A Study of State Changes in Chlorella: The Effect of Uncoupler and Energy Transfer Inhibitors

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Pre-illumination of *Chlorella* cells at room temperature with light primarily absorbed by PSII (650 nm) produces a state called state II. This is characterized by high fluorescence at 715 nm (high F715/F685) at liquid nitrogen temperature. Alternately if the cells are pre-illuminated by light primarily absorbed by PSI (710 nm) then state I with high fluorescence at 685 nm (low F715/F685) at 77K is produced. We have investigated the role of photophosphorylation in the development of state I/II in chlorella cells using uncoupler and energy transfer inhibitors. The results suggested that the development of state I depends upon the energization of the membrane. Both DCCD and TPTC which permit build up of proton gradient block the adaptation to state I indicating that proton gradient formation alone is not sufficient to develop state I. The data obtained by us as also published in the literature indicate that redox states of the electron transport carriers are responsible for the development of state II.

Introduction

The non-cyclic electron transport responsible for ATP and NADPH production is driven by the functioning in series of two photosystems. Besides, the cyclic phosphorylation catalyzed by photosystem I alone, produces additional ATP. The efficiency of photosynthesis measured as the rates of oxygen evolution will depend upon the relative rates of electron transport catalyzed by both the photosystems. The photosynthetic organisms such as algal cells achieve higher efficiency of photosynthesis by two ways under given light and temperature conditions:

(a) they synthesize the antenna pigments necessary for light harvesting by each of the photosystems such that the two light reactions are optimally balanced under the given growth conditions and (b) they regulate the distribution of the excitation energy between the two pigment systems such that part of the excitation energy arriving at the over-driven photosystem is diverted to the photosystem which is being driven at a slow pace. The latter operates as a fine tuning mechanism for efficient photosynthesis and has been the subject of intensive studies [1–3].

Abbreviations: CCCP, Carbonyl Cyanide *m*-chlorophenyl hydrazone; DCCD, dicyclohexyl carbodiimide; DCMU, 3-(3′,4′-dichlorophenyl)-1, 1-dimethyl urea; LHCP, light harvesting chlorophyll protein; PS, photosystem; TPTC, triphenyl tinchloride.

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The algal cells can be pre-conditioned by exposure to light primarily absorbed by PSII (light II) to produce a state called state II which is characterised by diversion of part of the excitation energy arriving at PSII to PSI. This results in increased efficiency of PSI reactions. Alternately if the algal cells are pre-illuminated by light primarily absorbed by PSI (light I) the organism acquires a state I characterised by diversion of more excitation energy to PSII. The state I/II phenomena have been explained by "α-hypothesis" according to Bonaventura and Myers [4] or by "spill-over hypothesis" suggested by Murata [5]. Irrespective of which hypothesis is preferred it is necessary to explain what types of modifications undergone by the photosynthetic membranes result in changes in the distribution of excitation energy between the two pigment systems. Since light I or II induces both redox changes and ATP synthesis it is evident that a conformational change brought about by light I or II through these processes may be responsible for adaptation to state I/II. In the present communication, we have investigated the relationship between the development of state I/II and the processes related to ATP synthesis by using uncoupler and energy transfer inhibitors. Our data demonstrate that energization of the membrane is essential for the development of state I. However, formation of "proton gradient" alone is not enough to produce state I. It appears that the state II is a relaxed state and may be brought about by redox changes.

Materials and Methods

Chlorella vulgaris was grown under autotrophic conditions at room temperature by bubbling air through it. The light was provided by 4 fluorescent tubes (20 W each). The algal culture grown for 24 h and in the log phase was used for all the experiments. The pre-illumination of algal cells was achieved by exposing the sample (3 – 5 ml) to either light I (obtained by passing the white light from a 500 W projector lamp through a Schott interference filter, 710 nm, 47.5% transmission) or light II (using 649.2 nm interference filter, 39.5% transmission). During pre-illumination the sample was maintained at 25 °C. In all the cases, the algal sample was allowed to relax for 10 min in the dark prior to pre-illumination.

