Biochimica et Biophysica Acta, 462 (1977) 86–101

BBA 47361

THE RELATIONSHIP BETWEEN THE ACTIVITY OF CHLOROPLAST PHOTOSYSTEM II AND THE MIDPOINT OXIDATION-REDUCTION POTENTIAL OF CYTOCHROME *b*-559

PETER HORTON and EDWARD CROZE

Division of Cell and Molecular Biology, State University of New York at Buffalo, Buffalo, N.Y. 14214 (U.S.A.)

(Received March 7th, 1977)

SUMMARY

The role of cytochrome *b*-559 in Photosystem II reactions has been investigated using hydroxylamine treatment of chloroplast membranes. Incubation of chloroplasts with hydroxylamine in darkness resulted in inhibition of water oxidation and a decrease in the amplitude of cytochrome *b*-559 reducible by hydroquinone. The loss of water oxidizing activity perfectly correlated with the decrease in amplitude of cytochrome *b*-559 reduction. Potentiometric titration of cytochrome *b*-559 after hydroxylamine treatment revealed a component with $E_{m_{7.8}}$ at +240 mV in addition to a lower potential species at +90 mV. This compared to control chloroplasts in which cytochrome *b*-559 exists in the typical high potential state, $E_{m_{7.8}} = +383$ mV, in addition to some of the low potential ($E_{m_{7.8}} = +77$ mV) form. Photosystem II activity could be further inhibited by incubation with hydroxylamine in the light. In these chloroplasts only low rates of photooxidation of artificial electron donors were observed compared to 'dark' chloroplasts. In addition, the hydroxylamine light treatment caused a further change in cytochrome *b*-559 redox properties; a single component, $E_{m_{7.8}} = 90$ mV is seen in titration curves. The role of cytochrome *b*-559 in Photosystem II functioning is discussed on the basis of these observations which suggest a dependence of photooxidizing ability of Photosystem II on the redox properties of this cytochrome.

INTRODUCTION

Since the observation was made that over 50 % of the chloroplast cytochrome *b*-559 has a midpoint oxidation reduction potential (E_m) of +360 [1, 2] and not approx. +100 mV as previously thought, there has been a continuous search for a function for this Photosystem II component. An oxidation of cytochrome *b*-559 by Photosystem I in the presence of FCCP [2], PMS [3], at low pH [4] and after pre-

Correspondence to be addressed to Dr. Peter Horton.

illumination with high intensity light [4] suggested that it might function in the main electron transport chain between the primary acceptor of Photosystem II and plastoquinone, perhaps after a light-induced decrease in E_m [2, 5, 6]. However, the oxidation kinetics were in each case much slower than expected for such a functional role and recent kinetic data also indicate sluggish reduction kinetics for the photo-oxidized cytochrome *b*-559 [7]. Alternative roles for cytochrome *b*-559 in Photosystem II functioning are warranted, particularly with respect to explaining the unusually high E_m state that appears to be necessary for its physiological role.

Many treatments which inhibit oxidation of water by Photosystem II also cause a decrease in the E_m of cytochrome *b*-559 [8, 9]. Although in most instances this appears to indicate a role for cytochrome *b*-559 in H_2O oxidation, there does exist observations where high potential cytochrome *b*-559 (i.e. ($E_m \approx +360$ mV) is not required for H_2O oxidation [8]. Nevertheless, it is of interest that such treatments often do affect both H_2O oxidation and cytochrome *b*-559, and more detailed investigation of these effects may tell us more about the nature of the high potential cytochrome *b*-559 state, pointing to possible functional roles in Photosystem II reactions. One of the most specific and delicate treatments that inhibits H_2O oxidation, is incubation in dilute hydroxylamine solution [10, 11]. Incubation in darkness inhibits H_2O oxidation while allowing Photosystem II induced oxidation of artificial donors. Hydroxylamine probably works by causing release of membrane associated manganese [10], suggesting an important role for this metal in water oxidation. Reports of the effect of hydroxylamine on cytochrome *b*-559 are inconsistent. Whereas Cramer and Bohme [12] reported a significant loss of the high potential form of cytochrome *b*-559 upon addition of hydroxylamine, Cox and Bendall [8] see no effect in their assays. This discrepancy probably arises due to the different methods for assaying 'high potential *b*-559', the former employing a more sensitive method. This discrepancy also perhaps indicates that the hydroxylamine effect is more subtle than merely inducing a gross conversion of a high potential to a low potential form as for instance occurs upon detergent or heat treatment of chloroplast membranes. This paper describes a detailed investigation of hydroxylamine inhibition of Photosystem II function and indeed shows the existence of a hydroxylamine-induced potential state of cytochrome *b*-559 midway between the high and low potential forms.

MATERIALS AND METHODS

Chloroplasts were isolated from pea seedlings (Improved Laxtons Progress) grown for 19–22 days at 20 °C under white fluorescent illumination (Intensity 13 J/m^2/s) in vermiculite moistened with Hoaglands solution. The photoperiod was 12 h. Chloroplast isolation was performed as described previously [12] except that the isolation medium consisted of just 0.4 M sorbitol and 50 mM Mops, pH 7.0. Hydroxylamine treatment was performed at 20–21 °C as described by Izawa and Ort [11]. Oxygen uptake using methyl viologen as acceptor was measured using a Yellow Springs oxygen sensor and amplifer with chloroplasts at a concentration of 20 μg chloroplasts per ml in a reaction medium containing 0.1 M sucrose/5 mM NaCl/2 mM $MgCl_2$/5 mM K_2HPO_4/50 mM Tricine/NaOH, pH 7.8. Electron donors were used at the concentrations described in the figure legends.

Cytochrome redox changes were measured using an Aminco DW-2 spectro-

photometer, with a measuring wavelength of 560 nm and the reference set at 540 nm. Band pass was 1.0 nm with an instrument time constant of 2.5 s. The photomultiplier tube was blocked by Balzars DT green and Corning 4-96 filters. Redox titration was performed using a cuvette similar to that described by Dutton [13], employing a Fisher combination redox electrode. Anaerobic conditions were maintained by blowing a stream of oxygen-free nitrogen (Union Carbide) scrubbed with alkaline dithionite solution as described by Clark [14]. The sample was continually stirred by a magnetic stirrer bar. The following combination of mediators were used: hydroquinone (10 μM); 2,5 dimethylquinone (10 μM); 1,2 napthoquinone (10 μM); 1,4 napthoquinone (20 μM); 2,5 dihydroxyl 1,4 benzoquinone (20 μM), 2-hydroxy 1,4 napthoquinone (20 μM) as well as the ferricyanide used as titrant. The potential was measured on a Beckman digital pH meter (Model 4500). Oxidative and reductive titrations were performed by successive additions of ferricyanide and dithionite respectively. The potential range +0.45 V to −0.2 V was covered. The E_h of the reference electrode was established using the quinhydrone electrode at pH 7.00 and 7.41. The concentration of chloroplasts was 80 μg chlorophyll/ml and the reaction medium was as for O_2 electrode experiments except that sucrose was omitted. The temperature was maintained at 22–23 °C. Each titration took approximately 60 min.

