Chapter 4

EFFECT OF ATRAZINE AND TMPD ON THE PHOTOOXIDATION AND PHOTOREDUCTION OF DIFFERENT POTENTIAL FORMS OF Cyt b-559 IN PHOTOSYSTEM II

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1. Introduction

Chloroplast cytochrome b-559 is known to be associated with photosystem II and though its role is not precisely understood, cyt b-559 functions probably both on the reducing and the oxidising sides of photosystem II (Butler and Matsuda, 1983). A high as well as a low potential form of cyt b-559 with mid-point potentials respectively from +350 mV to +400 mV and from +50 mV to +100 mV at pH 7.8 have been reported (Cramer and Whitmarsh, 1977). A range of redox forms may be observed in the intermediate region (Cramer and Crofts, 1982). The high potential cyt b-559 requires the structural integrity of the thylakoid membrane, and its disruption causes cyt b-559 to be modified to lower potential forms (Erixon et al., 1972). A heterogenous population of the high potential cyt b-559 has been reported (Selak et al., 1984).

Most of the cyt b-559 in the low potential form can be restored to the high potential form on incorporation of PS II particles into liposomes. The liposomal environment restores the normal variable fluorescence and increases the rate of oxygen evolution substantially (Matsuda and Butler, 1983). The purpose of the present work was to study the role of cyt b-559 in photosystem II particles and to analyse the function of different potential forms in the electron transport process. We determined the amount of reduced cyt b-559 and the kinetics of cyt b-559 photooxidation and reduction in isolated PS II preparation suspended in buffer (for low potential form of cyt b-559) and in PS II incorporated into liposomes (for relatively higher or intermediate potential form of cyt b-559), in the presence of an ADRY like regent, N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) and electron transport inhibitor atrazine.

2. Materials and Methods

Spinach leaves obtained from local market were used to isolate PS II particles according to the method of Ghanotakis et al. (1984).

Preparation of liposomes

A thin layer of Dimyrisitoyl phosphatidyl choline, DMPC (Sigma Chemicals Co., USA) was obtained by evaporation of the organic solvents under nitrogen in rotary evaporator. The lipid was then dispersed in 20 mM Mes-NaOH, pH 6.0 containing 15 mM NaCl and 5 mM MgCl₂. Lipid and PS II particles were sonicated separately before mixing in a lipid to Chl ratio of 20:1. Immediately after mixing, the suspension was frozen in liquid nitrogen and thawed in H₂O
at 25°C. TMPD or atrazine were added in buffer before freeze-thawing, thus ensuring that it was trapped or incorporated inside the liposomes along with the PS II particles. Absorbance spectra and light-minus-dark difference spectra of cyt b-559 were scanned in the double wave-length mode of Shimadzu UV-3000 spectrophotometer \( \lambda_{\text{ref}} = 570 \) and \( \varepsilon_{570-559} = 15 \ \text{mM}^{-1} \ \text{cm}^{-1} \) was used. Actinic white light was provided by the cross-illumination attachment of the instrument.

The rate (k) of light-induced absorbance changes of cyt b-559 at 559 nm was determined by steady state kinetics.

\[
k = \ln \left( \frac{C_k_1}{C_k_2} \right) \cdot \frac{u}{v_o}
\]

where, \( C_k_1 \) is the concentration of cyt b-559 in the reduced state without illumination of sample and \( C_k_2 \) is the concentration of cyt b-559 in the reduced state on illumination. \( v_o \) – volume of sample passed from time of illumination till measurement and \( u \) – is the flow rate.

The sample was passed through a flow cell at constant speed of 0.25 ml/sec and was illuminated outside the sample compartment with a tungsten halogen lamp (50 W).

In the experiments with TMPD, the changes in \( \Delta A_{559} \) were corrected for \( \Delta A_{570} \). All experiments were performed in dim light at room temperature and the samples were kept in dark.

3. Results

Figure 4.1 shows the absorption spectra and the light-minus-dark difference spectra of cyt b-559 in the isolated PS II particles.

Chemically induced difference redox spectra show that very little ascorbate reducible form of cyt b-559 is present in the isolated PS II (in buffer) (Fig.4.2). In PS II, incorporated into liposomes, the cyt b-559 was mostly in the ascorbate reducible form. These results are comparable to those of Matsuda and Butler (1983) who demonstrated that the high potential hydroquinone reducible form is found only in liposomes. However, in our liposome preparation we obtained an intermediate potential form which could be reduced by ascorbate. We attribute this to the nature of
Fig. 4.1 Absorption spectra (A) and light minus dark difference spectra (B) of cyt b-559 in isolated PS II. Measurement was done in dual wavelength mode $\lambda_{\text{ref}} = 570$ nm.
Fig. 4.2 Redox difference spectra of cyt b-559 in photosystem II particles in buffer and in photosystem II particles incorporated into liposomes. Assay mixture consisted of 20 mM Mes-NaOH, pH = 6.0, 15 mM NaCl and 5 mM MgCl₂ and PS II particles containing 50 µg Chl/ml. Additions were made sequentially of 0.2 mM potassium ferricyanide, 20 mM hydroquinone, 20 mM sodium ascorbate and a few grains of sodium dithionite.
lipid used in the two cases. We have used DMPC whereas in their work DGDG and PC was used.

