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

## VIII. PURIFICATION AND PROPERTIES OF THE $F_A$ FACTOR, THE PRODUCT OF THE *chl B* GENE

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SUMMARY

The isolation and purification of the product of the *chl B* gene of *Escherichia coli* K 12 from the *chl A* mutant have been attempted. The purified protein gives a single band in 10 % sodium dodecylsulfate/polyacrylamide gel electrophoresis. The molecular weight is estimated to be 35000. This protein, that we have named "$F_A$ factor", does not contain any lipid, has a strong tendency to lose its activity by polymerizing but can be kept in an active state when stored in buffer containing NaCl. The addition of purified $F_A$ protein to a soluble extract from the *chl B* mutant strain grown under anaerobiosis in the presence of nitrate initiates the "complementation reaction", i.e. the reconstitution of the nitrate reductase activity and the formation of particulate material similar to the native membrane with respect to the structure and enzymatic function. $F_A$ protein acts both on the rate of reconstitution and on the total amount of reconstituted enzyme. The complementation leads to the reconstitution of non-sedimentable nitrate reductase and to the formation of three types of particles of different buoyant densities (1.10, 1.18 and 1.23) the two lightest of which contain nitrate reductase. It is shown that $F_A$ factor is incorporated only into the particles of intermediate density. In vivo, this factor is located in the native membranes of *chl A*, *chl C*, *chl D* and wild-type strains, whatever the growth conditions, aerobiosis or anaerobiosis, and in the presence or absence of nitrate. Protein $F_A$ can be released from either of these membranes (native or reconstituted) by removing $Mg^{2+}$ or by subjecting Kaback's vesicles to mechanical treatments; in the case of 1.18-reconstituted particles and wild-type membranes, the release of $F_A$ protein does not exert any effect on the level of the nitrate reductase activity.

## INTRODUCTION

The observations previously reported by Azoulay et al. [1], dealing with the biochemical [1, 2] and morphological studies [3] of the *chl-r* mutants of *Escherichia*

*coli* K 12 had led these workers to assume the existence of an alteration of the cytoplasmic membrane in order to explain the pleotropic effect of this type of mutation.

More recently, a fine morphological analysis has allowed us to state a number of obvious significant differences between the membranes of wild-type and of mutant strains (Mutaftschiev and Azoulay, to be published). Moreover, the results that we obtained for the complementation between soluble fractions of the cell-free extracts of mutant strains *chl A* and *chl B* [4], and rediscovered by MacGregor and Schnaitman [5], allow us to confirm our assumption.

At the present time, the complementation is to be considered as an in vitro reconstitution of the nitrate-bound membranous respiratory system. From this point of view, our system is related to that described by Yamashita and Racker [6], who have succeeded in reconstituting the succinoxidase mitochondrial complex from its individual components, and to a certain extent to the reconstitution of the oxidative phosphorylation system [7, 8].

The incorporation of ATPase into particulate structures formed by complementation shows that this process leads to the reconstitution of membranous sites specific for the binding of this enzyme [9].

In one of the first papers dealing with complementation [4], we proposed that the product of the *chl B* gene could play a major part in the reconstitution of the nitrate-bound membranous respiratory system. Later on, using cell-free extracts from double mutants to perform complementation, we showed that the presence of all three products in an active state of the *chl A*, *chl B* and *chl C* genes were required in the reaction mixture for complementation to be successful [10]. We had also observed that the yields in reconstituted nitrate reductase and newly formed particulate material were always higher when using the soluble fractions of the *chl A* and *chl B* mutants, cleared of all sedimentable material instead of the crude extracts of the same mutants. This last result was confirmed recently by MacGregor and Schnaitman [5]. These workers had previously shown [11], as Onodera et al. [12] by polyacrylamide gel electrophoresis that the membranes of these mutants were modified with respect to the protein level.

Therefore, it can be assumed that complementation is accomplished from previously non-sedimentable fragments of cytoplasmic membranes, the solubilization of which results from breakage procedures.

Taking the importance of the product of the *chl B* gene into account, the aims of the present study are to define its role in the complementation process, to determine precisely its subcellular location in the wild-type strain and *chl-r* mutants and to verify to what extent the product of the *chl B* gene could be incorporated into reconstituted particles.

## MATERIALS AND METHODS

### *Organisms and growth conditions*

*E. coli* K 12, strain PA 601 (356 of our collection) sensitive to chlorate and its two mutants *chl A* (356.15) and *chl B* (356.24). These mutants which were described previously [13, 14], have the *nit*$^-$ *gas* $^-$ phenotype and are chlorate resistant.

Cells were grown and harvested as previously described [15].

*Preparation of cell-free extracts*

Washed cells were suspended in 0.05 M Tris · HCl buffer (pH 7.6), disrupted in a French pressure cell then centrifuged at 10 000 rev./min for 15 min in a Sorvall RC 2 B to remove cell debris. The crude extracts were then centrifuged twice at 220 000 $\times g$ (at the bottom of the tube) for 90 min. The pellets were discarded and the supernatants, cleared of all sedimentable material, constituted the soluble fractions from which the complementation studies were performed.

*Complementation*

Two different procedures were used, depending on the study to be done. (a) To perform standard complementation, as defined by Azoulay et al. [4], the supernatant extracts of the two mutants *chl A* and *chl B* were mixed under conditions described by these workers. The reaction mixture, containing equivalent amounts (15–30 mg protein/ml) of each extract was incubated in a vacuum at 32 °C. The reaction, which is completed after 120 min, can be stopped by sudden cooling. In order to separate soluble and particulate fractions, the complementation mixtures were centrifuged twice at 220 000 $\times g$ for 90 min when the reaction was completed. (b) We have devised an alternative procedure using "$F_A$ protein" (purified as described in Results) instead of the *chl* $A^-$ soluble fraction in reaction mixtures containing for instance 200 mg *chl* $B^-$ soluble fraction and 0.5–1.0 mg $F_A$ protein. The incubation time was the same as described above and reconstituted particles were sedimented in the same way as for standard complementation.