Manganese was determined by the method of Sandell [15]. The chloroplast suspension (vol. $\approx$ 0.4 ml, approx. 5 mg/ml) was dissolved in HNO_3 (2 ml)+H_2SO_4 (7 ml), heated, boiled with further addition of HNO_3 to fumes of H_2SO_4, diluted with H_2O and the manganese oxidized to permanganate using persulfate, periodate and silver nitrate. Absorption spectra were recorded between 450 and 600 nm on the DW-2 and the absorption at the 524 maximum measured and compared to standard permanganate solutions.

RESULTS

Incubation of chloroplasts for 20 min in 5 mM hydroxylamine at pH 7.5 causes a pronounced decrease in the amplitude of the absorbance decrease at 560 nm seen upon reduction with hydroquinone. Reducibility by hydroquinone is generally considered as an assay for high potential cytochrome *b*-559 [1, 2] and so the data in Fig. 1 seem to suggest that hydroxylamine causes a decrease in the E_m of this cytochrome. The observation that a large fraction of the cytochrome *b*-559 is autoxidized upon incubation with hydroxylamine is also suggestive of a decrease in E_m. Examination of electron transport activities using methyl viologen as acceptor indicated that these chloroplasts retained only 10 % of the control H_2O oxidising ability. The dependence on the concentration of hydroxylamine for the decrease in hydroquinone reducibility and inhibition of water oxidation is shown in Fig. 2. Both effects are saturated at 2 mM hydroxylamine; further increases in the concentration of hydroxylamine do not change the redox properties of cytochrome *b*-559 beyond those seen in Fig. 1. Over a large number of different experiments, using different incubation times and hydroxylamine concentrations, good correlation is seen between the extent of inhibition of water oxidation and decrease in amplitude of reduction of cytochrome *b*-559 by hydroquinone (Fig. 3). The size of the hydroquinone amplitude associated with complete inhibition of water oxidation is approximately 42 % of the control.

This would suggest that only part (i.e. 58 %) of the high potential cytochrome

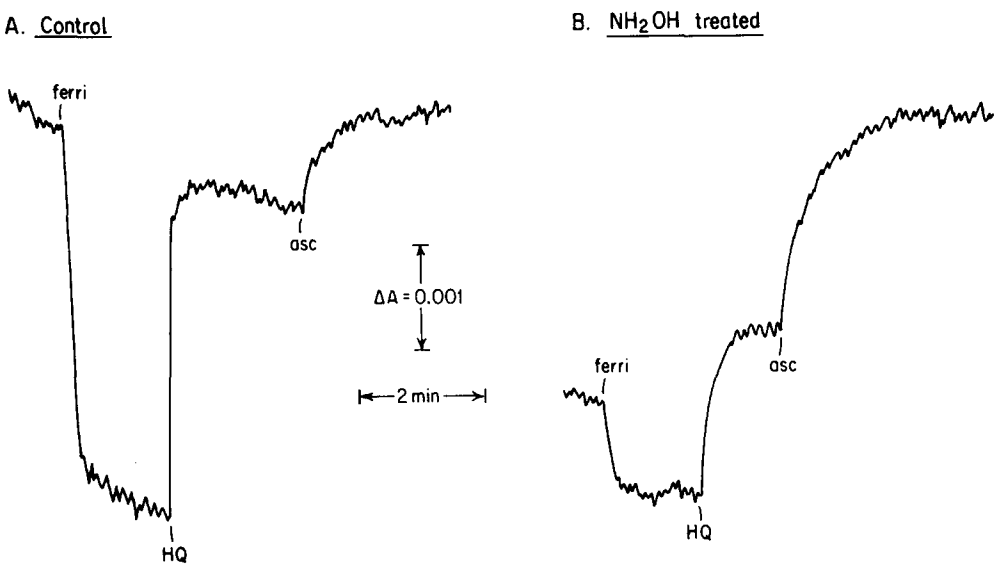

Fig. 1. Redox changes in cytochrome *b*-559 in control and hydroxylamine-treated chloroplasts. Chloroplasts (300 μg Chlorophyll/ml) were incubated at 21 °C for 20 min in a medium containing 0.2 M sucrose, 2 mM $MgCl_2$ and 10 mM Hepes/NaOH, pH 7.5 for the control and supplemented with 2 mM NH_2OH and 1 mM EDTA for treated chloroplasts. Measuring wavelength 560 nm, reference 540 nm. Ferricyanide 0.25 mM, hydroquinone 2 mM, ascorbate 4 mM. Chlorophyll concentration 80 μg/ml.

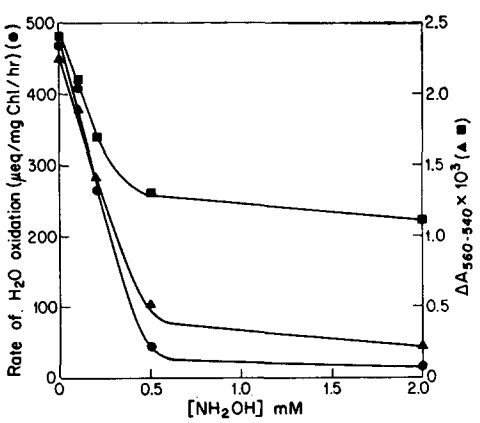

Fig. 2. Inhibition of water oxidation and alteration in cytochrome *b*-559 redox state after treatment with different concentrations of hydroxylamine. Incubation conditions were as in Fig. 1. Rate of water oxidation (●) was measured with methyl viologen (0.1 mM) as acceptor in the presence of NH_4Cl (5 mM) and at a chlorophyll concentration of 20 μg/ml. Absorbance changes at 560–540 nm were measured for reduction of ferricyanide-oxidized chloroplasts by 2 mM ferrocyanide (▲) or 2 mM hydroquinone (■) as described in Fig. 1 at chlorophyll concentration of 75 μg/ml.