The rate of light-induced absorbance change at $\lambda = 559$ nm was measured using a flow cell and steady state kinetic equation. Illumination was done outside the sample compartment and the dead time between illumination and measurement was 2 sec. It has been assumed that during this time the $\Delta A$ change due to dark relaxation of the redox state of cyt b-559 was negligible since the dark relaxation rate of light induced change at 559 nm has been reported to be very slow (Whitford et al., 1984). Compared to 2 sec dead time the $t_{1/2}$ of dark relaxation is slower by at least one order of magnitude.

The content of reduced cytochrome b-559 on illumination in presence of various concentrations of atrazine and TMPD are shown in Figs. 4.3 and 4.4. Photo-reduction of cyt b-559 low potential (in buffer) and of cyt b-559 intermediate potential (PS II in liposomes) was affected by the concentration of atrazine and TMPD used in the experiment (0.45 - 2.05 $\mu$M for atrazine and 4.5 - 20 $\mu$M for TMPD).

In presence of atrazine (Fig.4.3) the light induced reduction of cyt b-559 in PS II preparations in buffer decreased with increasing atrazine concentrations. In PS II incorporated in liposomes there was a relative increase in the amount of oxidised cyt b-559 on illumination (see Table 4.1) which increased with increasing concentrations of atrazine (Fig.4.3 and Table 4.1). From the above results we may conclude that the photoreduction of cyt b-559 (low as well as intermediate potential form) decreases with increasing concentrations of atrazine. The rate of photooxidation ($K_{ox}$) and photoreduction ($K_{red}$) is summarised in Table 4.1.

In presence of TMPD (Fig.4.4) content of reduced cyt b-559 (in PS II in buffer) on illumination depends on the concentration of TMPD(80x158). In PS II in liposomes, on illumination the cyt b-559 gets oxidised.

4. Discussion

Atrazine and TMPD affect the photoreduction and photooxidation of cytochrome b-559.

Presence of atrazine inhibits the photoreduction of cyt b-559 (Fig.4.3) indicating that cyt b-559 is reduced by the acceptor side of PS II (by either $Q_B$ or plastoquinone), since atrazine blocks the electron transport at $Q_B$. In the case
Fig. 4.3  Content of reduced cyt b-559 on illumination of PS II particles in presence of different concentrations of atrazine. The content of reduced cyt b-559 was 49.2% in dark (without any addition). The amount of chemically reduced cyt b-559 by dithionite was taken as 100% per cent reduced cyt b-559. The assay mixture contained 20. mM Mes-NaOH, pH 6.0, 15 mM NaCl and 5 mM MgCl₂. PS II particles corresponding to 10 μg Chl/ml were used. Other details are given in Materials and Methods.
Table 4.1 Per cent content of reduced cyt b-559 on continuous illumination and the rate of photoinduced $\Delta A_{559}$ in presence of various concentrations of atrazine and TMPD.

<table>
<thead>
<tr>
<th>PS II in Buffer</th>
<th>Reduced cyt b-559 (%)</th>
<th>Oxidised cyt b-559 (%)</th>
<th>$K_{\text{red}}10^{-3}$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrazine 0.45 µM</td>
<td>64.4</td>
<td>35.6</td>
<td>16.5</td>
</tr>
<tr>
<td></td>
<td>1.05 µM</td>
<td>58.2</td>
<td>41.8</td>
</tr>
<tr>
<td></td>
<td>1.5 µM</td>
<td>54.2</td>
<td>45.8</td>
</tr>
<tr>
<td></td>
<td>2.1 µM</td>
<td>51.0</td>
<td>49.0</td>
</tr>
<tr>
<td>TMPD 4.5 µM</td>
<td>49.6</td>
<td>50.4</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>7.5 µM</td>
<td>49.6</td>
<td>50.4</td>
</tr>
<tr>
<td></td>
<td>10.5 µM</td>
<td>52.7</td>
<td>47.3</td>
</tr>
<tr>
<td></td>
<td>13.5 µM</td>
<td>58.2</td>
<td>41.8</td>
</tr>
<tr>
<td></td>
<td>15.0 µM</td>
<td>59.7</td>
<td>40.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PS II in Liposomes</th>
<th>$K_{\text{ox}}10^{-3}$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td></td>
</tr>
<tr>
<td>Atrazine 0.45 µM</td>
<td>41.0</td>
</tr>
<tr>
<td></td>
<td>59.0</td>
</tr>
<tr>
<td></td>
<td>29.2</td>
</tr>
<tr>
<td></td>
<td>21.0</td>
</tr>
<tr>
<td>TMPD 4.5 µM</td>
<td>48.0</td>
</tr>
<tr>
<td></td>
<td>52.0</td>
</tr>
<tr>
<td></td>
<td>43.0</td>
</tr>
<tr>
<td></td>
<td>29.2</td>
</tr>
<tr>
<td></td>
<td>26.1</td>
</tr>
</tbody>
</table>