*Radioactive labeling of the cells*

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 $B_1$ [16] and a carbon source (glucose 2 g/l). Labeling was achieved by growing the cells for one generation in this medium containing either [$^{14}$C]leucine (0.01 mCi/l) in order to label the proteins, or [2-$^3$H]glycerol (1 mCi/l) and 0.5 mM glycerol in order to label the phospholipids. The labeled cells were harvested and treated as described above. Radioactivities were determined by liquid scintillation spectrophotometry.

*Nitrate reductase assay*

Nitrate reductase activity was measured by the rate of $H_2$ uptake, as described by Pichinoty and Piéchaud [17]. 1 unit of enzyme activity is defined as 1 $\mu$mole of $NO_3^-$ reduced per h.

*$F_A$ activity assay*

This was determined as the rate of nitrate reductase formed in a complementation mixture containing 0.15 ml of a crude extract of the *chl B* mutant and 5–20 $\mu$l of the sample depending on the concentration of this in $F_A$ protein. This mixture was introduced in the sidearm of a Warburg flask. Other reagents were the same as described previously. The system was incubated at 32 °C for 45 min before tipping. $F_A$ activity was expressed as reconstituted nitrate reductase units per ml or per mg protein of the sample.

*Assay of total lipids*

Total lipids were assayed according to the procedure of Folch et al. [18]. Proteins were precipitated by 15 % trichloroacetic acid then resuspended in a chloroform/methanol mixture (2 : 1, v/v). This was shaken overnight under a $N_2$ atmosphere then centrifuged at 12 000 $\times g$ for 10 min in order to sediment the proteins. The aqueous phase was removed from the supernatant with 0.05 M NaCl. The chloroform phase containing lipids was dried off, giving a lipid residue which was weighed.

*Polyacrylamide gel electrophoresis*

This was carried out according to the method of Davis [19] using 7.5 % (w/v) polyacrylamide gels (0.35 × 6 cm) with protein samples of 0.2–0.4 mg protein.

Sodium dodecylsulfate/acrylamide gel electrophoresis was performed according to Shapiro et al. [20]. Gels of 6 cm, prepared in tubes of 0.35 cm diameter, contained 10 % (w/v) acrylamide, 0.27 % (w/v) bis acrylamide, 50 % (v/v) 0.2 M phosphate buffer, 0.1 % (w/v) sodium dodecylsulfate. 0.17 % (v/v) *N,N,N',N'*-tetramethylenediamine and 0.075 % (w/v) ammonium persulfate were added for initiating and catalyzing the polymerization reaction. The gels and tank buffers were the same (0.1 M phosphate buffer containing 0.1 % sodium dodecylsulfate). Bromophenol blue was used to mark the solvent front. The amount of protein applied to the top of each gel was 150–200 $\mu$g protein. The gels were run at a current of 2 mA/tube until the complete migration of the indicator dye. Gels were stained for protein using Coomassie brilliant blue then destained by repeated washings of 7 % (v/v) acetic acid.

Both types of gels (with or without sodium dodecylsulfate) were scanned at 600 nm with a densitometer (modified Beckman DU spectrophotometer).

*Protein determination*

Protein concentration was assayed by the method of Lowry et al. [21] using bovine serum albumin as a standard.

## RESULTS

*Reconstitution of nitrate reductase activity by complementation*

For the sake of clarity, the designations Extracts A and B will be used throughout this report for the soluble extracts from *chl A* and *chl B* mutants, respectively. Neither of these extracts contains any nitrate reductase activity. When reaction mixtures containing equivalent amounts of Extracts A and B are incubated under the conditions described in Materials and Methods, the following facts are observed at the end of the reaction: (a) a reconstitution of nitrate reductase activity, the specific activity of which is 15–20 units/mg protein and from which 25–35 % is particulate, (b) from 7 to 10 % of the protein of the combined Extracts A and B becomes particulate, i.e. sedimentable after a double ultracentrifugation at 220 000 $\times g$ for 90 min of the incubated mixture. These results agree with our previous observations [4, 22] and those of MacGregor and Schnaitman [5].

When the sedimentable fraction is subjected to fractionation on sucrose density gradient (Fig. 1), it separates into three peaks of different buoyant densities: $d = 1.10$–$1.12$ (Peak I particles), $d = 1.18$ (Peak II particles) and $d \geqslant 1.22$ (Peak III particles). It is to be noticed that reconstituted nitrate reductase activity can be found only

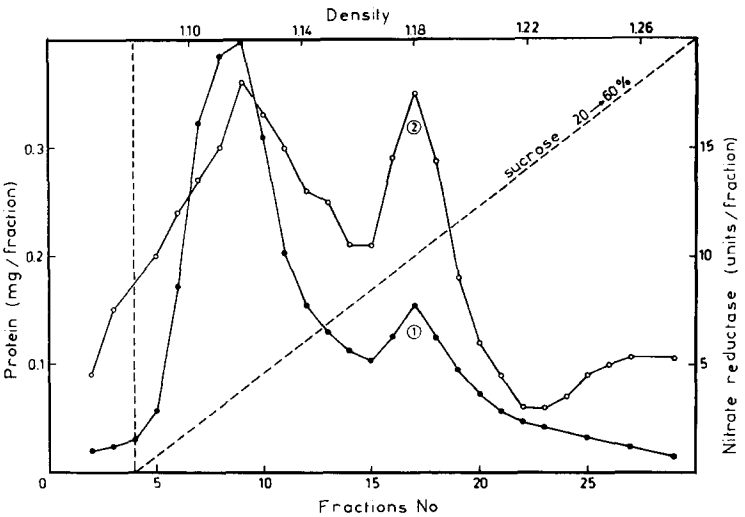


Fig. 1. Sucrose density sedimentation of reconstituted particles obtained by complementation between cell-free extracts of *chl A* and *chl B* mutants. The complementation mixture (9.5 ml) was incubated at 32 °C for 2 h then centrifuged at 220 000 × *g* for 90 min. The pellet (7.5 mg protein) was resuspended in 4 ml of 0.04 M Tris · HCl buffer (pH 7.6), layered onto a sucrose isopycnic gradient 20–60 % (w/v) then centrifuged at 85 000 × *g* for 14 h. Fractions of 1 ml were collected from top to bottom. Curve 1, reconstituted nitrate reductase activity (units per fraction); curve 2, protein (mg/fraction).

in the lightest particles (Peaks I and II) with specific activities of about 55 units per mg protein (Peak I) and 22 units per mg protein (Peak II). In other reports that are to be published in the same issue, we show that: (a) the lipid/protein ratios are different for the particles of Peaks I and II; the greater part of non-sedimentable nitrate reductase is constituted of a protein complex (estimated $M_r = 3\text{–}4 \cdot 10^6$) containing 3–5 % of lipids, among which are phospholipids, essentially phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol [23]; (b) ATPase present in a soluble state in Extracts A and B binds to particles of Peaks I and II [9].

