Light-Induced Oxidation-Reduction Reactions of Photosystem II in Dichlorophenyl-dimethyl Urea (DCMU) Inhibited Thylakoids

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Illumination of thylakoid membranes in the presence of 3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea (DCMU) causes the reduction of the primary quinone acceptor Q_A of photosystem II (PS II) and the storage of a positive charge on the donor side of the photochemical reaction center. These oxidation-reduction reactions are accompanied by characteristic changes of absorbance in the ultra-violet region of the spectrum. The PS II-related absorbance difference spectra (250–350 nm) were compared in control and hydroxylamine-treated thylakoid membranes, and in thylakoids suspended in the presence of carbonyl cyanide-*p*-(trifluoromethoxy)-phenylhydrazone (FCCP). The light *minus* dark difference spectra were dominated by the Q_A^- *minus* Q_A difference spectrum. Qualitatively, the three spectra were identical in the 300–350 nm region, however, they showed distinct differences in the 250–300 nm region. The latter arose because of different contributions from the donor side of PS II in the thylakoid membrane of the three samples. The result suggested that FCCP acts as the ultimate electron donor in DCMU-poisoned chloroplasts. Therefore, the absorbance difference spectrum in the presence of FCCP reflected a contribution from the Q_A^- *minus* Q_A component only. Deconvolution of the absorbance difference spectra of control and hydroxylamine-treated thylakoids yielded difference spectra attributed to the oxidation of a component on the donor side of PS II. This component did not conform with the known Mn(III) → Mn(IV) transition. Rather, it indicated the oxidation of a modified form of Mn in the presence of DCMU, probably a Mn(II) → Mn(III) transition. The results are discussed in terms of the use of DCMU-poisoned thylakoid membranes in the quantitation of the primary quinone acceptor Q_A by spectrophotometric approaches.

Introduction

The photochemical charge separation at photosystem II (PS II) causes the oxidation of the reaction center molecule P680 and the concomitant reduction of the primary electron-acceptor pheophytin molecule. In a temporal sequence of events, the negative charge from the pheophytin primary acceptor reduces the bound quinone acceptor Q_A molecule to the semiquinone anion form. The reduction of Q_A is accompanied by specific absorbance changes in the ultra-violet region of the spectrum [1–3]. It is completed within a few hundred picoseconds [4].

On the oxidizing or donor side of PS II, a number of different intermediates are known to act as electron donors to the $P680^+$. Electron donation occurs from the secondary donor Z which reduces $P680^+$ in the nanosecond time scale [5–7]. Electron donation to $P680^+$ could also originate from the auxiliary donor D. It is now believed that both Z and D are tyrosine (Tyr) molecules involved in the photosynthetic oxygen evolution process [8–10]. The photochemical oxidation of Z and D is responsible for the generation of the various forms of EPR signal II [11–13]. Optically, the oxidation of Z and/or of D is accompanied by absorbance difference changes in the ultra-violet region of the spectrum which are typical of Tyr^+ *minus* Tyr [14–16].

The ultimate electron donor on the oxidizing side of PS II is Mn atoms associated with the

Abbreviations: PS, photosystem; P680, reaction center of PS II; Q_A, primary quinone electron acceptor of PS II; Z and D, secondary PS II electron donors; Tyr, tyrosine; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea; ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y; FCCP, carbonyl cyanide-*p*-(trifluoromethoxy)phenylhydrazone.

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water-evolving complex. It is believed that four Mn atoms are contained in the water-splitting enzyme [17] and that they normally occur in the Mn(III) form [18]. Four successive electron-donation reactions from each water-evolving complex advance the oxidation state of each Mn atom to Mn(IV), a condition that is necessary and sufficient for the oxidation of two H_2O molecules, for the release of molecular oxygen and the return of the oxidation state of Mn to Mn(III) [18, 19]. The absorbance difference spectrum in the ultra-violet, generated upon the transition of Mn(III) to Mn(IV) is reported to be a broad positive absorbance band peaking at about 300 nm [14, 18]. However, the involvement of a Mn(II $\rightarrow$ III) transition in the water-oxidation process has not been ruled out [20].

