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A COMPARATIVE STUDY OF PHOTOSYNTHETIC ELECTRON TRANSPORT IN ALGAL CELLS AND SPINACH CHLOROPLASTS

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SUMMARY

Steady-state relaxation spectroscopy provides absorption data which may be used to calculate the rates of electron flow through intermediates of the electron transport chain. In chloroplasts, the calculated rates for P_{700} agree well with measured Hill reaction velocities.

The relaxation times of cytochrome and P_{700} are consistent with current theories of sequential electron transport from cytochrome to P_{700} in chloroplasts, but not in whole cells of a red and of a blue-green alga.

3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) inhibits all activity in chloroplasts while in intact cells electron transport through P_{700} and cytochrome continues even at a concentration of the inhibitor which eliminates O_2 evolution.

With 620-nm light, the amplitude of oxidized cytochrome increases upon addition of DCMU partly because its reduction rate declines and partly because quanta are diverted from Photosystem II to Photosystem I. Relaxation times also increase, but this increase can be reversed by further addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone.

INTRODUCTION

Photosynthesis of plants and algae may be suppressed through addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of O_2 evolution, or through genetic mutations at certain sites of the photosynthetic apparatus. Yet, under these conditions a number of energy-requiring cell processes are still driven by light (for references, see ref. 1). Among these processes are ion uptake, phototaxis, and various phosphorylation reactions together with inhibition of respiration. These observations seem to leave little doubt that O_2 evolving photoautotrophs possess a cyclic electron transport system capable of supplying energy for biosynthesis and other cellular work in the absence, and implicitly also in the presence, of photosynthesis. In this respect green plant photosynthesis resembles bacterial photosynthesis.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

We have investigated the cyclic electron transport system and its relationship to photosynthesis in intact cells of *Porphyridium cruentum* and *Anacystis nidulans* as well as chloroplasts by means of steady-state relaxation spectroscopy. This technique involves measurement of the amplitude and phase response of electron transport intermediates when this response is forced by modulated actinic light. The data, when applied to a suitable kinetic model, allow estimation of the rate of electron transport through the carriers even when no external parameter can be measured. For example, conversion of carbohydrate to amino acids is an energy-requiring process which can occur without appreciable transport of material into or out of the cell provided ATP is supplied by a photochemical reaction. Since, however, ATP synthesis is coupled to electron transport, biosynthetic and other ATP utilizing reactions could be indirectly monitored by relaxation spectrophotometry. To "calibrate" the method, we compared rates of Hill reactions with the rate computed from spectroscopic observations on P_{700}.

The data presented here provide spectroscopic evidence that intact cells possess a cyclic electron transport system which appears to be coupled to phosphorylation. Furthermore, the results provide an alternative interpretation of data which had formerly been understood on the basis of an interaction between the photoacts *via* electron transport. Chloroplasts as usually isolated do not have this cycle. Other differences in electron transport properties between chloroplasts and intact cells are discussed.

METHODS AND MATERIALS

A detailed description of the spectroscopic technique used for this work is published elsewhere[2]. With this method, intensity-modulated actinic light forces the electron transport carriers to respond with absorption changes which are modulated at the same frequency but which lag the actinic light. Two parameters are measured simultaneously: the amplitude of the response which is proportional to the concentration of the intermediate, and the magnitude of the phase delay which is a function of the reaction rate. The phase angle may be expressed as the 'relaxation time', the time required for the intermediate to go to the light-induced state and return to the dark state. Thus, the relaxation time is an observed parameter which cannot be translated into a rate constant without knowledge of the kinetic mechanism through which the carriers react. Nevertheless, it sets certain limits on the mechanism.

If one assumes a mechanism, the phase shift measurement provides the rate constant for the reaction and the degree of signal attenuation due to the modulation. These two parameters and the signal amplitude then permit calculation of the time-averaged electron flux through the carriers. In the present work, we assume only that P_{700} is oxidized in the light and reduced in the dark by a first-order reaction; the rate constant is then simply the inverse of the relaxation time.

Reduction of $Fe(CN)_6^{3-}$ and $NADP^+$ was measured in the same instrument at 420 and 340 nm, respectively. Near-red illumination was obtained with a broad-band interference filter whose transmission centered at 620 nm; the maximum intensity of this light at the cuvette was about 0.2 mW/cm^2. Far-red light was obtained with a Schott RG8 glass filter.

