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ASSOCIATION OF *PSEUDOMONAS* CYTOCHROME OXIDASE WITH LIPOSOMES

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Summary

Purified *Pseudomonas* cytochrome oxidase has been associated with asolectin liposomes by two different methods. Firstly, the enzyme was attached to liposomic membranes by adding it to a cholate-phospholipid dispersion and subsequently dialyzing the detergent out of suspension. In the second case the enzyme was adsorbed on the preformed liposomes when added to them after the dialysis.

A stimulation of the cytochrome oxidase activity approximately twenty-fold was observed by the first method. In contrast, the activation was absent in the second type of preparation, indicating that interaction between the enzyme and phospholipids is very different in the two types of vesicles.

The cholate-dialysis method for reconstitution of protein-phospholipid vesicles seems to lead to rather heterogenous preparations. These can be further fractionated, not only according to their size but also to the protein/phospholipid ratio, by gel chromatography.

Introduction

Phospholipids are arranged into membranous, vesicular structures when they are dispersed by sonication [1], or when the solubilizing detergent is removed from the phospholipid-detergent mixed micelle suspension [2,3]. These vesicles (liposomes) which usually consist of a single bilayer and are about 25 nm in diameter [1,3], have been used as supporting membranous structures for reconstruction of vectorially organized biochemical functions (for reviews, see refs. 4 and 5). Racker and his coworkers have shown that the enzymes and enzyme complexes which catalyze trans-membrane reactions (e.g. oxidative phosphorylation) regain the biological activity when incorporated into preformed liposomes containing lysophosphatidylcholine [6] or acidic phospholipids [7], or when attached to the forming phospholipid membranes during dialysis of the detergent (sodium cholate) [2].

Pseudomonas cytochrome oxidase (ferrocytochrome *c*-551 : O_2, NO_2^- oxidoreductase, EC 1.9.3.2) has been previously purified from acetone powdered bacteria [8]. The soluble enzyme is a dimer of two identical subunits [9,10]. The enzyme, which is one of the terminal oxidases of the denitrifying *Pseudomonas* cell [11,12], would be expected to interact with the cytoplasmic membrane. The cytochrome *c*-551 oxidase activity has shown to co-sediment with the cytoplasmic membrane fraction in the ultracentrifuge after disruption of the bacterial cells [13].

The purpose of this communication is to describe some details concerning the interaction of the purified enzyme with liposomic membranes. In general, cytochromes associated with membrane structures can be incorporated into liposomes. There seems to exist three kinds of interaction between membraneous structures and these proteins. These include: i, complexation mainly by electrostatic forces (as in the case of the mitochondrial cytochrome *c* [14,15]); ii, hydrophobic contact between an amphiphilic protein and the membrane (e.g., cytochrome b_5 [16—19]); and iii, transmembrane arrangement of a 'double amphiphilic' protein through the membrane (e.g., the mitochondrial cytochrome *c* oxidase [20,21]).

Pseudomonas cytochrome oxidase, which is highly water-soluble (in its dimeric form), and thus has to contain a large hydrophilic domain, is expected to belong to type 1 or 2 above. There is, however, plenty of evidence [22,23] that after solubilization many membrane proteins tend to aggregate to regular oligomeric structures (e.g. the octamer of cytochrome b_5 [24] when the solubilizing agent is removed, and these oligomers cannot be easily dissociated with the aid of mild detergents. It is therefore possible that an oligomerized membrane protein with the hydrophobic domains buried inside the quaternary structure interacts in a different way with membrane as compared to the monomer in vivo.

The interaction of the *Pseudomonas* cytochrome oxidase with asolectin liposomes seems to be very different when the purified protein is added to preformed vesicles as compared to the case where the enzyme is included in liposomes as they are formed. Activation of the catalytic activity is observed in the latter preparation. However, generation of a membrane potential in connection of the catalytic function is not observable.

Materials and Methods

Pseudomonas cytochrome oxidase and cytochrome *c*-551 were purified as previously reported [8]. Cholic acid (Merck) was recrystallized from ethanol, and [^{3}H]cholic acid was purchased from N.E.N. Asolectin was obtained from Nutritional Biochemicals Corp. (Ohiol, U.S.A.), Triton X-100 and sodium lauryl (dodecyl) sulphate from B.D.H., dimethylsuberimidate from Pierce, and Sepharose 4 B from Pharmacia. All other chemicals were of the highest grade available.

