Photoreactions of Cytochrome b_6 and Cytochrome f in Chloroplast Photosystem I Fragments

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Photosystem I fragments were prepared by digitonin treatment of spinach chloroplasts. The midpoint potential of cytochrome b_6 in the fragments is close to 0 V, showing a one electron transition. No cytochrome b_{559} was detectable, neither in difference absorption spectra nor in light-induced absorbance changes. In the absence of added cofactors only cytochrome b_6 photoreduction can be observed. This photoreduction is stimulated by ferredoxin. Ferredoxin-$NADP^+$ reductase appears not to be involved in cytochrome b_6 reduction. Photooxidation of cytochrome b_6 is dependent on plastocyanin addition and inhibited by DBMIB, a plastoquinone antagonist. Addition of plastocyanin restores cytochrome f photooxidation as well, reacting quite specifically in about equimolar concentrations to bound cytochrome f. The stimulation of cytochrome f oxidation is abolished by an antibody prepared against plastocyanin, indicating a surface location of plastocyanin in digitonin treated membranes.

Biphasic kinetics of dark-reduction of cytochrome f by ascorbate indicate that part of this cytochrome f is relatively inaccessible in the membrane. After preillumination a monophasic reduction is observed and the slowly oxidized component is absent. Illumination in the presence of plastocyanin causes a fast and complete reduction of cytochrome f, suggesting equilibration of cytochrome f with added plastocyanin, residing in the membrane surface. It appears that actinic light causes conformation and/or structural changes in the membrane of these digitonin fragments, influencing cytochrome f accessibility.

Introduction

The concept of two different photosystems operating in series in the electron transport of photosynthetic oxygen evolving organisms is now generally accepted [1]. Excitation of both photosystems leads to $NADP^+$-reduction and ATP-formation in a noncyclic pathway, whereas excitation of photosystem I alone results in a net-synthesis of ATP, suggesting a cyclic pathway, where electrons reenter the noncyclic chain between the two photosystems [2]. This general view was strengthened by the finding that fractionation of chloroplasts by various methods could physically separate photosystem I from photosystem II [3]. The fraction showing high rates of photosystem I activity contained a number of components, which have been characterized and partly isolated from chloroplasts. There is, however, disagreement on the participation of individual electron carriers and their interaction with the two primary photoproducts of the cycle. A number of components, including ferredoxin, Fd-$NADP^+$ reductase, cytochromes b_6, b_{559LP}, and f, plastocyanin, plastoquinone, have been implicated in this regard, although with considerable discordance.

In the view of a binary membrane model for the chloroplast thylakoid membrane, the action of the detergent digitonin is believed to separate both lipoprotein layers, each containing different parts of the electron transport system. The photosystem I fraction, showing densely packed globular 100 Å particles seems to represent the outer "half" of the grana membrane [4]. The phosphorylating capacity of this fraction [5] suggests a closed membrane vesicle, according to recent concepts of chemiosmotic energy conservation.

Digitonin treated chloroplasts [3] were used in order to isolate a relatively pure, but intact photosystem I fraction, with respect to photochemical activities. In particular, the pathways of cytochrome b_6 and f were investigated, which were shown to participate in cyclic electron transport [7, 33, 36, 39].

By reconstitution experiments, participation of other electron carriers lost upon digitonin treatment could be checked. The isolation procedure employed yielded a photosystem I fraction which was still

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Abbreviations: Fd, ferredoxin; Cyt, cytochrome; Chl, chlorophyll; PC, plastocyanin; DCPIP, 2,6-dichlorophenol-indophenol; DPC, 1,5-diphenylcarbazide; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; E_m, midpoint potential.

able to catalyze cyclic and non-cyclic phosphorylation with appropriate donor and acceptor systems [5].

Methods

Chloroplasts were prepared from spinach (variety: Atlanta), grown under controlled climate conditions as previously described [7]. Digitonin incubation followed by differential centrifugation was carried out according to the procedure of Boardman and Anderson [3]. The photosystem I fragments obtained were frozen in liquid nitrogen and stored at -80 °C. Photosystem I activity was checked by following the oxidation of 0.2 mM DCPIP at 600 nm in a Perkin-Elmer spectrophotometer (Mod. 124), equipped for side illumination [8]. With methylviologen (0.1 mM) as acceptor, rates of about 450 μmol/mg Chl × h at pH 7.8 were obtained. Using the same setup to measure DCPIP reduction with 1 mM DPC as donor, a rate of less than 1% of the above value for photosystem II activity could be measured.

Determination of chloroplast cytochromes by recording reduced minus oxidized difference spectra (Aminco, DW 2) was performed assuming an extinction coefficient (ε) of 20 $\text{mM}^{-1} \times \text{cm}^{-1}$ for the α-band of the cytochrome measured [9]. Triton X-100 (0.3%) was added to chloroplasts to convert cytochrome b_{559} to its low-potential form, facilitating the quantitative determination of all three cytochromes present [10].

From ferricyanide oxidized minus ascorbate reduced difference spectra the P 700 content was estimated assuming an ε of 64 $\text{mM}^{-1} \times \text{cm}^{-1}$ at 701 nm [11].