The measurement of state I/II was done by monitoring the fluorescence emission of the sample at liquid nitrogen temperature using Aminco-Bowman spectrophotofluorometer equipped with low temperature accessory (quartz dewar) and an X-Y recorder. The pre-treated sample was taken in a 3 mm dia glass-tube open at both ends and quickly frozen to liquid nitrogen temperature. The sample tube was inserted in a quartz dewar containing liquid nitrogen. The sample was excited by 440 nm and the emission spectrum from 650 to 750 nm was monitored. The ratio of peak heights at 715 nm and 685 nm (uncorrected) was calculated and used to indicate the development of state I/II as done earlier by others [5 – 7]. At low temperature the emission at 685 nm primarily originates from chlorophylls of PSII and hence indicates the extent of excitation energy arriving at the PSII reaction centre. The emission peak at long wavelength at low temperature originates in the chlorophylls of PSI and hence indicates the extent of excitation energy arriving at PSI reaction centre.

The optical density of the sample was monitored on Aminco DW-2A spectrophotometer and was less than 0.05 in a 1 cm path cuvette at 678 nm. The oxygen evolution of the sample was studied using Gilson oxygraph. The chemicals DCCD, TPTC, CCCP and DCMU were dissolved in 100% ethanol and added to the sample to give a desired concentration. In the sample the concentration of ethanol did not exceed 0.5% in any experiments.

Results and Discussion

The fluorescence emission spectra of algal cells are considerably affected by the pre-conditioning of the cells. The dark adapted or light II pre-treated sample shows similar spectra characterised by increased emission at 715 nm relative to 685 nm (Fig. 1 A and C). The pre-treatment of the sample by light I results in enhanced emission at 685 nm relative to 715 nm (Fig. 1 B). The data show that the pre-treatment with light I drives algal cells to state I whereas light II or dark incubation produces state II. The results are similar to those obtained by earlier workers [4 – 6, 8 – 11]. The similarity of emission spectra of dark adapted and light II adapted algal cells suggests that algal cells in the dark acquire a state which is similar to state II. Thus state II appears to be identical to a relaxed state. The ratio of F715/F685 for samples adapted to state II ranges between 1.0 to 1.2 whereas for algal cells in state I it is 0.8 or less.

The addition of DCMU to the algal cells prior to preillumination shows interesting changes (Table I). In these cells even the light II results in production

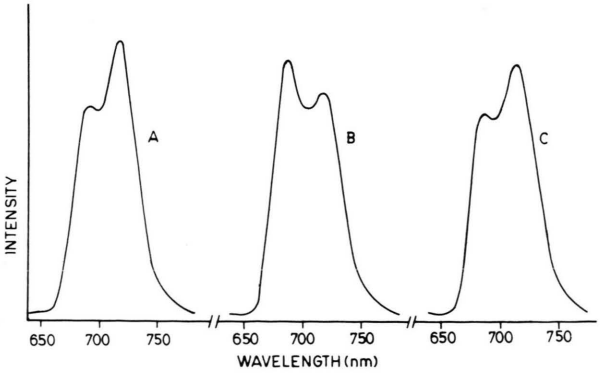

Fig. 1. Fluorescence spectrum of *Chlorella* at 77 K. A: Dark adapted; B: light I adapted; C: light II adapted.

Table I. Effect of DCMU on the development of state I/II in *Chlorella.*

Treatment	Pre-illumination (10 min)	Ratio F715/F685
nil	dark	1.3
nil	light I	0.8
nil	light II	1.0
DCMU	light II	0.7
DCMU	light II followed by dark	0.9

Note: DCMU concentration used was 1.5×10^{-5} M.

