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Purification of the Agent Inducing Lipid Peroxidation with Dihydroxyfumaric Acid in Rat Liver Mitochondria

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SUMMARY

Dihydroxyfumaric acid induces lipid peroxidation in rat liver mitochondria as reported previously. When the mitochondria were solubilized with 0.35% (W/V) sodium cholate, the supernatant itself could not catalyze lipid peroxidation with dihydroxyfumaric acid, but the precipitate slightly induced the reaction. The supernatant produced lipid peroxide in the presence of the precipitate and dihydroxyfumaric acid. The supernatant was heat sensitive contrary to the stability of the precipitate. An attempt was made to isolate active entity through a sephadex G-200 column and a DEAE-cellulose column, resulting in about 10-fold purification. At 408-410 nm the partially purified agent showed a maximum absorption, which disappeared rapidly after reduction with sodium dithionite and was slowly diminished with dihydroxyfumaric acid. The molecular weight was much larger than that of oxidized cytochrome c.

In 1963,Hochstein and Erster described an enzymatic lipid peroxidation system dependent upon the presence of NADPH,ADP and Fe^{3+} in rat liver microsomes(1). Since then, a NADPH-dependent lipid peroxidation system has been demostrated in rat liver mitochondria and beef heart submitochondrial particles(2-3).

We previously reported on some properties of DHF-induced lipid peroxidation system in rat liver mitochondria and microsomes(4-5). DHF-induced lipid peroxidation in mitochondria was heat sensitive. Therefore, we made an attempt to purify the heat-sensitive protein catalyzing the lipid peroxide formation with DHF.

METHODS

Solubilization of rat liver mitochondria

Mitochondria were isolated from the livers of male rats(200-300g) as previouly described(4). The mitochondria were suspended in 0.01M phosphate buffer(pH 7.5) containing 0.35%(W/V) sodium cholate and 0.15M KCl. After stirring for 40 min at 7°C, the suspension was centrifuged at 20,000 x g for 40 min. The supernatant, referred to as"Sup-1", was partially purified by a sephadex G-200 column and a DEAE-23 cellulose column. The precipitate, referred to as"Ppt-1", was washed two times in 0.01M phosphate buffer containig 0.15M KCl. In the present study on DHF-induced lipid peroxidation system, the Ppt-1 was used as substrate.

Estimation of lipid peroxidation

Thiobarbituric acid color reaction modified by Hunter et al(6) was used. Reaction mixture containing 0.5 mM DHF,Ppt-1 and Sup-1(or a fraction of each purification step) was shaken at 37°C under aerobic condition.

Abbreviations: DHF, Dihydroxyfumaric acid. MDA, Malondialdehyde.

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Other analytical methods

Protein was determined by the method of Lowry et al(7). Polyacrylamide disc gel electrophoresis was carried out according to the method described by Davis(8). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed following the method of Weber and Osborn(9).

MATERIALS

Dihydroxyfumaric acid, sodium cholate, cytochrome c(type III) and NADPH were obtained from Sigma Chemical Co., 2-thiobarbituric acid soium salt and NADH(grade II) were purchased from Nakarai Chemical Co., and Boehringer Mannheim GmbH, respectively. Sephadex and DEAE-23 cellulose were obtained from Pharmacia Fine Chemicals,Uppsala,and Whatman Biochemical.Ltd., respectively

RESULTS AND DISCUSSION

The mitochondria suspended in 0.01M phosphate buffer containing 0.35%(W/V) sodium cholate and 0.15M KCl were stirred for 40 min at 7°C, and then the suspension was centrifuged at 20,000 x g for 40 min. When only Sup-1 was incubated with DHF, malondialdehyde(MDA) formation was not observed, but Ppt-1 slightly induced the reaction with DHF. The addition of Sup-1 to the reaction mixture containing Ppt-1 and DHF stimulated MDA formation markedly (Fig.1). This MDA formation was inhibited by preincubation of Sup-1 at 100°C for 2 min. On the other hand, the preincubation of Ppt-1 at 100°C for 2 min had no effect on the reaction rate(Fig.2). At least one heat-sensitive enzyme may be involved in the DHF-induced lipid peroxidation. Thus, we attempted to isolate an active agent from Sup-1.

