SELECTIVE INCORPORATION OF CYTOCHROME OXIDASE INTO SMALL LIPOSOMES

Gera D. EYTAN and Rachel BROZA
Department of Biology, Technion-Israel Institute of Technology, Haifa, Israel

Received 10 November 1977

1. Introduction

A powerful technique in membrane research is reconstitution of biologically active vesicles from purified membrane proteins and phospholipids [1—5]. Functional reconstitution was achieved by either sonication of the proteins together with the lipids [3] or by dissolving them with detergents and subsequent removal of the detergents [1—3,5]. Recently, direct incorporation of proteins into liposomes has been described [6,7]. Purified proteins were incubated with preformed liposomes containing either low amounts of lysolecithin [6] or acidic phospholipids [7]. The incorporation occurred with no major perturbation of the liposomes. A drawback of all reconstitution procedures is the high lipid to protein ratios required for successful incorporation as compared to the composition of natural membranes. Whereas in the latter, the lipid to protein ratios vary between 0.25 and 1.0, the lipid to protein ratios optimal for reconstitution vary between 5 for the reconstitution of oligomycin sensitive ATPase [1], and 29 000 for band 3 of red blood cells [8]. Cytochrome oxidase is incorporated into a subpopulation of the liposomes while most of the liposomes are not suitable for incorporation [9]. In the present work, we have characterized this subpopulation. Liposomes were fractionated according to size and incubated with isolated cytochrome oxidase. Only the smaller liposomes with a diameter of approx. 22 nm were suitable for incorporation. Upon incorporation of the protein, the diameter of the liposomes increased to 22—35 nm. Cytochrome oxidase proteoliposomes prepared by cholate dialysis exhibited a similar size distribution.

2. Materials and methods

Cytochrome oxidase was prepared according to a modification of the procedure described [9,10]. The enzyme was further treated to exchange the Tween 80 bound to the enzyme with cholate. For this purpose the enzyme (10 mg/ml) was dissolved in potassium cholate (1%) and precipitated with ammonium sulfate (35% saturation). This procedure was repeated twice. Rat liver [^{32}P] phosphatidylcholine was prepared according to described procedures [11]. Egg phosphatidylcholine and cardiolipin were purchased from Sigma (St Louis, MO).

Liposomes were prepared by drying the phospholipid solutions under a stream of nitrogen gas. The phospholipids were redissolved in ether, dried and finally resuspended in a buffer. The suspension was sonicated in a bath type sonifier (80 W, 50 kHz) until the sample was clear. The buffer used in the present work was KCl (40 mM), EDTA (0.2 mM) in Hepes (*N*-2-hydroxyethyl-piperazine-*N'*-2 ethanesulfonic acid, 10 mM, pH 7.4). Cytochrome oxidase proteoliposomes were reconstituted either by cholate dialysis [2,3] or by direct incorporation [7].

Liposomes or proteoliposomes were fractionated according to size by Sepharose 4B chromatography. The column size was 0.9 × 140 cm and its flow rate was 4 ml/h. Fractions of 2 ml were collected. The void volume was determined with dextran blue. Liposomes were negatively stained as described [3]. The diameter of the liposomes was measured in electron micrographs at a magnification of ×100 000.

Cytochrome oxidase was determined polarographically essentially as described [3]. The assay medium

consisted of the buffer described above, cytochrome *c* (1 mg/ml) and ascorbate (20 mM). The respiratory control ratio was defined as the ratio of activity rate measured in presence of FCCP (carbonylcyanidetrifluoromethoxyphenylhydrazone, 2 μg) to that measured in its absence. Phospholipid concentrations were expressed as μmol phosphate/ml. They were determined by total ashing of samples, hydrolysis in HCl (0.5 N) and phosphate analysis [13].

