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MEMBRANE RECONSTITUTION IN *CHL-R* MUTANTS OF *ESCHERICHIA COLI* K 12

V. ATPase INCORPORATION INTO PARTICLES FORMED BY COMPLEMENTATION

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SUMMARY

Mechanical treatments of cell suspensions of *Escherichia coli* K 12 strain PA 601, and its two mutants *chl A*$^-$ and *chl B*$^-$, in a buffer without Mg^{2+} lead to partial solubilization of membrane-bound ATPase. After ultracentrifugation of cell-free extracts, ATPase can be recovered in the soluble fraction. Contrary to membrane ATPase, the soluble enzyme has the following properties: (1) it is insensitive to *N,N'*-dicyclohexylcarbodiimide; (2) heat-inactivation kinetics show a reactivation in the first 3 min and the half-time is 15 min; (3) ADP is a substrate. In the course of complementation between soluble fractions of mutants *chl A*$^-$ and *chl B*$^-$, a part of soluble ATPase is incorporated into the newly formed particles. The specific activity of these particles is nearly the same as that of native particles; the ATPase bound to native membrane and the ATPase bound to the newly-formed particles both have the same biochemical properties.

INTRODUCTION

Cell-free extracts of the *chl A*$^-$ and *chl B*$^-$ pleotropic mutants of *Escherichia coli* K 12, disrupted in a French press, freed from sedimentable material by ultracentrifugation, complement under well-defined conditions[1]. The complementation leads, on the one hand, to a restoration of nitrate reductase activity and, on the other hand, to particle formation. According to Azoulay *et al.*[1], the cytoplasmic membrane of these two mutants could be affected, and some elements normally lying at the membrane level can be recovered in soluble cell-free extracts. Schnaitman[2] and Onodera *et al.*[3] have shown that chlorate-resistant mutations have membrane structures.

Moreover, Mutaftschiev and Azoulay[4] have shown that particles formed during complementation show, under the electron microscope, distinctive membrane patterns with a trilaminar structure and vesiculation.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; KDO, 2-keto-3-deoxyoctulosonate.

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However, it remains to be determined whether these newly-formed particles have ATPase activity (EC 3.6.1.3) or if the biochemical properties of this enzyme are the same as those of *E. coli* native-membrane ATPase as described by Gunther and Dorn[5], Hafkenscheid and Bonting[6] and Evans[7], and generally the same as those of bacterial ATPases[8–16]. Besides, we have attempted to determine whether a reconstitution of ATPase activity takes place as in the case of nitrate reductase, or if this ATPase does exist in a soluble form in the supernatant extracts of the wild-type strain and of mutants *chl* A^- and *chl* B^- prior to complementation.

It should be emphasized that the methods used to prepare our extracts have a drastic effect on cytoplasmic membranes, more especially as the treated cells have a genetic defect at the membrane level. Under such conditions, the presence of enzyme activities and membrane components in the supernatants can be explained quite well. According to Evans[7], membrane ATPase is *N,N'*-dicyclohexylcarbodiimide (DCCD)-sensitive and Mg^{2+} and Ca^{2+}-dependent. In contrast, Roisin and Kepes[17] have shown that: (a) the membrane ATPase of *E. coli* exhibits an absolute requirement for Mg^{2+} in the neutral pH range, while Ca^{2+} is found able to activate ATPase at a more alkaline pH; (b) ADP inhibits ATP hydrolysis and transforms the Michaelian ATP concentration dependence into a sigmoid curve with increasing K_m and decreasing V. Solubilization of particulate ATPase by different means gives rise to an enzyme which is no longer sensitive to DCCD and may show the phenomenon of allotopy, as described by Racker[18] and Evans[7].

MATERIALS AND METHODS

Organisms and growth conditions

Escherichia 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[19,20] have the *nit*$^-$ *gas*$^-$ phenotype and are chlorate-resistant.

