BBA 46259

PHOTOREDUCTION OF CYTOCHROME b_{559} IN A PHOTOSYSTEM-II SUBCHLOROPLAST PARTICLE*

BACON KE, L. P. VERNON** AND T. H. CHANEY
Charles F. Kettering Research Laboratory, Yellow Springs, Ohio (U.S.A.)

(Received August 9th, 1971)

SUMMARY

A Photosystem-II reaction-center particle derived from spinach chloroplasts by Triton treatment contains only one kind of cytochrome, namely, cytochrome b_{559}, in the amount of slightly more than 2 per 100 total chlorophyll molecules. Cytochrome b_{559} is present in the oxidized form, has a standard redox potential of 58 mV, and undergoes photoreduction.

Photoreduction of cytochrome b_{559} and its re-oxidation in the dark can take place in the Photosystem-II particle without the addition of any exogenous electron donors such as diphenylcarbazide. The same reaction can even be better observed under anaerobic condition when oxidative photobleaching of bulk chlorophyll is prevented.

Whereas the photoreduction of 2,6-dichlorophenolindophenol catalyzed by the Photosystem-II particle and supported by diphenylcarbazide is completely inhibited by $2 \cdot 10^{-5}$ M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or carbonylcyanide-*m*-chlorophenylhydrazone, the photoreduction of cytochrome b_{559} is inhibited only 40 and 20 %, respectively.

The quantum efficiency of cytochrome b_{559} photoreduction is near unity. An Arrhenius plot yields an activation energy of 7.5 kcal/mole. The cytochrome reaction ceases when the reaction mixture becomes frozen. No photooxidation of cytochrome b_{559} at 77°K has been observed even when it was pre-reduced chemically.

Observations made thus far support the notion that cytochrome b_{559} is reduced by a reducing component formed in the electron-transport chain and re-oxidized by an oxidizing component formed on the oxidizing side of Photosystem-II. Thus, the light-induced redox reactions of cytochrome b_{559} form a small cycle around Photosystem-II. The nature for the lack of inhibition of cytochrome b_{559} photoreduction by DCMU is not yet clear.

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DPC, diphenylcarbazide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

* Contribution No. 439 from the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio, 45387, U.S.A. This work was presedted in part at the 2nd International Congress on Photosynthesis Research at Stresa, Italy, June 1971.

** Present address: Brigham Young University, Provo, Utah, U.S.A.

INTRODUCTION

Since cytochrome b_{559} was discovered[1,2], little information on its physical and chemical properties or its functional role in photosynthesis was known. RUMBERG[3,4], using alternating far red–red illumination of chloroplasts, obtained evidence for the reduction of a *b*-type cytochrome by Photosystem-II. LEVINE and coworkers[5,6], using chloroplast fragments from Chlamydomonas mutants, also demonstrated that cytochrome b_{559} was photoreduced by Photosystem-II. Similar conclusions were reached by other workers[7–9] for spinach chloroplasts.

The location of cytochrome b_{559} became better understood when it was found that this cytochrome was mainly concentrated in the heavy subchloroplast particles representing Photosystem-II[10–12]. A number of workers have reported on the standard redox potential of cytochrome b_{559}; the values fall into two groups, one lies at about +350 mV (refs. 13–16) and the other at +60 to +80 mV (refs. 12 and 17).

Although photoreduction of cytochrome b_{559} by Photosystem-II takes place at physiological temperatures, KNAFF AND ARNON[18] reported a photooxidation of this cytochrome at 77° K. The low-temperature photooxidation has subsequently been confirmed by others[16,19–21,34]. FLOYD *et al.*[21] observed a corresponding kinetics for the dark decay of the Photosystem-II reaction-center chlorophyll, P680, and the onset of the oxidation of cytochrome b_{559}, with a half time of 4.6 msec. From the low-temperature results, BOARDMAN *et al.*[16] and ERIXON AND BUTLER[19,20] suggested a direct electron donation from the cytochrome to the reaction-center chlorophyll at 77° K.

Although cytochrome b_{559} composition has been reported for subchloroplast particles[10–12], no investigations on the photochemical reactions have yet been made with these particles. Subchloroplast particles may have the advantage of providing an isolated Photosystem-II, and coupling of exogenous electron carriers may be controlled with these particles. In this paper, we wish to report some studies made on cytochrome b_{559} in a Photosystem-II subchloroplast particle isolated from spinach by the treatment of Triton X-100, and further purified by removing excess bulk chlorophyll. The composition and some properties of cytochrome b_{559} in these particles will be described. Photoreduction of cytochrome b_{559} and the effects of inhibitors suggest that cytochrome b_{559} may be located very close to the primary reductant and oxidant of Photosystem-II in this isolated particle.

EXPERIMENTAL

The Photosystem-II* subchloroplast particles were prepared from the "TSF-II" particle[11] by removing excess bulk chlorophyll[22]. Two or three TSF-II pellets[11] were homogenized with 25 ml cold 0.02 Tris (pH 8.0) containing 0.25 M sucrose and allowed to stand for 1 h. The suspension was then centrifuged at 105000 × *g* for 30 min. The supernate was decanted and then recentrifuged at 105000 × *g* for 30 min. As long as the resultant residue was large (more than a few mm in diameter), the supernate was centrifuged again until no precipitate remained. The supernate from the last centrifugation was passed through a 1-inch deep column (1-inch diameter) of Bioglass

* This particle has been designated as "TSF-IIa" in previous papers[22,25]. For the sake of simplicity, it is called Photosystem-II particle in the present paper.

