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MEMBRANE RECONSTITUTION IN *chl-r* MUTANTS OF *ESCHERICHIA COLI* K 12

VII. PURIFICATION OF THE SOLUBLE ATPase OF SUPERNATANT EXTRACTS AND KINETICS OF INCORPORATION INTO RECONSTITUTED PARTICLES

G. GIORDANO, C. RIVIERE and E. AZOULAY

Laboratoire de Structure et Fonction des Biomembranes, C.N.R.S. E.R. 143, U.E.R. de Luminy, 13288 Marseille Cedex 2 (France)

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SUMMARY

Membrane-bound ATPase (EC 3.6.1.3) of *Escherichia coli* K 12 is released in a soluble form by the mechanical treatments applied to the cells in order to break them. The purification of the soluble enzyme is described. The purified protein gives a single band in 7.5 % polyacrylamide gel electrophoresis. The molecular weight is estimated to be 350 000. The enzyme is cold-labile, Mg^{2+} dependent, insensitive to inhibition by *N,N'*-dicyclohexylcarbodiimide and specific for ATP and ADP. Membranes depleted of their ATPase activity by dilution in a buffer of low ionic strength and without Mg^{2+} are able to incorporate the purified ATPase only in the presence of 2–6 mM Mg^{2+}. ATPase binds to particles formed by complementation between supernatant extracts of *chl A* and *chl B* mutants. There are three kinds of particles of different buoyant densities (1.10, 1.18 and 1.23); ATPase binds only to the 1.10 and 1.18 particles. The kinetics of incorporation have been studied. ATPase begins to be incorporated into the 1.10 particles after 10 min of incubation up to a maximum at 20 min: from 30 min, ATPase is incorporated only into 1.18 particles and the amount of incorporated ATPase increases in proportion with the peak of 1.18 particles. These kinetics have a hyperbolic pattern. In order to explain the mechanism of assembly involved in complementation, two hypotheses are proposed.

INTRODUCTION

In a previous paper [1] we reported that a part of the ATPase (EC 3.6.1.3) activity present in a soluble state in cell-free extracts of the *chl A* and *chl B* pleotropic mutants of *Escherichia coli* K 12 was incorporated into membrane particles formed in the course of complementation between soluble fractions of these mutants. It was

Abbreviation: DCCD, *N,N'*-dicyclohexylcarbodiimide.

not then possible to present information on the mechanism of such an integration. Nevertheless, the particles formed during complementation are to be considered as membranous structures from both chemical and morphological points of view [2]. In this regard, the incorporation of ATPase into reconstituted particles appears to be a binding to membranes whose structure is already somewhat defined. Therefore, our system can be compared with those reported by Abrams and Baron [3] and Kagawa and Racker [4] for ATPase incorporation into cell membranes of *Streptococcus faecalis* or mitochondrial membranes of rat liver. In this respect, we reported that the solubilization of the membrane ATPase is accompanied by important changes of the properties of the enzyme and that, in addition, ATPase of reconstituted particles has the same properties as those of membrane-bound ATPase [1].

In the work described in this paper, we initially attempted to determine whether under suitable experimental conditions, the membranes of wild-type or mutant *chl-r* strains can be deprived of their ATPase activity as a whole, and then tried to explain the mechanism of binding of the soluble ATPase contained in cell-free extracts to such depleted membranes. It should be emphasized that complementation, which leads to a restoration of nitrate reductase activity [5, 6] and to the formation of particles [7] capable of binding this activity, the soluble ATPase and other proteins, is a complex phenomenon requiring successive events the sequence of which is hard to distinguish.

Consequently, it seemed advisable to follow the incorporation of the soluble ATPase into the reconstituted particles as a function of incubation time in order to confirm that complementation involves a sequence of increasingly complex organization stages.

MATERIALS AND METHODS

Organisms and growth conditions

E. coli K 12, strain PA 601 (356 of our collection) and its two mutants *chl A* (356.15) and *chl B* (356.24). These mutants, which were described previously [8, 9] have the *nit*$^-$ *gas*$^-$ phenotype and are chlorate resistant.

Cells were grown and harvested as previously described [10]. Washed cells were suspended in 0.05 M Tris buffer (pH 7.6), disrupted in a French pressure cell under a pressure of 15 000 lb/inch^2 or by sonication in a MSE Sonicator by four 30-s pulses at an amplitude setting of 7–8 μm, then centrifuged at 10 000 rev./min for 15 min in a Sorvall RC2 B to remove cell debris.

Preparation of membranes and soluble fractions

The crude extracts were centrifuged (Ti 50 rotor, Spinco Model L3) twice at 220 000 $\times g$ for 90 min. These, cleared of all sedimentable material, constituted the soluble fractions. The resulting pellets, suspended in 0.05 M Tris buffer (pH 7.6), gave the particulate fractions (6–12 mg protein/ml).

Moreover, membranes have been prepared as described by Kaback [11]. The treatment of cells by lysozyme and EDTA gives spheroplasts which are then lysed and centrifuged several times.

