Alteration of inner-membrane components and damage to electron-transfer activities of bovine heart submitochondrial particles induced by NADPH-dependent lipid peroxidation

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We investigated the changes of the inner-membrane components and the electron-transfer activities of bovine heart submitochondrial particles induced by the lipid peroxidation supported by NADPH in the presence of ADP-Fe³⁺. Most of the polyunsaturated fatty acids were lost as a result of the peroxidation, and phospholipids were changed to polar species. Ubiquinone was also modified to polar substances as the peroxidation proceeded. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed the disappearance of 27000-*M_r* and 30000-*M_r* proteins and the appearance of highly polymerized substances. Flavins and cytochromes were not diminished, but the respiratory activity was lost. The reactions of NADH oxidase and NADH-cytochrome *c* reductase were most sensitive to the peroxidation, followed by those of succinate oxidase and succinate-cytochrome *c* reductase. Succinate dehydrogenase and duroquinol-cytochrome *c* reductase were inactivated by more extensive peroxidation, but cytochrome *c* oxidase was only partially inactivated. NADH-ferricyanide reductase was not inactivated. The pattern of the inactivation indicated that the lipid peroxidation affected the electron transport intensively between NADH dehydrogenase and ubiquinone, and moderately at the succinate dehydrogenase step and between ubiquinone and cytochrome *c*.

Lipid peroxidation in mitochondria has mainly been studied in those of rat liver. McKnight & Hunter (1966) reported that the mitochondria modified by an Fe²⁺-induced lipid-peroxidation reaction lost matrix enzymes, but kept respiratory activities similar to those of non-phosphorylating submitochondrial particles. Pfeifer & McCay (1972) showed a loss of polyunsaturated fatty acids and an inactivation of respiratory activities in rat liver mitochondria during an NADPH-dependent lipid-peroxidation reaction. The inactivation of respiratory-chain enzymes such as NADH-cytochrome *c* reductase and succinate oxidase by peroxidation reactions has been reported (Tappel & Zalkin, 1959), but little is known about the sites of the respiratory chain that are sensitive to lipid peroxidation.

We decided to use bovine heart submitochondrial particles for studying mitochondrial damage exerted by lipid peroxidation, because the particles are well characterized as a unit of electron transfer. An NADH- and NADPH-dependent lipid peroxidation

has been shown to be catalysed by the respiratory-chain-linked NADH dehydrogenase of the particles (Takayanagi *et al.*, 1980; Takeshige *et al.*, 1980). We also studied the modification of ubiquinone by lipid peroxidation. Reports are available on modifications of the quinone side chain by illumination in the presence of oxygen (Snyder & Rapoport, 1969; Morimoto *et al.*, 1970) and on the photo-alteration of the quinone to polar substances by illumination *in vitro* and *in vivo* (Werbin *et al.*, 1974), but the destruction of the quinone by lipid peroxidation has been little studied.

We show in the present paper that the NADPH-dependent lipid-peroxidation reaction affected the electron-transport activity of bovine heart submitochondrial particles at several sites of the respiratory chain to different extents depending on the sites. The site between the NADH dehydrogenase and ubiquinone was most sensitive to the lipid peroxidation. The modification of ubiquinone to polar species and the loss of 27000-*M_r* and 30000-*M_r* proteins were observed.

Experimental

Submitochondrial particles

Bovine heart submitochondrial particles were prepared from slaughterhouse material by the method of Lee & Ernster (1967) as the non-phosphorylating EDTA-particles.

Lipid peroxidation

The submitochondrial particles (2 mg/ml) in a reaction mixture containing 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH 7.4 at 25°C, containing 1 mM-ADP and 0.1 mM-FeCl₃ were incubated at 25°C, shaken in the air. The reaction was started by the addition of NADPH (0.5 mM), and samples were taken at various time intervals, followed by the addition of 1 mM-MnCl₂ to the samples to stop the reaction. The samples were used for the assay of malondialdehyde by the thiobarbituric acid method of Bernheim *et al.* (1948) or for recovering the particles by centrifugation at 105 000 *g* for 60 min with 4 vol. of 0.25 M-sucrose at 4°C. The particles were suspended in the sucrose solution and used for analysis of the constituents and activities.

