Studies on cytochrome oxidase in carbon tetrachloride treated rats

P Padma & O H Setty*
Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India

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Cytochrome c oxidase was purified from control and CCl₄ treated rats and its kinetic properties were studied. The activity of the enzyme was inhibited by 51% in CCl₄ (4 g per kg body weight for 24 hr) treated rats. Studies on the kinetic properties showed that the Kₘ of the enzyme increased by 60% while Vₘₐₓ decreased by 44% in CCl₄ treated rats compared to controls. The content of cytochrome aa₃ was decreased by 34% while cytochrome b and c were not affected by CCl₄ treatment. Phosphatidylethanolamine and cardiolipin were decreased significantly by 40%, 49% and 60% respectively in CCl₄ treated rats. A decrease in the cytochrome aa₃ content and a change in the lipid environment of the membrane are probably responsible for a decreased rate of electron transfer from cytochrome c to oxygen.

Carbon tetrachloride, a well-studied hepatotoxin, causes lipid peroxidation in membrane, alters lipid metabolism, and decreases protein synthesis in the injured hepatocytes. During lipid peroxidation there is increased formation of free radicals. Biological membranes and subcellular organelles rich in polyunsaturated fatty acids are the major sites for the free radical mediated damage. Increased lipid peroxidation alters the lipid environment of the membrane and thus affects the activity of some enzymes.

Cytochrome oxidase, the terminal enzyme of the respiratory chain requires phospholipids for its optimal activity and any change in the lipid composition of the mitochondrial membrane decreases the activity of the enzyme as reported in ethanol fed baboons. Hence in the present study we investigated the effect of CCl₄ (which induces lipid peroxidation) on cytochrome c oxidase. The enzyme was purified from control and CCl₄ treated rats and the kinetic properties were studied. Furthermore we have examined the effect of CCl₄ on the cytochrome content and phospholipid composition of mitochondria.

Materials—Cytochrome c and Triton X-114 were obtained from Sigma Chemical company, St. Louis, MO, USA. DEAE-Sephadex was purchased from Pharmacia, Uppsala, Sweden. All other chemicals were obtained from commercial sources in India and were of analytical grade.

Treatment of rats—Male Wistar rats weighing 100 ± 20 g were used for the present study. Control group received mineral oil and the CCl₄ group received CCl₄ (4 g/ kg body weight) dissolved in 0.5 ml mineral oil through an intragastric tube and sacrificed 24 hr after the administration of CCl₄.

Isolation of mitochondria: Mitochondria from the liver were isolated by differential centrifugation according to the method of Lawrence and Davies. The isolation medium consisted of 70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 0.2 mM EDTA and BSA (0.36 mg/ml) adjusted to pH 7.4. The final pellet containing mitochondria was suspended in 0.25 M sucrose to a protein concentration of 15-20 mg/ml. Protein was measured by Biuret method using BSA as a standard. All solutions were prepared fresh daily in triple distilled water. The purity of the mitochondrial preparation was known from the activities of marker enzymes and the maximum contamination with other subcellular organelles was up to 5%.

Cytochrome oxidase was assayed using a Shimadzu-160A spectrophotometer. The activity of the enzyme was measured by following the rate of oxidation of ferrocytochrome c at 550 nm and the activity of the enzyme is expressed as nmol cytochrome c oxidized per min per mg protein.

The concentrations of cytochromes aa₃, b and c were estimated by difference spectra as described earlier using a Hitachi double beam spectrophotometer.

Mitochondrial lipids were separated by the procedure of Bligh and Dyer. Phospholipids were separated by thin layer chromatography using chloroform: methanol : water (65 : 25 : 4) as solvent, the sub-
classes of phospholipids were detected by exposing to iodine vapour and comparing with authentic standards. The individual phospholipids from TLC plate were scraped and digested with 60% perchloric acid. The inorganic phosphate was estimated by Fiske-Subbarow method.

Cytochrome oxidase was purified from liver mitochondria according to the method of Kadenbach et al. Mitochondria were solubilized by the addition of Triton X-114 and Triton X-100 and chromatographed on a DEAE-Sephacel column.

Statistical analyses were performed using Student’s t-test and a P value less than 0.05 was considered as significant.

Cytochrome c oxidase (E. C. 1.9. 3. 1), which is involved in the transfer of electrons from cytochrome c to oxygen was purified from control and CCl₄ administered rats and its kinetic properties were studied. The activity of the enzyme in controls and CCl₄ treated rats was 1702 ± 134 and 863 ± 164 nmol/min/mg protein, respectively (i.e., a 51% decrease in CCl₄ treated rats) showing that the rate of electron transfer at the last segment of the respiratory chain was affected.

