DSD is a homologue of cryptic DSD from *Burkholderia cepacia* and low-specificity D-threonine aldolase from *Arthrobacter* sp. strain DK-38, all of which have a cofactor binding motif of PHXK(T/A) in their N-terminal portions.

Key words: chicken kidney, D-serine, D-serine dehydratase, pyridoxal 5′-phosphate, proximal tubules.

Abbreviations: DAO, D-amino acid oxidase; NMDA, *N*-methyl-D-aspartate; DSD, D-serine dehydratase; PLP, pyridoxal 5′-phosphate; DTT, dithiothreitol; KPi, potassium phosphate; PBST, phosphate-buffered saline containing 0.1% Tween 20; GC-LALLS, low-angle laser light scattering measurement combined with gel chromatography; EST, expression sequence tag; RACE, rapid amplification of cDNA ends; PBS, phosphate-buffered saline.

D-Serine exists at high levels in distinctive regions of mammalian brains (*1–3*) where serine racemase is present (*4*), and is scarcely found in other brain regions where D-amino acid oxidase (DAO; EC 1.4.3.3) is present (*5–8*). Serine racemase is a dual-function enzyme that synthesizes D/L-serine from L/D-serine and also degrades L- and D-serine into pyruvate and ammonia (*9–12*). DAO degrades D-serine into 3-hydroxypyruvate, ammonia and H_2O_2.

N-Methyl-D-aspartate (NMDA) receptors play critical roles in excitatory glutamatergic synaptic transmission, and both hyper- and hypo-functions of these receptors are believed to cause serious dysfunction of mammalian brains (for recent reviews see *13–18*). NMDA receptors have a coagonist-binding site, which is distinct from the binding site for glutamate (*19*). D-Serine is one of the major physiological coagonists of NMDA receptors and the activity of the receptors is finely tuned by D-serine in various brain regions. Therefore, the synthesis, transport and degradation of D-serine need to be a concerted action.

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As with mammalian brains, non-mammalian vertebrate brains contain D-serine at substantial levels (*2*). Importantly, many non-mammalian vertebrates such as birds, reptiles and fishes have two D-serine-degrading enzymes, D-serine dehydratase (DSD; EC. 4.3.1.18) and DAO (*20*, *21* and our unpublished results). DSD catalyses the dehydration (α,β-elimination) of D-serine to aminoacrylate, which is rapidly hydrolysed to pyruvate and ammonia. Why these non-mammalian vertebrates need two D-serine-degrading activities remains unclear. Recently, using a simultaneous assay method for these D-serine-degrading enzyme activities (*21*), we have found that in chicken-brain homogenates D-serine is predominantly degraded by DSD, whereas in chicken kidney both DSD and DAO activities exist. These findings have important implications for understanding of the functional roles of D-serine in vertebrate brains including mammalian brains.

In this study, we purified and characterized DSD from chicken kidney, and also cloned the cDNA. This is the first pyridoxal-5′-phosphate (PLP)-dependent DSD purified from a eukaryote. Intriguingly, judging from its amino acid sequence, chicken DSD is distinct from the DSD protein coded by the *dsdA* gene (*22*), which most eubacteria produce when D-serine is available as an energy source. Chicken DSD showed a weak but

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significant homology in the primary structure of cryptic DSD from *Burkholderia cepacia* (*23*) and low-specificity D-threonine aldolase from *Arthrobacter* sp. strain DK-38 (*24*). We produced a polyclonal antibody for the purified enzyme. Immunohistochemical staining with this antibody revealed that DSD is produced in the proximal tubules of the chicken kidney.

EXPERIMENTAL PROCEDURES

Materials—Kidneys from adult male White Leghorn chickens were obtained from a local slaughter house and kept at about −35°C until use. D-Serine, L-serine, D-threonine, L-threonine, PLP, dithiothreitol (DTT), hydroxylamine, aminooxyacetate, 2,4-dinitrophenylhydrazine, cyanogen bromide, FAD and sodium pyruvate were all purchased from Wako Pure Chemical Industries (Osaka, Japan). 3-Methyl-2-benzothiazolinone hydrazone hydrochloride was obtained from Aldrich and bovine catalase was from Boehringer–Mannheim. All other chemicals were of analytical grade.

Assay of D-Serine Dehydratase—The 2,4-dinitrophenylhydrazine method (*25*) modified by us (*26*) was used to measure pyruvate production from D-serine. A standard assay mixture (200 μL) contained 50 mM potassium phosphate (KPi), pH 7.8, 50 mM D-serine and 10 μM PLP. The reaction was initiated by the addition of 5 μL of enzyme solution. After incubation at 37°C for 10 min, the reaction was stopped by the addition of 80 μL of 12.5% trichloroacetic acid, and then the resultant insoluble materials were removed by centrifugation. An aliquot of the supernatant (200 μL) was transferred to a microfuge tube and mixed with 40 μL of 0.1% 2,4-dinitrophenylhydrazine (2 M HCl solution). The mixture was incubated at room temperature for 10 min and then neutralized with the addition of 160 μL of 3.75 M NaOH. The absorbance at 450 nm was measured using a microplate reader (Titertek Multiscan MKII, Flow Lab. Inc., Mclean, VA, USA). A calibration curve was prepared by treating reaction mixtures containing known amounts of pyruvate (0–50 nmol) with 2,4-dinitrophenylhydrazine as described earlier without the addition of enzyme solutions. One unit of DSD activity was defined as the amount of enzyme producing 1 μmol of pyruvate per min under the standard assay conditions.