Complementation

The soluble cytoplasmic extracts of whole cells prepared as described above

were mixed under conditions defined by Azoulay et al. [12]. The reaction system, containing equivalent amounts (10–15 mg protein/ml) of the extracts of the two mutants was incubated in a vacuum at 32 °C. When the complementation was completed, after 120 min the mixture was centrifuged at 220 000 $\times g$ for 90 min. The particle-containing pellet was then resuspended in 0.05 M Tris buffer (pH 7.6).

It should be pointed out that all the extracts were dialysed against the same buffer for 2 h to eliminate any traces of endogenous phosphate.

Radioactivity labeling of the cells

The bacteria were grown in a complex medium as above described, then harvested by centrifugation at 10 000 $\times g$ for 10 min and suspended in a minimal medium supplemented with required amino acids and vitamin B_1 [13] and a carbon source. ^{14}C-labeling was achieved by growing the cells for one generation in this medium containing 0.228 μM [^{14}C]leucine (0.07 mCi/2 l). The labeled cells (10 g wet weight) were harvested and treated as described above. Radioactivities were determined by liquid scintillation spectrophotometry.

Gel electrophoresis

This was carried out according to the method of Davis [14] using 7.5 % (w/v) polyacrylamide gels (0.35 $\times$ 5 cm) with protein samples of 0.2–0.4 mg protein.

ATPase assay

ATPase activity was determined at 37 °C as the amount of inorganic phosphate (P_i) released by ATP hydrolysis in a 2-ml reaction system containing 4.4 mM ATP (sodium salt), 2.2 mM $MgCl_2$, 40 mM Tris · HCl buffer (pH 7.6) and enzyme sample (0.2–0.8 mg protein). Inorganic phosphate was assayed after 5–20 min of incubation according to Martin and Doty [15]. Phosphohydrolase activities were assayed in the same way by replacing ATP with ADP, GTP, CTP or Glc-6-*P* at the same molarity. 1 unit of enzyme activity is defined as 1 nmol of P_i released per min.

Protein determination

Protein concentration was measured by the Lowry procedure [16] using bovine serum albumin as a standard.

Chemicals

All reagents used were analytical grade.

RESULTS

Cell-free extracts of the wild-type strain 356 of *E. coli* K 12 and of the *chl-r* mutants grown anaerobically have an ATPase activity (50–80 units per mg protein) distributed throughout the particulate and soluble fractions [1]. We had previously shown that 11–14 % of the total phosphohydrolase activities bind to particles formed during complementation (carried out as described in the Materials and Methods section) and that these particles contain 7–8 % of the initial soluble protein in the reaction mixture.

Purification and properties of soluble ATPase from the supernatant extract of strain chl A⁻

The crude extract obtained by cell disruption was centrifuged for 90 min at 220 000 × *g*. The pellet was discarded and the supernatant was again centrifuged for 90 min at 220 000 × *g*. The last supernatant was the soluble fraction from which ATPase was isolated and purified. Because of the sensitivity of soluble ATPase to low temperatures, all subsequent steps were performed at room temperature.

This soluble fraction (168 mg protein) was applied to a column (38 × 2.5 cm) containing DEAE-cellulose equilibrated with 0.02 M Tris · HCl buffer (pH 7.7), 0.1 M NaCl. Elution was carried out with a linear NaCl gradient (0.1–0.4 M) in the same buffer. Active fractions were eluted between 0.225 and 0.285 M NaCl. A pool of the most active fractions was concentrated by ultrafiltration through a PM-30 Diaflo membrane filter. The fraction traversing the membrane filter did not contain any ATPase activity. The next step was a gel filtration. The concentrated fraction (2 ml) was layered onto a Bio-Gel A-5 M column (40 × 2.5 cm) equilibrated with 0.02 M Tris · HCl buffer (pH 7.7), 0.25 M NaCl and eluted with the same buffer. The ATPase activity is eluted as one single symmetrical peak (Fig. 1). The overall purification (Table I) is about 25-fold with a yield 14 % lower than that anticipated.

Polyacrylamide gel electrophoresis of the most active fractions (Fig. 1) concentrated at a volume of 2 ml shows a single protein band for fractions 40–48.

The molecular weight of ATPase was estimated approximately by chromatography on Bio-Gel A-5 M according to the procedure of Whitaker [17]. The internal standards used were horse-radish peroxidase (M_r 4 · 10^4), blue dextran (M_r 2 · 10^6), catalase of bovine liver (M_r 2.5 · 10^5) and ethanol dehydrogenase from yeast (M_r 1.5 · 10^5). The first two markers were determined by their specific absorption at 403 and 660 nm, respectively. The catalase was measured according to Kobayashi and

