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THE COUPLING FACTOR OF PHOTOPHOSPHORYLATION AND THE ELECTRIC PROPERTIES OF THE THYLAKOID MEMBRANE

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SUMMARY

The rate of ATP synthesis of illuminated chloroplasts is correlated with the electric conductance of their inner membranes. In agreement with previous studies it is shown that ATP synthesis is paralleled by an increased conductance of the thylakoid membrane. This conductance together with the ability to form ATP is abolished if chloroplasts are treated with an antibody against the coupling factor CF_1. It is not influenced by the fragmented monovalent antibody. This parallels the lack of influence of the fragmented antibody on ATP synthesis in contrast to its influence on hydrolysis and exchange reactions. We conclude that there are different sites for the interaction of the coupling factor with adenine nucleotides.

Extraction of the coupling factor is shown to increase the membrane conductance by more than two orders of magnitude. Reincorporation of the crude coupling factor partially restores the net conductance of the membrane (increase in resistance by a factor of 2.5), while a higher degree of restoration was observed for ATP synthesis and the proton conductivity of the membrane. We conclude that the extraction procedure opens different conductive channels in the membrane; a proton specific one, possibly associated with the binding protein for the coupling factor, plus other channels for "non-protons" which in contrast to the proton channel cannot be plugged by reincorporation of the coupling factor.

INTRODUCTION

The operation of a membrane-bound reversible ATPase in ATP formation and hydrolysis by chloroplast membranes is well established [1]. Extraction and reincorporation of this protein, designated CF_1, results in loss and restoration of the phosphorylating capacity of chloroplast membranes, respectively [2–5]. The ability of

Abbreviations: CF_1, coupling factor 1 for photophosphorylation; PMS, *N*-methyl phenazonium methosulfate.

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chloroplasts to take up protons under illumination is also abolished and restored by this treatment [6].

The isolated CF_1 protein has a multiple subunit structure with a total molecular weight of about 325 000 [7, 8]. CF_1 exhibits ATPase activity if properly activated [3, 7, 9]. An antiserum against CF_1, first prepared by McCarty and Racker [6], was shown to inhibit ATP formation, ATP hydrolysis and ATP $\leftrightarrow$ P_i exchange reactions in chloroplasts but had virtually no effect on electron transport or the light dependent proton uptake. It has been shown that a purified antibody against CF_1 abolishes the accelerated proton efflux and prevents the inhibition of electron transport by ATP in chloroplasts [10]. Another antibody against CF_1 (lettuce) was elicited in rabbits. This inhibited ATP synthesis, hydrolysis, ATP $\leftrightarrow$ P_i exchange reactions and coupled electron transport in chloroplasts [11, 12]. However, monovalent fragmented antibodies prepared from the same immunoglobulin fraction inhibited ATP formation only slightly, while ATP hydrolysis and ATP $\leftrightarrow$ P_i exchange reactions were strongly inhibited.

Studies on the electrochroic absorption change around 520 nm on flash excitation of chloroplasts revealed that the decay rate of these absorption changes reflects the electric conductance of the membrane [13–15]. It was demonstrated that the decay rate of these absorption changes is accelerated if ATP is synthesized [16, 17]. Moreover, partial dissipation of the electrochemical energy reduced ATP synthesis [17, 18]. Extraction of CF_1 increased the electrical conductivity and the proton permeability of the thylakoid membrane by two orders of magnitude [19]. These effects were at least partially reversed by reincorporation of CF_1 into extracted chloroplasts [19, 20].

In this communication we used an extraction procedure which gives a high degree of reconstitution [4]. We describe the effect of removal and reincorporation of CF_1 on the decay kinetics of the electrochroic absorption change, on proton translocation and ATP formation in chloroplast membranes. It is attempted to discriminate between specific effects due to extraction of membrane-bound CF_1 and unspecific side effects due to the extraction of other membrane components.

The effect of intact and fragmented anti-CF_1 on the electric properties of the membrane has also been investigated.

We find that the high degree of restoration in phosphorylating activity (continuous light) after reincorporation is paralleled only by partial restoration of the intrinsically low electric conductance of the membrane. Based on the observations on the influence on proton-carrying and non-proton-carrying ionophores on the phosphorylation rate under continuous light [17, 21] it is concluded that extraction of CF_1 not only increases the conductance for protons but also increases the conductance for other ions ("non-protons"). The first effect is largely reversible by reincorporation of CF_1. The second, a side effect can only be weakly reversed by reincorporation.

