Spontaneous excretion of D-alanine in urine in mutant mice lacking D-amino-acid oxidase

Ryuichi KONNO,*† Yoko NAGATA,† Akira NIWA* and Yoshihiro YASUMURA*

*Department of Microbiology, Dokkyo University School of Medicine, Mibu, Tochigi 321-02, and

†Department of Biochemistry, Sapporo Medical College, Sapporo 060, Japan

Urine from mutant mice lacking D-amino-acid oxidase contained a large amount of alanine compared with that from normal mice. Urinary alanine of the mutant mice was sensitive to D-amino-acid oxidase. H.p.l.c. showed that about 94 % of the urinary alanine had the D-configuration. These results suggest that D-amino-acid oxidase functions to decompose D-amino acid(s) in normal mice.

INTRODUCTION

D-Amino-acid oxidase (EC 1.4.3.3) catalyses the oxidative deamination of D-amino acids (stereoisomers of naturally occurring L-amino acids) to the corresponding α -oxo acids [1,2]. A variety of D-amino acids are metabolized by this enzyme, but L-amino acids are not. Higher animals have this enzyme in their kidneys, liver and brain. The catalytic mechanism and molecular properties of the enzyme have been elucidated in detail [3–5]. Amino acid sequences and complementary DNA sequences of the human and pig enzymes have been determined [6–8]. However, because D-amino acids are quite rare in higher animals, the physiological role of this enzyme is not known.

We have established a mutant mouse strain lacking D-amino-acid oxidase [9,10]. The mutant ddY/DAO⁻ mice excrete a large amount of methionine and alanine in urine compared with normal mice [11]. The methionine is mostly the D-isomer derived from their diet [12]. However, the configuration of alanine remained undetermined because separation of D- and L-isomers of alanine from other substances was poor. The reason for such a high rate of excretion of alanine was not clear. Meanwhile, Nagata *et al.* [13] found that the urine of the ddY/DAO⁻ mice fed on a diet devoid of supplementary DL-methionine still contained a large amount of D-amino acids. Therefore, alanine in the urine of the mutant mice was suspected to be the D-isomer. In the present work, the configuration of alanine was determined by derivatizing D- and L-isomers of alanine and separating them on a reversed-phase column as described by Marfey [14]. Implications of the results are discussed.

MATERIALS AND METHODS

Mice and urine collection

Normal ddY/DAO⁺ mice and mutant ddY/DAO⁻ [9,10] were raised on a commercial diet (Type NMF; Oriental Yeast, Tokyo, Japan). This diet contained supplementary DL-methionine [12], but it did not contain any other DL-amino acids. Mice were maintained at 24 ± 2 °C with a 12-h light/dark cycle. Male adult mice were kept in individual metabolic cages and urine was

collected overnight. They were given food and water *ad libitum*.

T.l.c. of urine with or without D-amino-acid-oxidase treatment

Urine from ddY/DAO⁻ mice was mixed with 2.5 μ l of a crystalline suspension of hog kidney D-amino-acid oxidase (activity 0.3 μ mol/min; Sigma, St Louis, MO, U.S.A.). After incubation for 1 h at 37 °C, 5 μ l of the solution was applied to a t.l.c. plate (silica gel 60; Merck, Darmstadt, Germany). Untreated urine from the ddY/DAO⁻ mice and urine from ddY/DAO⁺ mice (5 μ l each) was also applied to the plate. T.l.c. was done as previously described [15].

Analysis of the isomer composition of urinary alanine

Urine from 2–3 ddY/DAO⁻ mice was pooled (3–4 ml). Urinary amino acids were partially purified through deproteinization and cation-exchange chromatography as previously described [11,12]. The eluate from the cation-exchange resin was evaporated to dryness under reduced pressure. The dried residue was then dissolved in 3 ml of distilled water and applied to a column (1.8 cm × 4 cm) of Dowex 1-X8 (Muromachi Kagaku Kogyo, Tokyo, Japan). A flow-through fraction and 30 ml of distilled water wash were collected and evaporated to dryness. The residue was redissolved in 0.4 ml of distilled water and streaked on a preparative t.l.c. plate (LK 6; Whatman, Clifton, NJ, U.S.A.). It was chromatographed with butan-1-ol/acetic acid/water (3:1:1, by wt.). The plate was revealed under an ultraviolet lamp and a zone corresponding to standard alanine was scraped off. Alanine was extracted with 1 ml of distilled water. The solution was passed through a 0.2 μ m filter.

The configuration of urinary alanine was analysed according to the method of Marfey [14]. The above filtrate (100 μ l) was mixed with 200 μ l of a 1 % (w/v) solution of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, known also as Marfey's reagent; Pierce, Rockford, IL, U.S.A.) in acetone. To this solution, 40 μ l of 1 M-sodium bicarbonate solution was added, and incubation carried out at 40 °C for 1 h. After cooling to room temperature, 20 μ l of 2 M-HCl was added and the solution

Abbreviation used: FDAA, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide.

