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MEMBRANE RECONSTITUTION IN *chl-r* MUTANTS OF *ESCHERICHIA COLI* K12

IX. PART PLAYED BY PHOSPHOLIPIDS IN THE COMPLEMENTATION PROCESS

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SUMMARY

The supernatant extracts of the *chl A* and *chl B* mutants of *Escherichia coli* K 12, the phospholipids of which are labeled by growth in ^{32}P or [2-^{3}H]glycerol media, contain 20 times more radioactivity than the supernatant extract of the wild-type strain grown under the same conditions. We have observed that, after complementation, 80 % of the radioactivity previously contained by Extracts A and B is incorporated into reconstituted particles. The chromatography of ^{3}H-labeled Extract B on DEAE-cellulose and followed by gel filtration of radioactive fractions on Sephadex G-200 has shown that the phospholipids of Extract B are only bound to soluble proteins and not to fragments of membranes; it can be assumed that they have been solubilized in the form of a lipid-protein complex by cell breakage. When Extracts A and B are treated by phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) before being mixed together, an inhibition of the reconstitution of nitrate reductase activity which is proportional to the phospholipase C concentration and the length of treatment is observed. The analysis of lipids and phospholipids of particles (Peak I, Peak II and Peak III) formed during complementation and reconstituted nitrate reductase shows that their phospholipid contents (phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and phosphatidylserine) and especially that of Peak II ($d = 1.18$) are closely related to that of native particles from the wild-type strain. These results allow one to propose a hypothesis explaining the mechanism involved in complementation.

INTRODUCTION

The observations reported in the two previous papers [1, 2] dealing with the part played by different protein factors in the complementation between the soluble fractions of the *chl A* and *chl B* pleotropic mutants of *Escherichia coli* K 12, as also those formerly published by us [3] or other workers [4] allow one to confirm that

the pleotropic effect of the *chl-r* mutations is the result of an alteration of the membrane. The complementation is now to be considered, with respect to the molecular point of view, as an in vitro reconstitution of the nitrate-bound membranous respiratory system.

As stated by Rivière et al. [2], the complementation can be carried out with two systems. In system II, the reaction mixture contains equivalent amounts (15–30 mg protein per ml) of Extracts A and B [3]. System I uses purified F_A protein (50–100 μg/ml) instead of Extract A [2]. When incubated in a vacuum at 32 °C for 2 h, both systems lead to the reconstitution of nitrate reductase and the formation of sedimentable material (7–10 % of the protein of the reaction mixture). When subjected to fractionation on sucrose density gradient (isopycnic, 20–60 %), the sedimentable fraction separates into three peaks of different buoyant densities: Peak I (d = 1.10–1.12), Peak II (d = 1.18) and Peak III (d = 1.23). The reconstituted nitrate reductase activity is found only in Peak I and Peak II [2]. Moreover, we have shown that ATPase, present in a soluble state in Extracts A and B prior to being mixed, binds to the particulate structures (Peaks I and II) formed during the process. ATPase was purified and used as a membrane label to follow the formation of particles [1]. It was also seen that the population of reconstituted particles is heterogeneous, with respect to the morphological point of view [5].

The aims of the present study are to precisely determine the nature and the part played by phospholipid in the reconstitution process. With this object in mind, we have attempted to verify whether the mechanical treatments used for cell breakage lead to a partial solubilization of membranous lipids and phospholipids of the *chl-r* mutants.

MATERIALS AND METHODS

Organisms and growth conditions

E. coli K 12, strain PA 601 (356 of our collection) sensitive to chlorate and its mutants *chl A* and *chl B* (356.15 and 356.24). These mutants, which were described previously [6, 7], have the *nit*$^-$*gas*$^-$ phenotype and are chlorate resistant. Cells were grown and harvested as reported previously [8].

Preparation of membranes and soluble fractions

Cells were washed and suspended in 0.05 M Tris · HCl buffer (pH 7.6). After cell breakage in a French pressure cell or by sonication in a MSE sonicator, the crude extract was spun at 22 000 × g to remove whole cells and cell debris and then centrifuged twice at 220 000 × g for 90 min. The supernatants, cleared of all sedimentable material, constituted the soluble fractions. The pellets, suspended in 0.05 M Tris · HCl buffer (pH 7.6) gave the particulate fractions (6–12 mg protein per ml). For the sake of clarity, we will use the following designations throughout this report: Extracts WT, A and B for the soluble extracts from wild-type, *chl A*$^-$ and *chl B*$^-$ strains, respectively; Particles WT, A and B for the particulate fractions from wild-type, *chl A*$^-$ and *chl B*$^-$ strains, respectively.

