Effect of Lipid Depletion on the Kinetics of Microsomal NADH–Cytochrome c Reductase

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It has been known that liver microsomes possess an enzymatic activity catalyzing the reduction of exogenously added cytochrome c by NADH. Strittmatter and Velick (1956a) first isolated the NADH-cytochrome c reductase from rabbit liver microsomes, and observed that the electron transfer system from NADH to cytochrome c was assembled with NADH-cytochrome b5 reductase and cytochrome b5. NADH-ferricyanide reductase activity was also shown in liver microsomes, although the system was not physiological (Strittmatter and Velick 1956b). These enzymes are considered to be tightly bound to membranous components of microsomes (Ernster et al. 1962; Omura et al. 1965) and lipids might play an important role in such microsomal enzymes. In fact, requirement of lipids has been demonstrated for both NADH-cytochrome c reductase (Jones and Wakil 1967) and NADH-cytochrome b5 reductase (Rogers and Strittmatter 1973). However, little is known about the role of lipids in the regulation of microsomal enzymes. In the present paper, we examined the involvement of lipids in the microsomal NADH-cytochrome c reductase (NADH-cytochrom c oxidoreductase, EC 1.6.99.3) activity by means of kinetic studies.

MATERIALS AND METHODS

Chemicals. Horse heart cytochrome c and NADH were obtained from Boehringer-Soehne. Ferricyanide was of analytical grade. Phosphatidylserine and phosphatidylinositol, which were used as the electron acceptors, were obtained as described previously (Ishibashi and Imai 1974). Removal of lipids from the microsomes with 90% acetone resulted in 50% or more decrease of NADH-cytochrome c reductase activity. The decrease of the enzyme activity was a result of lowering of \( V_{\text{max}} \) and increase in \( K_m \). When sufficient amounts of phospholipids such as phosphatidylserine or phosphatidylinositol were added to the acetone-treated microsomes, the reductase activity was restored completely to the original level. On the other hand, when ferricyanide was used in place of cytochrome c as electron acceptor, the rate of reduction of ferricyanide was not affected by the lipid depletion and \( V_{\text{max}} \) and \( K_m \) for ferricyanide remained unchanged.

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inositol were purchased from Koch-Light Laboratories, Colnbrook, United Kingdom. The purity of these phospholipids was confirmed by thin layer chromatography.

**Preparation of microsomes.** Hen liver microsomes were prepared by the method of Jones and Wakil (1967). Lipids were removed from the microsomes by extraction with 90% acetone as described by Lester and Fleisher (1961). This treatment resulted in the loss of at least 80% of the microsomal phospholipid, when estimated in terms of lipid phosphorus. The phosphorous content of lipids was determined according to the procedure of Bartlett (1959). Protein was determined by the method of Lowry et al. (1951), using crystalline bovine serum albumin as reference protein.

**Solubilization of lipids.** Lipids were solubilized in aqueous medium by sonic oscillation (Jones and Wakil 1967).

**Enzyme assays.** The activities of microsomal enzymes were examined at 25°C in a cell of Hitachi recording spectrometer (model 356). For the assay of NADH-cytochrome c reductase, the incubation mixture contained 2.5-20 µM cytochrome c, appropriate amounts of microsomal protein and 80 mM Tris-HCl buffer (pH 7.5), in a final volume of 3.0 ml. The reaction was initiated by adding 2.5-25 µM NADH to the incubation mixture. The activity of NADH-cytochrome c reductase in either the intact or the acetone-treated microsomes was measured by increase in absorbance at 550 nm by employing the value of 2.1 x 10⁴ M⁻¹cm⁻¹ (Massey 1959) as the molar extinction coefficient for the reduction of cytochrome c.

For the NADH-ferricyanide reductase assay, the reaction mixture contained 20-50 µM potassium ferricyanide, appropriate amounts of microsomal protein and 80 mM Tris-HCl buffer (pH 7.5), in a final volume of 3.0 ml. The reaction was started by the addition of 10-50 µM NADH. The activity of NADH-ferricyanide reductase was determined spectrophotometrically by following the decrease in absorbance at 420 nm and employing the value of 1.02 x 10³ M⁻¹cm⁻¹ (Shellenberg and Hellerman 1958) as the molar extinction increment.