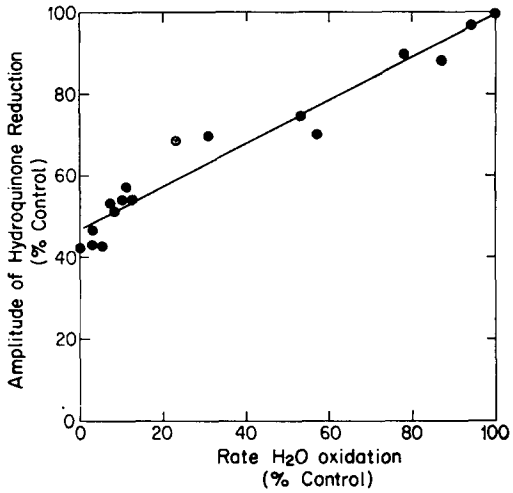

Fig. 3. Correlation between the decrease in amplitude of cytochrome *b*-559 reduction by hydroquinone and the inhibition of water oxidation in hydroxylamine-inhibited chloroplasts. Assays were as described in Figs. 1 and 2. Data, expressed as a percentage of control, uninhibited values were derived from different experiments where the extent of inhibition had been varied by alteration in hydroxylamine concentration, incubation time and incubation temperature.

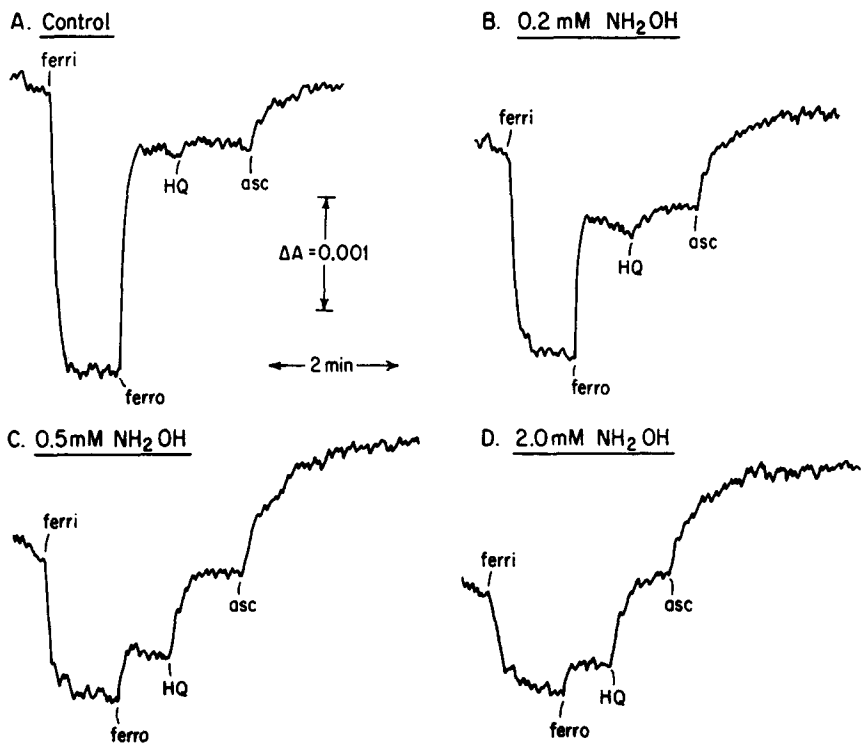

Fig. 4. Differing extents of reduction of cytochrome *b*-559 by ferrocyanide and hydroquinone after hydroxylamine treatment. Conditions as described under Figs. 1 and 2.

b-559 is affected by hydroxylamine treatment. However, an alternative explanation is indicated by the data in Fig. 4. Here ferrocyanide, a weaker reductant than hydroquinone (E_{m_7} ferri/ferrocyanide = +380 mV, E_{m_7} hydroquinone/benzoquinone +260 mV) was used to reduce oxidized cytochrome *b*-559. In control chloroplasts, as expected, the difference between absorbance decreases observed after ferrocyanide and hydroquinone addition is small. With hydroxylamine-treated chloroplasts, however, hydroquinone causes significantly more reduction than ferrocyanide. In fact, in the completely inhibited state ferrocyanide reducibility is negligible, this small amplitude possibly being due only to interference from cytochrome *f* reduction. Thus complete inhibition of H_2O oxidation by hydroxylamine causes virtually complete loss of ferrocyanide reducible cytochrome *b*-559 (see also Fig. 2). The difference in sensitivity of the amplitudes of reduction by hydroquinone and ferrocyanide suggests that the effect of hydroxylamine is to change the potential from a high (i.e. approx. +370 mV) to an intermediate state that is partially reducible by hydroquinone, but not at all by ferrocyanide. This state would have to be distinct from the low potential (E_{m_7} approx. +100 mV) form described previously [1, 2].

Potentiometric titration of the oxidation-reduction potential (E_h) of cytochrome *b*-559 in control and hydroxylamine-treated chloroplasts clearly showed the existence of a new potential species of cytochrome *b*-559. A titration of control chloroplasts is shown in Fig. 5. High potential cytochrome *b*-559 ($E_{m_{7.8}}$ = +383 mV) is clearly seen undergoing redox changes between E_h = +450 and E_h = +250 mV. A second component with an $E_{m_{7.8}}$ approximately +80 mV is seen; presumably this is