Plastoquinone was extracted from chloroplasts and photosystem I fragments according to the method of Barr and Crane [12]. After thin layer chromatography of the extract, the quinone content was determined in an oxidized minus borohydride-reduced difference spectrum, $\varepsilon = 15$ $\text{mM}^{-1} \times \text{cm}^{-1}$ at 255 nm.

The flavoprotein bound to chloroplasts and digitonin fragments was determined by (1.) NADP-photoreduction and (2.) the oxidase-reaction, using electron flow from NADPH to O_2 the presence of anthraquinone [14].

NADPH-photoreduction was measured in the Aminco DW 2 spectrophotometer equipped for side illumination, in the dual wavelength mode, with 366 nm as measuring and 400 nm as reference wavelength. With this setup, rates of 2 nmol NADPH formed per min could be measured. The reaction mixture contained in addition: 0.1 mM DAD, 1 mM sodium ascorbate, 0.3 μM plastocyanin, 0.3 μM ferredoxin, 15 μM NADP^+, and photosystem I fragments equivalent to about 30 μg chlorophyll in a total volume of 3 ml.

In the second method, measuring oxidase activity of Fd-NADP^+-reductase, dark oxygen uptake rates induced by flavoprotein bound to chloroplasts were compared with those of photosystem I fragments, normalized on a chlorophyll basis. Oxygen uptake was recorded by a YSI, Mod. 53, oxygen monitor at 25 °C. The standard reaction mixture contained in addition 0.1 mM sodium anthraquinone-2-sulfonate, 0.1 mM sodium azide, 0.6 mM NADP^+, 1 mM sodium isocitrate, 0.4 mg isocitrate-dehydrogenase. The reaction was started by adding NADP^+. Comparison of oxygen uptake rates of bound flavoprotein added with chloroplasts or photosystem I fragments allows an estimation of 65 – 80% flavoprotein still bound to the photosystem I fraction.

Isolation of ferredoxin, plastocyanin and Fd-NADP^+-reductase followed the standard procedures. The proteins were stored at concentrations of 30 to 100 μM at -20 °C until use. The absorbance ratios of protein to chromophore peak were 2.5 for ferredoxin, 2.0 for plastocyanin and 8.5 for Fd-NADP-reductase.

Light-induced absorbance changes were measured at 25 °C in an Aminco DW 2 spectrophotometer, dual wavelength mode. The actinic light intensity of wavelength 704 nm was 150 $\text{J} \times \text{m}^{-2} \times \text{s}^{-1}$. All absorbance changes were measured in the presence of 2×10^{-4} M sodium ascorbate. The bandwidth of the measuring beam was 1 nm for the difference spectra and 2 nm for light-induced absorbance changes compared to 570 nm reference [7]. The amplifier time constant was set to 2.5 sec or 100 msec (see figure legends).

Antiserum against spinach plastocyanin was a kind gift from Dr. G. Hauska. After purification by ammonium sulfate precipitation, it specificity was checked in a double diffusion test. Antiserum and control serum protein concentration was 27 mg/ml. Compared with purified preparations of ferredoxin, algal cytochrome f_{553} [13], Fd-NADP^+-reductase, the antiserum appeared to be monospecific against plastocyanin. The reactivity of the antibody was tested through ascorbate photooxidation by digitonin photosystem I fragments. This reaction was completely dependent on plastocyanin addition; the inhibition curve obtained with the plastocyanin antibody was similar to the one published in ref. 14, Fig. 1.

Results

1. *Cytochrome content; redox potential of cytochrome b_6; absence of cytochrome b_{559}*

The original studies of Boardman and Anderson [3] showed that after digitonin treatment of chloroplasts, two fractions are obtainable, with different pigment composition. Separation by differential centrifugation yielded a photosystem I fraction, containing cytochromes b_6 and f, whereas cytochrome b_{559} was believed to be associated with photosystem II [3]. This interpretation was substantiated by low temperature photooxidation of Cyt b_{559} by system II light [16], and the finding that upon further purification the photosystem II fraction still contained tightly bound Cyt b_{559} [17]. Later on, this view was modified mainly on the basis of low temperature difference spectra. It was proposed that a low potential form of cytochrome b_{559} appears to be associated with photosystem I digitonin particles and the cytochrome b_6-f complex, whereas the high potential form of Cyt b_{559} is associated with photosystem II [18, 19]. However, this interpretation disregards the splitting of the α-band of isolated Cyt b_6 at 77 °K [20], as well as the possibility of band shifts to shorter wavelength upon various treatments of cytochrome b_6 (personal observation).

Room temperature reduced minus oxidized difference spectra of a photosystem I fraction, obtained by digitonin treatment of chloroplasts, are shown in Fig. 1. Only the reduction of cytochrome f and cytochrome b_6 could be demonstrated; no ascorbate reducible b_{559} ($= b_{559LP}$) was found; furthermore, there was no apparent peak-shift to shorter wavelength or shoulder in the dithionite minus ascorbate reduced difference spectrum which would have indicated the presence of a low potential Cyt b_{559} not reducible by ascorbate. Cytochromes b_6 and f are present in a molar ratio of approximately 2 : 1. Addition of ferricyanide to the reference cuvette produced essentially the same spectra, indicating that both cytochromes were in the oxidized state.

