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INTERACTIONS BETWEEN PHOTOSYSTEM II COMPONENTS IN CHLOROPLAST MEMBRANES

A CORRELATION BETWEEN THE EXISTENCE OF A LOW POTENTIAL SPECIES OF CYTOCHROME *b*-559 AND LOW CHLOROPHYLL FLUORESCENCE IN INHIBITED AND DEVELOPING CHLOROPLASTS

PETER HORTON *, EDWARD CROZE ** and GREGORY SMUTZER

Division of Cell and Molecular Biology, State University of New York at Buffalo, Buffalo, N.Y. (U.S.A.)

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Summary

1. Chloroplasts inhibited by incubation with hydroxylamine in the light exhibit a low fluorescence yield upon illumination in the presence of dithionite sufficient to completely reduce the primary acceptor, Q. In the absence of magnesium ions, the fluorescence yield is the same as in control chloroplasts, suggesting that the reason for the low yield is a defect in the mechanism by which Mg^{2+} enhances the fluorescence. These chloroplasts were previously shown to contain only low potential ($E_{m7.8}$ = +80 mV) cytochrome *b*-559 (Horton, P. and Croze, E. (1977) Biochim. Biophys. Acta 462, 86—101).

2. In Photosystem II particles, in heat-treated chloroplasts and in trypsin-digested chloroplasts, high potential cytochrome *b*-559 is absent and the variable fluorescence yield is again low.

3. Peas grown under intermittent light contain only one-fifth of the content of high potential cytochrome *b*-559 seen in fully greened plants, yet show high rates of water to methyl viologen electron transport. Aquisition of the high potential cytochrome *b*-559 accompanies synthesis of chlorophyll *b*, the onset of Mg-stimulated fluorescence and an increased variable yield of fluorescence. A similar correlation was seen during greening of dark-grown barley.

4. It is proposed that the high potential state of cytochrome *b*-559 is due

* To whom correspondence should be addressed. Permanent address after August 1st, 1978: Biochemistry Department, University of Sheffield, Sheffield, U.K.

** Present address: Dept. of Biochemistry, Purdue University, West Lafayette, Ind., U.S.A.

Abbreviations: f_0, initial fluorescence yield; f_v, variable fluorescence yield; NH_2OH_{light} chloroplasts, chloroplasts inhibited with hydroxylamine plus light [2]; NH_2OH_{dark} chloroplasts, chloroplasts inhibited with hydroxylamine in darkness [2]; E_m, midpoint oxidation reduction potential; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

to the same membrane properties which allow cation enhanced variable fluorescence, so that the presence of low potential cytochrome *b*-559 is accompanied by a decrease in variable fluorescence yield.

Introduction

A normally functioning Photosystem II unit is defined as having the ability of photooxidize water, giving rise to the evolution of oxygen. In mature chloroplasts this functionality is accompanied by the presence of cytochrome *b*-559 with a midpoint oxidation reduction potential (E_m) of +380 mV [1—4]. When O_2 evolution is inhibited by various treatments [2,5] or in mutants [6], a lowering in the E_m of cytochrome *b*-559 is seen. This was recently characterized in hydroxylamine-treated chloroplasts as being a shift to an $E_{m7.8}$ of +240 mV [2]. This correlation does not indicate an obligatory role for cytochrome *b*-559 in water oxidation because the converse does not apply; it is possible the observe H_2O oxidation in the absence of the normal amount of the high potential (i.e., +380 mV) species (refs. 7 and 8, and see below). The association between O_2 evolution and cytochrome *b*-559 does imply, however, that the E_m value of the cytochrome responds, in a very sensitive way, to the organizational state of Photosystem II. For instance, in hydroxylamine-treated chloroplasts, cytochrome *b*-559 is successfully probing the photooxidizing ability of Photosystem II. It can be deduced from such observations that if an understanding of the factors affecting the E_m of cytochrome *b*-559 can be reached, our appreciation of the mechanism of Photosystem II will be greatly improved.

A second major property of Photosystem II in normal chloroplasts is chlorophyll fluorescence. Two kinds of changes in fluorescence yield are detectable upon illumination of chloroplasts. Firstly, there is a rise in fluorescence correlated to the closing of Photosystem II traps as the primary acceptor becomes reduced [9]. Secondly, there exist changes in the extent of this variable fluorescence yield dependent upon the concentration and valency of the cations present in the medium [10—13]. This latter effect is seen as a slow rise in the fluorescence yield when divalent cations are added to divalent cation depleted chloroplasts and is thought to represent a change in the partitioning of excitation between Photosystem II and Photosystem I. The exact meachanism of this effect remains to be established, although it seems to be related to the surface of the thylakoid membrane [13]. Cation effects on fluorescence are probably related to control of excitation distribution in vivo and ought to be associated with other events to confer Photosystem II specificity on the process (see ref. 14 for a brief review). It is of interest, therefore, to consider whether other components of Photosystem II (e.g., cytochrome *b*-559) show any responses during cation-regulated fluorescence. In this paper a relationship between the extent of the variable fluorescence yield and the redox behavior of cytochrome *b*-559 is described.