The content of reduced cyt b-559 was 49.2% in dark. Assay mixture was same as in Fig.3.

IP - intermediate potential.
Fig. 4.4 Content of reduced cyt b-559 on illumination of PS II particles in presence of different concentrations of TMPD. Other details are same as in Fig. 3.
of PS II incorporated in liposomes, illumination results in oxidation of cyt b-559 (Table 4.1). This may be due to the lipid environment which restores electron transfer from reduced cyt b-559 to the donor side (probably Z). The cytochrome redox state under continuous illumination would result from competition between reduction by plastoquinol (probably not directly from QB) and oxidation by PS II donor side.

The photoreduction of cyt b-559 was affected by TMPD. TMPD is known to accept electrons from QA and has been shown to destabilize the donor side of PS II in a manner analogous to other ADRY reagents (Velthuys, 1983). In the presence of a mixture of TMPD and TMPD+, Z+ oxidizes TMPD and QA- reduces TMPD+ (Agalidis and Velthuys, 1986). Like other ADRY reagents, TMPD is also oxidised by S2 state and may be responsible for the oxidation of reduced cyt b-559 (Ghanotakis et al., 1982). The photooxidation of TMPD and its subsequent rereduction by cyt b-559 has been demonstrated (Yerkes and Crofts, 1984). Recently a charge recombination pathway between QA and Z through a component C (suggested to be cyt b-559) has been considered (Tamura et al., 1986; Radmer et al., 1986).

Since our experiments were conducted under continuous illumination we presume that a steady state involving the photoreduction of TMPD+ by (1) QA- and (2) cyt b-559 and photooxidation of TMPD by Z+ and S2 is reached depending on the concentration of TMPD.

We observed that in PS II in buffer at low concentrations of TMPD (till 7.5 μM) very little photoreduction of cyt b-559 takes places and at higher concentrations the content of photoreduced cyt b-559 is increased (Fig.4.4) showing that up till 7.5 μM, TMPD is oxidising cyt b-559 and at higher concentrations it is reducing cyt b-559.

In presence of TMPD the cyt b-559 intermediate potential form (PS II in liposomes) gets oxidised on illumination (Table 4.1 and Fig.4.4) suggesting that only the intermediate potential form of cyt b-559 can reduce the donor side. Or in the liposomes the donor side function is restored facilitating the oxidation of cyt b-559, which is not taking place in PS II in buffer.

Our results may be best interpreted according to the following scheme (Fig.4.5) which represents the oxidation and reduction of cyt b-559 and the action of TMPD and atrazine in shifting the reaction (photoreduction of cyt b-559) to the right or to the left. This involves the function of cyt b-559 on both the
Fig. 4.5 Schematic representation of electron transfer in PS II and the involvement of cyt b-559 with other components.
reducing and oxidising sides of cyt b-559. The conversion of low potential form of cyt b-559 to high potential form by protonation (H\(^+\) taken from splitting of \(H_2O\)) has been suggested by Butler (1978; Butler and Matsuda, 1983) and may be affecting the electron transfer.

On the basis of our results we conclude that the photoreduction and photooxidation of cyt b-559 is affected by the concentrations of atrazine and TMPD. The photooxidation and photoreduction of cyt b-559 depends on its redox potential.

Liposomes made from DMPC were able to reconstitute an intermediate potential (ascorbate reducible) form of cyt b-559. The lipid environment might be a factor in determining the photoreduction and photooxidation of cyt b-559 by (1) restoring the integrity of OEC and PS II complex or (2) by modifying the environment in some way which facilitates e\(^-\) transfer from cyt b-559 to OEC component Z, or (3) restoring cytochrome b-559 to a relatively higher potential (ascorbate reducible form), which is able to reduce Z.

The PS II particles incorporated into liposomes provide a method of distinguishing absorbance changes due to photooxidation and photoreduction of different potential forms of cyt b-559.

5. References


Analysis of the $\alpha$ and $\beta$ centres of photosystem II in presence of atrazine and TMPD

Paliwal R. and G.S. Singhal (1987) in