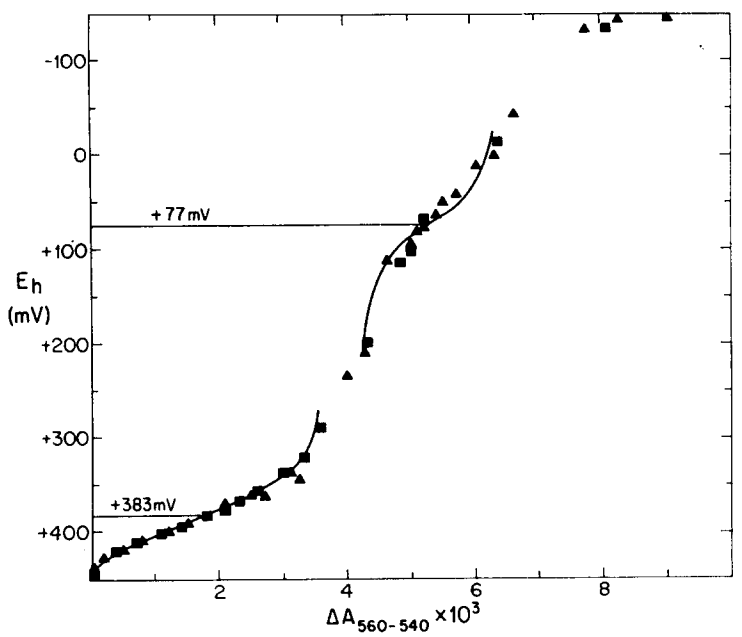

Fig. 5. Absorbance changes at 560–540 nm as a function of solution E_h during oxidative (▲) and reductive (■) titration of control chloroplasts incubated as in Fig. 1. Solid lines show Nernst plots (n = 1) with $E_{m_{7.8}}$ at 383 mV, amplitude 3.8 · 10^{-3} *A* and 77 mV, amplitude 2.0 · 10^{-3} *A*. Conditions exactly as described in the text.

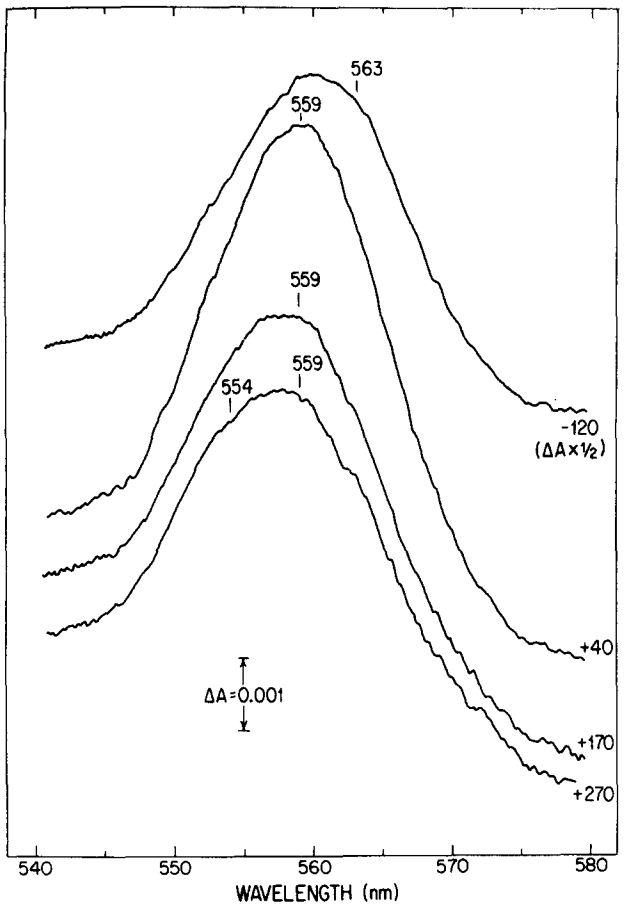

Fig. 6. Reduced-minus-oxidized difference spectra recorded during redox titration of control chloroplasts using DW-2 spectrophotometer in split-beam mode. The reference cuvette contained chloroplasts oxidized with 0.5 mM ferricyanide (E_h = +440 mV) and the sample was progressively reduced with dithionite. Redox potentials of the sample cuvette were measured as described in the text.

'low potential' cytochrome *b*-559. A third component with an $E_{m_{7.8}}$ at < 0 mV is seen and is probably due to cytochrome *b*-563 (see below). Difference spectra recorded over these potential ranges using a ferricyanide oxidized sample as a reference, show complete reduction of cytochrome *f* and a substantial amount of cytochrome *b*-559 at E_h = +275 mV, a maximum at 557–559 nm being observed; reduction of cytochrome *b*-559 is slightly increased as the potential is lowered to +170 mV (Fig. 6). Between +170 and +40 mV more reduction of cytochrome *b*-559 occurs and the spectrum now has a clear maximum at 559 nm. This corresponds to the reduction of the +80 mV species in Fig. 5. Most of the further reduction at potentials below +40 mV is due to cytochrome *b*-563, a pronounced shoulder at 563 nm being seen; contribution by this component is clearly sufficient to account for the 560 nm absorbance changes seen in the low potential (< 0 V) range in Fig. 5.

Titration of hydroxylamine treated chloroplasts does not show a large cyto-

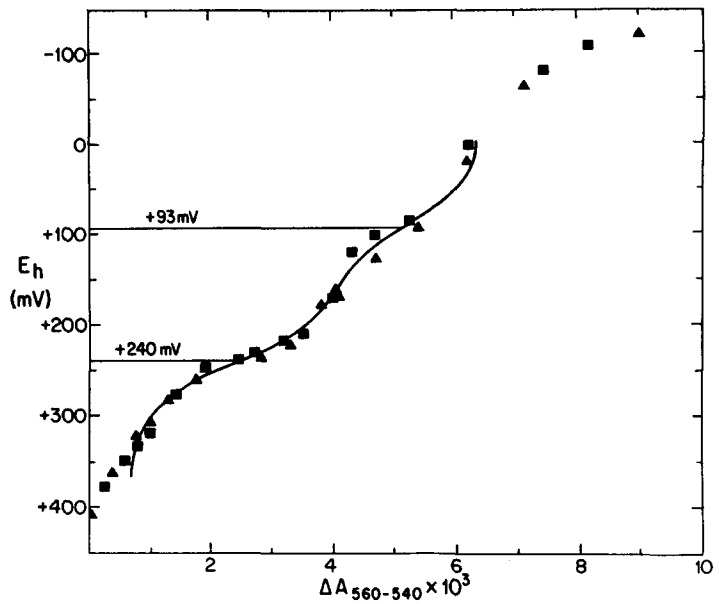

Fig. 7. Absorbance changes at 560–540 nm as a function of solution E_h during oxidative (▲) and reductive (■) titration of hydroxylamine-treated chloroplasts prepared as in Fig. 1, except that 5 mM hydroxylamine was used. Solid lines show Nernst Plots ($n = 1$) with $E_{m_{7.8}}$ at +240 mV, amplitude $3.5 \cdot 10^{-3}$ A and +93 mV, amplitude $2.5 \cdot 10^{-3}$ A. Conditions exactly as described in the text.

chrome *b*-559 component with $E_{m_{7.8}}$ +383 mV (Fig. 7). A residual amount may be present as indicated by the slight deviation from the Nernst curve at these potentials. Instead a component with $E_{m_{7.8}}$ at 240 mV is seen. This component is clearly distinct from low potential cytochrome *b*-559, which is seen following a discontinuity in the titration curve at approximately 150 mV. The titration below $E_h = +150$ appears to be similar to the control with a component at +90 mV. Fig. 8 shows difference spectra recorded during a reductive titration of hydroxylamine-treated chloroplasts. At $E_h =$ +280 mV negligible cytochrome *b*-559 (but all of the cytochrome *f*) is reduced, but with the E_h at +175 mV, considerable cytochrome *b*-559 is reduced. This spectrum now closely resembles the control spectrum of reduced cytochrome *f* + high potential cytochrome *b*-559 (see Fig. 6). It is interesting to note that the presence of a small amount of this new 240 mV species is perhaps the reason for the slight discontinuity in the control titration (Fig. 5).

