Degradation of Uric Acid in Fish Liver Peroxisomes

INTRAPEROXISOMAL LOCALIZATION OF HEPATIC ALLANTOICASE AND PURIFICATION OF ITS PEROXISOMAL MEMBRANE-BOUND FORM

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Urate-degrading enzymes such as uricase, allantoinase, and allantoicase are located in the peroxisomes of marine fish liver (Noguchi, T., Takada, Y., and Fuj iwara, S. (1979) J. Biol. Chem. 254, 5272-5275). On the basis of intraper oxisomal localization of hepatic allantoicase, 13 different fishes were classified into two groups: mackerel group and sardine group. Allantoicase is located on the outer surface of the peroxisomal membrane in the mackerel group and in the peroxisomal soluble matrix in the sardine group. The peroxisomal membrane enzyme and the peroxisomal matrix enzyme are not distinguishable on the basis of the number and molecular weight of the subunits, but differ in isoelectric point and electrophoretic mobility. The molecular weight of the fish allantoicase subunit is identical with that of the small subunit (allantoicase subunit) of amphibian allantoinase-allant oicase complex, suggesting that the subunit of fish allantoicase changed to the small subunit of the amphibian complex during evolution: allantoinase and allantoicase are present as a complex in amphibian liver (Noguchi, T., Fuj iwara, S., and Hayashi, S. (1986) J. Biol. Chem. 261, 4221-4223).

The end product of purine degradation varies from species to species (1). The degradation of purines to urate is common to all animal species thus far studied, whereas the degradation of urate is much less complete in higher animals. In some marine fishes, enzymes that convert purines to urate are located in the cytosol and those that convert urate to urea (uricase (EC 1.7.3.3), allantoinase (EC 3.5.2.5) and allantoicase (EC 3.5.3.4)) are present in the peroxisomes, suggesting that in purine degradation, peroxisomal enzymes have been lost during animal evolution (2). We have reported that uricase and allantoinase are located in the peroxisomal matrix and allantoicase in the peroxisomal membrane in some marine fish liver (2, 3). In the present study, fishes were found to be classified into two groups on the basis of intraperoxisomal localization of hepatic allantoicase: one with allantoicase located on the outer surface of liver peroxisomal membranes and the other with allantoicase located in the liver peroxisomal soluble matrix. Peroxisomal membrane allantoicase was purified from mackerel liver and compared with peroxisomal soluble matrix allant oicases from other fish livers.

MATERIALS AND METHODS

RESULTS

Intraperoxisomal Localization of Fish Liver Allantoicase—The intracellular distribution of allantoicase was examined by sucrose density gradient centrifugation with the postnuclear fractions of the livers of 13 different fishes (Figs. 1 and 2). In each case, the peroxisomes and mitochondria were separated; the peroxisomes, identified by their catalase activity (EC 1.11.1.6), were at a density of about 1.25 g/ml, and the mitochondria, identified by their glutamate dehydrogenase activity, at a density of about 1.18 g/ml. Acid phosphatase (used as a lysosomal marker) was distributed over a broad density range with a peak of about 1.19 g/ml, and the soluble top fraction formed presumably from broken peroxisomes (not shown). On the basis of sedimentation profiles of allantoicase, 13 different fishes were classified into two groups: sardine group and mackerel group. In the sardine group (sardine, bonito, young yellowtail, flying fish, yellow mackerel, gray mullet, carp, and pale chub), allantoicase was recovered both in the peroxisomes and in the soluble fraction (Fig. 1). In each case, the activity ratio of the peroxisomal fraction to the soluble fraction of allantoicase was nearly identical with that of catalase as the soluble matrix enzyme of the peroxisomes. These results suggest that hepatic allantoicase is located only in the peroxisomal matrix in the sardine group, because catalase activity in the soluble top fraction is from broken peroxisomes. In the mackerel group (mackerel, grunt, crucian carp, sunfish and large mouth bass), hepatic allantoicase was recovered only in the peroxisomal fraction, whereas catalase was recovered both in the peroxisomal fraction and in the soluble fraction from broken peroxisomes (Fig. 2). The results suggest that hepatic allantoicase is located in the peroxisomal membrane or core in the mackerel group.

Intraperoxisomal localization of allantoicase was examined in the livers of the two prototypes i.e. sardine and mackerel. Peroxisomal suspensions of fish livers from the sucrose density gradients were separately diluted with the same volume of 0.01 M pyrophosphate buffer, pH 9.0, which is known to break peroxisomes (15). After storage overnight at 4 °C, each suspension was subjected to sucrose density gradient centrifugation. Under these conditions, in rat liver, all of the catalase...
Allantoicase is located in the peroxisomal membrane or core in mackerel liver.

