# A COUPLING FACTOR FROM CHROMATIUM STRAIN D CHROMATOPHORES

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## 1. Introduction

The mechanism of photosynthesis in the obligatory anaerobic bacteria chromatium strain D differs in many respects from that of higher plants [1]. However, the bacterial chromatophores resemble chloroplasts in their lack of capacity for oxidative phosphorylation. It was therefore of interest to conduct a comparative study of photophosphorylation in chromatium strain D chromatophores. We attempted to study the partial reactions of photophosphorylation and to isolate a coupling factor. While this work was in progress [2] Baccarini-Melandri and Gest [3] reported a coupling factor from *Rhodopseudomonas capsulata*. In chloroplasts, the work on coupling factors is much more advanced than in chromatophores. Treatment of chloroplasts with EDTA in a low salt medium released a coupling factor [4]. The same factor could also be isolated from acetone powder of chloroplasts [5]. The coupling factor restored phosphorylation in EDTA washed chloroplasts [4, 5] and in nonphosphorylating subchloroplasts particles [5]. Both the chloroplasts and the coupling factor had a latent ATPase activity which could be activated by various treatments [5, 7]. An antiserum to the coupling factor inhibited phosphorylation and ATPase activity in the chloroplasts as well as ATPase activity in the isolated coupling factor [8]. These data indicate that the coupling factor is an enzyme which takes part in the terminal step of ATP synthesis in chloroplasts.

## 2. Methods

Chromatium strain D* bacteria were grown unaerobically on the medium of Hendley [9] supplemented with 0.2 percent malate and were illuminated for three days at 32°. The harvested bacteria were washed in tricine–NaOH, pH 7.8 0.1 M and stored under argon at −20°. The cells were ground with alumina in tricine–NaOH pH 7.8 0.1 M, then centrifuged at 12,000 *g* for 15 min to remove debris. The chromatophores were sedimented by centrifugation at 144,000 *g* for 1 hr and resuspended in a small volume of the grinding buffer. Chlorophyll content was determined by reading the absorbance at 850 nm using an extinction determined by Clayton [10].

Photophosphorylation was assayed in a reaction mixture containing: tricine–NaOH pH 7.8 33 mM; $^{32}$P-$K_2HPO_4$ pH 7.8 3.3 mM (containing $3 \times 10^6$ cpm); $MgCl_2$ 2.6 mM; ADP 1 mM; and chromatophores containing 50 μg bacteriochlorophyll in a total volume of 3 ml. The reaction was carried out in Warburg vessels under atmosphere of argon at 20°. The reaction was started by illumination at the intensity of 600 f.c. for 15 min and was stopped by addition of cold $CCl_3COOH$ to a final concentration of 3%. $^{32}$P-ATP formation was measured according to the method of Avron [11].

ATPase activity was assayed in the same reaction mixture as used for phosphorylation, however, ADP and Pi were omitted, $^{32}$P-ATP, 1.6 mM (containing

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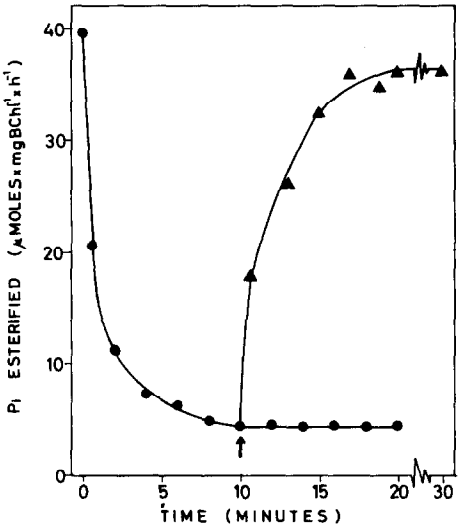


Fig. 1. Time course of inactivation and restoration of photophosphorylation activity in chromatophores. Chromatophore suspension was incubated in a medium containing tricine–NaOH pH 7.8, 3 mM. After 10 min, $MgCl_2$ 8 mM (final concentration) was added to the incubation medium. At various time intervals, as indicated, samples were withdrawn from the incubation medium and assayed for photophosphorylation activity. Reaction mixtures and assay conditions are described under Methods.

