Vol. 104, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1982 Pages 680-685

LIPOAMIDE DEHYDROGENASE IN VITAMIN B_2-DEFICIENT RAT LIVER

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Received December 1, 1981

The liver and the kidney of rats fed a B_2-deficient diet showed a decrease in lipoamide dehydrogenase(LADase) activity, 65% and 80% of those of rats fed on a B_2-supplemented diet, respectively, but the heart showed no decrease. The liver of B_2-deficient rats showed also a little decrease in the activity of glutathione reductase, while there were no differences in the activities of GOT and LDH in the liver between the B_2-deficient and B_2-supplemented rats. The activity of LADase in the cytosolic fraction of the liver, which was about one-tenth of that of mitochondrial fraction, was decreased in the B_2-deficient rats in a degree almost equal to the decrease in the activity in the mitochondrial fraction. The results obtained with single radial immunodiffusion test indicated that the decrease in the activity of LADase in B_2-deficient rats resulted from the decrease in the amount of the enzyme protein and that not only the livers of B_2-supplemented rats, but also of B_2-deficient rats contained no apoenzyme of LADase.

INTRODUCTION

Lipoamide dehydrogenase(LADase) is a component of α-ketoacid dehydrogenase complexes which consist of three kinds of enzymes and is located in the mitochondria(1-3). We suggested that the cytosolic LADase is different from the miotochondrial LADase, and that LADase is synthesized on free polyribosomes in rat liver(4). The final purpose of this work is to elucidate the mechanism of biosynthesis of LADase. Although Hamm et al(5) reported that in Arthrobacter oxidans, 6-hydroxy-D-nicotine oxidase is synthesized in vitro only when FAD covalently binds to the apoenzyme, the enzymatic processes involved in the formation of holoenzymes from nascent peptides and FAD are not clearly understood as yet. The present paper describes the change of LADase activity in the liver of vitamin B_2-deficient rat as a step to clarify the biosynthesis of LADase. The results showed that the decrease in the activity of LADase in B_2-deficient rat was due to the decrease in the amount of the enzyme protein and

0006-291X/82/020680-06$01.00/0

that not only the livers of B_2-supplemented rats, but also of B_2-deficient rat contained no apoenzyme of LADase.

MATERIALS AND METHODS

Animals. In order to get B_2-deficiency, male Wistar weighing 80 g were fed on the B_2-deficient diet for one month. As a control, male Wistar rats weighing 80 g were fed on the B_2-deficient diet supplemented with 30 mg of vitamin B_2/Kg of diet for one month. The amount of diet for the B_2-supplemented rats was limited to be the same amount of diet that B_2-deficient rats ate on the preceding day.

Purification of lipoamide dehydrogenase. Lipoamide dehydrogenase from rat liver was purified to homogeneity as described previously(4).

Subcellular fractionation. Subcellular fractionation was carried out as described previously(4). The homogenate was centrifuged at 900 x g for 15 min and the supernatant was centrifuged at 15,000 x g for 15 min to obtain the mitochondrial fraction. The post-mitochondrial supernatant was further centrifuged at 105,000 x g for 1 hr, and the precipitate was recovered as the microsomal fraction. The supernatant was used as the cytosolic fraction.

Preparation of antisera raised against purified mitochondrial LADase. A rabbit antibody against purified mitochondrial LADase were prepared as described previously(4). The antibody gave only one precipitation line with the crude mitochondrial and cytosolic extracts and the purified LADase on the Ouchterlony double diffusion test. These lines were completely fused.

Assay of enzyme activities. The activity of LADase was measured using lipoic acid as substrate by following the decrease in absorbance at 340 nm(6), taking a millimolar extinction coefficient of NADH as 6.2(7). The activities of succinate-cytochrome c reductase and glutathione reductase were measured according to the methods of Stotz(8) and Racker(9), respectively. The activities of lactate dehydrogenase(LDH) and aspartate aminotransferase(GOT) were measured according to Bernstein et al(10) and Schwartz(11), respectively. One unit of each enzyme was defined as the amount of 1 μmole NADH oxidized or 1 μmole cytochrome c reduced per min.

Other methods. The flavin content in the purified enzyme was measured as follows. The enzyme was boiled for 2 min and then quikly cooled. After the removal of the denatured protein by centrifugation, flavin content in the supernatant was measured with a Hitachi 203 fluorescence spectrophotometer, comparing to the fluorescence intensity of a FAD standard.