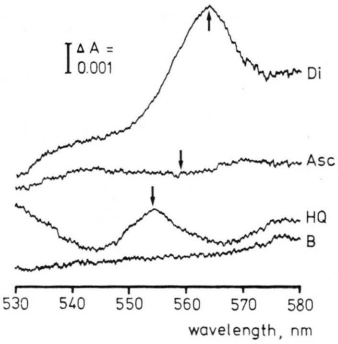

Fig. 1. Reduced minus oxidized difference spectra of a photosystem I fraction at room temperature. For details see Methods: B, baseline; HQ, hydroquinone reduced minus untreated; Asc, ascorbate reduced minus HQ reduced; Di, dithionite reduced minus ascorbate reduced. Arrows indicate wavelength maxima at 554 nm, 559 nm, and 563 nm, respectively, Chlorophyll concentration 100 μg/ml.

On the basis of a biphasic redox titration curve Knaff and Malkin [21] concluded that photosystem I fragments contain two b-cytochromes in approximately equimolar amounts, a low potential form of cytochrome b_{559} ($E_m = +110$ mV) and cytochrome b_6 ($E_m = -100$ mV); these E_m-values closely correspond to those found for the isolated enzymes [20].

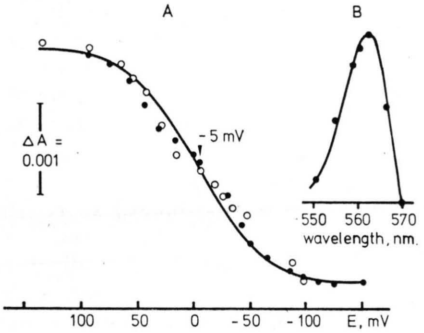

Fig. 2. A. Anaerobic titration of cytochrome b_6 as a function of redox potential. Details in ref. 22; (●), reductive titration with dithionite; (○), oxidative titration with ferricyanide; the mediators were 20 μM 2.4-naphthoquinone and 10 μM dihydroxy-p-benzoquinone. The calculated titration curve (—) assumes a midpoint of −5 mV and a one-electron transition. Chlorophyll concentration 150 μg/ml. B. Difference spectrum for the absorbance change titrated in Fig. 2, A. Ambient redox potential was changed from −100 mV to +250 mV and *vice versa* by addition of single aliquots of ferricyanide or dithionite. The absorbance change obtained is plotted as a function of wavelength; 20 μM 1,4-naphthoquinone was used as redox buffer and mediator. Chlorophyll concentration 110 μg/ml.

Anaerobic titration of the absorbance change at 563 nm relative to a 570 nm reference yielded a monophasic titration curve, with a midpoint potential of the component titrated at +5 mV (±10 mV), pH 7.8, and a slope corresponding to a one-electron transfer. The difference spectrum (Fig. 2 B) obtained by adding single aliquots of dithionite and ferricyanide to shift the potential from +250 mV to −100 mV identifies the titrated absorbance change as cytochrome b_6, with an absorption maximum at 563 nm. The asymmetry of the α-band in the difference spectrum of Fig. 2 B is also observable with purified Cyt b_6 (personal observation). This is consistent with the splitting of the α-band of Cyt b_6 at 77 °K. It should be noted that the midpoint potential of Cyt b_6 in this photosystem I fraction closely corresponds to the value found in coupled chloroplasts[22]. This value is about 100 mV more positive than that described in ref.[21]. It is known that a number of treatments, which change the energy state (*e. g.* uncoupling) and/or the intactness of the chloroplast membrane, cause a negative shift in the redox potential of b-cytochromes[22].

2. Content of other electron transport components; comparison of untreated chloroplasts

In an effort to further characterize this system, a quantitative estimation of the content of other electron transport components in the photosystem I fraction was carried out. These data are summarized in Table I.

Table I. Quantitative determination of different electron transport components in chloroplasts and photosystem I fragments.

Component *	Chloroplasts	Photosystem I fragments
Chlorophyll a/b	2.8	6.1
Cytochrome f	1.1 – 1.35	0.6 – 0.7
Cytochrome b_6	2.2 – 2.5	1.1 – 1.35
Cytochrome b_{559}	2.45 – 2.6	not detectable
Plastoquinone **	23.7	11.8
P 700	1.05	4.85
Fd-NADP$^+$ reductase	?	40 – 80% ***

* All values are normalized to 1 nmol of the component per 1 μmol chlorophyll.
** Average of 6 different determinations.
*** Determination: see Methods.

From the data of Fig. 1, it can be calculated that the photosystem I fraction contains about 0.6 nmol cytochrome f and 1.15 nmol cytochrome b_6 per 1 μmol chlorophyll; this indicates some loss in cytochrome content through digitonin treatment of chloroplasts. Since the estimation of cytochrome content by difference spectra does not require any isolation or fractionation procedures, these values are quite accurate estimates. It seems, therefore, that the high chlorophyll/cytochrome ratio, reflected also in the determination of other components, is a consequence of the spinach variety used and the growth condition, which does not recommend chlorophyll determinations as a reliable reference (comp. ref. 41).