Incubation of chloroplasts in hydroxylamine under illumination has been reported to inhibit Photosystem II function completely [16]. Photooxidation of artificial electron donors by Photosystem II was indeed found to be inhibited by the hydroxylamine plus light treatment. Photooxidation of diphenylcarbazide, hydroxylamine, catechol, potassium iodide and benzidine were all inhibited (Table I). All rates were sensitive to Diuron (10 μM) at a level of at least 90 % inhibition. The light-induced inhibition was dependent on a period of continuous light; illumination for 10 min caused approx. 2/3 inhibition and 90 % inhibition required 30 min of light.

Fig. 9 shows that the redox properties of cytochrome *b*-559 are changed by incubation with hydroxylamine in the light; hydroquinone reduction is almost com-

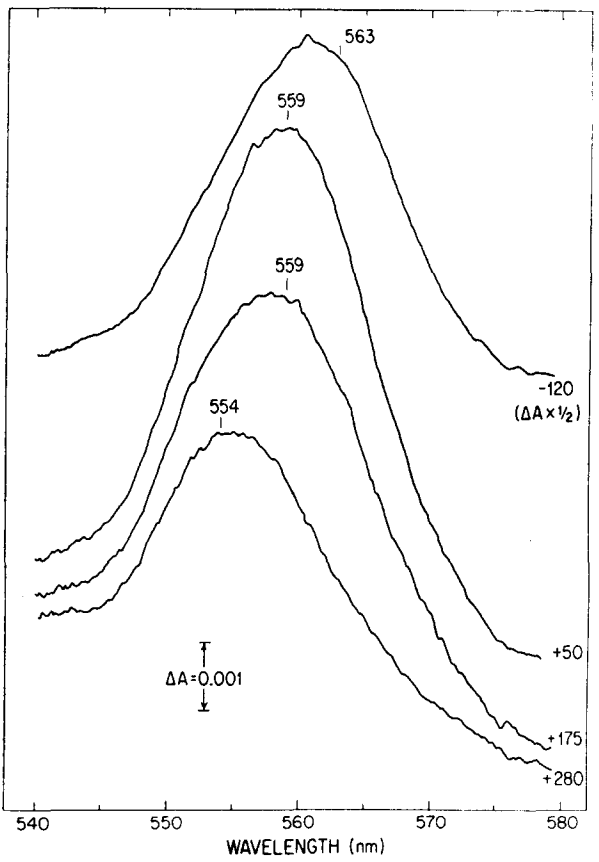

Fig. 8. Reduced-minus-oxidized difference spectra recorded during redox titration of hydroxylamine treated chloroplasts obtained as described in Fig. 6.

pletely inhibited, the residual amplitude at 560 nm being at least partially due to cytochrome *f* reduction. Note that the total amount of ascorbate-reducible cytochrome *b*-559 is decreased upon incubation with hydroxylamine in the light; this may be due to the inability of ascorbate to completely reduce the low potential form (see below). Potentiometric titration confirmed that hydroxylamine plus light has caused a further decrease in midpoint potential. A single component titration curve with $E_{m_{7.8}}$ +90 mV was now observed, indicating complete conversion of 'high potential' *b*-559 has occurred (Fig. 10).

Removal of manganese from the chloroplast membrane is generally accepted as the mode of inhibition by hydroxylamine [10]. Reports of the manganese content of chloroplasts vary from ≈ 60 chlorophylls/manganese to as high as 10 chlorophylls/manganese [17]. It was found that, if care is not taken to carefully wash the chloroplasts before manganese extraction, abnormally high ratios can be obtained in control chloroplasts. In particular, incubation for 20 min at 20 °C (i.e. the control in the hydroxylamine experiment) causes a fairly large decrease in the manganese content of the pea chloroplasts used in the present experiments, so as to give consistent values of

TABLE I

RATES OF PHOTOXIDATION OF PHOTOSYSTEM II DONORS BY HYDROXYLAMINE-TREATED CHLOROPLASTS

Chloroplasts (10 ml) were incubated with 5 mM hydroxylamine at 20 °C in darkness and under continuous white fluorescent illumination in 250 ml Erhlenmeyer flasks (light intensity 65 j/m² per s. Donor concentrations are hydroxylamine, 50 mM; potassium iodide, 20 mM; diphenyl carbazide, 0.5 mM; catechol, 0.5 mM plus 0.5 mM ascorbate; benzidine 0.5 mM. The acceptor was methyl viologen 0.1 mM plus sodium azide 0.2 mM. Rates are NH_4Cl uncoupled.

Donor		Rate of electron transport (μequiv./mg chlorophyll per h)	
		Dark	Light
Expt. 1	Hydroxylamine	279	56
	Potassium iodide	99	33
	Diphenyl carbazide	153	69
	Catechol/ascorbate	180	72
	Benzidine	54	12
Expt. 2	Hydroxylamine	222	81
	Potassium iodide	102	48
	Diphenyl carbazide	126	75
	Benzidine	36	20

64 chlorophyll/manganese (Table 3). Incubation with hydroxylamine decreases the Mn content to 41 % of the control, a ratio of 156 chlorophylls/manganese being obtained. The data show a reproducible release of manganese from the chloroplasts as found in previous studies [16]. Approximately one-half of the remaining manganese can be removed if incubation with hydroxylamine was carried out under continuous illumination; the manganese content decreased to about 20 % of the control, a chlorophyll/manganese ratio of 363 being determined.