Preparation of liposomes. Asolectin (40 mg/ml) was dispersed in 0.01 M Tris buffer (pH 7.5) containing 2% sodium cholate, 40 mM potassium chloride, and 1 mM dithiothreitol essentially as described by Kagawa and Racker [2]. When indicated, 3 mg of *Pseudomonas* cytochrome oxidase and 2.5 μCi of tritiated

cholate were added to 50 mg of asolectin. The dispersion was first incubated 20 min at 20°C, and then dialyzed against two 300 ml changes (5 h and 18 h) of 0.01 M Tris buffer at 4°C. Approx. 90% of the cholate was removed from the liposomic preparation during the dialysis, and almost the rest during the gel chromatography (Fig. 1).

In another series of experiments the enzyme (3 mg) was added to preformed (dialyzed) liposomes (50 mg phospholipid), which retained about 10% of the cholate initially present.

1.5 ml of the liposome suspensions was applied onto a 2 × 40 cm column of Sepharose 4 B, equilibrated with 0.01 M Tris buffer and saturated with soybean phospholipids before the first run. Fractions (1.8 ml) of eluate were collected and analyzed for protein [25], total phosphorus [26], and ^3H radioactivity. For protein analysis, 0.1-ml samples of the fractions were incubated in 0.5% sodium dodecyl sulphate prior to addition of the other reagents. The phospholipid concentrations were estimated assuming the mean molecular weight of 775.

Cross-linking of protein. Cross-linking of the purified and the liposome incorporated protein was carried out according to Davies and Stark [27]. 1.5 mg sodium dimethylsuberimidate was added to 0.5 ml 0.2 M Tris buffer (pH 8.5) containing 0.4 mg purified or liposome-incorporated protein. In the latter case, the liposomes for cross-linking were concentrated by ultracentrifugation at 45 000 rev./min for 2 h. The cross-linking was carried out for 3 h at 20°C, and the reaction was stopped by addition of sodium dodecyl sulphate (1%). Polyacrylamide gel electrophoresis was performed as described previously [27]; the gels were stained for protein with Amido black, and scanned with a Gilford scanning spectrophotometer at 570 nm.

Electron microscopy. Negative staining electron microscopy was performed with 1% potassium phosphotungstate (pH 7.0), or 1% uranyl acetate. Jeol JEM 100 B and Hitashi HU-11 electron microscopes were used with primary electron optical magnification of 45 000—80 000 diameters.

Sedimentation analysis. Sedimentation analysis was carried out in a Spinco Model E analytical ultracentrifuge equipped with a photoelectric scanner. The liposomic preparations were diluted to $A_{280} = 1.0$, and sedimentation was followed at 280 nm in 0.01 Tris buffer (pH 7.5) at the rotor speed of 60 000 revs./min. The sedimentation coefficients represented below are corrected only for the solvent viscosity and for temperature (20°C).

Determination of enzymic activity. The activity of the *Pseudomonas* cytochrome oxidase was determined with a Clark oxygen electrode in 0.01 M Tris buffer (pH 7.5) using ascorbate plus cytochrome *c*-551 as the electron-donating system.

Results and Discussion

Fractionation of liposomes by gel chromatography

Sodium cholate was removed from the detergent-phospholipid dispersion by dialysis in the presence or absence of the *Pseudomonas* cytochrome oxidase. In the latter case, the enzyme was added to the preformed liposomes after the dialysis step. Fig. 1 represents elution patterns of the protein-containing