2500 (BioRad Laboratories, Richmond, Calif.). The column was eluted with the same suspending medium. The deep-green fraction eluted from the column had the highest activity and was collected. The eluate was concentrated by partial freeze evaporation or by ultrafiltration, although the latter process reduced the activity by 1/3 to 1/2.

All chemicals were reagent grade where available and were used without further purification.

Absorption spectra and the oxidized-*minus*-reduced difference spectra were taken in a Cary Model 14R spectrophotometer. Fluorescence emission spectra were measured as described previously[23]. Methods for measuring light-induced absorption changes have been described previously[24]. A Kettering Model 68 radiometer was used for light intensity measurements.

CHARACTERISTICS OF THE PHOTOSYSTEM-II SUBCHLOROPLAST PARTICLES AND THE BOUND CYTOCHROME b_{559}

Some chemical and spectroscopic properties

The Photosystem-II particle is highly active in 2,6-dichlorophenolindophenol (DCIP) reduction when an exogenous electron donor such as diphenylcarbazide (DPC) is supplied, and the photoreaction is inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)[25]. Assay of Photosystem-II particle suspensions (20 μg chlorophyll per ml) by oxidized-*minus*-reduced difference spectroscopy at the highest sensitivity available on the Cary spectrophotometer (0.005 absorbance unit/inch) yielded no detectable P700. The particle also had a negligible $NADP^+$- reduction activity when the ascorbate–DCIP couple was used as the electron donor together with the necessary cofactors[25].

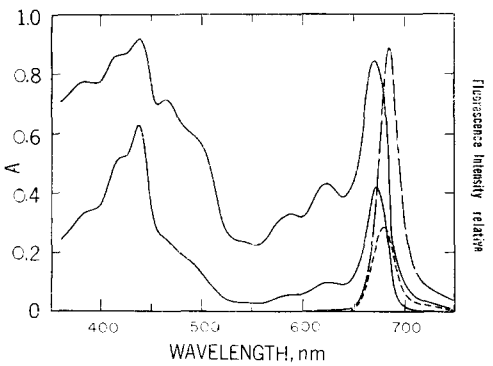


Fig. 1. Absorption spectra (solid curves) and fluorescence spectra (dashed curves) of the Photosystem-II particle in 0.01 M phosphate buffer (pH 6.4) at room (shorter curves) and liquid-nitrogen (taller curves) temperatures. Wavelength for fluorescence excitation, 435 nm; absorbance of the aqueous suspension at 670 nm, 0.1.

One anomaly, the high chlorophyll *a*/*b* ratio, has been previously reported[25]. This is evident from the absorption spectra (solid curves) in Fig. 1. Typical Photosystem-II particles have a chlorophyll *a*/*b* ratio of 8. Note that the low temperature only intensified the bands but did not bring out any additional band such as that of P700[26].

TABLE I

CYTOCHROME b_{559} CONTENT OF CHLOROPLASTS AND SUBCHLOROPLAST PARTICLES

Source	*Total number of chlorophyll molecules per cytochrome* b_{559}	*Reference*
Spinach chloroplasts	118[a]	10
	320	30
	450	12
	350	17
Digitonin-10K	120[b]	10
	320	30
French press-10K	113[b]	28
French press-10K/Digitonin-10K	164[c]	
Triton-"30P10"	300	12
TSF-II	100	11
Photosystem-II particle	43[d]	This work
Digitonin-144K	780[e]	10
Digitonin-144K	2650	30
TSF-I	—	31

[a] This ratio refers to total *b*-type cytochromes.

[b] The particle is relatively enriched in cytochrome b_{559}, but the ratio refers to total *b*-type cytochromes.

[c] B. Ke, unpublished experimental results.

[d] A higher cytochrome b_{559} content was estimated previously from the chemical difference spectrum at 77 °K (ref. 25).

[e] Estimated from original data given for total *b*-type cytochromes.

The particle used in the present work has the fluorescence properties shown by the dashed curves in Fig. 1. The fluorescence spectra at room temperature and 77 °K had practically a single emission band at 680 and 686 nm, respectively. There was little fluorescence at 735 nm, which was noticeable in TSF-II particles[23] as well as the previously prepared Photosystem-II particle[25]. More significantly, unlike the TSF-II particle, it lacked an emission band at 695 nm, which has often been attributed to the reaction-center chlorophyll of Photosystem II[23,27].

Cyrochrome b_{559} content and its redox properties

The TSF-II particles contain one cytochrome b_{559} per 100 total chlorophyll molecules[11], which represents an enrichment of 3–5 fold from the unfractionated chloroplasts. The present Photosystem-II particle was about twice further enriched in cytochrome b_{559}, thus making it the Photosystem-II subchloroplast particle with the highest cytochrome b_{559} content (see Table I); it contained no detectable cytochrome *f* or cytochrome b_6. Although cytochrome b_{559} is partitioned in digitonin-fractionated Photosystem-II particles, its content is not substantially different from the unfractionated chloroplasts. However, digitonin-fractionated 10K particles obtained from the (10K) lamellae fragments separated from a French-press homogenate[28] are enriched in cytochrome b_{559} by a factor of 2–3 (B.Ke, unpublished

experiment). Some literature data on cytochrome b_{559} content in various chloroplast and subchloroplast particles are listed in Table I.