Materials and Methods

Chloroplasts were isolated from peas as described previously [2]. Hydroxylamine inhibition of Photosystem II was performed by the procedure of Izawa

and Ort [15] with incubation being done in the light or dark [2]. Heat treated chloroplasts were prepared by incubation for 5 min at appropriate temperatures in 0.2 M sucrose, 2 mM $MgCl_2$ and 10 mM HEPES, pH 7.6 followed by addition of 2 vols cold medium before pelleting the chloroplasts by centrifugation. Incubation with trypsin was in the same medium for appropriate times at room temperature in 20 μg/ml trypsin (Sigma T8253). Digestion was terminated by addition of trypsin inhibitor (Sigma I-5) before centrifugation and washing. Chloroplasts lacking chlorophyll *b* and grana were prepared from peas grown for seven days in darkness followed by 48 h of intermittent light (90 s light every 100 min) according to the procedure of Armond, et al. [16].

Development of dark grown barley (variety Larker) was induced by exposing seedlings grown in complete darkness for 7 days at 21°C to continuous illumination from fluorescent tubes (warm white intensity 12 J/m^2 per s) at a temperature of 22°C. Etioplasts and developing chloroplasts were isolated by the method of Leese, Leech and Thompson [17].

Cytochrome absorption changes, redox titrations of cytochrome *b*-559 and rates of electron transport were measured exactly as described previously [2]. Chlorophyll fluorescence at 694 nm (Balzars interference filter) was excited using blue light (Corning 4-96, 5-58 and 1-75 glass filters) at intensities of 12 J/m^2 per s and <0.1 J/m^2 per s as described in figure legends. Induction curves were recorded using a Tracor Northern NS-570 after initiation of fluorescence by a Uniblitz Model 26 shutter (opening time 0.6 ms). Chlorophylls *a* and *b* were assayed by Arnon's method [18], using spectra recorded between 600 and 700 nm on an Aminco DW-2 spectrophotometer.

Results

Fluorescence changes in NH_2OH-treated chloroplasts

Chloroplasts with deficiencies on the donor side of Photosystem II, such as hydroxylamine-inhibited chloroplasts, do not reach the maximum amplitude of fluorescence even in high intensity light. Therefore, the true extent of the variable yield (f_v) is only seen upon reduction with dithionite. A probable explanation for the low f_v seen in the light is quenching by oxidized *P*-680 which may accumulate with insufficient electron donation [19,20]. Fluorescence yield changes upon addition of dithionite to control chloroplasts, chloroplasts incubated with NH_2OH in the dark, (NH_2OH_{dark}) and in the light (NH_2OH_{light}) are shown in Fig. 1. The maximum fluorescence yield in the

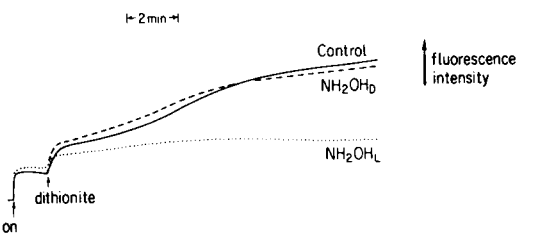

Fig. 1. Fluorescence yield in low intensity of control and hydroxylamine treated chloroplasts. NH_2OH_{dark} and NH_2OH_{light} refer to chloroplasts incubated for 30 min in 5 mM NH_2OH in darkness and continuous illumination respectively. Chlorophyll concentration 20 μg/ml, dithionite 3 mM.

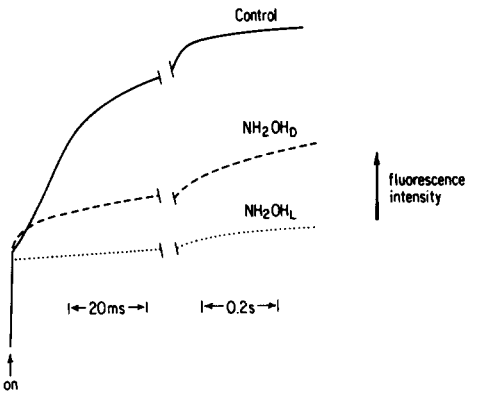

Fig. 2. Fluorescence induction curves of control and hydroxylamine treated chloroplasts. Samples as described in Fig. 1. Chlorophyll concentration 1 μg/ml; plus 20 μM diuron. Light intensity 12 J/m^2 per s.

control chloroplasts is approximately twice that of chloroplasts incubated in the light with NH_2OH. Dark-incubated samples were unchanged from the control. Fluorescence induction curves in high intensity light showed that the initial fluorescence level, f_0, corresponding to fully open Photosystem II traps, is unchanged by hydroxylamine treatment (Fig. 2). Control chloroplasts show a characteristic sigmoidal rise curve as the traps become closed. NH_2OH_{dark} chloroplasts show a much slower rise curve, that can be accelerated by addition of artificial electron donors. NH_2OH_{light} chloroplasts show only a small rise above the initial f_0 value. Fig. 3 shows the amplitudes of fluorescence induced by light with and without addition of an artificial donor. In contrast to NH_2OH_{dark} chloroplasts, NH_2OH_{light} samples show no stimulation of fluorescence by the donor. This is consistent with the inability of donors to restore Photosystem II electron transport [2,21], and the inhibition of Z to *P*-680 electron flow [20,22] in these chloroplasts. The fluorescence is kept close to the f_0 value, either by a $P\text{-}680^+Q^-$ back reaction as the only