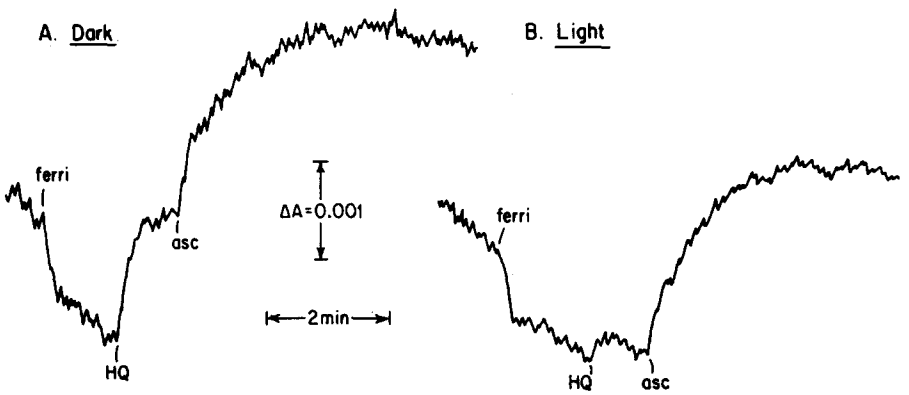

Fig. 9. Redox changes in cytochrome *b*-559 for chloroplasts incubated with hydroxylamine in darkness and under continuous illumination as in Table I. Measurements made as in Fig. 1.

TABLE II

DEPENDENCE OF INHIBITION OF PHOTOSYSTEM II DONOR PHOTOXIDATION ON ILLUMINATION TIME

Chloroplasts were treated with 5 mM hydroxylamine for a total time of 30 min. Conditions are as described under Table I with hydroxylamine as donor.

Illumination time (min)	Rate of electron transport (μequiv./mg chlorophyll per h)
0	126
10	36
20	24
30	12

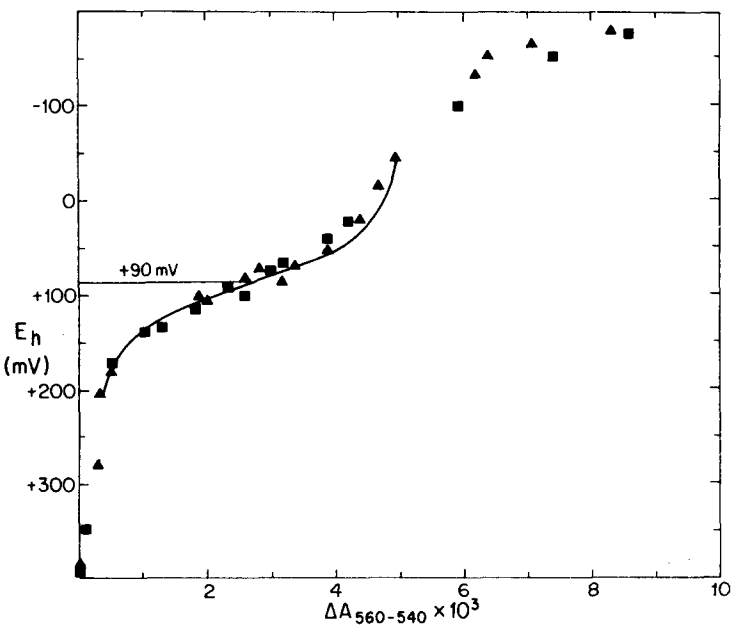

Fig. 10. Absorbance changes at 560–540 as a function of solution E_h during oxidative (▲) and reductive (■) titration of chloroplasts incubated for 30 min under continuous illumination with 5 mM hydroxylamine. Titration procedure was exactly as described in the text. Solid line is a Nernst plot ($n = 1$) with $E_{m_{7.8}}$ at +90 mV, amplitude $4.8 \cdot 10^{-3}$ A.

TABLE III

MANGANESE CONTENT OF CHLOROPLASTS IN CONTROL AND HYDROXYLAMINE TREATED CHLOROPLASTS

Chloroplasts were incubated for 30 min in 5 mM hydroxylamine as described in the text. Illumination conditions were as for the experiments in Fig. 9. Water oxidation rates for the chloroplasts were always > 90 % inhibited and light-treated chloroplasts showed approximately 70 % inhibition of donor oxidation. n is the number of different incubations. Values are ±S.E.M.

Chloroplasts	Manganese content (μmol chlorophyll/μg atom Mn)
Control	64±2 ($n = 6$)
Hydroxylamine (dark)	156±8 ($n = 5$)
Hydroxylamine (light)	363 (range 306–402) ($n = 3$)

DISCUSSION

Hydroxylamine effect on cytochrome b-559

Hydroxylamine treatment of chloroplasts has a pronounced effect on the midpoint potential of cytochrome *b*-559, causing a decrease from $E_{m_{7.8}}$ +383 mV to $E_{m_{7.8}}$ +240 mV when the incubation is done in darkness. No effect of hydroxylamine on the $E_{m_{7.8}}$ of cytochrome *f* or cytochrome *b*-563 was detected. With an $E_{m_{7.8}}$ of +240 mV, reducibility of cytochrome *b*-559 by hydroquinone would be finely balanced (E_{m_7} hydroquinone is 260 mV); thus addition of hydroquinone in excess and in the absence of prior oxidation, as performed in the experiment of Cox and Bendall [8], would probably establish a potential low enough to almost completely reduce the altered cytochrome *b*-559. On the other hand, addition of small amounts of hydroquinone (e.g. 0.3 mM in ref. 12 or 2 mM in Fig. 1) after prior oxidation with ferricyanide would be expected to reduce a species of E_m +240 mV only partially. Complete conversion of the control high potential cytochrome *b*-559 into this +240 mV species is caused by hydroxylamine treatments which inhibit water oxidation. A perfect correlation was seen between the presence of high potential cytochrome *b*-559 and the water oxidizing activity.

The +240 mV species of cytochrome *b*-559 is associated, then, with a Photosystem II unit which can not oxidize H_2O but is still able to function adequately using alternative donors to Photosystem II. Further inhibition of Photosystem II activity (by incubation with hydroxylamine in the light) results in the loss of this cytochrome *b*-559 redox species and the appearance of the more typical 'low potential' cytochrome *b*-559 species with an $E_{m_{7.8}}$ +80–90 mV. This is seen as a complete loss of hydroquinone reducibility and is accompanied by an inhibition of donor oxidation by Photosystem II.