Membranes have also been prepared according to the procedure described by Kaback [9]. The treatment of cells by lysozyme and EDTA gives spheroplasts which are then lysed giving membranes. These are harvested by centrifugation at

37 000 × *g*, suspended in 0.05 M Tris · HCl buffer (pH 7.6) and disrupted in a French pressure cell. The mixtures are then centrifuged twice at 220 000 × *g* for 90 min giving supernatants and membranous pellets.

Complementation

We have used two different systems containing either Extracts A+B [3] or Extract B+purified F_A protein for our studies [2]. After incubation in a vacuum at 32 °C for 2 h, which is sufficient time to complete the reaction, the mixture was centrifuged twice at 220 000 × *g* for 90 min in order to separate the soluble and particulate fractions. The particulate pellet was suspended in 0.05 M Tris · HCl buffer (pH 7.6) giving the reconstituted particulate fraction.

Determination of lipids and phospholipids

Total lipids of subcellular fractions were assayed by the procedure of Folch et al. [10]. Proteins were precipitated by 15 % (w/v) trichloroacetic acid then resuspended in 5 vol. or more of chloroform/methanol mixture (2 : 1, v/v). This was stirred overnight under a N_2 atmosphere at 4 °C then centrifuged at 12 000 × *g* for 10 min in order to sediment the proteins. The aqueous phase was removed from the supernatant with 0.05 M NaCl (0.2 vol. NaCl for 1 vol. aqueous phase). The chloroform phase was dried off, giving a lipid residue that was weighed and the lipid phosphorus content of which was determined according to Bartlett [11].

The fractionation of total lipids and phospholipids was achieved by thin-layer chromatography. Plates were prepared with Silica Gel G (Merck) and activated at 110 °C not less than 1 h before using. The developing solvents used were hexane/diethyl ether/chloroform/acetic acid (40 : 5 : 5 : 1, by vol.) for lipids and chloroform/methanol/acetic acid (60 : 25 : 4, by vol.) for phospholipids. The identification of individual lipids and phospholipids was carried out by comparison with lipids and phospholipids of analytical grade used as reference standards after exposing the plates to iodine vapours.

Radioactive labeling of the phospholipids

For labeling with ^{32}P, the bacteria were grown overnight in a complex medium containing 2 μCi/ml, then harvested and fractionated as described above. The phospholipids were extracted and analysed as above and the ^{32}P radioactivity of each of them was determined by liquid scintillation spectrophotometry.

For labeling with [2-^{3}H]glycerol, the bacteria were grown overnight in a complex medium and harvested by centrifugation as described above, then resuspended in a minimal medium supplemented with the required amino acids and vitamin B-1 [12] and a carbon source (2 g/l glucose). Labeling was achieved by growing the cells for one generation in this medium containing [2-^{3}H]glycerol (1–5 mCi/l depending on the work to be done) at the final concentration of 0.5 mM. The labeled cells were harvested and treated as described above. This labeling has the advantage of being specific for phospholipids, since when a very low amount of glycerol is added to a medium containing high quantities of another carbon source, glycerol kinase and glycerophosphate dehydrogenase are not induced; so the glycerol which enters into the cell can be used only for the synthesis of phospholipids. We have checked that the whole of the incorporated radioactivity can be extracted by chloroform. Individual

phospholipids separated by thin-layer chromatography were assayed for ^3H radioactivity by directly transferring the silica gel spots to counting vials of liquid scintillation spectrophotometry.

Polyacrylamide gel electrophoresis

This was carried out according to the method of Shapiro et al. [13] using 7.5 % (w/v) gels containing 0.1 % sodium dodecylsulphate. Samples containing 25 μg of membranous protein were solubilized by heating at 100 °C for 75 s in the presence of 2 % sodium dodecylsulfate and 50 mM 2-mercaptoethanol. After the addition of 50 mM sodium iodoacetate to the samples, they were layered over the gels and run at constant voltage and a current of 10 mA per gel at 4 °C for 5 h. Gels were stained for protein with Coomassie brilliant blue then destained by repeated washings with 7 % (v/v) acetic acid and scanned at 600 nm.

Nitrate reductase assay

Nitrate reductase activity was measured by the rate of H_2 uptake in a Warburg apparatus, as described previously by Pichinoty and Piéchaud [14]. 1 unit of enzyme activity is defined as 1 μmol of nitrate reduced per h.

Treatment of extracts with phospholipase C

1 ml of extract (10–15 mg protein) was incubated with stirring for 30 min at 37 °C with 10 I.U. of phospholipase C (Worthington Biochem. Corp.) in Trizma buffer (pH 7.3) containing 0.05 M $NaCl_2$ then centrifuged at 49 000 rev./min for 15 min in order to remove insoluble residue. The effect of phospholipase C attack was determined using the procedure of Fiske and Subbarow [15] to assay the released inorganic phosphorus.