Lipid analyses

The incubated sample was extracted with 20 vol. of chloroform/methanol (2:1, v/v) by the procedure of Folch *et al.* (1957). After 3 h, 0.2 vol. of 0.5% NaCl solution was added. The chloroform layer was evaporated off and the lipid residue was redissolved in a known volume of chloroform. Portions of the lipid were used for determination of phosphorus, for t.l.c. and for g.l.c. of fatty acid methyl esters. The lipid phosphorus was determined by the method of Fiske & SubbaRow (1925) after wet-ashing with conc. H₂SO₄. The phospholipid (10 µg of lipid P) was applied to a Silica Gel H thin-layer plate and developed with chloroform/methanol/water (65:25:4, by vol.). The plates were sprayed with Zinzadze reagent (Dittmer & Lester, 1964), and the spots showing a positive reaction were recorded densitometrically by the zigzag scanning mode with a Shimadzu CS-910 dual-wavelength TLC scanner at 650–470 nm. The phospholipids were identified by comparison with known standard samples. Fatty acid methyl esters were prepared by heating the lipid with BF₃/methanol in a boiling-water bath (Morrison & Smith, 1964) and extracted with n-hexane. The esters were analysed with a Shimadzu model GC-7APTF gas chromatograph equipped with a glass column (2 m × 3 mm) at 180°C. The packing was Chromosorb W (AW-DMCS; 80–100 mesh) with 5% Advance DS (Shimadzu). Methylation was complete, as checked by t.l.c., and the esters were determined quantitatively, with erucic acid as an internal standard.

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis

The incubated samples (2 mg of protein/ml) were solubilized by mixing the suspension with equal volume of a mixture containing 6% (w/v) sodium dodecyl sulphate, 10% (v/v) 2-mercaptoethanol, 20 mM-Tris/HCl buffer, pH 7.4, 4 M-urea, 2 mM-EDTA and 0.02% Bromophenol Blue, and incubated for 2 h at 37°C. The solubilized samples (60 µg of protein) were applied to 12.5% (w/v) polyacrylamide gel containing a constant ratio of acrylamide to methylenebisacrylamide (37.5:1). Preparation of the gel and the electrophoresis were done as described by Laemmli (1970). The following standard proteins were used as markers: albumin (*M_r* 66 000); ovalbumin (*M_r* 45 000); trypsinogen (*M_r* 24 000); β-lactoglobulin (*M_r* 18 400); lysozyme (*M_r* 14 300); RNA polymerase B subunits β' (*M_r* 180 000), β (*M_r* 140 000), x (*M_r* 100 000), α (*M_r* 42 000) and z (*M_r* 39 000). The gel was stained with 0.1% Coomassie Brilliant Blue R250.

Determination of cytochromes, flavins and ubiquinone

Cytochromes and flavins were determined from reduced-minus-oxidized difference spectra of the particles recorded on a Hitachi 340 spectrophotometer. Molar absorption coefficients used were $\Delta\epsilon_{562-575} = 22.0 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for cytochrome *b*, $\Delta\epsilon_{550-540} = 19.1 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for cytochrome *c* + *c*₁, $\Delta\epsilon_{465-500} = 11.0 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for flavins (Chance, 1957) and $\Delta\epsilon_{605-630} = 16.5 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for cytochrome *a* (Yonetani, 1959). Ubiquinone was measured spectrophotometrically with $\Delta\epsilon_{275(\text{ox.}-\text{red.})} = 12.25 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ after extraction with light petroleum (b.p. 30–60°C)/methanol as described by Pumphrey & Redfearn (1960) but without pyrogallol.

Preparation of the particles containing radioactive ubiquinone

A controlled amount of radioactive ubiquinone was re-incorporated into the submitochondrial particles by the method of Ernster *et al.* (1978). The particles (ubiquinone content 3.68 nmol/mg of protein), suspended in 0.15 M-KCl, were freeze-dried and extracted with n-pentane. The resulting ubiquinone-depleted particles (ubiquinone content 0.2 nmol/mg of protein) (100 mg) were treated with [3'-¹⁴C]ubiquinone-10 (185 nCi and 80 µM in 6 ml of n-pentane) at 4°C. The solvent was completely evaporated and the particles were homogenized in 25 mM-Hepes/NaOH buffer, pH 7.4, to a final protein concentration of 20 mg/ml. About 70% of the total ubiquinone (3.40 nmol/mg of protein) could be reduced by succinate in the presence of KCN, similarly to the intact particles. The activities of NADH-cytochrome *c* reductase and succinate-

cytochrome *c* reductase were restored to 81% and 84% of that of the original particles respectively by the re-incorporation.