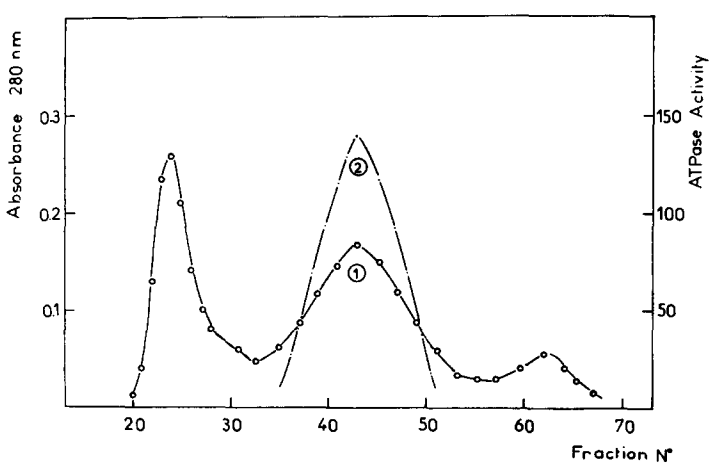

Fig. 1. Filtration on Bio-Gel A-5 M. A pool of the most active fractions from the chromatography on DEAE-cellulose was concentrated by ultrafiltration through a PM 30 Diaflo membrane. The 2 ml concentrated fraction was layered onto a Bio-Gel A column (40 × 2.5 cm). Elution was carried out with 0.02 M Tris · HCl buffer (pH 7.7), containing 0.25 M NaCl. The flow rate was 20 ml per h and fractions of 3 ml were collected. All steps were performed at 20 °C. Curve 1, absorbance 280 nm; curve 2, ATPase activity expressed as units (nmol P_i released/min) per fraction.

TABLE I

PURIFICATION OF ATPase FROM SOLUBLE FRACTIONS OF *chl A* MUTANT OF *E. COLI* K 12

	Total protein (mg)	Specific activity (units/mg protein)	Total activity (units)	Purification (-fold)	Yield (%)
Supernatant extract	168	50	8400	1	100
Chromatography on DEAE-cellulose	7.50	498	3730	10	44
Filtration on Bio-Gel A-5 M	0.96	1240	1180	25	14

Anraku [18]. The ethanol dehydrogenase was assayed as described by Racker [19]. From the elution positions of the standards and the enzyme, the molecular weight of ATPase was tentatively calculated to be $3.5 \cdot 10^5$ (Figs 3 and 4).

The purified enzyme was unstable and particularly cold-labile. The comparative stabilities of the soluble and particulate forms of the ATPase of *E. coli* K 12 are shown in Fig. 4. In the case of the particulate form obtained from the membrane fraction of the *chl* A^- strain as previously described [1] a loss of 25 % activity occurred after 5 days at 0 °C. On the other hand, for the soluble purified form (2 mg protein/ml), a loss of 85 % activity occurred within 24 h at the same temperature; at 20 °C, the loss was only 75 % activity after 5 days. No significant loss of activity was observed at 0 °C after 5 days or more when the enzyme was stored in 0.02 M Tris · HCl buffer (pH 7.7) containing 0.25 M NaCl and glycerol 40 % (v/v).

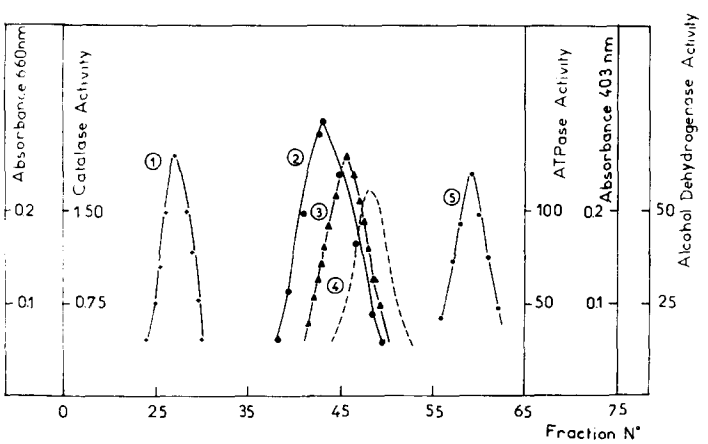

Fig. 2. Estimation of the molecular weight of ATPase by chromatography on Bio-Gel A-5 M. 2 ml of a mixture containing 2 mg purified ATPase, 5 mg horse-radish peroxidase, 5 mg yeast ethanol dehydrogenase, 1 mg beef-liver catalase and blue dextran was applied on a Bio-Gel A-5 M column (40 × 2.5 cm) equilibrated with 0.02 M Tris · HCl buffer (pH 7.7), containing 0.25 M NaCl. Elution was carried out with the same buffer at 20 °C. The flow rate was 10 ml per h and fractions of 3 ml were collected. Curve 1, blue dextran (absorbance 660 nm); curve 2, ATPase (units per fraction); curve 3, bovine liver catalase (units per fraction; 1 unit of catalase activity represents a decrease in the 230-nm absorbance of 0.01 per min); curve 4, ethanol dehydrogenase (nmol NADH released per min per fraction); curve 5, horse-radish peroxidase (absorbance 403 nm).