Protein determination

This was carried out by the procedure of Lowry et al. [16] using crystalline serum albumin as a standard.

Chemicals

All reagents used were analytical grade.

RESULTS

Analysis of the particles formed by complementation

We began to compare the chemical composition of the reconstituted particles to that of native particles prepared from the wild-type strain of *E. coli* K 12.

Polyacrylamide gel electrophoresis of the reconstituted particles shows the presence of about 15 protein bands (Fig. 1). Large differences with the profile of wild-type particles are seen. Two major bands of the latter are missing in the reconstituted particles and a major new band can be seen in these particles. At the present time, the differences cannot be interpreted; this will be the subject of future study.

The ratio total lipid to protein of the reconstituted particles is about 0.30–0.38 (0.30–0.34 for the wild-type particles) depending on the experiments and the ratio phospholipid to total lipid is about 0.57–0.67 (0.60–0.62 for the wild-type particles).

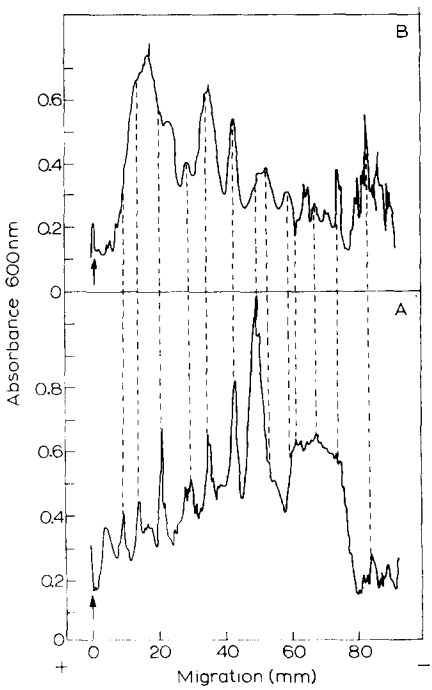

Fig. 1. Densitometry of gel electrophoretic patterns of proteins from native (A) and reconstituted (B) particles. Samples containing 25 μg protein were treated as described in Materials and Methods. Gels were scanned at 600 nm.

Phospholipids of reconstituted particles are phosphatidylethanolamine, phosphatidylglycerol, cardiolipin and phosphatidylserine, i.e. all the species found in the membrane of *E. coli*. Neutral lipids are mono-, di- and triglycerides and some traces of fatty acids; the concentrated chloroform solution of the neutral lipids is of a bright orange color produced by material tentatively identified as coenzyme Q by the method of Lester et al. [17].

Effect of phospholipase C upon complementation

We have resumed and improved a preliminary work of Azoulay [18] dealing with the effect of phospholipase C upon complementation. Our object was to study the part played by phospholipids in this reconstitution process. For these experiments, we have used purified phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) devoid of proteolytic activity.

The results of the first series of experiments were the following. (a) When Extracts A and B (15 mg protein per ml) are incubated with 10 I.U. phospholipase C per ml for 1 h before being mixed together, reaction mixtures which give very abnormal complementation are obtained: no formation of sedimentable material and very little amount of reconstituted nitrate reductase (2 units per mg protein, i.e. 5–10 % of that obtained with control untreated Extracts A and B). (b) When phospholipase C is added to the reaction mixture at the very beginning of incubation, the same observations can be made. (c) When phospholipase C is added after 45 min of incubation,

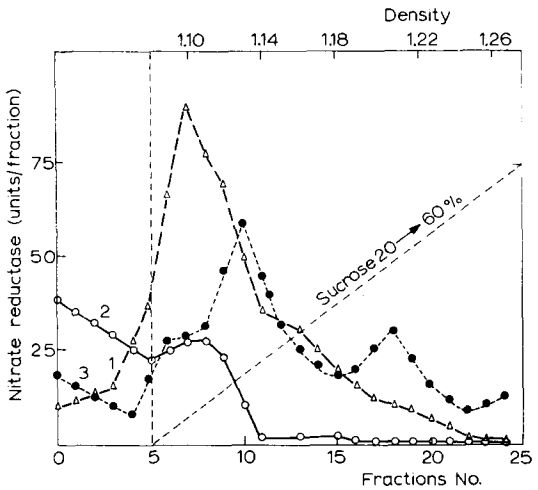

Fig. 2. Influence of treatment by phospholipase C on the reconstitution of nitrate reductase. Sucrose density gradient profiles showing the sedimentation characteristics of the reconstituted nitrate reductase in (curve 1) a standard complementation mixture incubated for 2 h, (curve 2) a standard complementation mixture incubated for only 10 min and (curve 3) a complementation mixture incubated for 2 h but carried out with Extracts A and B treated with 1.5 I.U. phospholipase C per ml for 30 min before mixing. The 9-ml mixtures contained 99 mg protein of each extract. 4 ml of each complemented mixture were layered on linear sucrose gradients (20–60 %, w/v, in preparation buffer) and centrifuged at 27 000 rev./min (SW 27 rotor) in a Spinco model L ultracentrifuge. Fractions of 1 ml were collected from top to bottom and assayed for nitrate reductase activity.