Radioactivity measurement

Samples (0.5 ml) were solubilized with 0.1 ml of 1 M-NaOH, and 10 ml of the toluene/Triton X-100 (2:1, v/v) mixture containing 0.06% diphenyl-oxazole was added with shaking. The emulsion was clarified by the addition of HCl and its radioactivity was counted in a scintillation counter (Aloka model LSC-703 Liquid Scintillation System). NaOH and HCl were omitted for the samples of light-petroleum/methanol extract.

Determination of enzymic activities

Enzymic activities were determined with a Hitachi 556 spectrophotometer at 37°C. The activity of NADH–ferricyanide reductase was measured by the decrease in A_{420} , NADH–cytochrome *c* reductase and succinate–cytochrome *c* reductase by the increase in A_{550} , cytochrome *c* oxidase by the decrease in A_{550} with 76 μ M-ferrocytochrome *c* at pH 7.0 (Yonetani & Ray, 1965), NADH–ubiquinone-1 reductase by the decrease in A_{340} (Hatefi, 1978) in the presence of antimycin A (0.4 μ g/mg of protein) with 0.12 mM-2,3-dimethoxy-5-methyl-6-n-pentyl-1,4-benzoquinone as ubiquinone-1 at pH 8.0, succinate–ubiquinone-2 reductase by the decrease in A_{660} (Hatefi & Stiggall, 1978) in the presence of antimycin A with 0.12 mM-2,3-dimethoxy-6-n-decyl-1,4-benzoquinone as ubiquinone-2 and 86 μ M-2,6-dichlorophenol-indophenol, succinate dehydrogenase by the decrease in A_{660} (Ackrell *et al.*, 1978) in the presence of antimycin A and 1 mM-KCN with 1.08 mM-phenazine methosulphate and 52 μ M-2,6-dichlorophenol-indophenol, and duroquinol–cytochrome *c* reductase by the increase in A_{550} (Nelson & Gellerfors, 1978) in the presence of KCN with the use of 30 μ M-cytochrome *c* and 60 μ M-duroquinol. Succinate oxidase was measured polarographically with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Reagents

Ubiquinones and radioactive ubiquinone-50 (specific radioactivity 48 μ Ci/mg) were gifts from Eisai Co., Tokyo, Japan. Ubiquinone analogues of ubiquinone-1 and -2 were used because of their chemical stability (Wan & Folkers, 1978). Phospholipids used as standards were obtained by silicic acid chromatographic separation of bovine heart mitochondrial lipid essentially by the method of Rathbone & Maroney (1963). Duroquinol was prepared by the reduction of duroquinone (tetramethyl-*p*-benzoquinone) in methanolic solution (Nelson &

Gellerfors, 1978). Erucic acid, albumin, trypsinogen, β -lactoglobulin, ovalbumin and lysozyme were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and RNA polymerase B was obtained from Seikagaku Kogyo Co., Tokyo, Japan. Other reagents were of analytical-reagent grade.

Results

Changes in phospholipids

We studied the changes of phospholipids caused by the NADPH-dependent lipid peroxidation in the presence of ADP-Fe³⁺ chelate. Table 1 shows malondialdehyde in the incubated samples, and lipid phosphorus and five major fatty acids in the phospholipid fraction after incubation of the particles with or without NADPH for 30 min at 25°C. A part of the lipid phosphorus was lost during the peroxidation, but amounts of saturated fatty acids and oleic acid were essentially unchanged. Polyunsaturated fatty acids were extensively degraded: approx. 90% of arachidonic acid and 80% of linoleic acid were lost during the peroxidation. Malondialdehyde was formed equivalent to about 10% of the polyunsaturated fatty acids degraded (about 450 nmol/mg of protein) on a molar basis.