Cells were grown and harvested as previously described[21]. Washed cells were suspended in 0.05 M Tris buffer (pH 7.6), disrupted in a French press under a pressure of 15000 lb/inch^2 or by sonication in a Branson sonifier by 4 pulses of 15 s at 9 kcycles, then centrifuged at 10000 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 at 82500 × *g* for 30 min (Ti 50 rotor, Spinco Model L4); the pellets were discarded and the supernatants were centrifuged twice again at 220000 × *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 per ml).

Moreover, membranes have been prepared as described by Kaback[22]. The treatment of cells by lysozyme and EDTA gives spheroplasts which are then lysed and centrifuged several times.

Complementation

The supernatant extracts were mixed under conditions described by Azoulay *et al.*[1]. The reaction system, containing equivalent amounts (10–15 mg protein per 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 220000 $\times g$ 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.

Assay procedures

ATPase activity was determined as the amount of inorganic phosphate realeased by ATP hydrolysis in a reaction system containing 4.4 mM sodium ATP, 2.2 mM $MgCl_2$, 40 mM Tris buffer (pH 7.6) and enzyme extract (0.2–0.8 mg protein). Inorganic phosphate was assayed after 5–20 min of incubation in 2-ml samples according to Martin and Doty[23]. 1 unit of enzyme activity is defined as 1 nmole of phosphate released per min.

Nitrate reductase activity was measured by the rate of H_2 uptake[24] in manometric systems containing H_2, 9 mM benzyl viologen, hydrogenase, 0.33 M phosphate buffer (pH 7), 0.25 M KNO_3 and enzyme extract. The specific activity is expressed as μmoles NO^-_3 reduced/mg protein per h.

Protein concentration was assayed by the method of Lowry *et al.*[25] with crystalline bovine serum albumin as a standard.

2-Keto-3-deoxyoctulosonate (KDO) was assayed according to the method of Weissbach and Hurwitz[26] modified by Cynkin and Ashwell[27] as reported by Colowick and Kaplan[28]. RNA was determined as described by Moule[29].

RESULTS

ATPase distribution among particulate and soluble extracts of wild-type and mutant strains

The crude extracts of wild-type and pleotropic mutants contain about 50–100 ATPase units per mg protein depending on conditions of growth and extraction. Table I gives the activities of the soluble and particulate fractions. It can be seen that ATPase is distributed equally between the soluble and particulate fractions in both wild-type and mutant strains.

TABLE I

ATPase DISTRIBUTION AMONG PARTICULATE AND SOLUBLE EXTRACTS OF WILD-TYPE AND MUTANT STRAINS

ATPase activities are expressed as nmoles of inorganic phosphate released by hydrolysis of ATP per min (units), as described in Materials and Methods. Specific activities are expressed as units per mg protein.

Fraction	*Specific activity*			*Total activity*			*% of total activity in crude extract*		
	356	*chl A⁻*	*chl B⁻*	*356*	*chl A⁻*	*chl B⁻*	*356*	*chl A⁻*	*chl B⁻*
Crude extracts	54	59	85	1700	3000	1900	100	100	100
Soluble fraction	40	50	70	800	1300	1000	47	43	55
Particulate fraction	73	60	75	800	1200	800	47	40	43

Phosphohydrolase activities of soluble and particulate fractions of wild-type and mutant strains

The pellets resulting from ultracentrifugation are resuspended in 0.05 M Tris buffer (pH 7.6) in order to obtain a protein concentration of about 10 mg/ml. These extracts, containing the major part of membrane material, are contaminated by fragments of lipopolysaccharide, revealed by the presence of KDO ($4 \cdot 10^{-9}$ moles per mg protein) and by ribosomal RNA (25%). Table II gives their phosphohydrolase activities. It can be seen that particulate fractions are specific for ATP and GTP and, at a lower level, of UTP and CTP; this pattern is quite similar to that of ATPases. On the other hand, the soluble fractions are able to hydrolyze ATP, AMP, ADP, phosphoenolpyruvate and glucose 6-phosphate, and have therefore several phosphohydrolase activities carried by one or several proteins.