Protein was determined by the method of Lowry et al(12) with bovine serum albumin as a standard.

Reagents. Cytochrome c and rotenone were purchased from Sigma Chemical Co., and NADH(grade II) was from Boehringer Mannheim GmbH. DL-lipoic acid was obtained from Wako Pure Chemical Industries, Ltd(Japan). Other chemicals used were commercial products of reagent grade.

RESULTS AND DISCUSSION

The LADase activities of liver, kidney and heart of rats fed a B_2-deficient and B_2-supplemented diets were shown(Table 1). LADase activities decreased significantly in liver and kidney of B_2-deficient rat, but the LADase did not decreased in heart. Hoppel et al(13) reported that the oxidation of pyruvate and α-ketoglutarate did not decrease in vitamin B_2-deficient rat liver. Therefore, it seems that LADase is not the rate-limiting enzyme in oxidation of pyruvate and α-ketoglutarate. The activities of LADase, glutathione

Table 1. LADase activities in the liver, kidney and heart of B_2-deficient and B_2-supplemented rats.

	Activity (Unit/g tissue)		
	Liver	Kidney	Heart
B_2-Deficiency	3.5 ± 0.2	4.1 ± 0.5	5.9 ± 0.6
B_2-Supplementation	5.2 ± 0.47	5.0 ± 0.4	5.7 ± 0.7
	$p<0.01$	$p<0.05$	

The values represent the mean(± S.D) of four rats.

reductase, succinate-cytochrome c reductase, aspartate aminotransferase and lactate dehydrogenase were measured in liver of B_2-deficient and B_2-supplemented rats(Table 2). LADase activity decreased significantly and glutathione reductase activity decreased a little in the B_2-deficient rat liver after one month, but the difference was statistically not significant. Succinate-cytochrome c reductase activity did not decrease. Hoppel et al(13) reported that in the liver of vitamin B_2-deficient rats the succinate dehydrogenase activity with 2.6-dichlorophenolindophenol as an electron acceptor decreases 50 %, but oxidation of succinate remained at a constant level, suggesting that succinate dehydrogenase is not rate-limiting in the case of succinate oxidation. Therefore, it is considered that succinate-cytochrome c reductase activity did not decrease in liver of B_2-deficient rat. Aspartate aminotransferase which has

Table 2. Several enzyme activities in liver of B_2-deficient and B_2-supplemented rats.

	LADase	Succinate-cytochrome c reductase	GOT	LDH	Glutathione reductase
B_2-Deficiency	2.94 ± 0.7	24.4 ± 3	87.5 ± 15	580 ± 68	7.4 ± 2.2
	(0.019 ± 0.0033)	(0.155 ± 0.011)	(0.6 ± 0.033)	(4.0 ± 0.6)	(0.046 ± 0.016)
B_2-Supplementation	5.12 ± 0.6	22.75 ± 1.5	83.0 ± 3.8	585 ± 134	8.6 ± 2.8
	(0.036 ± 0.003)	(0.15 ± 0.01)	(0.57 ± 0.054)	(4.2 ± 1)	(0.060 ± 0.023)
	$p<0.001$ ($p<0.001$)				

Each enzyme activity was measured as described in "MATERIALS AND METHODS". Values are the mean ± S.D. of five rats expressed as unit/g tissue and unit/mg protein in parenthesis. GOT; aspartate aminotransferase, LDH; lactate dehydrogenase.

Table 3. LADase activity in subcellular fraction of the liver of B_2-deficient and B_2-supplemented rats.

	Mitochondria	Cytosol	Microsomes
B_2-Deficiency	2.65 ± 0.52	0.2 ± 0.034	0.022 ± 0.0085
	(0.041 ± 0.004)	(0.0030 ± 0.0008)	(0.0013 ± 0.00035)
B_2-Supplementation	4.4 ± 0.74	0.36 ± 0.052	0.045 ± 0.012
	(0.060 ± 0.007)	(0.0080 ± 0.00066)	(0.0033 ± 0.00092)
	$p<0.01$	$p<0.01$	$p<0.05$
	($p<0.01$)	($p<0.001$)	($p<0.01$)

subcellular fractionation was carried out as described in "MATERIALS AND METHODS". Values are the mean ± S.D. of five rats expressed as unit/g tissue and unit/mg protein in parenthesis.

pyridoxal phosphate as the prosthetic group and lactate dehydrogenase which requires NAD as the coenzyme did not decrease.