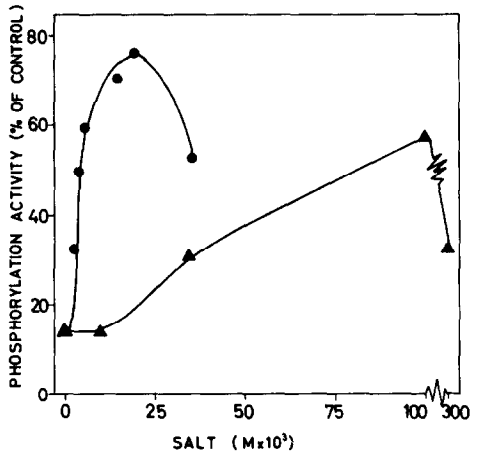


Fig. 2. The effect of the concentration of salt during incubation on restoration of photophosphorylation activity in resolved chromatophores. Chromatophores were incubated in a medium containing tricine–NaOH, pH 7.8, 3 mM. After 10 min of incubation either $MgCl_2$ (●—●) or NaCl (▲—▲) were added at concentrations as indicated. After another 10 min of incubation, phosphorylation activity of the chromatophores was assayed as described under Methods.

$7 \times 10^5$ cmp) was added and the bacteriochlorophyll content was increased to 200 μg/3 ml. Other assay conditions were the same as for phosphorylation but the reaction was carried in the dark.

ATP–Pi exchange activity was assayed under the same conditions as ATPase activity, however, non-radioactive ATP was used and $^{32}P$-$K_2HPO_4$ pH 7.8 3.3 mM (containing $2.5 \times 10^6$ cpm) was added.

Pyrophosphatase activity was assayed under the same conditions as ATPase, but with pyrophosphate replacing the radioactive ATP. Inorganic phosphate released from pyrophosphate was determined by the method of Ames [12].

Coupling factor was extracted by incubation of chromatophores in a medium containing tricine–NaOH pH 7.8 3 mM. After incubation for 10 min at room temperature the suspension was centrifuged at 144,000 *g* for 1 hr at 0°. Washed chromatophores were recovered from the pellet and the clear supernatant (Sup II) contained coupling factor activity. Protein content of Sup II was determined by the method of Herriot [13].

## 3. Results and discussion

Incubation of chromatophores in a medium containing tricine–NaOH pH 7.8 3 mM, resulted in an inactivation of photophosphorylation. The inactivation of phosphorylation was time dependent and was almost complete after 10 min of incubation (fig. 1). Unlike chloroplasts [4], the inhibition of phosphorylation in chromatophores did not require the presence of EDTA in the medium (experiment not shown here). Addition of magnesium to the medium, where inactivation took place, resulted in restoration of phosphorylation activity. The restoration increased with time reaching over 80 percent of the original activity after 10 min of incubation in the presence of magnesium ions. The restoration of phosphorylation was most effective at a concentration of 8 mM $MgCl_2$ (fig. 2). Incubation in the presence of NaCl gave partial restoration of phosphorylation, however, this salt was found to be less effective than $MgCl_2$. Inactivation of phosphorylation in low salt medium was prevented by the addition of 2 mM $MgCl_2$ to the incubation medium (not shown).

The possibility that a factor required for phos-

Table 1
Restoration of photophosphorylation activity to washed chromatophores by incubation with Sup II.

| Treatment of chromatophores | Incubation | Pi esterified (μmoles/mg BChl/hr) None | PMS |
|---|---|---|---|
| None | – | 77.0 | 98.0* |
| Washed in 3 mM tricine** | – | 0.5 | 1.0 |
| Washed in 3 mM tricine** | Sup II, 0 min*** | 8.2 | – |
| | Sup II, 10 min | 17.2 | 42.0 |
| | Sup II (boiled), 10 min | 1.0 | 1.2 |

* Phosphorylation was assayed as described in Methods but with the addition of PMS, 0.1 mM

** Washed chromatophores were obtained as described in Methods.

*** Incubation medium contained 8 mM $MgCl_2$ and Sup II equivalent to the amount obtained by washing of the chromatophores used in each assay. Photophosphorylation was assayed after the incubation time indicated in each treatment.