The complementation phenomenon thus defined needs the presence of both Extracts B and A in the reaction mixture (Table I). The efficiency of the reaction (measured by the specific activity of the reconstituted nitrate reductase, the velocity of the reconstitution reaction and the percent of soluble protein which became sedimentable) depends on the protein concentration of Extract B that is to give 15–30 mg protein/ml. It can be seen in Table I that the incubation of Extract B in the absence of Extract A does not give any reconstitution of nitrate reductase activity but leads to the aggregation in a particulate state of 3–5 % of protein.

A series of experiments was performed in order to know the effect of adding increasing amounts of Extract A to complementation mixtures containing a given amount of Extract B (14 mg protein) on the reconstitution of nitrate reductase activity. We have observed that the amount of reconstituted enzyme increases in a direct ratio up to a maximum of 23 units recorded for 4 mg Extract B protein. This result suggests that a protein factor contributed by Extract A plays an important part

TABLE I

PARTICULATE PROTEIN FORMATION AND REPARTITION OF THE RECONSTITUTED NITRATE REDUCTASE ACTIVITY AFTER INCUBATION OF *chl A* AND *chl B* EXTRACTS, SINGLY OR MIXED

| Reaction mixtures | % particulate protein* | Nitrate reductase activity** | | |
|---|---|---|---|---|
| | | % soluble | % particulate | |
| | | | $d = 1.10$ | $d = 1.18$ |
| Extract B | 3–5 | 0 | 0 | 0 |
| Extract A | 3–5 | 0 | 0 | 0 |
| Extracts B+A | 7–10 | 66 | 23 | 11 |
| Extracts B+$F_A$*** | 7–10 | 56 | 28 | 16 |

* Particulate protein is expressed as percent of total protein.

** Soluble and particulate nitrate reductase activities are expressed as percent of total reconstituted activity. The particulate form was fractionated by sucrose density sedimentation as described in Fig. 1.

*** In this mixture, purified protein $F_A$ was used instead of Extract A.

in this reaction. In order to verify such an assumption, we have attempted to separate this factor, named "$F_A$ factor" or "$F_A$ protein" (see refs 2 and 4).

*Purification of $F_A$ protein*

As we have shown above, the complementation needs the addition of a protein factor contributed by the soluble fraction of the *chl A* mutant to the reaction mixture. The purification of such a factor was attempted using the reconstitution of nitrate reductase activity as an assay method. 1 unit of $F_A$ protein was defined as 1 $\mu$mol of $NO_3^-$ reduced per h and the specific activity expressed as units per ml or per mg protein of Extract A or $F_A$ solution added in the complementation mixture under optimal conditions which we shall describe hereafter. The purification scheme is given in Table II. The *chl A* mutant was grown overnight in a 10-l complex medium. Cells were harvested, washed and disrupted as described in Materials and Methods

TABLE II

PURIFICATION OF $F_A$ PROTEIN FROM SOLUBLE FRACTION OF *chl A* MUTANT

The steps of the purification are described in detail in the Results section.

| | Total protein (mg) | Specific activity (units/mg protein) | Total activity (units) | Purification (-fold) | Yield (%) |
|---|---|---|---|---|---|
| Extract A | 468 | 271 | 126 828 | 1 | 100 |
| Chromatography on DEAE | 95 | 1148 | 109 072 | 4.2 | 86 |
| First filtration Sephadex G-75 | 19 | 1528 | 29 032 | 5.6 | 22.9 |
| Second filtration Sephadex G-75 | 5.6 | 3904 | 21 860 | 14.4 | 17.2 |

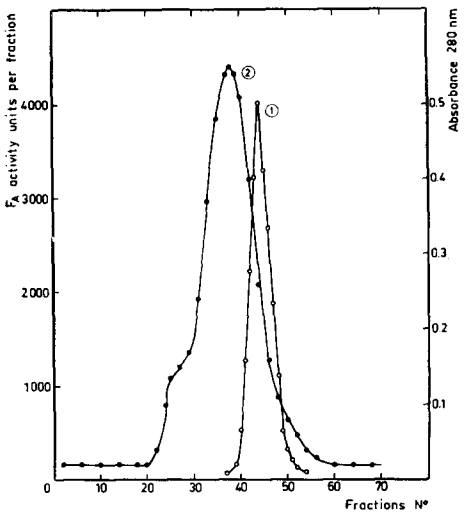


Fig. 2. Sephadex G-75 filtration of $F_A$ protein. A pool of the most active fractions from the first gel filtration, which was performed as indicated in Results, was concentrated by ultrafiltration through a PM 30 Diaflo membrane filter. The 5-ml concentrated fraction (3.8 mg protein) was layered onto a Sephadex G-75 column (114 × 1.64 cm) equilibrated with 0.02 M Tris · HCl buffer (pH 7.7) containing 0.25 M NaCl. Elution was with the same buffer, The flow rate was 20 ml/h and fractions of 3 ml were collected. Curve 1, $F_A$ activity (units per fraction); curve 2, absorbance (280 nm).

