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INTERMEMBRANE ELECTRON TRANSPORT IN THE ABSENCE OF ADDED WATER-SOLUBLE CARRIERS

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SUMMARY

Electron transport from untreated to mersalyzed microsomal vesicles at the level of NADH-cytochrome b_5 reductase or cytochrome b_5 has been demonstrated in the absence of added water-soluble electron carriers. A similar effect was shown in the systems "intact mitochondria – mersalyzed microsomes" and "mersalyzed mitochondria – untreated microsomes". No measurable electron transport between intact and mersalyzed particles of inner mitochondrial membrane was found. The obtained data suggest that the capability to carry out intermembrane electron transfer is specific for NADH-cytochrome b_5 reductase and/or cytochrome b_5, localized in microsomal and outer mitochondrial membranes.

INTRODUCTION

In previous papers [1, 2] we have described an intermembrane electron transfer between microsomal redox chain and mitochondrial cytochrome oxidase via added cytochrome *c*. Further study along this line revealed that in the same system there is a mechanism of intermembrane transfer of reducing equivalents requiring no water-soluble carrier.

MATERIALS AND METHODS

NADH was obtained from C. F. Boehringer, rotenone, succinate (disodium salt) and mersalyl were from Sigma Chemical Co., tris(hydroxymethyl)aminomethane and pronase were from Calbiochem.

150–200 g male rats were used in experiments on microsomes and mitochondria. The animals were starved for 12 h before decapitation. To obtain microsomes, rat liver was perfused by isolation medium (0.15 M cooled solution of KCl). A 25 % homogenate was prepared in a Dounce homogenizer with a Teflon pestle. Unbroken cells, nuclei and mitochondria were centrifuged for 20 min at $9600 \times g$ in a High-Speed 18 (MSE) centrifuge. The microsomal fraction was sedimented by means of

centrifugation of the postmitochondrial supernatant at 100 000 $\times g$ for 1 h in a VAC 601 (Janezki) centrifuge. All procedures for obtaining the microsomal fraction were carried out at +4 °C. The microsomal sediment was suspended in a small volume of the isolation medium. The protein content was determined as described by Lowry et al. [3] in the presence of 0.1 % sodium deoxycholate. Crystalline bovine serum albumin (fraction V, Sigma) was used as a standard. For method of preparation of rat liver mitochondria, see ref. [1]. Beef heart submitochondrial particles without factor F_1 (NaCl particles) were prepared as described in ref. 4.

The cytochrome b_5 reduction was monitored by a Hitachi 356 spectrophotometer at 408–424 nm. Anaerobiosis was achieved by means of the glucose oxidase system.

Oxygen uptake was measured with an LP-7 polarograph and a stationary platinum electrode covered with a Teflon film.

Treatment of microsomes, mitochondria or submitochondrial particles with mersalyl, an inhibitor for SH groups, was carried out at 4 °C for 30 min. Mersalyl concentration was $1 \cdot 10^{-6}$ M, protein concentration 10 mg/ml.

Pronase treatment of microsomes was performed at 4 °C for 12 h in the mixture containing 20 % glycerol, 1 mM dithiothreitol, 150 mM KCl, 50 mM Tris/HCl (pH 7.4), 0.04 mg pronase/mg microsomal protein. Then the mixture was centrifuged at 250 000 $\times g$ for 2 h. The sediment was washed with 150 mM KCl.

RESULTS AND DISCUSSION

In the first series of experiments, electron transfer between intact and mersalyl-treated microsomal membranes was studied. As is seen in Fig. 1A, NADH addition to anaerobic microsomes whose NADH-cytochrome b_5 reductase was inactivated by mersalyl pre-treatment, does not reduce cytochrome b_5. Subsequent addition of dithionite results in cytochrome b_5 being reduced (Curve 1). In the untreated microsomes, NADH induced almost complete cytochrome b_5 reduction (Curve 2). Curve 3 shows the response of the mixture of the mersalyl-treated and untreated microsomes. In this case, cytochrome b_5 was found to be reduced by NADH not only in the untreated, but also in mersalyl-inhibited microsomes. These data indicate that there is electron transfer from untreated to mersalyl-treated microsomes, which occurs at the levels of NADH-cytochrome b_5 reductase and (or) cytochrome b_5.

