FLAVIN ADENINE DINUCLEOTIDE REQUIREMENT FOR KYNURENINE HYDROXYLASE OF RAT LIVER MITOCHONDRIA[1]

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L-Kynurenine-3-hydroxylase, a monooxygenase, catalyzes the conversion of L-kynurenine to 3-hydroxy-L-kynurenine with the stoichiometric utilization of NADPH and molecular oxygen (De Castro et al., 1956; Saito et al., 1957). Quite recently, data (Okamoto et al., 1967) have been presented to show that kynurenine hydroxylase is localized in the outer membrane fraction of rat liver mitochondria, and that it is possible to achieve about 12-fold concentration of the enzyme activity as compared with that of the original mitochondrial preparation.

In this communication, the kynurenine hydroxylase activity of mitochondrial outer membrane fraction was investigated after acid ammonium sulfate treatment. Available evidence indicates that

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flavin adenine dinucleotide is required for the hydroxylase activity.

Experimental——All the procedures were carried out at 0 - 4°. The outer membrane fraction of rat liver mitochondria was prepared as previously described (Okamoto et al., 1967) and suspended in 0.25 M sucrose solution containing 0.1 mM EDTA with a glass homogenizer. To 3 ml of the suspension, which contained approximately 4.5 mg of protein per ml, were added 1.3 g of solid ammonium sulfate with continuous stirring, and the mixture was adjusted to pH 4.5 or 3.2 with 1 N acetic acid, and further stirred for 10 minutes. The suspension was then centrifuged at 105,000 X g for 20 minutes, and the pellet was suspended in 1.8 ml of 100 mM Tris-acetate buffer, pH 8.1, containing 0.25 M sucrose and 0.1 mM EDTA. The preparations thus obtained will be referred to as "pH 4.5 and 3.2 ammonium sulfate fractions", respectively. L-Kynurenine-3-^{14}C was prepared from L-tryptophan-3-^{14}C (obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England) according to the method of Hayaishi (1953), using partially purified tryptophan oxygenase (Ishimura et al., 1966) and rat liver formamidase (Knox, 1955). The purity of the ^{14}C-labeled-L-kynurenine was checked by chromatographic and spectrophotometric examinations. Upon paper chromatography, only one radioactive spot corresponding to L-kynurenine was obtained in several different solvent systems (Nishizuka et al., 1965). The specific activity was calculated to be 20,000,000 cpm per μmole on the basis of the optical density of L-kynurenine at 360 mμ (ϵ_{360} = 4,600, pH 7.0) (Hayaishi, 1953). Protein was determined by the method of Lowry et al. (1951).

Requirement of FAD for Kynurenine Hydroxylase in Experiments of NADPH Oxidation——As shown in Table I, the addition of FAD had no effect on the hydroxylase activity of outer membrane fraction.

Table I

Requirement of FAD for Kynurenine Hydroxylase

The reaction mixture contained 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. After a 5-min incubation with 10 μM FAD or 10 μM FMN, the reaction was started at 24° by the addition of kynurenine to the reaction mixture. The numbers represent the kynurenine-dependent NADPH oxidation expressed in mμmole per min per mg protein, as determined by a Shimadzu MPS-50L spectrophotometer. When boiled enzyme was used in place of pH 3.2 ammonium sulfate fraction, no activity was observed.

Addition	Enzyme Preparation		
	Native	Acid Ammonium Sulfate Treatment at	
		pH 4.5	pH 3.2
None	47.0	28.2	0
FAD	47.3	45.1	4.2
FMN	--	32.9	0.3

When the outer membrane fraction was treated with ammonium sulfate at pH 4.5, the treated fraction by itself had 60 per cent of the original activity. The activity was almost completely recovered upon the addition of 10 μM FAD, but FMN showed no significant effect. The fraction treated with ammonium sulfate at pH 3.2 was entirely devoid of kynurenine hydroxylase activity. On the addition of FAD, such a preparation showed about 9 per cent of the original activity (Table I). Further addition of boiled outer membrane fraction extract did not increase the enzyme activity. No significant activity was observed when FMN was added in place of FAD. Thus far, no conditions have been found which resulted in complete resolution without denaturing the enzyme protein. The concentration of FAD which gave the 50 per cent maximal enzyme activity was estimated to be approximately 0.3 μM (Fig. 1).