giving a crude extract which was then centrifuged twice at 220 000 × *g* for 90 min. The last supernatant, cleared of all sedimentable material, was the soluble fraction from which $F_A$ protein was isolated and purified. This soluble fraction was loaded on a DEAE-cellulose column (40 × 2.5 cm) equilibrated with 0.02 M Tris · HCl buffer (pH 7.7). Elution was carried out with a linear NaCl gradient (0–0.3 M) of 1 l in the same buffer. Active fractions were eluted between 0.14 and 0.18 M NaCl. A pool of the most active fractions was concentrated to 11 ml (8.6 mg protein/ml) by ultrafiltration with a PM-30 Diaflo membrane filter, using the Amicon apparatus under $N_2$ at 2.5–3 kg/cm$^2$. The fraction traversing the membrane filter did not contain any $F_A$ activity. The next step was gel filtration. The concentrated fraction was applied to a Sephadex G-75 column (114 × 1.64 cm) equilibrated with 0.02 M Tris · HCl buffer (pH 7.7) containing 0.25 M NaCl and eluted with the same buffer. The most active fractions were pooled again and concentrated to 19 mg protein/5 ml through a PM-30 Diaflo membrane filter. The concentrated fraction was then subjected to a new gel filtration under the same conditions as above. The elution profile is in Fig. 2. From Table II and Fig. 2, the following observations are to be made. (a) $F_A$ activity is eluted in a fraction a little lighter that the protein peak. In order to verify this point a series of successive gel filtrations was performed which gave the same result in all our purification experiments. We have observed that if gel filtrations are carried out in the absence of NaCl, the purified fractions precipitate and do not retain their activity. (b) The overall purification is about 14-fold when the purified fraction is compared with the *chl* $A^-$ soluble fraction; the yield is 17 %.

The purified $F_A$ protein is very stable and can be kept in an active state a very long time when stored at −30 °C in 0.02 M Tris · HCl buffer containing 0.25 M NaCl.

The molecular weight of $F_A$ protein was estimated approximately by gel filtration on Bio-Gel A-0.5 M according to the procedure of Whitaker [24]. The internal standards used were cytochrome $c_3$ of bovine heart ($M_r$ 12 270), horse-radish peroxidase ($M_r$ 43 000) and yeast ethanol dehydrogenase ($M_r$ 150 000). The first two markers were determined by their specific absorption at 547 and 403 nm, respectively. The ethanol dehydrogenase was assayed as described by Racker [25]. From the elution volumes of the standards and the $F_A$ protein, the molecular weight was tentatively calculated to be 35 000 (Fig. 3).

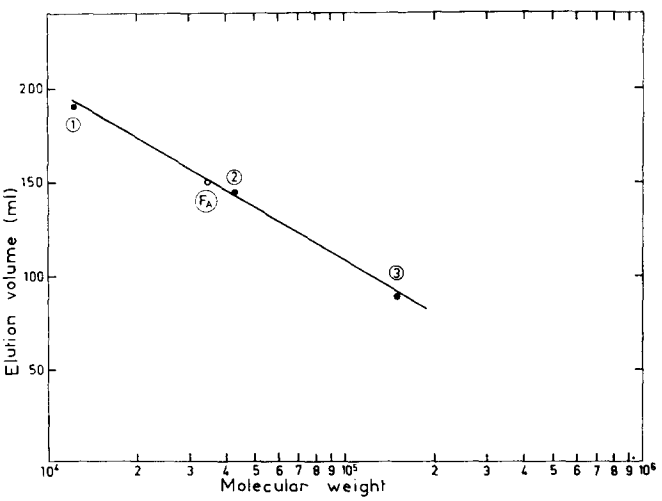


Fig. 3. Calibration curve for approximate estimation of the molecular weight of $F_A$ protein by chromatography on Bio-Gel A-0.5 M. 4 ml of a mixture containing 2.24 mg purified protein $F_A$, 5 mg horse-radish peroxidase, 5 mg yeast ethanol dehydrogenase, 5 mg bovine heart cytochrome $c_3$ was applied onto a Bio-Gel A-0.5 M column (114 × 1.64 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. The flow rate was 20 ml/h and fractions of 3 ml were collected. Elution volumes of cytochrome $c_3$ (1), peroxidase (2), ethanol dehydrogenase (3) and $F_A$ protein versus molecular weights. Semi-logarithmic plotting.

Polyacrylamide gel electrophoresis of the most active fractions shows the presence of seven protein bands of various intensities (Fig. 4A). This result was always obtained in several experiments. It could imply that the $F_A$ protein exists under several degrees of polymerization, the lightest of which being the active form ($M_r$ 35 000). The active fractions were subjected to sodium dodecylsulfate gel electrophoresis in order to depolymerize the associated units and, with this procedure, a single protein band was obtained (Fig. 4B).

We looked for the presence of lipids in the purified $F_A$ protein using the method of Folch et al. [18] and for the presence of phospholipids by purifying $F_A$ protein from *chl A*$^-$ cells grown in [2-$^3$H]glycerol minimal medium according to the procedure described above (Table II). The Folch analysis did not detect any lipid and the $F_A$ protein obtained from the cells labeled on phospholipids was not radioactive. Therefore, we assume that $F_A$ protein does not contain lipids.

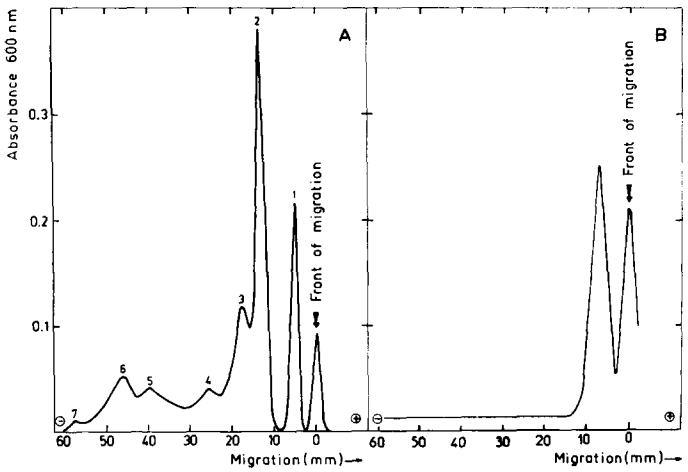


Fig. 4. Densitometry of gel electrophoretic patterns of purified $F_A$ protein in the absence (A) and in the presence (B) of sodium dodecylsulfate. The fractions No. 44 and 45 from the gel filtration on Sephadex G-75 were analysed on 7.5 % polyacrylamide gels (A) or on 10 % polyacrylamide gels containing 0.1 % sodium dodecylsulfate. (B) The gels were scanned at 600 nm with a densitometer (modified Beckman DU spectrophotometer) and the absorbance was recorded continuously.