Another piece of evidence of intermicrosomal electron transfer was obtained in the aerobic experiments (Fig. 1B). Again, addition of NADH to the mersalyl-treated microsomes was without effect (Curve I). NADH treatment of the uninhibited microsomes gave rise to a reduction of cytochrome b_5. Then cytochrome b_5 was reoxidized due to NADH exhaustion (Curve 2). In the sample containing a double amount of microsomes, half of which were mersalyzed (Curve 3), the higher level of cytochrome b_5 reduction was observed. Besides, cytochrome b_5 reoxidation proceeded much faster than in the previous case, the facts suggesting the mersalyl-treated microsomes do take part in NADH utilization.

The above data on mixtures of inhibited and noninhibited microsomes cannot be explained by redistribution of intact and mersalyl-inhibited NADH cytochrome b_5 reductases between two microsomal pools. If it were the case, preincubation of the treated and untreated microsomes before NADH addition would

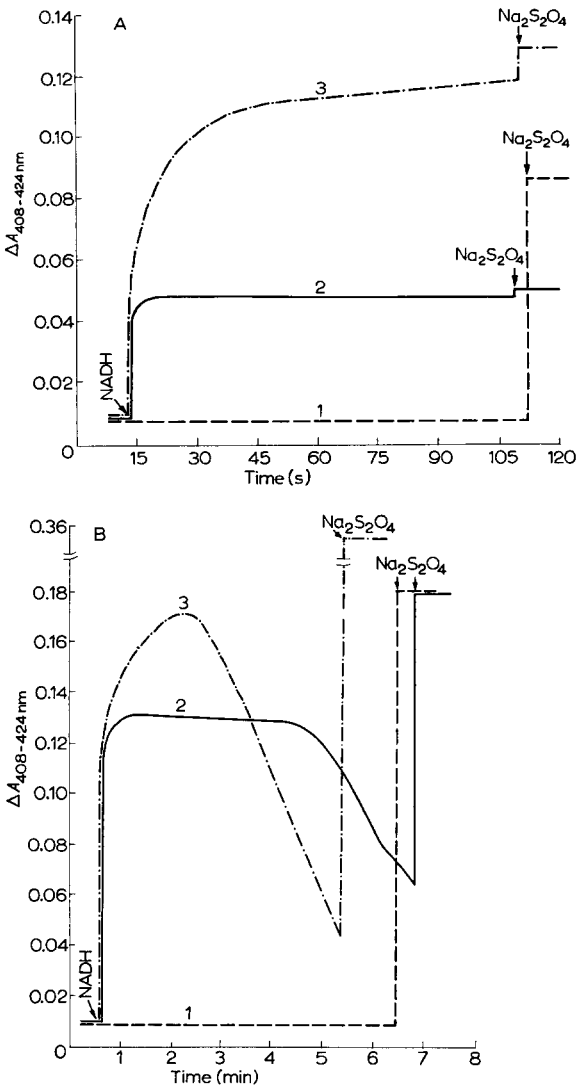

Fig. 1. Anaerobic (A) and aerobic (B) reduction of cytochrome b_5 in the mersalyl-treated and untreated microsomes. A, 3 ml incubation mixture contained 50 mM Tris/HCl buffer, pH 7.4, 33 mM glucose, glucose oxidase (50 units), catalase (500 units); 3 mg microsomal protein treated with mersalyl (Curve 1); 1.5 mg protein of intact microsomes (Curve 2); 1.5 mg protein of intact microsomes and 3 mg protein of mersalyzed microsomes (Curve 3). Microsomes were incubated for 5 min to obtain anaerobiosis, then the reaction was initiated by adding $1 \cdot 10^{-5}$ M NADH. B, 3 ml incubation mixture contained 50 mM Tris/HCl buffer, pH 7.4; 6 mg protein of microsomes treated with mersalyl (Curve 1); 6 mg protein of the untreated microsomes (Curve 2); 6 mg protein of untreated microsomes and 6 mg protein of microsomes treated with mersalyl (Curve 3).

have caused an increase in the reduction rate of cytochrome b_5 in the mersalyl-treated vesicles. In fact, a decrease of this rate was observed (Fig. 2), which was apparently due to redistribution of free mersalyl between two pools of microsomes and partial inhibition of NADH-cytochrome b_5 reductase in the intact microsomes.