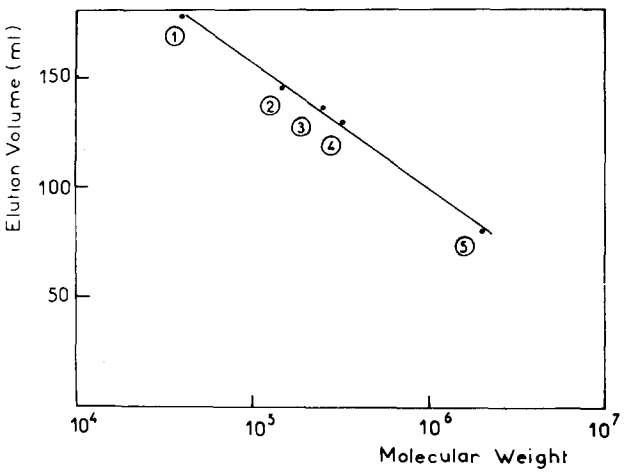

Fig. 3. Calibration curve for the estimation of the molecular weight of purified ATPase by chromatography on Bio-Gel A-5 M. Elution volumes of horse-radish peroxidase (1), yeast ethanol dehydrogenase (2), bovine liver catalase (3), ATPase (4) and blue dextran (5) versus molecular weights. Semi-logarithmic plotting.

The soluble purified enzyme shows other important differences from the particulate form. In Table II, a number of phosphate esters were tested as substrates. ADP was the only compound other than ATP that was hydrolysed. This new property, already reported by Davies and Bragg [20], is rather difficult to explain, for ADP is not a substrate but an inhibitor of the membrane enzyme [1, 21]. Moreover, it can

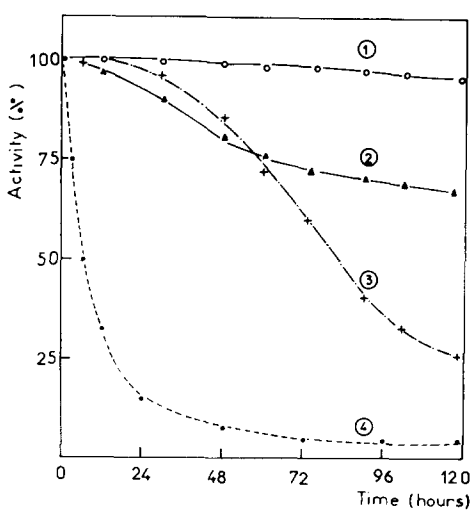

Fig. 4. Stabilities of membrane ATPase from wild-type strain and purified soluble ATPase from the *chl A*$^-$ mutant strain. Samples were stored in 0.05 M Tris · HCl buffer (pH 7.6), containing 40 % glycerol (curve 1) or in the same buffer without glycerol (curves 2–4). Particulate ATPase at 0 °C, curve 2. Soluble ATPase at 0 °C, with glycerol curve 1, without glycerol curve 4. Soluble ATPase at 20 °C, curve 3. ATPase activities were determined on samples taken at various times.

TABLE II

INHIBITION AND SUBSTRATE SPECIFICITY OF PURIFIED SOLUBLE ATPase

Reaction mixture	Specific activity (units/mg protein)	% activity **
3.3 mM ATP*	0	0
3.3 mM ATP	1200	100
3.3 mM ATP, 125 mM NaCl	1200	100
3.3 mM ATP, 1 mM or 0.1 mM DCCD	1100	92
3.3 mM ADP	336	28
3.3 mM AMP	0	0
3.3 mM glucose 6-phosphate	0	0
3.3 mM *p*-nitrophenylphosphate	0	0

* Reaction mixture without $MgCl_2$. All other mixtures contain 1.66 mM $MgCl_2$.

** Activities are expressed as percent of maximal activity measured with 3.3 mM ATP, 1.66 mM $MgCl_2$.

be seen in Table II that soluble ATPase activity was not inhibited by 1 mM DCCD (*N,N'*-dicyclohexylcarbodiimide). The other properties (K_m value for ATP, Mg^{2+} and Ca^{2+} dependence, kinetics of heat inactivation) are the same for soluble purified and particulate ATPases [1].

Preparation of membranes depleted of any ATPase activity

Such depleted membranes had been prepared previously by Abrams and Baron [3] and by Roisin and Kepes [21]. We have devised an alternative procedure (Fig. 5) using 1 mM Mg^{2+} in cell suspensions in order to protect membranes. This procedure, when applied to cells of the wild-type or of mutant *chl-r* strains gives depleted membranes still retaining a weak residual ATPase activity (5–15 units per mg protein).

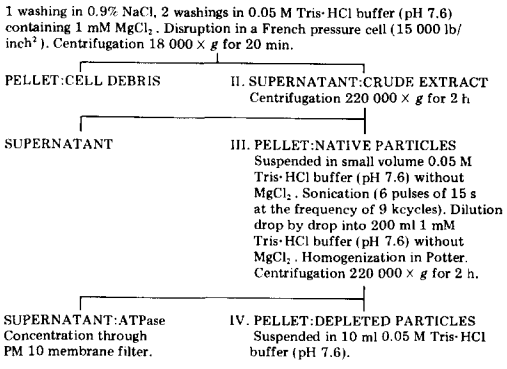

Fig. 5. Procedure of preparation of membranes depleted of any ATPase activity.