Cytochrome b_{559} in the Photosystem-II particle was present in the oxidized state. Under aerobic conditions, it was only partially (40–50%) reduced by $2 \cdot 10^{-2}$ M ascorbate; even dithiothreitol reduced only 70–90%. However, under anaerobic condition, the same amount of ascorbate reduced more than 80% of the total cytochrome. Fig. 2 shows a reduced-*minus*-oxidized difference spectrum of the Photosystem-II particle with $2 \cdot 10^{-3}$ M dithiothreitol present in the sample cuvette, and represents approximately 70% reduction of the total cytochrome b_{559}. Addition of dithionite to the sample cuvette brought about a complete reduction. With a given reductant in the sample cuvette, the difference spectrum in the α-band region was not affected by the presence of ferricyanide in the reference cuvette, indicating that cytochrome b_{559} was originally present in the completely oxidized state.

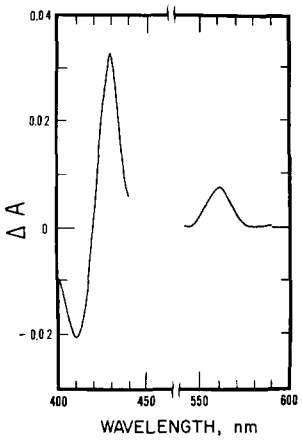


Fig. 2. Reduced-*minus*-oxidized difference spectrum of the Photosystem-II particle. Absorbance of the suspension at 675 nm, 1.5. The sample cuvette contained $2 \cdot 10^{-3}$ M dithiothreitol.

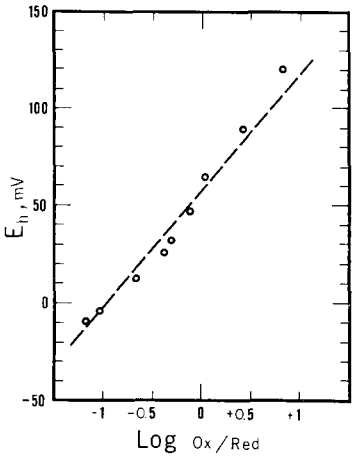


Fig. 3. Redox titration of cytochrome b_{559}. Redox buffer consisted of 10^{-3} M $FeCl_3$–0.5 M potassium oxalate in 0.1 M phosphate buffer at pH 7.0. 0.1 M dithionite in $2 \cdot 10^{-2}$ M NaOH was used as the titrant. A combination Pt–Ag/AgCl electrode was used for redox potential measurements. The potential of the Ag/AgCl reference electrode was separately determined with quinhydrone at several pH values to be 196 mV. Cytochrome b_{559} reduction was monitored at 560 nm in the Cary Model 14 spectrophotometer.

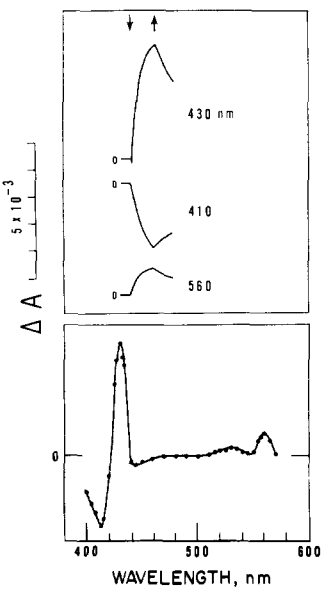
Since the Photosystem-II particle contains only one *b*-type cytochrome, a redox titration monitored by spectrophotometry was greatly simplified. A titration of the Photosystem-II particle carried out according to the procedure described by HILL[29] using a ferrous–ferric oxalate buffer and dithionite as the titrant. Total cytochrome b_{559} concentration in the suspension was separately determined by dithionite reduction. As shown in Fig. 3, the titration yielded a standard redox potential (E_0') of 58 ± 3 mV for cytochrome b_{559} at pH 7, which is in excellent agreement with that determined by HIND AND NAKATANI[12] for cytochrome b_{559} in their Triton-fractionated Photosystem-II fragment (30P10), and is consistent with the value reported by FAN AND CRAMER[17] for cytochrome b_{559} in unfractionated

spinach chloroplasts (*cf.* DISCUSSION). Note also that cytochrome b_{559} with an E_0' of + 58 mV accounted for all the cytochrome present in this Photosystem-II particle. This redox-potential value together with the effect of oxygen on ascorbate reduction indicates that cytochrome b_{559} in this subchloroplasts particle must be easily auto-oxidizable.

LIGHT-INDUCED REDUCTION OF CYTOCHROME b_{559}

Light-induced absorption changes and the light-minus-dark difference spectra

When the Photosystem-II particle was illuminated by red light in the absence or in the presence of an exogenous electron donor such as diphenylcarbazide (DCP) or 1,4-dihydroquinone (*vide infra*; also *cf.* Fig. 6), absorption changes corresponding to cytochrome b_{559} reduction could be observed. Fig. 4, top, shows light-induced absorption-change transients at 560, 430 and 410 nm in the Photosystem-II particles, and Fig. 4, bottom, shows the light-*minus*-dark difference spectrum derived from changes at the individual wavelengths. The complete agreement of the wavelength positions as well as the relative magnitudes of the light-*minus*-dark difference spectrum shown in Fig. 2 confirmed that a light-induced reduction of cytochrome b_{559} occurred. Longer illumination showed that the absorption increase reached a steady state in about 5–10 sec and decayed completely in about 20 sec (*cf.* Fig. 8).



