# PHOTOPHOSPHORYLATION: MECHANISM OF RECONSTITUTION BY COUPLING FACTOR 1 ($CF_1$)

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## 1. Introduction

Loss of photophosphorylation capacity by treatment of thylakoid membranes can be due to inhibition of ATP-synthetase, loss of ATP-synthetase, loss of an electron transport component, damage to the energy conserving reactions (e.g. $H^+$ pump), or alteration of the membrane (e.g. uncoupling due to high $H^+$ leakage). Since EDTA-treatment decreases $\Delta pH$ [1] and releases photosynthetic control [2,3], loss of photophosphorylation capacity may be due to uncoupling and not to a loss of ATP-synthetase itself. The discrepancy between total loss of phosphorylation capacity and only partial loss of $CF_1$, measured as $Ca^{2+}$-ATPase, has been noticed [4]. Residual amount of ATP-synthetase cannot be measured directly, and residual ATPase activity needs trypsin or heat activation to become manifest. After optimal activation 720 $\mu$mol of ATP are hydrolyzed per mg Chl X h at 37°C by summer spinach, whereas with saturating light even at 22°C ATP synthesis up to 1400 $\mu$mol/mg Chl X h can be observed in the PMS system.

Rebinding of $CF_1$ leads to a decrease in $H^+$ efflux [5] and inhibition of electron flow [6]; therefore residual ATP-synthetase may be activated by sufficient $\Delta pH$ again, and the added protein need not become catalytically active [7]. On the other hand thylakoids treated with silico tungsten acid, which depletes them entirely of the coupling factor as judged by ATPase activity and the presence of 'knobs', were reconstituted partially by a homogeneous preparation of soluble $CF_1$ [8].

We report herein that the degree of reconstitution of photosynthetic ATP-synthetase is indeed dependent on the residual amount of ATPase; but the reconstituted rate is higher than expected from the residual amount of $CF_1$, i.e. added $CF_1$ became an active ATP-synthetase again.

We also investigated the dependence of reconstitution on the amount of $CF_1$ added. If $CF_1$ first has to play a structural role [4] and reseal the membrane, before the residual and added ATP-synthetase can be energized by $\Delta pH$ (according to the chemiosmotic hypothesis), the reconstitution curve should be sigmoidal. This was reported [9] with chloroplasts, but surprisingly also with chromatophores from *Rhodospirillum rubrum* [10] and *Rhodopseudomonas capsulata* [11], although the latter resolved vesicles are not uncoupled. In most cases, however, hyperbolic curves have been reported, both for *Rhodospirillum rubrum* [12] and *Rhodopseudomonas capsulata* [13] and for chloroplasts [8,6].

We find that the discrepancy is probably due to different amounts of residual coupling factor, and to the light intensity used during the measurement of photophosphorylation.

## 2. Materials and methods

Spinach was grown in a growth chamber at 19°C with 13 h light (9000 lux) and 11 h dark or in the field.

Chemicals were of reagent grade (p.A.); tricine and EDTA from Merck; PMS from Serva; ATP, trypsin and trypsin inhibitor (Soybean) from Boehringer.

Chloroplast thylakoid systems were isolated in 0.4 M sucrose, 20 mM tricine–NaOH, pH 8.5, 10 mM

NaCl; shocked in 10 mM NaCl and resuspended in 0.1 M sucrose, 10 mM tricine–NaOH, pH 8.5, 10 mM NaCl. Standard procedures were used for chlorophyll determination [14], coupling factor isolation, activation by trypsin and measurement of soluble $Ca^{2+}$-ATPase [15], measurement of bound $Ca^{2+}$-ATPase [8], cyclic photophosphorylation with PMS [16], $^{32}P$ determination [17] and protein determination [18].

Light intensity during photophosphorylation was measured with an YSI-Kettering Model 65 radiometer.

One unit of $Ca^{2+}$-ATPase is defined as the amount that hydrolyzes 1 μmol ATP/min.

## 3. Results

Chloroplast thylakoid systems were treated with 0.75 mM EDTA [5,19] to remove $CF_1$. Addition of 10 mM NaCl prevented the loss of photophosphorylation capacity as described [2,3,20]; also no loss in bound $Ca^{2+}$-ATPase occurred (fig.1). At lower concentrations of NaCl phosphorylation capacity was more easily lost than $Ca^{2+}$-ATPase. Also the reconstituted activity of photosynthetic ATP-synthetase was dependent on the salt during EDTA-treatment, and correlated with the residual activity of $Ca^{2+}$-ATPase and not with the residual phosphorylation capacity. The reconstituted activity of ATP-synthetase exceeded clearly the residual activity of $Ca^{2+}$-ATPase. All conditions were optimal (light, conc. of PMS, ascorbate, BSA), and the amount of ATP-synthetase was limiting in the control (Berzborn and Müller, unpublished).