of state I with a ratio F715/F685 equal to 0.7. This ratio changes to 0.9 in the dark following the light II treatment. Thus the algal cells seem to be driven to state II in the dark even in the presence of DCMU. The concentration of DCMU used totally blocked oxygen evolution (data not shown). It may therefore, be assumed that non-cyclic electron transport must have been blocked. That the algal cells in the presence of DCMU are driven to state I by even light II was also observed earlier by Duysens [12] and Williams *et al.* [13]. It is known that in light I the cyclic photophosphorylation predominates with little, if any, non-cyclic electron transport. It is therefore, suggested that phosphorylation associated with the cyclic flow may be responsible for driving algal cells to state I in light I. The situation in the presence of DCMU and light II can be examined against this background. In DCMU treated material as there is no oxygen evolution, non-cyclic electron flow can be presumed to be completely blocked. However, the chlorophylls of PSI do absorb light II and can carry out cyclic photophosphorylation in the presence of DCMU. It is thus possible to explain development of state I even in light II and DCMU by suggesting that cyclic phosphorylation is responsible. The fact that in the dark it is driven to state II once again indicates that state II is similar to the relaxed state.

If it is true that phosphorylation is essential for adaptation to state I we should not get the development of state I in the presence of uncouplers of photophosphorylation in light I. It has been shown that CCCP is an uncoupler of photophosphorylation. This uncoupler at μM concentration was found to totally block CO_2 dependent oxygen evolution (data not presented) indicating that it enters the *Chlorella* cells and prevents ATP synthesis. Data in Table II show that at 3 μM concentration, it completely blocks the development of state I in light I. This observation is consistent with the proposal that for development of state I, conditions leading to phosphorylation are necessary. An interesting observation made was that cells that had already acquired state I in light I when subsequently treated with CCCP and left in the dark could not acquire state II (Fig. 2A). However, they were able to adapt themselves to state II in light II (Fig. 2B). Thus, in the presence of an uncoupler, dark state does not seem identical to state II and that state II can be developed by light II even if conditions do not permit ATP synthesis.

It is known that CCCP functions as an uncoupler by destroying the proton gradient. The data presented above, thus, do not tell whether the accumulation of proton gradient and membrane conformational changes associated with it are necessary for development of state I, or whether any other step involved in ATP synthesis is essential for it. In order to understand the involvement of proton gradient formation in developing state I, two other chemicals viz. DCCD and TPTC were used. Both these are known to inhibit ATP synthesis in isolated chloroplasts by blocking movement of protons through the proton channel of CF_0. They however allow the build up of proton gradient [14–15]. Thus if proton gradient formation alone would be enough for the development of state I, we would observe adaptation to state I in the presence of these energy transfer inhibitors. Data presented in Figs. 3 and 4 show that the algal cells could not acquire state I in

Table II. Percentage state change in *Chlorella* treated with different concentrations of CCCP.

CCCP Conc. [μM]	State I to state II in dark	State II to state I in light I
0.00	100.0	100.0
0.30	92.5	92.5
1.50	58.0	84.0
2.55	17.0	22.5
3.00	0.0	0.0

In studies on transfer from state I to II the cells were allowed to develop state I in light I. CCCP was then added and the samples left in the dark. In the second set of experiments involving adaptation to state I from state II the dark adapted sample was treated with CCCP and then transferred to light I. The change in the ratio of long wavelength to short wavelength fluorescence in the absence of uncoupler in light II and light I was considered as 100%.

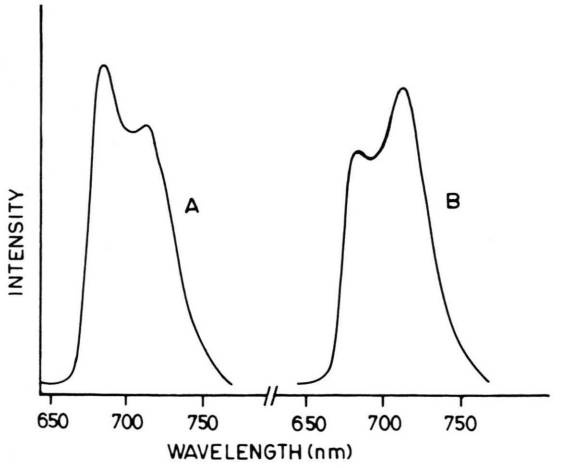

Fig. 2. Fluorescence spectrum of *Chlorella* at 77 K. A: light I or light I + dark in the presence of 3 μM CCCP. B: light I + 3 μM CCCP + light II.