*Contribution of $F_A$ protein to the reconstitution of nitrate reductase*

As shown above, complementation needs the addition of Extract A to Extract B. Table I shows that the complementation can also be performed when purified $F_A$ protein is used instead of Extract A. When reaction mixtures containing about 150 mg Extract B protein and 0.5 mg purified $F_A$ protein are incubated at 32 °C for 2 h, a reconstitution of nitrate reductase is obtained, the specific activity of which is 15–20 units/mg protein, and the aggregation in a particulate state of about 10 % of the soluble protein of the reaction mixture before incubation. This result shows that the complementation reaction is initiated by the addition of $F_A$ protein, which is not present in an active form in Extract B prepared from a strain, the *chl B* gene of which is altered by mutation.

The experimental results obtained when using $F_A$ protein are absolutely similar to those obtained with Extract A. In particular, this new complementation system also leads to three forms of nitrate reductase (non-sedimentable, particulate I and particulate II as described above) as it is seen in Table I. Moreover, the complementations initiated by the addition of the $F_A$ factor follow the same kinetics as those we described in a previous paper [26] for standard complementation (Extract A+ Extract B). It is seen in Fig. 5 that the kinetics of reconstitution of nitrate reductase activity depends on the concentration of the $F_A$ protein added to the mixture. The rate of reconstitution increases in a direct ratio to the amount of $F_A$ protein up to a maximum obtained over 60 $\mu$g $F_A$ protein for 2.55 mg protein of Extract B. It should be pointed out that for expressing the amount of reconstituted nitrate reductase it is necessary to use the concentration of Extract B protein which will give the maximal reconstitution for a given amount of $F_A$. These experimental criteria were defined precisely in a previous paper [4]. Therefore, the $F_A$ factor does act on the rate of reconstitution and on the total amount of enzyme reconstituted when the reaction is completed,

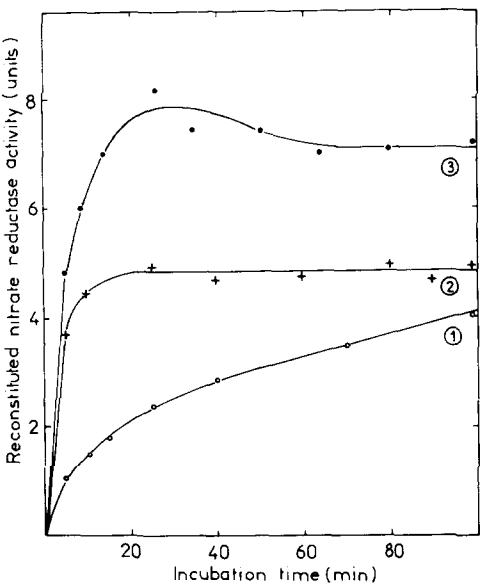


Fig. 5. Influence of $F_A$ protein concentration on the reconstitution of nitrate reductase activity. The reaction mixtures containing 2.55 mg protein of Extract B/0.2 ml and increasing amounts of $F_A$ protein were incubated in Warburg flasks under an atmosphere of $H_2$ at 32 °C for 100 min. Curve 1, 16 $\mu$g $F_A$ protein; curve 2, 33 $\mu$g $F_A$ protein; curve 3, 66 $\mu$g $F_A$ protein. Reconstituted nitrate reductase is expressed as units/mg protein.

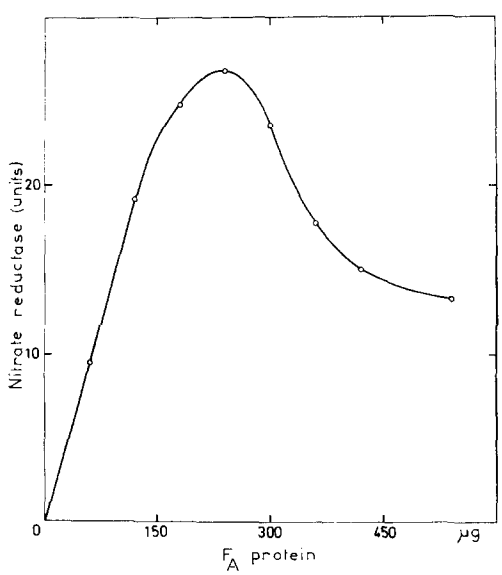


Fig. 6. Influence of $F_A$ protein concentration on the amount of reconstituted nitrate reductase activity. The reaction mixtures containing 2.65 mg protein of Extract B/0.1 ml and increasing amounts of $F_A$ protein were incubated in Warburg flasks under an atmosphere of $H_2$ for 45 min. The reconstituted activity is expressed as units per reaction mixture.

after 2 h of incubation. As it is seen in Fig. 6, the amount of reconstituted nitrate reductase increases in a direct ratio with the amount of $F_A$ protein. In addition, Fig. 6 shows that, over this $F_A$ amount, a lowering is observed; this result can be explained quite well, when it is recalled that the efficiency of the complementation reaction depends on the total protein content of the reaction mixture and that any dilution by addition of buffer leads to an inhibition [26].

The complementation carried out with purified $F_A$ protein has the same biochemical properties as standard complementation described in 1969 [4]: sensitivity to temperature (reversible inhibition at 0 °C, optimum at 32 °C, irreversible inhibition at 50 °C). Our previous observations [4] on the sensitivity of Extract A to temperature (half-time was 17 min at 50 °C) have been corroborated for purified $F_A$ protein, which, when incubated at 50 °C for 15 min, polymerizes as a whole and becomes inactive. Optimal pH is between 7.4 and 7.7. The complementation is inhibited at 50 % by $O_2$ bubbling for 15 min in Extract B, but can be carried out in tubes open to the air; these observations confirm, on the one hand, our previous results on the effect of $O_2$ [4] and, on the other, those of MacGregor and Schnaitman [5] on the possibility of performing complementation in the presence of air.