We then studied the phospholipid pattern of the particles by applying the extract to a Silica Gel H thin-layer plate, developing with chloroform/methanol/water (65:25:4, by vol.), spraying with Zinzadze reagent and measuring the phosphorus concentration densitometrically. The particles incubated without NADPH gave three peaks, corresponding to phosphatidylcholine, phosphatidylethanolamine and cardiolipin, with the percentages of total phospholipids being 40.5%, 38.2% and 17.6% respectively (Fig. 1a). These values are in good agreement with those obtained by Krebs *et al.* (1979) and Fleischer *et al.* (1961). The lipids extracted from the particles incubated with NADPH showed marked changes in the composition (Fig. 1b). The peaks corresponding to cardiolipin and phosphatidylethanolamine decreased greatly, whereas that of phosphatidylcholine changed less appreciably. New species were formed, which were indicated by the rise of troughs between the peaks and the appearance of a new peak more polar than phosphatidylcholine. The peak corresponding to phosphatidylcholine in the damaged particles should not be taken wholly as phosphatidylcholine, but should be regarded as modified species originated from cardiolipin and phosphatidylethanolamine, because about 80% of linoleic acid was lost during the peroxidation (Table 1), and phosphatidylcholine, the predominant phospholipid in mitochondria, contains linoleic acid by about 50% of

Table 1. *Changes in fatty acid composition of phospholipid extracted from the submitochondrial particles damaged by lipid peroxidation*

Bovine heart submitochondrial particles (2 mg/ml) in 25 mM-Hepes/NaOH buffer, pH 7.4 at 25°C, containing 1 mM-ADP and 0.1 mM-FeCl₃ with or without 0.5 mM-NADPH were incubated at 25°C for 30 min. Phospholipids were extracted and analysed for fatty acids by g.l.c. after methylation. The malondialdehyde is that of the suspension formed during the incubation.

Incubation	Fatty acids (nmol/mg of protein)					Lipid P (μ g/mg of protein)	Malondialdehyde formation (nmol/mg of protein)
	C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{20:4}		
Without NADPH	105	171	112	382	164	15.7	1.0
With NADPH	100	142	95	83	15	13.0	47

Fig. 1. *Changes in phospholipids extracted from the submitochondrial particles damaged by lipid peroxidation: t.l.c. analyses*

Bovine heart submitochondrial particles were incubated with or without NADPH as described in Table 1. Phospholipids (10 μ g of lipid P) were applied to a Silica Gel H thin-layer plate and developed with chloroform/methanol/water (65:25:4, by vol.). The spots showing a positive reaction for phosphorus were recorded densitometrically by the zigzag method at 650–470 nm. (a) Control incubation without NADPH; (b) incubation with NADPH. Key: CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Minor, minor components. Arrows indicate phospholipid peaks modified by the peroxidation.

total weight of its fatty acid moiety (Kagawa *et al.*, 1973). The decrease of phosphatidylethanolamine and the formation of more-polar phospholipids have been reported by May & McCay (1968) in rat liver mitochondria subjected to an NADPH-dependent lipid-peroxidation reaction.

Changes in membrane proteins

The submitochondrial particles were incubated with NADPH in the presence of ADP-Fe³⁺, and the membrane proteins were analysed by sodium dodecyl sulphate / polyacrylamide - gel electrophoresis (Fig. 2). As the lipid peroxidation proceeded, 27000-*M_r* and 30000-*M_r* bands decreased and were lost in 30 min, concomitant with slight decreases in some other bands. Bands of high-molecular-weight proteins, found at the top of the separating gel (lanes 5–8) and in the wells of the stacking gel (lanes 6–8), were observed in the particles incubated with NADPH. These may be due to the polymerization of the lost or diminished proteins. Degradation of proteins during the peroxidation is less likely, because no increase in low-molecular-weight bands was observed.

Changes in respiratory components

Table 2 shows the changes in the contents of cytochromes and flavins by incubating the particles with NADPH and ADP-Fe³⁺ for 30 min at 25°C under the same conditions as described for Table 1. The peroxidation caused no appreciable changes in the contents of flavins and cytochromes, except that cytochrome *b* decreased slightly (about 18%).