In the past, this laboratory employed light *minus* dark absorbance difference spectrophotometry for the measurement and quantitation of semiquinone anion formation in thylakoid membranes [21–23]. Thylakoid membranes were suspended in the presence of the electron-transport inhibitor dichlorophenyl-dimethyl urea (DCMU) and the oxidant potassium ferricyanide and were illuminated by continuous light. Each illumination resulted in a one-electron transfer to the primary quinone acceptor Q_A and in the storage of one positive charge on the oxidizing side of PS II. The semiquinone anion, formed under these conditions, is stable and it is accompanied by the typical difference spectrum with peaks at 320 nm (positive change) and 267 nm (negative change). Positive charges on the oxidizing side of PS II normally advance the oxidation state of Mn in the water-splitting enzyme [18–20]. In the presence of DCMU and potassium ferricyanide, however, positive charge accumulation on the donor side of PS II has not been reported. Similarly, the associated absorbance changes have not been characterized. This is a relevant question since DCMU and potassium ferricyanide are used widely by many laboratories in PS II-related research. The present work reports on light-induced absorbance difference spectra (250–350 nm region) of PS II in thylakoid membranes suspended in the presence of DCMU and potassium ferricyanide. The origin of these absorbance changes is discussed in terms of the oxidation-reduction reactions on the donor side of PS II.

Materials and Methods

Chloroplast thylakoid membranes were isolated from freshly harvested, hydroponically grown spinach (*Spinacia oleracea* L.) by grinding the leaves in a blender in a buffer containing 50 mM Tricine-NaOH (pH 7.8), 0.4 M sucrose, 10 mM NaCl and 5 mM $MgCl_2$. The slurry was filtered through miracloth and chloroplasts were precipitated by centrifugation at $5000 \times g$ for 5 min. The pellet was resuspended in a small amount of the isolation buffer to a Chl concentration of about 1 mg/ml using a Wheaton homogenizer. The sample was then kept in the dark on ice until use. Chlorophyll concentrations were determined in 80% acetone using the procedure of Arnon as previously described [21–23].

Hydroxylamine-treated thylakoids were obtained upon incubation of chloroplasts for 15 min at a Chl concentration of 0.5 mM in the presence of 20 mM NH_2OH and 2 mM Na-EDTA [22]. Control and NH_2OH-treated membranes were washed twice and finally resuspended in a small volume of the isolation buffer. All operations were carried out in dim light at 4 °C. Inside-out thylakoid vesicles [23] were prepared from thylakoid membranes upon Yeda Press treatment at 2000 lbs per square inch (13.7 MPa) and then kept in the dark on ice until use.

Absorbance-difference measurements in the ultra-violet region of the spectrum were performed with a laboratory-constructed difference spectrophotometer. The optical path length in the cuvette was 1.78 mm for the measuring beam and for the actinic beam it was 1.26 mm. Actinic excitation was provided in the green region of the spectrum by a combination of Corning CS 4-96 and CS 3-69 filters. The intensity incident to the sample was about 50 $\mu E \cdot m^{-2} \cdot s^{-1}$. Light *minus* dark absorbance difference spectra were measured in the 250–350 nm region. For the absorbance change measurements, control thylakoids were suspended in the presence of 20 μM DCMU and 2 mM potassium ferricyanide. Alternatively, thylakoids were suspended in 20 μM DCMU, 2 mM potassium ferricyanide and 5 μM FCCP. Hydroxylamine-treated thylakoids were suspended in the presence of 20 μM DCMU and 2 mM potassium ferricyanide. The amplitude of the absorbance change at each wavelength was corrected for the effect of particle flat-

tening [24] and for any residual contribution from P 700 [25].

The experimental protocol involved one pre-illumination of the samples (3 s duration at 50 $\mu E \cdot m^{-2} \cdot s^{-1}$) sufficient for the photoreduction of the primary quinone acceptor Q_A and for the oxidation of cytochrome *f* and of P 700 (presence of DCMU and ferricyanide). A subsequent 60 s dark incubation resulted in the relaxation of Q_A^-, probably *via* a back reaction with the oxidizing side of PS II. Each sample was then illuminated four times at a rate of once per minute and the resulting absorbance change signals were averaged to yield the specific measurement. The frequency and duration of illumination and the presence of DCMU ensured that the endogenous Fe^{2+} located between Q_A and Q_B remained in the reduced state throughout the measurement.

