Morphological properties of proteoliposomes reconstituted with the Na^+ pump methylmalonyl-CoA decarboxylase from *Veillonella alcalescens*

Manfred Rohde, Patricia Däkena, Frank Mayer and Peter Dimroth

Institut für Mikrobiologie der Universität Göttingen, Grisebachstrasse 8, D-3400 Göttingen and *Institut für Physiologische Chemie der Technischen Universität München, Biedersteiner Strasse 29, D-8000 München 40, FRG*

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Proteoliposomes reconstituted with purified methylmalonyl-CoA decarboxylase have a mean diameter of 62 nm and contain on average 9–10 enzyme molecules integrated in their membrane. Affinity labelling with avidin-gold complexes was used to determine the number of enzyme molecules which were oriented with the biotin prosthetic group exposed to the outside. About 50% of the proteoliposomes became labelled under optimized conditions, containing on average 3.4 avidin-gold complexes on their surface. These data from electron microscopy are in good agreement with those from biochemical analyses indicating the presence of about 7 enzyme molecules per average-sized proteoliposome from which about 1–2 enzyme molecules are properly oriented in the membrane to perform an inwardly directed Na^+ pumping.

Proteoliposome *Methylmalonyl-CoA decarboxylase* *Avidin-gold* *Na^+ pump*

1. INTRODUCTION

In certain anaerobic bacteria the exergonic decarboxylation of oxaloacetate, methylmalonyl-CoA or glutaconyl-CoA is coupled to an active transport of Na^+ through the cell membrane [1,2]. The proteins responsible are biotin-containing enzymes which can be readily purified by affinity chromatography on monomeric avidin-Sepharose columns [1,2].

Reconstitution of the purified decarboxylases into proteoliposomes has enabled a study of the vectorial process [1,2]. In tightly sealed proteoliposomes the decarboxylation of 1 mol methylmalonyl-CoA or oxaloacetate was reversibly coupled to the translocation of 2 mol Na^+ [3]. It was also shown that the Na^+ is pumped in an electrogenic fashion creating a membrane potential in addition to a concentration gradient [1,2]. However, to quantify these energetic parameters, a more detailed knowledge of the morphology of the proteoliposomes is required. It is extremely important to know what percentage of the volume enclosed by the proteoliposomal membranes is available to Na^+ pumping enzyme molecules since this volume must be used to calculate the concentration gradients which are established by the Na^+ pumps.

An approach to answer this question has been made by studying methylmalonyl-CoA decarboxylase-containing proteoliposomes by electron microscopy. The total number of enzyme molecules integrated in the membrane of an average-sized proteoliposome has been counted and compared to the number of enzyme molecules which are oriented with the substrate-binding site facing the outside, as determined by affinity labelling of the biotin prosthetic group with avidin-gold. From these data and those from biochemical analyses it is concluded that the internal volume of proteoliposomes which is available for Na^+ pumping is at least 46%, but probably a higher percentage of the total internal space.

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2. MATERIALS AND METHODS

2.1. *Reconstitution*

Methylmalonyl-CoA decarboxylase (EC 4.1.1.41) of *Veillonella alcalescens* was purified by affinity chromatography on monomeric avidin-Sepharose as described [4]. Reconstitutions were performed as described [3,5] with 6 mg soybean lecithin (Sigma, type II S) and 0.11 mg methylmalonyl-CoA decarboxylase. Oxaloacetate decarboxylase (EC 4.1.1.3) of *Klebsiella aerogenes* was reconstituted into liposomes by the same procedure and at the same protein to phospholipid ratio. As a control, liposomes were prepared by the same reconstitution procedure but in the absence of protein.

2.2. *Affinity labelling of proteoliposomes with avidin-gold complexes*

Egg white avidin was succinoylated as described [6,7], modified as follows: the lyophilized avidin (5 mg) was dissolved in 5 ml of a saturated solution of sodium succinate, pH 8.35. After cooling to 4°C, 5 mg succinic anhydride were added with gentle stirring for 1 h, followed by incubation overnight at 4°C. The solution was then incubated for 1 h at 23°C and dialyzed for 40 h against 5 mM K-phosphate buffer, pH 7.5.