P. cruentum was grown at 20° in the medium of BRODY AND EMERSON[3],

A. nidulans at 35–40° in the medium of HECKER[4]. Air containing 1 % CO_2 was continuously flushed through the cultures, from which aliquots were withdrawn and used for measurements without further preparation.

To isolate chloroplasts, approx. 100 g of deveined spinach leaves were ground in a Waring blender for 5 sec with 100 ml of medium containing 0.4 M sucrose, 10 mM NaCl, 0.1 mM EDTA, and 25 mM Tris–HCl buffer (pH 7.65). The slurry was squeezed through six layers of cheese cloth and centrifuged for 2 min at 500 × *g* to remove debris. Chloroplasts were sedimented by centrifugation at 800 × *g* for 15 min and were washed twice with 60 ml of 0.4 M sucrose.

Chlorophyll content was determined as described by ARNON[5]. The reaction medium contained 0.4 M sucrose, 10 mM NaCl, and 50 mM Tris–HCl buffer (pH 8.1); chlorophyll concentration was 25 μg/ml.

RESULTS

The spectrum of modulated absorptions obtained with chloroplasts in the presence of methyl viologen is shown in Fig. 1. The most prominent change at 700 nm is due to P_{700} (ref. 6), the "trapping" pigment of Photosystem I. Three pronounced

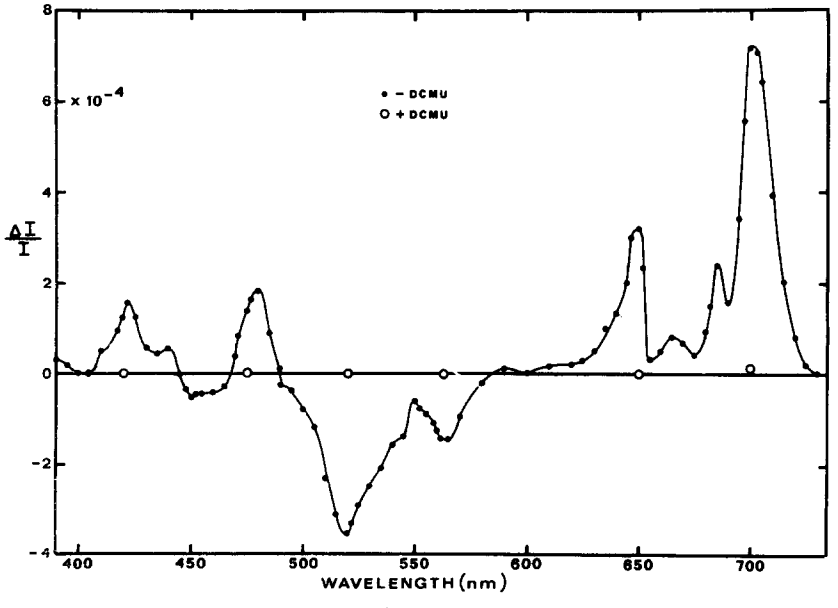

Fig. 1. Spectrum of modulated absorption changes in chloroplasts during a Hill reaction. Oxidant: methyl viologen (1 mM). Modulation frequency: 10 cycles/sec; white actinic light.

peaks at 650, 520 and 475 nm have been attributed to chlorophyll *b* (ref. 7). A distinct 420-nm peak which is probably cytochrome *f* occurs but its amplitude is considerably smaller than the 700-nm band. The absorption at 430 nm is reportedly the Soret band of P_{700} (ref. 6) whose magnitude should be approximately one-third of the 700-nm change[8]. However, such a ratio was never observed. All absorbance changes are completely inhibited with DCMU.

Table I contains relaxation times for the pigments absorbing at 700 and 420 nm during a chloroplast Hill reaction. The data, averages of ten or more measurements, indicate that the relaxation times are independent of the oxidant. Moreover, P_{700} always relaxes faster than cytochrome, an observation which supports the postulated sequential electron transport from cytochrome to the chlorophyll pigment.

TABLE I

OBSERVED RELAXATION TIMES OF P_{700} AND CYTOCHROME *f* DURING HILL REACTION OF CHLOROPLASTS

The reaction mixtures contained 0.25 mM $Fe(CN)_6^{3-}$, or 10 μM methyl viologen, or 0.5 mM $NADP^+$ together with 0.06 mg ferredoxin per 2 ml (Sigma, Type III from spinach). Modulation frequency: 10 cycles/sec; white actinic light.