Table 2
The effect of incubation in low salt medium on photophosphorylation, ATPase and ATP–Pi exchange activities.

| Treatment | Activity (μmoles Pi/mg BChl/hr) Phosphorylation | ATPase | ATP–Pi exchange |
|---|---|---|---|
| A) None* | 142.8 | 4.8 | 7.3 |
| B) Incubation in 3 mM tricine | 1.0 | 4.9 | 1.2 |
| C) B + incubation in 8 mM $MgCl_2$ | 61.5 | 4.8 | 2.6 |

* A) Photophosphorylation, ATPase and ATP–Pi exchange activities were assayed as described in Methods.
B) Assay of the activities was preceded by incubation for 10 min in 3 mM tricine.
C) Assay of the activities was preceded by incubation as in B which was followed by another 10 min incubation in the presence of 8 mM $MgCl_2$.

phorylation was released from the chromatophores during incubation in low salt medium was verified by the separation of the chromatophores from the medium after incubation. The washed chromatophores obtained after centrifugation did not have any phosphorylation activity (table 1). The supernatant fraction (Sup II) did not have any phosphorylation activity. However, incubation of the washed chromatophores with Sup II in the presence of magnesium ions restored phosphorylation activity. It is possible that the factor was a protein since Sup II contained protein and the factor was found to be heat labile and non dialyzable [14]. The restoration of phosphorylation was also dependent on the amount of Sup II which was added to the washed chromatophores. A non-specific effect of the proteins in the supernatant was ruled out by showing that incubation of bovine albumin in the presence of magnesium ions did not restore phosphorylation to the washed chromatophores [14].

The electron transport catalyst PMS (*N*-methylphenazonium methosulphate) stimulated phosphorylation in the untreated and in the resorted chromatophores but not in the washed particles (table 1).

An electron transport catalyst is not expected to stimulate phosphorylation in particles lacking a coupling factor. However, these data did not rule out the possibility that an electron carrier which is not bypassed by PMS was removed from the washed chromatophores. PMS would not be expected to catalyze electron transport if it did not bypass the removed carrier.

Chromatophores catalyze only cyclic electron transport, therefore it was difficult to test whether or not an electron transport carrier was removed by the treatment. However, an effect of washing on a partial reaction of phosphorylation, which does not depend on electron transport, could indicate whether or not a coupling factor was removed by this treatment. Indeed, the chromatophores catalyzed a dark ATP–Pi exchange reaction which was inactivated by incubation of the chromatophores in a low salt medium (table 2). Similarly to phosphorylation, addition of magnesium ions to the medium, where inactivation took place, resulted in restoration of ATP–Pi exchange activity. The exchange activity was very low in washed chromatophores but was not found in Sup II (not shown). ATP–Pi exchange activity was restored after incubation of the washed chromatophores with Sup II in the presence of magnesium ions.

Table 3
The effect of inhibitors on photophosphorylation, ATPase and ATP–Pi exchange activities.

| Addition | Concentration | Treatment of chromatophores | Percent of control rate of activity* | | |
|---|---|---|---|---|---|
| | | | Phosphorylation | ATPase | ATP–Pi |
| FCCP | $6.6 \times 10^{-4}$ M | None | 25 | 128 | 16.4 |
| FCCP | $6.6 \times 10^{-4}$ M | Restored** | 23.7 | 127 | 7.8 |
| DIO-9 | 20 μg/ml | None | 78 | 64 | 61 |
| DIO-9 | 20 μg/ml | Restored | 64 | 57 | 70 |
| Phlorizin | $5 \times 10^{-4}$ M | None | 73 | 77 | 61 |
| Phlorizin | $5 \times 10^{-4}$ M | Restored | 54 | 47 | 76 |
| HOQNO | $3.3 \times 10^{-4}$ M | None | 38 | 97 | 107 |
| HOQNO | $3.3 \times 10^{-4}$ M | Restored | 59 | 99 | 96 |

* The rate of activity without any addition was taken as 100 percent. Assay of the various activities was as described in Methods.
** The chromatophores were incubated for 10 min in 3 mM tricine then incubated for another 10 min in the presence of 8 mM $MgCl_2$.