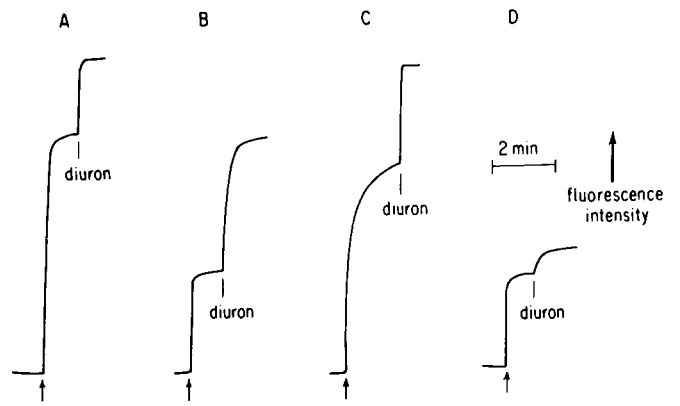

Fig. 3. Effect of electron donors on fluorescence yield under high intensity light. Chloroplasts were prepared as in Fig. 1. A, control; B, NH_2OH_{dark}; C, NH_2OH_{dark} + artificial donor (50 mM NH_2OH); D, NH_2OH_{light} ± artificial donor (50 mM NH_2OH). Chlorophyll concentration 5 μg/ml, diuron 20 μM, light intensity 12 J/m^2 per s.

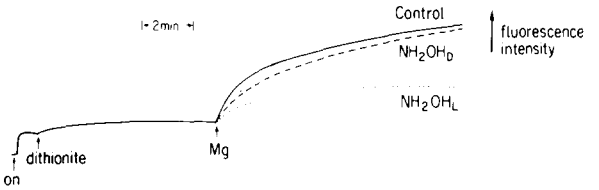

Fig. 4. The effect of Mg^{2+} on fluorescence yield in control and hydroxylamine treated chloroplasts. Conditions described in Fig. 1 except that following incubation chloroplasts were washed twice and resuspended in a Mg^{2+}-free medium. $MgCl_2$ concentration, 3 mM.

reaction available for reducing *P*-680$^+$ or because of quenching by oxidized *P*-680 [22].

Because f_0 is unchanged, NH_2OH_{light} treatment reduced f_v by a factor of approx. 4. In order to examine the reason for the low yield of variable fluorescence in the inhibited chloroplasts cation, effects were investigated, it is the increase in f_v induced by divalent cations which gives rise to the high yield in control chloroplasts [10—13]. Control and hydroxylamine chloroplasts were depleted by Mg^{2+} by washing twice in Mg^{2+}-free medium and their fluorescence characteristics were examined (Fig. 4). Prior to the Mg^{2+} addition, all three samples showed identical fluorescence intensity. On adding Mg^{2+}, however, the fluorescence yield of NH_2OH_{light} chloroplasts rose far less than either control or NH_2OH_{dark} chloroplasts. The final levels after several minutes in Mg^{2+} now exhibit the same differences as seen in Fig. 1. Thus the low fluorescence yield in NH_2OH_{light} chloroplasts is fully explainable in terms of a decrease in the ability of Mg^{2+} to stimulate fluorescence. Increasing the $MgCl_2$ concentration to 20 mM did not enhance the fluorescence above that seen with 3 mM.

The decrease in fluorescence amplitude in NH_2OH_{light} is accomplished by an inhibition of the photo-oxidizing ability of Photosystem II and by the formation of low potential ($E_{m7.8}$ + 80 mV) cytochrome *b*-559 [2]. Experiments were performed on other systems in which low potential cytochrome *b*-559 is present instead of the normal +380 mV species.

Heating of chloroplasts to 55°C has been shown to inhibit Photosystem II and to cause a decrease in E_m of cytochrome *b*-559 [4]. Table Ia shows the amplitude of reduction of cytochrome *b*-559 by ascorbate and hydroquinone which gives an approximate estimate of the proportions of low potential *b*-559 species, higher potential *b*-559 being reduced by addition of hydroquinone [2]. Complete lack of reduction by hydroquinone indicates absence of both the +380-mV or +240-mV species of cytochrome *b*-559 and indicates the presence of a single, low potential ($E_{m7.8}$ + 80 mV) form. This is similar to NH_2OH_{light} chloroplasts (Table Id). These chloroplasts show no photo-oxidizing ability in the presence of artificial donors. The fluorescence yield of chlorophyll is also reduced, again as seen in NH_2OH_{light} treated chloroplasts. Thus in heat-treated chloroplasts, loss of photochemical activity accompanies the fluorescence yield decrease and the formation of low potential cytochrome *b*-559. Trypsin treatment has been reported to act upon cytochrome *b*-559 before loss of H_2O oxidation. Data in Table Id show that incubation in trypsin

TABLE I

REDOX PROPERTIES OF CYTOCHROME *b*-559 AND CHLOROPHYLL FLUORESCENCE IN CONTROL AND TREATED CHLOROPLASTS

Heat treatment and trypsin digestions (10 min) were performed as described in the text. NH_2OH chloroplasts were obtained by incubation in 5 mM NH_2OH for 30 min as previously described [2]. Amplitudes of cytochrome *b*-559 reduction were determined by successive addition of ferrocyanide (2 mM), hydroquinone (2 mM) and ascorbate (4 mM) after prior oxidation with 0.25 mM ferricyanide [2]. Chlorophyll concentration was 80 μg/ml for chloroplasts and 60 μg/ml for photosystem II particles. Values f_0 and f_v were obtained from the amplitude of fluorescence in low intensity light before and after addition of dithionite, as described in Fig. 1.