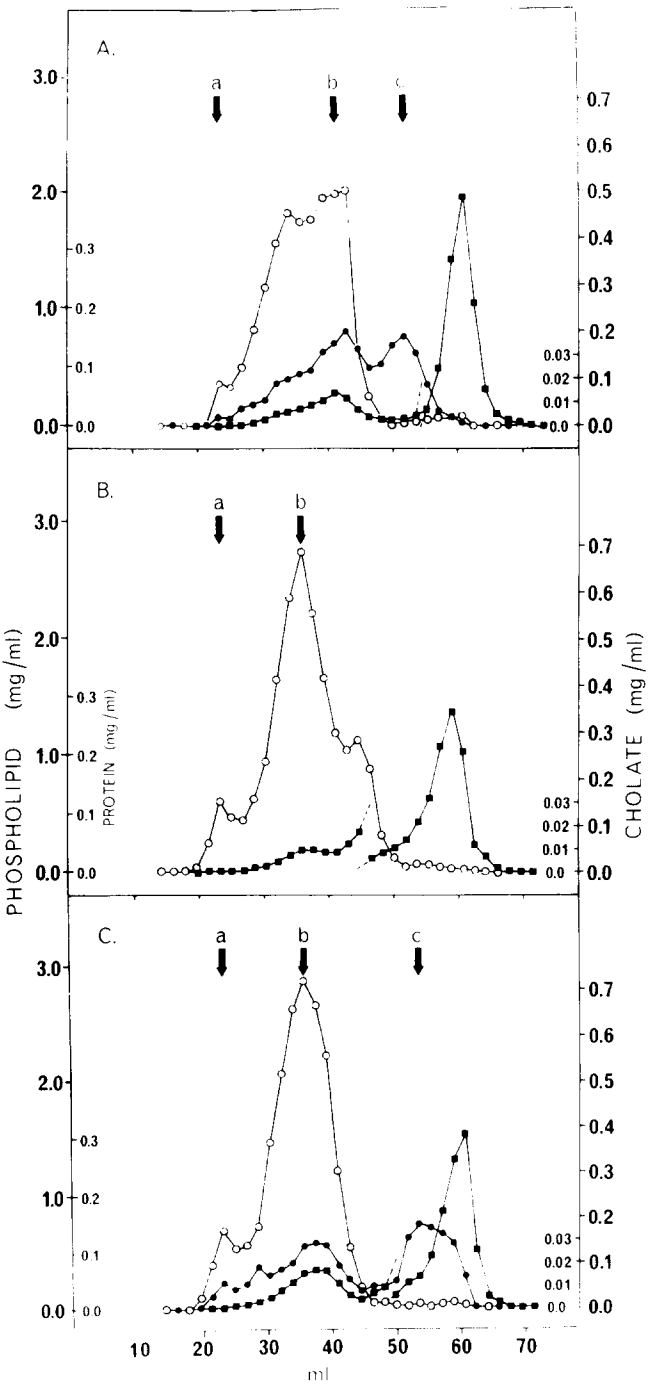

Fig. 1. Fractionation of liposomes by gel chromatography. Gel chromatography of *Pseudomonas* cytochrome oxidase-asolectin liposomes was carried out as described in Materials and Methods. The phospholipid (○———○), protein (●———●) and cholate (■———■) concentration of the fractions are represented in Parts A, B and C. Note that the scale for cholate concentration is shortened at approximately 55 ml of elution. The enzyme was added to phospholipid-cholate dispersion prior to dialysis (A), or after dialysis (C). Gel chromatography of asolectin liposomes without the enzyme is represented (B) for reference.

liposomes formed in the presence (Fig. 1A) and absence (Fig. 1C) of the enzyme. Elution pattern of the asolectin liposomes without the enzyme is shown in Fig. 1B. In all cases four main fractions can be resolved. The first, eluting at the void volume contains large multilamellar vesicles of undispersed phospholipid (Fig. 2a). The second fraction contains liposomes of somewhat variable size (Fig. 2 b, c, e). The third fractions contains the unbound protein (Fig. 2 d, f), and the fourth the cholate micelles.

In the conditions used (see Materials and Methods), approx. 64 and 55% of the total protein initially present was attached to liposomes when added to phospholipid before and after the dialysis step, respectively. The molar ratio of the dimeric enzyme (M_r = 120 000 [8,9]) to the phospholipid in the fractions b in Fig. 1A and 1C was approx. 1 : 2000 and 1 : 4000, respectively. These values do not represent a saturation point. In this kind of experiment the relative amounts of protein and phospholipid in the liposomes, as well as the relative amounts of bound and unbound enzyme, varied from one experiment to another. A clear-cut saturation value cannot, therefore, be given. However, asolectin liposomes seem to bind *Pseudomonas* cytochrome oxidase at a lower molar ratio than, for example, phosphatidylcholine vesicles bind cytochrome b_5 [19].