Light *minus* dark absorbance-difference rate-spectra were measured in the 240–340 nm region. The duration of sample illumination for these measurements was 10 s. Inside-out thylakoid vesicles were used for these measurements and were suspended in the presence of 250 μM DMQ and 50 μM gramicidin D (control), or 250 μM DMQ, 50 μM gramicidin D, and 1 mM $MnCl_2$ (Mn).

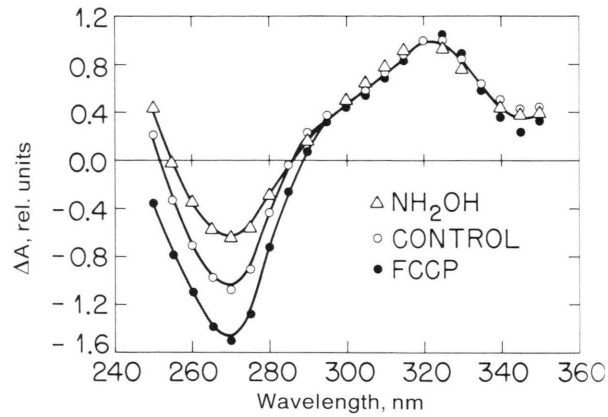

Fig. 1. Absorbance-difference spectra of intact spinach thylakoids suspended in the presence of DCMU and potassium ferricyanide (CONTROL) or DCMU, potassium ferricyanide, and FCCP (FCCP). Hydroxylamine-treated thylakoids with inactivated H_2O-splitting enzyme (NH_2OH) were measured in the presence of DCMU and potassium ferricyanide. The difference spectra were normalized to the same relative amplitude at 320 nm. The absolute absorbance change values at 320 nm were 1.45×10^{-3} (Control), 1.10×10^{-3} (NH_2OH), and 1.0×10^{-3} (FCCP). Chlorophyll (*a* + *b*) concentration in the cuvette was 200 μM. Note the differences among the various spectra in the 250–290 nm region.

Results and Discussion

The chemical compounds hydroxylamine (NH_2OH) and carbonyl cyanide-*p*-(trifluoromethoxy)phenyl-hydrazone (FCCP) have distinct but different effects on electron-transport reactions at the oxidizing side of PS II. Assessment of the effect of these compounds on PS II was provided from the comparison of the light-induced absorbance difference spectra of intact thylakoid membranes suspended in the presence of 20 μM DCMU and 2 mM potassium ferricyanide. Under these conditions, PS I and the electron-transport intermediates between the two photosystems do not undergo light-dependent oxidation-reduction reactions [22, 25]. Hence, only the charge separation reactions at PS II and electron-transport reactions on the oxidizing side of this photosystem will contribute to absorbance change measurements in the ultra-violet region of the spectrum. Fig. 1 shows light-induced absorbance difference spectra in the 250–350 nm region of untreated thylakoid membranes (control), of thylakoid membranes treated with hydroxylamine (NH_2OH), and of thylakoid membranes suspended in the presence of 5 μM carbonyl cyanide-*p*-(trifluoromethoxy)-phenyl-hydrazone (FCCP). It was determined that the amplitude of the absorbance change at 320 nm per charge separation was the same in the three samples [22]. Hence, the difference spectra in Fig. 1 were normalized to the same maximum value (1.0) at 320 nm. Visual inspection of the results suggested that the light-induced absorbance difference spectra in the 250–350 nm region (Fig. 1) are dominated by the contribution of the semiquinone anion *minus* quinone (Q_A^- *minus* Q_A) difference spectrum [2, 3].

