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ABNORMAL NADPH-CYTOCHROME P-450 REDUCTASE IN THE LIVER MICROSOMES OF RIBOFLAVIN-DEFICIENT RATS

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SUMMARY: When rats were kept on a riboflavin-deficient diet, NADPH-cytochrome c and NADPH-ferricyanide reductase activities of the liver microsomes (deficient microsomes) decreased to 27% and 40% of the corresponding controls. To elucidate the unbalanced decrease of these activities in deficient microsomes, enzymological and immunochemical properties of the NADPH-cytochrome P-450 reductase in the liver microsomes of riboflavin-deficient rats were compared with those of control rats. Judging from quantitative immunoprecipitation, the amount of the reductase protein in the deficient microsomes was 67% of control, whereas the FAD and FMN contents in the immunoprecipitates were 110% and 59% of control, respectively. When the reductase was purified from the deficient microsomes, it contained 18.0 and 10.9 nmoles of FAD and FMN, respectively, per mg of protein, while the control enzyme contained 14.5 and 14.3 nmoles of the flavins, respectively. These and other lines of evidence suggest the existence of an abnormal NADPH-cytochrome P-450 reductase, having unbalanced contents of FAD and FMN, in deficient microsomes.

INTRODUCTION: A liver microsomal flavoprotein, NADPH-cytochrome P-450 reductase (Fpt), contains one mole each of FAD and FMN (1) and is functional in supplying reducing equivalents to cytochrome P-450 (2). Fpt can catalyze lipid peroxidation *in vitro* in the presence of ADP-ferric iron complex (3).

When rats and mice were kept on a riboflavin-deficient diet, a significant decrease of NADPH-cytochrome c reductase activity of the liver microsomes was found (4,5). However, it was not clear whether the decrease of the reductase activity was due to the decreased amount of the enzyme or to other factors, such as the modification of flavin contents.

Abbreviations: Fpt, NADPH-cytochrome P-450 reductase; Anti-Fpt, antibodies against Fpt purified from the liver microsomes of control rats; T-C, NADPH-cytochrome c reductase; T-F, NADPH-ferricyanide reductase; Deficient microsomes, the liver microsomes of riboflavin-deficient rats; Control microsomes, the liver microsomes of control rats; Deficient Fpt, Fpt purified from deficient microsomes; Control Fpt, Fpt purified from control microsomes. Definition: Abnormal Fpt, the Fpt having unbalanced contents of FAD and FMN found in the liver microsomes of riboflavin-deficient rats.

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Recent kinetic studies (6) suggested separate roles of the two species of flavins in the reductase. The preparation of an FMN-depleted enzyme (7,8) confirmed that FAD served as an electron acceptor from NADPH and FMN as an electron donor to cytochrome P-450, respectively.

In this communication, we report the existence of an abnormal Fpt, having an altered ratio of FAD to FMN, in the liver microsomes of riboflavin-deficient rats, which will be called "deficient microsomes".

MATERIALS AND METHODS:

Riboflavin-deficient rats: Wistar-King male rats, 3-weeks old, were fed on a riboflavin-free basal diet for 5-6 weeks as reported previously (9). Control rats were fed on a diet to which 40 mg of riboflavin per kg of the basal diet had been added. In a recovery experiment, riboflavin (50 mg/kg) was intraperitoneally injected to the riboflavin-deficient rats which were then killed to prepare liver microsomes after suitable time intervals.

Purification of Fpt's from deficient and control microsomes: Detergent-solubilized Fpt was purified by the method of Yasukochi and Masters (10) with some modification. SDS-polyacrylamide gel electrophoresis of purified Fpt was performed as described by Laemmli (11).

Immunochemical studies of Fpt's in deficient and control microsomes: Trypsin-solubilized Fpt was purified from the liver microsomes of control rats according to Omura and Takesue (12) and antibodies against the purified Fpt (Anti-Fpt) were prepared as described by Noshiro and Omura (13). Immunodiffusion analysis and quantitative immunoprecipitation of Fpt in solubilized microsomes were performed essentially according to Harada and Omura (14).

Enzyme assays and other analytical methods: Enzymatic reductions of cytochrome c, detergent-solubilized cytochrome b_5 and ferricyanide by NADPH were measured essentially as described by Omura and Takesue (12). Flavin determinations of purified Fpt and immunoprecipitated Fpt from solubilized microsomes were performed according to Yagi (15) and Faeder and Siegel (16), respectively. Protein was determined by the method of Lowry *et al.* (17) using bovine serum albumin as the standard.

Reagents and biochemicals: NADPH and cytochrome c (from horse heart) were purchased from Boehringer Mannheim GmBH. 2',5'-ADP-Sepharose 4B was from Pharmacia Fine Chemicals. FAD and FMN were from Sigma Chemical Co.Ltd and purified by DEAE-cellulose chromatography (18) before use. Other chemicals used were of reagent grade.