no effect is seen, the reconstitution is the same as in the control. (d) When Extracts A and B are incubated with 10 I.U. phospholipase C for only 30 min before being mixed together, the reconstituted nitrate reductase activity is 50 % of the control.

Another series of experiments was performed using only 1–2 I.U. phospholipase C per ml for 30 min. Under such conditions, the inhibition observed is 20 % and reconstituted particles are obtained. In Fig. 2 the results of the analysis of the complementation mixture obtained after this treatment are seen. In the control experiment, after 10 min of incubation two kinds of nitrate reductase are obtained; the major part of the reconstituted enzyme is soluble and the minor part is in the Peak I particles, the buoyant density of which is 1.10; after 120 min of incubation, the distribution described previously [2] is observed. In the experiment performed with extracts pretreated with phospholipase C, it can be observed that the reconstituted activity is located in particles of which the density is appreciably heavier.

Incorporation of phospholipids into reconstituted particles

The ratio total lipid to protein of Extracts A and B is about 0.10–0.16 depending on the experiments. These lipids contain some phospholipids. Table I shows that, after complementation, these lipids are found in reconstituted particles. A series of more precise experiments was carried out using cells grown under anaerobiosis on ^{32}P medium containing nitrate to prepare Extracts A and B the phospholipids of which were determined by measuring the ^{32}P radioactivity in the methanol/chloroform mixtures of the extraction of these compounds. It is seen in Table II that a part of the

TABLE I

RATIOS TOTAL LIPIDS/PROTEIN AND PHOSPHOLIPID/LIPID OF EXTRACTS A AND B AND RECONSTITUTED PARTICLES

Complementation was performed by incubating a mixture of extracts A and B (10 mg protein of each per ml) at 32 °C for 30 min. The mixture was then centrifuged at 220 000 × *g* for 90 min in order to sediment the reconstituted particles. Total lipids were assayed by the method of Folch et al. as described in Materials and Methods. Phospholipids were determined according to Bartlett [11].

	Total lipids/protein	Phospholipid/lipid
Extract B	0.15–0.16	0.10–0.13
Extract A	0.10–0.12	0.09–0.10
Reconstituted particles	0.30–0.38	0.57–0.67
Complementation supernatant	0.04	Traces

TABLE II

INCORPORATION OF PHOSPHOLIPIDS LABELED BY ^{32}P OR [2-^{3}H]GLYCEROL INTO RECONSTITUTED PARTICLES

Complementation was carried out by incubating a mixture of Extracts A and B labeled by ^{32}P or [2-^{3}H]glycerol as described in Materials and Methods, then ultracentrifuged in order to separate reconstituted particles and complementation supernatant. ^{32}P and ^{3}H radioactivities were determined as described in Materials and Methods.

	^{32}P labeling		^{3}H labeling: ^{3}H radioactivity (cpm/mg protein)
	Total protein (mg)	Total radioactivity (cpm)	
Extract WT	—	—	10 000
Extract B	30	9 100	173 000
Extract A	35	16 700	230 000
Reconstituted particles	5	20 300	—
Complementation supernatant	59	2 900	60 000

radioactivity contained in Extracts A and B is not incorporated into the reconstituted particles.

Effect of mechanical treatments upon the membranes of wild-type and chl-r strains

The incorporation of a part of the phospholipids contained in Extracts A and B into the particles reconstituted by complementation imply that the treatments used for cell breakage lead to a partial solubilization of the membranes of the mutants *chl A* and *chl B*. This was studied in the following experiments. Membrane vesicles prepared by the method of Kaback [9] were disrupted in a French pressure cell then centrifuged twice at 220 000 × *g* giving membranous pellets and supernatants which were assayed for protein and total lipid. We have seen that the percent of solubilized protein is twice that obtained in the case of the wild-type strain as was shown by

Rivière et al. [2] and that a quarter of the lipids is solubilized in the case of mutants (almost none for the wild-type strain).