**Measurement of initial velocities.** In the kinetic investigation, the true Michaelis constant and maximal velocity were obtained by the double reciprocal plots at different concentrations of substrates. The velocities of reduction were measured at early linear stages which usually continued for 3 min after the initiation of the reaction.

**RESULTS**

**Effect of addition of phospholipid on the NADH-cytochrome c reductase activity in the lipid-depleted microsomes**

The initial velocity of NADH-cytochrome c reductase activity in either the intact or the acetone-treated microsomes increased linearly with the increase in the enzyme amount used as shown in Fig. 1, although the reductase activity in the acetone-treated microsomes was 50% or less of that in the intact microsomes.

When phosphatidylserine in micelle form was added to the acetone-treated microsome system, the rate of cytochrome c reduction was restored to almost the same level as for the intact microsomes as shown in Fig. 2. When a limited amount of phospholipid was employed, however, the line bent on the way and a new line of much smaller slope was obtained. The latter slope resembled that for the acetone-treated microsome system.

Essentially the same results were obtained in a similar experiment using phosphatidylinositol-containing micelles.
Lipid Depletion and Microsomal NADH-Cytochrome c Reductase

Fig. 1. Effect of lipid depletion on the NADH-cytochrome c reductase activity.
The activity was assayed as described in Materials and Methods except that the amount of microsomal protein was varied. The rate of reduction of cytochrome c was followed by increase in absorbance at 550 nm.
—, intact microsomes; ..., acetone-treated microsomes.

Fig. 2. Activation of NADH-cytochrome c reductase in the acetone-treated microsomes by the addition of phosphatidylserine.
The activity was assayed as described in Materials and Methods except that the amount of microsomal protein was varied. —, intact microsomes; ..., acetone-treated microsomes; ---, acetone-treated microsomes with phosphatidylserine micelles added (○, 32 µg of lipid phosphorus; ●, 16 µg of lipid phosphorus).

Effect of lipid depletion on the NADH-ferricyanide reductase activity

When ferricyanide was used in place of cytochrome c as electron acceptor, the NADH-ferricyanide reductase activity was not affected by acetone-treatment as shown in Fig. 3.

Kinetics of the reduction of cytochrome c in the intact and acetone-treated microsomes

The NADH-cytochrome c reductase activity of microsomes was assayed at different concentrations of either NADH (2.5–25 µM) or cytochrome c (2.5–20 µM) as variable substrate. The double reciprocal plots were made and the lines were all parallel for either cytochrome c or NADH as variable substrate both in the intact and acetone-treated microsomes (not shown as figures). These results indicated that the reaction of NADH-cytochrome c reductase in microsomes was kept up with a “Ping-Pong mechanism” (Cleland 1963; Strittmatter and Velick 1957; Hara and Minakamai 1971) even when lipid depletion occurred.

Fig. 4 shows the plots of reciprocals of the apparent maximal velocities against the reciprocals of the concentrations of cytochrome c. Based on the data in Fig. 4, $K_m$ values for cytochrome c and NADH and $V_{max}$ of the microsomal NADH-
Fig. 3. Effect of lipid depletion on the NADH-ferricyanide reductase activity.

The activity was assayed as described in Materials and Methods except that the amount of microsomal protein was varied. The rate of reduction of ferricyanide was measured by decrease in absorbance at 420 nm. ○, intact microsomes; ●, acetone-treated microsomes.

Fig. 4. Reciprocal plots of apparent $V_{\text{max}}$ versus the reciprocals of the concentrations of cytochrome c.

This diagram represents the replots of double reciprocal plots of initial velocities of NADH-cytochrome c reductase activity in the intact and acetone-treated microsomes against NADH concentrations at a series of fixed concentrations of cytochrome c. The concentrations of cytochrome c are intercepts on the ordinate at infinite concentrations of NADH. The reaction mixtures contained 129 and 167 µg protein of the intact (○) or acetone-treated (●) microsomes, respectively. The amounts of NADH and cytochrome c were varied as indicated in Materials and Methods.

cytochrome c reductase were calculated as shown in Table 1. The acetone treatment resulted in changes in $K_m$ for NADH from 2.5 to 8.3 µM and for cytochrome c from 6.3 to 10.0 µM and $V_{\text{max}}$ from 82.9 to 38.6 µM per min per mg protein.
TABLE 1. Comparison of kinetic parameters of NADH-cytochrome c reductase and NADH-ferricyanide reductase before and after acetone-treatment of microsomes