As far as the mechanism of the hydroxylamine induced alteration in the properties of cytochrome *b*-559 is concerned, the present experiments cannot distinguish between direct and indirect effects. The mode of inhibition of H_2O oxidation by this compound is generally accepted to be due to its ability to cause release of intrinsic manganese from the thylakoid membrane [10]. These experiments, together with the activation of inactive photosystem units by manganese [18], have led to the hypothesis that manganese is in some way involved in H_2O oxidation. However, the way in which manganese is involved and the nature of the binding sites is unknown. That hydroxylamine itself can act as a good electron donor to Photosystem II (in our experiments it is the most efficient of all the donors tested) suggests that its inhibitory mechanism involves an effect directly on the active center(s) involved in H_2O oxidation. For the inhibition of cytochrome oxidase by hydroxylamine, it has been proposed that an oxidation product (nitric oxide) can interact with a component of the oxidase [19]; a similar type of interaction with chloroplast manganese could be its mode of inhibition of H_2O oxidation.

The greater inhibition of photosystem function and further decrease in the E_m of cytochrome *b*-559 that results when chloroplasts are illuminated in the presence of hydroxylamine remains unexplained. The rate of inhibition of H_2O oxidation is also increased (data not shown, see also ref. 16). The inability to oxidize artificial donors and the almost complete removal of manganese suggests that the reaction centers themselves may be destroyed in the light. Alternative explanations are light-induced

accessibility changes in the vicinity of Photosystem II (e.g. 20) or inhibition by photooxidation products of hydroxylamine.

The experiments described in this paper suggest that manganese exists in close association with cytochrome *b*-559, much as the Cu is associated with cytochrome aa_3 in cytochrome oxidase. Removal of 50 % of the manganese causes a decrease in midpoint potential of about 150 mV. A further decrease of about the same magnitude occurs when approximately one-half of the remaining manganese is released. Possible contribution to the release of manganese in the light from increased transport of manganese present in the internal thylakoid lumen should not be ignored; Blankenship and Sauer demonstrate manganese release inside the chloroplast with only slow equilibration with the external solution [21]. However, the 80 % loss of manganese seen after incubation in the light is in excess of the 2/3 release consistently seen in "dark" experiments such as those in Table III or in previous work [16]; even when manganese in the inner chloroplast lumen is accounted for, dark release from the membrane is never more than 2/3 [21]. The use of EDTA during hydroxylamine incubation probably accounts for the apparent lack of membrane permeability problems in the present experiments; EDTA has been reported to increase the half-time of manganese diffusion across chloroplast membranes from 2.5 h to 750 ms [21]. This data does not, of course, rule out the cytochrome *b*-559 responses being indirect, resulting from membrane disorganization arising from manganese removal. However, the association of manganese with cytochrome *b*-559 is a reasonable hypothesis on which to base further studies on the search for its elusive function. It is known from fractionation studies that cytochrome *b*-559 is a major Photosystem II component [22, 23] and it has previously been suggested that a positive ionic environment (such as from manganese ion) could be a factor giving rise to the unusually high midpoint potential of this *b* cytochrome [6]. Removal of manganese would be predicted, if this were true, to decrease the midpoint potential and this is exactly what is observed.

The function of cytochrome b-559 in Photosystem II

Several hypotheses exist for the role of cytochrome *b*-559 in Photosystem II. One concerns its operation in a cyclic manner around Photosystem II. A new argument against this being a physiological cycle is the slow reduction kinetics of cytochrome *b*-559 [7]. In terms of this hypothesis, the observed effects of hydroxylamine would be explained in terms of overall effects on Photosystem II function, disrupting those organisational features which are the causes of the high potential state of cytochrome *b*-559.

A second hypothesis is that cytochrome *b*-559 is involved in those reactions which are essential for water oxidation itself. A role as a redox carrier has been rejected because of inconsistencies in the correlation between water oxidation and the presence of high potential cytochrome *b*-559 during trypsin inhibition [8] and during development of photosynthetic systems [24]. However, it seems advisable to carry out a more detailed investigation of these apparent inconsistencies; it is likely that it is naive to discuss just two potential species of cytochrome *b*-559, with hydroquinone reducibility defining just a single high potential form. The observation of a partly reducible species, with an intermediate E_m described in this paper, is a clear demonstration of the danger of too simplistic an interpretation of experiments using single reductants. It is possible that there can exist many different potential species of cytochrome *b*-559;

TABLE IV

SUMMARY OF THE RELATIONSHIP BETWEEN THE CONTENT OF CYTOCHROME *b*-559 REDOX SPECIES AND THE ACTIVITY OF PHOTOSYSTEM II

Chloroplasts	Redox form of cytochrome *b*-559		Photosystem II activity		Manganese content (%)
	% Total	$E_{m_{7.8}}$ (mV)	H_2O (%)	Donor (%)	
Control	58	+383	100	–	100
	31	+ 77			
Hydroxylamine (dark)	58	+240	< 5	100	41
	42	+ 93			
Hydroxylamine (light)	95	+ 90	0	10–20	18

such a suggestion was in fact made several years ago following observation of Tris-inhibited chloroplasts [25]. Thus, until careful potentiometric studies of cytochrome *b*-559 are made in more situations, it would be unwise to rule out its involvement in water oxidation.

Table IV summarises the observations made on the association between the redox properties of cytochrome *b*-559 and the functional state of Photosystem II. From such data it is impossible to implicate with any certainty a role for cytochrome *b*-559 in Photosystem II. The E_m is clearly an indicator of the functional state of photo-oxidizing ability of Photosystem II. The observations are explainable in terms of cytochrome *b*-559 acting as a carrier between water (as a +383 mV species) or artificial donor (as a +240 mV species) and P680. However, more information is required regarding the redox potentials of the intermediates on the oxidizing side of Photosystem II.

Some estimates of the E_m of the So $\rightarrow$ S_1 and $S_1 \rightarrow S_2$ states indicate fairly low values, within the range covered by cytochrome *b*-559 [26]. On the other hand, other calculations indicate much higher values of > 500 mV [27]. Studies on the photo-oxidation of cytochrome *b*-559 at 77 K after different numbers of pre-illumination flashes at 20 °C have shown a strong dependence of oxidation amplitude on flash number [28]; a periodicity of 4 was seen, suggesting interaction between the O_2 evolving system and cytochrome *b*-559. The fact that cytochrome *b*-559 can be oxidized by Photosystem II at 77 K [28–31] and in Tris-washed chloroplasts [28] also implicates a role as an electron donor to P680, although in the case of the low temperature oxidation evidence exists for cytochrome *b*-559 being an alternative donor [32].