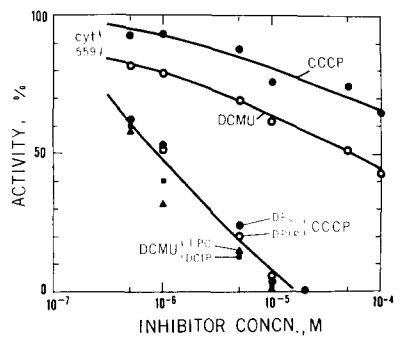


Fig. 4. Top: Light-induced absorption-change transients at several wavelengths representative of cytochrome b_{559}. The actinic light was isolated by an interference filter transmitting from 650 to 740 nm and had an intensity of approx. 10^5 ergs·cm^{-2}·sec^{-1}. Illumination time, 2 sec (between the two arrows); total time span for each signal trace, 5 sec. Bottom: Light-*minus*-dark difference spectrum. The solution contained: Photosystem-II particle in 0.01 M phosphate buffer (pH 6.4) with chlorophyll content of 10 μg/ml; diphenylcarbazide, $5 \cdot 10^{-4}$ M.

Fig. 5. Dependence of cytochrome b_{559}-reduction activity (measured at 560 nm) on inhibitor (DCMU and CCCP) concentrations. The lower curve shows the inhibitor effect on DCIP-reduction activity supported by DPC[32]. Other conditions same as in Fig. 4.

Effect of some inhibitors on cytochrome b_{559} reduction

Photosystem-II reductions are specifically inhibited by DCMU. This is true for DCIP reduction by Photosystem-II particles supported by the exogenous electron donors DPC[25] or 1,4-dihydroquinones[32]. On the other hand, the reduction of cytochrome b_{559}, as measured by the absorption increase at 560 nm is much less sensitive to DCMU. CCCP behaves similarly to DCMU for both cytochrome b_{559} or donor-supported dye reduction. Fig. 5 shows the inhibitory action of DCMU and CCCP on cytochrome b_{559} reduction; the inhibitor action on dye reduction[32] is reproduced in the same figure for comparison. At the inhibitor concentration for complete inhibition of dye reduction, cytochrome b_{559} was inhibited only about 40 % by DCMU (also see Fig. 6) and only 20% by CCCP. Among other inhibitors tested: 1 mM *o*-phenanthroline gave about 20% inhibition; 20 μM NH_2OH or 5–10 μM gramacidin were without any effect.

Photoreduction of cytochrome b_{559} independent of the presence of exogenous electron donors

Although the light-induced absorption-change transients, shown in Fig. 4, were obtained with an exogenous electron donor present, the reduction reaction can also take place under appropriate conditions without the presence of an electron donor (*vide infra*; also *cf.* Fig. 8). This may be illustrated with the following two examples.

In Fig. 6, the upper transients in the first column represent light-induced absorption changes due to cytochrome b_{559} reduction monitored at 560 nm without (dashed) and with (solid) DPC present, and the two transients were practically identical. The transients immediately below show changes with the same reaction mixtures which contained DCMU at $2 \cdot 10^{-5}$ M. Again, the presence of an electron donor had little effect on the signal, except the magnitudes of both signals were decreased by about 30% due to DCMU (*cf.* Fig. 5).

Absorption-change transients due to cytochrome b_{559} reduction in the second column were monitored at 430 nm, without and with DPC, and without (upper) and with (lower) DCMU present. With DPC present, the absorption increase at 430 nm appeared normal, as judged by its magnitude relative to that at 560 nm. However, without DPC present, the absorption increase appeared to be counteracted by an absorption decrease even before the light was turned off (at the arrow). With DCMU present, the situation was accentuated; even with DPC present, the magnitude was lowered by the inhibitory action of DCMU, but a premature decrease is also noticeable. Without DPC present, the rate of the counter decrease was so high that the net signal became very small. At higher DCMU concentrations, only a net decrease in absorption was observed.

Separate measurements revealed that the counter absorption decrease in the 430 nm region was due to a slow, irreversible photobleaching of the bulk chlorophyll. This photobleaching was retarded by electron donors such as DPC, but accentuated by Photosystem-II inhibitors such as DCMU and CCCP[32]. The absorption changes at 560 nm (the first column of Fig. 6), where the photobleaching had negligible effect on the cytochrome b_{559} absorption changes, conclusively demonstrated that cytochrome reduction took place independent of the presence of an exogenous electron donor. It is also of interest to note that when DPC was present in the reaction mixture, it was independently oxidized, presumably by the oxidant pool generated by Photosystem-II[32].