Procedure of Purification

Step.1 Sup-1 was concentrated by ultrafiltration through a Diaflo PM-10 membrane and was dialyzed overnight against phosphate buffer to remove sodium

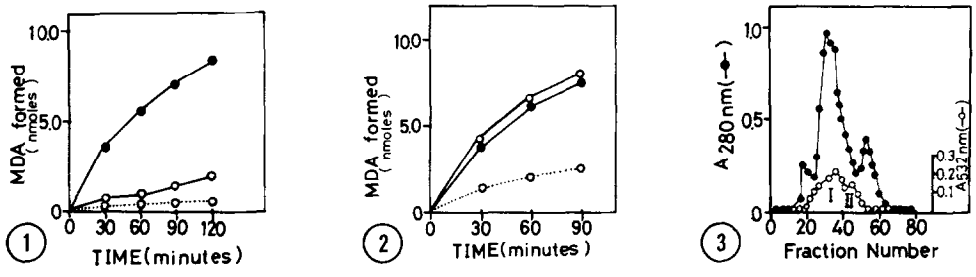

Fig. 1. DHF-induced lipid peroxidation in Sup-1 and Ppt-1. Mitochondria(1.5 mg protein/ml) were suspended in 0.01M phosphate buffer containing 0.35%(W/V) sodium cholate and 0.15M KCl, stirred for 40 min at 7°C, and then centrifuged at 20,000 x g for 40 min. The Ppt-1 was resuspended in 0.01M phosphate buffer(pH 7.5) containing 0.15M KCl, making the total volume equal to the starting suspension. o—o ; Ppt-1 + DHF(0.5 mM), o·····o ; Sup-1 + DHF(0.5 mM), ●—● ; Sup-1 + Ppt-1 + DHF(0.5 mM).

Fig. 2. Effects of heating on DHF-induced lipid peroxidation. Experimental details are described in the legend for Fig.1.The heated supernatant and precipitate were kept at 100°C for 2 min. ●—●; Control(Sup-1 + Ppt-1 + DHF), o—o; Heated Ppt-1 + Sup-1 + DHF, o·····o; Heated Sup-1 + Ppt-1 + DHF.

Fig. 3. Sephadex G-200 column chromatography of the Sup-1. Experimental procedure is described in the text. Fractions of 2.3 ml were used.

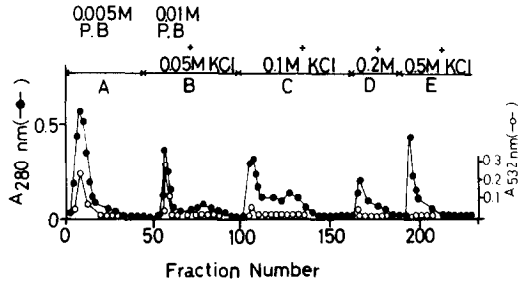

Fig. 4. DEAE-23 cellulose column chromatography of P-1.
Experimental procedure is described in the text. Fractions of 2.3 ml were used.
P.B ; phosphate buffer.

cholate. The dialysate added with 90% aceton was stirred for 20 min at -15°C, and then centrifuged at 5,000 x g for 5 min at -5°C. After the sediment was suspended in 0.01M phosphate buffer(pH 7.5) containing 0.15M KCl with constant stirring for 6 hr at 7°C, the solution was centrifuged at 20,000 x g for 40 mir Approximately 70-80% of the activity was recovered in the supernatant.

Step.II The supernatant concentrated by ultrafiltration through a Diaflo PM-10 membrane was applied to a sephadex G-200 column(2.2 x 44 cm) equilibrated with 0.01M phosphate buffer containing 0.15M KCl, followed by the elution with the same buffer. As shown in Fig. 3, the fractions containing MDA formation activity were eluted as two peaks(P-1 and P-II). The absorption spectrum of the P-II was similar to that of reduced cytochrome c. P-II was heat-insensitive(data are not shown). On the other hand, P-1 was heat-sensitive and combined together. Solid ammonium sulfate was slowly added to the combined solution with constant stirring to 60 % saturation. After the mixture was stirred for 20 min, it was centrifuged at 10,000 x g for 20 min. The precipitate was dissolved in a minimum volume of 0.005 M phosphate buffer and was dialyzed overnight against 5 liters of 0.005 M buffer.