Oxidant	τ_{700} (*msec*)	τ_{420} (*msec*)	*Ratio* τ_{420}/τ_{700}
$Fe(CN)_6^{3-}$	19	25	1.3
Methyl viologen	15	22	1.4
$NADP^+$	18	24	1.3

Calculated electron flow rates through P_{700} and rates of reduction of $NADP^+$ and $Fe(CN)_6^{3-}$ are compared in Table II. Both calculated and measured velocities are similar which would suggest that the electrons which pass through P_{700} arrive at the site of $NADP^+$ and $Fe(CN)_6^{3-}$ reduction. However, too exact comparison is futile at this time because of uncertainties in the extinction coefficient of P_{700}. These calculations are based on a millimolar extinction coefficient of 80, whereas SCHLIEPHAKE *et al.*[9] report a value of 40–42 based on an assumed stoichiometry of unity between P_{700} and $Fe(CN)_6^{3-}$ or dichlorophenolindophenol. A coefficient of 40 would raise the computed rates 2-fold. DCMU at the concentration used inhibits both calculated and measured flux to a similar extent.

TABLE II

COMPARISON OF MEASURED AND CALCULATED RATES OF ELECTRON FLOW

Conditions as in Table I. $Fe(CN)_6^{3-}$: 0.5 μM DCMU; pH 7.5; 20 cycles/sec; $NADP^+$: 1 μM DCMU; pH 8.1; 10 cycles/sec.

Parameter	*$Fe(CN)_6^{3-}$*		*$NADP^+$*	
	−DCMU	*+DCMU*	*−DCMU*	*+DCMU*
Amplitude ($\Delta I/I \times 10^5$)	33	4	24	2
τ_0 (msec)	18	15	17	19
v_{calc} (700 nm) (μequiv/mg chlorophyll per h)	39	5	15	1
v_{obs} (μequiv/mg chlorophyll per h)	42	5	23	0

Parts of the difference spectrum of *P. cruentum* are shown in Fig. 2. The long wavelength portion of the spectrum is dominated by P_{700}; smaller bands occur in the region of chlorophyll absorption. In the Soret region, the largest absorbance change at 420 nm is presumably due to cytochrome *f*; a broad, mostly featureless absorption from 500 to 600 nm makes measurement of its α-band difficult. No evidence

was obtained for participation of *b*-type cytochromes. The qualitative features of the spectrum remain after addition of DCMU.

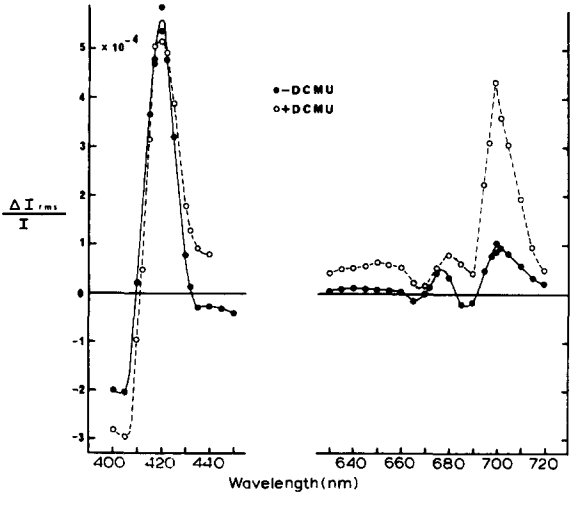

Fig. 2. Difference spectrum of *P. cruentum*. Modulation frequency: 7.5 cycles/sec; white actinic light.

Amplitudes of the 700- and 420-nm bands in far-red light of low intensity are shown in Table III. Obviously, the inhibition of O_2 evolution which is complete at this concentration of the inhibitor (5 μM), has little effect on electron transport through P_{700} and cytochrome. This observation confirms an earlier report by KE AND NGO[10] who used brief flashes of light.

TABLE III

EFFECT OF DCMU ON *P. cruentum* CELLS IN PHOTOSYSTEM-I LIGHT

Modulation frequency: 13 cycles/sec.