The difference spectra of the three samples were identical in the 290–350 nm region, in agreement with earlier findings [22]. However, significant differences existed in the 250–290 nm region, indicating that electron-transport reactions on the donor side of PS II may contribute differently in the three samples. These results further suggested that, in thylakoid membranes suspended in the presence of 20 μM DCMU and 2 mM potassium ferricyanide,

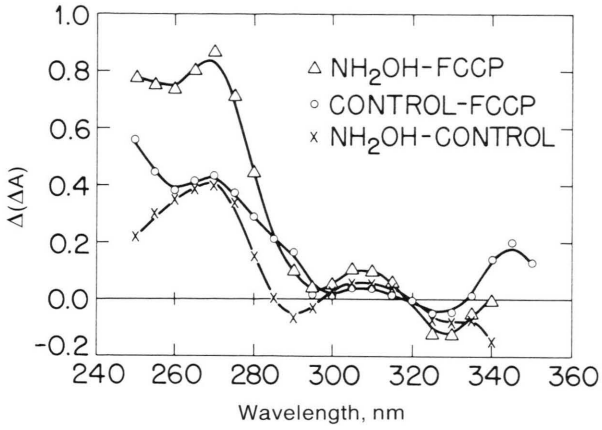

Fig. 2. Difference spectra derived from the results of Fig. 1. Control *minus* FCCP (○), NH_2OH *minus* FCCP (△), and NH_2OH *minus* control (×). Note that these difference spectra may suggest composite features of tyrosine-like oxidation superimposed on a broader positive absorbance change.

modification(s) of the electron-transport reactions on the oxidizing side of PS II are reflected on the light-induced difference spectra measured in the 250–290 nm spectral region but not in the 300–350 nm region.

To gain better insight into the PS II donor-side electron-transport reactions taking place in the three samples, we used the results of Fig. 1 to calculate the difference-difference spectra ($\Delta(\Delta A)$) between individual ΔA spectra. By this procedure, we hoped to cancel out contributions of the Q_A^- *minus* Q_A in the 250–350 nm region and, therefore, to expose absorbance difference changes in the 250–350 nm region occurring due to electron transport on the oxidizing side of PS II. Fig. 2 shows three sets of light-induced difference-difference ($\Delta(\Delta A)$) spectra. There are minor features in the 300–350 nm region but major differences in the 250–290 nm spectral region.

Under the experimental conditions employed in this work, *i.e.*, presence of DCMU and potassium ferricyanide, light-induced electron-transport reactions involve a single charge separation at PS II, resulting in photoreduction of the primary quinone acceptor Q_A. The positive charge on the photochemical reaction center is removed by electron donation from secondary donors to PS II. These include the secondary donors Z and D which are thought to be tyrosine molecules [8–10] and the cluster of Mn from the water-splitting enzyme [17, 18]. The cytochrome b_{559} is also known to act as electron donor to $P680^+$, however, all cytochrome b_{559} remained oxidized in the course of these light-induced measurements (presence of potassium ferricyanide [26]). Hence, the $\Delta(\Delta A)$ spectra shown in Fig. 2 could originate only from oxidation of tyrosine and/or from oxidation of Mn in the thylakoid membrane.

The results presented in the foregoing are evidence of unusual light-induced oxidation-reduction reactions on the donor side of photosystem II, occurring in thylakoid membranes suspended in the presence of DCMU and of potassium ferricyanide. The absorbance change spectra shown in Fig. 2 do not conform to a Mn(III) → Mn(IV) transition as this transition is accompanied by absorbance difference changes with a maximum at about 300 nm and a minimum at 250 nm [18]. Rather, the results of Fig. 2 suggest that the positive charge is stored, at least partly, on an unknown electron donor, probably a modified form of intrinsic Mn. However, the possibility cannot be excluded that, under our experimental conditions, extrinsic Mn(II) was oxidized by some of the reaction centers, thus providing the necessary electron for the reduction of Q_A. The extent of this unusual oxidation differed significantly among the three thylakoid membrane samples employed in this work. Hydroxylamine-treated samples showed the largest contribution, whereas thylakoid membranes suspended in the presence of FCCP showed the smallest, if any at all, oxidation of this component. This property is in agreement with the ADRY nature of FCCP [28–31] and suggests that FCCP plays a direct role as electron donor on the oxidizing side of PS II [30–32].

