NADPH-DEPENDENT REDUCTASE SOLUBILIZED FROM MICROSOMES BY PEROXIDATION AND ITS ACTIVITY

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Summary

Isolated rat liver microsomes were subjected to enzymatic or non-enzymatic lipid peroxidation in vitro. NADPH-dependent cytochrome c reductase activity was released from the microsomes into the media during peroxidation. This activity could be recovered from the media by DEAE-cellulose chromatography. The recovered enzyme retained high activity for the reduction of cytochrome c and a lower level of activity for the reduction of cytochrome P-450. The active fractions were capable of enzymatically supporting the peroxidation of isolated mitochondria in the presence of organically complexed Fe^{+3} and NADPH, and in this respect the specific activity was found to be about ten times higher than in microsomes.

Previous studies in our laboratories have demonstrated that, relative to other hepatic microsomal enzymes, NADPH-cytochrome c reductase was unaffected by rather severe conditions of lipid peroxidation (1). Furthermore, we were struck by the similarity between deoxycholate-treated microsomes and those which had undergone lipid peroxidation (1). We therefore have studied the possibility that intact NADPH-cytochrome c reductase can be solubilized from the hepatic microsomes of rats by lipid peroxidation as has been reported for deoxycholate treatment (2). Herewith are presented the results of these studies.

Methods

Male unstarved rats of the Sprague-Dawley strain weighing 150-200 g were killed by decapitation and the liver were immediately removed, perfused with ice-cold 0.25 M sucrose and weighed. After mincing, the livers were homogenized in 5 volumes of the cold sucrose solution with a glass-teflon homogenizer. The microsomes were prepared as described by Dallner (3), washed once with 0.15 M KCl and subsequently resuspended in ice-cold 0.25 M sucrose to a final

Table I. Malondialdehyde formation and the release of protein and phospholipid from isolated rat liver microsomes after *in vitro* lipid peroxidation[a]

Fraction	MDA[b]	Protein mg/ml	Protein %	Phospholipid mg/ml	Phospholipid %	PLP/protein
Control						
supernatant	15	0.15	15	0.02	4	0.10
pellet	-	0.89	85	0.48	96	0.54
Peroxidized						
supernatant	92	0.35	30	0.03	7	0.09
pellet	-	0.81	70	0.43	93	0.53

a The peroxidation system consisted of 0.03 M Tris-KCl buffer, pH 7.5, 0.2 mM sodium pyrophosphate, 0.02 mM ferric chloride, 0.2 mM ascorbic acid (or NADPH) and the microsomes from 100 mg of liver wet weight per ml. The control incubates were the same except for the omission of the Fe^{+3} pyrophosphate and electron donor. The incubations were carried out on 4 ml samples with continuous shaking at 37° for 20 minutes.

b The malondialdehyde (MDA) content of the media after peroxidation is given in nm MDA/mg protein in the 105,000 x g supernatant of the incubate.

volume containing the microsomes from 1 gm liver/ml. For later use the mitochondria were also isolated from the original homogenates by the method employed by Johnson and Lardy (4). The mitochondria were washed once with 0.25 M sucrose and once with 0.15 M KCl and then resuspended in 0.15 M KCl to a final concentration representing 2 gm liver wet wt/ml.

Lipid peroxidation of 0.3 ml aliquots of the microsomal suspension was carried out as we have described previously using either ascorbic acid or NADPH as the electron source (Table I). The incubates from 3 tubes were pooled and 0.5 ml aliquots were taken for assay for malondialdehyde content by the thiobarbituric acid reaction (5). The remaining pooled incubates were centrifuged at 105,000 x g for 2 hours and the resulting supernatants were decanted and pooled. Aliquots were taken for the determination of protein and phospho-

Table 2. Effect of lipid peroxidation on cytochrome c reductase activity of the 105,000 x g supernatants and pellets obtained after incubation[a].