Another example is shown in Fig. 7, where mammalian ferrocytochrome *c* was used as the electron donor for Photosystem-II[32]. The top row shows absorption-change transients at 560, 556.5 and 550 nm in the absence of added electron donors, and all transients represent cytochrome b_{559} reduction. The bottom row shows changes at the same wavelengths except the reaction mixture contained 10 μM ferrocytochrome *c*. From the signals at 560 and 556.5 nm, it is obvious that cytochrome b_{559} reduction took place unaffected by the presence of mammalian ferrocytochrome *c*. However, the steady absorption decrease at 550 nm clearly showed that cytochrome *c* was independently oxidized. At 550 nm, the small positive absorption increase due to cytochrome b_{559} reduction would have been masked by the large absorption decrease due to cytochrome *c* oxidation. The wavelength 556.5 nm is an isosbestic point for cytochrome *c* redox changes[33], therefore no contribution by cytochrome *c* oxidation is expected at this wavelength, and the transient at 556.5 nm was indeed unaffected, with or without cytochrome *c* present. Mammalian cytochrome *c* oxidation contributes a small absorption increase at 560 nm, which would account for the slightly larger absorption increase at 560 nm in the presence of cytochrome *c* than in its absence.

Photoreduction of cytochrome b_{559} under anaerobic conditions

Since it is known that cytochrome b_{559} is autooxidizable, the question arises whether the dark decay of the absorption change was caused by reoxidation of the photoreduced cytochrome by oxygen. It was also shown earlier that absorption changes in the Soret region may be influenced by changes due to oxidative photo-

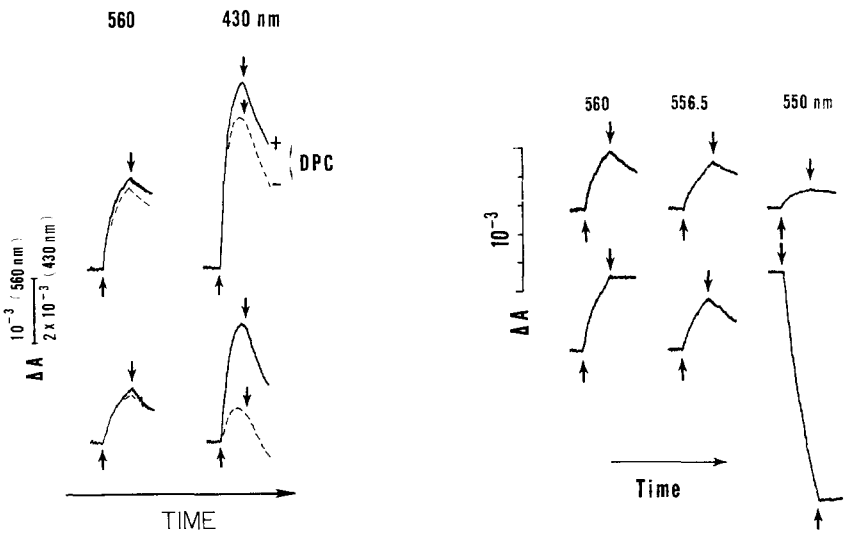


Fig. 6. Light-induced absorption-change transients measured at 560 and 430 nm in the absence and in the presence of the exogenous electron donor (DPC) and/or DCMU. Solid curves, with DPC; dashed curves, without DPC. Transients at top, no DCMU; transients at bottom, with 10^{-5} M DCMU. Chlorophyll, 11 μg/ml. Illumination conditions same as in Fig. 4.

Fig. 7. Light-induced absorption changes at 560, 556.5 and 550 nm in the Photosystem-II particles in the absence (top row) and in the presence (bottom row) of mammalian ferrocytochrome *c*. Chlorophyll, 12 μg/ml; cytochrome *c* (when present), 10 μM. Illumination conditions same as in Fig. 4.

bleaching of bulk chlorophyll. For these reasons, the effect of oxygen on the light-induced reduction of cytochrome b_{559} and its back reoxidation were examined. For this examination, we have chosen a Photosystem-II particle preparation which had a more prominent photobleaching reaction even in the presence of an electron donor. Under the aerobic conditions that are usually used for these investigations, the light-induced absorption changes at 430 nm shown in Fig. 8, top, were observed. The absorption decrease due to chlorophyll bleaching in this sample is almost as fast as that shown in Fig. 6 where DCMU was also present. However, when the sample was made anaerobic by adding glucose, glucose oxidase, catalase and ethanol to remove and trap the dissolved oxygen, the 430-nm absorption change became completely normal, without any evidence of an interference by an absorption change due to chlorophyll bleaching. This signal also shows that the reduction reached a steady state in about 10 sec and decayed completely in about 25 sec. It is also worth noting that, under anaerobic conditions, the same light-induced reduction of cytochrome b_{559} can be brought about without the presence of any exogenous electron donor.

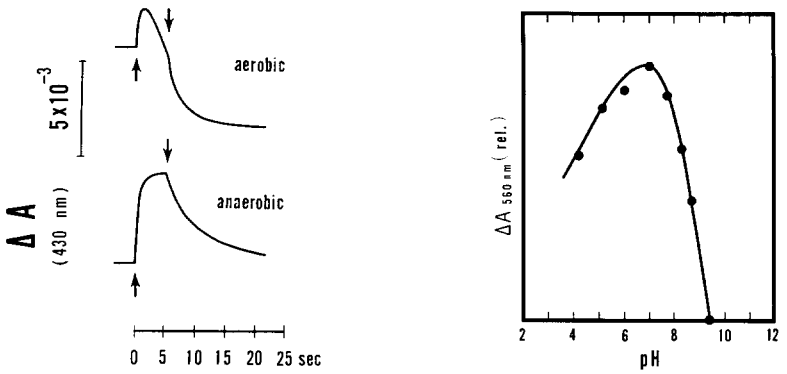


Fig. 8. Top: Light-induced absorption changes in a Photosystem-II particle at 430 nm under aerobic condition. Bottom: same as the top except under anaerobic condition. Photosystem-II particle suspended in 0.01 M phosphate buffer (pH 6.4). Chlorophyll concentration, 12 μg/ml; diphenylcarbazide, $5 \cdot 10^{-4}$ M. Illumination conditions same as in Fig. 4. Illumination duration, 5 sec; total time span, 25 sec.