To determine whether adventitious or extrinsic Mn might be involved in electron donation in thylakoid membranes suspended in the presence of DCMU and potassium ferricyanide, we worked with inside-out thylakoids and attempted to induce electron donation from artificially added Mn ($MnCl_2$). The experimental conditions employed was inside-out thylakoid membranes from spinach suspended in the presence of dimethyl-benzoquinone (DMQ), to act as artificial electron acceptor, and gramicidin D. Samples were illuminated for a fixed period of time in the absence (CONTROL) or presence (Mn) of 1 mM $MnCl_2$ and the rate of

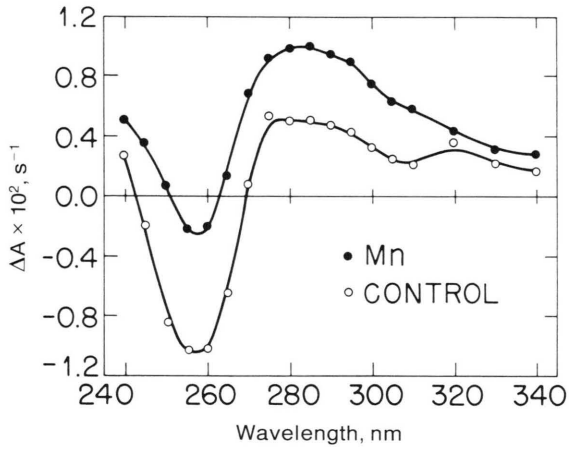

Fig. 3. The rate of absorbance change as a function of wavelength. Inside-out thylakoid membrane vesicles were suspended in the presence of DMQ and gramicidin D (open circles) or DMQ, gramicidin D, and $MnCl_2$ (solid circles).

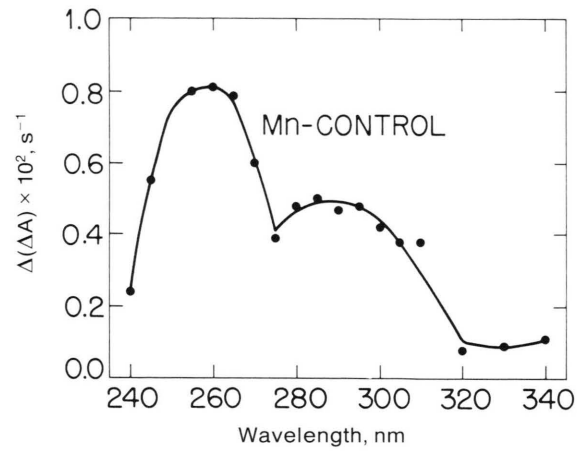

Fig. 4. Difference spectrum derived from the results of Fig. 3 with inside-out membrane vesicles: Mn *minus* Control. The difference spectrum suggests composite features of two different oxidation transitions.

the absorbance change ($\Delta A \cdot t^{-1}$) was measured in the 240–340 nm region. Fig. 3 shows the light-induced absorbance difference spectra generated under these experimental conditions. Under these conditions, absorbance change contributions from the advancement of the S-states should be minimized because several turnovers of the H_2O-splitting enzyme during the course of these measurements should result in cancellation of the contribution from the advancement of individual S-states. Nevertheless, some contribution from the advancement of the S-states might be expected from PS II complexes with partially damaged H_2O-splitting enzyme in these inside-out thylakoids. In the control sample (open circles), the difference spectrum reflects the accumulation with time of reduced dimethyl-benzoquinone ($DMQH_2$, see [33]). The difference spectrum in the presence of $MnCl_2$ (solid circles) is dominated by the $DMQH_2$ *minus* DMQ component but, it is evident that additional superimposed changes occur.

To gain better insight into the differences in electron donation between the two samples, we used the results of Fig. 3 to calculate the difference-difference spectrum ($\Delta(\Delta A) \cdot t^{-1}$). By doing so we hoped to cancel out common components and, therefore, to expose fully the specific contributions in the presence of Mn. Fig. 4 shows the resulting difference-difference spectrum (Mn *minus* control) which appears to be composed of two broad absorbance difference bands. One of them is peaking at about 257 nm, it indicates a slower absolute rate of DMQ reduction in the Mn *versus* the control sample, and is attributed to less efficient electron transport to DMQ in thylakoids suspended in the presence of 1 mM $MnCl_2$. The second component is a broadly positive absorbance difference band, peaking at about 290 nm, and is attributed to oxidation of Mn. Deconvolution of the results in Fig. 4 was obtained upon subtraction of the DMQ *minus* $DMQH_2$ difference spectrum [33] from the composite spectrum of Fig. 4. Fig. 5 shows the composite spectrum (dashed line), and, upon deconvolution, the DMQ *minus* $DMQH_2$ component (solid circles) and the remaining broad band peaking at about 290 nm (open circles). The broad band peaking at about 290 nm is similar to the absorbance difference spectrum attributed to the advancement of Mn(III) to Mn(IV) [14, 18]. It is possible that $MnCl_2$ (1 mM) inhibits the complete turnover of the Mn redox cycle and results in the accumulation of the higher Mn oxidation states in the thylakoid membrane. Alternatively, one may argue that Fig. 5 (open circles) shows a Mn(III) *minus* Mn(II) difference spectrum derived from the oxidation of extrinsic Mn after ligation with components of the thylakoid membrane. Indeed, evidence has been presented [34] to suggest that certain ligand centers