Kinetic data was obtained at 37°C using 50 mM KPi, pH 7.8, containing varying amounts of D-serine (0–10 mM), D-threonine (0–50 mM), L-serine (0–100 mM) or L-threonine (0–100 mM). The effects of pH on the activity of purified DSD were examined at 37°C and at an ionic strength of 0.15 M (adjusted with NaCl) using the buffer solutions containing 50 mM D-serine and 0.05 M KPi (pH 5.5–8.0) or 0.05 M Tris–HCl (pH 8.0–11.2).

The enzymatic production of pyruvate was confirmed both by the lactate dehyrogenase method (*11*) and by our HPLC method (*21*). The presence or absence of serine racemase activity in the purified DSD was examined using the method reported by Hashimoto *et al*. (*27*).

Protein Assay—Protein contents were determined by the Bradford method using a Bio-Rad protein assay dye solution using bovine serum albumin as a standard (*28*). The subunit concentrations of the purified DSD samples were calculated using the subunit molecular weight of 40,398.

Purification of D-Serine Dehydratase—Unless otherwise stated, all of the procedures were conducted at about 4°C and all buffers contained 1 mM DTT. HPLC was performed at room temperature. Chicken kidneys (360 g) were homogenized in 3 vol. of 10 mM KPi (pH 7.2) containing 50 μM PLP using a blender. After 10-min incubation on ice, the homogenates were centrifuged at 17,700 × *g* for 20 min. The resultant supernatant was diluted 2-fold with cold 10 mM KPi, pH 7.2 (crude extract). The crude extract was applied to a DEAE-Sepharose column (5 × 18.5 cm, Pharmacia) pre-equilibrated with 10 mM KPi, pH 7.2. The column was washed with 600 mL of the equilibration buffer, and then the bound proteins were eluted with 800 mL of 50 mM KPi, pH 7.2. The active fractions were pooled (DEAE-Sepharose), and brought to 30% ammonium sulfate saturation with continuous stirring. After 30-min incubation, the solution was centrifuged at 10,000 × *g* for 20 min to remove precipitates. The supernatant was increased to 40% ammonium sulfate saturation and incubated for 30 min. The precipitates were collected by centrifugation at 10,000 × *g* for 20 min, and resuspended in 6 mL of 10 mM KPi, pH 7.2, containing 50 μM PLP. The suspension was dialysed twice against 2 L of 1.0 mM KPi, pH 7.2, containing 50 μM PLP. After about 20 h dialysis, insoluble materials were removed by centrifugation to give a clear enzyme solution (ammonium sulfate). The enzyme solution was applied to a hydroxyapatite column (1.5 × 8 cm, Nacalai Tesque, Kyoto) pre-equilibrated with 1.0 mM KPi, pH 7.2, containing 50 μM PLP. After washing the column with 30 mL of the equilibration buffer, the enzyme was eluted with 5 mM KPi, pH 7.2, containing 50 μM PLP. The active fractions were pooled (hydroxyapatite). The hydroxyapatite fraction was applied to a Mono Q HR5/5 column (Pharmacia) pre-equilibrated with 10 mM KPi, pH 7.2. The column was washed with 3 mL of the equilibration buffer, and then the enzyme was eluted with a linear gradient of KPi buffer, pH 7.2 (10–100 mM/15 mL) at a flow rate of 0.5 mL/min. The active fractions were pooled (Mono Q), and concentrated to about 0.1 mL using an Amicon Ultra filter (30,000 MWCO). An aliquot of the concentrated Mono Q fraction (20 μL) was applied to a TSKgel SuperSW3000 column (4.6 × 300 mm, Tosoh) pre-equilibrated with 20 mM KPi, pH 7.2, containing 50 μM PLP. The column was developed with the same buffer at a flow rate of 0.2 mL/min. This gel filtration step was repeated until all the concentrated Mono Q fraction was applied to the column, and all the active fractions were pooled (SuperSW3000). Using an Amicon Microcon YM-30 filter, the pooled fractions were concentrated to about 0.1 mL and stored at −30°C.

SDS–Polyacrylamide Gel Electrophoresis—SDS–PAGE was performed as described by Laemmli (*29*) using a 12.5% gel. Proteins in the gel were stained with a Coomassie Brilliant Blue staining kit (Wako Pure Chemical Industries). For amino acid sequencing and western blot analysis, the separated proteins were

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transferred onto a PVDF membrane (Clear Blot Membrane-P, ATTO) using a semi-dry blotter (AE-6677-S, ATTO).