It was confirmed that EDTA-treated thylakoid systems decrease rapidly in their residual phosphorylation capacity, even in the presence of $Mg^{2+}$ [6,21]. This decrease in activity was much slower, if the particles were diluted into the medium for reconstitution, described in fig.1; then also reconstitution of phosphorylation was still possible: EDTA-particles, suspended in the reconstitution medium for 30 min at 0°C followed by addition of $CF_1$ and incubation for 10 min at 24°C, showed 55% reconstitution of ATP-synthetase; 70% reconstitution was observed, if $CF_1$ was added immediately and incubated for 10 min only (photophosphorylation of control: 1170 μmol ATP/mg Chl × h; and of EDTA-particles, if measured immediately, 10% of control).

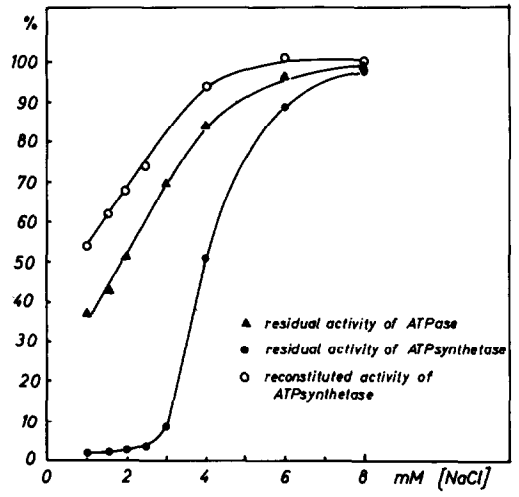


Fig.1. Dependence of residual activity of photosynthetic ATP-synthetase, residual activity of $Ca^{2+}$-ATPase, and reconstituted ATP-synthetase on the salt concentration during EDTA-treatment. (Thylakoid suspension was diluted 1:20 to 0.1 mg Chl/ml with 0.75 mM EDTA, and the pH adjusted to 7.2 with NaOH; after 10 min at 0°C the particles were spun down for 5 min at 27 000 × *g*, and resuspended in 0.1 M sucrose, 10 mM tricine–NaOH, pH 8.5, 10 mM NaCl, and immediately used for measurement of photophosphorylation or reconstitution. Reconstitution with $CF_1$ was performed at 24°C for 10 min in a volume of 0.5 ml containing; 1 mg BSA; 10 μmol tricine–NaOH, pH 7.8; 1 μmol ATP-NaOH pH 7.2; 1 μmol EDTA–NaOH, pH 7.2; 5 μmol $MgCl_2$; 50 μg chlorophyll and coupling factor protein with about 2 units of $Ca^{2+}$-ATPase. For photophosphorylation 0.1 ml of reconstitution mixture (10 μg chlorophyll) were used. Volume 1 ml, containing 50 μmol tricine–NaOH, pH 8.5; 50 μmol NaCl; 5 μmol $MgCl_2$; 3 μmol ADP, 2 μmol $P_i$; 2 μmol Na–ascorbate; 1 mg BSA, $^{32}P_i$ and 0.1 μmol PMS. Light intensity; 7 × $10^5$ erg/cm² × *s* red light (> 610 nm, Schott filter R610). Control rates were: Phosphorylation for 1 min at 22°C: 890 μmol ATP/mg Chl × h; $Ca^{2+}$-ATPase for 5 min at 37°C: 460 μmol $P_i$/mg Chl × h).