TABLE II

PHOSPHOHYDROLASE ACTIVITIES OF PARTICULATE AND SOLUBLE FRACTIONS OF WILD-TYPE AND MUTANT *chl A*$^-$ AND *chl B*$^-$ STRAINS

Enzymatic activities are expressed as nmoles of P_i released per min (units) per mg protein.

Substrates	*Soluble fractions*		*Particulate fractions*	
	Specific activity	%*	*Specific activity*	%*
ATP	60	100	80	100
ADP	30	50	0	0
AMP	20	30	0	0
GTP	–	–	80	100
UTP	–	–	24	30
CTP	–	–	24	30
Phosphoenolpyruvate	15	25	0	0
Glucose 6-phosphate	20	30	0	0

* Percentage activity determined by ATP hydrolysis.

It should be emphasized that all these activities have been assayed at the same pH (7.6) and with the same substrate concentration as for ATPase determinations.

Moreover, the results for the wild-type and the mutants *chl A*$^-$ and *chl B*$^-$ are exactly the same.

Biochemical study of wild-type strain particulate ATPase

The particulate fraction of wild-type strain, having ATPase activity as mentioned above, has been subjected to fractionation by the following procedure: 4 ml of particulate fraction (10 mg protein/ml) are layered onto a sucrose isopycnic gradient 20–60% (w/v) in 0.05 M Tris buffer (pH 7.6) then centrifuged at $85000 \times g$ for 14 h in an SW 25 rotor. The whole ATPase activity is concentrated in the lightest fractions whose density ranges from 1.16 to 1.18 (Fig. 1). This location is similar to that previously described in the case of nitrate reductase[1]. These partially purified fractions have been analysed. They are devoid of any cell wall contamination with respect to

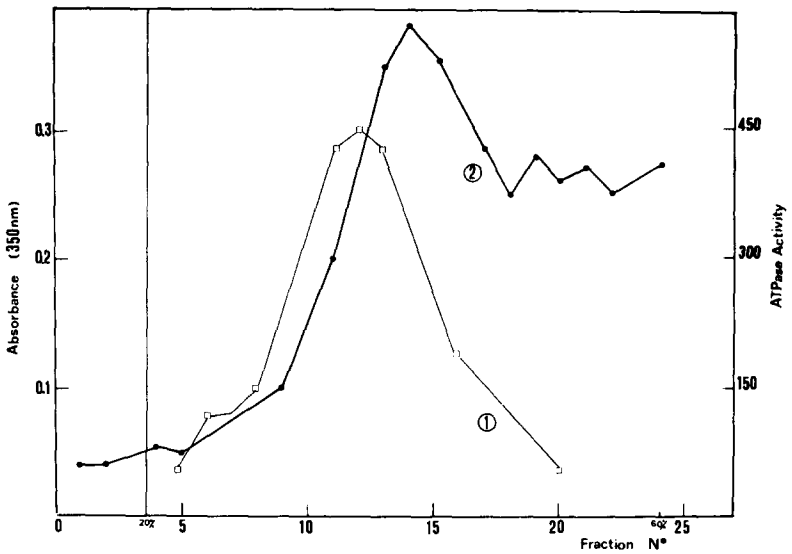

Fig. 1. Sucrose density sedimentation of particulate fraction of *E. coli* K 12 strain 356. 4 ml (40 mg protein) of particulate fraction was layered onto a sucrose isopycnic gradient of 20–60% (w/v). Centrifugation was performed at 85000 × *g* for 14 h. Curve 1, ATPase activity expressed as nmoles P_i released per fraction per min. Curve 2, absorbance at 350 nm.