Western Blot Analysis—Tissues (1 g each) were homogenized in 3 vol. of 10 mM KPi (pH 7.2) containing 50 μM PLP using a glass homogenizer. Aliquots of the homogenates (20 μg protein) were loaded and run on a 12.5% SDS–PAGE gel, and the separated proteins were transferred onto a PVDF membrane. The membrane was blocked in 5% (w/v) fat-free milk in phosphate-buffered saline containing 0.1% Tween 20 (PBST). Anti-DSD polyclonal antibody (see later) was diluted 1:10,000 in 5% fat-free milk in PBST and incubated with the membrane for 2 h at room temperature. The membrane was washed four times with PBST. Horseradish peroxidase conjugated-secondary antibody (goat anti-rabbit IgG, Amersham, Buckinghamshire, UK) was diluted 1:5,000 in 5% fat-free milk in PBST and incubated with the membrane at room temperature for 1 h. The immunoreactive bands were detected with an enhanced chemiluminescence detection system (Perkin Elmer Life Science, Boston, MA).

Amino Acid Sequence Determination—Purified DSD and the peptides produced from the enzyme by in-gel treatment with trypsin (*30*) or cyanogen bromide (*31*) were analysed for N-terminal amino acid sequences on an Applied Biosystems model 477A gas-phase sequencer. The tryptic peptides extracted from the gels were separated by HPLC on a 3C18 column (2 × 100 mm, Tosoh). The cyanogen-bromide cleaved peptides were separated by SDS–PAGE, and then electro-transferred onto a PVDF membrane.

Molecular-Weight Determination—The molecular weight of native DSD was determined by low-angle laser light scattering measurement combined with gel chromatography (GC-LALLS) (*32*) as described previously (*26*) using a TSKgel SuperSW3000 column (4.6 × 300 mm). The molecular-weight standards used were bovine ribonuclease A (M_r = 13,700) ovalbumin monomer (M_r = 45,000), bovine albumin monomer (M_r = 66,300), and catechol 2,3-dioxygenase (*Pseudomonas putida*, homotetramer of M_r = 140,624); the first two proteins were from Sigma and the third was purified by the reported method (*33*).

The subunit molecular weight of DSD was determined by matrix-assisted laser desorption mass spectrometry using a Voyager-DE RP mass spectrometer (PerSeptive Biosystems) and α-cyano-4-hydroxycinnamic acid as a matrix-forming material. Catechol 2,3-dioxygenase (*Pseudomonas putida*, subunit molecular weight of 35,156) was used for mass calibration.

Cloning of Chicken DSD cDNA—Using the *tblastn* program (*34*), the amino acid sequences determined for the peptide fragments of the purified chicken DSD (see the sequences highlighted with bold letters in Fig. 6) were compared to the chicken expressed sequence tags (ESTs) database. When the peptide sequence RERCRALGVRLRPHVKTHKTLEGG was used as a query, one EST (BX929724) consisting of 832 bp was found to encode an identical sequence. In addition to the query sequence, this EST contained a start codon, and the deduced N-terminal amino acid sequence was identical to that determined for the purified enzyme (MWLGALLDTLPTPA). Therefore, on the basis of the nucleotide sequence of this EST, we designed the following primer set: forward primer, 5′-ATGTGGTTG GGTGCCCTCCTGGACACG-3′; reverse primer, 5′-GTTG GCGATGGCCTGGGCCAGCTCCAG-3′. Total RNA was extracted from chicken kidney using an RNeasy Midi kit (Qiagen). The first strand cDNA was prepared from the total RNA using Super Script III First Strand Synthesis System (Invitrogen). The initial 5′- region of the DSD gene was amplified from the cDNA by PCR using a Platinum *pfx* DNA polymerase kit (Invitrogen) and the primer set described earlier. The PCR conditions were as follows: denaturation at 94°C for 15 s; 35 cycles of 94°C for 15 s, 58°C for 30 s and 72°C for 60 s. The PCR product was cloned into pCR4-TOPO vector (Invitrogen) and sequenced using an ABI Prism 310 DNA Sequencer (Applied Biosystems) and a DYEnamic ET terminator kit (Amersham). The nucleotide sequence of the PCR product encoded all three peptide sequences of the purified DSD (Fig. 6). The complete DSD gene was determined by 3′-rapid amplification of cDNA ends (3′-RACE) using a Gene Racer kit (Invitrogen). The primer set for the primary PCR was 5′-AGAGCCGGCGT GCGCCCGACG-3′ and a GeneRacer 3′ Primer, and that for the second PCR was 5′-GCCCTGGAGCTGGCCCAGG CCATC-3′ and Gene Racer 3′ nested Primer. The nucleotide sequence of the PCR product was determined as described earlier.

Analysis of Kinetic Data—DSD showed Michaelis–Menten kinetics for all substrates used. Therefore, we analysed the kinetic data using a [S]/*v* versus [S] plot (Hanes–Woolf plot, *26*), where *v* is the initial velocity and [S] is the substrate concentration. The turnover number (k_{cat}) was calculated by dividing the maximal velocity (V_{max}) by the subunit concentration of the enzyme supposing that all the subunits contained PLP in their active sites, namely being in holo-form.

Polyclonal Antibody Preparation—A polyclonal antibody against purified chicken DSD was produced in a rabbit by KYA Co. (Kyoto, Japan) using the manufacturer's standard methods. Briefly, primary immunization was carried out by subcutaneous injection with the purified DSD (1 mg) in Freund's complete adjuvant emulsion. Booster immunizations were repeated every 2 weeks using Freund's incomplete adjuvant. After the third booster immunization, the rabbit was euthanized by exsanguination under general anaesthesia and the blood was collected. Anti-chicken DSD rabbit IgG was purified from the rabbit serum using a protein A-Sepharose column (Pharmacia) according to the manufacturer's instructions, and stored at −20°C until use. The protein concentration of the purified antibody solution was 3.3 mg/mL.