3. Results

3.1. *Liposome size requirements for activation and incorporation of cytochrome oxidase*

The distribution pattern of liposomes, prepared from phosphatidylcholine and cardiolipin is shown in fig.1. Multilamellar vesicles, emerging with the void volume, comprised less than 5% of the phospholipids loaded on the column. The single layered liposomes, retained by the column, appeared as a broad asymetric peak. Examination of the different fractions with an electron microscope revealed clear separation of the liposomes according to size. The diameter of the majority of the liposomes ranged from 18–40 nm (fig.1). Analysis of the lipid composition of the different fractions revealed no heterogeneity in the phosphatidylcholine to cardiolipin ratio. In order to check the capacity of the differently sized liposomes to participate in activation and reconstitution of cytochrome oxidase, the enzyme was incubated with ten times its weight of fractionated liposomes. Full activation occurred with liposomes whose diameter ranged from 18–40 nm. The multilamellar vesicles and the largest single-layered liposomes were not suitable for activation of the enzyme. Functional incorporation of the enzyme, resulting in both activation and respiratory control, required liposomes with a diameter of approx. 22 nm. Smaller and larger liposomes, although suitable for activation of the enzyme, were not satisfactory for reconstitution of respiratory control (fig.1).

3.2. *Size of cytochrome oxidase proteoliposomes*

In order to determine the size of cytochrome oxidase proteoliposomes, the purified enzyme was incorporated into preformed liposomes. The resulting mixture of proteoliposomes and protein-free liposomes were analyzed by chromatography in a Sepharose column (fig.2). Under these conditions cytochrome oxidase is incorporated only into 5–10% of the liposomes [9] and thus it is not surprising that the size distribution of all liposomes was not significantly changed by incorporation of the protein. The incorporated enzyme was not distributed uniformly among all liposomes but was present only in proteoliposomes with diameters of 25–35 nm. Thus, upon incorporation, the size of the functionally active liposomes increased from 22 nm to 25–35 nm. A

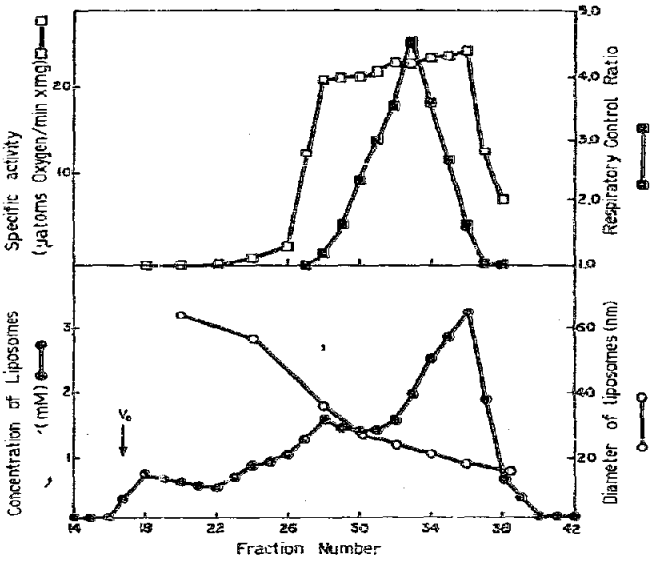

Fig.1. Selective incorporation of cytochrome oxidase into 22 nm liposomes. Liposomes were prepared with [^{32}P]-phosphatidylcholine (64 μmol, 0.5 μCi) and cardiolipin (16 μmol) by sonication to clarity in 1 ml buffer. The liposomes were fractionated in a Sepharose column and the fractions were analyzed for phospholipid content by radioactivity counting. The diameter of the liposomes was measured in electron micrographs. Samples of the fractions were diluted with the buffer to a final phospholipid concentration of 0.5 mM. Cytochrome oxidase (0.05 mg/ml) was added and incubated for 30 min at room temperature. Samples of the incubation were withdrawn and assayed for activity in the absence and presence of FCCP (2 μg). The specific activity of the enzyme before fractionation was 7 μatoms oxygen/min mg. After incorporation of this enzyme into a 20-fold excess of unfractionated liposomes, its activity in presence of uncoupler increased to 25 μmol oxygen/min mg and its respiratory control ratio became 5.2. The recovery of phospholipid from the column was 92%.