Binding of soluble purified ATPase to depleted membranes

When a reaction mixture containing depleted membranes, purified ATPase, 0.05 M Tris · HCl buffer (pH 7.6), 2 mM Mg^{2+} is incubated for 2 h at 32 °C, it can

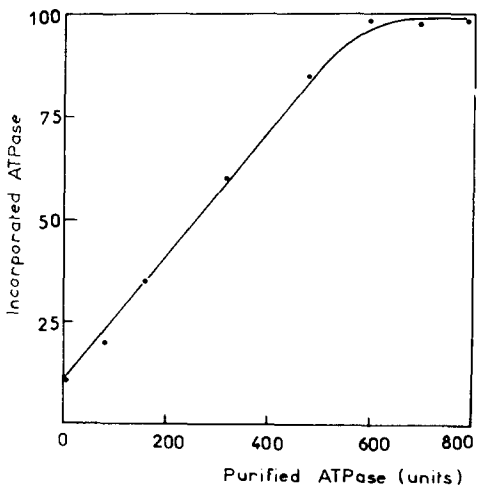

Fig. 6. Binding of purified soluble ATPase to depleted membranes. The reaction mixtures (4 ml) containing 4 mg depleted membranes, increasing amounts of ATPase, 2 mM $MgCl_2$ and 0.05 M Tris · HCl buffer (pH 7.6), were incubated at 32 °C for 2 h then centrifuged at 220 000 × *g* for 90 min. The amounts of bound ATPase were determined on the pellets suspended in the same buffer and expressed as units per mg protein.

be seen that a part of the enzyme is bound to the membranes. Fig. 6 shows an experimental titration of one component with the other. When increasing amounts of soluble ATPase are added to a given amount of depleted membranes, the binding of soluble ATPase increases in a direct ratio up to a maximum of 100 units/mg protein, a value approaching the specific activity of native membranes of this bacterium [1]. If Mg^{2+} is not added to the reaction mixture, no incorporation is observed. We have also seen that the binding of soluble ATPase to depleted membranes increases in a direct ratio with the Mg^{2+} concentration up to a maximum obtained for 2–6 mM Mg^{2+}. When soluble ATPase is incubated in the presence of 2–6 mM Mg^{2+} without the membranes, no formation of sedimentable material can be obtained by sedimentation at 220 000 × *g*. On the other hand, soluble ATPase cannot bind to non-depleted membranes. Therefore, the binding is to be regarded as specific.

Another series of similar experiments has been carried out using soluble fractions from either mutant instead of purified ATPase. In this case, we have observed that the depleted membranes are able to incorporate a part of the phosphohydrolase activities contained in these fractions. We can assume that this part is to be considered as the soluble ATPase contained in these fractions. It can be seen, as in the preceding experiments, that the number of ATPase units bound/mg membrane protein increases in a direct ratio with the amount of supernatant up to a maximum of 90 units bound for 700 units of supernatant ATPase activity. It should be emphasized that, in these two series of experiments, one with the purified enzyme, the other with a supernatant, the binding depends on the presence and concentration of Mg^{2+} and on the amount of enzyme (purified or unpurified) introduced into the reaction mixture. For saturating concentrations of enzyme, it results in membranes, the specific activity of which approaches that of native particles [1].

In a previous paper [1], we had shown that only a part of the ATPase con-

tained in soluble fractions of mutants *chl A* and *chl B* was incorporated into particles formed during complementation. We had observed that 65 % of the phosphohydrolase activities contained in the soluble fractions before the complementation remained in a soluble state when the reaction was over. We thought that these activities still retained some ATPase. In order to verify this assumption, we have carried out the following experiments. In a series of reaction mixtures similar to those described above, increasing amounts of a supernatant resulting from the ultracentrifugation of a complementation mixture (to sediment reconstituted particles) were added to a given amount of depleted membrane. Such a complementation supernatant retains phosphohydrolase activities. These experiments show that depleted membranes incorporate at the same rate, ATPase from complementation supernatant and ATPase from soluble fractions of wild-type or mutant strains. This result means that among the phosphohydrolase activities of the complementation supernatant some ATPase can be found which was not incorporated into reconstituted particles. Moreover, we have observed that ATPase bound to depleted membranes recovers the properties of the native membrane ATPase (sensitivity to DCCD, cold resistance).

Effect of osmotic shock upon membranes

chl-r mutations cause great weakness of the cytoplasmic membrane. In order to attain a more accurate understanding of complementation, we have used, in the preparation of membranes of *chl-r* mutants, two procedures less drastic for membranous material but sufficiently selective to prevent cell-wall and cytoplasmic contamination. In the first series of experiments [1], suspensions of membranes prepared according to the procedure of Kaback [11] were subjected to sonic waves (7 pulses of 15 s). Such a treatment leads to the solubilization of 20–30 % of membrane protein (10–15 % only in the case of the wild-type strain). Among proteins thus released is an ATPase which we have subsequently isolated. It has the same properties as the ATPase isolated and purified from whole cells (insensitivity to DCCD, cold-lability, capacity for ADP hydrolysis).

In the second series of experiments, with membranes depleted of any ATPase activity and prepared as described above (Fig. 5), we have studied the effect of removing Mg^{2+} by dilution in a buffer of lower molarity without Mg^{2+} upon reconstituted particles and native membranes from wild-type or *chl-r* mutant strains; the results of these experiments (Table III) allow us to establish the two following facts: (1) with this treatment, the percentage of protein released by membranes of *chl-r* mutants is 2–3 times that released by membranes of the wild-type; (2) ATPase is released to a large extent (over 70 %) from the three types of membranes. It should be pointed out that newly formed particles and mutant *chl-r* membranes show the same patterns of release of ATPase and protein.