Fig. 9. Cytochrome b_{559} photoreduction activity measured at 560 nm *vs.* pH. Chlorophyll concentration, 10 μg/ml. Solution was made anaerobic by the glucose/glucose oxidase trap. Illumination conditions same as in Fig. 4.

pH dependence

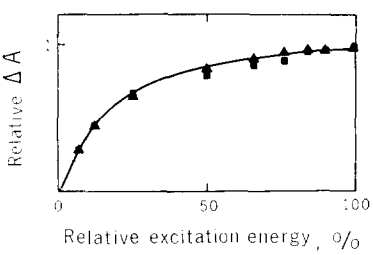
The pH dependence of cytochrome b_{559} photoreduction was examined under anaerobic conditions by measuring the light-induced 560-nm absorption changes. Without an exogenous electron donor present, the optimum pH was near 6.5–7.0; the reaction was practically nil above pH 9 (Fig. 9). With 1,4-dihydroquinone as the donor, the entire curve appeared to shift toward the lower pH; the maximum was near 6, the upper limit was near pH 8.5.

Excitation-intensity dependence and quantum yield

The rate dependence of cytochrome b_{559} photoreduction on excitation intensity is shown in Fig. 10. For this study, the excitation light was isolated with a 10-nm wide

interference filter with a maximum transmission at 670 nm, coinciding with the maximum absorption by the sample. At low intensities, the rate increased linearly with intensity, and reached an optimum rate at a higher intensity.

An approximate estimation showed that the quantum yield for the reaction was near unity. The quantum yield was estimated by comparing the actual quantum flux and the rate of cytochrome b_{559} reduction calculated from the absorption changes. The absorbed energy was measured from the difference by first placing the Kettering Model 68 radiometer probe immediately behind a cuvette containing only the buffer and then behind the cuvette containing the subchloroplast suspension used in the absorption-change measurements. At a quantum flux of 3.6 nEinsteins$\cdot$cm$^{-2}\cdot$sec^{-1}, 3.5 and 2.7 nmoles$\cdot$cm$^{-2}\cdot$sec^{-1} of cytochrome b_{559} was reduced as estimated from absorption increases at 430 and 560 nm, respectively (20 l$\cdot$mmole^{-1} was used as the millimolar extinction coefficient for the 560-nm band of cytochrome b_{559}; the extinction coefficient for the 430-nm band was estimated from Figs. 2 and 4).



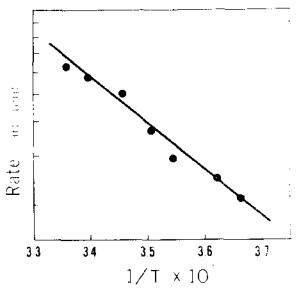


Fig. 10. Cytochrome b_{559} photoreduction activity plotted *vs.* excitation intensity. 100% intensity corresponds to an incident flux of $5.7\cdot10^{16}$ quanta (at 670 nm)$\cdot$cm$^{-2}\cdot$sec^{-1}.

Fig. 11. An Arrhenius plot of the log of cytochrome b_{559} photoreduction rate *vs.* reciprocal temperature. Experiments conducted in a thin-path (1.8 mm) cuvette inside a Dewar. Sample temperature was measured with a thermocouple. Chlorophyll concentration, 96 μg/ml. Illumination conditions same as in Fig. 4.

Temperature dependence and energy of activation; and the absence of photooxidation of cytochrome b_{559} at 77°K

The rate of cytochrome b_{559} photoreduction was sensitive to temperature. An Arrhenius plot for the temperature range from 25° to about 0° is presented in Fig. 11. From the slope of the plot, the activation energy for cytochrome b_{559} reduction was estimated to be 7.5 kcal/mole. Below the freezing temperature (which is usually slightly below 0° depending on the rate of cooling), the cytochrome reaction apparently ceased, and a new, rapid and reversible absorption change presumably associated with the Photosystem-II reaction-center chlorophyll occurred, which persisted even at 77°K (B.Ke report in preparation).

Separate experiments in an attempt to observe cytochrome b_{559} photooxidation were performed by first adding 10^{-2} M dithiothreitol to the sample prior to freezing to bring the cytochrome b_{559} to the reduced state. An identical Photosystem-II suspension was placed in the reference cuvette which contained some ferricyanide. The samples were rapidly frozen to 77°K, and the oxidized-*minus*-reduced difference spectrum taken, which showed the difference α-band of cytochrome b_{559} at 556 nm.

The sample containing the reduced cytochrome b_{559} was then irradiated with red light for 2 min while it was maintained at 77°K. The difference spectrum taken after irradiation was identical to that before irradiation, indicating that cytochrome b_{559} in this subchloroplast particle was not photooxidized at 77°K, contrary to that observed with unfractionated chloroplasts[16,18-21,34,35].

