FUSION OF CELLS AND PROTEOLIPOSOMES: INCORPORATION OF BEEF HEART CYTOCHROME OXIDASE INTO RABBIT ERYTHROCYTES

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1. Introduction

Biochemical research of plasma membranes has been hampered by the limited possibilities of manipulation of its composition and components. Once proteins are solubilized, many membrane functions such as solute transport, response to stimuli and cellular adhesion are lost. Recently, procedures have been devised for reconstitution of transport functions of membrane proteins [1,2]. A promising approach to functional research of other plasma membrane proteins could be their isolation and reinsertion into cells lacking them, thus allowing their bioassay.

The most promising experimental approach seems to be initial reconstitution of the protein into a proteoliposome by one of the available procedures [1,2] and subsequent fusion of the latter with cells. Fusion of liposomes and cells has been studied extensively [3]; however proteoliposomes have not yet been fused with cells. Here, beef heart cytochrome oxidase was incorporated into proteoliposomes [4,5], the proteoliposomes were fused with rabbit erythrocytes and the presence of cytochrome oxidase in the erythrocyte membranes was demonstrated with ferritin-conjugated antibodies.

2. Materials and methods

Phosphatidylcholine and cardiolipin were purchased from Sigma Chemical Co. Phosphatidylethanolamine was purified from soybean lipids according to [6]. Rat liver ^{32}P-labeled phospholipids were prepared according to [7]. Cytochrome oxidase was prepared from beef heart mitochondria by the method in [8,9]. Cytochrome oxidase antiserum was prepared in rabbits according to [10]. Ferritin-conjugated goat anti-rabbit IgG was purchased from Miles Yeda Ltd. The buffer used throughout the work contained 128 mM KCl, 32 mM NaCl, 20 mM Hepes (*N*-2-hydroxyethylpiperazine-*n'*-2-ethanesulfonic acid (pH 7.6)) and 0.5 mM EDTA.

Rabbit erythrocytes were prepared by drawing blood into an equal volume of buffer containing heparin (0.1 mg/ml). The erythrocytes were washed 3 times with buffer. Liposomes were prepared by drying a phospholipid solution under a stream of nitrogen gas. The lipids were redissolved in ether, dried and resuspended in buffer. The suspension was sonicated in a bath-type sonifier (80 W, 50 kHz) until samples became clear. The pH was readjusted to 7.6 whenever necessary. Cytochrome oxidase (1 mg/ml) vesicles were reconstituted by direct incorporation as in [5]. Concentrations of phospholipids and liposomes were expressed in terms of their phosphate content which was determined by total washing of samples, hydrolysis in 0.5 N HCl and phosphate analysis as in [11]. Liposome concentration was equivalent to 25 mM P_i. In some experiments, the proteoliposomes were made radioactive by preparing them with ^{32}P-labeled phospholipids.

The standard fusion procedure was as follows: radioactive cytochrome oxidase vesicles (5 mM) and erythrocytes (30% v/v) were incubated for 1 h at room temperature in presence of 20 mM $CaCl_2$. EDTA (40 mM) was added and the erythrocytes were separated from excess proteoliposomes by cen-

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trifugation into a Ficoll (10% in buffer) cushion. The ^{32}P content and cytochrome *c*-dependent oxygen consumption were determined both in the cell pellets and in the supernatants. Cytochrome oxidase activity was assayed polarographically essentially as in [12].

Proteoliposome-treated erythrocytes were prepared for electron microscopic observation by a method in [13]. Termination of fusion was followed by 2 washes with buffered sucrose (0.3 M). The suspensions were fixed with glutaraldehyde. After further washes, these samples (0.5 ml, 40% v/v) were incubated with cytochrome oxidase antiserum (0.2 ml) for 30 min at 30°C. This amount of antiserum was enough to inactivate all cytochrome oxidase activity introduced into the cells. The cells were washed, treated with ferritin-conjugated goat anti-rabbit IgG and prepared for thin sectioning and observation in the electron microscope as in [13].