Additions	*420 nm*		*700 nm*	
	Amplitude ($\Delta I/I \times 10^4$)	τ(*msec*)	*Amplitude* ($\Delta I/I \times 10^4$)	τ(*msec*)
None	2.9	16	0.37	19
+ DCMU	2.6	18	0.45	24

The relaxation times also increase upon addition of DCMU. Although the magnitude of this increase varies from culture to culture, it is generally small and never exceeded a factor of 2. More importantly and in contrast to chloroplasts, in all cases and both in the absence and presence of DCMU, the relaxation times at 700 nm are equal or greater than those at 420 nm. As will be argued below, this observation does not allow sequential electron transport from cytochrome to P_{700}.

Illumination of the cells with light which principally excites phycobilins

provided the data of Fig. 3. Under these conditions, DCMU causes an increased amplitude of the cytochrome modulation and a longer relaxation time. In addition, the flux calculations demonstrate that twice as many electrons are now passing through cytochrome. Distinct effects could be observed only at 420 nm because the actinic light intensity was too low for an accurately measurable P_{700} signal. FORK AND AMESZ[11] have previously studied the low intensity response of P_{700} and cytochrome.

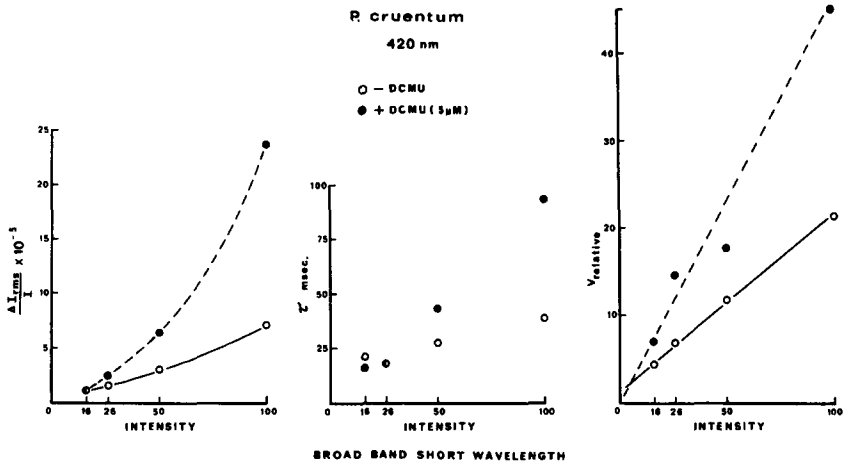

Fig. 3. Effect of DCMU on relaxation parameters in *P. cruentum*. Modulation frequency: 2.5 cycles/sec; broad band 620-nm actinic light.

As shown in Table IV similar results were obtained with *A. nidulans*. Again, DCMU provokes a larger amplitude and an increased relaxation time and again the calculations indicate a doubling of the flux. When the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) is added to the poisoned cells, the relaxation time reverts to that observed with unpoisoned cells and the amplitude decreases. The lower amplitude is mainly due to the lower relaxation time and only partially to inhibition by CCCP. Computed flux data indicate that the inhibition is approx. 20 % at the highest concentration of the uncoupler which was studied.

TABLE IV

EFFECTS OF DCMU AND CCCP ON *A. nidulans* CELLS IN PHOTOSYSTEM-II LIGHT

Modulation frequency: 7.5 cycles/sec.

Additions	*Amplitude* ($\Delta I/I \times 10^5$)	τ(*msec*)	$v_{relative}$
None	4	13	3.5
DCMU (5 μM)	13	37	7.0
DCMU + CCCP (12.5 μM)	10	21	6.6
DCMU + CCCP (25 μM)	8	17	6.0
DCMU + CCCP (40 μM)	6	12	5.6

DISCUSSION

The experiments with chloroplasts demonstrate that the steady-state relaxation technique allows calculations from spectral data of electron flow velocities through intermediate carriers. Subject to an accurate extinction coefficient for P_{700}, the calculated rates for this pigment are at least of the same order of magnitude as those measured directly.

Other data presented here show that there exist considerable differences in the kinetic behavior of chloroplasts and whole cells. For example, many experimental results have been interpreted in terms of a sequential electron transport from cytochrome to P_{700}. The relaxation times of these pigments in chloroplasts are consistent with this formulation because cytochrome relaxes more slowly than P_{700}. A necessary consequence is, however, that electron transport from cytochrome to P_{700} is slow compared to the reaction which reduces cytochrome.

