ISOLATION OF A MEMBRANE-BOUND PROTEIN HAVING COUPLING FACTOR CAPACITY AS WELL AS ADP–P_i EXCHANGE AND ADPase ACTIVITIES FROM *RHODOPSEUDOMONAS VIRIDIS*

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1. Introduction

It was earlier observed by García et al., that a crude preparation of coupling factor isolated from *Rhodopseudomonas capsulata* showed a high dark phosphate esterification activity [1]. It was then postulated that this incorporation could be the result of an as yet unknown ADP–P_i exchange reaction, and no relationship was established between the coupling activity and the dark esterification activity.

We report here on the purification of a protein from *Rhodopseudomonas viridis* having ADP–P_i exchange activity, Mg^{2+} activated ADPase activity and the ability of restoring a light-induced phosphate esterification capacity when rebound to uncoupled membranes.

2. Materials and methods

Rhodopseudomonas viridis was grown as previously described [2].

2.1. *Coupling factor purification*

The coupled membranes were prepared by sonicating the cells suspended in 100 mM glycyl–glycine buffer, pH 8.0, 2.5 mM $MgCl_2$. They were sonicated at 70 W output in a Branson sonifier, in the cold for 5 min.

Cell debris was eliminated by centrifugation for 20 min at 15 000 × *g* and the membranes recovered by centrifugation at 144 000 × *g* for 90 min. The membranes were washed twice with the same buffer. They were finally resuspended in the same buffer to a concentration of 1 mg Bchl/ml.

The membrane suspension was added dropwise to 20 vol. of cold acetone (–20°C) and the protein was filtered through Whatman No. 1 paper. The dried powder was extracted twice with 50 mM Tris, pH 8.0, at room temperature, and the combined extracts centrifuged 1 h at 100 000 × *g*. The supernatant was fractioned with ammonium sulfate and the protein, precipitating between 45–55% saturation, was recovered. The protein was resuspended in 50 mM Tris, pH 8.0, and passed through a Sephadex 6B column equilibrated with the same buffer. The fractions having ADPase and ADP–P_i exchange activities were pooled and passed again through an identical column.

The fractions having maximum ADPase and ADP–P_i exchange activities were pooled, concentrated and run in a linear sucrose gradient 40–25% sucrose in an SW50 rotor at 45 000 rpm for 19 h.

The coincident activities of ADPase and ADP–P_i exchange were pooled, passed through a DEAE–Sephadex A-25 column and eluted using a linear NaCl gradient between 0–1.0 M in 50 mM Tris pH 8.0.

The protein fractions containing ADP–P_i exchange activity were pooled and passed through a similar DEAE–Sephadex A-25 column. The resulting protein having ADP–P_i exchange, ADPase and recoupling of photophosphorylation activities were used as such. The yield was 12% of the initial ADPase activity representing 0.3–0.5% of the initial protein.

2.2. *Uncoupled membranes*

The uncoupled membranes were prepared by two

different procedures according to Baccarini-Melandri et al. [3] or Fisher et al [4]. The final pellet thus obtained was resuspended in 50 mM glycyl–glycine buffer pH 8.0 containing 2.5 mM Mg^{2+} which was also 50% in glycerol. These membranes were totally devoid of photophosphorylation capacity.

2.3. *Disc-gel electrophoresis*

It was run as described by Davis [5]. The acrylamide concentration was 5% and the electrophoresis was carried out in the cold for 90 min.

2.4. *Enzymatic activities*

2.4.1. Recoupling

The recoupling of the membranes was carried out by preincubation 30 min at 30°C of 12 μg Bchl (uncoupled membranes) in a mixture containing: 5 μmol Tris, pH 7.5, 0.37 μmol $MgCl_2$, 2.8 μmol glucose, 0.5 μmol ATP and 1.0 mg of bovine serum albumin in a final volume of 0.1 ml and variable amounts of enzyme.