Cytochrome c oxidase was purified on DEAE-Sephacel column, purity of the preparation was checked by measuring the heme a content and the enzyme activity. The content of heme a in mitochondria and purified cytochrome oxidase was 0.21 and 4.1 nmol/mg protein respectively. Kinetic properties of the purified enzyme were studied in control and CCl₄ treated rats using reduced cytochrome c as substrate. There was no significant difference in Kₘ and Vₘₐₓ of control and CCl₄ treated rats at 15°C and 25°C. But at 37°C the Kₘ increased by 60% (from 2.5 to 4.0 μM) while Vₘₐₓ decreased by 44% (22,000 to 12,320 nmole/min/mg protein) in CCl₄ treated rats compared to controls (Fig. 1). This partly explains the significant decrease in the activity of cytochrome oxidase in CCl₄ treated rats.

The concentration of cytochrome aa₃, which serves as an electron carrier between cytochrome c and molecular oxygen was significantly decreased (34%) in CCl₄ treated rats as compared to controls. The concentration of cytochrome b and c were not affected on CCl₄ administration (Table 1). As respiratory process involves the transport of electrons via cytochromes to molecular oxygen, any decrease in the cytochrome concentration would affect the rate of electron transport via respiratory chain and thereby alter the energy production of mitochondria.

The effect of CCl₄ on phospholipid composition of mitochondria was studied. Phosphatidylcholine, phosphatidylethanolamine and cardiolipin decreased significantly by 40%, 49% and 60% respectively (Table 1). Phosphatidylcholine and phosphatidyl ethanolamine have been shown to be essential for the normal functioning of cytochrome oxidase in ethanol fed baboons. Cardiolipin, a polyunsaturated phospholipid located in the inner mitochondrial membrane is prone to lipid peroxidation under conditions of oxidative stress. Presence of cardiolipin has been shown to activate the cytochrome chain and influence ATP synthase.

Na⁺-K⁺ATPase activity of cardiac sarcolemma was decreased when exposed to superoxide or other oxy radicals. The decrease in the enzyme activity was associated with decrease in Vₘₐₓ and increase in Kₘ. The SH group content of the enzyme was also proportionately decreased and this change in Vₘₐₓ and Kₘ was related to the SH content of the enzyme. The

![Graph](image-url)
of the extract.

Table I—Effect of administration of CCl₄ on cytochrome content and phospholipid composition of mitochondria

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>CCl₄ treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochromes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aα₄</td>
<td>100 ± 17</td>
<td>66 ± 11*</td>
</tr>
<tr>
<td>b</td>
<td>100 ± 11</td>
<td>107 ± 4</td>
</tr>
<tr>
<td>c</td>
<td>100 ± 2</td>
<td>97 ± 11</td>
</tr>
<tr>
<td>Phospholipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>100 ± 9</td>
<td>60 ± 5**</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>100 ± 17</td>
<td>51 ± 5***</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>100 ± 15</td>
<td>40 ± 2***</td>
</tr>
</tbody>
</table>

P values: *=< 0.05, **< 0.01, ***< 0.005 vs corresponding control.

Results are expressed as percent control and are the mean ± SD of six animals. The control values for cytochrome aα₄, b and c are 0.196 ± 0.033, 0.18 ± 0.021 and 0.334 ± 0.001 nmoles/mg protein respectively. The control value for phosphatidylcholine, phosphatidylethanolamine and cardiolipin are 27.0 ± 2.5, 17.5 ± 3 and 13.0 ± 2.0 µg phospholipid phosphorous/g tissue.

apparent Kₘ and Vₘₐₓ of UDP-glucuronosyl transferase towards 1-hydroxyprene was significantly changed due to the administration of carcinogenic chemicals in rats. This change in Vₘₐₓ and Kₘ was implicated to the major changes in the hepatic expression of the enzyme. The decrease in Vₘₐₓ and increase in Kₘ that is observed in the present study due to the administration of carbon tetrachloride might be related to the changes in the expression of the enzyme.

It has been reported recently that the administration of carbon tetrachloride abolishes the phosphorylation of ADP to ATP and the prior administration of aqueous extract of P. fraternus would prevent the carbon tetrachloride induced inhibition on ATP. This protective effect might be due to the antioxidant property of the extract.

The following factors together contribute to a significant decrease in the activity of cytochrome oxidase in CCl₄ treated rats. These are:

1) A change in the lipid environment of the membrane (a significant decrease in the levels of cardiolipin) probably led to a decreased rate of electron transfer from cytochrome c to oxygen.

2) A significant decrease in the level of cytochrome aα₄, which is important for the transfer of electrons from cytochrome c to oxygen.

3) A decrease in Vₘₐₓ and increase in Kₘ, probably due to a change in the expression of the enzyme also leads to a decreased activity of the enzyme.

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References