The P 700 content was obtained using the extinction coefficient of 64 mM at 701 nm in ascorbate-reduced minus ferricyanide-oxidized difference spectra[11]. They show that P 700 is in the reduced state, enriched to about 5-fold in this photosystem I fraction. This is also reflected by the high Chl a/Chl b ratio of 6.1 *vs* 2.8 in untreated chloroplasts.

Plastoquinone content was determined by a quantitative extraction method and thin layer chromatography as described by Barr and Crane[12]. The data show that the photosystem I fraction still contains a considerable amount of plastoquinone decreased to about half the value found for chloroplasts. Similar results were obtained for Triton-subchloroplast particles[23].

Ferredoxin was absent, as indicated by the inability of photosystem I fragments to photoreduce NADP$^+$ in the presence of saturating amounts of plastocyanin and Fd-NADP-reductase with DAD/ascorbate as donor system. Similarly, an estimate of bound flavoprotein was attempted. With saturating amounts of ferredoxin and plastocyanin present, there was some NADP$^+$-photoreduction activity, which could be stimulated by adding isolated flavoprotein. The saturation curve obtained, extrapolated to zero reductase content, gave a value of 35 – 40% for flavoprotein still bound to photosystem I. Furthermore, another enzymatic method was used to determine the amount of bound flavoprotein. It makes use of the fact that the reductase catalyzes electron transport from NADPH to oxygen in the dark, mediated by anthraquinone. This experiment was suggested by Dr. E. F. Elstner[14]. Comparing the rates of oxygen uptake in the dark of chloroplast and digitonin fragmented chloroplasts in an NADPH-regenerating system, a value of 65 – 85% for flavoprotein still bound to photosystem I was found. The discrepancy between these two estimates is under investigation. In any case, the results show that there are appreciable amounts of flavoprotein ($\geqq$ 40% of the control) still bound to the photosystem I fraction.

An estimation of the plastocyanin content was attempted by EPR-spectroscopy. The data show that the photosystem I fraction is depleted in plastocyanin, in agreement with the results on plastocyanin dependence of Cyt f/or Cyt b_6 photooxidation. A 20% residual content[21], however, would have escaped detection due to the limited resolution of the instrument, and interference by other paramagnetic signals at $g = 2.05$ and 77 °K.

3. Photoreactions of cytochrome f

We shall first concentrate on cytochrome f photoreactions measured at 554 nm. The tracings in the upper part of Fig. 3 A show that cytochrome f photooxidation is only observed after addition of plastocyanin. The difference spectra of Fig. 3 B clearly reveal that in the absence of plastocyanin only an absorbance increase at 563 nm could be detected. This will be discussed in more detail below. Addition of plastocyanin produces an additional absorbance peak at 554 nm, indicating cytochrome f photooxidation. The specificity of plastocyanin binding is demonstrated by plotting the effect of plastocyanin concentration on restoration of cytochrome f photooxidation. Fig. 4 shows a biphasic saturation curve, with a break point at a level of about 60% cytochrome f photooxidized; this plastocyanin concentration (marked by an arrow in Fig. 4) corresponds to the total amount of cytochrome f present in these fragments, suggesting a stoichiometric (1 : 1) relationship between the two components.

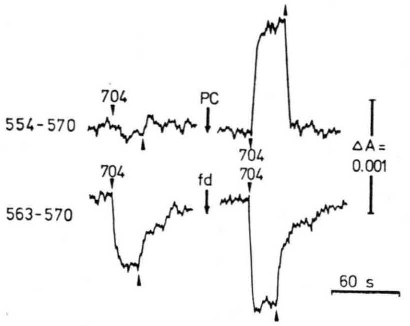

Fig. 3 A. Light-induced absorbance changes of cytochrome f (554 − 570 nm) and cytochrome b_6 (563 − 570 nm) before and after addition of plastocyanin (PC) and ferredoxin (Fd), respectively. Chlorophyll concentration 74 μg/ml; 0.14 μM plastocyanin or ferredoxin was added where indicated. Downward arrows: 704 nm light on; upward arrows: light off. Upward deflection: absorbance decrease relative to a 570 nm reference.

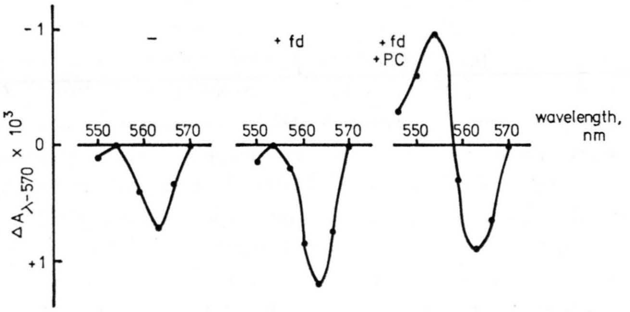

Fig. 3 B. Difference spectra of the light-induced absorbance changes in the absence and presence of plastocyanin and plastocyanin plus ferredoxin; conditions as in Fig. 3 A.