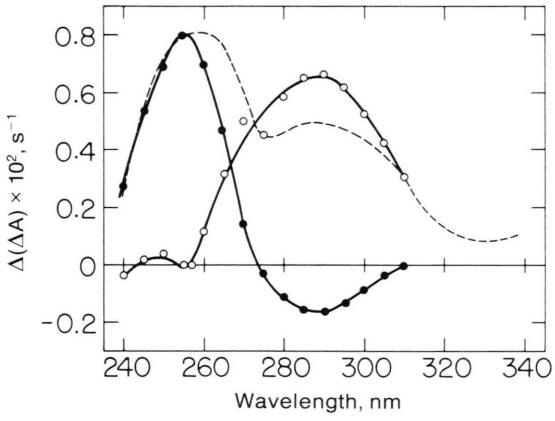

Fig. 5. Deconvolution of the Mn *minus* Control difference spectrum into a DMQ *minus* $DMQH_2$ component (solid circles) and a broad absorbance difference band peaking at about 290 nm (open circles).

undergoing a Mn(II) → Mn(III) transition will also generate absorbance difference spectra similar to that of Fig. 5 (open circles). The second alternative is supported by the amplitude of the absorbance change shown in Fig. 5 (open circles) which suggests the oxidation of one donor equivalent per about 30 Chl molecules, a value much higher than can be accounted for by Mn associated with the H_2O-splitting complex.

In summary, results from this work suggest that electron-transfer reactions on the donor side of photosystem II are modified upon the addition of different chemical compounds to suspensions of thylakoid membranes. These modifications involve alteration and/or inhibition in the advancement of the S-states, and/or electron donation from other intermediates on the donor side of PS II. For example, in the presence of DCMU and of potassium ferricyanide, illumination of thylakoid membranes resulted in the oxidation of a component yielding a broad band difference signal peaking at a wavelength less than 280 nm. The origin of this signal is unknown, however, one could suggest oxidation of a modified form of Mn in the thylakoid membrane. One may also consider oxidation of the secondary auxiliary donor D which is responsible for the EPR signal II_{slow} [12, 13].

It is of interest to note the effect of FCCP on the electron-transport reactions at PS II. The results of Fig. 1 and Fig. 2 support the contention that FCCP catalyzes a non-specific electron donation on the oxidizing side of PS II [30–32] and, thus, it prevents the light-induced oxidation of D and/or of Mn. As already proposed by other authors [31, 32], exogenous reductants of unknown origin are involved in the prompt relaxation of the oxidized form of FCCP, thereby ensuring that FCCP does not accumulate in the oxidized form. This function is consistent with the known property of FCCP as an ADRY agent [29–32].

The results have direct bearing on the application of the semiquinone anion measurement (ΔA_{320}) in the quantitation of PS II centers in thylakoid membrane preparations [21]. A question on the use of this technique in the quantitation of PS II was the possible contribution of absorbance change at 320 nm from the advancement of the S-states. The results provide evidence that such overlaps of absorbance change do not occur when thylakoid membranes are suspended in the presence of 20 μM DCMU and 2 mM potassium ferricyanide. Rather, when measured in the presence of DCMU and ferricyanide, light-induced absorbance change measurements at 320 nm (ΔA_{320}) are a fairly clear indicator of semiquinone anion formation and they can be safely used in the quantitation of PS II in thylakoid membranes.

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