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

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Received 19 August 1976

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 ΔpH [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 μmol of ATP are hydrolyzed per mg Chl × h at 37°C by summer spinach, whereas with saturating light even at 22°C ATP synthesis up to 1400 μmol/mg Chl × 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 ΔpH 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 ΔpH (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).

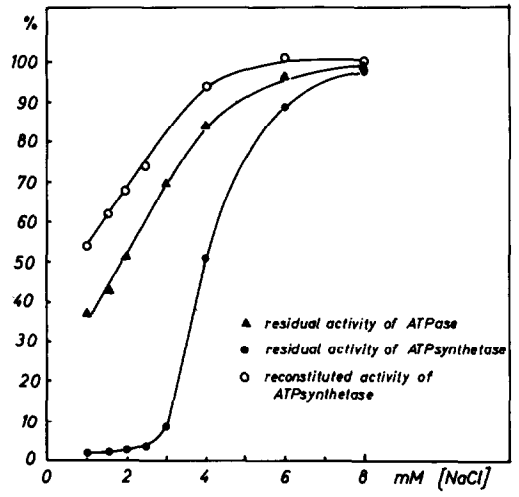

Fig.1. Dependence of residual activity of photosynthetic ATP-synthetase, residual activity of Ca^{2+}-ATPase, and reconstituted ATP-synthetase on the salt concentration during EDTA-treatment. (Thylakoid suspension was diluted 1:20 to 0.1 mg Chl/ml with 0.75 mM EDTA, and the pH adjusted to 7.2 with NaOH; 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

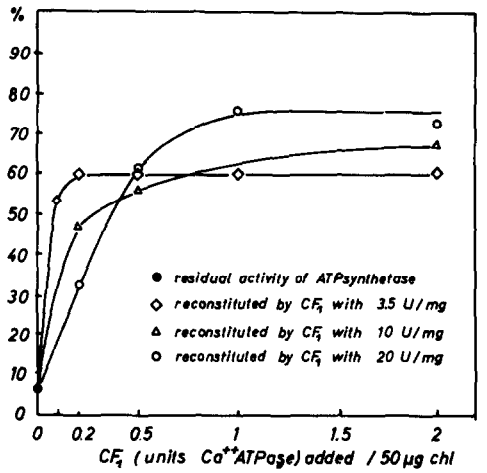

Fig.2. Dependence of reconstituted activity of photosynthetic ATP-synthetase on the amount of CF_1 added. (EDTA treatment, reconstitution and measurement of PMS mediated cyclic photophosphorylation as in fig.1). 100% control rate: 920 µmol ATP/mg Chl × h at 7 × 10^5 erg/cm² × s.

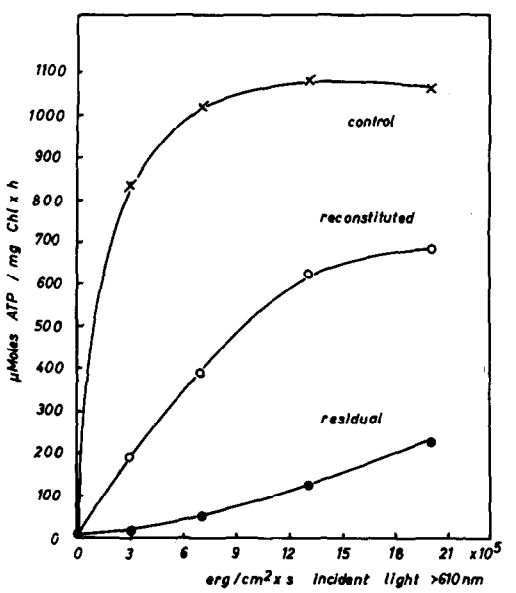

Fig.3. Dependence of photophosphorylation on incident light intensity. (EDTA treatment, reconstitution and measurement of PMS mediated cyclic photophosphorylation as in fig.1). Residual activity of Ca^{2+}-ATPase was below 40% of the control; specific activity of CF_1 preparation used for reconstitution 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-

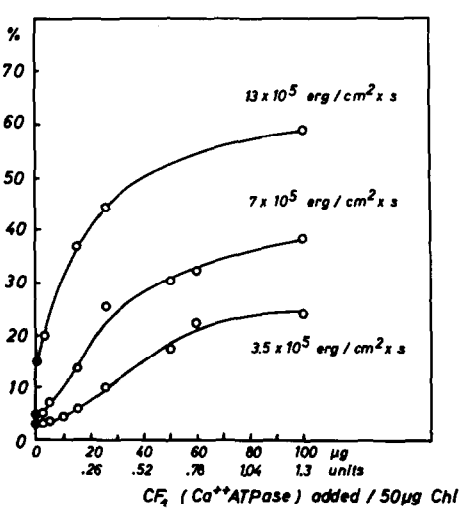

Fig.4. Dependence at different light intensities of reconstituted activity of photosynthetic ATP-synthetase on the amount of CF_1 added. (EDTA treatment, reconstitution and measurement of PMS mediated cyclic photophosphorylation as in fig.1). Phosphorylation rate is shown in % of the activity of untreated 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 7×10^5 erg/cm^2 × s, which saturate the control, to 20×10^5 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 δ and ϵ 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

The accurate and skillful technical assistance of Miss M. Tobien is much appreciated. A financial support by the Deutsche Forschungsgemeinschaft is acknowledged.

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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