Phospholipids of Extracts A and B

The labeling of phospholipids by growing the cells on [2-^{3}H]glycerol medium allows one to obtain extracts in which the phospholipids can be easily assayed by radioactivity counting without extraction by methanol/chloroform. Table II shows that the radioactivity is 20 times higher in Extracts A and B than in the extract of the wild-type strain and that, after complementation, 80 % of the radioactivity of Extracts A + B is found in the reconstituted particles. This incorporation was also shown in the following experiments. Extract B labeled with [2-^{3}H]glycerol was layered onto a DEAE-cellulose column equilibrated with 0.02 M Tris · HCl buffer (pH 7.7). Elution was carried out with a linear NaCl gradient (0–0.8 M) in the same buffer. Protein fractions having concentrated the bulk of radioactivity were eluted between 0.1 and 0.3 M NaCl (Fig. 3, curves I_{pr} and I_{pl}). A control experiment carried out with a ^{3}H-labeled Extract of the wild type is not observable at the same molarity as the elution fractions containing high amounts of protein and radioactivity (Fig. 4). A complementation experiment of "system I" type [2] was carried out between ^{3}H-labeled Extract B and 50 μg F_A protein; when the incubation was completed, the mixture was centrifuged at 220 000 × g and the supernatant subjected to chromatography on DEAE-cellulose under the same conditions as above: the elution profile (Fig. 3, curves II_{pr} and II_{pl}) did not show the fractions containing protein and phospholipids which are characteristic of Extract B.

The most radioactive protein fractions from another experiment of the chroma-

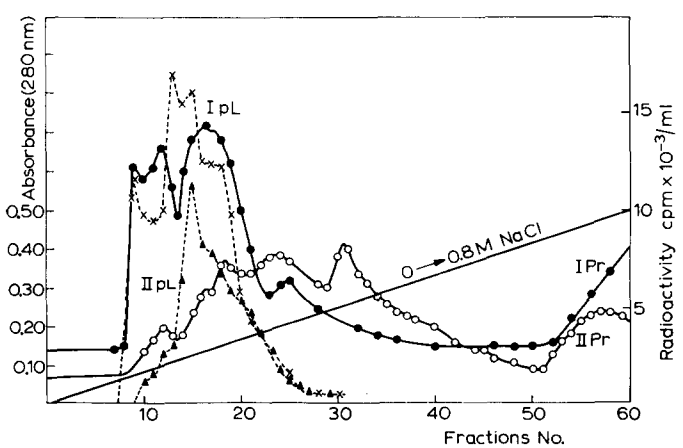

Fig. 3. Phospholipids of Extract B before and after complementation. Extract B having its phospholipids labeled by [2-^{3}H]glycerol as described in Materials and Methods was used to bring about a complementation with 50 μg F_A protein. When incubated for 2 h, the mixture was centrifuged at 220 000 × g for 2 h. 240 mg protein of ^{3}H-labeled Extract B and 205 mg protein of a complementation supernatant were then layered onto DEAE-cellulose columns (17 × 2.5 cm) equilibrated with 0.02 M Tris buffer (pH 7.7). Elutions were with linear NaCl gradients (0–0.8 M) in the same buffer. Fractions of 3 ml were collected. Curve I_{pr}, protein of ^{3}H-labeled Extract B (absorbance at 280 nm); curve I_{pl}, radioactivity of ^{3}H-labeled Extract B; curve II_{pr}, protein of complementation supernatant; curve II_{pl}, radioactivity of the complementation supernatant.

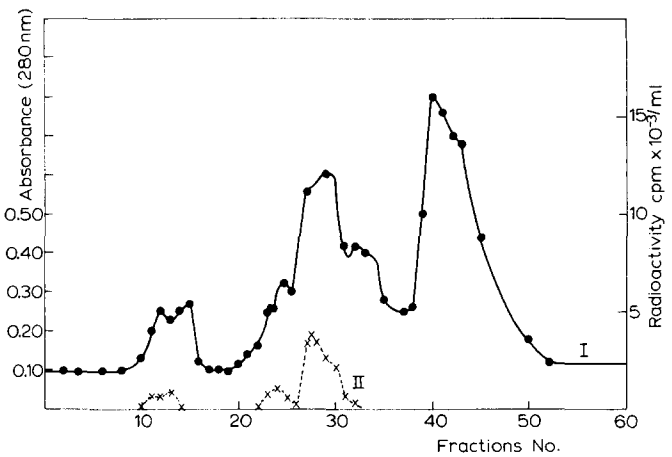

Fig. 4. Phospholipids of wild-type extract labeled by [2-^{3}H]glycerol. The chromatography of 110 mg protein of this extract on DEAE-cellulose was carried out under the same conditions as in Fig. 3. Curve I, protein; curve II, ^{3}H radioactivity.