Chloroplasts	Amplitude of cyt *b*-559 reduction ($A_{560-540} \cdot 10^3$)			Fluorescence amplitudes (arbitrary units)		
	+ferro	+HQ	+asc	f_0	f_v	f_v/f_0
a Heat 25°C	1.9	0.6	1.0	17	70	4.1
Heat 45°C	0.2	0.7	2.5	15	44	2.9
Heat 55°C	0	0	2.7	15	21	1.4
b Trypsin	0	0.1	1.4	14	20	1.4
Control	2.1	0.6	0.8	17	66	3.9
c Triton photosystem II	0	0	2.7	21	44	2.1
d NH_2OH_{light}	0	0.3	2.2	11	10	0.9
NH_2OH_{dark}	0.2	1.4	2.0	11	35	3.2
Control	2.2	0.4	0.9	10	38	3.8

for 10 min causes complete loss of higher potential forms of cytochrome *b*-559 and a 70% decrease in the f_{max} value obtained upon reduction with dithionite or a 82% decrease in f_v. These chloroplasts retained 32% of the rate of water oxidation.

Triton Photosystem II particles, prepared by the method of Vernon and Shaw [23] also show a decreased fluorescence yield and the presence of low potential cytochrome *b*-559 (Table Ic). The extent of the decrease in yield depends on whether Mg^{2+} is included in the reaction mixture. Mg^{2+} has been shown to quench chlorophyll fluorescence in these particles [24,25].

Cytochrome b-559 and fluorescence in developing chloroplasts

Arnzten and co-workers have shown that dark grown pea seedlings greened under an intermittent light regime show normal levels of photochemical activity but were characterized by a low, Mg^{2+}-independent fluorescence yield [16,26]. Table II shows data from pea seedlings grown under similar conditions. Rates of reduction of methyl viologen were approx. twice control (24 h continuous light) values and Photosystem I rates about 3 times higher. High chlorophyll *a/b* ratios were seen in "flashed" plants, indicating the absence of the light harvesting chlorophyll *a/b* protein [16,26]. The maximum fluorescence yield seen upon reduction with dithionite is 36% lower than in fully-developed chloroplasts (data not shown), and is insensitive to Mg^{2+} addition. All the features compare favorably with previous data [16,26]. Fig. 5 shows absorption spectra of "flashed" peas in the cytochrome region. The content of ferrocyanide and hydroquinone reducible cytochrome *b*-559 is clearly very low as seen from the small absorbance change at 559 nm. Cytochrome

TABLE II

ELECTRON TRANSPORT AND FLUORESCENCE PROPERTIES OF PEAS GROWN UNDER INTERMITTENT LIGHT

Rates of electron transport were measured at 20°C using a Clark electrode as previously described [2] and using methyl viologen as acceptor. H_2O to methyl viologen rates were corrected for O_2 uptake insensitive to diuron (10 μM). Photosystem I was assayed using diaminodurene (0.4 mM) and ascorbate (1 mM). All rates are NH_4Cl uncoupled. f_v and f_0 were determined from induction curves + $MgCl_2$ (3 mM) with and without dithionite at a chlorophyll concentration of 1 μg/ml and light intensity 12 J/m^2 per s.

Chloroplasts	Rate of electron transport (mol O_2/mg Chlorophyll per h)		$\frac{f_v}{f_0}$ (+ Mg)	$\frac{f_v + Mg}{f_v - Mg}$	$\frac{\text{Chlorophyll } a}{\text{Chlorophyll } b}$
	Whole chain	Photosystem I			
Intermittent light	288	5777	1.46	1.01	∞
Intermittent + 5 h continuous light	271	3825	1.80	1.80	6.8
Intermittent + 24 h continuous light	178	2130	2.20	2.20	4.1

f dominates the hydroquinone-ferricyanide spectrum, but is less predominant in ferrocyanide-ferricyanide spectra, it being only slightly reduced by this high potential reductant [27]. Thus the small amount of cytochrome b-559 in this spectrum can now be more accurately quantified. Redox titration as described in ref. 2 showed that 90% of cytochrome b-559 could be reduced between E_h = +150 and 0 mV (data not shown). Table III compares the con-

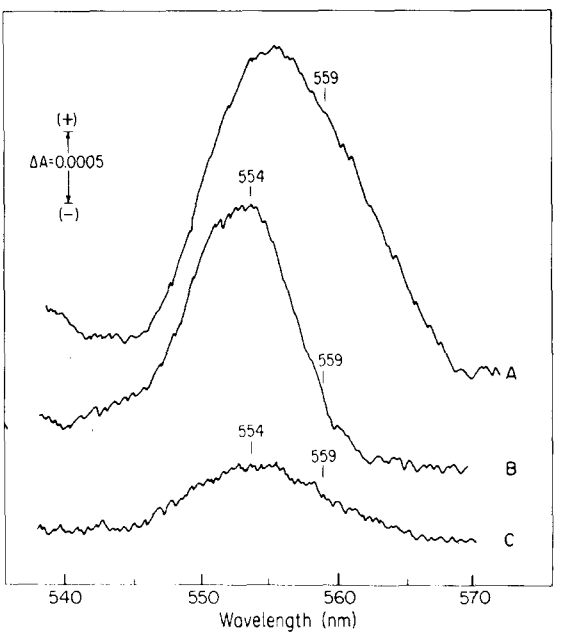

Fig. 5. Reduced-minus-oxidized difference spectra of chloroplasts isolated from "flashed" peas (grown under intermittent light): A, ascorbate (3 mM) minus ferricyanide; B, hydrquinone (2 mM) minus ferricyanide; C, ferrocyanide (2 mM) minus ferricyanide. Chlorophyll concentration 4 μg/ml; ferricyanide, 0.25 mM.