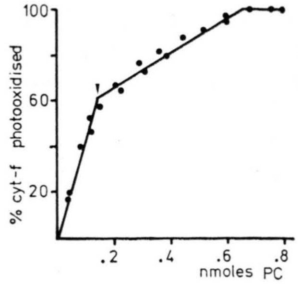

Fig. 4. Effect of plastocyanin concentration on cytochrome f photooxidation. Chlorophyll concentration 80 μg/ml. The 100% value corresponds to an absorbance change equivalent to the total amount of cytochrome f present. The arrow indicates an equimolar ratio of cytochrome f to added plastocyanin.

In order to further characterize the plastocyanin stimulated Cyt f reaction, the question of location of the bound (Cyt f) and added component (PC), which seemed to interact quite specifically, relative to the water/membrane interface was investigated.

Horton and Cramer[25] used the polar oxidant ferricyanide as a probe to characterize the relative accessibility and changes in accessibility of cytochrome f. They found that ferricyanide causes a biphasic dark oxidation of cytochrome f, which changed to a fast monophasic oxidation after freeze-thaw and a slow monophasic oxidation after preillumination of the chloroplasts. It was concluded that a trigger for a conformational change in the cytochrome and/or the membrane, responsible for alterations in the kinetics of Cyt f chemical oxidation, could be the photooxidation of the cytochrome itself.

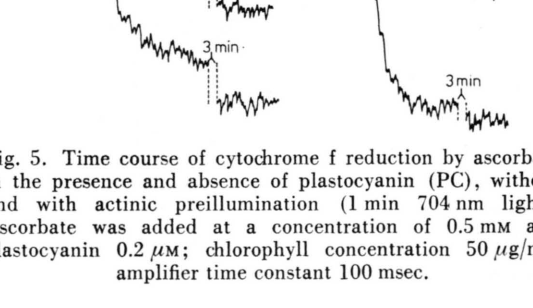

Fig. 5. Time course of cytochrome f reduction by ascorbate in the presence and absence of plastocyanin (PC), without and with actinic preillumination (1 min 704 nm light). Ascorbate was added at a concentration of 0.5 mM and plastocyanin 0.2 μM; chlorophyll concentration 50 μg/ml; amplifier time constant 100 msec.

Fig. 5 shows that addition of 0.5 mM ascorbate to a dark adapted photosystem I sample causes a biphasic reduction of Cyt f, with one half to two thirds of the total absorbance change as fast as the time required for mixing ($\approx$ 1 sec). Reduction is completed after about 3 – 4 min (note the break in the time scale in Fig. 5). The failure of Horton and Cramer[25] to observe different kinetics of ascorbate-reduction after addition of ferricyanide may have been a consequence of ferricyanide treatment of the chloroplasts themselves. Addition of ferricyanide to the photosystem I fraction prior to reduction by ascorbate also abolished the kinetic differences in dark reduction of cytochrome f. Preillumination with far-red light changes the biphasic reduction kinetics in accordance with the results found in chloroplasts, showing a slower ($\tau_{1/2} \approx 9$ sec) monophasic reduction by ascorbate. The half reduction time is about twice as fast as the value found for chloroplasts, indicating some removal of a membrane barrier through digitonin treatment. It should be noted though that in this case photooxidation of cytochrome f could not trigger a conformational change, since it is dependent on plastocyanin addition in these fragments. In the presence of added plastocyanin, the kinetics of Cyt f dark reduction are not changed (Fig. 5, lower part). However, after preillumination with far-red light, a fast monophasic reduction of cytochrome f is seen. This could be interpreted by a movement of the cytochrome f complement closer to the membrane surface or more likely by a facilitated equilibration of cytochrome f through added plastocyanin, residing on the membrane surface. This is further substantiated by looking at chemical oxidation-reduction of added plastocyanin before and after preillumination, measured as absorbance change at 590 nm with a 550 nm reference. The data indicate (not shown here) that both ferricyanide and ascorbate oxidize or reduce plastocyanin within the mixing time, independent of preillumination.

A further experiment to test the location of added plastocyanin would be the use of a specific antibody against plastocyanin and its effect on cytochrome f oxidation. The reactivity of the monospecific antibody was tested through ascorbate photooxidation by photosystem I particles, which is completely dependent on plastocyanin addition (see Methods). In Fig. 6, the effect of antibody addition to photosystem I fragments on cytochrome f photooxidation is shown. On the right, the figure demonstrates the well known effect of plastocyanin addition, stimulating Cyt f photooxidation with a biphasic saturation curve and a break point at about at 1 : 1 molar ratio of cytochrome f and plastocyanin. The left part of the figure shows that addition of antibody inhibits completely Cyt f oxidation, whether plastocyanin is added at equimolar or higher concentrations, to completely saturate Cyt f photooxidation. Addition of control serum with equal protein concentration produced a minor effect. The results show that even after illumination added plastocyanin, with its antigenic determinants, remains accessible to the antibody in accordance with the data on chemical oxidation reduction (see above).