The binding of enzyme to the asolectin liposomes increases the sedimentation velocity from 2.8 S to 14.7 S. These values correspond to fractions b of Fig. 1B and A respectively. The unbound enzyme sediments slower than the purified dimeric *Pseudomonas* cytochrome oxidase. The sedimentation coefficient corresponding to fraction c of Fig. 1A was found to be 5.8 S (uncorrected for phospholipid and cholate present) whereas that of the purified enzyme is about 7.0 S [9]. The enzyme associated with liposomes could also be detected with the aid of ferritin-conjugated antibodies (see the insert in Fig. 2c), which were purified from rabbits immunized with the *Pseudomonas* cytochrome oxidase (Saraste, M. and Kuronen, T., unpublished).

The liposomic fraction usually contained vesicles of varying size. The minimal diameter observed in the electron micrographs was approximately 25 nm, which is in agreement with the previously reported values [1,3] for unilamellar liposomes. The heterogenous appearance of the liposomes is more apparent in the preparations which were formed in the absence of the enzyme (Fig. 2 b, e). The non-uniform nature of the liposome preparations was also revealed in sedimentation velocity determinations (not shown). Some liposomes having a uniform appearance, which could be occasionally observed in the electron micrographs are included in Fig. 2e (see the insert).

The purified *Pseudomonas* cytochrome oxidase cannot be resolved by negative staining with phosphotungstate [10]. The stain gave, however, a resolvable contrast to proteinous fraction c (Fig. 1A). In Fig. 2d, aggregates without uniform shape or size are observable. These are probably similar to the protein-detergent-phospholipid complexes produced in solubilization procedures of membrane proteins [22].

The cholate-dialysis method for reconstitution of liposomes [2] seems to result in a heterogenous vesicle preparation. To achieve reproducible results, the chromatographic fractionation of the crude liposome preparations was found to be necessary. The *Pseudomonas* cytochrome oxidase containing