3. Results and discussion

Cytochrome oxidase proteoliposomes were prepared with constant amount of cardiolipin and varying amounts of phosphatidylcholine and phosphatidylethanolamine. The vesicles were incubated with erythrocytes in presence of calcium ions. As shown in fig.1, only vesicles containing more than 50 mol% phosphatidylethanolamine became associated with erythrocytes. Both the phospholipids and the enzyme activity became attached to the cells. No enzyme activity was lost upon association with the cells. The proton pump found in proteoliposomes was lost upon incorporation into cells. This was expected since erythrocytes are permeable to protons. In order to preclude the possibility that the association of the proteoliposomes with erythrocytes occurred by transfer of individual components of the proteoliposomes to the cells, the 3 phospholipids included in the proteoliposomes were radioactively labeled. As shown in fig.2, the pattern of incorporation of the

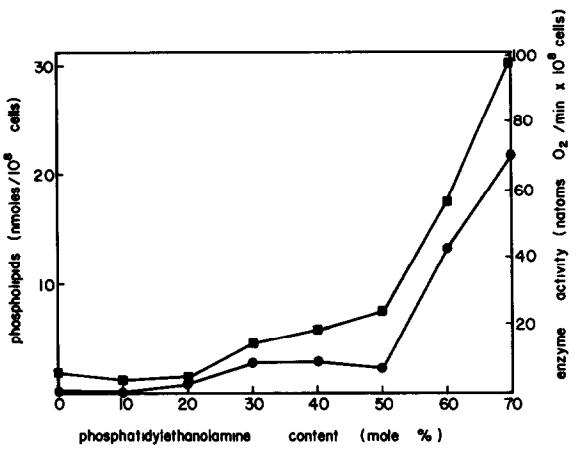

Fig.1. Effect of proteoliposome composition on fusion with cells. Liposomes (25 mM P_i) were prepared with cardiolipin (30 mol%) and varying amounts of phosphatidylcholine and phosphatidylethanolamine. $^{32}P_i$-labeled phosphatidylcholine and phosphatidylethanolamine were included in the liposomes. Cytochrome oxidase (1 mg/ml) was incorporated into the liposomes as described. The resulting proteoliposomes were incubated with erythrocytes in the presence of 20 mM $CaCl_2$ for 1 h at room temperature. The cells were washed as described and assayed for associated phospholipid radioactivity (■) and cytochrome oxidase activity (●).

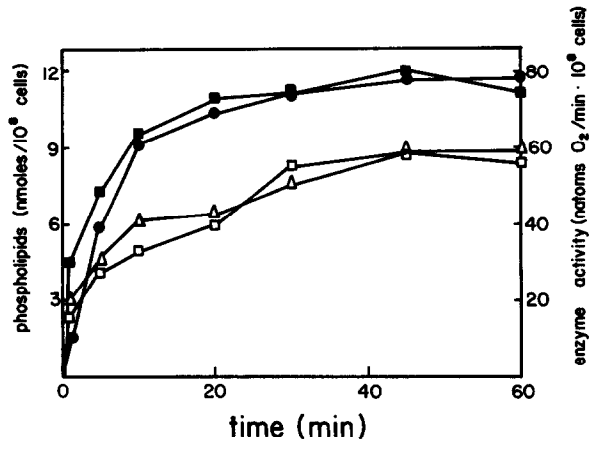

Fig.2. Time course of incorporation of proteoliposome components into cells. Cardiolipin (10 μmol), phosphatidylethanolamine (7.5 μmol), phosphatidylcholine (7.5 μmol) and radioactive rat liver phospholipids were dried. The specific radioactivities of the phospholipids were 0.01, 0.04 and 0.04 μCi/μmol, respectively. Buffer (1 ml) was added and the liposomes were formed by sonication to clarity. Cytochrome oxidase (1 mg/ml) was incorporated and the resulting proteoliposomes were incubated with cells (at 1:5, v:v) with 10 mN $CaCl_2$ (10 mM). After the indicated intervals, samples were withdrawn into 20 mM EDTA containing buffer and washed. The enzyme activity (●) associated with cells was determined. The cell lipids were extracted, separated by thin-layer chromatography and the radioactivity in the extracted spots corresponding to cardiolipin (■), phosphatidylethanolamine (□) and phosphatidylcholine (△) was counted.

individual phospholipid species and cytochrome oxidase activity was similar, indicating that whole proteoliposomes became associated with the erythrocytes. The association was rapid at room temperature and practically completed within 30 min. The association was dependent on Ca^{2+}. Maximal binding was achieved at 15–20 mM $CaCl_2$. Under these conditions 25–40 nmol phospholipids became attached to 10^8 cells. This corresponds to 30–48 × 10^3 enzyme molecules incorporated into 1 erythrocyte.