Treatment of chloroplasts with antibody against CF_1 is shown to inhibit both phosphorylation and the accelerated decay of the electric potential under flash excitation in the presence of ADP. This confirms the dependence of ATP-synthesis on proton translocation down the electric gradient under these conditions.

MATERIALS AND METHODS

Isolated chloroplasts were prepared from greenhouse-grown spinach leaves using standard procedures [4, 22]. Resolved and CF_1-reincorporated particles were prepared as described [4].

Absorption changes of chloroplast preparations suspended in an aqueous medium were measured in a rapid kinetic spectrophotometer [23]. The samples were illuminated with saturating Xenon-flashes (half-time of duration $\tau_{\frac{1}{2}} = 15$ μs, wavelength interval 630 to 680 nm) short enough to turn over each light reaction only once ("single turnover flash"). To improve the signal-to-noise-ratio the signals were excited repetitively by periodic flashes and averaged in a CAT 1000 on-line computer [24].

For the experiments depicted in Figs. 1 and 2 we used a 2 cm cuvette containing 15 ml of the following reaction mixture: 10 mM KCl, 67 μM benzylviologen as artificial electron acceptor and 1 mM tricine pH 8. For the pH measurements the buffer was replaced by the indicator cresol red at a final concentration of 30 μM [25].

Anti-CF_1 (lettuce) immunoglobulin fractions were prepared as described [11]. Fragmented anti-CF_1 was prepared from the immunoglobulin fraction by papain digestion [26]. Chloroplasts, containing 50 nmol of chlorophyll in 10 mM tricine pH 8, were incubated in a final volume of 0.3 ml for 5 min at 0 °C with the indicated amounts of anti-CF_1. Afterwards the incubation medium was suspended in 4.7 ml of the final reaction medium: 20 mM tricine pH 8, 20 mM KCl, 3.3 mM inorganic phosphate, 50 μM benzylviologen and 0.5 mM ADP as indicated.

Photophosphorylation was determined by the $^{32}P_i$ method [22] in a volume of 3.1 ml containing: 60 μmol tricine pH 8, 45 μmol KCl, 10 μmol $MgCl_2$, 10 μmol P_i (containing 10 μC ^{32}P), 4 μmol ADP pH 8, 0.03 μmol PMS and 0.2 μmol chlorophyll. It was illuminated for 1 min with white light at 20 °C. In the case of Table 1 photophosphorylation was determined enzymatically [27, 28].

RESULTS

Extraction and reincorporation of CF_1

The loss of ATP formation capacity upon extraction of chloroplasts with EDTA, depends on the particle concentration during incubation for CF_1 removal. In Fig. 1 the activities of resolved particles after EDTA extraction at several chlorophyll concentrations are compared. Extractions at high chlorophyll concentrations diminished the loss of ATP formation capacity under continuous illumination. This was paralleled by only partial inhibition of the alkalisation in the outer phase and by a less accelerated rate of relaxation of the electrochroic absorption change at 520 nm. Particles extracted at low chlorophyll concentrations, having almost completely lost their ATP synthesizing capacity, show a net acidification rather than alkalisation in single flash pH measurements with benzylviologen as electron acceptor (see also refs. 19 and 30). Reincorporation of supernatants containing CF_1 and Mg^{2+} into high chlorophyll extracted particles resulted in reconstituted particles with a rate of phosphorylation of 78 percent as compared with the non-treated chloroplasts. However, as shown in Fig. 2, the restoration of ATP synthesis under continuous light was paralleled by a less efficient restoration of the pH change and of the relaxation kine-