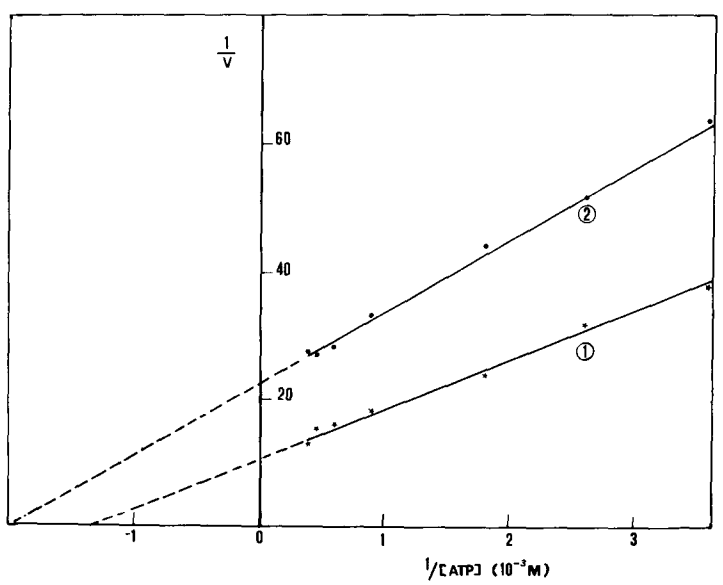

Fig. 2. Influence of ADP concentration on velocity of ATP hydrolysis catalysed by particulate fraction of strain 356. Lineweaver–Burk plot in the presence (Curve 2) and absence (Curve 1) of 1 mM ADP.

lipopolysaccharide (*i.e.* absence of KDO) but have an ATPase activity of about 500 units, 10 times more that of particulate fraction before fractionation (50 units).

The biochemical properties of membrane ATPase have been studied with these partially purified fractions. Under our experimental conditions, ATP hydrolysis

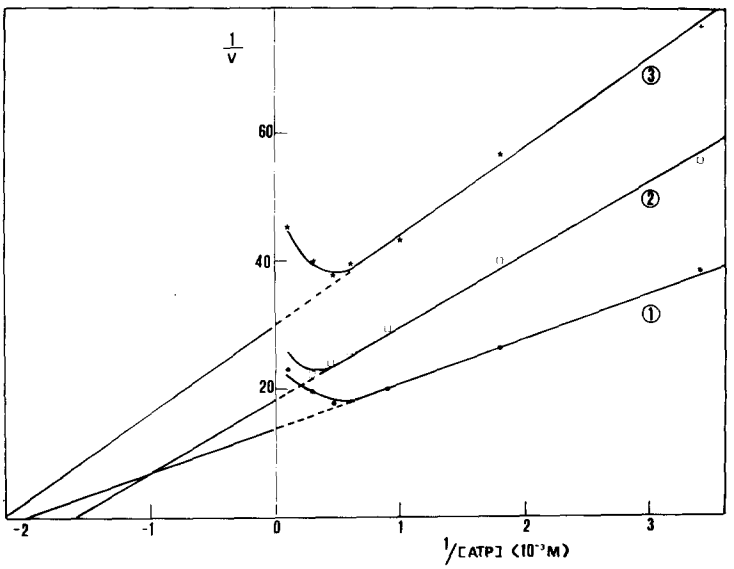

Fig. 3. Dependence of initial velocity of ATP hydrolysis on ATP concentration. Lineweaver–Burk plot with (1) complementation particles, (2) particulate fraction of mutant *chl B*— and (3) soluble fraction of mutant *chl A*—.