Incorporation kinetics of ATPase into particles formed by complementation

The population of reconstituted particles shows a large structural heterogeneity, as stated by Mutaftschiev and Azoulay [2]. When subjected to fractionation on sucrose density gradient, they separate into three peaks of different buoyant densities (1.10, 1.18, over 1.23). We had shown [1] that ATPase activity is located in the two light bands, of which only the 1.18 band can be regarded as identical to native particles from the wild-type strain with respect to buoyant density and morpho-

TABLE III

SOLUBILIZATION OF PROTEINS AND ATPase BY REMOVING Mg^{2+} IN MEMBRANES FROM WILD-TYPE STRAIN, *chl A* MUTANT STRAIN AND RECONSTITUTED PARTICLES

ATPase activity is expressed as total units (nmol P_i released per min). Percentages are expressed as ratios of protein and ATPase amounts contained in native membranes or in newly formed particles. Treatment applied to membranes is described in Fig. 5. Protein content of membranes: * before treatment; ** after treatment. *** Protein released by the treatment.

	Wild-type			Mutant *chl A*			Reconstituted particles		
	Native membrane*	Depleted membranes**	Depletion supernatant***	Native membrane*	Depleted membranes**	Depletion supernatant***	Particles*	Depleted particles**	Depletion supernatant***
mg protein	22	18.7	3.3	34	24	10	11.5	7.54	4.2
%	100	85	15	100	70.5	29.5	100	65	35
ATPase activity,	1320	168.3	980	1700	168	1340	1276	80	1067
%	100	12.7	74.2	100	9.8	78.8	100	5.2	83.6

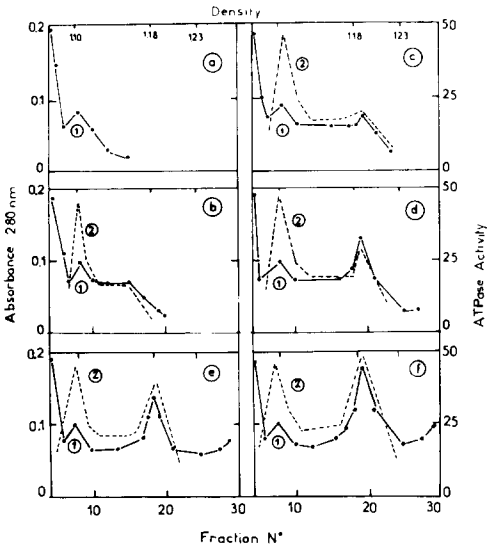

Fig. 7. Incorporation kinetics of the soluble ATPase of supernatant extracts into particles formed by complementation. Sucrose density sedimentation of reconstituted particles removed at various times. The complementation mixture (24 ml) contains 270 mg protein, 18 000 ATPase units and 0.05 M Tris · HCl buffer (pH 7.6). Samples of 4 ml were taken at 10 min (a), 20 min (b), 30 min (c), 40 min (d), 60 min (e) and 120 min (f), layered onto a sucrose isopycnic gradient 20–60 % (w/v) then centrifuged at 85 000 × *g* for 14 h. Curves 1, absorbances (280 nm); curves 2, incorporated ATPase activities (units per fraction).

logy [2]. Thus, using ATPase as a membrane label, we have followed its incorporation into reconstituted particles as a function of incubation time during complementation in a standard reaction mixture [12] containing 10 mg protein/ml of each soluble fraction of *chl A* and *chl B* mutants and 18 000 ATPase units. Six samples (4.5 ml) taken at various times were subjected to fractionation on sucrose density gradient (Fig. 7) and the particulate protein and ATPase activity in the resulting fractions assayed. In Fig. 7, it is seen that the amount of reconstituted particles increases in proportion to the incubation time. The 10-min particles have a buoyant density equal to 1.10 (Fig. 7a): the densities of the 40-min, 60-min and 120-min particles range from 1.18 to 1.23 (Figs 7d–7f). There is no further increase in the light particles ($d = 1.10$) after 10 min, but the amount of 1.18 particles increases with time up to a maximum of about 2 h. No ATPase activity is found in the 10-min sample. ATPase activity begins to be incorporated from the 20-min sample in the lightest particles only (Figs 7a and 7b). At this stage the rate of bound activity is a maximum: after 30 min of incubation, the amount of ATPase incorporated into reconstituted particles increases in proportion to the peak of heavy particles of density 1.18 (Figs 7c–7f). It should be pointed out that the amount of light particles ($d = 1.10$) is a maximum at 10 min and also that these particles bind a given amount of ATPase which does not increase beyond 20 min.