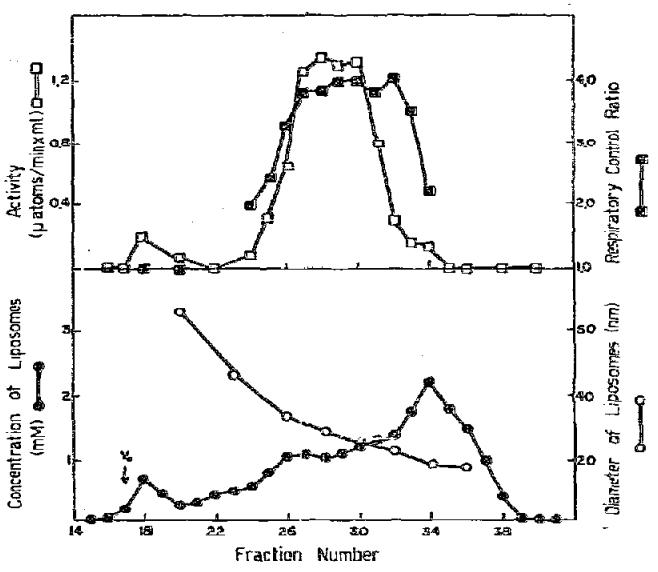

Fig.2. Size distribution of cytochrome oxidase proteoliposomes prepared by direct incorporation. Liposomes (40 mg/ml) were prepared as described in the legend to fig.1. The liposomes were incubated with cytochrome oxidase (2 mg/ml) for 30 min at room temperature and the resulting mixture was fractionated on a Sepharose column. Samples were withdrawn and assayed for phospholipid concentration, liposome size, activity and respiratory control. The specific activity of the reconstituted enzyme was 25 μatoms oxygen/min mg and its respiratory control ratio was 5.5. The recovery of phospholipid and activity after the column was 96% and 84%, respectively.

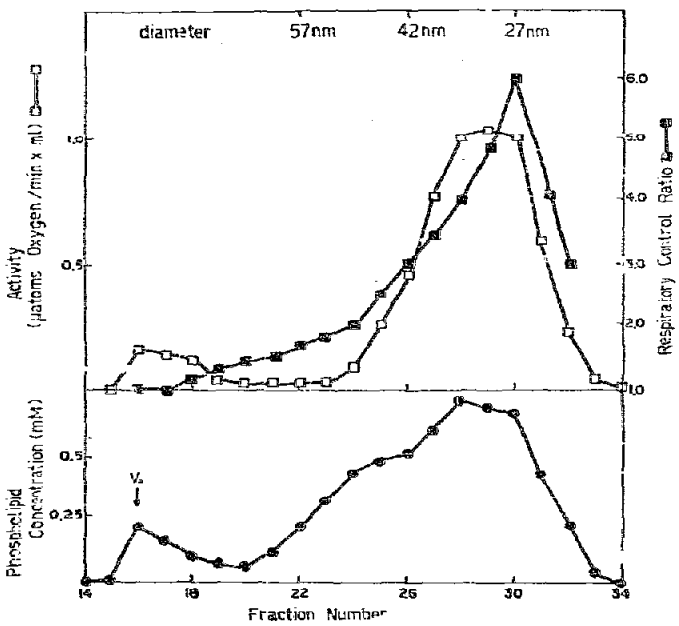

Fig.3. Size distribution of cytochrome oxidase proteoliposomes prepared by cholate dialysis. Cytochrome oxidase proteoliposomes were prepared from the following mixture: [^{32}P]phosphatidylcholine (20 μmol, 0.25 μCi), cardiolipin (5 μmol), cytochrome oxidase (1 mg) and potassium cholate (20 mg) in 1 ml buffer, by dialysis overnight against 200 vol. buffer. The resulting proteoliposomes exhibited respiratory control ratio of 8.0 and an uncoupled activity of 18 μatoms oxygen/min mg. The vesicles were fractionated in a Sepharose column. Samples of the fractions were tested for phospholipid content, respiratory control of the enzyme and its uncoupled activity. Respiratory control decreased upon gel filtration. The recovery of phospholipid and uncoupled activity was 94% and 92%, respectively.

small amount of enzyme was eluted from the column with the void volume, but as it exhibited no respiratory control, it probably was in an aggregated form and was not incorporated into liposomes.

Cytochrome oxidase proteoliposomes prepared by cholate dialysis were analysed in a similar fashion. The liposomes prepared by this procedure were larger than those prepared by sonication without detergents (fig.3). However, the size distribution of the enzyme-containing vesicles was similar to that of the proteoliposomes prepared by direct incorporation of the enzyme.

