ON THE SUBMITOCHONDRIAL LOCALIZATION OF L-KYNURENINE-3-HYDROXYLASE[1/]

Hiroshi Okamoto[2/], Shozo Yamamoto, Mitsuhiro Nozaki and Osamu Hayaishi

Department of Medical Chemistry
Kyoto University Faculty of Medicine
Kyoto, Japan

Received December 28, 1966

Since the discovery (De Castro et al., 1956) of a mitochondrial enzyme system capable of hydroxylating L-kynurenine to 3-hydroxy-L-kynurenine, earlier investigations (Saito et al., 1957; Stevens and Henderson, 1959) indicated that L-kynurenine-3-hydroxylase was associated with the mitochondrial fraction, but exact localization of this enzyme has not been established. Extensive studies of cell fractionation have been worked out by De Duve et al. (1955). From results using their method[3/] and from

---

1/ This investigation has been supported in part by research grants from the National Institutes of Health (CA 04222), the Jane Coffin Childs Memorial Fund for Medical Research, the Squibb Institute of Medical Research, and the Scientific Research Fund of the Ministry of Education of Japan.

2/ California Foundation for Biochemical Research Postgraduate Fellow.

3/ In experiments to be published elsewhere, we separated the heavy and light mitochondrial fractions according to the method of De Duve et al. (1955) and examined the distribution of L-kynurenine-3-hydroxylase in relation to heavy and light mitochondrial marker enzymes, namely monoamine oxidase (Baudhuin et al., 1964) and acid phosphatase (Appelmans et al., 1955), respectively. L-Kynurenine-3-hydroxylase came down predominantly with the heavy mitochondrial fraction.

electron microscopic observations[4/] on preparations as described below, we have confirmed that L-kynurenine-3-hydroxylase is exclusively localized in rat liver mitochondria.

In the present communication, submitochondrial distribution of L-kynurenine-3-hydroxylase in rat liver mitochondria was investigated after mild digitonin treatment under isotonic conditions. Monoamine oxidase, cytochrome oxidase and malate dehydrogenase in the submitochondrial fractions were also assayed to serve as reference enzymes. The mitochondrial L-kynurenine-3-hydroxylase activity could be detected when an intact mitochondrial preparation was used, and was found in the outer membrane fraction of mitochondria.

Rat liver mitochondria, prepared by the method of Sone and Hagihara (1964), were washed three times with 0.25 M sucrose. Subfractionation of the mitochondria by digitonin treatment was carried out by essentially the same method as described by Schnaitman et al. (1966). A 2% digitonin solution was prepared by adding digitonin (Sigma Chemical Co.) to warm 0.25 M sucrose and mixing for 30 minutes in a boiling water bath. The resulting solution remained clear for about 60 minutes at 0°. To 7 ml of the mitochondrial suspension, which contained 200 mg protein in 0.25 M sucrose, in an ice bath, 1.8 ml of the cold 2% digitonin solution was added with continuous stirring. The suspension was further stirred for 20 minutes at 0° and then diluted by the addition of 30 ml of cold 0.25 M sucrose. The diluted suspension was fractionated by differential centrifugation as follows: the suspension was centrifuged at 9,500 X g for 10 minutes and the su-

4/ Electron microscopic observations of the mitochondrial preparations used indicated no evidence for the presence of either lysosomes or microbodies. Details will be published shortly.

pernatant fluid and fluffy layer were decanted and combined. The decanted liquid was then centrifuged at 40,000 X g for 10 minutes. This resulted in a brown pellet and a yellow supernatant fluid. The supernatant fluid was further fractionated by centrifugation at 105,000 X g for 90 minutes, resulting in a firm reddish brown pellet and a clear, pale yellow supernatant fluid. Monoamine oxidase was selected as an outer membrane marker (Schnaitman et al., 1966), cytochrome oxidase as an inner membrane marker (Brosemer et al., 1963), and malate dehydrogenase as a matrix marker according to Schnaitman et al. (1966).

Effect of Hypotonic Treatment on Intact Mitochondria——As shown in Table I, with intact mitochondrial preparations, only

Table I

Experiments with Intact and Hypotonically Treated Mitochondria

The kynurenine hydroxylase activity of intact mitochondria was measured at 23° in the presence of 0.25 M sucrose by the method of Saito et al. (1957) in an assay system containing 80 mM Tris-acetate buffer, pH 8.1, 10 mM potassium chloride, 0.14 mM NADPH, 0.1 mM L-kynurenine, and enzyme preparation, in a final volume of 3 ml. The enzyme activity of hypotonically treated mitochondria was measured after preincubation of mitochondria in 80 mM Tris-acetate buffer, pH 8.1, for 25 minutes at 23°, by the same assay system. The numbers represent the decrease in optical density at 340 mμ during a 3 minute period, as determined by a Shimadzu MPS-50L spectrophotometer. This instrument is designed to allow spectrophotometry with turbid specimen.

Mitochondria	derived from g liver	Without kynurenine	With kynurenine	Δ
Intact	0.12	0.005	0.038	0.033
	0.24	0.009	0.072	0.063
Hypotonically treated	0.12	0.020	0.057	0.037
	0.24	0.033	0.101	0.068

little oxidation of NADPH was observed as judged by the decrease in absorbancy at 340 mμ and with hypotonically treated mitochon-

drial preparation NADPH was rapidly oxidized, in good agreement with previous reports (Lehninger, 1951; Okamoto et al., 1966). However, once kynurenine was added to these reaction mixtures, there occurred a rapid oxidation of NADPH.