Fraction	NADPH-dependent		NADH-dependent	
	nm/min/ml	nm/min/mg	nm/min/ml	nm/min/mg
Control				
supernatant	1	12	1	10
pellet[b]	57	86	900	1360
Peroxidized				
supernatant	8	28	3	10
pellet[b]	47	97	550	1040

a The activities are expressed in terms of nm substrate reduced/min per ml of fraction or per mg of protein in the fraction

b Pellets were resuspended in a volume of Tris-KCl buffer equal to the volume of the supernatants for enzyme assay

lipid content as described previously (1). The pellets obtained after centrifugation were also pooled and analyzed for protein and lipid content. Aliquots of both the supernatants and pellets were also reserved for enzyme assays. Incubates from microsomal suspensions not subjected to peroxidation were treated in the same manner and served as controls.

Approximately 150 ml of the supernatant from the peroxidized microsomes containing a total of 48 mg of protein was applied to a DEAE-cellulose (DE-52, Whatman) column (25 x 2.5 cm) which had been equilibrated with 0.1 M Tris-HCl buffer, pH 7.68-7.72. Continuous gradient elution was carried out with 500 ml of 0-0.5 M KCl in the Tris-HCl buffer. Chromatography was conducted at 4°C and 3.5 ml fractions were collected. The absorbance of the fractions so collected was read at 280 nm. NADH and NADPH cytochrome c reductase activities in the pellets, supernatants and fractions were measured by the method of Dallner (3) while cytochrome P-450 reductase activity was measured as described by Diehl et al. (6). These assays were performed in an Aminco DW-2 UV-VIS spectrophotometer.

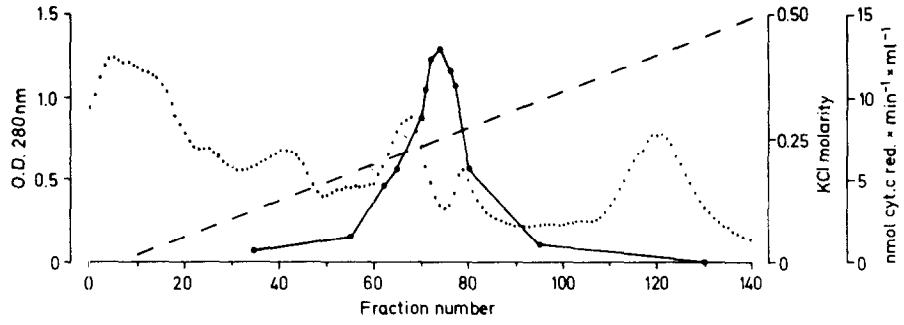

Fig. 1. Elution profile obtained from DEAE-chromatography of 105,000 x g supernatant from peroxidized microsomes (.....) 280 nm absorbance of the eluated fractions. (————) NADPH cyt. c red. activity. (- - -) KCl molarity.

Results and Discussion

The protein, phospholipid and malondialdehyde content of peroxidized and control supernatant and pelleted fractions of a representative experiment are presented in Table 1. In this experiment ascorbic acid was the electron donor but equivalent results were obtained when the peroxidation was carried out enzymatically using NADPH. It can be seen that after peroxidation the protein content of the supernatants of the incubates was about double that of non-peroxidized control incubates. The phospholipid (PLP) content was similarly affected so that the PLP/protein ratio stayed rather constant. This ratio was, however, always much lower than that found in the pellet or in fresh microsomes. The specific activity of NADPH-cytochrome c reductase in the peroxidized supernatant material was increased 2-fold in the face of the similarly increased protein content of that fraction (Table 2). A low level of activity was also seen in the supernatants of control incubates. This could be attributed to the presence of unsedimented microsomal particles. The apparently increased specific activity of the peroxidized pellet could be accounted for by the loss of protein from that fraction (Table 1). The specific activity of NADH-cytochrome c reductase remained constant in the peroxidized supernatant compared to controls even though there was about a 24% loss of this activity from the peroxidized pellet. Apparently there was a real loss

Table 3. Reductase activity recovered from the peroxidized supernatants of Table 2 by DEAE-chromatography and its support of the peroxidation of mitochondria[a].