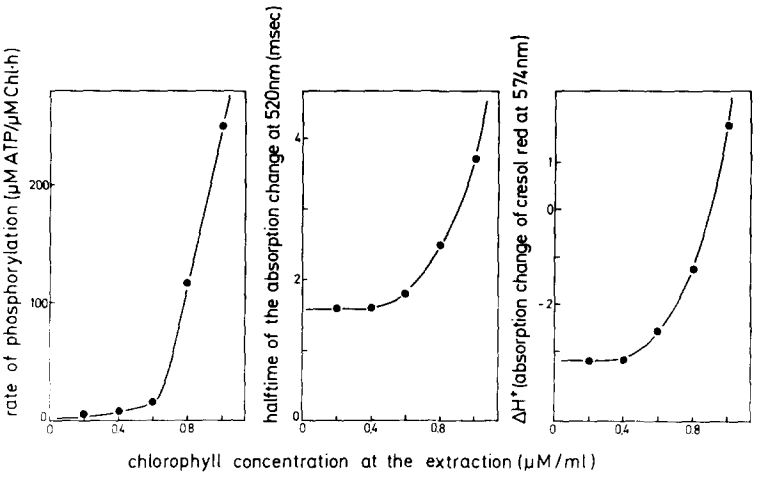

Fig. 1. Dependence of the phosphorylation rate (continuous light), the half time of the decay of the electrochroic absorption change at 520 nm and the pH amplitude indicated by the pH-indicating dye cresol red (both after flash excitation) upon the concentration of chlorophyll during removal of CF_1. The resolved particles were obtained by suspending the isolated chloroplasts in 0.4 M sucrose and 1 mM EDTA (pH 7.8) at the indicated chlorophyll concentration. After 10 min. incubation at 4 °C they were centrifuged for 15 min at 20 000 × g, washed once in 0.4 M sucrose and resuspended in a medium containing 0.4 M sucrose, 1 mM $MgCl_2$ and 1 mM tricine pH 8. For the measurements of the absorption change and ATP formation see Materials and Methods. The activities of the control chloroplasts (treated at 0.6 μM chlorophyll/ml with 0.4 M sucrose, no EDTA but 1mM tricine pH 8 and 1 mM $MgCl_2$): $\dot{A}TP = 423$ μM ATP/μM Chl · h, $\tau_{\frac{1}{2}}^{520} = 22$ ms, pH $= 4 \cdot 10^{-4}$.

tics of the 520 nm absorption change after single flash excitation. Although the decay rate of the electrochroic absorption change was not slowed down perfectly to the rate of the control, the result is qualitatively satisfying. Under conditions where the ATP rate was restored to 78 percent of the control, the decay rate of the electric field was slowed down by a factor of 2.3 as compared with extracted chloroplasts, although it still remained 4 times more rapid than the control.

Further investigation of the "reversed" pH change observed in extracted chloroplasts, not observed in steady state illumination with PMS or pyocyanine as cofactor [4] but evident with PMS in NaBr extracted chloroplasts [29], showed the net production of one half to one proton per electron. One likely possibility investigagated was that the extraction procedure deactivates the superoxide dismutase [30]. Thus in the case of benzylviologen as acceptor, O_2^- instead of H_2O_2 would be the terminal reduced product. This is indeed the case since addition of superoxide dismutase to an extracted chloroplast preparation eliminates the acidification. These results are reported elsewhere [30].

Antibodies against CF_1

It has been reported that the decay of the electric field under flash excitation is accelerated if ATP is synthesized [16, 17, 31]. We were curious as to the effect of the antibody against CF_1 on the special conductance channel associated with ATP synthesis. Fig. 3 summarizes the result of the action of anti-CF_1 in relation to the kinetics of the absorption changes at 520 nm.

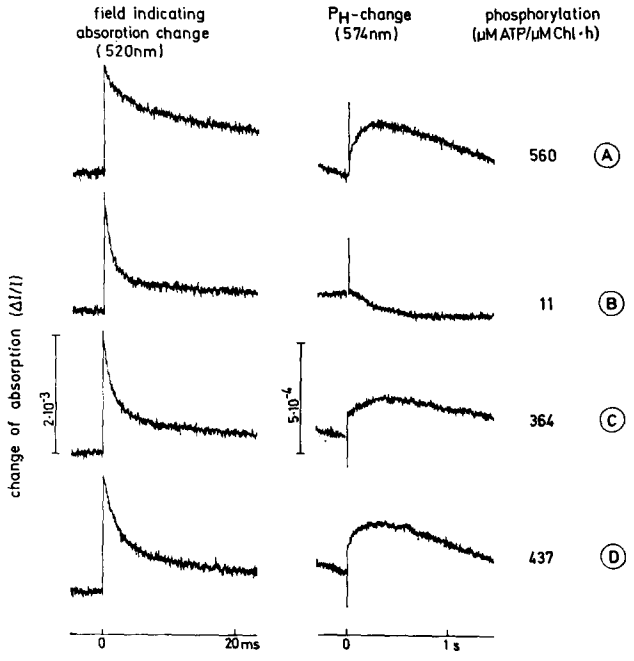

Fig. 2. Restoration of phosphorylation, the decay of the absorption change at 520 nm and the alkalisation in the outer phase after reincorporation of supernatant to EDTA extracted chloroplasts. (A) control; (B) extracted at 0.5 μM Chl/ml; (C) 0.5 equivalents supernatant; (D) 1 equivalent supernatant added. Equivalent means that the separated extracted chloroplasts are incubated with their respective supernatant in the presence of 10 mM $MgCl_2$. 0.5 equivalent means an analogue incubation in a supernatant diluted 1 : 1 with 0.4 m sucrose.