*Part played by $F_A$ protein in the reconstitution of membranous particles*

In a first series of experiments, the proteins of the *chl A* mutant were labeled by growing this strain in minimal medium with [$^{14}$C]leucine. The labeled Extract A was used to perform successive complementations in the following way: to a low amount of $^{14}$C-labeled Extract A is added a given amount of Extract B, incubation and ultracentrifugation are performed as described in Materials and Methods and reconstituted particles are assayed for protein and radioactivity; to the supernatant is added the same amount of Extract B as in the first step and this mixture is once again incubated then centrifuged, and so on. This procedure is repeated until the time when a further addition of Extract B does not cause a further formation of particulate material. Fig. 7 shows that the saturation is not reached after four successive additions; moreover, the amount of particulate protein increases with the amount of Extract B protein introduced in the mixture, while the incorporated radioactivity decreases after the first complementation down to 0.4 % of the total radioactivity at the last complementation. Moreover, we have observed that the amount of the reconstituted nitrate reductase incorporated into the newly formed particles increases with the amount of Extract B added to the reaction mixture. The results of this series of experiments shows that Extract A, which contains the $F_A$ protein, as we have shown above, contributes proteins other than $F_A$ factor in the first complementation, which agrees with the results of MacGregor and Schnaitman [5]. But, in the following complementations, the incorporated radioactivity is very low and this is in complete discordance with the conclusions drawn from results similar to those of the first complementation by these workers. We can assume that the *chl A* mutant is contributing an "assembly factor" only, at least when $F_A$+Extract B mixtures are used, while the *chl B* mutant is contributing the bulk of the protein present in the particulate fraction.

In the preceding experiments, the radioactivity incorporated into the reconstituted particles was too low for stating whether $F_A$ protein is or is not incorporated into these particles. In order to clarify this question, we have purified the $F_A$ protein according to the procedure described above from *chl A*$^-$ cells grown in $^{14}$C-labeled

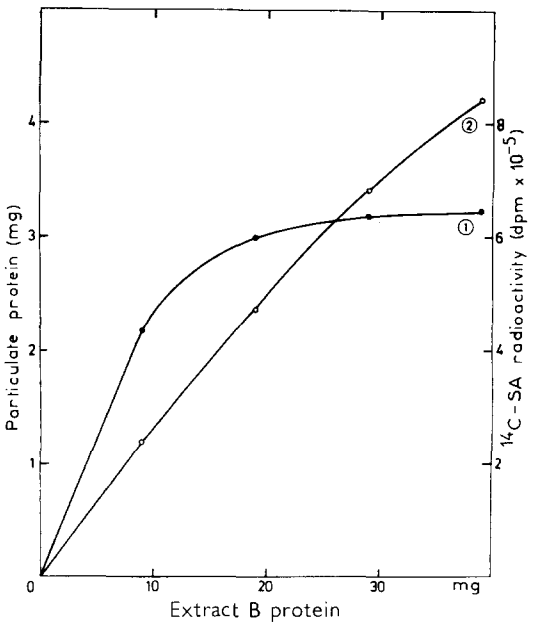


Fig. 7. Contribution of *chl A* and *chl B* mutants to the particulate fraction formed by complementation, *chl A*$^-$ was grown in minimal medium containing [$^{14}$C]leucine. *chl B*$^-$ was grown under normal conditions. To a given amount of $^{14}$C-labeled Extract A (12.5 mg protein) was added 9.75 mg protein of Extract B; incubation and ultracentrifugation were performed as described in Materials and Methods and newly formed particles were assayed for protein and radioactivity. The same amount of Extract B as in the first step was added to the supernatant and this second mixture was once again incubated and centrifuged and the particulate pellet assayed for protein and radioactivity. This procedure was repeated four times. Curve 1, particulate protein formed at each step (mg per system); curve 2, incorporated $^{14}$C radioactivity (dpm per mg protein).

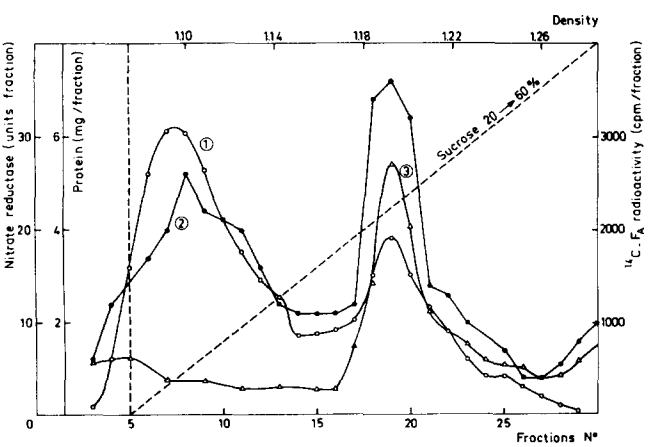


Fig. 8. Sucrose density sedimentation of the particulate fraction formed by complementation between purified $F_A$ protein and the soluble fraction of the *chl B* mutant. The complementation mixture containing 190 mg protein of Extract B and 1.2 mg $^{14}$C-labeled $F_A$ protein ($7 \cdot 10^4$ cpm) was incubated at 32 °C for 2 h then centrifuged twice at 220 000 × *g* for 90 min. The pellet (95 mg protein) was resuspended in 5 ml of 0.04 M Tris · HCl buffer (pH 7.6), layered onto a sucrose isopycnic gradient 20–60 % (w/v) then centrifuged at 85 000 × *g* for 17 h. Fractions of 1 ml were collected from top to bottom. Curve 1, nitrate reductase activity (units per fraction); curve 2, protein (mg per fraction); curve 3, incorporated $^{14}$C-labeled $F_A$ protein (cpm/fraction).