Immunohistochemistry—Chicken kidney was fixed with 10% formalin for 6 h and then embedded in paraffin. Each tissue section (3 μm) was mounted on a silane-coated glass slide, deparaffinazed in xylene, rehydrated in graded alcohols, and washed with phosphate-buffered saline (PBS). In the following treatments, exhaustive washing of the tissue sections with PBS was always performed between the previous treatment and the next. The mounted sections were first incubated in 0.01 M

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citrate (pH 7.0) at 120°C for 10 min using an autoclave, and then treated with 3% (v/v) H_2O_2 in methanol for 20 min at room temperature. The resultant tissue sections were incubated with 1:1,000 diluted anti-DSD antibody in PBS for 2 h at room temperature. As a negative control, the tissue sections were also incubated in PBS without the primary antibody. Following a 30-min incubation with a secondary antibody [Histofine Simple Stain MAX PO (MULTI), Nichirei Co., Tokyo, Japan] at room temperature, the immunoreactivity was visualized with a 3,3′-diaminobenzidine solution (DAB) (Nichirei Co., Tokyo, Japan). Finally, the sections were counterstained with haematoxylin.

RESULTS

DSD Purification—The results of a typical purification procedure are shown in Table 1. The DEAE-Sepharose chromatography step resulted in 24-fold purification with 56% yield. At the Mono Q chromatographic step, a slight inactivation of the enzyme occurred. This inactivation was probably due to the deprivation of the cofactor PLP from the enzyme, because we could not add PLP in the elution buffer due to its strong binding to the resin. The enzyme was reactivated at the final gel filtration step, in which the elution buffer contained PLP. Only a single active peak of DSD appeared during all of the chromatographic steps performed for the purification. The purified DSD had a specific activity of 1.39 μmol/min/mg protein, and was resolved into two protein bands (40,000 and 40,400 kDa) of equal abundance on SDS–PAGE (Fig. 1A). Both of the proteins had an identical amino terminal amino acid sequence of MWLGALLDTLPTPA. On the other hand, under the resolution power of the mass spectrometer used, only single peak centred at m/z value of 40,000 was obtained (data not shown). The molecular weight of the native enzyme was estimated to be 77,000 by GC-LALLS (Fig. 1B). These results suggest that native chicken DSD exists as an αα′-type heterodimer. However, since the corresponding cDNA was homogeneous (see later), it remains to be determined whether this heterogeneity shown by the purified enzyme is physiological or artificial. The enzyme was stable with no loss of activity when stored at −20°C in the presence of 50 μM PLP for at least 2 weeks.

The absorption spectrum of the purified DSD (Fig. 2) showed maxima at 280 and 416 nm (A_{280}/A_{416} ratio of 10.2), giving the enzyme a characteristic yellow colour. The absorption peak around 416 nm suggested that chicken DSD contains PLP as a cofactor. In fact, hydroxylamine and aminooxyacetate, which inactivate PLP, inhibited the enzyme activity (Fig. 3). The content of PLP per DSD subunit (mol/mol) was estimated to be about 0.4–0.5 by supposing a molar extinction

Table 1. **Purification of D-serine dehydratase from chicken kidney (360 g).**

Purification step	Total volume (mL)	Total activity (U)	Protein content (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	2,350	133	32,850	0.0040	100	1
DEAE-Sepharose	218	74	785	0.094	55.7	24
Ammonium sulfate	7.5	61.5	118	0.52	46.2	130
Hydroxyapatite	16.0	13.6	12.5	1.09	10.2	273
Mono Q	1.4	3.0	3.3	0.91	2.3	228
SuperSW3000	0.72	2.5	1.8	1.39	1.9	348

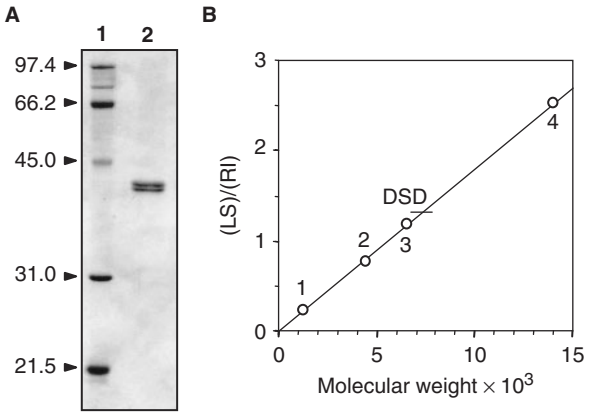

Fig. 1. **SDS–PAGE and GC-LALLS analyses of DSD purified from chicken kidney.** (A) SDS–PAGE was performed using a 12.5% gel. Proteins ware stained with Coomassie Brilliant Blue. Lane 1, molecular mass markers. The respective molecular masses (kDa) are shown on the left side. Lane 2, pooled fractions after TSKgel Super SW3000 chromatography (2 μg purified protein). (B) Low-angle laser light scattering measurement combined with gel chromatography. The purified native enzyme (20 μg) was applied to a TSKgel SuperSW3000 column (4.6 × 300 mm) and the elution was detected by a low-angle laser light scattering photometer and a differential refractometer in that order. The ratio of the output of the light scattering photometer (LS) to that of the refractometer (RI) was plotted against molecular weight: molecular weight markers: 1, bovine ribonuclease A (M_r = 13,700); 2, ovalbumin monomer (M_r = 45,000); 3, bovine albumin monomer (M_r = 66,300); 4, catechol 2,3-dioxygenase (M_r = 140,624). The mean (LS)/(RI) value (1.32, n = 3) for the enzyme is shown as a horizontal bar.