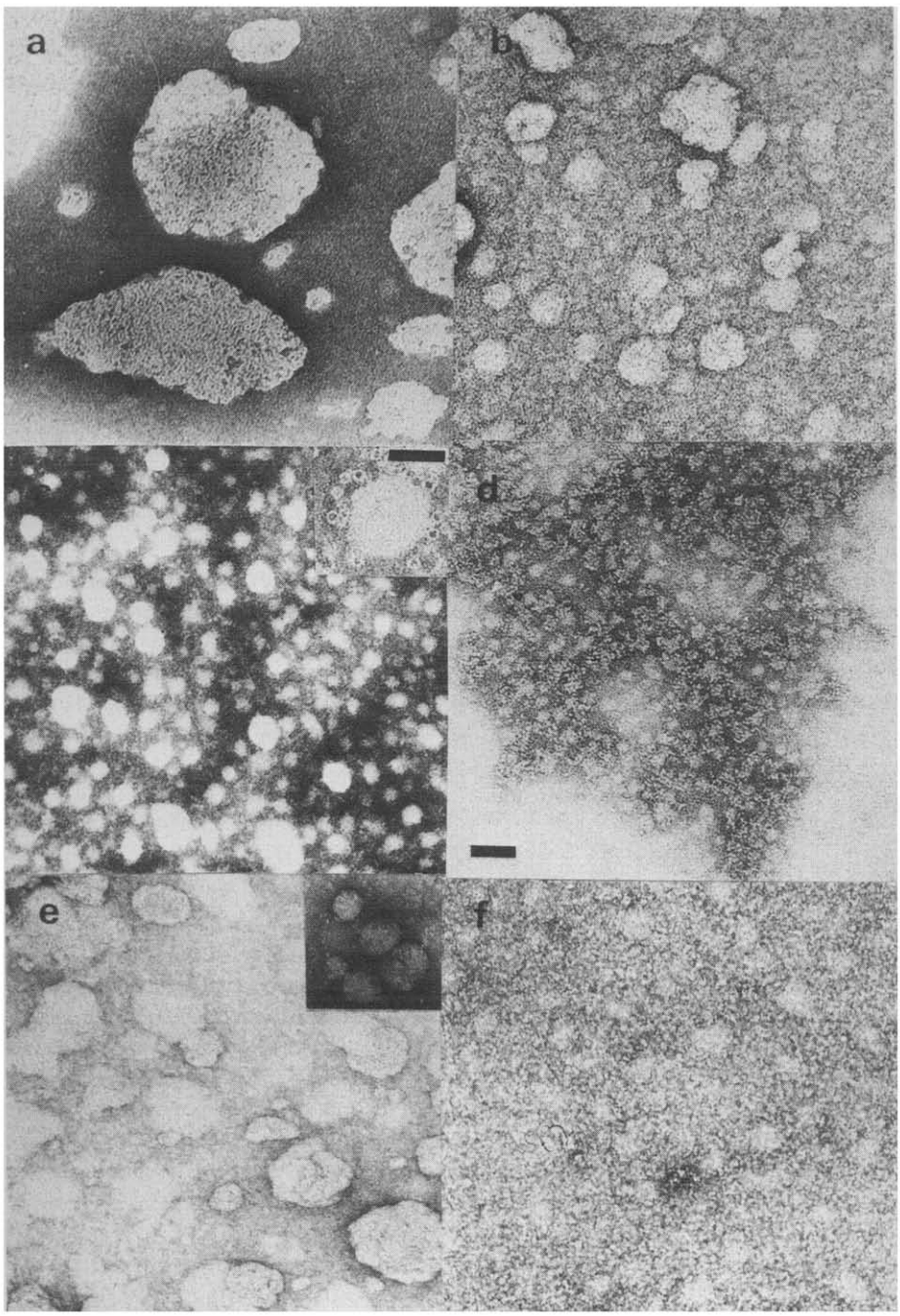

Fig. 2. Electron microscopy of fractionated liposomes. Electron micrographs of the different fractions from the Sepharose gel chromatography (Fig. 1) were obtained by negative staining with 1% potassium phosphotungstate (a, c and d) or 1% uranyl acetate (b, e and f). Magnification is × 140 000 (except the insert in c, which is magnified × 160 000 diameters), the bar representing 50 nm. The micrographs correspond to the fractions indicated in Fig. 1: a to the fraction B-1, b to B-b, c to A-b, d to A-c, e to C-b, and f to C-c. See text for further explanation.

liposomes were fractionated not only according to their vesicle diameter but also to their protein/phospholipid ratio (Fig. 1A).

Quaternary structure of Pseudomonas cytochrome oxidase in liposomes

A liposome preparation similar to the fraction b in Fig. 1A was subjected to cross-linking by dimethyl suberimidate. Fig. 3 shows the scans of the polypeptides after gel electrophoresis in the presence of dodecyl sulphate. No distinction between the general cross-linking pattern of the purified enzyme and the enzyme attached to liposomes was observed. A large amount of the dimeric polypeptide was present in both cases.

It can be concluded (compare scans B and C in Fig. 3) that *Pseudomonas* cytochrome oxidase is dimeric when incorporated to the liposomes. Similar cross-linking experiment after incubation of the pure enzyme in 2—5% sodium cholate at 37°C for 2 h gave no indication of dissociation of the dimer (data not shown).

Activation of Pseudomonas cytochrome oxidase in liposomes

A large increase of the specific activity was observed after incorporation of the *Pseudomonas* cytochrome oxidase into liposomes. The activation occurred only when the enzyme was incorporated during the formation of the liposomes (Table I). Only a two-fold activation, as compared to the more-than 20-fold activation in the former case, was observed with the enzyme adsorbed onto preformed liposomes.

Addition of Triton X-100 to the respiring vesicles caused a rapid inhibition of the rate of respiration to the level of that of the free enzyme. In contrast, Triton X-100 inhibits the free enzyme only slightly (Table I). The inactivation