This suspension was centrifuged at 40 000 rpm for 60 min on top of a layer of 20% sucrose in 50 mM Tris, pH 8.0, also containing 3.7 mM $MgCl_2$. This permits a net separation between excess protein and recoupled membranes.

The pellet was resuspended in 0.1 ml of 50 mM Tris, pH 8.0, 3.7 mM $MgCl_2$.

2.4.2. Photophosphorylation

Photophosphorylation was started by adding the recoupled membranes to 1.0 ml of a dilution mixture containing: 30 μmol Tricine, pH 8.0, 3.7 μmol $MgCl_2$, 18 μmol glucose, 1.7 μmol ADP, 3.3 μmol phosphate (with a specific activity of 7.5×10^5 cpm/μmol P_i), 100 μg yeast hexokinase, 0.4 μmol Na succinate and 1.3 nmol phenazine methosulfate (PMS). Incubation was at 30°C under saturating light conditions for varying lengths of time.

Photophosphorylation activity was determined as described before [6].

2.4.3. ADP–P_i exchange activity

The activity was measured in 1.16 ml final volume containing: 30 μmol Tricine, pH 8.0, 1.7 μmol ATP, 3.3 μmol phosphate (specific activity 7.5×10^5 cpm/μmol), 3.4 μmol $MgCl_2$. The reaction was started by the addition of the enzyme. The incubation was carried out at 30°C.

2.4.4. ADPase and ATPase activities

Mg^{2+} activated ADPase was determined in 0.8 ml of a mixture containing: 40 μmol glycyl–glycine, pH 8.0, 3 μmol ADP and 3 μmol $MgCl_2$.

Ca^{2+} activated ATPase was determined in 0.8 ml of a mixture containing: 40 μmol glycyl–glycine, pH 8.0, 3 μmol ATP and 3 μmol $CaCl_2$. In both cases the incubation was carried out at 30°C.

2.5. *Analysis of the products of the ADP–P_i exchange*

After the reaction was completed, the reaction mixture was rapidly cooled to 0°C and immediately passed through a Dowex 1-Cl^- column (0.7×11 cm). The elution was carried out stepwise. Initially 20 ml of 10 mM HCl were passed and 1 ml fractions were collected. Then a linear gradient (60 ml) of NaCl between 0 and 200 mM in 10 mM HCl was started and again 1 ml fractions were collected. In each fraction radioactive phosphate was determined. The columns were previously calibrated with the corresponding standards.

2.6. *Other analytical procedures*

Bchl was determined as described before [7]. Protein was determined by the methods of Lowry et al. [8]. Phosphate was measured as described [9]. Radioactivity was determined with a liquid scintillation spectrometer. The solvent used was a mixture of 1 vol. of Triton X-100 and 2 vol. of toluol containing 2,5-diphenyloxazole (PPO) and dimethyl 1,4 bis (2-(4-methyl-5-phenyloxazole))-benzene (dimethyl POPOP) This mixture will tolerate up to 1.0 ml of water with no appreciable quenching [10].

3. Results and discussion

During the purification of the coupling factor from *Rps viridis*, we observed that this activity was always accompanied by two others, namely an ADP–P_i exchange activity and a Mg^{2+} activated ADPase.

As shown in table 1 these two last activities maintain a constant ratio throughout the different purification steps and are always associated with the recoupling capacity.

Moreover, fig.1 shows the last step of the purification procedure of our coupling factor protein showing how the ADPase, ADP–P_i exchange activity and the recoupling capacity overlap.

Table 1

Purification step	Mg^{2+} activated ADPase (μmoles P_i released/h X mg protein)	ADP–P_i exchange (μmoles P_i incorporated/h X mg protein)	ADP–P_i exchange /ADPase
Ammonium sulfate 45–55%	3.88	4.4	1.13
First Sepharose 6B	10.1	11.1	1.10
Second Sepharose 6B	15.8	17.0	1.70
Sucrose gradient	17.0	18.5	1.08
First DEAE–Sephadex A-25	18.7	20.6	1.10
Second DEAE–Sephadex A-25	19.0	23.5	1.23

In all cases the recoupling activity was exclusively associated to the UDP–P_i exchange and ADPase activities. When the recoupling is performed using saturating amounts of protein the photophosphorylating capacity of the recoupled membranes oscillates between 1–2 μmol ATP/h X mg Bchl.