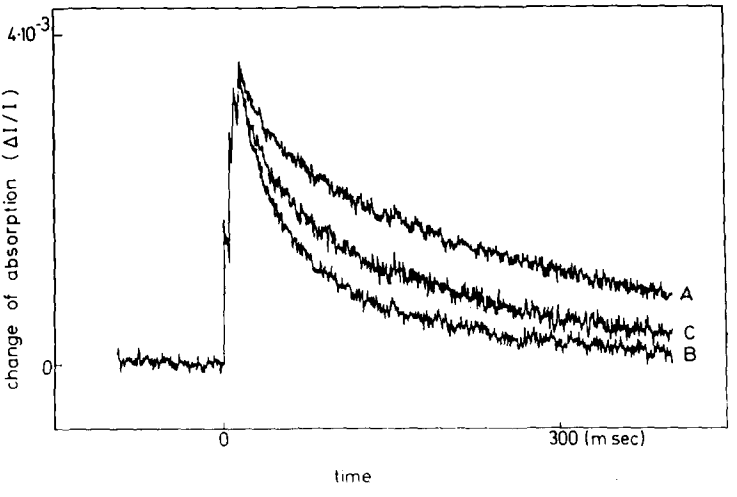

Fig. 3. Time course of the absorption changes at 520 nm on excitation with short flash groups. A group of four flashes was given in a time interval of 18 ms with a repetition rate for the group of 0.1 s^{-1} (A) chloroplasts incubated without anti-CF_1, decay rate without added ADP; (B) chloroplasts incubated without anti-CF_1 prior to sampling, decay rate with added ADP; (C) chloroplasts incubated with 100 μg anti-CF_1, decay rate with added ADP.

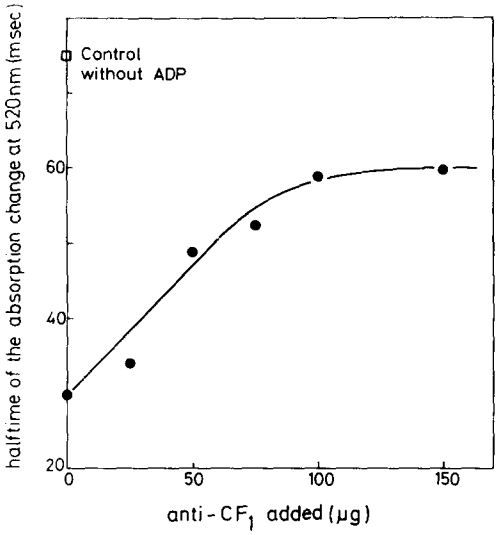

Fig. 4. Half time of the decay of the electrochroic absorption change at 520 nm in dependence on the amount of anti-CF_1 added during incubation. The control was measured without added ADP. In this case incubation with anti-CF_1 has no effect.

In the control experiment without ADP and anti-CF_1 the decay of the electrochroic absorption change is slow. After addition of ADP there is marked acceleration. However, after incubation of chloroplasts with anti-CF_1 the acceleration by ADP is reversed to 70 percent. On the other hand, incubation with anti-CF_1 had practically no effect on the absorption change in the absence of ADP.

A titration experiment of the acceleration of the decay of the electrochroic absorption change with increasing amounts of antibodies is depicted in Fig. 4. We find that anti-CF_1 is a much more specific agent for the reversal of the ADP+P_i induced increase of the conductivity than other poisons like phloridzin or Dio-9. In contrast to anti-CF_1, these agents sometimes accelerate the electric field decay even more.

TABLE I

EFFECT OF MULTIVALENT AND FRAGMENTED ANTIBODIES ON THE ACCELERATION OF THE DECAY OF THE ABSORPTION CHANGE AT 520 NM BY ADP (EXCITATION BY A SHORT GROUP OF FLASHES) COMPARED WITH THEIR EFFECT ON LINEAR PHOSPHORYLATION FROM H_2O TO BENZYLVIOLOGEN (CONTINUOUS LIGHT; ENZYMATIC ASSAY).