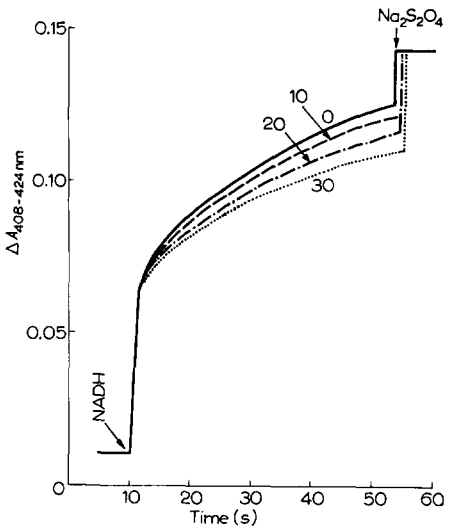

Fig. 2. The effect of the preincubation time on the rate of intermembrane electron transfer in the mixture of untreated and mersalyzed microsomes (anaerobic conditions). The incubation mixture contained 50 mM of Tris/HCl buffer pH 7.4, 100 μM glucose; glucose oxidase (50 units), catalase (500 units), 1.8 mg of protein of microsomes treated with mersalyl (for the condition of the treatment by mersalyl see Fig. 1) and 1.8 mg of intact microsomes in a total volume 3 ml was incubated at 25 °C. Duration of preincubation (min) is shown above the curves. Addition: 10 μM NADH.

Intermembrane electron transfer was found to be abolished by a pronase pretreatment. In the mixture of mersalyzed microsomes and pronase-treated microsomes, NADH addition did not induce cytochrome b_5 reduction (Fig. 3).

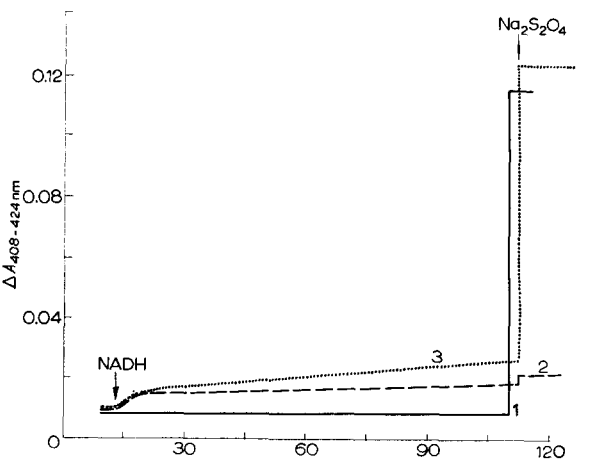

Fig. 3. Absence of intermembrane electron transfer in the mixture of mersalyzed and pronase-treated microsomes. Curve 1, mersalyzed microsomes (1 mg protein/ml); 2, pronase-treated microsomes (1 mg protein/ml); 3, mixture of mersalyzed microsomes (1 mg protein/ml) and pronase-treated microsomes (1 mg protein/ml). Other conditions as in Fig. 2.

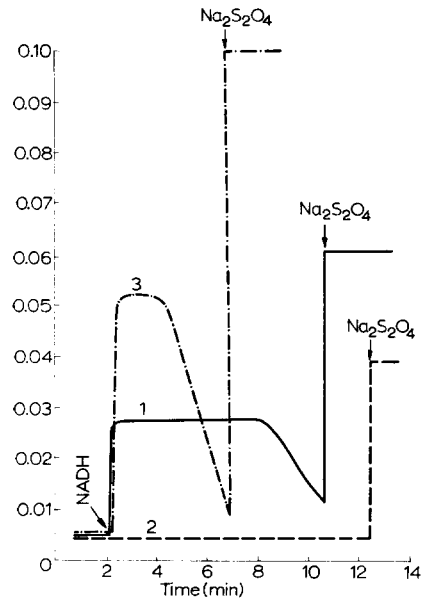

Fig. 4. Aerobic reduction and oxidation of cytochrome b_5 in the mixture containing intact mitochondria and microsomes treated with mersalyl. The incubation mixture (3 ml) contained 50 mM Tris/HCl buffer, pH 7.4, $5 \cdot 10^{-6}$ M rotenone; and 3 mg protein of microsomes treated with mersalyl. Curve 1, 4 mg mitochondrial protein; Curve 2, 3 mg protein of microsomes treated with mersalyl; Curve 3, 4 mg mitochondrial protein and 3 mg mersalysed microsomes. The reaction was initiated by adding 10 μM NADH to the incubation mixture.

