NADPH-DEPENDENT LIPID PEROXIDATION CATALYZED BY PURIFIED NADPH-CYTOCHROME C REDUCTASE FROM RAT LIVER MICROSOMES

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Summary - A purified preparation of rat liver microsomal NADPH-cytochrome c reductase has been shown to catalyze the NADPH-dependent peroxidation of isolated microsomal lipid. In addition to ADP and ferric ion required for NADPH-dependent lipid peroxidation in whole microsomes, this system requires high ionic strength and a critical concentration of EDTA. The peroxidation activity can be inhibited by superoxide dismutase suggesting that the superoxide anion, produced by this flavoprotein, is involved in the lipid peroxidation reaction.

Rat liver microsomes will catalyze an NADPH-dependent peroxidation of endogenous lipids (1). Tam and McCay (2) have shown that this reaction, which requires ferric ion and a chelating agent such as ADP or pyrophosphate, involves the transient formation of phospholipid peroxides which undergo further oxidation to a variety of products including malondialdehyde. Several lines of evidence suggest that this lipid peroxidation system is related to the mixed function oxidase of liver microsomes responsible for the oxidation of drugs, pesticides, polycyclic hydrocarbons, fatty acids and steroids: 1, both reactions require NADPH; 2, there is an apparent competition for reducing equivalents (3); and 3, there is an increase in lipid peroxidation activity when drug hydroxylation activity is induced by the administration of barbituates (4).

The NADPH-dependent flavoprotein, proposed to be involved in both lipid peroxidation and drug metabolism, is NADPH-cytochrome c reductase (1,5). In this report, we will present evidence to show that a purified, protease-solubilized form of this enzyme will catalyze the peroxidation of isolated microsomal lipid. In collaborative experiments done in Dr. M. J. Coon's laboratory at the University of Michigan, we have shown that this enzyme will also anaerobically reduce cytochrome P_{450} and support benzphetamine hydroxy-

lation with a cytochrome P_{450} fraction and added phosphatidylcholine (to be published in collaboration with Dr. Coon). Strobel and Coon (6) have proposed that the superoxide anion is involved in the hydroxylation mechanism and have shown that other superoxide generating systems will support hydroxylation when combined with cytochrome P_{450} and lipid. We have shown that this protease-solubilized enzyme is an excellent superoxide generator (7), and we will show in this report that the NADPH dependent peroxidations of lipid, catalyzed by this enzyme, can be inhibited by superoxide dismutase.

Methods

The preparation of rat liver microsomes and the purification of the microsomal NADPH-cytochrome c reductase have been previously reported (7,8). The purified reductase would reduce 55.7 μMoles of cytochrome c $min^{-1} mg^{-1}$ protein (0.3 M phosphate buffer, pH 7.5 at 25° C).

Total microsomal lipid was extracted under anaerobic conditions by the method of Folch et al. (9) and the amount of lipid was measured as lipid phosphorous by the method of Bartlett (10). Aqueous suspensions of lipid were prepared by sonication under anaerobic conditions using a Branson Model S 125 sonifier. This process produces multilayered lamellar structures commonly referred to as liposomes (11).

The NADPH-dependent peroxidation of unsaturated lipid, catalyzed by the purified NADPH-cytochrome c reductase, was assayed in the following manner. A suspension of liposomes (0.5 μMoles lipid phosphorus per ml) was incubated in a Dubnoff shaker (37° C), under an atmosphere of air in a reaction mixture containing 0.25 M Tris-HCl (pH 6.8 at 37° C), 0.25 M NaCl, 5×10^{-5} M EDTA, 2 mM ADP, 0.12 mM $Fe(NO_3)_3$, and 0.2 mM NADPH. Malondialdehyde concentrations, in aliquots removed from the incubation mixture at various times during the course of the reaction, were assayed by chromogen formation with thiobarbituric acid (12). The non-enzymatic rate of malondialdehyde production was determined prior to addition of the enzyme to the incubation mixtures, and

Table I. Requirements for the peroxidation of lipids by NADPH-cytochrome c reductase. The complete system in this table contained 0.15 μg ml^{-1} of NADPH cytochrome c reductase which was capable of reducing 7.1 nMoles of cytochrome c $min^{-1}ml^{-1}$ under the conditions used for lipid peroxidation. The deoxycholate-solubilized NADPH-cytochrome P_{450} reductase fraction (2.3 μg protein ml^{-1}) was capable of reducing 4.4 nMoles of cytochrome c $min^{-1}ml^{-1}$ under the conditions used for lipid peroxidation.

Description	Activity as nMoles Malondialdehyde $min^{-1}ml^{-1}$
Complete	1.79
Boiled enzyme	0.00
Minus NADPH	0.04
Minus ADP - Fe^{3+}	0.00
Minus EDTA	0.05
With deoxycholate-solubilized reductase	1.29

the rate of malondialdehyde production subsequent to enzyme addition is corrected for this non-enzymatic rate.

Results

The requirements for the NADPH-dependent peroxidation of isolated microsomal lipid, by purified NADPH cytochrome c reductase, are listed in Table I. The use of a deoxycholate-solubilized NADPH-cytochrome P_{450} reductase fraction, isolated by Strobel *et al.* (13), was equally as effective as the purified reductase on the basis of its NADPH-cytochrome c reductase activity. The time course of the reaction and the effect of ionic strength can be seen in Figure 1. The high ionic strength buffer required for maximal activity also decreases the non-enzymatic rate of peroxidation measured prior to the addition of the enzyme. This reaction also requires a critical concentration of EDTA (5×10^{-5} M) because higher concentrations approaching the ferric ion concentration (1.2×10^{-4} M) will inhibit the reaction (Figure 2). The use of buffers and reagents which had been passed through a column of Chelex 100 chelating resin had no effect on the requirement for EDTA.