Step. III The dialysate was applied to DEAE-23 cellulose column(1 x 15 cm) equilibrated with 0.005M buffer. After washing the column with 0.01M phosphate buffer, elution was carried out stepwise with 0.05M to 0.5M KCl. The elution profile is shown in Fig.4. The most purified peak B(P-B) eluted with 0.01M phosphate buffer containing 0.05M KCl was still proved to contain a few contaminations by subjecting it to a polyacrylamide disc gel electrophoresis. The procedure of purification is summarized in Table I. P-B was purified about ten times with a recovery of 0.1%.

The absorption spectra of the partially purified P-B in the oxidized and reduced state are shown in Fig.5. Oxidized form of P-B shows a maximum at 408-410 nm. When the protein was reduced with sodium dithionite, the absorption disappeared. According to Okuda et al(10), rat liver ferredoxin

Table I. Summary of purification procedure

Fractions	Protein (mg)	Specific activity (MDA nmoles/60 min/mg protein)	Total activity (MDA nmoles/60 min)	Protein recovery(%)
1.Supernatant (Sup-1)	350	32	11,200	100
2.Aceton treatment (90% aceton)	60	130	7,800	17
3.Sephadex G-200	16	74	1,184	4
4.60% Saturation of $(NH_4)_2SO_4$	9	105	648	2.5
5. DEAE-23 cellulose	0.4	298	119.2	0.1

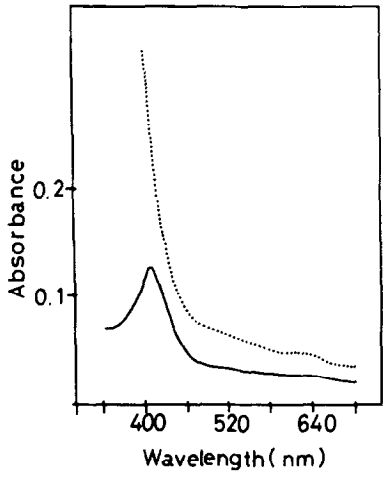

Fig. 5. Absorption spectra of the partially purified P-B.
———— ; oxidized form, ... ; reduced form.

Table II. Effects of various inducers.

Additions	Formed MDA (nmoles/mg protein/60 min)	
	P-B	P-II
DHF(0.5 mM)	50	7.6
Ascorbate(0.5 mM)	40	10
NADPH(0.5 mM)	0	0
NADH(0.5 mM)	0	0

P-B and P-II were incubated with Ppt-1 and DHF, Ascorbate,NADPH or NADH.

has an apparent molecular weight of 12400 and shows a maximum absorption at 408 nm with a small shoulder around 452nm, and this absorption disappeares after reduction with sodium dithionite. The molucular weight of P-B estimated

Table III. Effects of KCN, CO and H_2O_2 on P-B and P-II.

Reaction mixture	Concen.	Rate of MDA formation (%)	
		P-B	P-II
Control		100	100
+ KCN	0.5 mM	110	132
+ CO	bubbled for 3 min	110	77
+ H_2O_2	0.08 M	120	0
	0.8 M	150	0

from electrophoresis on sodium dodecly sulfate polyacrylamide gel was much larger than that of oxidized cytochrome c(mol. wt. 12384). P-B was slowly reduced by DHF, but was rapidly reduced by NADH or NADPH. When P-B or P-II was incubated with NADPH or NADH instead of DHF, the MDA formation was not observed(Table II). Table III shows effects of H_2O_2, KCN and CO on P-B and P-II. H_2O_2 completely inhibited MDA formation in P-II, but stimulated the reaction in P-B . In rat liver intact mitochondria, DHF-induced lipid peroxidation reported in the previous paper was not inhibited with H_2O_2 and was heat-sensitive. Therefore, P-B may play a main role in DHF-induced lipid peroxidation in mitochondria.

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