Colloidal gold of a defined diameter range (4–5 nm) was prepared as described [8]. A mixture of 2.5 ml of 0.6% tetrachloroauric acid and 0.7 ml of 0.2 M K_2CO_3 was poured into 120 ml distilled and filtered water to which a solution consisting of 0.8 ml ether and 0.2 ml ether saturated with white phosphorus was added. During a 15 min incubation at room temperature the colour of the solution changed to reddish brown and became deep red during the subsequent boiling for 5 min. The colloidal gold was stabilized by adsorption of succinoylated avidin at pH 2.5, the amount required being determined as in [9]. Succinoylated avidin (1.25 mg) was added to 5 ml colloidal gold with continuous stirring. After 30 min, the avidin-colloidal gold complexes were collected by centrifugation in an airfuge (Beckman) at $100\,000 \times g$ for 15 min. The pellet was washed 3 times with phosphate-buffered saline (5 mM K-phosphate, pH 7.5, 100 mM NaCl) containing 0.2 mg polyethylene glycol per ml to remove free avidin. The final pellet made up of avidin-colloidal gold complexes was resuspended in 2 ml phosphate-buffered saline.

Proteoliposomes or phospholipid vesicles (as a control) were incubated at 30°C for 4 h under occasional shaking with a mixture of 40 μl of the suspension of avidin-gold complexes in phosphate-buffered saline (see above) and either 0.5 ml of 30 mM K-phosphate, pH 8.9, containing 1 mM Na_2SO_4, 0.5 mM dithioerythrol and 1.5 mM NaN_3, or 0.5 ml of 50 mM K-phosphate buffer, pH 7.0.

2.3. *Electron microscopy*

Carbon support films were floated off a sheet of mica onto the surface of samples containing aggregated proteoliposomes in the buffers described above. After 30 s the carbon support with adhering proteoliposomes was transferred to an aqueous staining solution containing 4% uranyl acetate, pH 4.5, picked up after a few seconds with a copper grid, blotted dry with filter paper, and analyzed immediately in the electron microscope at low magnification and low electron dosage.

Negative staining using an aqueous solution containing 4% uranyl acetate, pH 4.5, was performed as described [10]. 'Shallow-stain' conditions [11] were achieved by immediately and completely blotting dry the grid containing the mounted and negatively stained particles.

2.4. *Electron micrography*

Samples were observed and electron micrographs were taken with a Philips EM 301 electron microscope at an accelerating voltage of 80 kV and at magnifications ranging from 13 700 to 44 600. Magnifications were calibrated as described [12].

Diameters of vesicles were measured with a calibrated ruler from prints with a calibrated final magnification of 44 000. Surface areas of negatively stained, flattened proteoliposomes and phospholipid vesicles were measured from prints using a graphics tablet in combination with an Apple computer. Overall numbers of enzyme molecules incorporated into proteoliposomes were estimated by counting of white dots present in projections of flattened proteoliposomes. These dots were absent in control samples of negatively stained phospholipid vesicles (not shown). The number of avidin-gold complexes attached per flattened proteoliposome, and the ratio of proteoliposomes without at-

tached avidin-gold complexes as compared to vesicles with bound avidin-gold complexes were determined from negatively stained samples by counting from prints. Aggregations of avidin-gold complexes occasionally observed were assumed to indicate the presence of only one enzyme particle; therefore, they were counted as a single label. The numbers of avidin-gold complexes attached to proteoliposomes were corrected by subtraction of the number of avidin-gold complexes found on the surface area of the background corresponding to the sum of the evaluated surface area of projections of flattened proteoliposomes. Background labelling was low.

3. RESULTS AND DISCUSSION

3.1. *Dimensions of proteoliposomes and number of integrated enzyme molecules*

As shown in fig.1, proteoliposomes reconstituted with methylmalonyl-CoA decarboxylase appear to be well preserved (they are sphere-like) under the mounting, staining and imaging conditions applied. Fig.2 gives their size distribution as measured from these samples. The majority are 30–100 nm in diameter and the mean value for the proteoliposome diameter is 62 nm, corresponding to a volume of 1.24×10^5 nm^3 and a surface area of 1.16×10^4 nm^2. Similar results were obtained with proteoliposomes reconstituted with oxaloacetate decarboxylase (not shown).

From samples as shown in fig.3, i.e. negatively stained, flattened proteoliposomes, an average number of 9–10 integrated enzyme particles (recognized by their size and white appearance, features not present in phospholipid vesicles used as controls, not shown) per vesicle can be estimated. This number includes both possible orientations of integration of the enzyme particles.