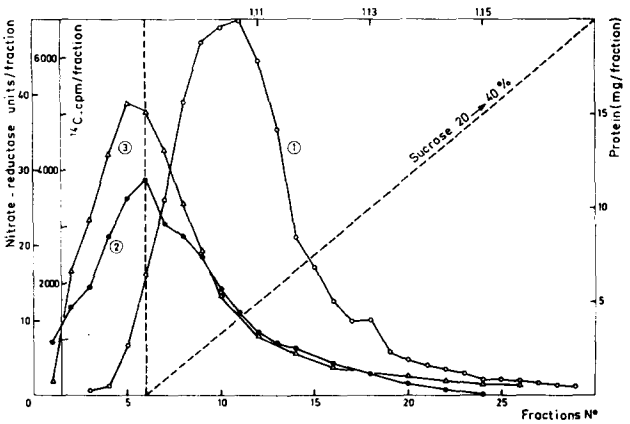


Fig. 9. Sucrose density sedimentation of the non-sedimentable fraction formed by complementation between purified $F_A$ protein and the soluble fraction of *chl B* mutant. The complementation mixture described under Fig. 8 was centrifuged twice at 220 000 $\times g$ for 90 min. The supernatant (6 ml) was layered onto a sucrose isopycnic gradient 20–40 % (w/v) then centrifuged at 85 000 $\times g$ for 17 h. Fractions of 1 ml were collected from top to bottom. Curve 1, nitrate reductase activity (units per fraction); curve 2, protein (mg per fraction); curve 3, $^{14}$C-labeled $F_A$ protein (cpm/fraction).

medium. The $^{14}$C-labeled $F_A$ protein thus obtained ($5.8 \cdot 10^4$ cpm/mg protein) was added to the unlabeled Extract B. When incubated at 32 °C for 2 h, the mixture was centrifuged twice at 220 000 $\times g$ for 90 min. 34 % of the radioactivity of the mixture was in the particulate pellet and 66 % in the supernatant. The particulate fraction, when subjected to fractionation on sucrose isopycnic gradient 20–60 % (w/v), separates into three peaks of different buoyant densities (Fig. 8). The only peak to be strongly radioactive is that of the Peak II particles, the density of which is 1.18–1.20. The supernatant fraction was also subjected to fractionation on sucrose isopycnic gradient 20–40 % in order to separate the non-sedimentable reconstituted nitrate reductase. It is seen in Fig. 9 that the enzyme is completely separated from the peak of radioactivity. This last result which shows that $F_A$ protein does not incorporate into reconstituted nitrate reductase was corroborated by the following experiment. This non-sedimentable nitrate reductase was partially purified by a double gel filtration on Bio-gel A-5 M and we have observed that it was not radioactive (unpublished result).

*Location of the $F_A$ protein*

In order to determine the location of $F_A$ factor, we have performed the following experiments. We have prepared membranes using two gentle procedures, less drastic for membranous material than cell breakage in a French pressure cell. The first method described by Kaback [27] gives membranous vesicles (10–20 mg protein/ml) which were sonicated (7 pulses of 15 s under 9 kcycles). The second one, which we described in detail in another paper [9], consists of depletion of the membranes of their ATPase activity using a dilution in a buffer of lower ionic strength and containing no $Mg^{2+}$. These depleted membranes are then sedimented by ultracentrifugation at 220 000 $\times g$ and the resulting supernatants (which we designate by "depletion supernatants") are concentrated by ultrafiltration with a PM-10 Diaflo membrane filter under $N_2$ at 3 kg/cm$^2$. The effects of sonication of Kaback's vesicles or removing

$Mg^{2+}$ on membranes, which have been studied for the wild-type, *chl A*$^-$ and *chl B*$^-$ strains and the reconstituted particles, are the following: (a) 10–15 % of membranous protein are solubilized for the wild-type strain, 20–30 % for the *chl-r* strains and the reconstituted particles. (b) The nitrate reductase bound to the membranes of the wild-type strain or the reconstituted particles is never solubilized. (c) In another paper [9], we show that ATPase is released from the three kinds of membranes.

We have attempted to see whether proteins playing a part in complementation were released from the membranes by these treatments. To this end, we have used the supernatants obtained by ultracentrifugation of the sonicated Kaback's vesicles and the depletion supernatants to perform cross complementations with Extracts A and B obtained from the standard procedure. For the sake of clarity, we will use the following designations. Extracts WT, A and B are soluble extracts of cells processed with the French pressure cell in buffer without $Mg^{2+}$ of wild-type, *chl A*$^-$ and *chl B*$^-$ strains, respectively. Vesicle Extracts A and B are centrifugation supernatants of Kaback's vesicles sonicated without $Mg^{2+}$ of *chl A*$^-$ and *chl B*$^-$ strains, respectively. Depletion extracts WT, A, B and Rec are supernatants obtained by centrifugation of previously depleted particles [9] for particles of wild-type, *chl A*$^-$ and *chl B*$^-$ strains and reconstituted particles, respectively. Table III illustrates the observations that we have made. (a) Depletion extracts A, WT and Rec are able to complement with Extract B, which implies that $F_A$ factor is present in these depletion supernatants. (b) The mixture Depletion Extract B+Extract A does not give any reconstitution. (c) The level of reconstituted nitrate reductase depends on the mixtures used, but for the Depletion Extract A+Extract B mixture, it is as much as the control Extract A+Extract B mixture. These results show, on the one hand, that $F_A$ protein is located

TABLE III

RECONSTITUTION OF NITRATE REDUCTASE ACTIVITY WITH ELEMENTS RELEASED FROM MEMBRANES

Meaning of designations: Extracts WT, A and B: soluble extracts of cells processed with the French pressure cell, wild-type, *chl A*$^-$ and *chl B*$^-$ strains, respectively. Vesicle Extracts A, B and WT: centrifugation supernatants of sonicated Kaback's vesicles, *chl A*$^-$, *chl B*$^-$ and wild-type strains, respectively. Depletion Extracts A, B, WT and Rec: centrifugation supernatants of particles depleted by removing $Mg^{2+}$ by dilution in a low ionic strength buffer, particles of *chl A*$^-$, *chl B*$^-$ and wild-type strains and reconstituted particles, respectively. The *chl B* mutant was grown anaerobically in the presence of nitrate.

| Reaction mixtures | Reconstituted nitrate reductase | |
|---|---|---|
| | Total activity (units) | Specific activity (units/mg protein) |
| Extracts B+A (control) | 14.9 | 6.8 |
| Extract B+Depletion Extract A | 15.4 | 7.7 |
| Extract B+Depletion Extract WT | 9.9 | 3.2 |
| Extract B+Depletion Extract Rec | 8.6 | 2.8 |
| Extract B+Vesicle Extract A | 8.5 | 3.4 |
| Extract B+Vesicle Extract WT | 7.6 | 2.8 |
| Extract A+Depletion Extract B | 0.8 | 0.35 |
| Extract A+Depletion Extract WT | 0.8 | 0.28 |
| Extract A+Vesicle Extract B | 1.2 | 0.4 |

in the membranes of wild-type and *chl A*$^-$ strains and in the membranes formed by complementation, and on the other, that $F_A$ protein can be released from these membranes by diluting them in a buffer without $Mg^{2+}$ as is the case for ATPase [9]. Such a result confirms the observations made above in the incorporation of $F_A$ protein into the reconstituted Peak II particles, the density of which is 1.18 (Fig. 8). It is also seen in Table III that identical results were obtained with centrifugation supernatants of sonicated kaback's vesicles.