Another postulated function for cytochrome *b*-559 in Photosystem II is that it has a proton-binding role in water oxidation [6, 33]. This was inferred from the effects of pH on the E_m of cytochrome *b*-559 [34, 35]. Such a role may not require direct redox participation in water oxidation, but is still consistent with the hydroxylamine effects described above if we assume that the E_m of cytochrome *b*-559 reflects its general condition or functionality.

The potential of cytochrome b-559 in active chloroplasts

There would appear to be two cytochrome *b*-559 molecules per reaction center

of Photosystem II (see review, ref. 33). Reduction by hydroquinone and ascorbate have suggested 80–90 % is a high potential species. However, this would appear to be an over-estimation. Titrations of cytochrome *b*-559 in normal, water-oxidizing chloroplasts indicate 58 % is in the +383 mV form and 31 % as a low potential species (Fig. 5). The exact amount and E_m of the latter species is uncertain but would appear to approximately +80–90 mV. At this potential ascorbate only partially reduces cytochrome *b*-559. Dithionite is a more appropriate reductant but data can become complicated by contribution from reduction of cytochrome *b*-563. The question remains as to what this low potential species represents. Is it a normal physiological component or a contribution from contaminating, inactive chloroplast fragments? The latter is the generally accepted explanation mainly because disruptive treatments do transform cytochrome *b*-559 into a low potential state [8, 9]. However, this is hard to reconcile with measurements on highly active CO_2-fixing chloroplasts which contain a ratio of approximately 1 : 1 high potential to low potential cytochrome *b*-559 [36]. An alternative explanation is that the low and high potential titration E_m values are not due to different molecular species, but due to the presence of two interacting cytochrome *b*-559 heme groups. The concept that the presence of two closely associated cytochrome moieties can result in the appearance of unexpected redox behaviour has been elaborated in a recent review on cytochrome oxidase [37].

ACKNOWLEDGEMENTS

We wish to thank Dr. William Cramer for making available unpublished manuscripts and Mrs. Lynn Leistner for typing the manuscript. This research was supported in part by a Faculty Research Fellowship and a Grant-in-aid from the SUNY Research Foundation and a grant from the National Science Foundation (PCM 7609669).

REFERENCES

1 Bendall, D. S. (1968) Biochem. J. 109, 46P
2 Cramer, W. A., Fan, H. N. and Böhme, H. (1971) J. Bioenerg. 2, 289–303
3 Knaff, D. B. and Arnon, D. I. (1971) Biochim. Biophys. Acta 226, 400–408
4 Horton, P. and Cramer, W. A. (1975) Biochim. Biophys. Acta 396, 310–319
5 Horton, P. and Cramer, W. A. (1976) Biochim. Biophys. Acta 430, 122–134
6 Cramer, W. A. and Horton, P. (1975) Photochem. Photobiol. 22, 304–308
7 Whitmarsh, J. and Cramer, W. A. (1978) Biochim. Biophys. Acta, in press
8 Cox, R. P. and Bendall, D. S. (1972) Biochim. Biophys. Acta 283, 124–135
9 Wada, K. and Arnon, D. I. (1971) Proc. Natl. Acad. Sci. U.S. 68, 3064–3068
10 Cheniae, G. M. and Martin, I. F. (1970) Biochim. Biophys. Acta 197, 219–239
11 Izawa, S. and Ort, D. R. (1974) Biochim. Biophys. Acta 357, 127–143
12 Cramer, W. A. and Böhme, H. (1972) Biochim. Biophys. Acta 256, 358–369
13 Dutton, P. L. (1971) Biochim. Biophys. Acta 226, 63–80
14 Clark, W. M. (1960) Oxidation-reduction Potentials of Organic Systems, Williams and Wilkens, Baltimore
15 Sandell, D. (1959) Colormetric Determination of Traces of Metals, p. 606, Interscience, New York
16 Cheniae, G. M. and Martin, I. F. (1971) Plant Physiol. 47, 568–575
17 Cheniae, G. M. (1970) Annu. Rev. Plant Physiol. 21, 467–498
18 Cheniae, G. M. and Martin, I. F. (1971) Biochim. Biophys. Acta 259, 167–181
19 Blokzijl-Homan, M. F. J. and Van Gelder, B. F. (1971) Biochim. Biophys. Acta 234, 493–498

20 Giaquinta, R. T., Dilley, R. A. and Anderson, B. J. (1973) Biochem. Biophys. Res. Commun. 52, 1410–1417
21 Blankenship, R. E. and Sauer, K. (1974) Biochim. Biophys. Acta 357, 252–266
22 Ke, B., Vernon, L. P. and Chaney, T. H. (1972) Biochim. Biophys. Acta 254, 345–357
23 Boardman, N. K. and Anderson, J. M. (1967) Biochim. Biophys. Acta 143, 187–203
24 Henningsen, K. W. and Boardman, N. K. (1973) Plant Physiol. 51, 1117–1125
25 Erixon, K., Lozier, R. Butler, W. L. (1972) Biochim. Biophys. Acta 267, 375–382
26 Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 292, 772–785
27 Kok, B., Radmer, R. and Fowler, C. F. (1975) in Proc. 3rd Int. Cong. Photosynthesis, (Avron, M., ed.), pp. 485–496, Elsevier, Amsterdam
28 Vermeglio, A. and Mathis, P. (1973) Biochim. Biophys. Acta 314, 57–68
29 Knaff, D. B. and Arnon, D. I. (1969) Proc. Natl. Acad. Sci. U.S. 63, 956–962
30 Erixon, K. and Butler, W. L. (1971) Photochem. Photobiol. 14, 427–434
31 Boardman, N. K., Anderson, J. M. and Hiller, R. C. (1971) Biochim. Biophys. Acta 234, 126–136
32 Vermeglio, A. and Mathis, P. (1973) Biochim. Biophys. Acta 292, 763–771
33 Cramer, W. A. and Whitmarsh, J. (1977) Annu. Rev. Plant Physiol. 28, in press
34 Horton, P. and Cramer, W. A. (1975) FEBS Lett. 56, 244–347
35 Horton, P., Whitmarsh, J. Cramer, W. A. (1976) Arch. Biochem. Biophys. 176, 519–524
36 Heber, U., Boardman, N. K. and Anderson, J. M. (1976) Biochim. Biophys. Acta 423, 275–292
37 Wikstrom, M., Harmon, H. J., Ingledew, W. J. and Chance, B. (1976) FEBS Lett. 65, 259–277