Changes in ubiquinone

Fig. 3 shows the relation between malondialdehyde formation and the content of ubiquinone-10, which was extracted from the particles with light-petroleum/methanol mixture and partitioned into the light-petroleum layer. As the malondialdehyde formation proceeded, the content of ubi-

Fig. 2. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the submitochondrial particles

The particles were incubated as described in Table 1 except for the incubation time. Electrophoretic procedures are described in the Experimental section. Lane 1, unincubated particles; lane 2, incubated without NADPH for 30 min; lane 3, incubated with NADPH and MnCl_2 for 30 min; lanes 4, 5, 6, 7 and 8, incubated with NADPH for 0, 5, 10, 20 and 30 min respectively. The values given under the lanes are malondialdehyde contents in nmol/mg of protein.

quinone-10 in the particles decreased. Incubation for 120 min with NADPH caused a decrease in ubiquinone-10 of about 80%. A possibility, however, remains that the content could be underestimated if a part of the quinone were reduced by the incubation. We therefore investigated whether the quinone extracted from the particles could be re-oxidized with Fe^{3+} (Kröger & Klingenberg, 1966). The reduced ubiquinone-10 that was extracted from the submitochondrial particles incubated with succinate and KCN was re-oxidized completely by Fe^{3+} in 10 h, whereas the spectrum of the extract from the particles damaged by the peroxidation did not change over 15 h (Fig. 4).

We then prepared submitochondrial particles the ubiquinone of which was replaced by radioactive [$3'$ - ^{14}C]ubiquinone, and investigated a possible release of the quinone from the particles during the peroxidation. Most of the radioactivity was recovered in the precipitate, even after a prolonged incubation of 120 min, suggesting that ubiquinone-50 and its peroxidation products remained in the

particles. When the particles were extracted with light-petroleum/methanol mixture, 85% of the radioactivity was extracted and 15% remained with the residue. Of the radioactivity in the extract, only 18% of that recovered in the particles was found in the light-petroleum layer, most (67%) being found in the methanol layer (Table 3). This observation may indicate that ubiquinone-50 was modified by the lipid peroxidation to polar products that cannot be extracted with light petroleum.

Inactivation of electron-transfer reactions by the peroxidation

In order to define the sites that are sensitive to the lipid peroxidation, we incubated the particles with NADPH in the presence of ADP-Fe^{3+} , stopped the reaction with 1 mM- MnCl_2 , collected the particles and assayed the rate of electron-transfer reactions. As shown in Fig. 5(a), both NADH oxidase and NADH-cytochrome *c* reductase activities were most sensitive to the peroxidation: they were inactivated to less than 50% in 2 min and to about

Table 2. *Changes in flavins and cytochromes of the submitochondrial particles by lipid peroxidation*

Incubation conditions were the same as described in Table 1. The submitochondrial particles were collected by centrifugation. The values are the means $\pm$ S.E.M. for four separate preparations. The recoveries of the particles after the centrifugation were $75 \pm 1\%$ and $76 \pm 2\%$ (means $\pm$ S.E.M., $n = 3$) for the samples incubated with or without NADPH respectively.

Incubation	Flavins (nmol/mg of protein)	Cytochromes (nmol/mg of protein)		
		<i>a</i>	<i>b</i>	<i>c + c₁</i>
Without NADPH	1.14 ± 0.03	1.01 ± 0.07	0.83 ± 0.05	0.84 ± 0.04
With NADPH	1.16 ± 0.06	0.92 ± 0.06	0.68 ± 0.04	0.82 ± 0.05

Fig. 3. *Changes of ubiquinone content of the submitochondrial particles during lipid peroxidation*

The submitochondrial particles were incubated as described in Table 1, except for the incubation time. At the time indicated, malondialdehyde in the suspension and ubiquinone extracted by light petroleum/methanol were determined. Ubiquinone content was expressed as the percentage of the initial content (3.91 nmol/mg of protein). Control incubation without NADPH: ○, malondialdehyde; □, ubiquinone. Incubation with NADPH: ●, malondialdehyde; ■, ubiquinone.

