IMMOBILIZATION OF MITOCHONDRIAL MEMBRANE IN SEPHAROSE 4B *VIA* PROTEOLIPOSOME RECONSTITUTION AND ITS D-β-HYDROXYBUTYRATE DEHYDROGENASE ACTIVITY

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Introduction

Membrane enzymes participating in central cell-metabolism in biological membranes have much potential for the production of biologically active compounds. Their practical application requires them to be immobilized on an appropriate support since the enzymes essentially require phospholipids or phospholipid bilayer structures for their optimal reactivity and stability. Liposomes, phospholipid bilayer vesicles, provide a suitable support, but are seriously limited because of their deficiency in inherent instability and handling[3]. One of the promising techniques to overcome these deficiencies is to immobilize the liposome in the pores of gel beads. This technique has been developed by Wallstén *et al.*[6] We have immobilized liposome-reconstituted γ-glutamyl transpeptidase (a membrane enzyme) in Sepharose CL-6B gel beads by applying the method of Wallstén, and have demonstrated the usefulness of the liposome-gel hybrid system as a support[1]. This system can be also be expected to be applicable to the immobilization of whole cell membranes as well as purified enzymes. Such immobilization would optimize the enzymes by providing them with nature's preferred environment. For engineering purposes, this is preferable because great difficulties are often encountered in the separation and purification of membrane enzymes.

The purpose of this study was to immobilize mitochondrial membrane fractions in Sepharose 4B gel beads and to examine the catalytic availability of the membrane with the guidance of the activity of D-β-hydroxybutyrate dehydrogenase (DBH, D-3-hydroxybutyrate:NAD^+ oxidoreductase, EC 1.1.1.30), which is originally localized on the inner face of inner mitochondrial membrane.

1. Experimental

1.1 Materials

Mitochondrial membrane fraction (23.9 mg/ml of proteins and 9.9 mg/ml of phospholipids) was prepared from pig liver according to the method of Hogeboom[2]. Egg yolk phospholipids, DL-3-hydroxybutyric acid, and NAD^+ were purchased from Wako Pure Chemicals. Sepharose 4B and Sephacryl S-500 were products of Pharmacia.

1.2 Immobilization of mitochondrial membrane fraction

Procedures used were similar to those previously described[1]. The membrane fraction was dispersed in a 50 mM Tris-HCl (pH 7.5) solution containing 200 mM saccharose, 1 mM EDTA, 4.5 μM rotenone, and 100 mg/ml cholate. The protein and phospholipid concentrations of the resultant dispersion (termed MF dispersion) were adjusted to 5 and 30 mg/ml, respectively, with egg yolk phospholipids. Sepharose 4B gel equilibrated with the MF dispersion was dialyzed against dialysis buffers containing 1 mM EDTA and 200 mM saccharose. After the separation of the gel and its supernatant and the subsequent washing of the gel with the dialysis buffers, the gel was subjected to protein and phospholipid analysis as reported elsewhere[1].

1.3 Enzyme assay

Proteoliposomes and MF dispersion were assayed for DBH reaction essentially according to Sekuzu *et al.*[4] The reaction mixtures contained in the final 1.0 ml volume: 50 mM Tris-HCl (pH 8.1), 0.5 mM EDTA, 2.5 mM dithiothreitol, 3 mM NAD^+, 20 mM DL-3-hydroxybutyric acid, 200 mM saccharose, and a suitable amount of proteoliposomes or MF dispersion. In order to prevent the reoxidation of NADH by the action of electron transfer chain, inhibitor rotenone had been contained in the MF dispersion before the proteoliposome reconstitution. The accumulation of NADH was examined by change in absorbance at 340 nm on a spectrophotometer, using an extinction coefficient of $6.27 \times 10^3\ M^{-1}\ cm^{-1}$ for NADH.