Three preparations of $CF_1$, with different specific activity in $Ca^{2+}$-ATPase were used for reconstitution (fig.2); they differed only slightly in purity as judged from the staining intensity after SDS polyacrylamide gel electrophoresis (data not shown). The thylakoids used contained 60% residual $Ca^{2+}$-ATPase; thus 60% of reconstituted ATP-synthetase were expected, if the added $CF_1$ only decreases $H^+$ leakage. The reconstitution exceeded that level. The additional reconstituted ATP-synthetase correlated with the specific activity

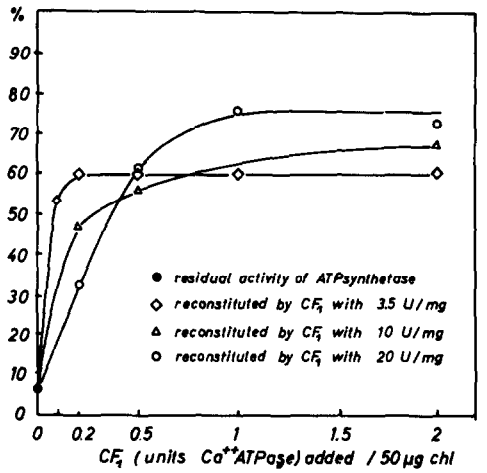


Fig.2. Dependence of reconstituted activity of photosynthetic ATP-synthetase on the amount of $CF_1$ added. (EDTA treatment, reconstitution and measurement of PMS mediated cyclic photophosphorylation as in fig.1). 100% control rate: 920 µmol ATP/mg Chl × h at 7 × $10^5$ erg/cm² × s.

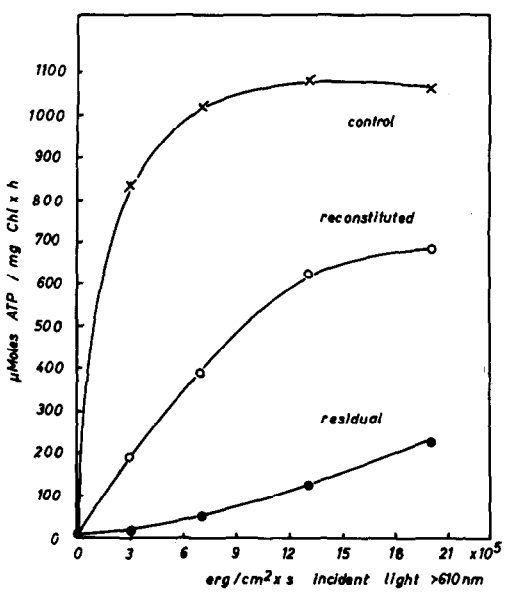


Fig.3. Dependence of photophosphorylation on incident light intensity. (EDTA treatment, reconstitution and measurement of PMS mediated cyclic photophosphorylation as in fig.1). Residual activity of $Ca^{2+}$-ATPase was below 40% of the control; specific activity of $CF_1$ preparation used for reconstitution was 13 units/mg; the amount of $CF_1$ added to EDTA treated thylakoids with 50 µg chlorophyll contained 2.6 units $Ca^{2+}$-ATPase.

in $Ca^{2+}$-ATPase of the $CF_1$ preparation added, if saturating amounts were used. With low amounts the preparation with 3.5 units reconstituted better, if the different preparations were compared on the basis of activity in $Ca^{2+}$-ATPase; if the coupling activity was compared on a protein basis, however, also with low amounts the better preparation reconstituted best.

The degree of reconstitution of photosynthetic ATP-synthetase was determined at incident light intensities, which exceeded the amount necessary to saturate the control thylakoid systems under our conditions (fig.3). Both the residual and the reconsti-

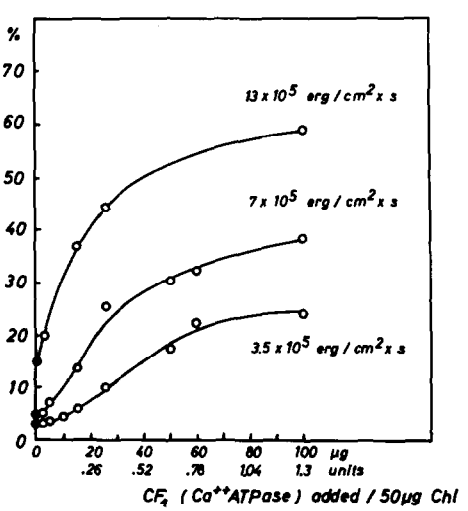


Fig.4. Dependence at different light intensities of reconstituted activity of photosynthetic ATP-synthetase on the amount of $CF_1$ added. (EDTA treatment, reconstitution and measurement of PMS mediated cyclic photophosphorylation as in fig.1). Phosphorylation rate is shown in % of the activity of untreated thylakoids at the respective light intensities; the control rates were: 900 µmol ATP esterified/mg Chl × h at 3.5 × $10^5$ erg/cm² × s; 1310 µmol at 7 × $10^5$ erg/cm² × s and 1230 µmol at 13 × $10^5$ erg/cm² × s. Control thylakoids contained 8 units of $Ca^{2+}$-ATPase/mg Chl. EDTA-treatment removed 65% or 0.26 units/50 µg Chl.

tuted activity in ATP-synthetase were not saturated with this light intensity; the degree of reconstitution could be doubled if the light intensity was increased from $7 \times 10^5$ erg/cm$^2$ × s, which saturate the control, to $20 \times 10^5$ erg/cm$^2$ × s red incident light.