TABLE III

CYTOCHROME CONTENT OF PEAS GROWN UNDER INTERMITTENT LIGHT

Cytochrome contents were determined from spectra of the type shown in Fig. 5B and C using previously published extinction coefficients [7]. The relative concentration of Q was estimated from the area over the fluorescence rise curves obtained by illumination in the presence of diuron [28].

	Cytochrome content (mol/mol)		
	Intermittent	+5 h	+24 h
Chlorophyll *a* + *b*/*f*	96	152	502
Chlorohyll *b*/*f*	—	20	99
Chlorophyll *a* + *b*/*b*-559	750	247	444
Chlorophyll *b*/*b*-559	—	32	87
b-559/*f*	0.13	0.62	1.13
Relative $[Q]^{-1}$	0.3	0.5	1.0
f/Q	1.6	1.7	1.0
b-559/Q	0.2	1.0	1.1

tents of cytochromes *f* and high potential cytochrome *b*-559 together with an estimate of the relative Q content obtained from fluorescence rise curves [28]. In the "flashed" plants, before exposure to continuous illumination, the content of cytochrome *f* is approx. 1.6 per reaction center. This probably reflects an excess of cytochrome *f* which is synthesized even without illumination [7,29]. After 5 h of continuous light the Photosystem II unit is more developed as the *a*/*b* ratio falls to 6.0 and f_v/f_0 increases to 1.8. The cytochrome *f* per reaction center remains close to 2, indicating that during this period maturation of pre-existing Photosystem II units is occurring [16,26]. After 24 h the ratio has decreased to approx. 1 cytochrome *f* per 500 chloroplasts, i.e., 1 per reaction center, as seen in normal chloroplasts, presumably new Photosystem II units have now been synthesized. The behavior of high potential cytochrome *b*-559 is markedly different. In the "flashed" plants 1 high potential cytochrome *b*-559 per 8 cytochrome *f* is present, indicating only 1 per 4—5 reaction centers. By 5 h, however, the content of cytochrome *b*-559 had increased dramatically to 1 high potential cytochrome *b*-559 per 1.7 cytochrome *f* or 1 per Photosystem II reaction center. After 24 h, as more Photosystem II units are formed, the *b*-559 content increases so that the cytochrome *b*-559 per *f* ratio increases to 1.1. Thus the amount of high potential cytochrome *b*-559 does not correlate at all with the photosynthetic activity during the initial period of development. Using the data in Tables II and III, the rate of Photosystem II activity is $2.5 \cdot 10^5$ μmol O_2/mol *b*-559 per h in "flashed" plants, $0.7 \cdot 10^5$ at 5 h and $0.8 \cdot 10^5$ after 24 h. Even after 24 h, the content of high potential cytochrome *b*-559 is still less than that of control, light-grown plants, where more than 1 cytochrome *b*-559 (high potential) per cytochrome *f* are observed [30]. The data suggest that the aquisition of high potential cytochrome *b*-559 during chloroplast differentiation is not associated with development of photosynthetic electron transport but with the accumulation of chlorophyll *b* and the appearance of the high fluorescence yield that results from Mg^{2+}-stimulated fluorescence. It seems from Table III that about one-fifth of the 24-h content of chlorophyll *b* is associated with the

TABLE IV

PROPERTIES OF CHLORPLASTS ISOLATED FROM GREENING BARLEY

Chloroplasts were isolated from dark grown seedlings exposed to different lengths of continuous illumination. Light grown barley refers to seedlings grown for 10 days under a 12-h photoperiod at 21°C. Contents of cytochrome *f* and high potential cytochrome *b* (*b*-559$_H$) were determined from hydroquinone minus ferricyanide difference spectra as previously described [7]. Fluorescence yields were measured as in Table I.

Illumination time (h)	Chlorophyll content		*f*/Chlorophyll (×10^3)	*b*-559$_H$/Chlorophyll (×10^3)	*b*-559$_H$/Chlorophyll *b* (×10^3)	*b*-559$_H$/*f*	Fluorescence yield (f_v/f_0)
	(*a* + *b*) (g,g, fresh wt.)	*a*/*b*					
2	0.3	∞	88.0	0	—	0	0.3
4	1.2	∞	16.1	0	—	0.01	0.4
6	5.1	∞	6.78	0.19	—	0.03	1.1
10	13.8	9.6	2.75	0.25	3.6	0.09	1.6
18	51.0	4.2	2.07	0.95	4.9	0.46	2.3
32	144	3.4	1.49	1.42	6.3	0.95	2.8
Light grown	272	3.1	1.29	1.61	6.0	1.23	3.0

aquisition of 1 mol of high potential cytochrome *b*-559 per reaction center.