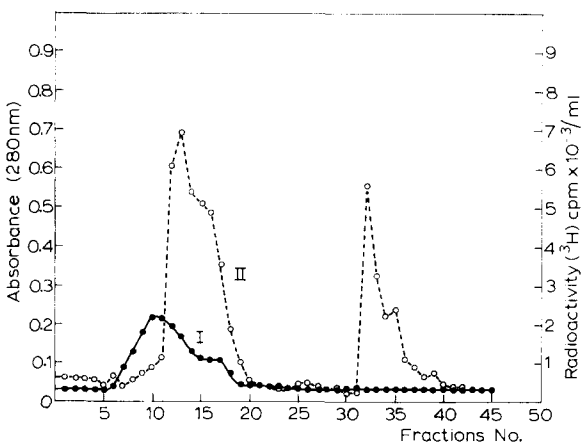

Fig. 5. Phospholipids of Extract B. 136 mg protein per 6.5 ml of Extract B labeled by [2-^{3}H]glycerol as described in Materials and Methods were subjected to chromatography on DEAE-cellulose under conditions described in Fig. 3. The most radioactive protein fractions eluted between 0.15 and 0.30 M NaCl were pooled and concentrated by ultrafiltration through a Diaflo membrane filter XM 50 then layered onto a Sephadex G-200 column (25 × 2.5 cm) equilibrated with 0.02 M Tris buffer (pH 7.7), containing 0.3 M NaCl. Elution was with the same buffer and fractions of 9 ml were collected. Curve 1, protein; curve 2, ^{3}H radioactivity.

tography of ^{3}H-labeled Extract B on DEAE-cellulose were pooled and concentrated by ultrafiltration then subjected to a gel filtration on a Sephadex G-200 column (Fig. 5). The elution profile shows that the major part of the phospholipids remains bound to protein eluted beyond the void volume and that about 30 % of the radioactivity is eluted at the end of filtration and is not bound to any protein.

Phospholipids of the particles formed by complementation

We have determined the lipid and phospholipid contents of the reconstituted particles obtained by complementation carried out with system I. The results in Table III allow one to make the following observations. (a) The ratio total lipid to protein is higher for Peak I particles (0.42) than for Peak II particles (0.30); the determination was not made for Peak III particles because of the low percentage of these among the total reconstituted particles. (b) Phosphatidylethanolamine and phosphatidylserine are in very similar concentrations in Peaks I and II; phosphatidylglycerol and cardiolipin of the Peak I particles are about 2-fold that observed for the Peak II particles. (c) The phospholipid content of Peak III is completely different, since a very low amount of phosphatidylethanolamine and a very high content of phosphatidylglycerol is observed. It is to be remembered that the Peak III particles have no nitrate reductase activity [2], do not bind ATPase [1] and are a minor part (10 %) of the total reconstituted particles.

TABLE III

PHOSPHOLIPIDS OF PARTICLES FORMED BY COMPLEMENTATION

The reconstituted particles (three families of different densities) were assayed for their total lipid to protein ratio and their phospholipid content according to Materials and Methods.

	Particulate fractions		
	Peak I ($d = 1.10$)	Peak II ($d = 1.18$)	Peak III ($d = 1.23$)
Total lipid/protein	0.42	0.30	—
Phospholipid content (% total):			
Phosphatidylethanolamine	63	68.6	2.6
Phosphatidylglycerol	19	11	68
Cardiolipin	13	6.6	19
Phosphatidic acids	—	9.6	8.6
Phosphatidylserine	3.7	3.5	1.2

Phospholipids of the non-sedimentable nitrate reductase

We have used a ^{3}H-labeled Extract B prepared as described in Materials and Methods in order to carry out complementation with purified F_A protein. After incubation, the mixture was centrifuged twice at 220 000 $\times g$ for 90 min. The resulting supernatant contains a non-sedimentable nitrate reductase activity and radioactivity corresponding to membranous phospholipids solubilized by cell breakage and which were not incorporated into the particles reconstituted during the complementation process. We have attempted to partially purify the non-sedimentable nitrate reductase in order to ascertain whether this enzyme is actually bound to phospholipids. This was carried out by two successive gel filtrations on Bio-Gel A-5 M (Fig. 6). The elution profile shows that the major part (90 %) of the nitrate reductase activity is indeed bound to radioactivity and, therefore, seems to be a protein-lipid association the molecular weight of which was tentatively estimated to be about $3 \cdot 10^6$–$4 \cdot 10^6$ with respect to the elution volume on our column (76 $\times$ 2.7 cm). The protein fractions containing nitrate reductase activity and ^{3}H radioactivity were then pooled and con-

centrated in order to analyse the phospholipid content. Table IV shows that the major part (73 %) of the phospholipids associated to the non-sedimentable nitrate reductase is phosphatidylethanolamine.