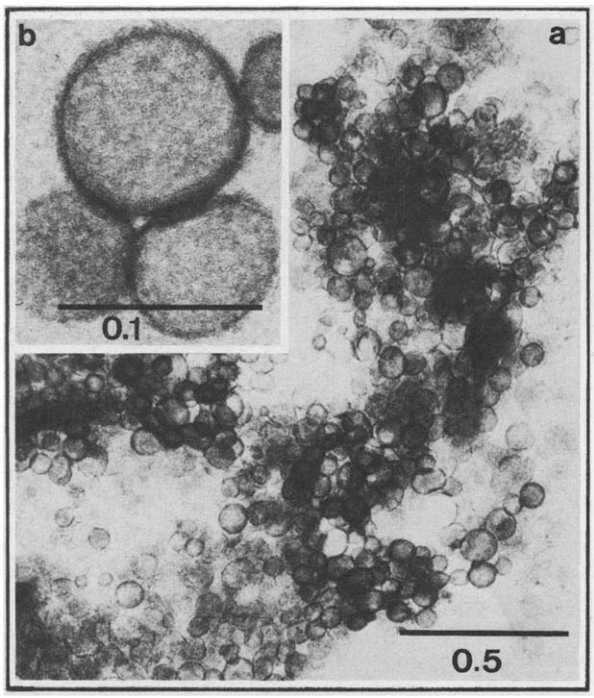

Fig.1. Aggregate of proteoliposomes stained with uranyl acetate. The vesicles appear to be well preserved (they are sphere-like). Dimensions are given in μm.

3.2. *Orientation of methylmalonyl-CoA decarboxylase in the proteoliposomes*

The orientation of enzyme molecules in the membrane was determined using affinity labelling with avidin-gold complexes. Only those enzyme molecules will attain labelling which have the biotin prosthetic group exposed to the outer surface. Enzyme with this orientation will catalyze decarboxylation of substrate applied from the outside and Na^+ translocation from the outside to the inside. The results shown in fig.4 indicate binding of avidin-gold to proteoliposomes reconstituted with methylmalonyl-CoA decarboxylase.

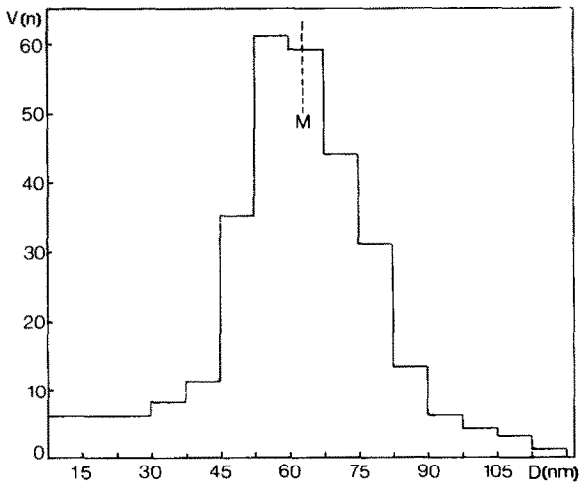

Fig.2. Size distribution of proteoliposomes as depicted in fig.1. The mean diameter is 62 nm. D (nm), diameter; V (n), number of vesicles.

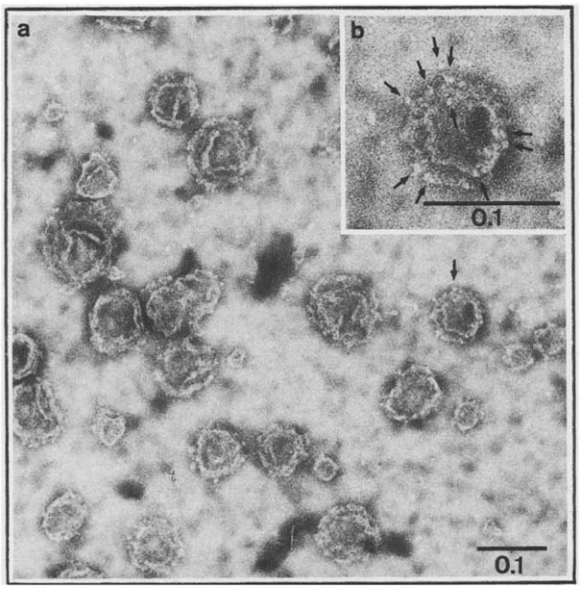

Fig.3. Negatively stained, flattened proteoliposomes. Arrows pointing to white dots interpreted to be projections of integrated enzyme molecules. Dimensions are given in μm.