Fraction	NADPH-cyt. c		P-450		MDA Production	
	nm/min/ml	nm/min/mg	nm/min/ml	nm/min/mg	nm/min/ml	nm/min/mg
No. 74	13	73	0.02	0.13	1.8	10.0
Microsomes	114	98	-		12.6	1.1

a The mitochondrial peroxidation system consisted of 0.03 M Tris-KCl buffer, 0.02 M ADP, 0.15 mM ferric chloride, 0.3 mM NADPH, 0.1 ml DEAE fraction or 0.05 ml microsomal suspensions and mitochondria from 0.5 gram liver in a final volume of 0.5 ml. As control served the same incubation medium using 0.1 ml of fraction No. 130.

of this enzyme activity because none was recovered in any of fractions obtained after chromatography. All of the changes described above were related to the extent of peroxidation as measured by malondialdehyde production.

A typical elution profile of the DEAE chromatographed supernatant fraction is given in Fig. 1. The reductase activity was recovered in fractions 55 to 95 with the highest specific activity occurring in fraction 74 (Table 3). It should be mentioned that this fraction also contained trace amounts of cytochrome b_5 but no detectable malondialdehyde.

When the capacity of the recovered enzyme to reduce purified cytochrome P-450 was assayed, a low level of activity was found (Table 3). This rate of reduction of P-450 occurred without the addition of extra lipid or substrate. There was no measurable phospholipid in the DEAE fractions employed. In one experiment where four active fractions were combined, the reduction of P-450 occurred at a rate of 0.18 nmol/min/ml, again with no lipid or substrate supplementation.

Finally, the reductase, solubilized by peroxidation itself, was capable of supporting the enzymatic lipid peroxidation of the membranes of isolated mitochondria (Table 3). When small amounts of NADPH and organically complexed iron were added to mitochondria together with varying amounts of the active fractions malondialdehyde production was detected. The malondialdehyde production was related to the amount of enzyme activity added and was not seen in preparations in which either the iron, the NADPH or the enzyme were omitted. In contrast to the finding of Pederson and Aust (7, 8) who used protease and deoxycholate solubilized enzyme for liposomal peroxidation, there was no apparent requirement of EDTA for the support of peroxidation in our system and the addition of EDTA in amounts above 1 μM was always inhibitory. These observations are consistent with the current state of understanding of the mechanism of lipid peroxidation.

In order to investigate the potential significance of these findings in intact cells, preliminary studies have begun using isolated hepatocytes in

which lipid peroxidation is initiated by the addition of the ADP-Fe^{+3} complex. To date they have demonstrated a similarly increased NADPH-cytochrome c reductase activity in the 105,000 x g supernatant obtained from these cells after sonication. This did not occur in similarly treated control hepatocytes. Whether this can also be demonstrated in the case of lipidperoxidation supported by pro-oxidants such as carbon tetrachloride remains to be examined.

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References

1. Högberg, J., Bergstrand, A., Jakobsson, S.V. (1973) Eur. J. Biochem., 37, 51-59

2. Ernster, L., Siekevitz, P., Palade, G. (1962) J. Cell Biol., 15, 541-562

3. Dallner, G. (1963) Acta Pathol. Microbiol. Scand. Suppl. 166

4. Johnson, D., Lardy, H.A. (1967) Enzymol. 10, 94

5. Bernheim, F., Bernheim, M.L.C., Wilbur, K.M. (1948) J. Biol. Chem., 174, 257-264

6. Diehl, H., Schadelin, J., Ullrich, V. (1970) Hoppe-Seylers Z. Physiol. Chem., 351, 1359-1371

7. Pederson, T.C., Aust, S.D. (1972) Biochem. Biophys. Res. Commun., 48, 789-795

8. Pederson, T.C., Buege, J.A., Aust, S.D. (1973) J. Biol. Chem. 248, 7134-7141