Both phosphorylation and ATP–Pi exchange activities were inhibited by the uncoupler FCCP and by the energy transfer inhibitors phlorizin and DIO-9 (table 3). The inhibitors and the uncoupler inhibited the two activities in the untreated as well as in the restored chromatophores. However, the electron transport inhibitor (HOQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide) [15] inhibited phosphorylation but not ATP–Pi exchange activity. As previously indicated, inactivation of phosphorylation could be caused by a loss of either an electron transport carrier or of a coupling factor. ATP–Pi exchange activity was not dependent on electron transport since the activity took place in the dark and was not inhibited by an electron transport inhibitor. Therefore, a loss of an electron transport carrier would not be expected to inhibit the exchange. It seems likely that during incubation in a low salt medium a coupling factor was released from the chromatophores.

The chromatophores catalyzed a dark ATPase activity which was related to photophosphorylation by the action of uncouplers and inhibitors. The uncoupler FCCP, which inhibited phosphorylation, stimulated ATPase activity. The energy transfer inhibitors DIO-9 and phlorizin inhibited both phosphorylation and ATPase activities (table 3). However, the dark ATPase activity was not affected by incubation in a low salt medium (table 2); the washed chromatophores had almost the same ATPase activity as the untreated particles, and Sup II had practically no ATPase activity. It seems that an ATPase which was related to photophosphorylation was not released from the chromatophores together with the coupling factor. On the assumption that ATPase activity in the chromatophores was catalyzed by the same enzyme which catalyzed photophosphorylation, and since the rate of ATPase activity was only a fraction of the rate of phosphorylation, it is suggested that most of the enzyme which catalyzed phosphorylation was in the form of a latent ATPase. It is possible that the coupling factor which was removed from the chromatophores by low salt treatment was this latent ATPase while the active ATPase remained in the chromatophores.

Results of substrates specificity study of ATPase activity in chromatophores are given in table 4. Phosphate was released from ATP and ADP but not from AMP. The lack of AMP hydrolysis indicates that an ATPase rather than a phosphatase catalyzed the hydrolysis of ATP. The incapacity of the washed chromatophores to hydrolyze ADP might indicate that ADP was hydrolyzed via its conversion to ATP by adenylate kinase. When adenylate kinase was washed away only ATP could be hydrolyzed by ATPase (table 4).

Table 4.
Substrate specificity of phosphate release activity in chromatophores, Sup I and Sup II.

| Treatment | Pi released (μmoles/mg BChl/hr) | | | |
|---|---|---|---|---|
| | ATP* | ADP | AMP | PPi |
| None | 5.9 | 4.6 | 0.4 | 45.0 |
| Incubated 10 min in 3 mM tricine | 4.4 | 4.4 | 0.6 | 42.2 |
| Washed in 3 mM tricine | 3.6 | 0.3 | 0.0 | 34.5 |
| Sup I (equivalent)** | 0.2 | 0.8 | 0.1 | 17.1 |
| Sup II (equivalent) | 0.22 | 0.5 | 0.15 | 14.5 |

* Reaction conditions were as described for the assay of pyrophosphate in Methods except for the substrates which were varied as indicated.

** Rates of the activities of Sup I and Sup II were calculated on the basis of the bacteriochlorophyll content of the chromatophores which were used for the preparation of the two supernatants. Sup I and Sup II contained 1.3 mg and 0.3 mg protein, respectively.

The chromatophores catalyzed a pyrophosphate activity which had a considerably higher rate than ATPase activity. Pyrophosphatase activity was also found in the supernatant which remained after the isolation of the chromatophores (Sup I), in the washed chromatophores and in Sup II (table 4). The pyrophosphatase activity was neither inhibited by energy transfer inhibitors nor stimulated by uncouplers which affected phosphorylation or ATPase activity. The data presented above make it difficult to relate pyrophosphatase to phosphorylation in chromatium chromatophores.

Light-induced pH changes in washed chromatophores were only partially inhibited [14]. Similar results were obtained in sonicated chromatophores from *Rhodopseudomonas capsulata* [3]. It is hard to imagine that washing in a low salt medium inactivated electron transport since pH changes which require light-induced electron transport were not inhibited by this treatment. ATP–Pi exchange activity, which does not require electron transport, was restored by Sup II. It is therefore suggested that a coupling factor rather than an electron transport carrier was removed from the chromatophores during incubation in a low salt medium.

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