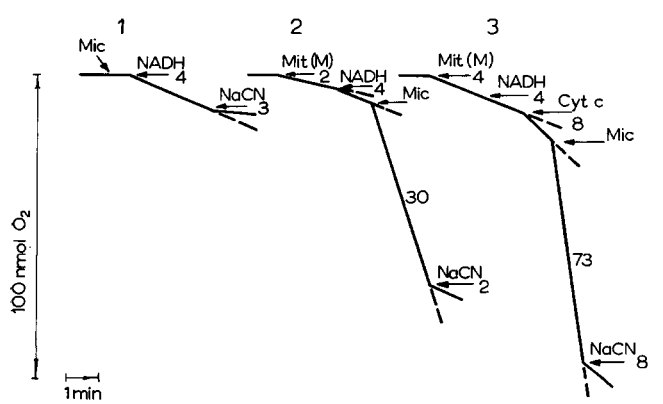

Fig. 5. NADH oxidation in the system of mersalyzed mitochondria and untreated microsomes. Incubation mixture contained 50 mM Tris/HCl buffer, pH 7.4. Additions: 1 mM NADH, 1 mM NaCN, 10 μM cytochrome *c* (cyt. *c*), 1 mg microsomal (mic) protein (Curve 1), 8 mg protein of mitochondria (mit) treated with mersalyl and 1 mg microsomal protein (Curve 2), 1 mg protein of mitochondria treated with mersalyl and 0.05 mg microsomal protein (Curve 3). Figures above the curves show the rate of oxygen uptake in nmol $\cdot$ mg^{-1} $\cdot$ min^{-1}.

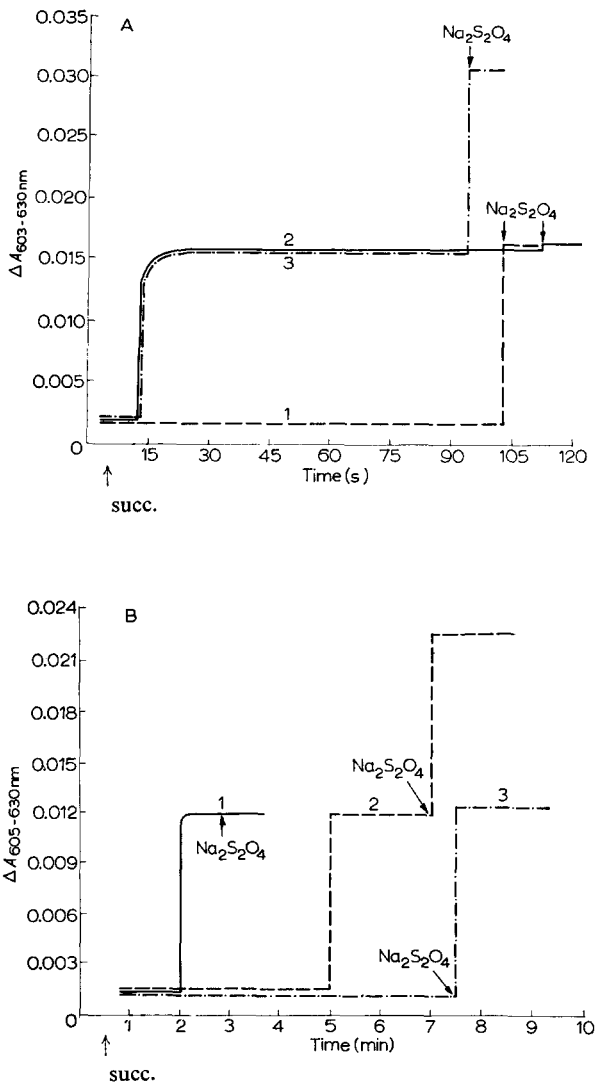

Fig. 6. Reduction of cytochromes $a+a_3$ in the system containing untreated submitochondrial particles and those treated with mersalyl. A. Anaerobic conditions. 3 ml of incubation mixture contained 50 mM Tris/HCl buffer, pH 7.4; NaCN 2 mM, glucose 100 μM, 50 units of glucose oxidase activity, rotenone $5 \cdot 10^{-6}$ M. Curve 1, 6 mg of protein of submitochondrial particles treated with mersalyl (the conditions of mersalyl treatment see fig. 4); Curve 2, 6 mg of protein of the intact particles; Curve 3, 6 mg of protein of particles treated with mersalyl and 6 mg of protein of the intact particles. The reaction was initiated by adding 10 mM succinate (succ.). B. Aerobic conditions. 3 ml of incubation mixture contained 50 ml Tris/HCl buffer, pH 7.4; rotenone $5 \cdot 10^{-6}$ M. Curve 1, 5 mg intact particles; Curve 2, 5 mg protein of the particles treated with mersalyl and 5 mg protein of the intact particles; Curve 3, 5 mg submitochondrial particles treated with mersalyl. The reaction was initiated by adding 10 mM succinate.

