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The presence of free D-amino acids in mouse tissues

Y. Nagata, T. Akino and K. Ohno[a]

Department of Biochemistry, Sapporo Medical College, and [a]Hokkaido Geriatric Institute, Sapporo 060 (Japan)
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Summary. The presence of free D-amino acids in mouse kidney, liver, brain, heart, lung, thymus and serum has been shown with an enzymic microdetermination method. The D-amino acid levels were higher in the extracts of kidney and liver than in those from other organs.
Key words. D-amino acid; D-amino acid oxidase; mouse tissue.

It is generally believed that mammals neither synthesize nor possess D-amino acids (DAA) whereas microorganisms, some insects, marine invertebrates and higher plants contain DAA[1]. Only D-Asp is known to exist in metabolically stable proteins[1] and tissues[2]. We have observed considerable amounts of free DAA in human plasma from patients with renal diseases[3], using an enzymic method[4] which detects neutral DAA. In the present study, the investigation was extended to mouse tissues.

Materials and methods. a) Reagents. D-amino acid oxidase (DAO, EC 1.4.3.3) from porcine kidney was obtained from Sigma (St. Louis, MO, USA) as a crystalline suspension in ammonium sulfate. It was freed of ammonium ions by passage through a Sephadex G-25 column equilibrated with 0.019 M sodium pyrophosphate buffer, pH 8.3 (PPB) resulting in solution of 0.3 mg/ml protein concentration. Catalase (EC 1.11.1.6), as a purified powder (10,000–25,000 units/mg protein) from bovine liver, was also purchased from Sigma. All other chemicals were analytical-grade products.

b) Preparation of tissue extracts. Normal, healthy infant (4-week-old), adult (19-week-old) and old (11-month-old) BALB/cA female mice were used. Each group consisted of 5 litters. The mice were kept on a normal diet and fasted 16–19 h prior to sacrifice by bleeding from the axillary vessels under anesthesia with ether. After rinsing with phosphate-buffered saline, pH 7.4 (PBS), to remove blood, the liver, kidney, brain, heart, lung and thymus were homogenized as whole organs, each with 4–5 vol. of PBS in a glass homogenizer in an ice bucket, at 1,000 rpm for 1 min. The homogenate was centrifuged at 160,000 × g for 15 min at 4 °C, and the supernatant extracts passed through a 'Centricut' ultramembrane filter (Kurabo, Osaka, Japan) to remove substances larger than 10,000 dalton by centrifuging at 5,000 × g for 1 h at 4 °C. The filtrate was used for the DAA assay.

c) DAA determination. DAO catalyzes the oxidation of DAA to produce α-keto acids; $R-CH(NH_2)-COOH + O_2 + H_2O \rightarrow R-CO-COOH + NH_3 + H_2O_2$. Details of the assay method are described elsewhere[4]. Briefly, three reaction tubes were prepared as follows (table 1): tube 1 consisted of 40 µl of the tissue extract diluted to 1–5 mg of protein per ml, 40 µl of solution A (consisted of 1.2 ml of 0.19 M PPB, pH 8.3, 30 µl of 0.5 mg/ml FAD, and 120 µl of 6 mg/ml catalase), 10 µl of water, and 10 µl of 0.019 M PPB, pH 8.3. Tube 2 contained a similar mixture to tube 1 except that DAO in PPB substituted for the 10 µl-buffer, and tube 3 contained DAO in PPB and 1 mM D-Ala, substituted respectively for the buffer and water. D-Ala was employed as the standard substrate throughout the present experiments. All amino acids were measured as alanine equivalents although other amino acids led to different molar absorbances in the kidney extract: D-Ala, 100%; D-Ser, 28.4%; D-Thr, 4.3%; D-Phe, 92.2%; D-Met, 72.3%; D-Leu, 83.2%; D-Ile, 51.4%; D-Val, 55.3%; D-Trp, 53.1%.