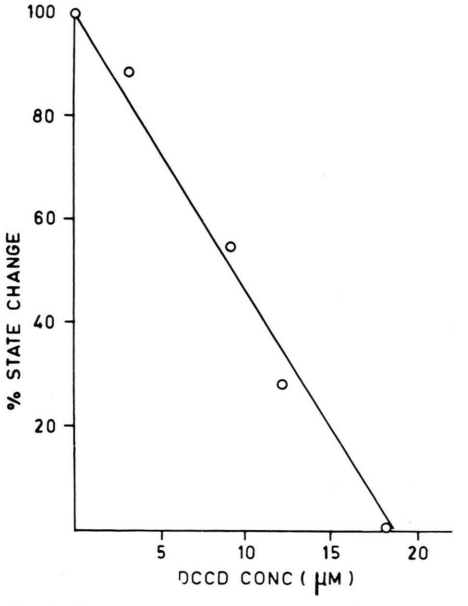

Fig. 3. Percentage state change in *Chlorella* cells from dark adapted state (similar to state II) to state I in the presence of different concentrations of DCCD.

light I in the presence of these compounds. At less than 20 μM DCCD (Fig. 3) and 4 μM TPTC (Fig. 4) the development of state I was totally blocked. It was therefore, concluded that proton gradient formation alone is not enough for the development of state I and some other processes associated with ATP synthesis may be necessary for its development. We have observed that CO_2 dependent oxygen evolution is completely blocked by 20 μM DCCD and 4 μM TPTC (data not shown) indicating that these compounds do penetrate the cell and inhibit oxygen evolution.

When the cells were allowed to develop state I and then DCCD was added, the state II could not be developed in the dark as is evident from Fig. 5 A. The ratio F715/F685 remained low. However, the cells were able to acquire state II in light II (Fig. 5 B). Identical results were obtained with TPTC. This observation is similar to that made in the case of the uncoupler CCCP, and once again suggests that the dark state is not always identical to state II. The fact that light II can drive the algal cells to state II even when photophosphorylation is blocked by either uncouplers or energy transfer inhibitors shows that for the development of state II, photophosphorylation is not essential.

The data presented in this communication bring out 3 major points concerning the development of state I/II.

1. Under normal conditions the state II is similar to the 'relaxed' state.
2. State I development depends upon some processes other than proton gradient formation associated with phosphorylation.

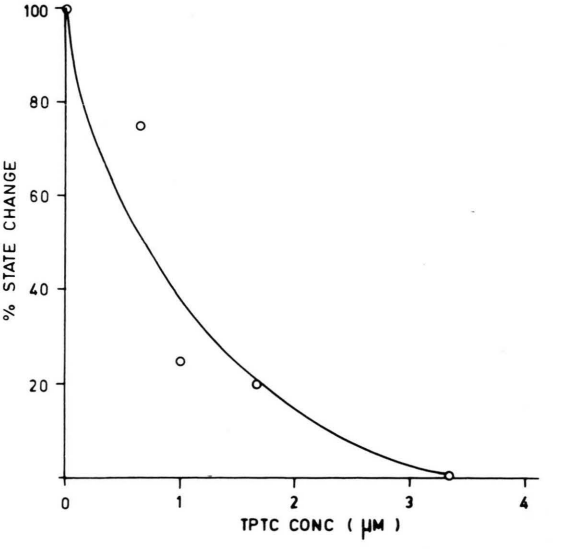

Fig. 4. Percentage state change in *Chlorella* cells from dark adapted state (similar to state II) to state I in the presence of different concentrations of TPTC.