4. Discussion

We have shown that out of all liposomes formed by sonication, only the smaller ones with a diameter of approx. 22 nm, are suitable for incorporation of cytochrome oxidase. This explains, at least in part, the high lipid : protein ratios required for reconstitution of cytochrome oxidase. The size distribution of proteoliposomes formed by direct incorporation and cholate dialysis is almost identical. Thus, despite the apparent difference between the two procedures, the actual insertion of the protein into the vesicles must be similar in both cases. It seems that in the cholate dialysis procedure, liposomes are first formed by partial dialysis, and only after further dialysis is the protein inserted into the already formed liposomes.

The size range of liposomes suitable for incorpora-

tion of cytochrome oxidase is extremely narrow. Similar size requirements, although less stringent, were described for other functional assays of phospholipid model systems. Fusion occurs in proteoliposomes containing acidic phospholipids with diameters up to 100–200 nm [14]. Fusion of liposomes larger than 200 nm can be induced by generation of osmotic pressure across the liposome membranes [15]. Phospholipid exchange protein catalyzes the transfer of phosphatidylcholine from sonicated neutral liposomes but not from multilamellar vesicles [11]. Inclusion of acidic phospholipid, e.g., cardiolipin, facilitates the transfer of phosphatidylcholine also from multilamellar vesicles [16].

Small liposomes, containing more than one phospholipid, are distinguished by the high curvature and the asymetry of their membranes. Structural studies with nuclear magnetic resonance [17], differential scanning calorimetry [18] and phospholipases [19] indicated that the high curvature causes a looser and less tightly-packed organization of the phospholipids. Introduction of acidic phospholipids into the vesicles may cause a further perturbation of the packing of the membrane, due to electrostatic repulsion of the anionic head groups.

The stringent size requirements of cytochrome oxidase incorporation into liposomes may indicate that also in vivo nascent membrane polypeptides are inserted preferentially into curved regions of biological membranes, e.g., apices of the mitochondrial cristae and chloroplast thylakoids. The present work might explain the difficulties encountered in incorporation of membrane proteins into planar black lipid membranes.

Acknowledgements

We would like to thank Professor Y. Avi-Dor and Mr C. Julian for their valuable comments.

References

[1] Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477–5487.
[2] Hinkle, P. C., Kim, J. J. and Racker, E. (1972) J. Biol. Chem. 247, 1338–1339.
[3] Racker, E. (1972) J. Memb. Biol. 10, 221–235.
[4] Racker, E. (1973) Biochem. Biophys. Res. Commun. 55, 224–230.
[5] Hilden, S., Rhee, H. M. and Hokin, L. E. (1974) J. Biol. Chem. 249, 7432–7440.
[6] Eytan, G., Matheson, M. J. and Racker, E. (1975) FEBS Lett. 57, 121–125.
[7] Eytan, G. D., Matheson, M. J. and Racker, E. (1976) J. Biol. Chem. 251, 6831–6837.
[8] Ross, A. H. and McConnell, H. M. (1977) Biochem. Biophys. Res. Commun. 74, 1310.
[9] Carroll, R. C. and Racker, E. (1977) J. Biol. Chem. in press.
[10] Yonetani, T. (1966) Biochem. Prep. 2, 14–20.
[11] Johnson, L. W. and Zilversmit, D. B. (1975) Biochim. Biophys. Acta 375, 165–175.
[12] Castle, J. D. and Hubbell, W. L. (1976) Biochemistry 15, 4818–4837.
[13] Ames, B. N. and Dubin, D. Y. (1960) J. Biol. Chem. 235, 769–775.
[14] Miller, C. and Racker, E. (1976) J. Memb. Biol. 26, 319–333.
[15] Miller, C., Arvan, F., Telford, J. N. and Racker, E. (1976) J. Memb. Biol. 30, 271–282.
[16] DiCorleto, P. E. and Zilversmit, D. B. (1977) Biochemistry 16, 2145–2150.
[17] Sheetz, M. P. and Chan, S. I. (1972) Biochemistry 11, 4573–4583.
[18] DeKruiff, B., Cullis, P. R. and Radda, G. K. (1975) Biochim. Biophys. Acta 406, 6.
[19] Dawson, R. M. C. and Hauser, H. (1967) Biochim. Biophys. Acta 137, 518–523.