Chapter 4

Establishing a relationship between progress of $F_{\text{PSI}}/F_{\text{PSII}}$ and stacking arrangement of thylakoids under continuous irradiance of white light
Establishing a relationship between progress of $F_{\text{PSI}}/F_{\text{PSII}}$ and stacking arrangement of thylakoids under continuous irradiance treatment.

Introduction

The efficiency of photosynthetic light energy conversion depends largely on the molecular architecture of the photosynthetic membranes, which also determines the migration of the excitation energy in the pigment system and its supply to the reaction centers. Most regulatory functions operate during photosynthesis, by adjusting the composition and/or the structure of the pigment system, in order to fine-tune the photosynthetic functions. For instance, redistribution of the excitation energy between the two photosystems is achieved via substantial reorganizations, e.g. the movement of a pool of mobile LHCII antenna proteins associated with PSII, and structural changes at the level of supercomplexes and remodeling of the membrane ultrastructure (Allen et al, 2011; ChUARTZMAN et al, 2008; FINAZZI et al, 2002; G.C. PApAgeoriou, 2011; IWAI et al, 2008; IWAI et al, 2010; Matsubara et al, 2011; Tikkanen et al, 2006; Varkonyi et al, 2009). LHCII in vivo and in vitro is also capable of undergoing thermo-optically driven reorganizations, i.e. structural changes induced by the dissipation of excess excitation energy (Dobrikova et al, 2003; Garab et al, 1988). The regulation of light-harvest, operating via the light-induced acidification of the lumen, i.e. via the formation of the light-induced trans-membrane proton gradient, is of particular importance since it is involved in the regulation of utilization vs. dissipation of the excitation energy in the light harvesting pigment system. The short-term regulatory mechanism like qE controls the magnitude of dissipation of the singlet excited state of chlorophyll a, and by this means protects plants against processes of photodegradation (Briantais et al, 1979; Horton et al, 1996; WraIGHT & Crofts, 1970). Although the molecular mechanism of photoprotection is still debated, it is most widely agreed that it requires and relies on the flexibility of the structure and/or composition of the light harvesting apparatus, i.e. it involves some kind of reversible structural changes either at the level of individual light harvesting or auxiliary complexes, such as PsbS, or/and at the level of their macroassembly (Allen et al, 2011; de Bianchi et al, 2011; Horton & Ruban, 2005; ilioaia et al, 2011; Lambrev et al, 2007; Li et al, 2009; Pascal et al, 2005; Ruban et al, 2007). Excess light leads to formation of a trans-thylakoid pH gradient which in turn also stimulates the xanthophyll cycle (Gilmore et al, 1995).

It has been already reported that at low pH (pH~5.5), the fluorescence of PSII decreases with concomitant increase in the fluorescence of PSI, resulting in an increase in the $F_{\text{PSI}}/F_{\text{PSII}}$ ratio (Jajoo et al, 2012; Singh-Rawal et al, 2010). This study indicated that the distribution of the excitation energy between the two photosystems can be altered by varying the pH, possibly by a rearrangement of the complexes between the stacked and nonstacked regions.

Here we have tried to establish a relationship between $F_{\text{PSI}}/F_{\text{PSII}}$ and stacking arrangements of thylakoids since both the phenomena were studied under similar conditions of prolonged irradiance and thus a comparative study could provide additional information regarding them. A reciprocal relationship between the changes in $F_{\text{PSI}}/F_{\text{PSII}}$ and the stacking arrangement of thylakoids for initial 30 minutes has been established. Also, both the oscillation of $F_{\text{PSI}}/F_{\text{PSII}}$
and alterations of stacking pattern of thylakoids under prolonged irradiance exposure were governed by trans-thylakoid ∆pH processes. These observations also prompted us to find out how acidification of luminal pH in absence of irradiance is contributing to the observed oscillation of $F_{PSI}/F_{PSII}$ and variations in $A_{580}/A_{678}$ occurring in presence of continuous irradiance. Mimicking effect of lowering of luminal pH and continuous white light irradiance were noticed on both the progress of $F_{PSI}/F_{PSII}$ and $A_{580}/A_{678}$.

**Results**

4.1. Comparative study between variations in extent of stacking and $F_{PSI}/F_{PSII}$

As already mentioned, the alterations in degree of stacking (Chapter 2, Figure 2.2 and 2.5) and the earlier report of oscillation of $F_{PSI}/F_{PSII}$ (Chapter 1, Figure 1.1), were occurring under identical conditions of continuous irradiance of white light and for clarification, only the initial decreasing phase observed during study of variations in extent of stacking has been compared with the initial increasing phase observed during the study of progress of $F_{PSI}/F_{PSII}$ at 77 K.

a. Comparison of progress of $A_{580}/A_{678}$ with $F_{PSI}/F_{PSII}$

Firstly we fitted the initial decrease in extent of stacking with the best-fit polynomial equation such that first derivative of the fitted equation indicated whether the phenomenon was an increasing or decreasing function (the initial 30 minutes data have been taken into account since a fixed pattern of decreasing tendency was noticed upto ~30 minutes time-interval). The progress of $A_{580}/A_{678}$ as function of continuous irradiance appeared to be a decreasing function ($dy/dx<0$) for 200, 500 and 800 µE m$^{-2}$ S$^{-1}$ intensity (y denotes the change in $A_{580}/A_{678}$ and x denotes the change in time in the best-fit equation) (Figure 4.1a, Table 4.1). However for 100 µE m$^{-2}$ S$^{-1}$ intensity, the progress of $A_{580}/A_{678}$ as function of continuous irradiance appeared to be an increasing function ($dy/dx>0$) (Figure 4.1a, Table 4.1) (Data taken from Chapter 2, Figure 2.2).