Fig. 4. *Oxidation of ubiquinone, extracted from the submitochondrial particles peroxidized or reduced with succinate, by FeCl₃*

Ubiquinone was extracted from the submitochondrial particles (10mg of protein) either incubated with succinate for 3 min in the presence of KCN or incubated with NADPH for 120 min as described in Table 1. The extracted ubiquinone was re-oxidized and determined spectrophotometrically (Kröger & Klingenberg, 1966). The extract was dried by evaporation and oxidized by $12 \mu\text{M-FeCl}_3$ in 5 ml of heptane/ethanol (1:4, v/v) in the dark. At the time indicated, the oxidized ubiquinone in the sample (1 ml) was determined spectrophotometrically from the absorption decrease at 280–289 nm caused by NaBH_4 with the molar absorption coefficient (oxidized-minus-reduced) of $8.8 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The ubiquinone determined after the oxidation of the extract for 15 h was 3.98 nmol/mg of protein for the particles incubated with succinate and KCN. ○, Ubiquinone extracted from the particles reduced with succinate; ●, quinone from the peroxidized particles.

Table 3. *Distribution of ubiquinone radioactivity in methanol and light-petroleum layers*

The submitochondrial particles containing [3^{14} C]ubiquinone-10 were incubated with or without NADPH as described in Table 1 for 120 min and centrifuged at 105 000 *g* for 60 min. The particles were resuspended in water, and a 1 ml portion (1 mg of protein) was mixed with 4 ml of methanol and 5 ml of light petroleum, and centrifuged. The radioactivities in the methanol and the light-petroleum layers were separately measured. Ubiquinone-10 in the light-petroleum layer was spectrophotometrically determined. The values in parentheses were the percentage recoveries of either ubiquinone-10 based on that of the original particles (3.40 nmol/mg of protein) or the radioactivity based on those in the recovered particles (661 and 803 c.p.m./mg of protein for the radioactivity incubated with and without NADPH respectively). The radioactivity of the original particles was 805 c.p.m./mg of protein.

Incubation	Ubiquinone-10 (nmol/mg of protein)	Radioactivities (c.p.m./mg of protein)		Malondialdehyde formation (nmol/mg of protein)
		Methanol layer	Light-petroleum layer	
Without NADPH	3.27 (96%)	27 (3%)	747 (93%)	2.1
With NADPH	0.70 (21%)	440 (67%)	116 (18%)	53

Fig. 5. *Changes in electron-transfer activities of the submitochondrial particles during lipid peroxidation*

The submitochondrial particles were incubated with NADPH or without NADPH in the presence of ADP and FeCl₃ as described in Table 1. The samples were taken at the times indicated, and the reaction was stopped by the addition of MnCl₂. Malondialdehyde in the suspension was determined and the submitochondrial particles were recovered by centrifugation. Malondialdehyde formation during the incubation with NADPH is shown in (a) (Δ), and the formation during the incubation without NADPH was 0.8 nmol/mg of protein at 30 min. The electron-transfer activities are expressed as percentages of those of the controls (means of three experiments). Essentially no inactivation was observed when the particles were incubated without NADPH. The reactions and their specific activities at zero time (given in parentheses as nmol/min per mg of protein, means $\pm$ S.E.M., $n = 3$) were as follows. (a) $\circ$, NADH-cytochrome *c* reductase (1319 $\pm$ 21); $\bullet$, NADH oxidase (844 $\pm$ 36); $\square$, succinate-cytochrome *c* reductase (1061 $\pm$ 69); $\blacksquare$, succinate oxidase (700 $\pm$ 84, oxygen consumption). (b) $\square$, Succinate dehydrogenase (1293 $\pm$ 88); $\blacksquare$, succinate-ubiquinone-2 reductase (880 $\pm$ 126); $\circ$, NADH-ubiquinone-5 reductase (1116 $\pm$ 41). (c) $\circ$, NADH-ferricyanide reductase (18 400 $\pm$ 200); $\square$, cytochrome *c* oxidase (3049 $\pm$ 382); $\blacksquare$, duroquinol-cytochrome *c* reductase (653 $\pm$ 68).

10% of the original activity in 10 min. Succinate oxidase and succinate-cytochrome *c* reductase activities were similarly inactivated when the particles were incubated for more than 10 min, but at the early phase of the incubation, for example at 2 min, 80% of the activities remained. This may be explained by a supposition that electron transport between NADH and ubiquinone is most sensitive to the peroxidation. The supposition can be confirmed by the observation shown in Fig. 5(b) that the activity of NADH-ubiquinone-5 reductase was more sensitive to the peroxidation than were those of succinate dehydrogenase and succinate-ubiquinone-10 reductase. Comparison of Figs. 5(a) and 5(b) also suggests that the electron transfer between ubiquinone and cytochrome *c* is sensitive to the peroxidation, and the inactivation becomes apparent at the late stage of the incubation. This is in agreement with the observation shown in Fig. 5(c) that duroquinol-cytochrome *c* reductase activity, which represents the activity of ubiquinone-cytochrome *c* reductase, was moderately inactivated. Cytochrome *c* oxidase activity was only partially inactivated even after long incubation, and NADH-ferricyanide reductase activity was essentially resistant to the peroxidation.