	Half time of the decay of the absorption change at 520 nm (ms)	Phosphorylation rate (continuous light) (mμM ATP/μM Chl · s)
Chloroplasts	30	65.3
Chloroplasts + 100 μg anti-CF_1	61	21.2
Chloroplasts + 200 μg fragmented anti-CF_1	37	54.8

The effect of fragmented anti-CF_1 on the acceleration of the decay of the absorption change at 520 nm with ADP is compared with the effect of multivalent anti-CF_1 (Table I). In agreement with prior studies [12] it is evident that the fragmented antibody is a less potent inhibitor of photophosphorylation. This is paralleled by the small effect on the ADP-induced acceleration of the electric field decay.

DISCUSSION

The results presented above relating to the effect of extraction and reincorporation of CF_1 into chloroplast membranes on the phosphorylation rate in continuous light, the total electric conductance and the proton permeability, clearly demonstrate that extraction opens up a highly conductive channel in the membrane. It can be plugged up again by reincorporation of CF_1 (in agreement with prior studies [19, 20]). This provides evidence that it is associated with the receptor site, possibly the binding protein, for CF_1 in the membrane and not just due to an unspecific damage of the membrane during the extraction procedure.

The increase of the electric conductance by two orders of magnitude upon extraction, paralleled by an increase of the proton permeability, makes it very probable that the conducting channel opened upon extraction of CF_1 is specific for protons. Prior studies support this conclusion since no specificity for any other ion was revealed [19].

One side effect of the extraction procedure which masked the effect on the proton uptake could be attributed to the concomitant deactivation of the superoxide dismutase as mentioned above [30].

The parallelism between restoration of photophosphorylation (continuous light) after reincorporation of CF_1 and the restoration of the slower decay of the electric potential was quantitatively less satisfactory. However, this cannot be considered as an argument against the correlation between ATP synthesis and an extra proton flux down the electrochemical gradient as demonstrated in prior studies [17]. One result from these studies bears on the apparent imperfect correlation between ATP synthesis (continuous light) and the electric conductance (flash excitation) reported in this paper.

It was shown that valinomycin while increasing the potassium permeability of the thylakoid membrane competitively inhibits the synthesis of ATP upon flash excitation of chloroplasts [17]. However, it does not inhibit ATP-synthesis in continuous light [16, 20]. This can be explained as follows; introduction of a potassium channel into the thylakoid membrane by valinomycin dissipates the electric part of the electrochemical gradient induced by flash excitation. Since the valinomycin induced conductance and the synthesis of ATP compete for this energy, ATP synthesis is inhibited under these conditions. In contrast, under continuous light, valinomycin should not affect the steady state level of the electric potential or of the pH difference across the membrane, both of which determine the capacity for ATP synthesis. This is because the electrochemical potential of the proton is determined by the dynamic balance between the rate of electrogenic proton pump and the proton permeability of the membrane. Valinomycin does not act on either of these variables but only on the potassium permeability.

In analogous fashion the apparent discrepancy in the degree of the restoration

of the electric conductance and of the ATP yield can be understood as follows; reincorporation of the coupling factor to extracted chloroplasts largely restores the low proton permeability of the thylakoid membrane. However, it does not fully plug up the overall electric leak conductivities. In comparison to control chloroplasts reconstituted chloroplasts are more leaky, the leak in conductivity being mainly due to ions other than protons. Thus reconstituted chloroplasts resemble chloroplasts treated with valinomycin for higher potassium conductivity. It is probable that the leaks which are not abolished upon reincorporation are due to unspecific damage of the membrane structure during the extraction and reincorporation procedure but not to the intrinsic properties of the enzyme system.

The inhibition by anti-CF_1 of the ADP accelerated decay of the electric potential gives evidence that the synthesis of ADP under flash excitation is coupled to a translocation of protons across a special pathway through CF_1 as postulated by Mitchell [32]. Interaction of CF_1 with fragmented anti-CF_1, shown to affect mainly ATP-utilizing reactions but not the decay rate of the electric potential or ATP synthesis, may reflect the nature of two different catalytic sites on CF_1, one specialized for ATP synthesis and another one for ATP-utilizing reactions.

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