The analysis of our purest enzyme preparation by non-dissociationg disc-gel electrophoresis (tubes 85–89 from the gradient shown in fig.1) shows in each of them the presence of a single protein band, thus providing strong evidence that the three activities are produced by a single protein (fig.1 inset).

The isolated protein showed no Ca^{2+} or Mg^{2+} dependent ATPase activity.

When the identity of the products of the ADP–P_i exchange was investigated by ion exchange chromato-

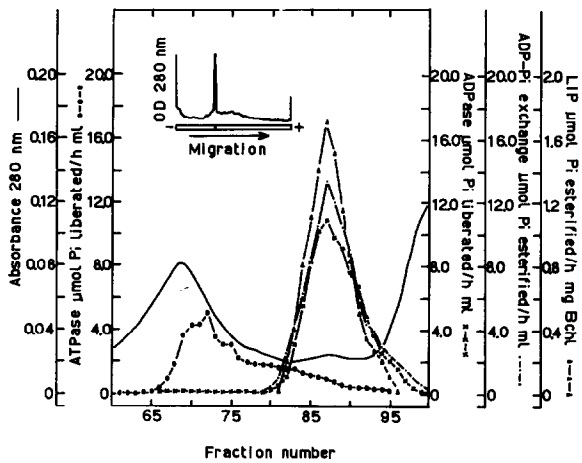

Fig.1. Last step of purification of ADPase isolated from *Rps. viridis*. The DEAE A-25 column was carried out as described under Materials and methods. (○——○) Ca^{2+} activated ATPase. (△——△) Light induced phosphate esterification capacity of recoupled membranes. (· —— ·) ADP–P_i exchange activity. (X——X) Mg^{2+} activated ADPase. (——) Protein pattern, absorbance at 280 nm. For details of activity measurements, see Materials and methods.

Fig.1 inset. Non-dissociating disc-gel electrophoresis. The sample used in this experiment represents the pool obtained after mixing tubes number 85–89 of the gradient shown in fig.1. The protein present in the isolated fractions 85–89 also gives a single protein band when analyzed by an identical disc-gel electrophoresis system. The gel was scanned at 280 nm (tracing) and stained with amido black (gel pattern).

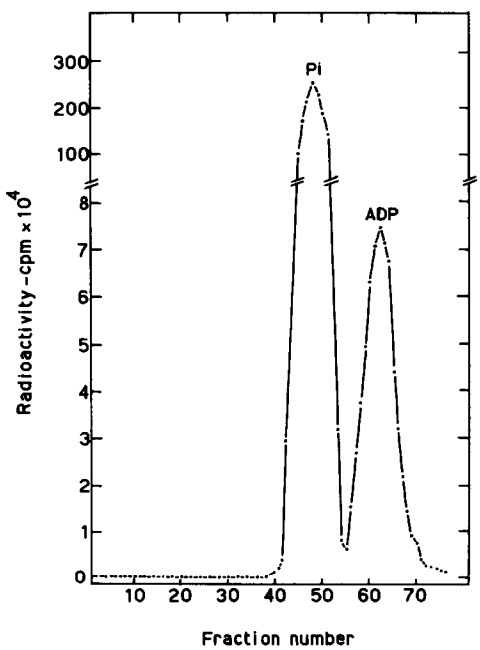

Fig.2. Products of the ADP–P_i exchange. The Dowex 1-Cl^- was run as described under Materials and methods. The reaction was carried out in three times, the volume mentioned under Materials and methods and the amount of protein was 90 μg. The incubation time was 20 min at 30°C.

graphy, a method that allows a clear separation between P_i, AMP, ADP and ATP, the results shown in fig.2 were obtained. Thus two radioactive peaks were detected. The most prominent peak corresponds to excess radioactive phosphate. [^{32}P]ADP was the main radioactive product representing approximately 99% of the total incorporation. No exchange activity was obtained when ADP was replaced by ATP or AMP.