In order to confirm that the effects observed in this work were indeed fusion and not adhesion, we have utilized electron microscopy. Observation of the cells after incubation with proteoliposomes in the presence of $CaCl_2$ revealed almost no vesicles either adhering to the cell membranes or included in the cell matrix. We have localized the incorporated enzyme in the cell membrane. For this purpose, rabbit antibodies against the bovine cytochrome oxidase were prepared and used in combination with ferritin-conjugated goat anti-rabbit antibodies. Erythrocytes were incubated with cytochrome oxidase vesicles in the presence of 20 mM Ca^{2+}. Subsequently, the cells were washed and treated for visualization of incorporated enzyme molecules. As shown in fig.3d,e the cells were labeled with ferritin marking the enzyme location. The number of ferritin molecules bound to each thin section of the cells ranged in the hundreds. The ferritin appeared in clusters of ~5 molecules. This was expected since it has been shown that several molecules react with 1 cytochrome oxidase molecule [14]. Moreover, it has been shown that each molecule of Rh antigen is labeled with several ferritin molecules [15]. Another noteworthy feature is that the ferritin molecules are not located next to the membrane but at 20–45 nm. This is explained by the combined length of the 2 antibody molecules; the protruding part of the cytochrome oxidase and the ferritin molecule is ~40 nm [16,17]. The erythrocyte membrane is clearly visible and no other membrane structures are associated with it. The amorphous material next to the membrane is the antibodies. The ferritin clusters were randomly distributed around the cell membranes. In order to rule out the possibility that the ferritin labeling was due to non-specific binding of antibodies or even to specific labeling of some component of the rabbit cells, we have fused protein free liposomes with the cells and treated them as above. In this case only very few ferritin molecules were observed in thin sections of the cells (fig.3a,b) and no amorphous material was observed. The insertion of the proteoliposome enzyme into the cell membranes was easier to observe in ghosts occasionally formed during the treatment of the cells with antibodies. There, the membranes were not obscured by the dense packing of the hemoglobin and it was clear that the ferritin molecules were indeed associated with the cell membranes and not with vesicles tightly bound to the cells. In ghosts the ferritin label appeared on both faces of the membranes (fig.3).

An intriguing feature is that in ghosts the ferritin labels both surfaces of the membrane. The lysis of the cells to form ghosts did not occur during the actual fusion of the cells with the proteoliposomes, but upon their further handling. Thus, the proteoliposomes approached the erythrocyte membranes only from outside. The presence of ferritin label on both surfaces of the membranes is interesting as fusion of vesicles and cells is assumed not to involve scrambling of the membranes sidedness. Cytochrome oxidase is oriented unidirectionally in proteoliposomes with its cytochrome *c* oxidizing site exposed to the medium [5,9,12]. Since all the activity of the enzyme remains evident after fusion with cells, the orientation of the enzyme probably remains consistantly uni-

Fig.3. Fusion of proteoliposomes with erythrocytes. Electron microscopy. Liposomes were prepared with cardiolipin (30 mol%), phosphatidylethanolamine (60 mol%), and phosphatidylcholine (10 mol%) as described. Cytochrome oxidase (1 mg/ml) was incorporated into part of the liposomes. The resulting proteoliposomes and protein-free liposomes were fused with erythrocytes as in section 2. After fusion was terminated, samples were treated by the suitable ferritin-conjugated antibodies and processed for electron microscopic observation as described. Almost no ferritin was associated with cells fused with protein-free liposomes (a,b). On the other hand, the cells fused with proteoliposomes were labeled extensively with ferritin (d,e). During the prolonged treatment of the cells, after the termination of the fusion, some lysis occurred and ghosts were formed. Such ghosts of cells fused with protein-free liposomes and proteoliposomes are shown in (c) and (f), respectively.