† To whom correspondence should be addressed.

was filtered through a 0.2 μ m membrane. D-Alanine, L-alanine and DL-alanine obtained from Sigma, Ajinomoto (Tokyo, Japan) and Wako (Osaka, Japan) respectively were treated in the same manner and used as the standards.

An h.p.l.c. system (Millipore; Waters, Milford, MA, U.S.A.) was used for separation and quantification of the alanine derivatives. After being degassed, 5 μ l of the above filtrate was applied to a reversed-phase column (3.9 mm $\times$ 150 mm, Nova-pak C18; Waters). The alanine derivatives were eluted using a mobile phase consisting of A (0.05 M-triethylamine phosphate, pH 3.0) and B (acetonitrile). Linear gradients were programmed as follows: 0–10 min, 100:0 (A/B, v/v)–82:18; 10–35 min, 82:18–77:23; 35–45 min, 77:23–60:40; 45–50 min, 60:40–100:0. The gradient between 18 and 23% (v/v) acetonitrile was made gentle because the alanine derivatives were eluted between these concentrations of acetonitrile. The flow rate was 2 ml/min and the detector was set at 340 nm.

RESULTS

T.l.c. showed that urine from ddY/DAO⁻ mice lacking D-amino-acid oxidase has two extra spots which were faint in urine from normal ddY/DAO⁺ mice (Fig. 1, lanes 1 and 2). We previously showed that these spots were alanine and methionine [11]. The methionine was mainly the D-isomer [12]. The configuration of alanine was examined in this study. Urine of the ddY/DAO⁻ mice was incubated with D-amino-acid oxidase and chromatographed on t.l.c. plates. Fig. 1 (lane 3) shows

Fig. 1. Thin-layer chromatogram of urine with or without treatment with D-amino-acid oxidase

Lane 1, untreated urine of a normal ddY/DAO⁺ mouse; lane 2, untreated urine of a mutant ddY/DAO⁻ mouse lacking D-amino-acid oxidase; lane 3, D-amino-acid-oxidase-treated urine of the mutant mouse; lane 4, standard alanine; lane 5, standard methionine.

Fig. 2. H.p.l.c. showing D- and L-isomer compositions of alanine

Alanine was derivatized and the resulting D- and L-alanine derivatives were separated by h.p.l.c. (a) Standard DL-alanine. (b) Alanine purified from urine of the ddY/DAO⁻ mice lacking D-amino-acid oxidase. Note that sensitivity of the recorder was reduced in (b) to one-eighth of that of (a) because of the presence of a large amount of D-alanine compared with the L-isomer.

that the spots corresponding to alanine and methionine almost disappeared after treatment with the enzyme. This result suggested that alanine as well as methionine was mostly the D-isomer. Then, the D- and L-isomer compositions of alanine in the urine from the ddY/DAO⁻ mice were quantitatively determined. The large spot migrating just below alanine consisted of some amino acids and probably other ninhydrin-positive materials. The nature of these substances was not determined.

Fig. 2(a) shows an h.p.l.c. chromatogram of standard DL-alanine treated with FDAA. The D- and L-diastereomers of alanine were clearly separated on a reversed-phase C18 column. The L-alanine derivative was eluted prior to the D-alanine derivative. A large peak between them was a hydrolysed derivatizing reagent. This elution pattern was consistent with that reported by Marfey [14]. In addition to these three peaks, there was one peak before and one peak after the peak of the L-alanine derivative. These substances were not identified. They seemed to be reagent-derived materials, because the peaks were also observed even when distilled water was mixed with the FDAA reagent and processed in the same manner as the DL-alanine solution (results not shown).

Alanine was purified from urine of the ddY/DAO⁻ mice by deproteinization and ion-exchange chromatography followed by t.l.c. It was derivatized with the FDAA reagent and analysed by h.p.l.c. One of the chromatograms is shown in Fig. 2(b). Though there was a variation between samples, most of the urinary alanine ($94.0 \pm 1.5\%$, mean $\pm$ s.d., $n = 7$) was the D-isomer.

The abundance of D-alanine over the L-isomer in the urine from the ddY/DAO⁻ mice was checked by changing the procedure. Amino acids purified from urine of the ddY/DAO⁻ mice were first derivatized with the FDAA reagent and then chromatographed by two-dimensional t.l.c. to clearly separate the alanine derivative from other amino acid derivatives. An h.p.l.c. analysis of the alanine

derivative extracted from the spot on the t.l.c. plates also showed that most of alanine was the D-isomer.