A similar lack of correlation between high potential cytochrome *b*-559 and Photosystem II activity was described by Henningsen and Boardman [7] during study of the light induced development of dark grown barley seedlings. Data obtained from a similar investigation are shown in Table IV. At times less than 6 h, the amount of high potential cytochrome *b*-559 was insignificant, the chlorophyll *b* content was too low to be detectable by the assay used and the f_v/f_0 ratio remained less than 1. After 6 h, which corresponds to the end of the lag phase in chlorophyll accumulation the chlorophyll *a*/*b* ratio began to fall while the f_v/f_0 and the content of high potential cytochrome *b*-559 increased. During the period of rapid development (between 6 and 32 h) the amount of high potential cytochrome *b*-559 correlates well with the decrease in the chlorophyll *a*/*b* ratio and the appearance of high yields of f_v. At the end of 32 h of greening, the chlorophyll *a*/*b* ratio, f_v/f_0 and the content of high potential cytochrome *b*-559 all approached the values seen in light grown plants. The cytochrome *f*/chlorophyll ratio was high initially because normal contents of cytochrome *f* were present in etioplasts and the ratio therefore decreases as chlorophyll is synthesized.

It should be noted that, in the case of the continuous greening system, that the f_v/f_0 increase will also reflect association of chlorophyll with newly synthesized photosystems, particularly in the early phases of development; the very low ratio observed during the first few hours is probably an indication that the chlorophyll is not yet associated with functional Photosystem II units. Thus, a decrease in f_0 is observed (e.g., between 6 h and 10 h the f_0 value decreases by 34%) during these stages.

Discussion

Earlier data had shown a perfect correlation between the presence of high potential cytochrome *b*-559 and the ability to photooxidize water [2].

Further, formation of low potential cytochrome *b*-559 ($E_{m7.8}$ + 80 mV) was accompanied by loss of the ability to photooxidize even artificial donors. These observations, made using NH_2OH incubation in the light or dark to separate two inhibition states of Photosystem II, can be duplicated using heat treatment of chloroplasts; at 45°C chloroplasts could photooxidize donors but not water and showed about 40% of control amplitude of hydroquinone reduction, equivalent to NH_2OH_{dark} chloroplasts. Incubation at 55°C caused formation of low potential cytochrome *b*-559 and inhibited artificial donor oxidation, this time equivalent to NH_2OH_{light} chloroplasts.

Accompanying the inhibition of photo-oxidizing activity in NH_2OH_{light} and 55°C chloroplasts is a dramatic decrease in the maximum fluorescence yield which is apparently due to a decreased ability to respond to addition of divalent cations. The problem posed is whether any or all of these three correlated phenomena, the low potential cytochrome *b*-559, low fluorescence yield and absence of photo-oxidizing capability, are in any way directly related. Trypsin treatment was able to separate the latter from the former two. Thus, trypsin-treated chloroplasts are able to photooxidize water and artificial donors under conditions when high potential cytochrome *b*-559 is completely absent. This confirms the earlier observation of Cox and Bendall [8]. In trypsin-treated chloroplasts a single, low potential cytochrome *b*-559 species is present and simultaneously the fluorescence yield is decreased. Recently, both Arntzen (personal communication) and Forti [31] have shown a similar effect of trypsin on Mg^{2+}-stimulated fluorescence. These experiments establish a link between the high-potential state of cytochrome *b*-559 and divalent cation-induced elevation of chlorophyll fluorescence.

Experiments on developing chloroplasts have strengthened this argument. Plants greened under intermittent light contain active photosystems, but lack chlorophyll *b* granal stacks and are characterized by a low f_v which is unaffected by divalent cations [16,26]. There is, correspondingly, a reduced number of divalent cation binding sites which are inferred to be on the missing chlorophyll *a/b* light-harvesting complex [26]. The above data show that the content of high potential cytochrome *b*-559 in such chloroplasts is only a fraction of that in normal chloroplasts; it is estimated that there is only one molecule of high potential cytochrome *b*-559 per 5 Photosystem II reaction centers or 1 per 8 molecules of cytochrome *f*. When the seedlings are subsequently exposed to continuous light, chlorophyll *b* is synthesized, the fluorescence yield increases and the content of high potential cytochrome *b*-559 approaches normal values (at 24 h, one per cytochrome *f* is measured). In chloroplasts isolated from peas grown under intermitent light, high rates of H_2O to methyl viologen electron flow are seen in the absence of normal amounts of high potential cytochrome *b*-559. A similar observation was made on beans grown under a ms flash regime [32]. It seems unlikely that high potential cytochrome *b*-559 has an obligatory role in water oxidation. Instead, the high potential state seems to be related to the increased complexity of the chloroplast membrane system as chlorophyll *b* is being synthesized and grana are forming. The same correlation is seen during greening of etiolated barley in continuous light (Table IV and ref. 7); again low potential cytochrome *b*-559 accompanies the low fluorescence state.