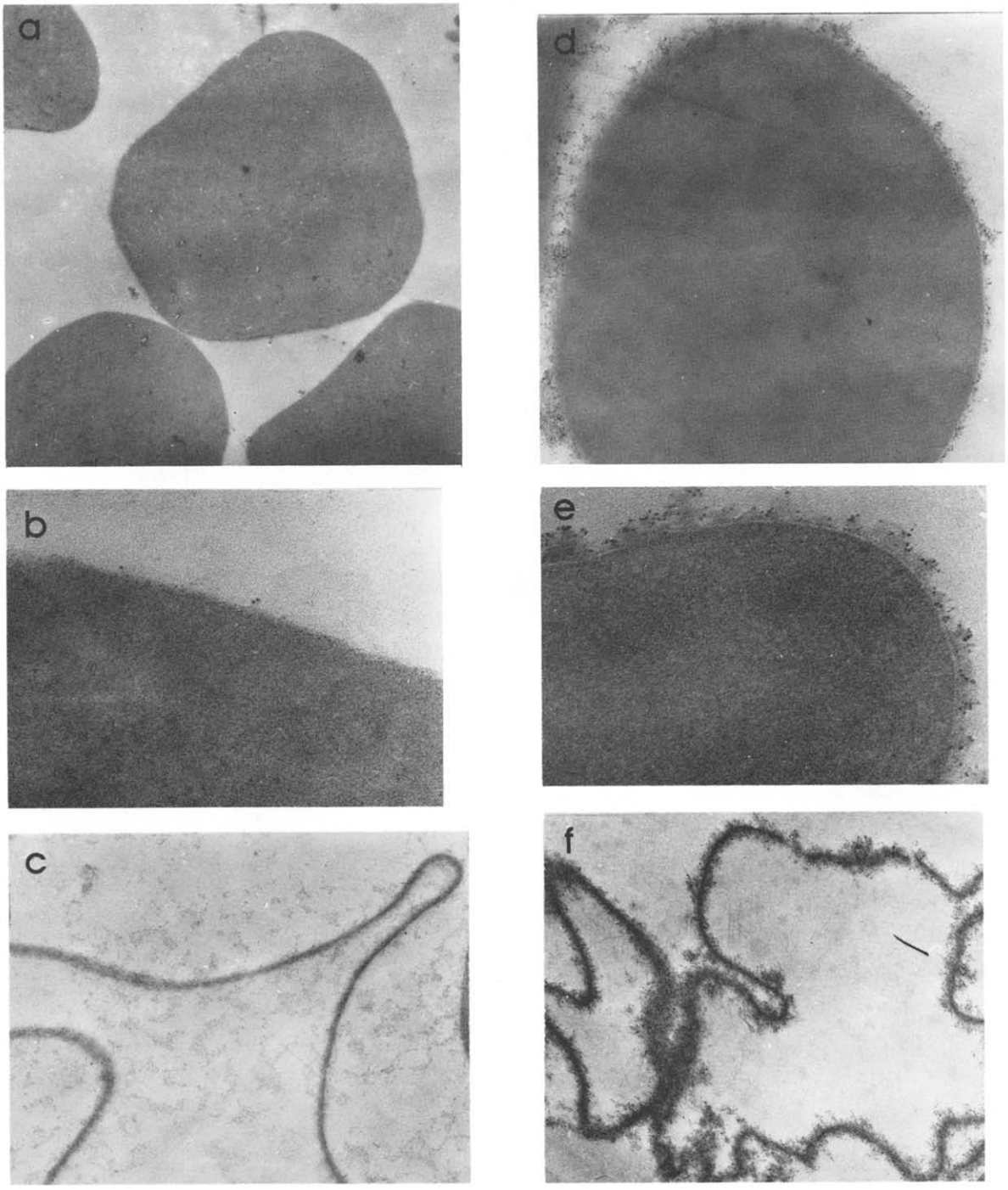

Fig.3

directional with the cytochrome *c* site exposed to the medium. The label on both membrane surfaces seems to be due to the transmembraneous orientation of the enzyme. It has been demonstrated that antisera reacted with both surfaces of the enzyme in membranes [10,19]. Both were shown to depend on Ca^{2+} and presence of an acidic phospholipid and phosphatidylethanolamine in the vesicles.

Acknowledgements

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