Next, subcellular fractionation of the liver was carried out as described in "MATERIALS AND METHODS" and LADase activity in each fraction was measured with B_2-deficient and B_2-supplemented rats(Table 3). As shown in Table 3, the LADase activity in each fraction of the liver of B_2-deficient rats decreased in an almost same degree. LADase activity in supernatant was about one-tenth to one-fifteenth of that in mitochondria independently of B_2-deficient and B_2-supplemented rats. LADase activity in microsomes was very low. As reported previously(4), using succinate-cytochrome c reductase activity as a marker enzyme of mitochondria, the contamination of mitochondria into microsomes was about 3 %. On the other hand, the contamination of mitochondria into cytosol was about 2 %, juding from the distribution of the glutamate dehydrogenase as a marker enzyme of mitochondria. LADase activity in cytosol is somewhat higher than what can be explained by the contamination of mitochondria into cytocol(4). Moreover, since the degree of the inhibition by antiserum is different between cytosolic and mitochondrial LADase activities as reported earlier(4), we consider LADase in cytosolic fraction is of cytosolic origin. Figure 1 shows the result of single radial immunodiffusion test of the mitochondrial and cytosolic extracts, and the purified LADase. The plots of the

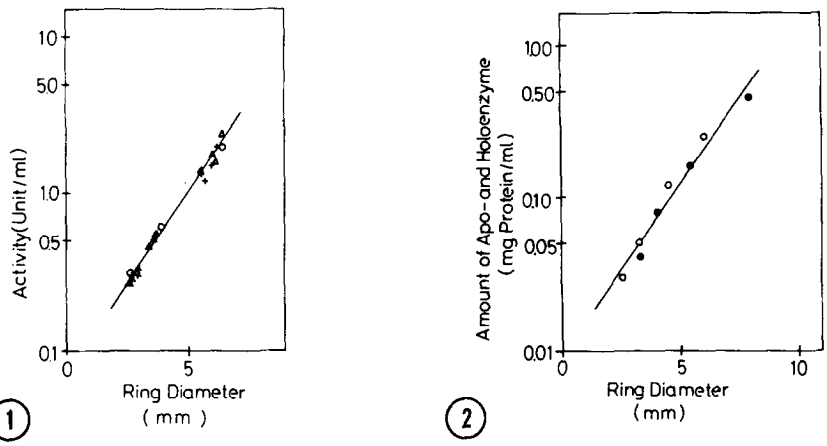

Figure 1. Single radial immunodiffusion of the purified LADase and the crude mitochondrial and cytosolic extracts from the liver of B_2-deficient and B_2-supplemented rats.

Mitochondrial(+) and cytosolic(×) extracts of liver of B_2-deficient rat, mitochondrial(△) and cytosolic(▲) extracts of liver of B_2-supplemented rat, and the purified mitochondrial LADase(○).

Figure 2. Single radial immunodiffusion of the purified LADase and apoenzyme.

Apoenzyme(○) was prepared from the purified LADase(●) according to the method of Veeger et al(13). Apoenzyme preparation shown in Figure contained about 15 % holoenzyme and 50 % apoenzyme returned to holoenzyme by the addition of FAD juding from LADase activity. Therefore, Figure shows that anti-LADase antibody not only reacted with holoenzyme, but also with apoenzyme and denatured apoenzyme which could not return to holoenzyme by the addition of FAD.

activities of purified LADase, which is considered to be holoenzyme from its FAD content(4), versus the ring diameters formed exhibited linear relationship and the plots of LADase activities in the mitochondrial and cytosolic extracts from both B_2-deficient and B_2-supplemented rats versus the ring diameters stood on the line depicted by the purified enzyme, suggesting that the decrease of LADase activity in the liver of B_2-deficient rats reflects the decrease in amount of enzyme protein. From this result and the facts that the addition of FAD to the liver homogenate from B_2-deficient rats caused no increase in LADase activity and that antiserum against the purified LADase could react and precipitate the apoenzyme of LADase as well as the holoenzyme(Fig.2), we consider that even the liver of B_2-deficient rats contains no apoenzyme of LADase. Following two possible concepts for the decrease in LADase activity in B_2-deficient rat will be presented from the result described above; 1. protein synthesis of LADase may be regulated by

vitamin B_2. 2. it may involve the quick degradation of the synthesized precursor or apoenzyme of LADase. Further detailed studies on the mechanism of the decrease of LADase in vitamin B_2-deficient rats are in progress.

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