The DBH activity of gel-entrapped proteoliposomes was examined by use of a fluidized bed reactor (12 × 150 mm) equipped with a water jacket. One milliliter of the gel

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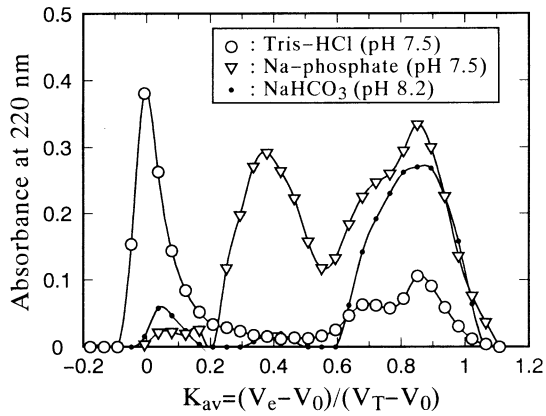

Fig. 1 Sephacryl S-500 gel column chromatogram of proteoliposomes reconstituted with various dialysis buffers, where V_e is the elution volume, V_0 the elution volume of human red blood cells, and V_T the total volume of the column

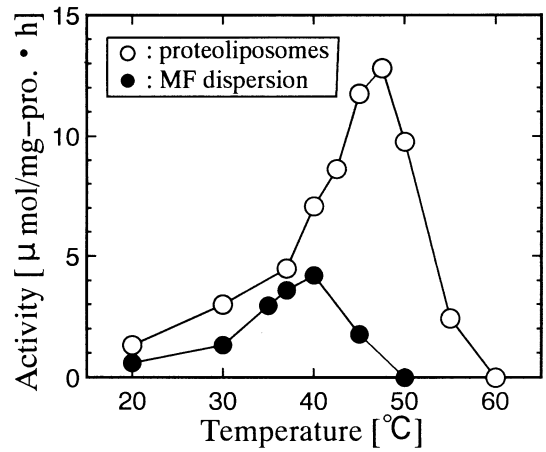

Fig. 2 Effect of temperature on D-β-hydroxybutyrate dehydrogenase activity

was loaded in the reactor, and 22 ml of the assay mixture was circulated by a peristaltic pump at 37°C at a flow rate of 0.5 ml/min. An aliquot of the mixture was withdrawn at regular time intervals, and changes in absorbance were measured. Gel-specific activity was calculated by

$$\text{Gel-Specific Activity}\,[\mu\text{mol/ml-gel}\cdot\text{h}] = \frac{dA}{dt} \times \frac{1}{6.27\times10^3} \times \frac{V_t}{V_g} \times 10^3 \quad (1)$$

where A is absorbance, V_g the gel volume, and V_t the total volume of the gel and the assay mixture.

2. Results and Discussion

The immobilization was performed with several dialysis buffers, and proteoliposome reconstitution in gel supernatants was examined on a Sephacryl S-500 gel column chromatography. As **Fig. 1** shows, a 50 mM Tris-HCl (pH 7.5) dialysis buffer caused increases in absorbance at 220 nm around void fractions, in which proteins and phospholipids were determined to be in a ratio of 2.1. This suggests that the reconstitution of large proteoliposomes occurs. Sodium hydrogen carbonate (pH 8.2) and Na-phosphate (pH 7.5) buffers did not give such large proteoliposomes. Colorimetric analysis of Sepharose 4B gels obtained in these buffers showed that few proteins and phospholipids existed in the gels of the latter two buffers. On the contrary, 0.67 mg/ml-gel of proteins and 0.28 mg/ml-gel of phospholipids were found in the gel of the Tris-HCl buffer. The relationship between the immobilization and the constitution of proteoliposomes was consistent with the previous study[1]: enzyme-bound liposomes having K_{av} values of about 0.4 on a Sephacryl S-500 column are not immobilized on Sepharose 4B but are immobilized on Sepharose CL-6B, which has pores smaller than Sepharose 4B. These results indicate that mitochondrial membrane of pig liver was immobilized in the pores of the gel beads in the form of proteoliposomes with the Tris-HCl buffer.

The proteoliposome immobilization was also confirmed by dye calcein entrapment. Thus, the gel beads and their supernatant, which were obtained in the same way except for dye calcein instead of saccharose in MF dispersion, showed a rapid increase in fluorescence by the addition of detergent Triton X-100 (data not shown). Since calcein fluorescence is self-quenching in the vesicle interior, it is clear that the proteoliposomes are immobilized in Sepharose 4B.