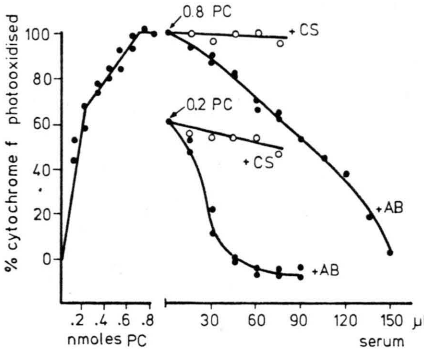

Fig. 6. Effect of a monospecific plastocyanin antibody on cytochrome f photooxidation; with plastocyanin (0.2 nmol) added in about equimolar concentrations to bound cytochrome f to give 60% of total cytochrome f photooxidized and plastocyanin (0.8 nmol) added in excess to give complete cytochrome f photooxidation. CS, control serum; AB, antibody; these were added at a 60% and 100% level of cytochrome f photooxidation. Chlorophyll concentration 90 µg/ml.

It has been claimed that the interaction of externally added plastocyanin and the membrane might be mainly electrostatic, since addition of 0.26 M NaCl strongly inhibited the light-induced oxidation of cytochrome f[26]. Fig. 7 confirms the apparent inhibition of the far-red light induced absorbance decrease measured at 554 nm when comparing the lower two tracings. This, however, may be attributed to a baseline-shift induced by 0.26 M NaCl as evident from comparison of the tracings in the upper part of the figure; the absolute stimulation of cytochrome f oxidation by plastocyanin remains the same.

4. Photoreactions of cytochrome b_6

As already mentioned far-red illumination of the photosystem I fraction obtained by digitonin treat-

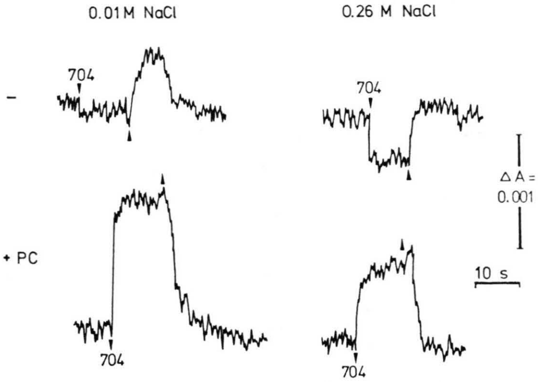

Fig. 7. Effect of NaCl on plastocyanin-stimulated photooxidation of cytochrome f. Conditions as in Fig. 3, A; 0.28 μM plastocyanin (PC) was added where indicated; chlorophyll concentration 100 μg/ml; amplifier time constant 100 msec.

ment of chloroplasts causes an absorbance increase with a maximum at 563 nm (Fig. 3 A and spectrum of Fig. 3 B) indicating cytochrome b_6 reduction. Furthermore, it is shown in Fig. 3 that the rate and extent of this photoreduction could be enhanced by addition of ferredoxin. Similar to the plastocyanin dependent cytochrome f oxidation, a saturation curve for ferredoxin stimulated cytochrome b_6 reduction was attempted. However, due to the smaller effect of ferredoxin addition on b_6-reduction and the limited instrument resolution, only a rough estimate could be made. By decreasing the incident light intensity to 2.5% of its original value, the absorbance change measured at 563 nm was markedly decreased both in rate and extent. In this case addition of ferredoxin produced a larger effect, enhancing photoreduction of Cyt b_6 (see Fig. 8); from these data a requirement of about 0.3 μM ferredoxin was estimated to completely saturate the reaction. This is about twice as much as the amount of plastocyanin required for complete cytochrome f oxidation. Since there is no absolute dependence of Cyt b_6 reduction on ferredoxin addition, although the enzyme is completely absent in this fraction (see preceding paragraph, Table I), the role of ferredoxin in cyclic electron transport remains open to question.

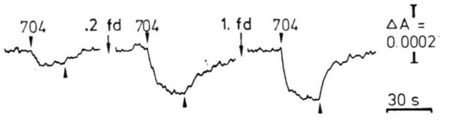

Fig. 8. Stimulation of cytochrome b_6 reduction by ferredoxin at low light intensities. Ferredoxin concentration added was 0.2 nmol and 1.0 nmol as indicated. Chlorophyll concentration 66 μg/ml.

Addition of Fd-NADP$^+$-reductase, did not influence Cyt b_6 redox reactions nor did a reductase antibody, added at a concentration to inhibit 70% of a transhydrogenase activity with isolated Fd-NADP reductase, *i. e.* 66.5 pmol; this amount corresponds roughly to the value of flavoprotein still bound to photosystem I fragments used in this experiment, under the assumption that there is a 1 : 2000 flavoprotein/Chl ratio in the chloroplasts, used through all experiments (unpublished observation).