The reaction was started by the addition of DAO. After a 10-min incubation at 37 °C, the reaction was stopped

Table 1. Composition of the reaction tubes for the DAA assay

	Tube 1	Tube 2	Tube 3
40 µl	Extract	Extract	Extract
40 µl	Solution A	Solution A	Solution A
10 µl	Distilled water	Distilled water	D-Ala
10 µl	PPB	DAO	DAO

by 50 µl of 1 mM 2,4-dinitrophenylhydrazine in 1 M HCl, and a subsequent incubation at 37 °C for 10 min was followed by the addition of 0.35 ml of 0.6 M NaOH. Light absorption of the resulting hydrazone at 445 nm was measured after standing at room temperature for 5 min. The amount of free, inherent DAA in the sample was calculated as follows:

$$\text{DAA (nmol/g)} = \frac{A_2 - A_1}{A_3 - A_2} \times \frac{10\,(\text{nmol})}{v_a\,(\text{ml})} \times \frac{v_b\,(\text{ml})}{w\,(\text{g})}$$

where A_1, A_2 and A_3 indicate the optical absorbances of tubes 1, 2 and 3, respectively; v_a, the volume of the extract added to the tubes; v_b, the volume of the tissue extract of an organ; w, the wet weight of the tissue (the organ). The positive control, tube 3, was employed in each assay, since the color formation of D-Ala added to the extracts was shown not to be constant; it was dependent upon the extract used and on other experimental conditions. The Michaelis constant (K_m) was 2.8×10^{-3} M in the assay system. Protein concentration was determined according to Lowry et al.[5] using a bovine serum albumin standard.

Results. Various amounts of D-Ala added to the liver and kidney extracts were measured to see if they were detectable quantitatively, since the extracts might include substances that affect the reactions, such as an abundance of free L-amino acids (LAA), although substances larger than 10,000 dalton, which include DAO and other enzymes, should have been removed from the extracts. As shown in figure 1, the standard curve in the presence of the liver or kidney extract coincides with the PBS-control curve, indicating that the extracts had no influence on the D-Ala assay. This suggested that quantitative detection of free, inherent tissue DAA, as little as 0.2 nmol, was indeed feasible. This was further confirmed by the results that the concentrations of DAA were parallel to the dilution when the liver and kidney extracts were diluted with PBS 1:5, 1:10 and 1:20, respectively (fig. 2). In addition, the reaction proceeded linearly with time, as has been shown previously[6].

Table 2 shows free DAA levels of the tissue extracts. Free DAA were found in all the tissues examined, i.e., the kidney, liver, brain, heart, lung, thymus and serum. Among them, higher free DAA levels were observed in the kidney and liver. The levels seem to have increased with age in the kidney. As for the brain, if the cerebellum or the brain stem had been measured instead of the whole brain, higher DAA levels would have been observed. The ratio of free DAA to whole free amino acids (data were cited from Munro[7]) was estimated to be approximately 1% for the kidney and liver of adult and old mice.

Table 2. Free D-amino acid levels of mouse tissues. Values are mean ± SEM (n = 5). In each of the five experiments a different animal was used. ND, not determined; g, wet weight of the tissues

Mice	D-Amino acids (nmol/g) 4-week-old	19-week-old	11-month-old
Kidney	84 ± 20	155 ± 34	221 ± 54
Liver	159 ± 44	136 ± 16	238 ± 37
Brain	63 ± 8	43 ± 8	63 ± 8
Heart	31 ± 11	36 ± 15	24 ± 12
Lung	30 ± 5	ND	33 ± 3
Thymus	14 ± 6	ND	ND
Serum	26 ± 3	11 ± 3	25 ± 4

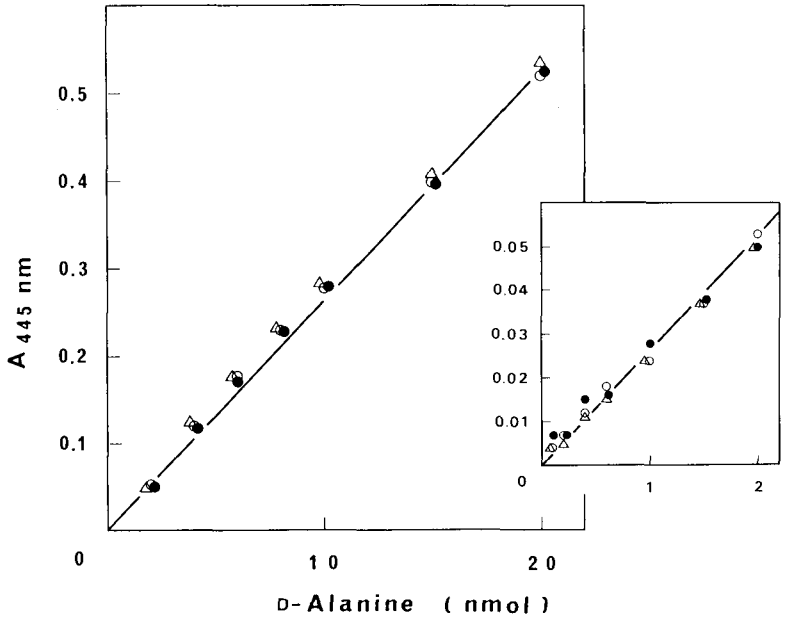

Figure 1. Effect of tissue extracts on detection of D-Ala. D-Ala was added in various quantities to reaction tubes containing D-amino acid oxidase, Solution A, and either PBS (○), kidney extract (△) or liver extract (●). Values after subtraction of D-Ala blank are plotted.