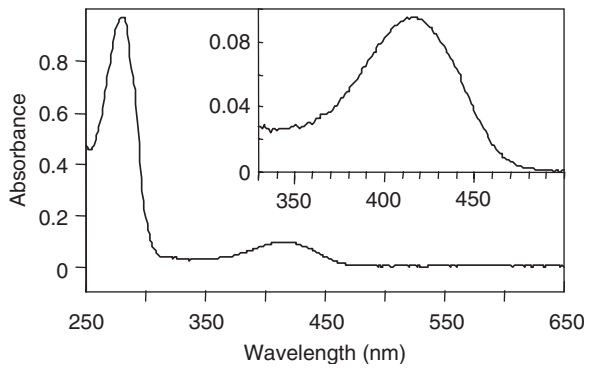

Fig. 2. **Absorption spectrum of chicken DSD.** The spectrum of DSD was measured in 20 mM KPi (pH 7.5) containing 1 mM DTT and 10 μM PLP using a PhamaSpec UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan). The DSD concentration was 1.2 mg/ml. The absorption of the buffer itself was subtracted from that of the DSD solution. The inset is an enlargement of the spectrum in the visible wavelength region.

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coefficient of 6–8 $mM^{-1}cm^{-1}$ at 416 nm for the bound PLP (*35*). We failed to quantify the enzyme-bound PLP according to the method described by Wada and Snell (*36*), partly due to the limited amounts of the purified enzyme.

Kinetic Properties of Chicken DSD—All the steady-state kinetics data for D-serine and alternative substrates fitted to the Michaelis–Menten equation with good quality (for D-serine, see the best-fit curve in Fig. 4). The K_m and k_{cat} values are summarized in Table 2. The K_m value for L-serine (12.3 mM) was about 100-fold higher than that for D-serine (0.13 mM). Chicken DSD showed no activity for L-threonine at concentrations as high as 100 mM, whereas the K_m value for D-threonine was 0.31 mM. These results suggested that the substrate binding site of chicken DSD is highly specific for the D-configuration (2*R* isomer). The k_{cat} values for D- and L-serine and D-threonine were 0.81, 0.20 and 0.051 s^{-1}, respectively. These results suggested that the size of the side chain group of the substrate strongly affects the dehydration step in the ES complex. The specificity constant (k_{cat}/K_m) for D-serine was $6.19 \times 10^3\ M^{-1}s^{-1}$, 38- and 390-fold higher than those for D-threonine and L-serine, respectively. We could not detect significant levels of serine racemase activity for the purified chicken DSD (data not shown).

Figure 5 shows the pH-dependence of DSD activity. The pH optimum was about 8.7. The bell-shaped dependence suggested that the enzyme requires at least two ionizable groups, one protonated and one deprotonated, for the catalytic reaction. The activities were negligible below pH 5.5 and the relatively high activities remained at pH higher than 11.

Nucleotide Sequence of the cDNA and Predicted Amino Acid Sequence—The nucleotide sequence of the DSD gene and the deduced amino acid sequence are presented in Fig. 6. The cDNA contains 1128 bp coding for a 376-amino-acid protein with the molecular weight of 40,398. This is in agreement with the molecular weight estimated by mass spectrometry within the experimental

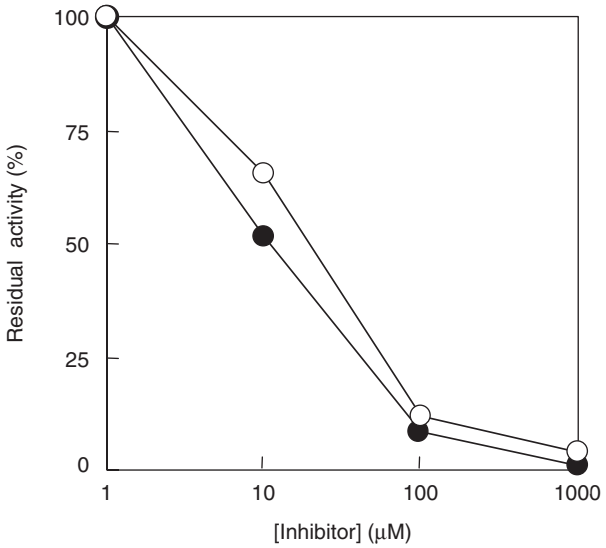

Fig. 3. **Inhibition of chicken DSD by hydroxylamine (open circle) and aminooxyacetate (filled circle).** The purified DSD (3.5 μg, 200 μL) was incubated at 37°C for 30 min in 50 mM KPi (pH 7.8) in the presence of the inhibitors. The residual enzyme activity was measured under standard assay conditions.