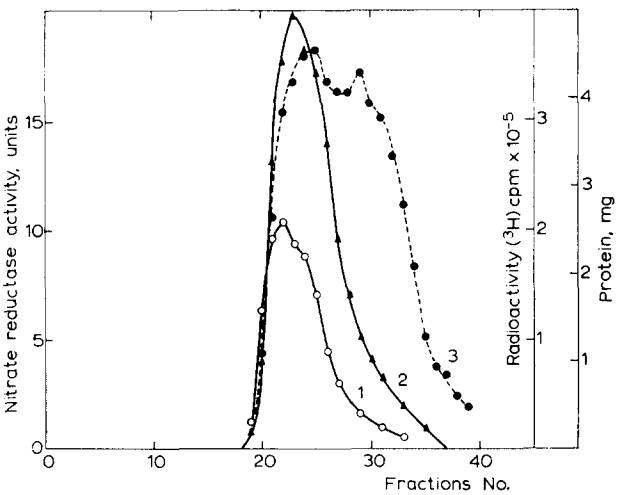

Fig. 6. Elution profile of non-sedimentable reconstituted nitrate reductase obtained by filtration on Bio-Gel A5M. The complementation mixture (13–16 mg protein per ml) containing soluble fractions from *chl B* mutant labeled by [2-^{3}H]glycerol and unlabeled *chl A* mutant was incubated at 32 °C for 2 h then centrifuged twice at 220 000 × *g* for 90 min. 11 ml of the last supernatant were layered onto a Bio-Gel A-5 M column (76 × 2.7 cm) equilibrated with 0.02 M Tris buffer (pH 7.7). Elution was carried out with the same buffer. The flow rate was 10 ml/h and fractions of 8 ml were collected. A pool of the most active fractions was concentrated by ultrafiltration through a PM 30 Diaflo membrane filter. The 5-ml concentrated fraction was applied on the same column as above and elution was under the same conditions. In the 8-ml fractions ^{3}H radioactivity (curve 1), nitrate reductase activity (curve 2) and protein (curve 3) were determined.

TABLE IV

PHOSPHOLIPID CONTENT OF THE NON-SEDIMENTABLE NITRATE REDUCTASE

The radioactive non-sedimentable nitrate reductase prepared as described in Fig. 6 was subjected to quantitative analysis by thin-layer chromatography as described in Materials and Methods. The sample containing 30 mg total protein and 1.1 mg total lipid was resuspended in 0.5 ml CH_3Cl. 30 μl of this suspension was deposited on the plate. The radioactivity indicated for cardiolipin is one-third of that actually recorded (3 glycerol/mol) and the radioactivity indicated for phosphatidylglycerol is one-half of that recorded (2 glycerol/mol).

Phospholipid	^{3}H radioactivity (cpm)	% of total phospholipid content
Cardiolipin	36 817	14.2
Phosphatidylglycerol	29 525	11.4
Phosphatidylethanolamine	189 617	73
Phosphatidylserine	3 300	1.3

DISCUSSION

Effect of the chl-r mutations

It is shown that the phospholipid content of Extracts A and B is 20 times higher than for Extract WT and that the mechanical treatments used for cell breakage release some lipids and solubilize twice as much membranous protein in the case of *chl-r* mutants than as for the wild-type strain. These facts can be compared to the effect of removing Mg^{2+} by dilution upon the membranes of the wild-type and *chl-r* strains [1, 19]: we have seen that membranous vesicles, obtained by lysis of spheroplasts of the wild-type strain grown in anaerobiosis in the presence of nitrate, release some proteins, especially F_A factor and ATPase, but retain wholly nitrate reductase, when subjected to such a treatment; in the case of spheroplasts prepared from *chl-r* strains grown under the same conditions, the release of proteins is 2-fold stronger [2]. Since mechanical treatments of *chl* A^- and *chl* B^- cells give Extracts A and B containing some components able to reaggregate in order to reconstitute the polyenzymatic system bound to nitrate reductase [20], one can state that the effect of *chl-r* mutations is exerted at the membrane level. Therefore, the results reported in the present paper confirm our first hypothesis as to the nature of *chl-r* mutations [21], which was later taken again by Schnaitman [22], Onodera et al. [23] and Glaser and DeMoss [24]. From the fluid mosaic model of Singer and Nicolson [25], we can assume that nitrate reductase and probably most of the polyenzymatic complex of which it is a component are an integral part of the membrane (intrinsic protein), for they can never be released by an osmotic shock (removal of Mg^{2+} by dilution) alone and the solubilization needs detergents [26, 27]. From this model, it can be assumed, in the case of *chl-r* mutations, that the components of the polyenzymatic system could not correctly aggregate inside the membrane and, therefore, could be themselves as extrinsic proteins easily removed from the membrane without using detergents. Thus, it can be understood why the mixture of Extracts A and B leads to the reconstitution of the nitrate-bound membranous respiratory system.