Discussion

NADPH-dependent lipid peroxidation in bovine heart submitochondrial particles results in a remarkable loss of arachidonic acid and linoleic acid. Phospholipids were converted into polar forms, total lipid phosphorus being decreased only a little. Ubiquinone was modified to polar forms that cannot be extracted with light petroleum. Most of the modified ubiquinone was recovered in the methanol layer by the extraction with light-petroleum/methanol mixture, but a part of the ubiquinone seemed to be attached to the membrane residue. The conversion of ubiquinone into polar forms may be ascribed to the peroxidation of the isoprenoid side chain, in analogy with illumination experiments (Snyder & Rapoport, 1969; Morimoto *et al.*, 1970), and the firm attachment to the membrane is explained by interaction of the carbonyl residues of the product with the membrane. Loss of proteins from the particles was not apparent, but sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed the disappearance of 27 000- M_r and 30 000- M_r bands and the appearance of highly polymerized species. The polymerization may be due to cross-linking of the proteins with malondialdehyde (Chio & Tappel, 1969). Changes in electron carriers such as cytochromes and flavins were not apparent, but the respiratory activities of the submitochondrial particles were extensively lost.

Various sites of the respiratory chain were damaged by the peroxidation. The reaction between NADH dehydrogenase and ubiquinone was highly sensitive to the peroxidation, and the succinate dehydrogenase reaction and the reaction between ubiquinone and cytochrome *c* were moderately sensitive. The loss of respiratory activities due to the peroxidation in the submitochondrial particles could be attributed to modifications of lipids and proteins. Decrease in ubiquinone concentrations, however, may not be responsible for the decreased respiratory activities, but may accelerate the NADH-dependent lipid-peroxidation reaction, as discussed in a previous paper (Takayanagi *et al.*, 1980). A factor responsible for the loss of electron-transfer activities may be the changes in phospholipids. Two sites of the respiratory chain have been shown to be inactivated by treatment of electron-transfer particles with phospholipase: one between NADH dehydrogenase flavoprotein and ubiquinone, and another between cytochromes *b* and *c* (Minakami *et al.*, 1964). Extensive inactivation of NADH-ubiquinone reductase and somewhat moderate inactivation of ubiquinone-cytochrome *c* reductase have been reported on depletion of phospholipids from bovine heart complexes I and III (Fry & Green, 1981). They have shown the absolute requirement of the enzymic activities for cardiolipin, which is shown in the present paper to be destroyed by peroxidation. Another mechanism of inactivation of electron transfer is the destruction of thiol groups by lipid peroxides (Lewis & Wills, 1962), because succinate-ubiquinone reductase has been shown to be resistant to the depletion of phospholipids (Yu *et al.*, 1978) and succinate dehydrogenase is a typical thiol enzyme. NADH-ubiquinone reductase is also sensitive to thiol inhibitors (Minakami *et al.*, 1964). Further, the present study shows the disappearance of 27 000- M_r and 30 000- M_r bands in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis during the peroxidation. These proteins might be concerned with electron-transfer activities. We have no other information about the proteins than their molecular weights, but a possibility that they are iron-sulphur proteins cannot be ruled out because iron-sulphur proteins have been assigned to 29 000–30 000- M_r and two other higher- M_r bands in Complex I (Ragan, 1976) and a 26 000–27 000- M_r band in Complex II (Davis & Hatefi, 1971) and Complex III (Nelson & Gellerfors, 1978) in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

We have shown the alteration of membrane components and the damage to electron-transfer activities of bovine heart submitochondrial particles by the NADPH-dependent lipid-peroxidation reaction in the presence of physiological concentrations of adenosine polyphosphate and Fe^{3+} . Similar

lipid-peroxidation reactions and damage to mitochondria may happen in some pathological conditions.

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