The following considerations extend this explanation: since the photochemical oxidation of P_{700} is fast compared to its chemical reduction, τ_{700} may be viewed as the time required for only the reduction of this pigment. The oxidation of cytochrome through sequential electron transport ought then to require a similar interval, leaving only a fraction of τ_{420} for cytochrome reduction. Calculations based on a sequential kinetic model[2], using the data of Table I, indicate that cytochrome is reduced in approx. 5 msec.

Whole cells show different kinetics. Here, τ_{700} is always larger than τ_{420} which means, in terms of the sequential formulation, that the oxidation and reduction of cytochrome (τ_{420}) is a faster event than its oxidation alone (τ_{700}), an impossible situation. Furthermore, the large signal amplitude at 420 nm requires that cytochrome be oxidized relatively faster than it is reduced; again, the relaxation times indicate that this requirement is not met by sequential electron transport. Independent action of P_{700} and cytochrome would satisfy both the data from chloroplasts and from whole cells.

DCMU has a small effect upon electron transport with intact cells in Photosystem-I light of low intensity, although O_2 evolution is inhibited by the concentration used[10,13]. This observation has long been the operational definition for "cyclic" electron transport. Moreover, this cycle appears to be coupled to the generation of ATP or a high energy intermediate since a number of energy-requiring cell processes also persist in the presence of the inhibitor.

In contrast, DCMU inhibits both the calculated and measured electron flow in chloroplasts to a similar extent. It is not clear why a DCMU-insensitive flux does not occur in this case and why it is necessary to add exogeneous cofactors such as phenazine methosulfate to observe cyclic transport.

The steady-state amplitude of oxidized cytochrome in Photosystem-II light increases considerably upon addition of DCMU. Such an observation has previously been reported by DUYSENS *et al.*[12] and AMESZ[13] who interpreted their data in terms of an interaction between the two light reactions of photosynthesis by way of electron transport: red light oxidized cytochrome through Photosystem I and simultaneously reduced it through Photosystem II. The net result with Anacystis was a low amplitude of oxidized cytochrome. DCMU supposedly inhibited the reduction by blocking Photosystem II activity and thereby caused an increased steady-state level of the oxidized pigment.

The increase in relaxation time at 420 nm upon addition of DCMU indicates, indeed, that the reduction of cytochrome is slower but not completely inhibited. Furthermore, the flux calculations demonstrate that the increased amplitude of oxidized cytochrome is not only due to a slower reduction but also to a 2-fold increase in the number of electrons passing through cytochrome. Since this phenomenon does not occur in Photosystem-I light, we suggest that DCMU acts to divert, in an unknown manner, quanta from Photosystem II to Photosystem I. KE AND NGO[10] reached similar conclusions from their data.

DUYSENS *et al.* corroborated their interpretation by showing that the oxidation level of cytochrome in Photosystem-I light could be decreased with additional illumination of Photosystem-II light. When DCMU blocked electron flow in Photosystem II, the decrease was not observed. We show here, however, that it is possible to decrease the oxidation level of cytochrome with the uncoupler CCCP even though reducing equivalents from Photosystem II are blocked by DCMU. In addition, the relaxation time of the inhibited cells is restored to that of the untreated cells.

Our results with intact organisms may be explained by a mechanism in which cytochrome functions solely in a cyclic electron transport system on the oxidizing side of a coupled energy conversion step (cross-over, see CHANCE and WILLIAMS[14]). The rate of reduction at this site determines the oxidation level of cytochrome: the smaller the rate, the higher the oxidation level. In turn, the rate of reduction is determined by the degree of utilization of the high-energy intermediate formed here, such that a rapid utilization results in a high rate. Hence, Photosystem II is envisioned to act on the relaxation time and implicitly the oxidation level of cytochrome by utilizing this intermediate rather than by contributing reducing equivalents. When DCMU is added, Photosystem II is inhibited, utilization of the intermediate declines and its "pool" fills. The consequence is a larger relaxation time (smaller rate constant) and a higher oxidation level for cytochrome. Further addition of the uncoupler CCCP mimics utilization of the high-energy intermediate thereby returning the relaxation time to that of unpoisoned cells and decreasing the oxidation level of cytochrome. The hypothesis is quite speculative and certainly less definitive than the commonly accepted series formulation[15]. In the author's opinion, however, the series formulation cannot be satisfactorily amended to rationalize these results.

ACKNOWLEDGEMENTS

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