It has been shown in chloroplasts that under anaerobic conditions chemically reduced cytochrome b_6 can undergo photooxidation mediated by plastoquinone [7]. The tracings in Fig. 9 A show – from left to right – first the well known Cyt b_6 reduction, induced by far-red light, and its reoxidation in the dark under anaerobic conditions. Addition of dithionite to lower the redox potential of the medium to approximately –200 mV completely abolishes this light induced change. Addition of plastocyanin in a concentration range that fully saturates Cyt-f oxidation restores Cyt b_6 photooxidation, indicating that both cytochromes are oxidized through the same pathway, which requires plastocyanin. Addition of 2 μM DBMIB, which completely inhibits electron transport through the plastoquinone [27] still present in this photosystem I fraction (see Table I) inhibits also plastocyanin dependent Cyt b_6 photooxidation.

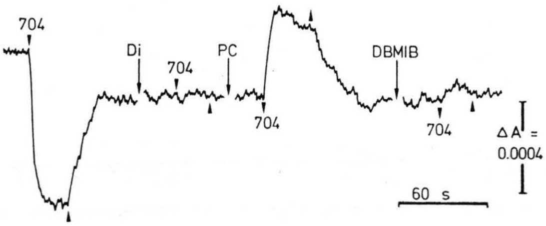

Fig. 9 A. Light-induced absorbance changes of cytochrome b_6 under anaerobic conditions. Addition of dithionite lowered the redox potential of the medium to about –200 mV; addition of 14.5 nM plastocyanin restores cytochrome b_6 oxidation; the absorbance change was abolished by 2 μM DBMIB. Chlorophyll concentration 33 μg/ml..

The difference spectra of Fig. 9 B show that under anaerobic conditions in the presence of a strong reductant it is Cyt b_6 whose oxidation is

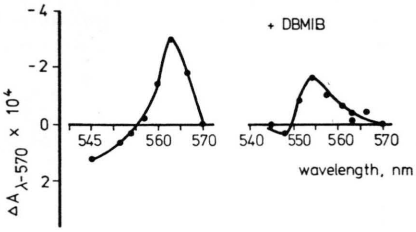

Fig. 9 B. Difference spectra for the light-induced absorbance changes obtained as in Fig. 9 A, in the presence of plastocyanin and plastocyanin plus DBMIB, as indicated. Chlorophyll concentration 30 μg/ml.

stimulated by plastocyanin, as indicated by a maximum at 563 nm. Cytochrome f does not appear in the spectrum under these conditions, which means that it functions in the pathway of cytochrome b_6 oxidation, maintaining its redox state. Since only marked shifts in the redox level of an individual compound are measured with the dual wavelength method, intermediates can only be detected when the reduction-oxidation pathways are either inhibited by a specific inhibitor or missing component, or accelerated by addition of the solubilized redox component.

Addition of DBMIB, which is believed to block plastoquinone oxidation and hence the pathway between cytochrome b_6 and f, results in a peak-shift to 554 nm of the light minus dark difference spectrum. Oxidation of cytochrome f by photosystem I is now more favoured than its reduction by plastoquinone and cytochrome b_6, respectively.

Discussion

Although the existence of cyclic electron transport involving photosystem I now appears to be well documented in photosynthetic green plants and algae [28], there seems to be less agreement on its role, especially regarding whether cyclic phosphorylation is required stoichiometrically for photosynthetic CO_2 fixation [29].

An endogenous cyclic phosphorylation can be demonstrated in isolated chloroplasts, capable of fixing CO_2 at high rates, without addition of cofactors and/or electron transport enzymes [30]. In osmotically broken chloroplasts an artificial cofactor has to be added with special requirements as to lipid solubility, redox potential and proton-carrying properties to give high rates of ATP formation [31]. The reduction and oxidation kinetics of cytochrome b_6 by photosystem I on the other hand appear sufficiently fast to exclude unknown enzymes having been discarded or inactivated during osmotic shock [32]. At present, it is not known why chloroplasts lose their ability to form ATP in cyclic electron transport when they lose their outer membrane. In addition, there is considerable disagreement on participation and interaction of intermediate electron transport enzymes between the two primary photoproducts of photosystem I.

The results obtained with photosystem I digitonin fragments show that Cyt b_6 and Cyt f oxidation require plastocyanin, which is lost during preparation. On the basis of saturation curves for plastocyanin in these reactions, a specific interaction of added plastocyanin with the membrane and cytochrome f can be postulated. Plastocyanin restores electron flow when added in about equimolar concentrations to the cytochrome f content. Similar results were obtained with pyridine-treated photosystem I particles [34]. The inhibitor studies with a monospecific plastocyanin antiserum show that plastocyanin, whether added in equimolar concentrations or in excess, remains accessible to the antibody. The notion that plastocyanin is adsorbed by the membrane mainly be electrostatic interaction [26] could not be confirmed. Addition of 0.26 M NaCl had no influence on restoration of cytochrome f oxidation by plastocyanin.

Using sonicated chloroplasts, Hauska *et al.* [15] concluded that externally added plastocyanin creates an artificial electron flow, sensitive to the antibody, whereas plastocyanin *in situ* could only be inhibited when sonic oscillation was performed in the presence of the antibody.

This is in contrast to the results of Schmid *et al.* [35], where a monospecific plastocyanin-antiserum inhibited photosynthetic electron transport and did agglutinate chloroplasts. They concluded that plastocyanin should be located in the outer thylakoid membrane surface. The experiments with digitonin-treated chloroplasts used here can, however, not decide whether or not externally added plastocyanin creates an artificial electron flow.