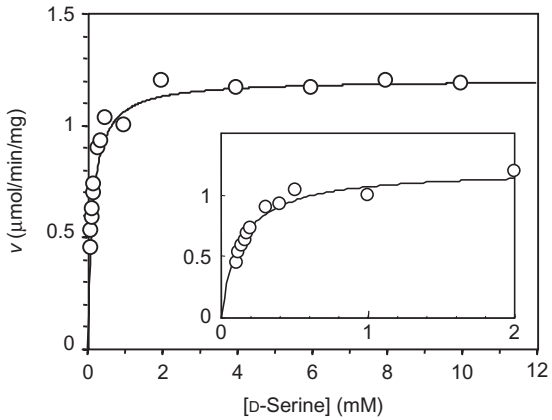

Fig. 4. **Dependence of the initial velocity of chicken DSD on the D-serine concentration.** The steady-state reactions were performed at 37°C in 50 mM KPi, pH 7.8. The final DSD subunit concentration was 186 nM. The lines drawn in the figures are made on the basis of the Michaelis–Menten equation using the corresponding best-fit values of K_m and k_{cat} listed in Table 2.

Table 2. **Kinetic parameters of chicken DSD for hydroxyamino acids.**

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
D-Serine	0.131 ± 0.017	0.811 ± 0.004	$(6.19 \pm 0.25) \times 10^3$
D-Threonine	0.312 ± 0.096	0.0512 ± 0.0002	$(1.64 \pm 0.50) \times 10^2$
L-Serine	12.3 ± 1.8	0.197 ± 0.006	16.0 ± 2.3
L-Threonine[a]	–	0.0	–

[a]The enzyme showed no activity up to 100 mM L-threonine.
The kinetic parameters for DSD were determined at 37°C with 50 mM KPi, pH 7.8. The data was fitted to the Michaelis-Menten equation and the best-fit values for the parameters are listed with the standard deviation.

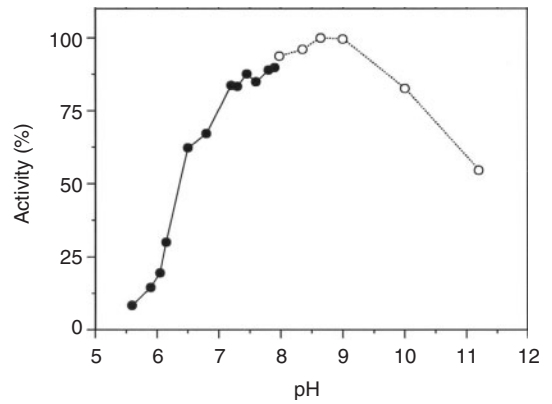

Fig. 5. **pH-Dependence of chicken DSD activity at 37°C.** The reaction mixtures (200 μL) contained 50 mM D-serine in the following buffers (ionic strength 0.15 M, adjusted with NaCl): 0.05 M KPi (pH 5.5–8.0, filled circle) and 0.05 M Tris–HCl (pH 8.0–11.2, open circle). The reaction was started by the addition of 1.5 μg of purified DSD.

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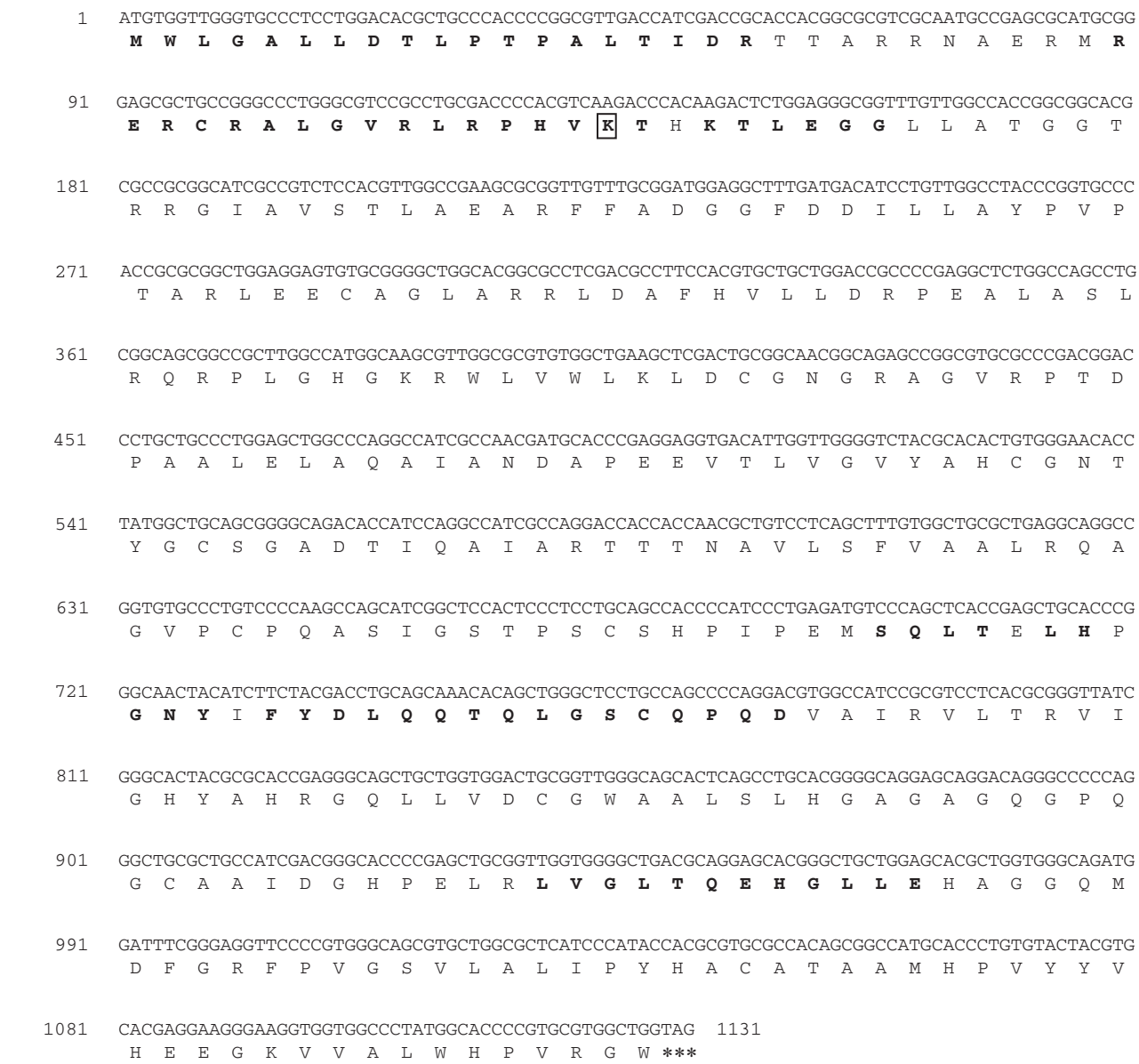

