RACEMIZATION OF AMINO ACIDS BY MAMMALIAN TISSUES

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We have demonstrated previously that D-amino acids occur in the free amino acid pools in tissues of marine invertebrates in diverse phyla (Preston, R.L. Comp. Bioch. Physiol. 87B: 55-62, 1987; Preston, R.L. Comp. Bioch. Physiol. 87B: 63-71, 1987). Furthermore, we showed that D-amino acids were transported and metabolized by invertebrate tissues. The principal route of metabolism utilizes a racemase, an enzyme that interconverts D- and L-amino acids (Preston, R.L., McQuade, B., Oladokun, O. and Sharp, J., Bull. MDIBL 36: 86, 1997). In mammals, D-amino acids have also been detected in rather low concentrations in blood, urine and tissues but their presence has been attributed to diet or bacterial contamination (Armstrong, D. W., Gasper, M., Lee, S. H., Zukowski, J. and Ercal, N., Chirality 5: 375-378, 1993). However, recent investigations (Nagata, Y., Horiike, K. and Maeda, T. Brain Research 634: 291-295, 1994) showed that D-serine occurs in mammalian brain. Furthermore, the presence of D-serine could not be explained by bacterial contamination (they used germ free mice in some experiments) or dietary sources. One possible source of D-amino acids in mammalian tissues could be synthesis via racemases, a mechanism we have observed to be very common in marine invertebrates. We report here the detection of endogenous racemase activity in porcine brain and bovine heart. To our knowledge, this is the first reported evidence for the occurrence of these enzymes in mammalian tissues.

Commercially available acetone extracts of a variety of mammalian tissues (Sigma Chemical Co., St. Louis, MO) were analyzed for the presence of D-amino acids and racemase activity. The extracts were suspended in deionized water (10:1 v/w), homogenized and centrifuged at 15,000 x g for 5 min. In some cases, the supernatant was clarified by filtering through a 0.2 μm membrane filter. In most cases an aliquot of the supernatant was used for D-amino acid and racemase analysis. However, in some experiments the supernatant was treated with 80% ethanol to precipitate proteins. This was centrifuged, the pellet washed 3 times in 80% ethanol and then extracted with deionized water to solubilize the racemase. We have shown with invertebrate tissues that this procedure recovers racemase activity while removing most other proteins. D-Amino acid content and racemase activity were measured using a coupled enzyme assay. Typical assay conditions to detect neutral D-amino acids were: tissue extract (50 μl), tetrasodium pyrophosphate (NaPP) buffer saturated with the chromophoric peroxidase substrate, *o*-dianisidine (50 mM NaPP, pH 8.5; 110 μl), D-amino acid oxidase (0.06 units; 20 μl) and horseradish peroxidase (0.02 mg; 20 μl) and water, 50 μl. The reaction was run at room temperature in a 96 well microplate and the product formation (oxidized *o*-dianisidine) measured at 490 nm using an ELISA plate reader. The absorbance is read every 10 min initially and at longer intervals for up to 8 hours. The racemization reaction proceeds rather slowly and the rates of reaction were usually calculated from the slopes of time courses plotted from these data. All measurements were done in quadruplicate and a variety of positive (containing added D-amino acid) and negative controls were included. The racemase assay used a similar procedure except that 200 mM L-alanine was added to the extract and the formation of D-amino acid measured. This assay can detect D-amino acids at concentrations from 0.05 mM or higher. Two mammalian tissues, porcine brain and bovine heart, showed racemase activity. Typical data are presented in Table 1 with a series of controls.

Condition*	0.5 mM D-Ala	200 mM L-Ala	Perox.	DAO	$A_{490} \pm$ S.E.**	Ratio***
Porcine Brain						
Extract only	-	-	-	-	0.022 ± 0.001	-
Assay enzymes only	-	-	+	+	0.022 ± 0.001	-
L-Alanine only	-	+	+	+	0.028 ± 0.001	-
D-Alanine only	+	-	+	+	0.401 ± 0.016	-
Positive control (D-ala added)	+	-	+	+	0.448 ± 0.002	10.2
D-Amino acid content	-	-	+	+	0.052 ± 0.001	1.2
Endogenous DAO assay	+	-	+	-	0.032 ± 0.001	0.7
Racemase assay	-	+	+	+	0.098 ± 0.015	2.2
Bovine Heart						
Extract only	-	-	-	-	0.010 ± 0.002	-
Assay enzymes only	-	-	+	+	0.022 ± 0.001	-
L-Alanine only	-	+	+	+	0.028 ± 0.001	-
D-Alanine only	+	-	+	+	0.401 ± 0.016	-
Positive control (D-ala added)	+	-	+	+	0.418 ± 0.005	13.1
D-Amino acid content	-	-	+	+	0.025 ± 0.001	0.8
Endogenous DAO assay	+	-	+	-	0.022 ± 0.001	0.7
Racemase assay	-	-	+	+	0.093 ± 0.002	2.9

* See text below for explanation of the experimental conditions. All samples contained NaPP buffer pH 8.5 with saturating concentrations of the chromophoric reagent, *o*-dianisidine. Abbreviations: D-ala = D-alanine; L-ala = L-alanine; Perox. = horseradish peroxidase; DAO = D-amino acid oxidase.