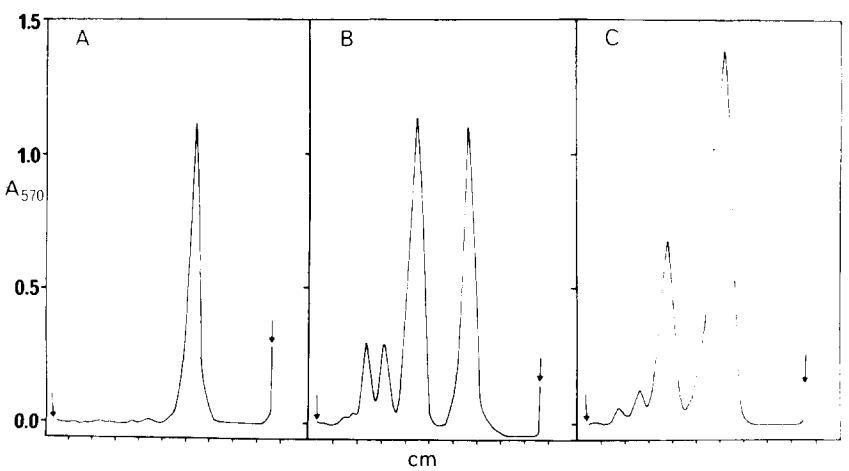

Fig. 3. Cross-linking of *Pseudomonas* cytochrome oxidase in liposomes. Cross-linking of the purified (B) and the liposome-bound (C) enzyme by dimethyl suberimidate was performed as described in Materials and Methods. The cross-linked proteins were analyzed by gel electrophoresis in the presence of sodium dodecyl sulphate. The gels were stained for protein and scanned for absorbance. The scanning pattern of a non-cross-linked preparation (A) is shown for reference. The bands from left to right correspond to the monomeric, dimeric, trimeric and tetrameric enzyme.

TABLE I

ACTIVITY OF *PSEUDOMONAS* CYTOCHROME OXIDASE IN LIPOSOMES

Activity of *Pseudomonas* cytochrome oxidase was determined with a Clark oxygen electrode equipped with a pen recorder. The total volume of the cuvette was 1.2 ml. The reaction was carried out in 0.01 M Tris buffer (pH 7.5) containing 5 μM cytochrome *c*-551, and 10—30 μg of enzyme protein. The reaction was started with addition of 0.5 M sodium ascorbate (10 μl). After 3—5 min, 25 μl 10% Triton X-100 was added (to final concentration of 0.2%). The values of specific activity represented in the table are the means of three parallel determinations and are given in μmol O_2/min per mg protein.

Preparation	Activity	
	— Triton X-100	+ Triton X-100
Purified enzyme	0.25	0.21
Fraction b/A	5.36	0.21
Fraction c/A	0.31	0.27
Fraction b/C	0.49	0.27
Fraction c/C	0.30	0.18

of the liposome-bound enzyme is probably a consequence of delipidation of the enzyme protein by the detergent.

The difference in the degree of activation between the two types of *Pseudomonas* cytochrome oxidase containing liposomes indicates that the phospholipid bilayer not only directs the complex formation of cytochrome *c*-551 and the enzyme, but also probably induces a conformational change in the enzyme protein (by analogy perhaps, to cytochrome *c* [15]) and causes activation. While the former aspect may be present in both types of preparation, the latter and larger aspect of activation is manifested only when the enzyme has been attached to the liposomic membrane during its formation. Clearly the interaction between the enzyme and the phospholipid membrane is different in these two preparations.

Generation of membrane potential

The generation of membrane potential was not observed to be coupled to the enzymic activity in liposomes. Membrane potential was determined indirectly measuring the possible changes in transmembrane distribution of synthetic lipophilic anions and cations [28] (phenyldicarboundecaboron and tetrapentylamine, respectively, were used in these studies). This method, as well as the more direct determination of potential changes in liposomes fused with a black membrane [29], led repeatedly to the conclusion that membrane potential is not generated in preparations corresponding to the fraction b in Fig. 1A (when the enzyme is oxidizing cytochrome *c*-551 as described in legend to Table I). This was further confirmed using safranine as a probe for membrane potential generation [30,31] and nitrite as the electron acceptor.

It is thermodynamically feasible that the transfer of an electron from cytochrome *c*-551, which has the mid-point potential of 0.28 V [32], to the physiological electron acceptor nitrite [33] (the redox potential of the NO_2^-/NO couple is 0.58 V at pH 7) is capable of oxidative phosphorylation. The fact that the purified dimeric enzyme can not be reconstituted to liposomes in such

way that an electric potential is generated indicates that: 1, the monomeric enzyme interacts differently with the cytoplasmic membrane; or 2, there are additional components to the enzyme to modify this interaction in vivo. However, phospholipid requirement for the activity of even the dimeric enzyme makes it clear that a membrane environment, and attachment to a membrane structure, is not unnatural for this enzyme.

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