The present experiments, therefore, indicate clearly an intimate relationship between the midpoint potential of cytochrome *b*-559 and the divalent cation induced fluorescence increase. Both these seem to be peripheral features of Photosystem II, in the sense that neither are required for photosynthesis to occur. The magnesium effect is only to allow "tuning" of the light harvesting process for optimum efficiency, particularly in low intentsity light. The question arises as to the reason for the association of the cytochrome *b*-559 response to this phenomenon. Several alternative suggestions can be made. One is that cytochrome *b*-559 is in some way related to the State II-State I transition that is probably the normal role of the Mg^{2+} effect; thus, could high potential cytochrome *b*-559 be involved in the mechanism by which energization of Photosystem II triggers increased efficiency of energy transfer to Photosystem I? In this context the proton-linked functioning of cytochrome *b*-559 could be of importance [27,33—35] as also may be the Photosystem II-induced membrane conformational change that seems to involve cytochrome *b*-559 [36]. It is of interest that low pH-induced inhibition of Mg^{2+} stimulated fluorescence [37] occurs over the pH range 6 to 4.5, exactly the same range as the low pH-induced decrease in the E_m of cytochrome *b*-559 [27].

An alternative and more probable explanation, however, is that the two effects are associated because of a close interaction in the chloroplast membrane. The cation induced fluorescence increase would appear to involve properties of the outer surface [38]. The trypsin inhibition of Mg^{2+}-stimulated fluorescence, which may involve an effect directly on the chlorophyll *a/b* protein (Arntzen, C.J., personal communication) again indicates an outer surface phenomena. Cytochrome *b*-559 is also sensitive to trypsin and accessibility studies have previously suggested a location close to the outer surface [39]. In addition, cytochrome *b*-559 is located close to the reaction center of Photosystem II, as evidenced by its Photosystem II-induced photooxidation at 77 K [40—42] and by its presence in reaction center submembrane preparations [43]. The light-harvesting unit is also closely bound to Photosystem II reaction center as shown by fractionation studies [44] and by examination of particles seen in freeze-fractures of chloroplast membranes [45]. Thus it is not unreasonable to expect that, even though their functions may be unrelated, cytochrome *b*-559 and the light-harvesting assembly may be simultaneously affected by perturbation of the membrane. It is now possible to suggest a mechanism by which cytochrome *b*-559 achieves high potential during membrane assembly. Cation induced fluorescence changes are interpreted in terms of cation binding to the chlorophyll proteins themselves [46] or in terms of the electric double-layer which arises as a result of elctrostatic interaction between surface negative charges and cations [13]. The latter can rationalize the divalent/monovalent cation antagonism [13,38] in terms of positive charge density. Thus it is possible that membrane surface charge density, made more positive when the membrane is able to show cation-regulated fluorescence, is the primary factor in establishing the high-potential state of cytochrome *b*-559. Because removal of Mg^{2+} from chloroplasts sufficient to elicit a large drop in fluorescence does not measurably alter the E_m of cytochrome *b*-559 (Horton, P., unpublished data), the sensitivity to charge density appears not to be the same. While this data still does not estab-

lish a role for cytochrome *b*-559 in photosynthesis, the dynamic nature of membrane surface properties would add to this model the possibility of alterations in its redox properties during different physiological transitions and could be the basis for as yet undefined regulatory mechanisms in photosynthesis.

A final point worthy of discussion is the rate of the excitation which is not fluoresced in these low fluorescent chloroplasts. In normal chloroplasts, absence of Mg^{2+} stimulates utilization of excitation by Photosystem I, whose quantum yield is thereby enhanced [12]. In low fluorescent chloroplasts (e.g., NH_2OH_{light}), we have been unable to demonstrate enhancement of Photosystem I quantum yield. Thus excitation appears to be quenched in a non-radiative decay process unassociated with energy transfer to Photosystem I. Since it is f_v which is specifically quenched, quenching at the level of reaction center chlorophyll (i.e., a k_d process) ought to be involved according to Butler's model [47,48]. Thus the increased quenching in the low fluorescent chloroplasts could be the reason for and not the result of the decreased stimulation of fluorescence by Mg^{2+}. Quenching at reaction center chlorophyll due to oxidized *P*-680 has been proposed from low temperature measurements [49] and from μs fluorescence yield changes following flash excitation [20,50,51]. That chloroplasts inhibited by NH_2OH_{light} would have an elevated steady state *P*-680$^+$ level is not inconceivable and could provide an explanation of low fluorescence yield. However, quenching by *P*-680$^+$ seems not to be the explanation, since the effect is still seen in low light and in the presence of excess dithionite. In addition, both developing chloroplasts and trypsin-inhibited samples show the same phenomenon and here electron transport to *P*-680 can proceed at near normal levels. Nevertheless, the appearance of a quenching process when low potential cytochrome *b*-559 is formed is an alternative explanation of the lowered f_v and the reduced degree of Mg^{2+}-stimulation of fluorescence.

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References

1 Bendall, D.S. (1968) Biochem. J. 109, 46
2 Horton, P. and Croze, E. (1977) Biochim. Biophys. Acta 462, 86—101
3 Knaff, D.B. and Arnon, D.I. (1971) Biochim. Biophys. Acta 226, 400—408
4 Erixon, K., Lozier, R. and Butler, W.L. (1972) Biochim. Biophys. Acta 267, 375—382
5 Wada, K. and Arnon, D.I. (1971) Proc. Natl. Acad. Sci. U.S. 68, 2064—3086
6 Epel, B.L. and Butler, W.L. (1972) Biophys. J. 12, 922—929
7 Henningson, K.W. and Boardman, N.K. (1973) Plant Physiol. 41, 1117—1125
8 Cox, R.P. and Bendall, D.S. (1972) Biochim. Biophys. Acta 283, 124—135
9 Duysens, L.N.M. and Sweers, H.E. (1963) in Microalagae and Photosynthetic Bacteria (Jap. Soc. Plant Physiol., eds.), pp. 353—372, Univ. of Tokyo Press
10 Gross, E.L. and Hess, S.C. (1973) Arch. Biochem. Biophys. 159, 832—836
11 Homann, P. (1969) Plant Physiol. 44, 932—936
12 Murata, N. (1969) Biochim. Biophys. Acta 189, 171—181
13 Barber, J. and Mills, J. (1976) FEBS Lett. 68, 288—292
14 Horton, P. (1976) Int. J. Biochem. 7, 597—605
15 Izawa, S. and Ort, D.R. (1974) Biochim. Biophys. Acta 357, 127—143