Fig. 5, in the Miniprint, shows inactivation profiles of allantoicase by the antibody against sardine liver allantoicase using the peroxisomes and the peroxisomal extracts from sardine or mackerel liver. In each case, allantoicase activities of the peroxisomal extract were inactivated by the antibody. On the other hand, allantoicase activity of the peroxisomes from mackerel liver was inactivated by the antibody, whereas the activity of the peroxisomes from sardine liver was not inactivated. These findings show that hepatic allantoicase is located on the outer surface of the peroxisomes in mackerel liver and in the soluble matrix of peroxisomes in sardine liver.

All fishes of the sardine group were found to be identical with sardine and all of the mackerel group with mackerel with respect to the intraperoxisomal localization of hepatic allantoicase.

**Purification of Peroxisomal Membrane Allantoicase from Mackerel Liver**—We have purified and characterized the peroxisomal soluble matrix allantoicase from sardine liver (12). In the present study, the purification of the peroxisomal membrane allantoicase from mackerel liver was carried out and compared with the peroxisomal soluble matrix allantoicases of other fishes. The peroxisomal membranes were prepared from the peroxisomal fraction of mackerel liver as described under “Materials and Methods.” Allantoicase could be solubilized from the peroxisomal membrane by treatment with n-octyl-β-D-thioglucoside. An approximately 1,500-fold purification was achieved from liver homogenates with a yield of about 2.1% (Table I, Miniprint). The enzyme preparation may be stored at -20 °C for at least 5 weeks without loss of activity, and there was little loss with storage at 0-4 °C for at least 2 weeks. The enzyme preparation showed a single protein-staining band upon polyacrylamide gel electrophoresis at pH 9.0 (Fig. 6). The molecular weight of the enzyme was estimated to be approximately 100,000 by sucrose density gradient centrifugation. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate gave a single protein band in the soluble fraction.

In the soluble matrix is recovered in the soluble top fraction, uricase in the core is located at a density of 1.23 g/ml, and NADH-cytochrome-c reductase in the membrane is distributed over a broad density range with a peak of about 1.17 g/ml (8, 16). Different sedimentation profiles of allantoicase activity were observed between sardine liver and mackerel liver. In the sardine, allantoicase as well as catalase were completely solubilized and recovered only in the soluble top fraction, showing that allantoicase is located only in the peroxisomal soluble matrix (Fig. 3, Miniprint). In contrast, in the mackerel, allantoicase was not solubilized and was distributed over a broad density range with a peak of about 1.17 g/ml, whereas catalase is completely solubilized and recovered only in the soluble top fraction (Fig. 4, Miniprint). In fish liver, uricase is not a marker enzyme of the peroxisomal core and NADH-cytochrome-c reductase is not a marker enzyme of the peroxisomal membrane, because uricase is located in the peroxisomal soluble matrix, and NADH-cytochrome-c reductase has not been demonstrated in the peroxisomal membrane (2, 3). However, the present results suggest that

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![Fig. 1. Subcellular distribution of allantoicase in sardine liver.](image1)

![Fig. 2. Subcellular distribution of allantoicase in mackerel liver.](image2)

![Fig. 6. Polyacrylamide gel electrophoresis of allantoicases purified from sardine and mackerel liver.](image3)

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TABLE II

Properties of peroxisomal membrane allantoicase and peroxisomal matrix allantoicase

| Properties | Liver allantoicase | Peroxisomal membrane | Peroxisomal matrix |
|------------|--------------------|----------------------|--------------------|
| Number of subunits | 2 | 2 | 2 |
| M<sub>s</sub> of subunit | 48,000 | 48,000 | |
| pH optimum | 6.5-6.7 | 6.5-6.7 | |
| K<sub>a</sub> for allantoate (mM) | 7.0 | 4.5 | |
| Isoelectric point<sup>a</sup> | 8.05 | 6.20 | |
| Electrophoretic mobility | 0.38 | 0.62 | |

<sup>a</sup>See Ref. 14.

![Diagram of ALN and ALC interactions](image)

Fig. 8. Changes of allantoinase and allantoicase in intracellular localization and molecular structure during animal evolution. ALN, allantoinase; ALC, allantoicase; ALNC, allantoinase-allantoicase complex.

component that had an estimated molecular weight of about 48,000, showing that the enzyme consists of two identical subunits each with a M<sub>s</sub> = 48,000 (Fig. 6).

Some physical, enzymatic, and immunological properties of peroxisomal membrane-bound allantoicase purified from mackerel liver were compared with the peroxisomal soluble matrix allantoicase purified from sardine liver (Table II). The two enzymes were not distinguishable with respect to the number and molecular weight of the subunit and pH optimum. In contrast, they differed in K<sub>a</sub> for allantoate, isoelectric point, and electrophoretic mobility.