The role of the superoxide anions produced by the purified reductase (7) was investigated by examining the effect of erythrocuprein, the protein which

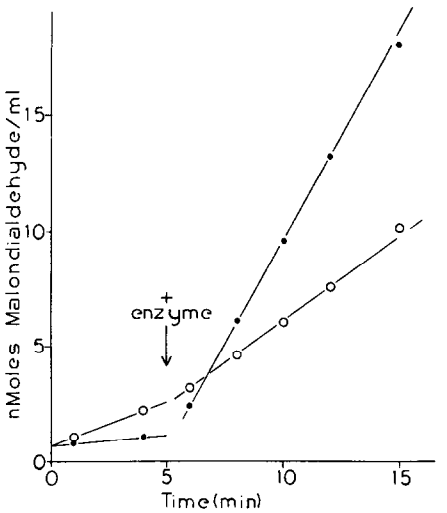

Fig. 1.

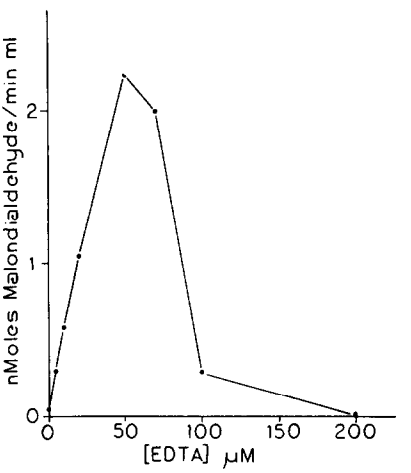

Fig. 2.

Figure 1. NADPH-dependent lipid peroxidation catalyzed by NADPH-cytochrome c reductase and the effect of ionic strength. (•) 0.25 M Tris-HCl and 0.25 M NaCl; (o) 0.05 M Tris-HCl with no NaCl.

Figure 2. Lipid peroxidation as a function of EDTA concentration.

catalyzes the dismutation of the superoxide anion (14), on the rate of peroxidation. The results of these experiments (Table II) demonstrate that the peroxidation reaction can be inhibited by more than 50% by erythrocuprein. If the erythrocuprein was heated in a boiling water bath for 5 min, which destroyed all superoxide dismutase activity as assayed by its ability to inhibit epinephrine oxidation (7), no inhibition of lipid peroxidation was observed. The effect of catalase indicates that the peroxide produced does account for some of the peroxidation but the amount of inhibition by catalase is always less than 20%. When catalase is included in the incubation mixture, the inhibition by erythrocuprein is even greater since the peroxidation produced by peroxide is now eliminated.

Discussion

Rat liver microsomes catalyze an NADPH-dependent peroxidation of endogenous

Table II. The inhibition of lipid peroxidation by erythrocuprein (Pentex) in the absence and presence of 100 μg/ml of catalase (Sigma Type C-100). The use of 50 μg/ml of catalase produced the same amount of inhibition.

Conditions	Minus Catalase		Plus Catalase	
	nMoles Malondialdehyde $min^{-1}ml^{-1}$	%	nMoles Malondialdehyde $min^{-1}ml^{-1}$	%
Control	1.46	100	1.28	100
+ 50 μg erythrocuprein ml^{-1}	.95	65	.61	48
+100 μg erythrocuprein ml^{-1}	.75	51	.45	35
+200 μg erythrocuprein ml^{-1}	.68	47		

lipids in the presence of ferric ion and a chelating agent. The results in this communication demonstrate the role of the microsomal NADPH-cytochrome c reductase in this process by the fact that the purified flavoprotein will catalyze the peroxidation of isolated lipid under similar conditions, if EDTA is also included in the reaction mixture. Lipid peroxidation catalyzed by this enzyme is inhibited by erythrocuprein suggesting that the superoxide anion is involved in the reaction. Further evidence for the role of NADPH-cytochrome c reductase in both lipid peroxidation and drug metabolism was obtained by showing that the NADPH-cytochrome P_{450} reductase fraction isolated by Strobel et al. (13) would catalyze lipid peroxidation in our system.

The requirement for EDTA is the most significant difference between the NADPH-dependent lipid peroxidation reaction in whole microsomes and the reaction catalyzed by the purified enzyme. This suggests that the microsomal system contains some component which performs the same function as the EDTA in our system. The inhibition produced by higher levels of EDTA suggests that ferric ion with ADP as a ligand is still required.

The demonstrated production of superoxide by the purified reductase (7) and the ability of erythrocuprein to inhibit the peroxidation reaction suggest that it is the superoxide anion, or a reactant derived from it, which initiates the peroxidation produced by the NADPH oxidase activity. As has been shown with whole microsomes by Pfeifer and McCay (15), a radical-like component is

produced by the peroxidation reaction which will lyse erythrocytes included in the reaction mixture. The peroxidation reaction catalyzed by the purified reductase with isolated lipid will also cause lysis of included erythrocytes, but the omission of lipid from the reaction mixture resulted in almost no hemolysis indicating that the component causing lysis is not the superoxide radical (to be published).

Since erythrocuprein is reported to quench singlet oxygen (16) which is produced by the non-enzymatic dismutation of superoxide (17), singlet oxygen may be the peroxidation initiator. The reaction of singlet oxygen with olefins to form allylic peroxides is a well known reaction (18) and it has recently been shown that soybean lipoxidase, which catalyzes the formation of peroxides of unsaturated fatty acids, will also catalyze other reactions characteristic of those produced by singlet oxygen (19). We intend to continue our investigation of the role of the superoxide anion in the lipid peroxidation reaction and also determine if other components of the microsome are involved.

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