16 Armond, P.A., Arntzen, C.J., Biantais, J.M. and Vrnotte, C. (1976) Arch. Biochem. Biophys. 175, 54—63
17 Leese, B.M., Leech, R.M. and Thompson, W.W. (1972) in Proc. 2nd Int. Cong. Photosynthesis (Forti, G., Avron, M. and Melandri, A., eds.(, pp. 1485—1494, Junk, The Hague
18 Arnon, D.I. (1949) Plant Physiol. 24, 1—15
19 Mohanty, P., Zilinskas, B. and Govindjee (1972) FEBS Lett. 20, 273—276
20 Jurinsic, P. and Govindjee (1977) Biochim. Biophys. Acta 461, 253—267
21 Cheniae, G.M. and Martin, I.F. (1971) Plant Physiol. 47, 568—575
22 Govindjee, Jurinsic, P. and Wraight, C. (1977) 4th Int. Cong. Photosynthesis Abstr. (Coombs, J., ed.), p. 136, U.K. Science Committee, London
23 Vernon, L.P., Ke, B. and Shaw, E.R. (1967) J. Biol. Chem. 241, 4101—4109
24 Prochaska, L.H. and Gross, E.L. (1977) Arch. Biochem. Biophys. 181, 147—154
25 Horton, P. and Croze, E. (1977) FEBS Lett. 81, 259—263
26 Davis, D.J., Armond, P.A., Gross, E.L. and Arntzen, C.J. (1976) Arch. Biochem. Biophys. 175, 64—70
27 Horton, P. and Cramer, W.A. (1975) FEBS Lett. 56, 244—247
28 Malkin, S. and Kok, B. (1966) Biochim. Biophys. Acta 126, 413—432
29 Plesnicar, M. and Bendall, D.S. (1973) Biochem. J. 136, 803—812
30 Cramer, W.A. and Whitmarsh, J. (1977) Annu. Rev. Plant Physiol. 28, 133—172
31 Gerola, P. and Benedetti, E., Gorlashi, F.M., Jennings, R.C., Rizzi, S., and Forti, G. (1977) 4th Int. Cong. Photosynthesis Abstr. (Coombs, J., ed.), p. 128, U.K. Science Committee, London
32 Strasser, R.J. and Cox, R.P. (1974) Plant Sci. Lett. 3, 19—23
33 Horton, P., Whitmarsh, J. and Cramer, W.A. (1976) Arch. Biochem. Biophys. 176, 519—524
34 Horton, P. and Cramer, W.A. (1976) Biochim. Biophys. Acta 430, 122—134
35 Cramer, W.A. and Horton, P. (1975) Photochem. Photobiol. 22, 304—308
36 Giaquinta, R., Dilley, R.A., Anderson, B.J. and Horton, P. (1974) J. Bionerg. 6, 167—177
37 Mills, J. and Barber, J. (1975) Arch. Biochem. Biophys. 170, 306—314
38 Mills, J.D., Telfer, A. and Barber, J. (1976) Biochim. Biophys. Acta 440, 495—505
39 Horton, P. and Cramer, W.A. (1974) Biochim. Biophys. Acta 368, 348—360
40 Knaff, D.B. and Arnon, D.I. (1969) Proc. Natl. Acad. Sci. U.S. 63, 956—962
41 Erixon, K. and Butler, W.L. (1971) Photochem. Photobiol. 14, 427—434
42 Boardman, N.K., Anderson, J.M. and Hiller, R.A. (1971) Biochim. Biophys. Acta 234, 126—136
43 Ke, B., Vernon, L.P. and Chaney, T. (1972) Biochem. Biophys. Acta 256, 345—356
44 Arntzen, C.J. and Ditto, C.L. (1976) Biochim. Biophys. Acta 449, 259—274
45 Armond, P.A. and Arntzen, C.J. (1977) Plant Physiol. 59, 398—404
46 Davis, D.J. and Gross, E.L. (1975) Biochim. Biophys. Acta 387, 557—567
47 Butler, W.L. and Kitajima, M. (1975) Biochim. Biophys. Acta 376, 116—125
48 Butler, W.L and Kitajima, M. (1975) in Proc. 3rd Int. Cong. on Photosynthesis (Avron, M., ed.), pp. 13—24, Elsevier, Amsterdam
49 Okayama, S. and Butler, W.L. (1972) Biochim. Biophys. Acta 267, 523—529
50 Den Haan, G.A., Duysens, L.N.M. and Egberts, D.J.N. (1974) Biochim. Biophys. Acta 368, 409—421
51 Mauzerall, D. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1358—1362