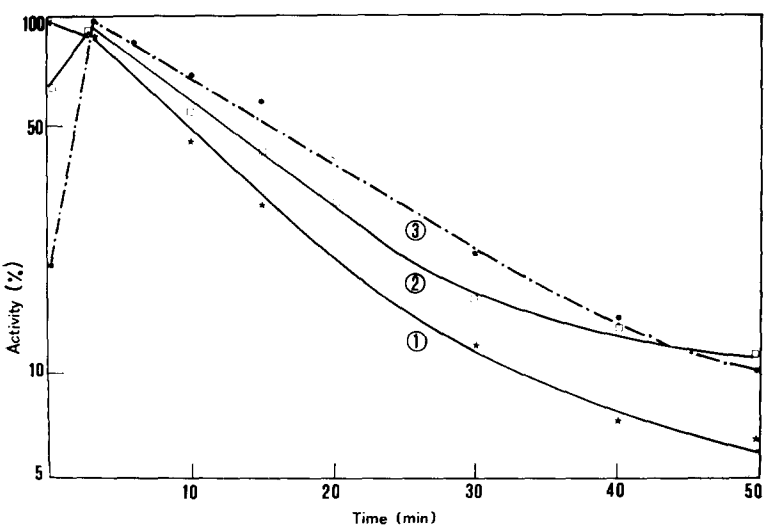

Fig. 4. Heat-inactivation kinetics of particulate (1) and soluble fraction of mutant *chl A*— (3) and of complementation particles (2). Incubation of 8–10 mg protein is performed at 64 °C. Aliquots are pipetted at different times, cooled to 0 °C, diluted and transferred into a reaction system. The 3 min needed for enzyme reactivation are not included in the values given for half-times of the 3 activities. ATPase activity is expressed as% of maximal activity. Semi-logarithmic plotting.

liberates ADP which accumulates; this fact explains the break in the slope observed after 25 min of incubation when 25% of the ATP added is hydrolysed. It might be an inhibition by the reaction product which is not hydrolysed by the particulate fraction, as shown in Table II. This inhibition is quite significant: 24% for 0.5 mM ADP and 43% for 1 mM ADP; it has a mixed pattern: V and K_m of the enzyme are both modified and its K_i equals 0.5 mM (Fig. 2).

The study of variation of ATPase activity depending on substrate concentration is shown Fig. 3 (Curve 2). The K_m of the particulate enzyme for its substrate is 0.6 mM. We have shown an inhibition effect by excess of substrate.

Heat inactivation takes place at temperatures higher than 55 °C and the inactivation kinetics pattern differs from that of first-order reactions. Besides, after 30 min at 64 °C the remaining activity is about 10% that of the initial level. Half-time is 7 min at 64 °C (Fig. 4, Curve 1).

Oligomycin has no effect upon bacterial ATPases. 10^{-4} M DCCD, with inhibitory effects as described by Evans[7], lowers the ATPase activity of membrane particles by 80% (Table III). It can be seen that sodium (either acetate or Cl^-) has no effect upon membrane ATPase of *E. coli* K 12 strain 356, contrary to the observations of Evans[7].

TABLE III

INHIBITION OF PARTICULATE (NATIVE AND NEWLY FORMED) AND SOLUBLE ATPases

Inhibitors	*Inhibition (%)**		
	Particulate native enzyme	*Particulate newly-formed*	*Soluble enzyme*
ADP 0.3 mM	—	14	0
0.5 mM	29	42	0
1.0 mM	43	—	0
DCCD 0.1 mM	82	70	8
Na^+ 125 mM	0	0	0

* % inhibition refers to activity relative to the case of ATP hydrolysis.

Membrane ATPase is Mg^{2+} dependent. The crude extracts have an endogenous activity (10%) which no longer persists after dialysis against 0.05 M Tris buffer at pH 7.6. After dialysis the addition of Ca^{2+} results in a restoration of ATPase activity at pH 7.6, the level of which ranges from 20–25% of that measured with Mg^{2+}, depending on protein content and experiments.