DISCUSSION

The alanine in urine of the ddY/DAO⁻ mice was mostly the D-isomer. The ratio of L-isomer to D-isomer was about 1:15.7. This result is quite consistent with our previous finding that the ddY/DAO⁻ mice excreted about 12.4-fold more alanine in the urine than did normal ddY/DAO⁺ mice [11]. It seems that the mutant mice could not metabolize D-alanine due to the lack of D-amino-acid oxidase, so that they constantly excreted it in urine, which caused an accumulation of D-alanine more than 10-fold above the normal level. The present result is also consistent with the results of Nagata *et al.* [13] that urine of the ddY/DAO⁻ mice fed a diet devoid of supplementary DL-methionine contained about 13.4-fold more D-amino acids than that of the ddY/DAO⁺ mice.

The ddY/DAO⁻ mice excreted D-methionine in urine (Fig. 1; [12]). This was because a commercial mouse diet contained supplementary DL-methionine. The D-isomer was unmetabolized and excreted. However, because their diet was not supplemented with DL-alanine, the source of the D-alanine that was also excreted in a large quantity in urine is not clear. D-Alanine should have an exogenous origin because D-amino acids are quite rare in the metabolism of higher animals. One possible source is bacteria inhabiting the alimentary tract. Peptidoglycan of bacterial cell walls is known to contain a large amount of D-alanine [16]. It is likely that components released from bacteria are absorbed through the intestine of the host animals. Hoeprich [17] showed that serum of mice and guinea pigs contained D-alanine whereas serum of germ-free counterparts did not. Nagata *et al.* [18] showed that human plasma also contained a small amount of D-amino acids.

The result that mutant mice lacking D-amino-acid oxidase spontaneously excreted a large amount of D-alanine in urine, whereas normal mice did not, suggests that the enzyme is actively eliminating D-amino acid(s) *in vivo*. It may be one of the physiological functions of this enzyme. Nagata *et al.* [13] have also suggested that the

enzyme is involved in the catabolism of D-amino acids in the body.

We thank Mr. Brian Beffort for advice in preparing this manuscript.

REFERENCES

1. Krebs, H. A. (1935) *Biochem. J.* **29**, 1620–1644
2. Meister, A. (1965) in *Biochemistry of the Amino Acids*, 2nd edn., vol. 1, pp. 297–304, Academic Press, New York
3. Bright, H. J. & Porter, D. J. T. (1975) in *The Enzymes* (Boyer, P. D., ed.), 3rd edn., vol. 12, Part B, pp. 421–505, Academic Press, New York
4. Yagi, K. (1980) in *New Horizons in Biological Chemistry* (Koike, M., Nagatsu, T., Okuda, J. & Ozawa, T., eds.), pp. 257–270, Japan Scientific Societies Press, Tokyo
5. Miura, R. & Miyake, Y. (1988) *Bioorg. Chem.* **16**, 97–110
6. Ronchi, S., Minchiotti, L., Galliano, M., Curti, B., Swenson, R. P., Williams, C. H., Jr. & Massey, V. (1982) *J. Biol. Chem.* **257**, 8824–8834
7. Fukui, K., Watanabe, F., Shibata, T. & Miyake, Y. (1987) *Biochemistry* **26**, 3612–3618
8. Momoi, K., Fukui, K., Watanabe, F. & Miyake, Y. (1988) *FEBS Lett.* **238**, 180–184
9. Konno, R. & Yasumura, Y. (1983) *Genetics* **103**, 277–285
10. Konno, R. & Yasumura, Y. (1984) *J. Neurochem.* **42**, 584–586
11. Konno, R., Isobe, K., Niwa, A. & Yasumura, Y. (1988) *Biochim. Biophys. Acta* **967**, 382–390
12. Konno, R., Isobe, K., Niwa, A. & Yasumura, Y. (1988) *Metab. Clin. Exp.* **37**, 1139–1142
13. Nagata, Y., Konno, R., Yasumura, Y. & Akino, T. (1989) *Biochem. J.* **257**, 291–292
14. Marfey, P. (1984) *Carlsberg Res. Commun.* **49**, 591–596
15. Konno, R. & Yasumura, Y. (1988) *Lab. Anim. Sci.* **38**, 292–295
16. Wheat, R. W. (1988) in *Zinsser Microbiology* (Joklik, W. K., Willett, H. P., Amos, D. B. & Wilfert, C. M., eds.), 19th edn., pp. 63–76, Appleton & Lange, Norwalk, CT
17. Hoeprich, P. D. (1965) *J. Biol. Chem.* **240**, 1654–1660
18. Nagata, Y., Akino, T., Ohno, K., Kataoka, Y., Ueda, T., Sakurai, T., Shiroshita, K. & Yasuda, T. (1987) *Clin. Sci.* **73**, 105–108

Received 14 March 1989/21 April 1989; accepted 27 April 1989