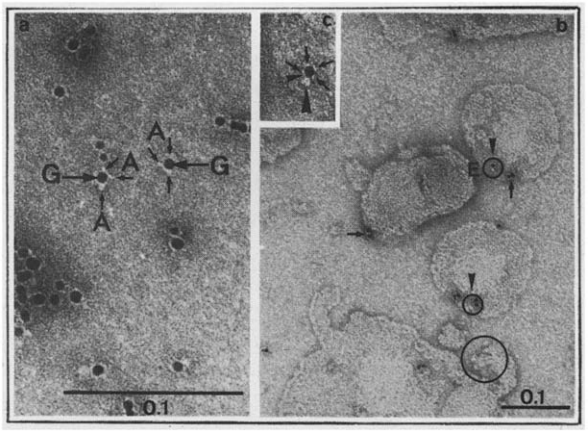

Fig.4. (a) Avidin-gold complexes shown at high magnification. G, colloidal gold; A, avidin surrounding the colloidal gold. Dimensions are given in μm. (b) Avidin-gold complexes attached to negatively stained, flattened proteoliposomes. The small circles with arrowheads indicate situations where the enzyme molecules are recognized as white dots with attached avidin-gold complexes. The arrows point to bound avidin-gold complexes attached to proteoliposomes without clearly recognizable enzyme molecules. The large circle surrounds an area where white dots (enzyme molecules) are seen without attached avidin-gold complexes. Dimensions are given in μm. (c) Higher magnification of the area marked E in (b). Small arrowhead, colloidal gold; arrows, avidin; large arrowhead, enzyme molecule. The colloidal gold has a diameter of about 4.5 nm.

The amount of avidin-gold complexes which became attached to the proteoliposomes was dependent upon experimental conditions. The highest amount of labelling (46% of all proteoliposomes present) was obtained after stabilizing the avidin-gold complexes in buffer at pH 2.5 and incubation of these complexes with proteoliposomes in buffer at pH 8.9. Only 34% of the proteoliposomes became labelled if the incubation was performed in buffer of pH 7.0.

The number of avidin-gold complexes bound per proteoliposome was determined by evaluating 400 proteoliposomes from 2 independent sets of experiments. After correction for background labelling (cf section 2), those proteoliposomes carrying attached avidin-gold complexes (fig.4) on average had 3.2 complexes bound to their surface. This value, as compared to the overall estimated number of enzyme molecules integrated into an average proteoliposome (see above), indicates that about 30% of the integrated enzyme molecules in an average proteoliposome are oriented with their biotin-carrying subunit to the outside.

One could conclude from the results reported here that only about 50% of the proteoliposomes carry enzyme molecules in the proper orientation for Na^+ pumping from the outise to the inside. This value, derived from the number of proteoliposomes becoming labelled with avidin-gold complexes should be taken as a minimum number, since the percentage of labelling is dependent upon experimental conditions (see above) and since due to sterical factors part of the externally oriented biotin may not be accessible to binding of avidin-gold.

The number of enzyme molecules per proteoliposome as counted from the electron micrographs is in good agreement with the amount determined by biochemical analyses. About 9 μg protein per mg phospholipid were incorporated during reconstitution [5]. With an estimated M_r for methylmalonyl-CoA decarboxylase of 125 000 (sum of M_r values of the 4 subunits [4]) the number of enzyme molecules per mg phospholipid was calculated to be 4×10^{13}. The number of proteoliposomes per mg phospholipid was calculated

to be 6×10^{12} from the inner diameter of an average-sized proteoliposome (62 nm minus 2×4 nm for the lipid bilayer) and the experimentally determined internal volume of proteoliposomes (0.5 μl per mg phospholipid [5]). From these values it is calculated that a proteoliposome on average should contain approx. 7 decarboxylase molecules. About 20% of the enzyme has been shown to be oriented with the substrate-binding site facing the outside [5]. Therefore, approx. 1.4 decarboxylase molecules per proteoliposome should on average be oriented in such a way as to perform an inwardly directed Na^+ transport. Taking these results together with those from affinity labelling with avidin-gold we conclude that at least 46%, probably a number closer to 100% of the proteoliposomes are capable of performing Na^+ pumping from the outside to the inside.

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