Part played by phospholipids in the complementation process

Our results show that the complementation carried out with either system I or system II leads to the formation of sedimentable particles into which 80 % of the phospholipids contained previously in Extracts A and B are incorporated. The phospholipid content of the reconstituted particles is similar to that of the membranes of the wild-type strain from both qualitative and quantitative points of view. This observation explains quite well the inhibitory effect of phospholipase C upon the reconstitution process; it is to be reminded that phospholipase C actually acts only when it is added to Extracts A and B before being mixed together or to the reaction mixture at the very beginning of incubation. Such a result implies that phospholipids play a role in the earliest stages of the process. When low amounts of phospholipase C or short contact times are used, an attending lowering of the inhibitory effect and changes of the buoyant density of particulate nitrate reductase are observed. This means that phospholipids play both parts, qualitative and quantitative. It should be noticed that the phosphoesterasic activity of phospholipase C is maximal for phosphatidylethanolamine; this implies that phosphatidylethanolamine is the major phospholipid.

The reconstitution of nitrate reductase by complementation also needs the presence of divalent cations. In previous reports [3, 19], we had seen that complementation requires Mg^{2+} and that this is contained by Extracts A and B; in this respect, our system is to be compared with those of other workers [28–32] who had shown that Mg^{2+} is necessary for the reconstitution of sarcoplasmic and mitochondrial membranes. We had also seen that, after dialysis, the addition of Mg^{2+} to complementation mixtures exerts an effect upon the rate of reconstitution of the nitrate reductase activity. This result can be explained if one assumes that the amount of the lipids incorporated into the reconstituted membranes depends upon the concentration of Mg^{2+} [29, 33–35].

State of phospholipids in Extracts A and B

The complementation process requires that the phospholipids contained in Extracts A and B are in a well-defined state. Therefore, it is desirable to ascertain the state of the phospholipids in the supernatant extracts. We had previously shown that complementation is a function of the saline content and dilution of the reaction mixture [19, 3]. This allows one to think that lipids and phospholipids released from the membrane of *chl B* mutant and contained in Extract B are in a non-micellar state; such an assumption is confirmed by the morphological observations of Mutaftschiev and Azoulay [5] who had seen only very small aggregates under the electron microscope in negative staining, and also by the fact that the chromatography on DEAE-cellulose of Extract B labeled by [2-^{3}H]glycerol gives protein fractions having concentrated the bulk of the radioactivity and after filtering these pooled and concentrated fractions on Sephadex G-200 the phospholipids remain bound to protein of low molecular weight; it is pointed out that such protein-phospholipids aggregates are seen neither in Extract WT nor in the supernatant obtained by centrifugation of an incubated complementation mixture. These results allow one to assume that the phospholipids of Extracts A and B which take part in complementation are neither in a micellar state nor associated to membranous fragments. Taking their chromatographic behavior into account, they are to be thought of as strongly aggregated lipid-protein complexes.

The effect of phospholipase C shows very precisely that phospholipids play their part in the earliest states of the process, not only in the formation of particles but also in the reconstitution of nitrate reductase activity. It is to be noticed that phospholipase C has no effect upon the activity of the reconstituted enzyme, in either the soluble or the particulate form and that native nitrate reductase of the wild-type strain still remains active when separated from membrane lipids [36]. One can think that the complex formed during complementation is quite stable and integrates phospholipids in a manner which makes them not accessible to the action of phospholipase C. We have shown that complementation gives rise to three types of particles, the phospholipid contents of which are different and to a non-sedimentable nitrate reductase aggregated to phospholipids the major part of which is phosphatidylethanolamine.

From our results taken as a whole, it can be assumed that lipid-protein aggregates go through a sequence of increasingly complex organization stages, in which the stoichiometry of the different components plays a major role. As is the case for the reconstitution of mitochondrial membranes [37, 38], the first aggregation con-

trolled by F_A protein could give rise to a complex essentially containing phosphatidylethanolamine and capable of binding to other proteins in order to lead to vesiculate structures which in their turn could have specific binding sites for ATPase [1] and cytochromes *b* [3].

At the present time it remains to ascertain the nature of the process by which Peak I particles are transformed into Peak II particles whose buoyant density, composition and morphology approximate the most closely those of native membrane vesicles.

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