RESULTS:

NADPH-cytochrome c and NADPH-ferricyanide reductase activities in deficient and control microsomes: In order to clarify the mechanism of decrease of NADPH-cytochrome c reductase activity in deficient microsomes, we determined NADPH-cytochrome c (T-C) and NADPH-ferricyanide (T-F) reductase activities of the microsomes and compared them with those of control microsomes. As shown in Table 1, T-C and T-F activities in deficient microsomes decreased to 27% and 40% of control level, respectively. Moreover, T-F/T-C of deficient microsomes was 1.6, while that of control was 1.0. This ratio of the defi-

Table 1 Effects of Riboflavin Deficiency and Supplementation on NADPH-Cytochrome c (T-C) and NADPH-Ferricyanide (T-F) Reductase Activities of the Liver Microsomes of Riboflavin-deficient Rats

Feeding Condition	T-C	T-F	T-F/T-C
	U/mg protein (% of control)		(% of control)
Control	0.242 ± 0.013	0.253 ± 0.011	1.04 ± 0.05
Deficient	0.065 ± 0.010 (27%)	0.102 ± 0.015 (40%)	1.57 ± 0.04 (151%)
Deficient +Riboflavin (60 hours)	0.175 ± 0.005 (72%)	0.180 ± 0.017 (71%)	1.03 ± 0.12 (99%)

Each value represents the mean ± S.D. of three rats.

cient microsomes was further increased up to about 5 as the feeding with the deficient diet was continued for additional 2-3 weeks (data not shown). After riboflavin injection, T-C and T-F activities recovered to almost control levels in a few days and T-F/T-C decreased to 1.0, the control value.

Vermilion and Coon (7,8) reported that an FMN-depleted Fpt could not donate electrons to cytochrome P-450 and some other electron acceptors including cytochrome c and 2,6-dichlorophenolindophenol. They also noticed that the modified Fpt retained ferricyanide reductive activity. Therefore, the observed unbalanced ratio of T-F/T-C suggested an alteration in the contents of FAD and FMN in the Fpt of deficient microsomes.

Immunochemical comparison of Fpt's in deficient and control microsomes: The effect of Anti-Fpt on T-C and T-F activities of deficient and control microsomes were examined. As shown in Fig. 1, the inhibitions of T-C and T-F activities of deficient microsomes by Anti-Fpt were very similar to those of control microsomes. When solubilized deficient and control microsomes were examined by Ouchterlony double immunodiffusion analysis using Anti-Fpt, both microsomal samples showed a continuous single precipitation line (data not shown), suggesting that Fpt's in deficient and control microsomes are immunologically indistinguishable.

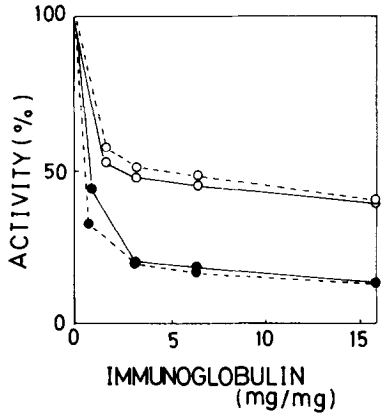

Fig. 1. Effect of Anti-Fpt on NADPH-Cytochrome c (T-C) and NADPH-Ferricyanide (T-F) Reductase Activities of the Liver Microsomes of Riboflavin-deficient and Control Rats
Microsomes (0.4 mg protein for T-C and 1 mg protein for T-F) were preincubated in 2 ml of reaction mixtures with various amounts of Anti-Fpt as indicated in the figure for 10 min at 25° C, and then T-C and T-F activities were measured.
--●--, T-C activity in deficient microsomes; —●—, T-C activity in control microsomes; --○--, T-F activity in deficient microsomes; —○—, T-F activity in control microsomes.

In order to explain the unbalanced activities of T-C and T-F of deficient microsomes, the amount of immunoprecipitable Fpt and the contents of FAD and FMN in the Fpt of deficient microsomes were determined by quantitative immunoprecipitation method. As shown in Table 2, the contents of immunoprecipitable Fpt and the total flavin in the immunoprecipitates obtained from deficient microsomes were 67% and 84% of respective controls. However, the FAD and FMN contents in the immunoprecipitates were 110% and 59%, respectively. After riboflavin injection (60 hours), total flavin content in the immunoprecipitates did not appreciably alter, but T-C and T-F activities of the microsomes recovered to almost control levels (Table 1). The ratio of FAD to FMN in the immunoprecipitates also decreased to about 1.0. These results indicated that the unbalanced activities of T-C and T-F of deficient microsomes were caused by an alteration in the ratio of two types of flavins in Fpt molecule, suggesting the possibility of formation of an FMN-depleted Fpt in deficient microsomes.