To get more information on the relative location of cytochrome f and plastocyanin in the membrane, the effect of charged redox reagents on oxidation-reduction kinetics of an individual component was investigated. Using ferricyanide as a probe for accessibility of chloroplast cytochrome f, Horton and Cramer [25] found that there is a considerable fraction of cytochrome f which is relatively inaccessible in the membrane. By studying the effect of actinic illumination and electron transport inhibitors on the accessibility of cytochrome f to ferricyanide, they concluded that the photooxidation of cytochrome f itself may be responsible for alterations in the kinetics of cytochrome f chemical oxidation.

In the photosystem I fragments used here, there are also two pools of cytochrome f, distinguishable on the basis of dark-reduction kinetics. Part of the cytochrome f complement remains relatively inaccessible to the chemical probe. Since ascorbate also produces biphasic reduction kinetics, it seems that both oxidation and reduction sites of cytochrome f exhibit similar behaviour. After illumination, part of the cytochrome f complement seems to move closer to the aqueous interface, as indicated by a slow monophasic reduction. This change of accessibility could not be triggered by cytochrome f photooxidation, which occurs only in the presence of plastocyanin in these fragments. Addition of plastocyanin does not change dark reduction kinetics, but after preillumination, a fast (within mixing time ≈ 1 sec) monophasic reduction of cytochrome f is seen. This is interpreted by a facilitated equilibration of the reductant with cytochrome f through plastocyanin residing on the membrane surface. This interpretation does not contradict the data of Horton and Cramer[25], where KCN treatment inhibited dark oxidation of cytochrome f, assuming that Cyt f is reduced or oxidized chemically *via* plastocyanin, which is destroyed upon KCN treatment.

The data find additional support by fast dark chemical redox kinetics of plastocyanin, which could not be resolved with this setup, whether or not the fragments were illuminated (data not shown).

Having discussed the pathway of cytochrome f oxidation, we will now turn to cytochrome b_6 photoreactions. From studies on well-coupled isolated spinach chloroplasts it was concluded that cytochrome b_6 appears to be a specific component of cyclic electron transport; it could be photoreduced and photooxidized by photosystem I alone, in a DCMU-insensitive reaction pathway[36, 7]. Furthermore, it was demonstrated that the pathway of cytochrome b_6 oxidation includes plastoquinone and a site of energy conservation[7]. This study extends these observations, showing that it is possible to obtain a photosystem I fraction with a photoactive cytochrome b_6 component. Moreover, cytochrome b_6 had a redox potential close to that obtained in coupled chloroplasts[22]. No evidence for the presence and/or activity of cytochrome b_{559LP} could be found. Exogenously added ferredoxin stimulated cytochrome b_6 reduction; although it was not dependent on the presence of ferredoxin, this stimulation was more pronounced at low light intensities. It was estimated that ferredoxin saturates cytochrome b_6 reduction at about twice the amount necessary for plastocyanin to saturate cytochrome b_6 or f oxidation.

Addition of soluble flavoprotein had no effect on cytochrome b_6 reduction. Since this photosystem I fraction still contains considerable amounts of bound flavoprotein, its action as a cytochrome b_6 reductase could not be ruled out. However, an antibody against Fd-NADP$^+$-reductase did not inhibit cytochrome b_6 reduction, but there might be unidentified flavoproteins reacting in this pathway. Evidence so far accumulated, suggesting a role of flavoprotein in cyclic electron flow, on the other hand is based mainly on the ability of isolated flavoprotein to act as a cytochrome f-NADPH reductase[37]. Whether or not this reaction occurs in the chloroplast electron transport system remains to be answered.

A role for ferredoxin in cyclic electron flow was suggested since it catalyzed cyclic ATP-formation in broken chloroplasts in the absence of other added cofactors[38]. However, to measure ATP formation, ferredoxin had to be added in about 100-fold excess to other electron carriers. It appears that this ferredoxin catalyzed ATP formation uses the native energy conservation site through plastoquinone; it is the only cyclic reaction so far described to be completely inhibited by DBMIB[27].

Cytochrome b_6 oxidation, as already mentioned, requires plastocyanin addition, showing a similar dependence as did cytochrome f oxidation. Cytochrome b_6 oxidation in the presence of plastocyanin was inhibited by DBMIB, indicating an involvement of plastoquinone. This agrees with the earlier statement that photosystem I fragments still contain about half the amount of plastoquinone found in chloroplasts.

The results reported here are consistent with the idea that there are several electron transport enzymes which are common to open-chain and cyclic electron transport[39, 7]. Regarding the development of photosynthesis, it was hypothesized[40] that the ancestral photosynthetic organism possessed only cyclic electron transport. This cyclic system might have been very similar to what can be isolated as photosystem I complex (still capable of electron transport and ATP formation) from "modern" oxygen evolving organisms. The development of a second photosystem communicating with the original cyclic chain *via* the plastoquinone pool gives an idea why enzymes in the pathway of plastoquinone oxidation are shared by both cyclic and non-cyclic electron transport[39, 33, 7].

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