Binding of ATPase to particulate fractions formed by complementation

The supernatant extracts of wild-type strain 356 have phosphohydrolase activities as previously stated (Tables I and II). The complementation, carried out by mixing soluble fractions of mutant strains *chl* A^- and *chl* B^-, leads to the reconstitution of particles. After 2 h, when the complementation is over, the mixtures are centrifuged to collect the particle-containing pellets. These pellets contain, in a particulate state,

TABLE IV

ATPase ACTIVITY BEFORE AND AFTER COMPLEMENTATION OF CELL-FREE EXTRACTS FROM MUTANTS *chl A*⁻ AND *chl B*⁻ OF *E. coli* K 12

	Protein (mg/ml)	*Total protein content (mg)*	*Specific activity**	*Total activity*	%**	
					Protein	*Activity*
chl A⁻ supernatant	10.7	48.15	64	3081	57	56
chl B⁻ supernatant	7.8	35.10	68	2386	43	44
Complementation supernatant	9.6	77.8	50	4050	93	74
De novo particles	1.35	5.7	130	741	7	13.6

* Specific activities are expressed as ATPase units/mg protein.

** % protein and % activity are expressed as fractions of total protein and total activity in the reaction mixture before starting complementation.

7–8% of initial soluble protein contained in the reaction mixture (Table IV). It should be emphazised that: (1) the production of particles does need the mixing of the soluble fractions of the two mutants; (2) the incorporation of soluble ATPase into these particles does need such a mixing. Soluble fractions of either wild-type or mutant strains are not able to lead to such a result when isolated.

The results of several experiments identical to that described in Table IV allow us to establish the two following facts: (1) 11 –14%of the total ATPase binds to particles during complementation whereas the complementation supernatant retains only 65–75% of the initial activity of the reaction mixture; (2) the specific activity of the newly-formed particles is 80–130 units, just a little above the level in native particles.

The *de novo* formed particles have been layered onto an isopycnic 20–60% sucrose gradient and centrifuged under the conditions previously described for native particles (Fig. 5). They separate on the sucrose gradient giving three protein peaks as assayed by the method of Lowry *et al.*[25] but one single turbidity peak (absorbance at 350 nm). ATPase activity is in the two light bands with a specific activity of 350 units (*i.e.* 70% of the activity of native particles).

Comparative study of soluble and membrane-bound ATPases

The ATPase bound to newly formed particles catalyses ATP and GTP hydrolysis at the maximal rate, but UTP and CTP are hydrolysed at only 30% of the maximal rate. These particles have no phosphohydrolase activity, contrary to supernatant extracts of the mutants *chl* A^- and *chl* B^-. Inhibitory effects of ADP and DCCD (Table II) agree with these results.

Before complementation, the soluble ATPase is not inhibited by 10^{-3} and 10^{-4} M DCCD; its kinetics of heat inactivation at 64 °C begins by a reactivation in the first 3 min and its half-time is 13 min (Fig. 4, Curve 3). The incorporation of ATPase into newly-formed particles leads to DCCD sensitivity and a new heat inactivation at 64 °C with a weaker reactivation and a half-time of 9 min (Fig. 4, Curve 2).

ATPases of newly-formed particles and native particles both have the same following properties: K_m for substrate (ATP), need for Mg^{2+} with a maximal activity