Incorporation of ^{14}*C-labeled ATPase*

These last results allow us to suppose that the structure of reconstituted parti-

cles progresses through successive stages. An attempt to verify this assumption was made using a much more sensitive method. The *chl A*$^-$ mutant was grown in [^{14}C]-leucine minimal medium, the soluble ATPase from the cell-free extract was purified according to the procedure described above (Table I). The [^{14}C]ATPase ($1.5 \cdot 10^5$ cpm/mg protein) thus obtained was introduced into a standard complementation mixture. Six samples were removed at various times and centrifuged at 220 000 $\times g$ for 90 min to sediment the reconstituted particles. The former were then washed and resuspended in 1 ml 0.05 M Tris · HCl buffer (pH 7.6) and subjected to fractionation on a sucrose density gradient. Fig. 8 shows the kinetics of evolution of the reconstituted particles. It is seen, as in the preceding experiment, that the amount of light particles is a maximum at 10 min. The radioactivity begins to be incorporated into the light particles after 10 min up to a maximum incorporation at 20 min (Figs 8a and 8b). From 30 min, the radioactivity is incorporated only into heavy particles of density 1.18 (Figs 8c–8f). In Fig. 9, it is seen that the kinetics of incorporation of ^{14}C-labeled ATPase (Fig. 9, curve 1) and unlabeled ATPase (Fig. 9, curve 2) are almost the same. The only difference, due to a higher sensitivity of the method using radioactive label, is the presence of a small incorporation into the 10-min particles. Moreover, the kinetics of incorporation of both labeled and unlabeled ATPases always begins with a lag, the length of which cannot be determined precisely by our techniques. The incorporation kinetics then increases in a hyperbolic manner up to a maximum achieved

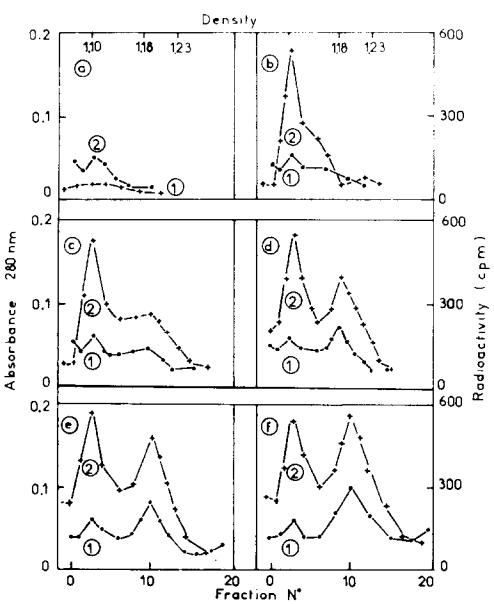

Fig. 8. Incorporation kinetics of ^{14}C-labeled purified soluble ATPase into particles formed by complementation. Sucrose density sedimentation of reconstituted particles removed at various times. The complementation mixture (27 ml) contains 240 mg protein, 12 000 unlabeled ATPase units, 2940 [^{14}C]ATPase units ($3.48 \cdot 10^5$ cpm) and 0.05 M Tris · HCl buffer (pH 7.6). Samples of 4 ml were taken at 10 min (a), 20 min (b), 30 min (c), 40 min (d), 60 min (e) and 120 min (f), layered onto a sucrose isopycnic gradient 20–60 % (w/v) then centrifuged at 85 000 $\times g$ for 14 h. Curves 1, absorbances (280 nm); curves 2, incorporated [^{14}C]ATPase activities (cpm/fraction).

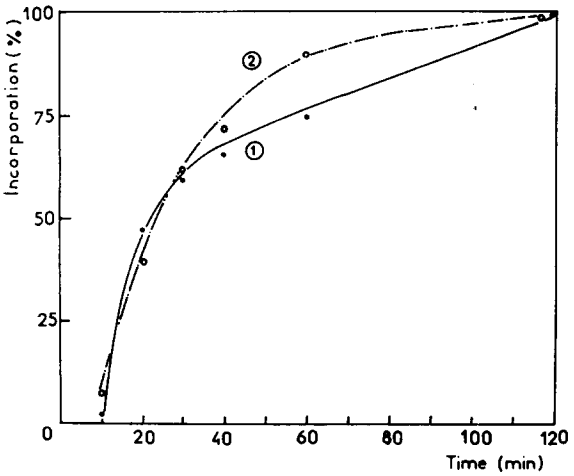

Fig. 9. Comparison of incorporation kinetics of labeled and unlabeled ATPases into particles formed by complementation. The labeled and unlabeled complementation mixtures had the composition described in Figs 8 and 7, respectively. Samples of 4 ml taken at various times and named in the two preceding figures were centrifuged at 220 000 × *g* for 90 min in order to sediment the newly formed particles. Curve 1, incorporated [^{14}C]ATPase (percent of maximal activity); curve 2, incorporated unlabeled ATPase (percent of maximal activity).

at 120 min. In all these incorporation experiments, it should be emphasized that the ATPase bound to light and heavy particles is sensitive to 1 mM DCCD, a property typical of membrane ATPases. Such a property appears at the same time as the first particles ($d = 1.10$) having an ATPase activity.

DISCUSSION

The results described in this report confirm our previous observations on the presence of ATPase in the soluble fractions of mutants *chl A* and *chl B* and on the binding of this enzyme to particles formed by complementation between these fractions. The purification of the ATPase contained in a soluble state by these extracts has allowed us to precisely determine the properties of this enzyme, especially molecular weight (roughly estimated to $3.5 \cdot 10^5$), Mg^{2+} requirement, cold-lability and resistance to DCCD, the last two of which allow one to distinguish the soluble from the particulate form [22–24].