Finally the dependence of reconstitution on the amount of $CF_1$ added was determined. Both hyperbolic and sigmoidal curves were obtained, depending on the light intensity during measurement (fig.4). At least 5 times the amount of removed $CF_1$ had to be added into the reconstitution mixture to saturate the reconstitution.

## 4. Discussion

Avron [3,19] and Vambutas and Racker [22] concluded that the enzyme responsible for photophosphorylation could be isolated from EDTA-extracts of chloroplast thylakoids. The fact that an antiserum against the purified $Ca^{2+}$-ATPase inhibited photophosphorylation [5] supported the hypothesis that $Ca^{2+}$-ATPase and photosynthetic ATP-synthetase are activities of the same protein. The structural role of the coupling factors $CF_1$ from chloroplast [4] and $F_1$ from mitochondria [23] was recognized and led to the search for entirely resolved thylakoids [8,24]. No method has been described which provides good reconstitution after complete removal of $Ca^{2+}$-ATPase from the membrane [4,8,9,24–27,7]. Unspecific damage to the membrane by extraction of a lipid [25,26] and an additional coupling factor $CF_2$ [28] have been discussed.

Our results in fig.1 confirm both the finding [8] that the reconstituted activity of photosynthetic ATP-synthetase is dependent on the residual activity of $Ca^{2+}$-ATPase, and the finding [8] that the reconstituted ATP-synthetase exceeds the level of residual ATPase, i.e. the added $CF_1$ exerts its structural role or coupling activity, but also becomes a catalytically active ATP-synthetase.

Since the thylakoids are uncoupled after EDTA-treatment [1,2,29], residual activity of ATP-synthetase cannot be used to measure residual amount of the enzyme. This may also apply to the treatment with NaBr [24]. Reconstitution, however, is highly dependent on the potential activity [8,30,31] and amount of residual $CF_1$, which in turn is dependent on the conditions during EDTA-treatment, in particular the concentration of monovalent or divalent [3,20] salts. This might explain why higher concentrations of neutralized EDTA [27] or higher chlorophyll concentrations, i.e. less dilution of the thylakoid suspension during EDTA-treatment [6] yield particles which can be reconstituted better.

Incomplete reconstitution could be due to a wrong orientation of rebound $CF_1$ molecules, which might prevent $H^+$ leakage [29] but not recover catalytic activity. The results in fig.2 suggest that partially denatured $CF_1$ can have coupling activity. In this case the reconstitution may be even more dependent on the amount and activity of the residual ATP-synthetase. With saturating amounts of $CF_1$ the reconstitution is dependent on the specific activity (as $Ca^{2+}$-ATPase) of the added enzyme [22] and fig.2. This supports the notion that the added $CF_1$ is at least part of the photosynthetic ATP-synthetase.

If one extrapolates the dependence of reconstitution on the specific activity of the $CF_1$ preparation added, it follows that a preparation of at least 30 units/mg is needed for complete reconstitution. It has to be investigated, whether the procedure which includes a sucrose density centrifugation step [15], will yield this result. Measurement of the fluorescence ratio [15,9] is helpful for monitoring purity, but not as a measure for high coupling activity or active ATP-synthetase. On the other hand $CF_1$ preparations with high activity as $Ca^{2+}$-ATPase may have no coupling activity, e.g. if the small peptides $\delta$ and $\epsilon$ are missing [32].

Since the degree of reconstitution is not only dependent on the amount and activity of residual $CF_1$ (fig.1) and the amount and specific activity (fig.2) of added $CF_1$, but also on the light intensity during measurement (fig.3 and 4), the discrepancies in the literature concerning the shape of reconstitution curves can be explained.

## Acknowledgements

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## Note added in proof

In accordance with our results (fig.1) a recent paper by H. Hesse, R. Jank-Ladwick and H. Strotmann (Z.f. Naturf. 31c, 445–451, 1976) describes the effects of $Ca^{2+}$ on the resolution and reconstitution of phosphorylation.

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