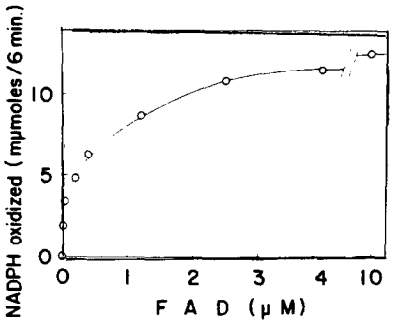

Fig. 1. Effect of FAD Concentration on Kynurenine Hydroxylase Activity. The pH 3.2 ammonium sulfate fraction (0.6 mg protein) was incubated for 5 min at 24° with varying amounts of FAD in the same reaction mixture of Table I. The reaction was started by the addition of 0.1 mM L-kynurenine.

Chromatographic Determination of 3-Hydroxykynurenine Formed—In order to study more fully the participation of FAD in the formation of 3-hydroxykynurenine from kynurenine, experiments were performed with the pH 3.2 ammonium sulfate fraction using L-kynurenine-^{14}C as substrate. The reaction mixture (1 ml) contained 3.6 mμmoles of L-kynurenine-^{14}C (72,000 cpm), 0.1 μmole of cold L-kynurenine, 10 μmoles of potassium chloride, 0.3 μmole of NADPH, 50 μmoles of Tris-acetate buffer, pH 8.1, and pH 3.2 ammonium sulfate fraction (0.6 mg protein). The reaction mixture containing 5 mμmoles of FAD (or FMN) or none was incubated aerobically at 24° for 3 hours, centrifuged, and 0.15 ml of the supernatant solution of the reaction mixture was applied on a paper with carrier 3-hydroxy-L-kynurenine and L-kynurenine. Separation of 3-hydroxy-L-kynurenine and L-kynurenine was carried out by ascending paper chromatography on Whatman No. 3 filter paper by use of a mixture of methanol, n-butanol, benzene, water (2:1:1:1 volume) containing 1 per cent 15 N ammonium hydroxide solution (De Castro et al., 1956) as a solvent.

As shown in Table II, with the addition of FAD, the amount of 3-hydroxykynurenine formed was increased, being accompanied with stoichiometric disappearance of kynurenine. On the contrary, when

Table II

Effect of Flavin Nucleotides on 3-Hydroxykynurenine Formation

The experimental conditions are described in the text. The area on the chromatogram corresponding to 3-hydroxy-L-kynurenine or L-kynurenine as determined by fluorescence (De Castro et al., 1956) under a Mineralight lamp was cut off and the radioactivity was assayed by a Packard Tri-Carb liquid scintillation spectrometer (Wang and Jones, 1959) with 0.5 per cent 2,5-diphenyloxazole and 0.03 per cent 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene in toluene as the counting solution. Numbers represent millimicromoles per total reaction mixture.

Addition	Kynurenine Consumed	3-Hydroxykynurenine Formed
	mμmole	mμmole
None	2.1	2.0
FAD	32.7	30.0
FMN	4.2	1.7

FMN had been added in place of FAD, the amount of 3-hydroxykynurenine was only about 6 per cent of that found in the presence of FAD, and this value was essentially similar to that obtained in the absence of cofactors. Similar results were obtained when 3-hydroxykynurenine formation in the course of the reaction was determined by the increase in optical density at 400 mμ[3/].

Discussion——The impairement of conversion of kynurenine to 3-hydroxykynurenine by riboflavin deficiency has already been indicated by the data relating to the nutritional studies on the effect of vitamin B_2 on tryptophan metabolism (Porter et al., 1948; Charconnet-Harding et al., 1953; Mason, 1953; Henderson et al., 1951, 1955; Stevens and Henderson, 1959). However, the participation of flavins in the hydroxylating system has not yet been definitely established. Our present experiments with the use of the acid am-

3/ Unpublished observation.

monium sulfate-treated mitochondrial outer membrane fraction revealed the possible co-enzyme property of FAD for the kynurenine hydroxylase. Recently, the reactivation of kynurenine hydroxylase of acetone-treated rat liver mitochondria by phospholipids has been demonstrated by Mayer and Staudinger (1967). Although further investigation is necessary to determine the exact role of factors responsible for the kynurenine hydroxylation, the data reported in this communication may provide clues toward elucidating the conditions under which the kynurenine hydroxylating system operates.

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