Purification and properties of Fpt's from deficient and control microsomes: The T-F/T-C ratios in the process of purification of Fpt's from deficient and control microsomes are shown in Table 3. In the purification of Fpt

Table 2 Effects of Riboflavin Deficiency and Supplementation on the Contents of Fpt, Total Flavin, FAD and FMN in the Fpt Immunoprecipitates of the Liver Microsomes of Riboflavin-deficient Rats

Feeding Condition	Fpt	Total Flavin	FAD	FMN
	μg/mg Ms (% of control)	nmoles/mg Fpt (% of control)		
Control	7.6	20.3	10.3	10.3
Deficient	5.1 (67%)	17.4 (84%)	11.3 (110%)	6.1 (59%)
Deficient +Riboflavin (60 hours)	6.5 (85%)	19.6 (95%)	8.6 (83%)	11.0 (107%)

Each value represents the mean of two rats.
Ms, microsomal protein; Fpt, immunoprecipitated NADPH-cytochrome P-450 reductase.

from deficient microsomes, T-F/T-C was 4.4-4.8 until the step of DE-52 chromatography, but it was lowered to 1.3 after 2',5'-ADP-Sepharose 4B affinity chromatography. On the other hand, the corresponding ratio of control Fpt was always 1.0. These results suggested that the affinity of abnormal Fpt from deficient microsomes to 2',5'-ADP-Sepharose 4B was different from that of control Fpt. Judging from SDS-polyacrylamide gel electrophoresis, the purified Fpt from deficient microsomes (deficient Fpt) was almost homogeneous (data not shown).

As shown in Table 4, NADPH-dependent reductions of cytochrome c and detergent-solubilized cytochrome b_5 by deficient Fpt were 67% and 64%, respec-

Table 3 T-F/T-C Ratios of Fpt's in the Process of Purification from the Liver Microsomes of Riboflavin-deficient and Control Rats

Purification Steps	T-F/T-C Deficient Fpt°	Control Fpt
Microsomes	4.6	1.0
Solubilized Microsomes	4.4	1.1
DE-52 Eluate	4.8	1.0
ADP-Sepharose Eluate	1.3	1.0

°, Deficient Fpt was prepared from the rats which were fed on a riboflavin-deficient diet for 7-8 weeks.
The T-C activities of deficient and control Fpt's at the step of ADP-Sepharose eluate were 23.0 and 37.0 U/mg protein, respectively.

Table 4 Catalytic Activities of Fpt's Purified from the Liver Microsomes of Riboflavin-deficient and Control Rats

Fpt	Cyt. c	Cyt. b_5	Ferri-cyanide
	U/mg protein (% of control)		
Control	34.3	0.14	34.0
Deficient	23.0 (67%)	0.09 (64%)	29.6 (87%)

tively, of those of control Fpt, but the ferricyanide reduction activity of deficient Fpt was 87% of control. To explain the unbalanced T-C and T-F activities of deficient Fpt, FAD and FMN contents in deficient Fpt were determined. As shown in Table 5, the total flavin content of deficient Fpt was 28.9 nmoles /mg of protein, which was almost the same with that of control Fpt. The FAD and FMN contents of deficient Fpt were 18.0 and 10.9 nmoles/mg of protein, respectively, while those of control Fpt were 14.5 and 14.3 nmoles/mg of protein, respectively. These results suggested that deficient Fpt contained an abnormal Fpt, in which FMN was replaced by FAD, as well as a normal Fpt having one mole each of FAD and FMN.

DISCUSSION: Determination of FAD and FMN contents in the Fpt purified from deficient microsomes suggested that the Fpt preparation contained an abnormal Fpt, in which FMN was replaced by FAD, in addition to normal Fpt having one mole each of FAD and FMN. The unbalanced activities of T-C and T-F observed with the deficient microsomes could be explained by this presumption, since an FMN-depleted Fpt could not donate electron to cytochrome c (7).

Table 5 Determinations of Total Flavin, FAD and FMN Contents in Fpt's Purified from the Liver Microsomes of Riboflavin-deficient and Control Rats

Fpt	Total Flavin	FAD	FMN
	nmoles/mg protein		
Control	28.8	14.5	14.3
Deficient	28.9	18.0	10.9

Each value represents the mean of triplicate determinations.

Fass and Rivlin (19) reported that the decrease of FMN was higher than that of FAD in the livers of riboflavin-deficient rats. We also observed such a tendency in the liver microsomes of riboflavin-deficient rats (data not shown). Vermilion and Coon (7) reported that FAD and riboflavin could bind to an FMN-depleted Fpt. These results together with ours suggest that the amount of FMN in the livers of riboflavin-deficient rats is not enough to saturate the binding-site of Fpt molecules in the microsomes, while a relatively excess amount of FAD is available to be inserted to the FMN binding-site of the molecules.

We noticed that a significant amount of T-F activity from deficient microsomes was lost in the purification of Fpt at the step of 2',5'-ADP-Sepharose 4B affinity chromatography. The affinity of abnormal Fpt from deficient microsomes to 2',5'-ADP-Sepharose 4B might be different from that of control Fpt. However, judging from the properties of the purified Fpt preparations, a considerable amount of abnormal Fpt which probably contained two moles of FAD per mole of enzyme was present in the Fpt preparation from the deficient microsomes. The isolation of abnormal Fpt and the elucidation of its physiological significance in the livers of riboflavin-deficient rats are now in progress.

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