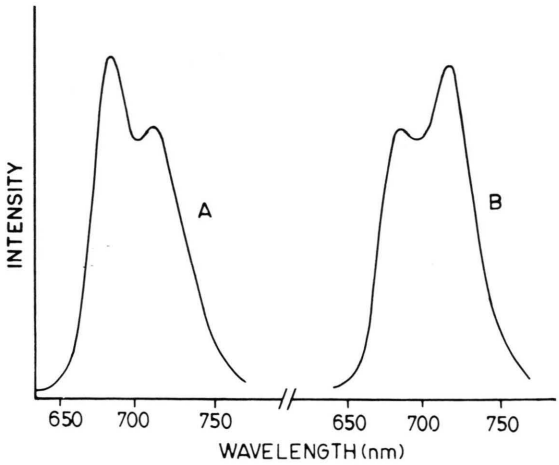

Fig. 5. Fluorescence spectra of *Chlorella* at 77 K. A: light I or light I + dark in the presence of 20 μM DCCD. B: light I + DCCD + light II.

3. State II development proceeds in the absence of any processes associated with photophosphorylation but light II is essential particularly when ATP synthesis is blocked.

The first point is in agreement with several earlier reports. The second and third conclusions are newer observations and need to be discussed in the light of suggested involvement of LHCP phosphorylation in excitation energy distribution in chloroplasts [16]. The data presented here show that energization of the membrane leading to ATP synthesis is necessary for the development of state I only. Therefore, state I is an energized state and may involve LHCP phosphorylation. However, it has been shown by Allen and Bennett [17] that LHCP phosphorylation is DCMU sensitive. The results obtained by us show that state I is developed in the presence of DCMU. Thus, state I cannot be associated with LHCP phosphorylation. The question of whether state II development is associated with LHCP phosophorylation could be examined. Our results show that light II induces a state II in algal cells even in the presence of uncoupler CCCP or energy transfer inhibitors DCCD and TPTC. If ATP is not available to the cells the LHCP phosphorylation cannot proceed because it is catalyzed by a kinase using the substrate ATP. Therefore, the development of state II even in the presence of inhibitors of ATP synthesis leads one to conclude that LHCP phosphorylation may have nothing to do with the development of state II. It may be mentioned here that ATP required for phosphorylation of LHCP in intact cells cannot come from mitochondrial electron transport either, because CCCP, DCCD and TPTC are also inhibitors of mitochondrial ATP synthesis.

There is one observation made by Bennett [18] which is relevant in this connection. He has shown that LHCP is phosphorylated in light even in the presence of CCCP if ATP was added externally. Thus if prior to addition of CCCP, DCCD or TPTC, the cell had an accumulated reservoir of ATP one could still expect LHCP phosphorylation in light II in the presence of ATP synthesis inhibitors. This possibility indeed exists in our experimental procedure since we had pre-illuminated cells in light I before adding the inhibitors. Thus cyclic phosphorylation may have produced enough ATP which could be subsequently used for LHCP phosphorylation and this may be related, if not responsible for state II development. If the LHCP phosphorylation was responsible for the development of state II, it becomes difficult to reconcile the observations that (a) state II or a membrane conformation giving identical fluorescence pattern as state II does develop in the dark and (b) LHCP phosphorylation does not proceed in the dark. In fact LHCP is dephosphorylated in the dark [19]. These considerations suggest that LHCP phosphorylation is not always related to state II development. Considering that under certain conditions (*e.g.* in the presence of CCCP, DCCD or TPTC) dark incubation does not develop state II while light II can still induce state II, it is reasonable to conclude that redox changes brought about by light II are responsible for adaptation to state II. These redox changes may bring about certain membrane conformational changes that are essential for the development of state II.

Recent studies by Ried and Reinhardt [11] on quantum requirement of state I/II transition led them to conclude that the state change is due to the redox state of an electron carrier located near the middle of the electron transport chain. Also Ley and Butler [7] after a careful consideration of different observations suggested that a certain component linking PSII and PSI must be reduced to develop state II. It thus appears that redox changes rather than LHCP phosphorylation may be responsible for the development of state II.

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