DISCUSSION

By means of chloroplast fractionation by Triton treatment, it is apparently possible to obtain a stable subchloroplast particle representative of Photosystem II, as characterized by its ability in catalyzing electron transport to typical Hill acceptors from suitable electron donors. Furthermore, the subchloroplast particle is highly enriched in cytochrome b_{559}, and the latter undergoes light-induced redox reactions without the presence of any external electron carriers. As shown in Fig. 4, under steady-state illumination, where transient absorption changes associated with the faster-reacting primary components are presumably not seen, the subchloroplast particles yield a light-*minus*-dark difference spectrum of almost exclusively reduced cytochrome b_{559}.

The redox potential of +58 mV measured for cytochrome b_{559} in the Photosystem-II particle is in excellent agreement with the value reported by HIND AND NAKATANI[12] for their Triton-fractionated heavy particles. However, this value is substantially more negative than that reported more recently by several other workers[13-16,32] for cytochrome b_{559} in unfractionated chloroplasts. The existence of cytochrome b_{559} in more than one redox form has been reported previously[13]. At this writing, reports from several laboratories[34-37] have further confirmed this: intact chloroplasts apparently contain both the high- and low-potential forms of cytochrome b_{559}; the high-potential form is apparently sensitive to detergent[34-36], organic solvents[34-35], Tris washing[36], aging [36], and to heat treatment[34-35], and is transformed into the low-potential form by any of these treatments. CRAMER *et al.*[37] further showed that the redox potential of cytochrome b_{559} is dependent on the coupling state of the chloroplasts to photophosphorylation, *i.e.* in coupled chloroplasts, cytochrome b_{559} has a more positive E'_0 value of +360 mV, whereas in the uncoupled chloroplasts, the E'_0 assumes a value of +60 to +80 mV. The experimentally determined redox potential of +58 mV for cytochrome b_{559} in the Triton-fractionated Photosystem-II subchloroplast particles is consistent with these findings.

It is also worth noting that among the observations made on photooxidation of cytochrome b_{559} at 77°K, several groups[15,16,34,35] have demonstrated that it was the high-potential cytochrome b_{559} that underwent the low-temperature photooxidation. Our finding that the cytochrome b_{559} in the Photosystem-II particle, with a redox potential of +58 mV, even pre-reduced chemically before the reaction mixture was frozen, did not undergo photooxidation at 77°K, further indicates that the low-temperature photooxidation may be a very unique property of the high potential cytochrome b_{559}.

Of more interest is the fact that cytochrome b_{559} in the particicle undergoes a photoreduction, similar to that found to occur in intact chloroplasts[4-9]. The fact that photoreduction occurs without the presence of an exogenous electron donor suggest that cytochrome b_{559} in the Photosystem-II particle must have been reduced

by the reducing component formed in or near the primary photochemical stage involving a charge separation. The reversibility of the reaction, *i.e.* reoxidation of photoreduced cytochrome b_{559}, under anaerobic condition and in the absence of an added electron acceptor suggests that the cytochrome is oxidized by the oxidizing component also formed in or near the primary photochemical reaction. Thus, the light-induced redox reaction of cytochrome b_{559} forms a small cycle around Photosystem II.

The DCMU insensitivity of cytochrome b_{559} photoreduction in the Photosystem-II particle is also remarkable. The site of DCMU action is generally considered to be after the primary electron acceptor, Q. Thus, the inhibitor data would suggest that either cytochrome b_{559} receives electrons more directly prior to Q, or that a structural derangement occurred in the Photosystem-II particle as a result of detergent treatment, and thus the electron-transport pathway leading to the bound cytochrome b_{559} was not inhibited by the usual amount of DCMU. It should be noted, however, that electron transport to an externally added, diffusable electron acceptor such as DCIP was still subject to DCMU blocking, albeit at a relatively high inhibitor concentration. Presently available data do not enable us to decide which mechanism might be operative in cytochrome b_{559} photoreduction.

A closer interaction of cytochrome b_{559} with the photochemically formed reducing component seems to be supported by the high quantum efficiency found for the reaction. Further examination of the rapid kinetics of cytochrome b_{559} and the Photosystem-II reaction-center chlorophyll, P680, which is being planned, might shed some light on this problem. In this connection, it may be of interest to note that for the low-temperature photooxidation of cytochrome b_{559} a risetime of 4.6 msec was found by FLOYD *et al.*[21], which corresponds to the decay of the 680-nm absorption decrease attributed to P680. In this case, the reaction-center chlorophyll P680 was assumed to be the primary electron donor of Photosystem II.

Suggestions have been made previously that cytochrome b_{559} may not be located on the main electron-transport chain between the two photosystems. The first such suggestion was made by RUMBERG[4] from flash-kinetic evidence. Based on evidence from uncoupler effects, HIND[8] suggested that cytochrome b_{559} may be located on one of two alternative non-cyclic electron-transport pathways. Hind found that one class of uncouplers, CCCP and antimycin A, which operates on the basal pathway, greatly promotes the oxidation of cytochrome b_{559} by Photosystem I, whereas uncouplers of the other class, which includes amines, do not affect cytochrome b_{559} turnover. As a result of recent findings on low-temperature photooxidation of cytochrome b_{559} by Photosystem II, other suggestions[34,35,38] have been put forward for the physiological role of cytochrome b_{559} in chloroplasts. Whether photoreduction of cytochrome b_{559} in the Photosystem-II particles represents a physiologic reaction, as has also been observed previously in chloroplasts, awaits further clarifications.