** The absorbance at 490 nm was read against a NaPP blank after 3.5 hours incubation. Note: A statistical comparison (t-test) of the absorbance values for each experimental condition compared with the respective control conditions (extract only) for both porcine brain and bovine heart reveals that all values are significantly different from the control at the $p < 0.005$ ($n = 4$) with the exception of the "Assay enzymes only" and "L-alanine only" conditions for porcine brain..

*** Ratio = A_{490} each condition/[A_{490} (extract only control) + A_{490} (Assay enzymes only)] The denominator compensates for the intrinsic color of the extract and the assay enzymes.

Racemase activity was detected in this coupled enzyme assay as an increase in chromophore production (oxidized *o*-dianisidine) after addition of L-alanine compared with baseline endogenous D-amino acid content or contamination. The controls for this coupled enzyme assay are very important since there are multiple sources of D-amino acid (and interfering tissue pigments) apart from racemase activity. The details of the conditions employed for this assay were as follows: "Extract only" = Tissue extract with NaPP buffer only. This was a control for endogenous color of the extract. "Assay enzymes only" = This controls for the slight intrinsic color of DAO and peroxidase. "L-Alanine only" = commercial L-alanine sometimes contains low concentrations of D-alanine as a contaminant. This was a control for reagent contamination. "D-Alanine only" = D-Alanine was added at a final concentration of 0.5 mM. This condition confirmed that the assay enzymes were active and was the condition of

comparison for determination of the presence of endogenous inhibitors. "Positive Control (D-ala added)" = Tissue extracts may contain endogenous inhibitors for the enzymes (DAO and peroxidase) used for this assay. D-Alanine was added to the tissue extract and compared with the condition without extract ("D-Alanine only") to control for this possibility. "D-Amino acid content" = This condition measured the presence of endogenous D-amino acids. "Endogenous DAO assay" = Many mammalian tissues contain endogenous DAO. The presence of this activity could deplete free D-amino acid pools below limits detectable with this assay. This would not necessarily interfere with the racemase assay however. "Racemase assay" = 200 mM L-alanine was added to tissue extract to drive the racemization in the direction of D-amino acid production. An increase in the D-amino acid content after L-alanine addition implies enzymatic conversion of L-alanine to D-alanine.

Both the porcine brain extract and the bovine heart extract showed comparatively little intrinsic color ("Extract only" controls). The "L-alanine only" control had very little if any D-amino acid contamination (0.028 $\pm$ 0.001 O.D. units) compared with the "Assay enzymes only" condition (0.022 $\pm$ 0.001 O.D. units). The "Positive control (D-ala added)" condition showed comparatively little inhibition due to endogenous inhibitors. In porcine brain extract, the positive control corrected for the added absorbance due to intrinsic color in the extract (0.448 – 0.022 = 0.426 O.D. units) slightly exceeded (by 6%) the absorbance without extract (0.401 O.D. units). In bovine heart extract, the positive control corrected for the added absorbance due to intrinsic color in the extract (0.418 – 0.010 = 0.408 O.D. units) was nearly the same as the absorbance without extract (0.401 O.D. units).

The apparent D-amino acid content was very low in both porcine brain and bovine heart extracts, very close to the lower limit of sensitivity of this assay (<0.05 mM). Neither extract exhibited detectable endogenous DAO activity (ratios of reaction rate relative to the appropriate controls being 0.7 in both cases). Finally, racemase activity was clearly detectable in both tissues. In porcine brain, the racemase activity exceeded that of the controls by 2.2 fold and in bovine heart the racemase exceed controls by 2.9 fold. In both cases the absorbance values are statistically different from the "negative" controls ($p< 0.001$; $n = 4$). Perhaps a better comparison is with the "D-Amino acid content" condition that includes the low level D-amino acid contamination in the tissue. In this case, the racemase activity is statistically significant from this "control" at $p<0.001$ for porcine brain and $p<0.05$ for bovine heart.

Although the absolute racemase activities in these comparatively crude tissue extracts were low, we feel we have demonstrated unequivocally the presence of racemase activity in mammalian tissue. We have repeated these experiments at least a dozen times and have obtained similar results.. We have done further experiments at Illinois State University using fresh bovine heart and pig brain and have, as might be expected, demonstrated racemase activity in these preparations. To our knowledge, this is the first report of racemase activity in mammalian tissues.

The physiological role of racemases and D-amino acids in mammalian tissues is unknown but several functions are possible: utilization as a source of nutrition, use as precursors for antibiotics and use as neurotransmitters. D-Amino acids may be acquired from food or intestinal flora and the presence of racemase (converting D- to L-amino acids) provides a nutritional benefit for those tissues with the enzyme. Many antibiotics (e.g. valinomycin and gramicidin) have D-amino acids as part of their structures. D-Serine has been shown to be a potent activator of the NMDA receptor and therefore it could have a substantial impact on neural function at comparatively low concentrations (Nagata, Y., Horiike, K. and Maeda, T. Brain Research 634: 291-295, 1994). It is also possible that D-Amino acids and racemases may be part of an entirely new sort of cellular metabolic or regulatory pathway. If this is true, further research in this area could lead to new approaches in understanding normal heart and brain function. (Bryan McQuade, Rory McCaw and Seneca Cancel were Hancock County Scholars funded by the SETH Program, NSF DBI9531348. Drew Sommerville was supported by an American Heart Association Undergraduate Summer Fellowship. Also supported in part by NIEHS grant ESO3828-11).