As was mentioned before, when rebound to the membrane the protein restores light-induced phosphorylation.

Maximum values of photophosphorylation obtained with the reconstituted system (enzyme + uncoupled membranes) oscillate between 1–2 μmol ATP/h mg Bchl. This reaction was 80% inhibited in the presence of antimycin (1.5×10^{-4} M) indicating that this photophosphorylation reaction requires an operative electron transport chain. Values of 7–10 μmol ATP/h mg Bchl for photophosphorylation were obtained in intact membranes of this bacterium.

There is considerable information showing that in mitochondria [11,12], plant chloroplasts [13,14] and bacteria, both photosynthetic and non-photosynthetic [15–18], an ATPase is responsible for the last step during electron transport-mediated energy conservation. Our results, however, would indicate the possibility that at least in *Rhodopseudomonas viridis* an enzyme having ADP–P_i exchange and ADPase activities reestablishes the capacity for electron transport mediated phosphate esterification. It is yet uncertain whether this present photophosphorylation capacity indeed involves ATP formation through the direct phosphorylation of added ADP or it only represents an increased ADP–P_i exchange reaction activated by light.

Moreover, as shown in fig.1, a membrane-bound Ca^{2+}-dependent ATPase was also obtained during the purification of our enzyme. However, this enzyme presents no recoupling activity under the conditions used in the assay.

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References

[1] García, A. F., Drews, G. and Kamen, M. D. (1974) Proc. Natl. Acad. Sci. USA 71, 4213–4216.
[2] Pucheu, N. L., Kerber, N. L. and García, A. F. (1974) Arch. Microb. 101, 259–272.
[3] Baccarini-Melandri, A. and Melandri, C. A. (1971) in: Methods in Enzymology (San Pietro, A. ed.) Vol. 23, pp. 556–561 Academic Press, New York.
[4] Fisher, R. R. and Guillory, R. J. (1969) FEBS Lett. 3, 27–30.
[5] Davis, B. J. (1964) Ann. N.Y. Acad. Sci. 121, 404–425.
[6] Avron, M. (1960) Biochim. Biophys. Acta 40, 257–272.
[7] Clayton, R. K. (1963) in: Bacterial Photosynthesis (Gest, H., San Pietro, A. and Vernon, L. P. eds.) pp. 495–500, Autioch Press, Yellow Springs.
[8] Lowry, O. H., Rosebrough, N. H., Farr, A. L., Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[9] Taussky, H. and Shorr, E. (1953) J. Biol. Chem. 202, 675–685.
[10] Andersen, L. E. and Mc Clure, W. O. (1973) Anal. Biochem., 51, 173–179.
[11] Pullman, M. E., Penepsky, H. S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329.
[12] Penepsky, H. S., Pullman, M. E., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3330–3336.
[13] Vambutas, V. K. and Racker, E. (1964) J. Biol. Chem. 240, 2660–2667.
[14] Adolfsen, R. and Moudrianakis, E. N. (1971) Biochemistry 10, 2247–2253.
[15] Baccarini-Melandri, A., Gest, H. and San Pietro, A. (1970) J. Biol. Chem. 245, 1224–1226.
[16] Johansson, B. C., Baltscheffsky, M. and Baltscheffsky, H. (1973) Eur. J. Biochem. 40, 109–117.
[17] Bogin, E., Higashi, T. and Brodie, A. F. (1970) Arch. Biochem. Biophys. 136, 337–351.
[18] Melandri, B. A., Baccarini-Melandri, A., Gest, H. and San Pietro, A. (1971) in: Energy Transduction in Respiration and Photosynthesis (Quagliarello, E., Papa, S. and Rossi, C. S. eds) pp. 593–597, Adriatica Editrice.