On Ouchterlony double diffusion analysis (17), the antibody against sardine allantoicase produced a single band of precipitin against both sardine allantoicase and mackerel allantoicase (Fig. 7, Miniprint). However, the antibody produced a spur between the two precipitin lines.

Molecular weights of allantoicase subunits in the peroxisomal extracts of other fish livers were examined by immunoblotting (18) using the antibody against sardine liver allantoicase. Yellow mackerel was examined as a representative of the sardine group with the peroxisomal soluble matrix allantoicase, and grunt as a representative of the mackerel group with the peroxisomal membrane-bound allantoicase. In each case, the molecular weight of the allantoicase subunit was identical with those of sardine or mackerel allantoicase subunit.

**DISCUSSION**

We have reported that allantoinase and allantoicase are different proteins in marine fish and invertebrate liver, whereas the two enzymes form a complex in amphibian liver (4, 12). This allantoinase-allantoicase complex consists of four subunits (48,000 × 2 + 54,000 × 2). Allantoinase activity of allantoinase-allantoicase complex is from the large subunit (allantoinase subunit) with a M<sub>s</sub> = 54,000, and allantoicase activity of the allantoinase-allantoicase complex is from the small subunit (allantoicase subunit) with a M<sub>s</sub> = 48,000. Fish allantoinase is a single peptide with M<sub>s</sub> = 48,000, and fish allantoicase consists of two identical subunits each with a M<sub>s</sub> = 54,000. The molecular weight of the large subunit of amphibian allantoinase-allantoicase complex is identical with mackerel liver allantoinase, and the small subunit is identical with the subunit of the peroxisomal soluble allantoicase from sardine liver (12). On the basis of intraperoxisomal localization of hepatic allantoicase, 13 fishes were classified into two groups: the sardine group with allantoicase within the peroxisomal soluble matrix and the mackerel group with allantoicase bound to the peroxisomal membrane. The peroxisomal matrix allantoicase of the sardine group and the peroxisomal membrane-bound allantoicase of the mackerel group were not distinguishable in number and molecular weight of the subunit.

Recently, we found that the subcellular localization of allantoinase varies among fishes, and amphibian allantoinase-allantoicase complex is located only in the cytosol: hepatic allantoinase is located both in the peroxisomes and in the cytosol in marine fishes (sardine, mackerel, grunt, bonito, young yellowtail, flying fish, and yellow mackerel), and only in the cytosol in fresh water fishes (crucian carp, sunfish, large mouth bass, gray mullet, carp, and pale chub). On the basis of the present and previous data (2–4, 12), changes of allantoinase and allantoicase in the molecular structure and intracellular localization during animal evolution are shown in Fig. 8. Fish liver allantoinase is a single peptide with a M<sub>s</sub> = 54,000 and is located both in the peroxisomes and in the cytosol, or only in the cytosol. Fish liver allantoicase consists of two identical subunits with a M<sub>s</sub> = 48,000 and is located in the peroxisomal soluble matrix or on the outer surface of the peroxisomal membrane. The evolution of fishes to amphibians resulted in the dissociation of allantoinase into subunits and in the association of allantoinase with the subunit of allantoicase. This amphibian enzyme was lost by further evolution.

**REFERENCES**

1. White, A., Handler, P., and Smith, E. L. (1973) Principles of Biochemistry, McGraw-Hill Book Co., New York
2. Noguchi, T., Takada, Y., and Fujiwara, S. (1979) J. Biol. Chem. 254, 5292-5297
3. Fujiwara, S., Ohashi, H., and Noguchi, T. (1987) Comp. Biochem. Physiol. 86B, 23-26
4. Takada Y., and Noguchi, T. (1983) J. Biol. Chem. 258, 4762-4764
5. Sinha, A. K. (1972) Anal. Biochem. 47, 389-394
6. Beaufay, H., Bendall, D., Baudhuin, P., and de Duve, C. (1959) Biochem. J. 73, 623-628
7. Bergmeyer, H. U., and Bernt, E. (1974) in Methods and Enzymatic Analysis (Bergmeyer, H. U., ed) pp. 752-758, Academic Press, Orlando, FL
8. Donaldson, R. P., Tolbert, N. E., and S阿拉伯enberger, C. (1972) Arch. Biochem. Biophys. 152, 199-215
9. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1955) Biochem. J. 60, 604-617
10. Osmundsen, H., Neat, C. E., and Norum, K. R. (1979) FEBS Lett. 99, 292-294
11. Niswesojo, P., and Hultin, H. (1976) Eur. J. Biochem. 67, 87-94
12. Noguchi, T., Fujiwara, S., and Hayashi, S. (1986) J. Biol. Chem. 261, 4221-4223
13. Watanabe, A., and Price, C. A. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6304-6308
14. Noguchi, T., Okuno, K., Takada, Y., Minatogawa, Y., Okai, K., and Kido, R. (1978) Biochem. J. 169, 113-122
15. Leighton, F., Poole, B., Lazarow, P. B., and de Duve C. (1969) J. Cell Biol. 41, 521-535