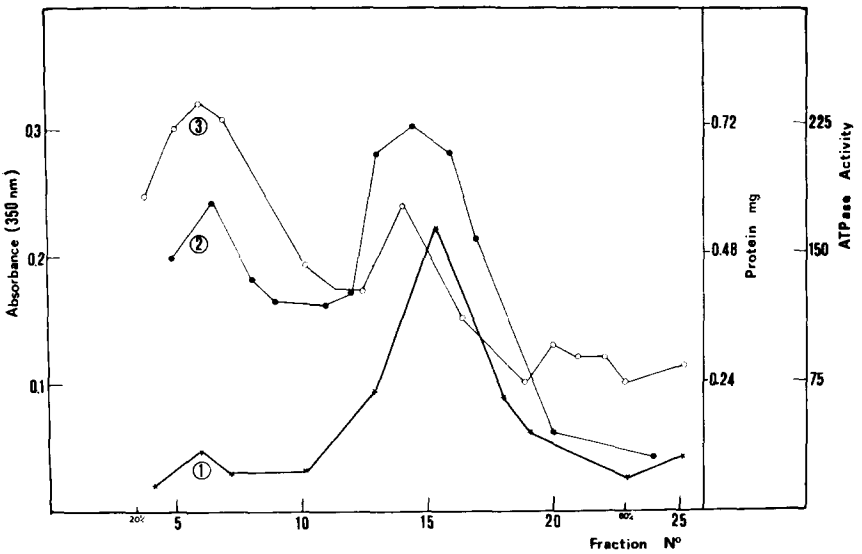

Fig. 5. Sucrose density sedimentation of newly-formed particles obtained by complementation between cell-free extracts of mutants *chl A*— and *chl B*—. Sucrose isopycnic gradient 20–60% (w/v); centrifugation performed at 85000×*g* for 14 h. Curve 1, absorbance 350 nm. Curve 2, ATPase activity expressed as nmoles P_i released per fraction per min. Curve 3, protein concentration, mg per fraction.

observed for a rate $[Mg^{2+}]/[ATP]$ equal to 0.5 (Fig. 6), inhibition by excess of substrate. It should be emphasized that these properties are the same for soluble and particulate (native or newly-formed) ATPases (Figs 3 and 6).

The most important differences between soluble and particulate ATPases lie in sensitivity to DCCD, in the influence of ADP, which inhibits the particulate form but is a substrate for the soluble one, and in the kinetics of heat-inactivation.

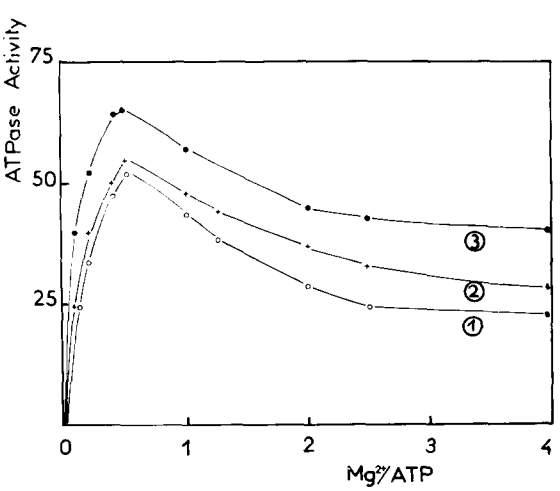

Fig. 6. Influence of Mg^{2+} concentration on velocity of ATP hydrolysis in the presence of (1) soluble fraction of mutant *chl A*—, (2) particulate fraction of mutant *chl B*— and (3) complementation particles. ATPase activity is expressed as nmoles P_i released/mg protein per min.

In order to know more acurately the nature of the phosphohydrolase reactions given by the soluble fractions, we have undertaken to purify these enzymatic proteins. Table V shows the purification procedure comprising the following steps. The supernatant fraction is layered onto a DEAE-cellulose column (3 cm × 30 cm) equilibrated with 0.02 M Tris buffer (pH 7.7). Elution is carried out with a linear NaCl gradient (0 to 0.8 M) in the same buffer. Active fractions eluted between 0.20 and 0.22 M NaCl are combined, dialysed and layered once again onto a DEAE-cellulose column equilibrated with the same buffer. Elution is carried out with an NaCl gradient (0 to 0.4 M). ATPase activity, eluted by the same NaCl concentration, separates as one single peak. Table V shows that the ATPase has been purified about ten times with a yield of 50%.

This partially purified enzyme protein retains the ability to perform ADP hydrolysis.

TABLE V

PARTIAL PURIFICATION OF SOLUBLE ATPase

Fraction	*Total protein (mg)*	*Specific activity (units/mg protein)*	*Total activity (units)*
Soluble fraction	160	42	6800
1st chromatography on DEAE-cellulose	36	140	5100
2nd chromatography on DEAE-cellulose	7.7	425	3100

Solubilization of membrane ATPase

On the basis of the preceeding observation, we postulate that the treatments applied to whole cells (wild-type or mutant strains) lead to partial solubilization of membrane-bound ATPase. In order to verify this assumption, we have prepared membranes under different conditions. Thus, suspensions of membranes (10–20 mg protein per ml) prepared from *chl* A^- and *chl* B^- mutants according to the method of Kaback[22] are submitted to sonic waves (7 pulses of 15 s). After this treatment, we observe that 20–30% of membrane proteins and 25–40% of membrane-bound ATPase are solubilized.