<table>
<thead>
<tr>
<th>NADH-linked reductase</th>
<th>Kinetic parameters</th>
<th>Microsomes</th>
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<tr>
<td></td>
<td></td>
<td>$K_m$ for NADH ($\mu$M)</td>
<td>$K_m$ for electron acceptor ($\mu$M)</td>
<td>$V_{max}$ ($\mu$M/min/mg protein)</td>
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<td></td>
<td>acetone-treated</td>
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<td>10.9</td>
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<tr>
<td>NADH-ferricyanide reductase</td>
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<td>354.5</td>
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<td></td>
<td>acetone-treated</td>
<td>8.5</td>
<td>51.9</td>
<td>353.7</td>
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Kinetic constants were determined from bisubstrate kinetic analysis of initial rate data for NADH-cytochrome c reductase activity given in Fig. 4. Kinetic constants for NADH-ferricyanide reductase were obtained with the same technique as for NADH-cytochrome c reductase. The reaction conditions were as given in Fig. 4 and all experiments in this table were made with a single microsomal preparation.

Kinetics of the reduction of ferricyanide

Kinetic parameters of the NADH-ferricyanide reductase activity in microsomes were estimated in the same way as for the NADH-cytochrome c reductase activity and are shown in Table 1. Acetone-treatment of microsomes apparently had no effect on $V_{max}$ and $K_m$ for ferricyanide, whereas it significantly elevated the $K_m$ for NADH.

DISCUSSION

It has been postulated by several investigators that lipids might play an important role in microsomal electron transfer functions (Jones and Wakil 1967; Rogers and Strittmatter 1973). Jones and Wakil (1967) studied the lipid requirement of the microsomal NADH-cytochrome c reductase of hen liver, and showed that a combination of two different phospholipids, lecithin and lysolecithin, restored completely the decreased reductase activity of the acetone-treated microsomes, although a single addition of lecithin failed to restore the decreased activity.

The present study revealed, however, that the activity of the acetone-treated NADH-cytochrome c reductase was completely restored to the original intact level by addition of sufficient amounts of either phosphatidylserine or phosphatidylinositol (Fig. 2). On the other hand, the activity of NADH-ferricyanide reductase was not affected by the acetone-treatment (Fig. 3), in agreement with the observation by Jones and Wakil (1967).

Then we investigated kinetically the properties of NADH-cytochrome c reductase in the intact microsomes, and compared them with those in the acetone-treated microsomes. The results indicated that the lipid depletion from microsomes decreased the $V_{max}$ significantly and increased the $K_m$ values for either NADH or
cytochrome c. Therefore, the decrease of the reductase activity in microsomes by the acetone-treatment might be attributable to the changes of the kinetic parameters of either \( K_m \) or \( V_{max} \).

In the NADH-ferricyanide reductase system, the \( K_m \) value for ferricyanide as well as the maximal velocity of the reductase in the lipid-depleted microsomes was essentially the same as in the intact microsomes. It has been shown that exogenous ferricyanide accepts electron directly from microsomal flavoprotein, but not through cytochrome \( b_5 \) (Strittmatter and Velick, 1956b). These situations suggest that the lipid depletion of microsomes seems to have affected the rate of electron transfer between flavoprotein and exogenous cytochrome c, but not between NADH and flavoprotein in the NADH-cytochrome c reductase.

Recently Rogers and Strittmatter (1974) indicated that cytochrome \( b_5 \) and cytochrome \( b_5 \) reductase are randomly distributed in the microsomal membrane. They (1973) also suggested that in the lipid-free microsomes the protein might exist as a heterogeneous mixture of high molecular weight aggregates, probably due to hydrophobic interactions. Consequently the lipid depletion could modify the spacial arrangement of both flavoprotein and cytochrome \( b_5 \) in microsomes.

In our case the addition of enough amounts of either phosphatidylserine or phosphatidylinositol restored the NADH-cytochrome c reductase activity in the acetone-treated microsomes to nearly the same level as that in the intact microsomes. As discussed above, the lipid-depletion of microsomes seems to have affected the rate of electron transfer between flavoprotein and exogenous cytochrome c. Therefore, interaction of phospholipids with microsomal NADH-cytochrome c reductase would have an important role, especially in the reduction of cytochrome \( b_5 \).

References