Results, similar to those in Fig. 1, were obtained when mitochondria, instead of untreated microsomes, were added to mersalyzed microsomal preparation (Fig. 4).

The data in Fig. 4 may be interpreted as an indication of intermembrane electron transfer from mitochondria to microsomes. Fig. 5 demonstrates the electron transfer in the opposite direction, i.e. from microsomes to mitochondria. Rat liver mitochondria were treated with mersalyl to inhibit electron transfer chains of the inner and outer membranes. Under these conditions, added NADH induced a very slow oxygen consumption. Subsequent addition of untreated microsomes stimulated significantly the rate of respiration which was sensitive to cyanide. Further stimulation of oxidation rate required cytochrome *c* addition (cf. Curves 2 and 3).

In the last series of experiments, an attempt to demonstrate electron transfer between untreated and mersalyzed submitochondrial particles was undertaken. The data are shown in Fig. 6. One can see (Fig. 6A, Curve 1) that succinate addition to the untreated anaerobic submitochondrial particles induced fast and complete reduction of cytochromes $a+a_3$ (Curve 2). In the particles treated with mersalyl in a concentration (0.1 μmol mersalyl per mg protein) that induces complete inhibition of succinate oxidase, cytochrome $a+a_3$ reduction occurred after addition of dithionite but not of succinate (Curve 1). When the mixture of equal amounts of mersalyzed and intact particles was used (Curve 3), it was found that succinate reduced only a half of the total cytochrome $a+a_3$ pool. Similar results were obtained in the experiments carried out in aerobic conditions (Fig. 6B).

These data indicate that intermembrane electron transfer between submitochondrial particles cannot take place in the conditions used. The fact that in the same conditions electrons can be transported between microsomal vesicles or between microsomes and the outer membrane of mitochondria suggests that catalysis of intermembrane transfer of reducing equivalents may be specific for NADH-cytochrome b_5 reductase flavoprotein and (or) cytochrome b_5, localized in the microsomal and outer mitochondrial membrane.

It may be important to note in this connection that molecules of NADH-cytochrome b_5 reductase and cytochrome b_5 are composed of two unequal parts: the larger one (3/4 of the enzyme molecule) hydrophilic, containing a flavin or a heme group responsible for the catalytic function, and the smaller one, hydrophobic, requiring the protein to be anchored to a membrane [5, 6]. Apparently, intermembrane electron transfer occurs between two molecules of these redox carriers belonging to two different membrane vesicles. This process proves possible due to the fact that prosthetic groups of the membrane-bound reductase and cytochrome b_5 are situated outside the membrane.

Two alternative mechanisms of intermembrane oxidoreduction can be considered. (1) Both the reductase and cytochrome b_5 are immobile in the membrane, and electron transfer from one membrane to another occurs in a site of occasional collision of two vesicles. (2) The reductase and (or) cytochrome b_5 can diffuse along the membrane, so that exchange of electrons between two vesicles coming into contact proves possible for carriers localized not only in the site of collision but also in other areas of the vesicles. Taking into account the great difference in sizes of proteins and microsomal vesicles or mitochondria we can conclude that the latter mechanism seems to be much more probable than the former one. In this connection, data of Strittmatter's

group [5–7] indicating that NADH-cytochrome b_5 reductase and cytochrome b_5 diffuse quickly along microsomal membrane should be noted.

An intriguing possibility is that the biological function of NADH-cytochrome b_5 reductase and cytochrome b_5 consists of transport of reducing equivalents from one part of cytosol to another by means of lateral diffusion and intermembrane transfer. Assuming this point, we may suggest that different regions of protoplasm are equilibrated with respect to reducing equivalent of -0.3 V redox potential by means of NADH-cytochrome b_5 reductase, and of about zero redox potential by means of cytochrome b_5. Within a single cistern of endoplasmic reticulum or a single mitochondrion, this process involves lateral diffusion of the above float-like proteins in the plane of the membrane. Lateral diffusion of the reductase and cytochrome b_5, supplemented with intermembrane electron transfer, allowed the single vesicle limitation to be overcome and the overall intracellular membrane service for reducing equivalent transport to be organized.

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