We have devised a procedure using the removing of Mg^{2+} by dilution in a buffer of lower molarity without Mg^{2+} to deplete membranes of any ATPase activity. The binding of soluble purified ATPase to these depleted membranes needs only 2–6 μmol Mg^{2+} per mg protein excluding "nectin". Such a situation differs from that described in *S. faecalis* by Abrams and Baron [3, 25]. This contradiction could be explained assuming that in the case of *E. coli* K 12 and/or of our procedure for obtaining depleted membranes, the protein factor(s) needed for the incorporation of soluble ATPase remains strongly bound to membranes. Our results agree with the situation described in *Micrococcus lysodeikticus* [26, 27] and in *Bacillus megatherium* [28, 29] with respect to the need for Mg^{2+}. Moreover, ATPase bound to depleted

membranes recovers the properties of the native membrane enzymes. The specific activity of such membranes is near that of native membranes (about 100 units). This result implies that there is a very close stoichiometry in the binding of soluble ATPase to depleted membranes. Nevertheless, on account of the heterogeneity of the membrane particles [2], it was not possible for us to calculate the number of binding sites.

In the same way, the soluble ATPase incorporated into particles formed during complementation recovers the properties of the native membrane enzyme and, as above, the specific activity of the reconstituted particles is almost that of native membranes. This result allows us to suppose that membrane structures in the process of formation make new binding sites for ATPase. We know that the incorporation of ATPase into depleted membranes needs Mg^{2+}; therefore, we can assume that the complementation mixture contains sufficient Mg^{2+} to allow the binding of ATPase to reconstituted particles under optimal conditions.

Azoulay et al. [12] and MacGregor and Schnaitman [6] had shown, that only a part of reconstituted nitrate reductase is incorporated into reconstituted particles. In the same way, only a part of soluble ATPase is incorporated into reconstituted particles (the lightest of which have buoyant densities of 1.10 and 1.18). The part of ATPase which fails to be incorporated can be explained, assuming that a part of reconstituted particles aggregates to give abnormal structures not having the binding sites for ATPase (heavy particles, density over 1.23). This assumption can be better appreciated when it is recalled that the part of ATPase which is not incorporated when complementation is complete can still bind to depleted membranes.

If we compare the totality of our results of a model of the in vitro reconstitution of enzymatically active membranes, to the model of reconstitution of mitochondrial membranes, we are able to envisage, as Yamashita and Racker [30] and Tzagoloff et al. [31] for a succinoxidase complex reconstitution and Kopaczyk et al. [32] and Bulos and Racker [33] for an oxidative phosphorylation reconstitution, that the associations needed for the reconstitution of active membranes go through a sequence of increasingly complex organization stages. That is the reason why we have selected ATPase as a label using its allotopic properties [1, 18, 23] to try to understand the mechanism of the formation of membrane structures during complementation. The principal interest of our study lies in the fact that we have precisely defined the membranous origin of some protein factors playing a part in this phenomenon, such as ATPase and cytochrome *b* [2]. In the second place, we have shown that ATPase is incorporated following hyperbolic kinetics, which begin after 10 min of incubation when the first particles become visible. ATPase binds first to light particles ($d = 1.10$) then to heavier particles ($d = 1.18$). The analysis of this binding leads us to propose two hypotheses on the mechanism of incorporation of ATPase into reconstituted particles. According to the first, soluble ATPase could initially be incorporated into light particles which afterwards aggregate to give heavier particles; this assumption implies that the rate of formation of light particles is equal to the rate of their aggregation into heavier particles, when accounting for the constant amount of light particles and the amount of ATPase so bound. According to the second hypothesis, soluble ATPase could be incorporated independently into several kinds of particles; this assumes the existence of two different mechanisms for the aggregation of particles. Such mechanisms should be independent from one another and have rates dependent on the concentrations in the reagents needed by the aggregation of each type of parti-

cles. The first of them should give rise to the lightest particles up to a quickly achieved maximum. The second should slowly give rise to the heavier particles. In both assumptions, the main difficulty is due to the fact that light particles are formed in an early stage at a maximal rate and bind simultaneously a maximal amount of ATPase. In the second hypothesis, it is difficult to understand which reagents could make each kind of particles and why the two stages would be fully independent from one another, but in favor of the last hypothesis, it is to be noticed that the reconstituted particles separate into three bands of different buoyant densities. It seems that this result implies that each type of particle can be organized from different reagents.

The differences between the two classes of particles could be due to the fact that their organization is also monitored by different specific factors. The stoichiometric aspects of complementation are not to be neglected, for this phenomenon needs a complex mixture for which few parameters are known at present. Our first hypothesis implying a sequence of increasingly complex stages in the organization of particles cannot be fully discarded, for heavy particles ($d = 1.18$) begin to appear and increase when a given amount of light particles is achieved. This last result is to be compared with the observations of Yamashita and Racker [30] on the reconstitution of the succinoxidase complex from its individual components: these workers have shown that reconstitution is a relatively slow process and that the adding order and concentration of the different factors in the reaction mixture are of great importance in the evolution of reconstitution.

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