Fig. 6. **Nucleotide sequence of the chicken DSD cDNA and deduced amino acid sequence.** The asterisk shows a translational stop codon. The amino acid sequences in bold letters are those determined for the purified enzyme by the amino acid sequencing. Probable PLP-binding lysine (K45) is enclosed in a box.

error. All of the four peptide sequences of the purified enzyme determined by Edman degradation (shown in bold letters in Fig. 6) coincided with corresponding deduced peptide sequences.

The predicted amino acid sequence showed no similarity to those of inducible DSD (dsdA) from *Escherichia coli* (*22*) or mammalian L-serine dehydratase (EC. 4.3.1.17) (*37*). However, cryptic DSD from *Burkholderia cepacia* (GenBank U41162, 23), low-specificity D-threonine aldolase from *Arthrobacter* sp. strain DK-38 (GenBank, AB010956) (*24*), and a hypothetical alanine racemase-like protein (GenBank, YP_275478) showed weak but significant similarity in primary structure to chicken DSD (Fig. 7). Importantly, all of these proteins share a common sequence motif of PHXK(T/A) (X = G, A or V) in the N-terminal portion. In the case of the low-specificity D-threonine aldolase (*24*), the lysine residue in the motif is confirmed to be the binding site of PLP, suggesting that Lys45 of chicken DSD is the PLP-binding site.

Tissue Distribution of DSD—The tissue distribution of chicken DSD was analysed by western blot analysis (Fig. 8). We found the highest level of DSD expression in the kidney, followed by liver and cerebellum. The heart had no detectable levels of DSD expression. These results are consistent with the tissue distribution of DSD activity determined by our HPLC method (*21*).

Localization of DSD in the Chicken Kidney—The cellular localization of DSD in the chicken kidney was

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Fig. 7. **Comparison of the amino acid sequence of chicken DSD with those of other PLP-dependent enzymes.** The sequences were aligned using GENETYX-Mac version 11.2.0 (Software Development, Tokyo, Japan). The accession numbers of the primary structures of chicken DSD, cryptic DSD from *Burkholderia cepacis* (*B. cepacia* DSD), low-specificity D-threonine aldolase from *Arthrobacter* (LS DTD) and a hypothetical alanine racemase (Hypothetical Alr) are AB284370, U41162, AB010956 and YP_275478, respectively. The PLP-binding lysine residue is marked by an asterisk. The boxed amino acid sequences indicate a distinct PLP-binding motif shared by these enzymes.

analysed by immunohistochemistry. As shown in Fig. 9, only the epithelial cells in the proximal tubules expressed DSD, and other cells showed no significant levels of DSD.

DISCUSSION

Our recent finding that chicken brain homogenates contain substantial levels of DSD activity and no detectable levels of D-serine oxidase activity (*21*) prompted us to purify and characterize chicken DSD. This is the first report on vertebrate DSD. Intriguingly, chicken DSD is distinct from the ubiquitous bacterial enzyme coded by *dsdA* gene (*22*), whose enzymatic properties have been investigated in detail. The chicken DSD shows significant sequence similarity with the cryptic DSD first isolated from *Burkholderia cepacia* (GenBank U41162). These two enzymes have a PLP-binding motif of PHXK(T/A), which is also found for other PLP-dependent enzymes such as low-specificity D-threonine aldolase from *Arthrobacter* sp. Strain DK-38 and a hypothetical alanine racemase-like protein (GenBank YP_275478) (Fig. 7). Therefore, chicken and cryptic DSD together form a new DSD family. Enzymatic and biochemical properties of the cryptic DSD are poorly understood. The present study is the first characterization of DSD belonging to this family. The specificity constant of chicken DSD for D-serine is 390-fold higher than that for L-serine, indicating that the enzyme is highly specific for D-serine (Table 2).