*Regulation of the $F_A$ protein*

By the techniques we have just described, we were able to show that $F_A$ protein is present in an active state in reconstituted particles, wild-type, *chl A*$^-$, *chl C*$^-$ and *chl D*$^-$ strains, whatever the growth conditions, aerobiosis or anaerobiosis, and in the presence or the absence of nitrate. The levels of reconstituted enzyme are always the same for the extracts of the different strains, provided that reaction mixtures contain the same amounts of soluble Extract B from *chl B*$^-$ strain grown in anaerobiosis in the presence of nitrate.

## DISCUSSION

The results reported in the present paper permit some conclusions to be drawn about the nature of $F_A$ protein and the part played by it in the complementation process. We had previously shown that $F_A$ factor is to be considered as the product of *chl B* gene [10].

From the experimental point of view, the complementation can be realized with two different experimental systems. The first of these consists of a mixture of the soluble fractions from *chl A*$^-$ and *chl B*$^-$ strains in equivalent amounts incubated at 32 °C for 2 h; it is the "standard complementation" previously described by Azoulay et al. [2, 4]. In the second system, Extract A is replaced by the $F_A$ protein purified from Extract A itself. This new system, when incubated at 32 °C for 2 h, leads to the same results as the first one, i.e. reconstitution of nitrate reductase activity and formation of sedimentable particles (containing a part of the reconstituted activity) distributed among two families of different buoyant densities. With this system, it is shown without doubt that (a) The complementation is initiated by the introduction of active $F_A$ protein into the reaction mixture. (b) The $F_A$ factor is incorporated neither into the soluble enzymatic complex, nor into the light particles (Peak I) of density 1.10, but only into the heavy particles (Peak II), of density 1.18–1.20. The last observation, which is very important for an understanding of complementation, is to be compared with our previous results showing that complementation begins with the reconstitution of non-sedimentable elements [3, 22, 26] and with the formation of light particles to which soluble ATPase binds [9]. In this connection, complementation differs from the artificial systems of reconstitution using liposomes and from those consisting of reaggregating detergent-solubilized membranes by dialysis against $Mg^{2+}$-containing buffer [28], in so far as an enzymatic reconstitution was not obtained. Our system can be compared with the reconstitution of mitochondrial membranes [6–8] because it needs a sequence of increasingly complex organization stages with respect to morphology [3], lipid/protein ratio and phospholipid content [23] and buoyant density [9]. The peculiar nature of the complementation is the major

part played by $F_A$ protein which differs from the "coupling factors" described by Racker [29] in the fact that the coupling factors confer allotopic properties to the enzymes bound to the reconstituted membranes; in our system, in the absence of $F_A$ protein, the incubation leads to an aggregation having neither the enzymatic properties of the native membranes nor the binding sites for the fixation of soluble ATPase (Frelat et al., unpublished results).

We have shown in this report that $F_A$ protein exhibits the following properties. (a) It has a strong tendency to polymerize, which leads to its total inactivation. (b) Its molecular weight is rather low (about 35 000). (c) It does not contain any lipid. (d) It is present in an active state in the membranes of wild-type and *chl-r* strains except in the *chl-B*$^-$ strain. (e) It is synthesized in these strains whatever the growth conditions, aerobiosis or anaerobiosis, with or without nitrate. (f) It is heat-inactivated and the optimal pH ranges from 7.4 to 7.7.

From our results taken as a whole, it can be supposed that $F_A$ factor acts as an enzymatic protein, especially as the velocity of the reconstitution depends on the concentration of $F_A$ protein in the reaction mixtures.

At the present time this supposition remains to be definitely confirmed and to be stated precisely what kind of reaction is catalysed by $F_A$ protein. Nevertheless, we can imagine that it acts on the quaternary structure of proteins, so that changes of conformation can be produced allowing some proteins to be aggregated to result in an active nitrate reductase. If we add that according to Lam and Nicholas [30], Tanigushi and Itagaki [31] and Forget [32], nitrate reductase contains molybdenum and iron which have a part to play at the level of the active site and that we have shown that the products of the *chl A* and *chl C* genes are required for a good reconstitution [10], it is possible that $F_A$ protein brings about these two proteins giving rise to the soluble nitrate reductase the molecular weight of which is 250 000 [22]. This soluble enzyme is found when complementation is stopped after the first minutes of incubation or if it is performed with extracts treated by phospholipase C prior to incubation [22].

We have also shown that membranes prepared from the wild-type strain grown anaerobically in the presence of nitrate release $F_A$ protein without affecting nitrate reductase when subjected to an osmotic shock by removing $Mg^{2+}$. In the same way, we have seen that, in the course of complementation, $F_A$ protein is incorporated only into Peak II particles ($d = 1.18$) from which it can be released by the same treatment as above without affecting the nitrate reductase contained in these particles. Therefore, one of the major conclusions to be drawn from our results is that $F_A$ protein specifically located in the cytoplasmic membrane and absent in an active form in the *chl B* mutants, is needed for the reconstitution of soluble or sedimentable nitrate reductase but not for the expression of the enzyme and that it is incorporated into only membranous structures, having completed a certain stage of organization, and capable of binding it. These results, in conjunction with those reported in the next paper [23] dealing with the nature of the phospholipids incorporated in the course of complementation, open a wide field for investigations in order to understand the morphogenesis of the active membranous structures, as far as the morphogenesis is controlled by specific protein factors.

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