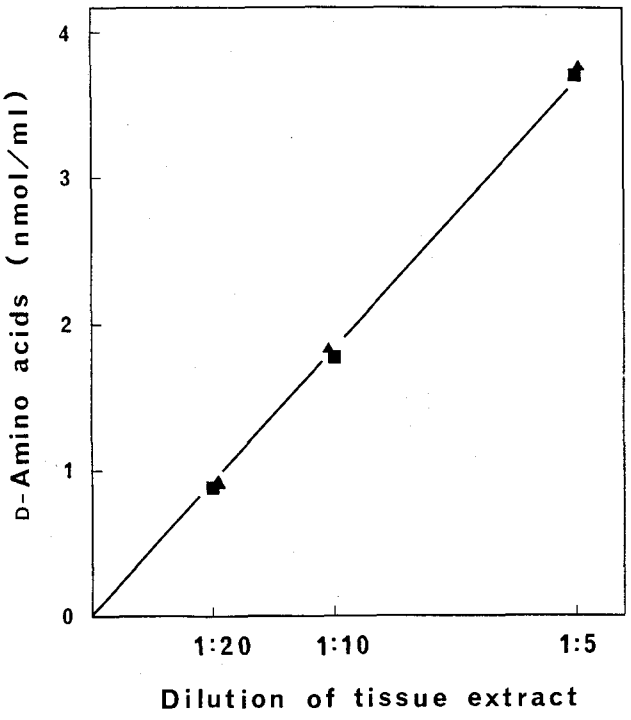

Figure 2. Dilution of tissue extracts vs. their inherent free D-amino acid concentration. Free D-amino acids were measured after dilution of kidney (▲) or liver (■) extract with PBS 1:5, 1:10 or 1:20, and the D-amino acid concentration of the diluted extract was obtained.

Discussion. The present assay method detects only D-isomers of free neutral amino acids such as Ala, Met, Pro, Tyr, Ile, Leu, Phe, Ser, Val and Trp, and also Gly and L-Pro weakly, because of the specificity of DAO[8]. The rate of color formation due to Gly was 1/200 of that due to D-Ala, and that due to L-Pro was far less than that for Gly in our assay. Gly and L-Pro are not likely to have been measured as DAA, because there was no difference in the free Gly and Pro levels (Hitachi Amino Acid Analyser L8500) of the kidney between the DAO-lacking mutant and the control mice, whereas the free DAA level of the mutant was measured to be 6-fold higher than that of the control (data not shown).

The amino acids observed in the present study were probably present as free amino acids, because no enzyme has been found in mammals that can hydrolyze peptides containing DAA residues. In addition, only the D-form of amino acids present in the tissues could have been measured, since no racemase that might produce DAA from their L-enantiomers is known in mammals. It is not clear whether the DAA are of endogenous origin, or come from exogenous sources like gut bacteria.

The existence of free DAA has been demonstrated in animal tissues. This suggests that the physiological role of DAO is, in part, to catalyze the oxidative deamination of free DAA. The physiological role of the enzyme remains obscure, in spite of its wide distribution in various organs of many animals.

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Prothymosin alpha is not a nuclear polypeptide

O. E. Tsitsiloni[a], P. P. Yialouris[a], K. Sekeri-Pataryas[b] and A. A. Haritos[a]*

[a] *Zoological Laboratory, Faculty of Sciences, University of Athens, GR-157 84 Athens (Greece) and* [b] *Department of Biology, National Research Center of Natural Sciences 'Demokritos', Aghia Paraskevi, GR 153 10 Athens (Greece)*
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Summary. Using a radioimmunoassay for the NH_2-terminus of prothymosin alpha, the crossreactive material was measured in subcellular fractions of calf thymus and liver. No significant amount of crossreactive material was found in the nucleus. This provides experimental evidence against a recent hypothesis, based on structural evidence, that prothymosin alpha is a nuclear polypeptide.
Key words. Prothymosin alpha; thymosin alpha 1.

Prothymosin alpha is a polypeptide of approximately 110 residues which has been isolated and sequenced from mammalian tissues[1-5]. It has a wide tissue[6-8] and phylogenetic distribution[9,10]. It has been related to cell