<sup>3</sup>S. Hayashi, S. Fujiwara, and T. Noguchi, unpublished data.
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16. Hsieh, B., and Tolbert, N. E. (1976) J. Biol. Chem. 251, 4408-4414

17. Ouchterlony, O. (1949) Acta Pathol. Microbiol. Scand. 26, 291-304

MATERIALS AND METHODS

Methods: — larvae (Salmo gairdneri), young whitefish (Salmo gairdneri), trout (Salmo gairdneri), brown trout (Salmo gairdneri), and pike perch (Stizostedion lucioperca) were used. Peroxisomes were isolated from the livers of these fish. Peroxisomal fractions were prepared by differential centrifugation and by a discontinuous gradient of sucrose. The isolated peroxisomes were washed with 50 mM potassium phosphate buffer, pH 7.5, and resuspended in 50 mM potassium phosphate buffer, pH 7.5. The peroxisomal fractions were then analyzed by SDS-PAGE and immunoblotting.

Immunoblotting: — SDS-PAGE was performed using a 12% polyacrylamide gel. The peroxisomal fractions were then transferred to a nitrocellulose membrane and probed with antibodies against peroxisomal proteins. The blots were then reacted with secondary antibodies conjugated to horseradish peroxidase and visualized with an enhanced chemiluminescence detection system.

Other Methods: — Determination of protein, isoelectric focusing, polyacrylamide gel electrophoresis in the presence or absence of sodium dodecyl sulfate, and determination of protein concentration by immunoblotting were performed as described previously.

Supplemental Material: — Determination of hepatic allantoinase and purification of its peroxisomal membrane-bound form

Degradation of Uric Acid in Fish Liver Peroxisomes:

INTRODUCTION

By

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Fig. 3 In vitro peroxosomal localization of uricase-like activity. Peroxosomal fractions (fractions 3-10) were incubated with uricase (0.5 U/mg protein) and uric acid (10 mM) for 1 h at 37°C. Uricase activity was measured by the decrease in uric acid concentration. The results are expressed as the ratio of uricase activity in the peroxisomal fraction to that in the non-peroxisomal fraction.

Fig. 4 In vitro peroxosomal localization of uricase activity. Peroxosomal fractions (fractions 3-10) were incubated with uricase (0.5 U/mg protein) and uric acid (10 mM) for 1 h at 37°C. Uricase activity was measured by the decrease in uric acid concentration. The results are expressed as the ratio of uricase activity in the peroxisomal fraction to that in the non-peroxisomal fraction.
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Fig. 5 Immunization of peroxisomal allantoinase with the antibody raised against saurine allantoinase. A shows the immunization of saurine allantoinase, and B shows that of mackerel allantoinase. Details of the immunization are described in the text. Peroxisomes were prepared from saurine and mackerel liver and were solubilized by the addition of nonyl-β-thioglycolamide at the final concentration of 1.25%. ●, peroxisomes; ○, peroxisomal extract.

Fig. 7 Ouchterlony double diffusion analysis of the peroxisomal membrane allantoinase and the peroxisomal matrix allantoinase. The center well (I) contained 25 µl of antiserum against saurine allantoinase. Peroxisomal matrix allantoinase purified from saurine liver. Wells 2 contained the peroxisomal membrane allantoinase from saurine liver. Wells 3 contained the peroxisomal membrane allantoinase from mackerel liver.

| TABLE 1 |
| --- | --- | --- | --- | --- |
| Purification of allantoinase from mackerel liver | Details of purification and assay methods are given in the text. | | | |
| Protein | Total activity | Specific activity | Yield | Purification |
| Homogenate | 7655 | 371 | 0.41 | 100 | 1 |
| LiCl (peroxisome-enriched fraction) | 2067 | 143 | 0.074 | 63.2 | 1.5 |
| Peroxisomal membrane | 106.9 | 154 | 1.54 | 41.2 | 31.8 |
| Membrane extract | 12.56 | 123 | 9.82 | 33.2 | 200 |
| Sepharose 6-200 | 3.30 | 73.9 | 22.42 | 19.9 | 464 |
| DEAE-cellulose | 1.29 | 56.3 | 43.56 | 15.2 | 880 |
| Hydroxylapatite | 0.63 | 40.6 | 64.58 | 11.0 | 1337 |
| Polycrylamide gel electrophoresis | 0.11 | 8.13 | 73.91 | 2.19 | 1530 |