The DBH reaction was determined first by using proteoliposomes in the gel supernatant at 37°C which gave a specific activity of 0.45 μmol/mg-protein·h, which was comparable to that of MF dispersion. **Figures 2** and **3** show the temperature dependence of the DBH reaction. As Fig. 2 shows, the proteoliposome constitution raised the optimal temperature and yielded DBH activity of about 3 times higher than the MF dispersion at each optimal temperature. Figure 3 shows the remaining DBH activity after the preincubation at 50°C at pH 7.5. The enzyme in the proteoliposomes is much more stable than that in MF dispersion and doubles in activity with a 20-min pre-incubation. The unexpected activation was thought to be due to the rearrangement of membrane components. Thus, the proteoliposomes were quite effective for DBH activity and stability.

The Sepharose 4B-entrapped proteoliposomes were examined next for their catalytic availability by use of a fluidized bed reactor. As **Fig. 4a** shows, the absorbance raises linearly with time when rotenone and both NAD^+ and D-3-hydroxybutyric acid were contained in the proteoliposomes and in the reaction mixture, respectively. Control experiments in the absence of NAD^+ and rotenone showed that this increase is attributed to NADH formation and not to the leakage of proteoliposomes. Figure 4b shows the reusability of the gel-entrapped proteoliposomes after the used gel beads were recovered. In the presence of saccharose, the gel-entrapped contents of proteins and phospholipids (0.67 and 0.28 mg/ml-gel, respectively) were unchanged even after the third operation, and the constant gel-specific activity of about 0.12 μmol/ml-gel·h was obtained at each operation. In the absence of saccharose,

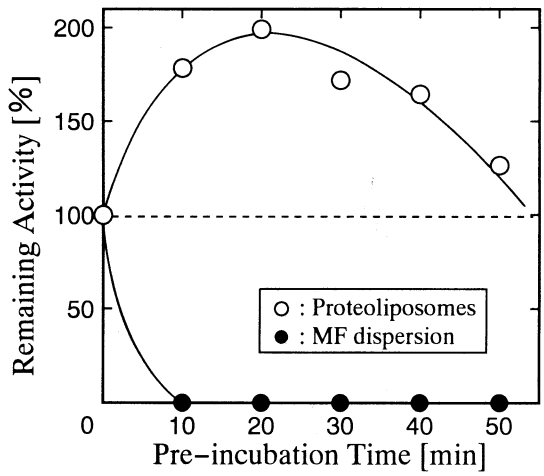

Fig. 3 Effect of pre-incubation time at 50°C on the remaining activity of D-β-hydroxybutyrate dehydrogenase

however, the repeated operation lowered the DBH activity and completely abolished after the second operation. The disappearance of the activity was due to the disruption of proteoliposome membrane by osmotic pressure, since neither proteins nor phospholipids were found in the gel beads. From these data, it is concluded that the gel-entrapped proteoliposomes are capable of exhibiting catalytic activity and are relatively stable and reusable as long as an isotonic solution was employed. Although Strasser *et al.*[5] immobilized submitochondrial particles on concanavalin A-Sepharose through the binding of the particles-containing glycoproteins and the concanavalin A, the present system permits cell membranes to be easily immobilized in Sepharose gel without any attachments and to display the catalytic function of membrane enzymes.

Conclusion

The crude mitochondrial membrane of pig liver was

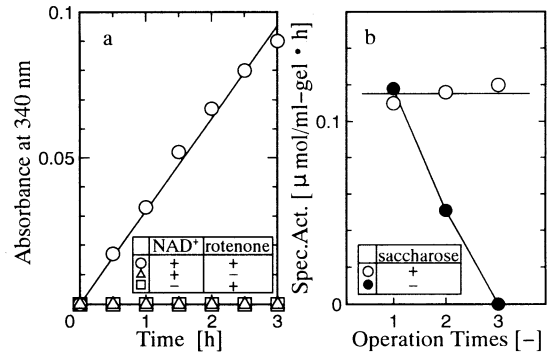

Fig. 4 D-β-Hydroxybutyrate dehydrogenase reaction of Sepharose 4B-entrapped proteoliposomes: a) time course and b) reusability

immobilized in Sepharose 4B gel in the form of proteoliposomes and had the activity of DBH, a membrane enzyme. The gel-immobilized proteoliposomes were relatively stable and reusable and effective for the activity and stability of DBH.

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