The PLP-dependent enzymes are classified according to their fold type (*38*). The 'classical' *E. coli* D-serine

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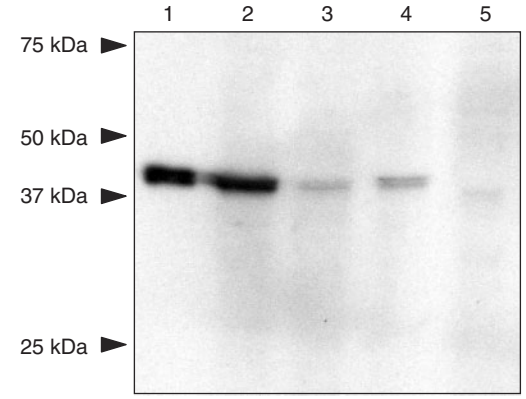

Fig. 8. **Tissue distribution of chicken DSD.** Tissue homogenates (20 μg protein) were subjected to western blot analysis using 12.5% SDS–PAGE gel and anti-chicken DSD antibody. Molecular sizes are indicated on the left in kDa. Lane 1, purified DSD (2 μg/lane); Lane 2, kidney; Lane 3, liver; Lane 4, cerebellum; Lane 5, heart.

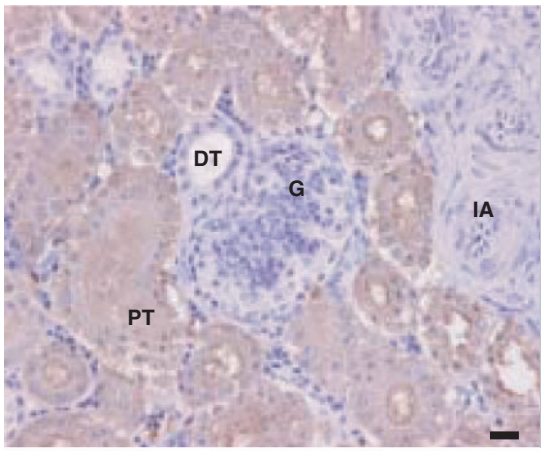

Fig. 9. **Cellular localization of DSD in the chicken kidney.** Chicken DSD in the kidney was visualized using anti-chicken DSD antibody. The kidney cells were also stained with haematoxylin. IA, intralobular artery; G, glomerulus; PT, proximal tubule; DT, distal tubule. The scale bar corresponds to 10 μm.

dehydratase (dsdA), mammalian L-serine dehydratase and mammalian serine racemase are fold type II enzymes, whereas bacterial alanine racemase, which is homologous to the cryptic D-serine dehydratase and low-specificity D-threonine aldolase, belongs to Fold type III family. Therefore, the vertebrate D-serine dehydratase reported here is expected to be a fold type III enzyme.

It is reported that mammalian serine racemases have L- and D-serine dehydratase activities in addition to their racemase activities (*9–12*). Both the racemase and the dehydratase activities of mammalian serine racemases are equally activated by ATP or divalent metal cations such as Mg^{2+} and Ca^{2+} (*11*). For example, the k_{cat}/K_m values of activated mouse serine racemase for L-serine racemization and for D-serine dehydration are 4.5 and 4.7 $M^{-1}s^{-1}$, respectively (*12*), the latter value being about 1300-fold lower than that of the chicken DSD elucidated here. We examined the effects of these cations and ATP on chicken DSD activity. Chicken DSD was not activated by these divalent ions or ATP, and also not inhibited by EDTA (data not shown). We also examined whether chicken DSD has serine racemase activity. The enzyme showed no detectable levels of serine racemase activity even when 10 mM D-serine or L-serine was used as a substrate. In addition, no alanine racemase or aspartate racemase activities were detectable for chicken DSD (data not shown). These results, together with the dissimilarity in the primary structure from mammalian serine racemase family enzymes, indicate that chicken DSD is entirely different from mammalian racemases.

Chicken kidney contains about 10-fold higher levels of DSD activity compared to liver and brain (*21*). Immunohistochemical examination revealed that DSD is produced exclusively in the proximal tubule epithelial cells. A similar distribution in the kidney is known for mammalian DAO (*39*). In some mammals including rat (*40*), D-serine has toxic effects on kidney proximal tubules. It is unknown whether D-serine is nephrotoxic in chickens. Physiological roles of DSD in the chicken kidney are still unclear.

In conclusion, we have carried out the first reported purification of a vertebrate DSD. The DSD forms a new subfamily of dehydratase together with cryptic DSD from *Burkholderia cepacia*. To examine the physiological roles of DSD, especially in the vertebrate brain, overexpression of the DSD in *E. coli*, further biochemical and immunohistochemical studies are under way.

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