ACKNOWLEDGEMENTS

Support for this work by a National Science Foundation Grant (GM-8460) is gratefully acknowledged.

REFERENCES

1 H. LUNDEGARDH, *Physiol. Plant.*, 15 (1962) 390.
2 H. LUNDEGARDH, *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 1587.
3 B. RUMBERG, *Biochim. Biophys. Acta*, 102 (1965) 354.
4 B. RUMBERG, in J. B. THOMAS AND J. C. GOEDHEER, *Currents in Photosynthesis*, Donker Press, Rotterdam, 1966.
5 R. P. LEVINE, D. S. GORMAN, M. AVRON AND W. L. BUTLER, *Brookhaven Symp. Biol.*, 19 (1966) 143.
6 R. P. LEVINE AND D. S. GORMAN, *Plant Physiol.*, 41 (1966) 1293.
7 W. A. CRAMER AND W. L. BUTLER, *Biochim. Biophys. Acta*, 143 (1967) 332.
8 G. HIND, *Photochem. Photobiol.*, 7 (1968) 369.
9 G. BEN HAYYIM AND M. AVRON, *Eur. J. Biochem.*, 14 (1970) 205.
10 N. K. BOARDMAN AND J. M. ANDERSON, *Biochim. Biophys. Acta*, 143 (1967) 181.
11 L. P. VERNON, B. KE, H. H. MOLLENHAUER AND E. R. SHAW, in H. METZNER, *Progress in Photosynthesis Research*, Vol. 1, Tübingen, 1969, p. 131.
12 G. HIND AND H. Y. NAKATANI, *Biochim. Biophys. Acta*, 216 (1970) 223.
13 D. S. BENDALL, *Biochem. J.*, 109 (1968) 40P.
14 I. IKEGAMI, S. KATOH AND A. TAKAMIYA, *Biochim. Biophys. Acta*, 162 (1968) 604.
15 D. B. KNAFF AND D. I. ARNON, *Biochim. Biophys. Acta*, 226 (1971) 400.
16 N. K. BOARDMAN, J. M. ANDERSON AND R. G. HILLER, *Biochim. Biophys. Acta*, 234 (1971) 126.
17 H. N. FAN AND W. A. CRAMER, *Biochim. Biophys. Acta*, 216 (1970) 200.
18 D. B. KNAFF AND D. I. ARNON, *Proc. Natl. Acad. Sci. U.S.*, 63 (1969) 956.
19 K. ERIXON AND W. L. BUTLER, *Biophys. Soc. Meeting*, Abstr. TPM-D2, 1971.
20 K. ERIXON AND W. L. BUTLER, *Biochim. Biophys. Acta*, 234 (1971) 381.
21 R. A. FLOYD, B. CHANCE AND D. DEVAULT, *Biochim. Biophys. Acta*, 226 (1971) 112.
22 L. P. VERNON, S. KLEIN, F. G. WHITE, E. R. SHAW AND B. C. MAYNE, paper presented at *2nd Int. Conf. on Photosynthesis, Stresa, Italy, June 1971.*
23 B. KE AND L. P. VERNON, *Biochemistry*, 6 (1967) 2221.
24 B. KE, R. W. TREHARNE AND C. MCKIBBEN, *Rev. Sci. Instrum.*, 35 (1964) 296.
25 L. P. VERNON, E. R. SHAW, T. OGAWA AND D. RAVEED, *Photochem. Photobiol.*, 14 (1971) 343.
26 W. A. CRAMER AND W. L. BUTLER, *Biochim. Biophys. Acta*, 153 (1968) 889.
27 N. K. BOARDMAN, S. W. THORNE AND J. M. ANDERSON, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 586.
28 P. V. SANE, D. J. GOODCHILD AND R. B. PARK, *Biochim. Biophys. Acta*, 216 (1970) 162.
29 R. HILL, in K. PAECH AND M. V. TRACEY, *Modern Methods of Plant Analysis*, Vol. 1, Springer, Berlin, 1956.
30 D. I. ARNON, H. Y. TSUJIMOTO, B. D. SWAIN AND R. K. CHAIN, in K. SHIBATA, A. TAKAMIYA, A. T. JAGENDORF AND R. C. FULLER, *Comparative Biochemistry and Biophysics of Photosynthesis*, University of Tokyo Press, 1968.
31 L. P. VERNON, B. KE AND E. R. SHAW, *Biochemistry*, 6 (1967) 2210.
32 B. KE, manuscript in preparation.
33 E. MARGOLIASH AND N. FROHWIRT, *Biochem. J.*, 71 (1959) 570.
34 D. S. BENDALL AND D. SOFROVA, *Biochim. Biophys. Acta*, 234 (1971) 371.
35 D. S. BENDALL AND D. SOFROVA, paper presented at *2nd Int. Conf. on Photosynthesis, Stresa, Italy, June 1971.*
36 K. WADA AND D. I. ARNON, *Annu. Meet. Am. Soc. Plant Physiol., Pacific Grove, Calif., August 1971*, Abstr. No. 196.
37 W. A. CRAMER, H. N. FAN AND H. BÖHME, *J. Bioenerg.*, in the press.
38 R. G. HILLER, J. M. ANDERSON AND N. K. BOARDMAN, paper presented at *2nd Int. Conf. on Photosynthesis, Stresa, Italy, June 1971.*