Intact mitochondria catalyzed kynurenine-dependent NADPH oxidation approximately as rapidly as did hypotonically treated mitochondria and these kynurenine-dependent NADPH oxidations depended on the amounts of mitochondria used. These results corresponded closely with experiments measuring 3-hydroxy-L-kynurenine formation as detected by the increase in optical density at 400 mμ (Saito et al., 1957; Stevens and Henderson, 1959).

Fractionation of Mitochondria by Digitonin Treatment——In Table II are listed the distribution patterns recorded in the present experiments. To allow a closer comparison with the reference enzymes, the patterns for kynurenine hydroxylase are shown together with those observed for monoamine oxidase in parallel experiments. Fifty per cent of the kynurenine hydroxylase activity was found in the 105,000 X g pellet, and the increase in specific activity was about 12-fold that of the unfractionated preparation. On the contrary, almost all the activity of cytochrome oxidase and malate dehydrogenase was found in other fractions, indicating that these have well been separated from the kynurenine hydroxylase activity by the differential centrifugation procedure.

Discussion——The present evidence of the correlative appearance of monoamine oxidase activity and kynurenine hydroxylase activity in digitonin-treated mitochondrial preparation, considered together with electron microscopic findings[5] on the 105,000 X g pellet, suggest strongly the localization of L-kynurenine-3-hydroxy-

5/ Unpublished observation.

Table II

Fractionation of Mitochondria by Digitonin Treatment

Protein was determined by the method of Lowry et al. (1951). Kynurenine hydroxylase was measured by the same assay system as in Table I, and expressed in mμmoles NADPH oxidized per minute per mg protein. Monoamine oxidase was assayed by a modification of the method of Tabor et al. (1954) by following the formation of benzaldehyde spectrophotometrically at 250 mμ in an assay system containing 5 mM benzylamine hydrochloride and 50 mM phosphate buffer, pH 7.5, and expressed in mμmoles benzaldehyde produced per minute per mg protein. Cytochrome oxidase was assayed polarographically by measuring the O_2 consumption with the use of a rotating Pt electrode in an assay system containing 75 mM phosphate buffer, pH 7.5, 0.03 mM cytochrome c, 3.75 mM sodium ascorbate, and 0.3 mM N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride, and expressed in μ atoms oxygen consumed per minute per mg protein. Malate dehydrogenase was determined by the method of Ochoa (1955) in an assay medium containing 1 mM amytal to inhibit respiratory oxidation of NADH, and expressed in μmoles NADH oxidized per minute per mg protein. In order to insure full activity all fractions excepting 105,000 X g (S) were sonicated for about 1 minute prior to assaying for cytochrome oxidase and malate dehydrogenase (Schnaitman et al., 1966). Sonic disruption was not required for the estimation of kynurenine hydroxylase (Saito et al., 1957) and monoamine oxidase (Schnaitman et al., 1966). (P) and (S) indicate pellet and supernatant, respectively.

Fractions	Protein		Kynurenine hydroxylase		Monoamine oxidase		Cytochrome oxidase		Malate dehydrogenase	
	mg	%	sp.act.	%	sp.act.	%	sp.act.	%	sp.act.	%
Unfractionated (digitonized)	200	100	4.29	100	10.2	100	0.36	100	2.01	100
9,500 X g (P)	61.6	32	1.34	9.6	3.2	9.7	0.48	41	2.59	40
40,000 X g (P)	45.2	23	7.08	37	15.1	33	0.78	49	1.43	16
105,000 X g (P)	8.4	4.2	49.8	49	141	58	0.40	4.7	0.37	0.8
105,000 X g (S)	83.8	42	0.06	0.6	0.49	2.0	0.00	0.0	2.26	47
Recovery		100		96		103		95		104

lase in the outer membrane. Furthermore, this result is consistent with the appearance of L-kynurenine-3-hydroxylase activity in intact mitochondria.

The data reported in this communication may provide clues toward elucidating the conditions under which the kynurenine hydroxylating system operates, as well as to understanding mitochondrial outer membrane function.

Acknowledgments——We are indebted to Dr. Yasuhisa Nakamura, Department of Anatomy, Kyoto University Faculty of Medicine, for his kind collaboration with electron microscopy, and to Dr. Bernard J. Finkle for his kind help in the preparation of this manuscript.

REFERENCES

Appelmans, F., Wattiaux, R., and De Duve, C., Biochem. J., 59, 438 (1955).
Baudhuin, P., Beaufay, H., Rahman-Li, Y., Sellinger, O. Z., Wattiaux, R., Jacques, P., and De Duve, C., Biochem. J., 92, 179 (1964).
Brosemer, R. W., Vogell, W., and Bücher, Th., Biochem. Z., 338, 854 (1963).
De Castro, R. T., Price, J. M., and Brown, R. R., J. Am. Chem. Soc., 78, 2904 (1956).
De Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F., Biochem. J., 60, 604 (1955).
Lehninger, A. L., J. Biol. Chem., 190, 345 (1951).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).
Ochoa, S., in S. P. Colowick and N. O. Kaplan (Editors), Methods in enzymology, Vol. I, Academic Press, Inc., New York, 1955, p. 735.
Okamoto, H., Ichiyama, A., and Hayaishi, O., Arch. Biochem. Biophys., in press (1966).
Saito, Y., Hayaishi, O., and Rothberg, S., J. Biol. Chem., 229, 921 (1957).
Schnaitman, C., Erwin, V., and Greenawalt, J., J. Cell Biol., submitted (1966).
Sone, N., and Hagihara, B., J. Biochem. (Tokyo), 56, 151 (1964).
Stevens, C. O., and Henderson, L. M., J. Biol. Chem., 234, 1191 (1959).
Tabor, C. W., Tabor, H., and Rosenthal, S. M., J. Biol. Chem., 208, 645 (1954).