The study of this solubilized enzyme shows that it has the same properties as the ATPase isolated from supernatant extracts obtained from whole cells by drastic methods and an especially wide activity range towards different phosphate esters, particularly ADP, contrary to membrane ATPase of kabackosomes, the specificity of which is very restricted.

Moreover, we have carried out two cross-complementations. The first of them by mixing the supernatant of *chl* A^- mutant prepared by extrusion under high pressure (French press) and the supernatant of *chl* B^- mutant obtained by sonication of membrane vesicles; the second one by mixing the supernatant of sonicated *chl* A^- membranes and the extrusion supernatant of *chl* B^- mutant. After 2 h of incubation we observe a formation of particles in both cases and a reconstitution of nitrate reductase

activity (1.2 units in the first system and 2 units in the second). Simultaneously, ATPase activity is incorporated in newly-formed particles in a proportion ranging between 8 and 10%.

DISCUSSION

We had previously shown that complementation between soluble fractions of cell-free extracts of mutant strains *chl* A^- and *chl* B^- results in the restoration of membranous nitrate reductase and in the reconstitution of particles having most of the characteristics of particles of membranous origin. We thought that chlorate-resistant mutants were affected at the level of anaerobic electron transport systems. The results presented for ATPase in this report suggest that this system of membrane reconstitution is of more general extent. Moreover, they imply that the relationship between the different products of the various genetic loci of chlorate-resistant mutations could have an effect on structure and function of membrane, as suggested by Rolfe and Onodera[30].

Mechanical treatments of cell suspensions, carried out under well-defined conditions of pH and ionic strength, lead to a partial solubilization of membrane-bound ATPase. The same treatments, applied to membrane vesicles obtained from spheroplastes by mild procedure, result also in a solubilization of ATPase. It should be emphasized that soluble fractions have phosphohydrolase activities; at the present time, the purifications were not carried out to the point of stating the nature of these activities precisely. Nevertheless, the first results allow us to think that solubilized membrane ATPase is, in all likelyhood, able to perform ADP hydrolysis. Moreover, we have stated the differencies between the two forms of ATPase; our results agree with those of Evans[7] and Abrams and Baron[11] and on the effect of DCCD and those of Davies and Bragg[31] on the part played by ADP. We have shown that the membrane-bound enzyme is Mg^{2+}-dependent, which agrees with the results of Roisin and Kepes[17]; but contrary to these workers, we have not observed that the inhibition of ATP hydrolysis by ADP follows a sigmoid curve.

The main interest of our study lies in the fact that, in the course of complementation, the previously released ATPase incorporates itself into newly-formed particles; when the phenomenon is over, the soluble fraction has lost 30% of its total activity while the particulate fraction has gained 15% of the total activity. This pattern looks like the allotopy of Racker[18] observed in the case of the reattachement of previously released ATPase to mitochondrial membrane. In addition, ATPase of newly-formed particles has the same properties as those of membrane-bound ATPase (need for Mg^{2+}, sensitivity to DCCD and ADP, specificity for ATP) and they can be considered as the same enzyme. Therefore, complementation can be regarded as a true reconstitution according to the criteria of Razin[32].

At the present time it remains to be determined whether the ATPase bound to newly-formed particles is to be considered on the whole as solubilized membrane ATPase and to explain why it integrates into two types of vesicles of different buoyant densities. It seems that the stoichiometry of the complementation reaction is not very precise, which leads to an accumulation of some enzyme proteins, as we had previously seen in the case of nitrate reductase.

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