The progress of $F_{PSI}/F_{PSII}$ as a function of continuous irradiance appeared to be an increasing function since $dy/dx>0$ for 200, 500 and 800 µE m$^{-2}$ S$^{-1}$ intensity (Figure 4.1b, Table 4.1). However for 100 µE m$^{-2}$ S$^{-1}$ intensity, the progress of $F_{PSI}/F_{PSII}$ as function of continuous irradiance appeared to be a decreasing function (Data taken from Chapter 1, Figure 1.1).

**Summary**

*Thus a reciprocal relationship was observed for the progress of $A_{580}/A_{678}$ and $F_{PSI}/F_{PSII}$ as a function of continuous irradiance for 30 minutes for 200, 500 and 800 µE m$^{-2}$ S$^{-1}$ intensity of irradiance.*
Figure 4.1: Relationship between alterations in extent of stacking and $F_{PSII}/F_{PSI}$. Progress of $A_{580}/A_{678}$ vs time (minutes) (a); progress of $F_{PSII}/F_{PSI}$ vs time (minutes) (b); variations in chlorophyll concentration of heavy fraction pellet vs time (minutes) (c) as a function of continuous irradiance has been fitted to the best fit polynomial equation using MATLAB R2013b software and the first derivative of the fitted equation indicated whether the phenomenon was an increasing or decreasing function. The $R^2$ square values are as follows: 0.9956, 0.9946, 0.0047, 0.993 (a), ~1 (b, for all four intensities), ~1 (c, for all four intensities).
b. Comparison of variations in extent of stacking for digitonin treated thylakoids with progress of $F_{PSI}/F_{PSII}$

A comparative study was also made for digitonin treated thylakoid. The estimation of chlorophyll concentration of digitonin treated thylakoid appeared to be a decreasing function ($dy/dx<0$) with the progress of irradiance for all intensities tested (Figure 4.1c, Table 4.1) ($y$ denotes the change in % Chlorophyll and $x$ denotes the change in time in the best-fit equation) (Data taken from Chapter 2, Figure 2.5).

It might be that, for absorption spectroscopy intact thylakoid membranes were used. But for estimation of extent of stacking, detergent, digitonin was used. Digitonin affect the integrity of the membrane fractions by being more susceptible to unstacked thylakoids. Since estimation of extent of stacking (the chlorophyll estimation was executed on heavy fraction pellet of digitonin treated thylakoids) was performed mainly on stacked membrane fractions and not on intact thylakoids so this might be one of the reasons for the different observations of thylakoids exposed to 100 $\mu$E m$^{-2}$ S$^{-1}$ intensity of irradiance.

<table>
<thead>
<tr>
<th>$\mu$E m$^{-2}$ S$^{-1}$</th>
<th>Extent of stacking $A_{580}/A_{678}$</th>
<th>$F_{PSI}/F_{PSII}$</th>
<th>Extent of stacking % Chlorophyll of pellet</th>
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</tr>
<tr>
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<td>$dy/dx&lt;0$</td>
<td>$dy/dx&gt;0$</td>
<td>$dy/dx&lt;0$</td>
</tr>
</tbody>
</table>

*Table 4.1: Variations in $F_{PSI}/F_{PSII}$ and stacking pattern of thylakoids for initial 30 minutes*

Conclusions

*Reciprocal relationship exists between progress of $F_{PSI}/F_{PSII}$ and variations in stacking arrangements of thylakoids at least for initial 30 minutes for 200, 500 and 800 $\mu$E m$^{-2}$ S$^{-1}$ intensity of irradiance.*
4.2. Effect of acidification of lumen pH in determining the kinetics of \(F_{\text{PSI}}/F_{\text{PSII}}\) and \(A_{580}/A_{678}\)

a. Effect of acidification of thylakoid lumen pH on kinetics of \(F_{\text{PSI}}/F_{\text{PSII}}\)

Since generation of trans-thylakoid \(\Delta\)pH is an important determinant of both \(F_{\text{PSI}}/F_{\text{PSII}}\) and \(A_{580}/A_{678}\), so we thought to check the effect of acidification of thylakoid lumen pH on both the phenomena.

Firstly the fluorescence emission spectra were taken at different pH range of 5.0-8.0 and \(F_{\text{PSI}}/F_{\text{PSII}}\) or \(F_{722}/F_{685}\) ratio from the baseline normalized spectra of the treated thylakoids were plotted against pH (Figure 4.2). It was observed that on decreasing the pH from ~8 to 5, there was an overall increase in the amplitude of the plot of \(F_{\text{PSI}}/F_{\text{PSII}}\) versus pH treatments. The amplitude of the oscillation was also noticed to be increased with the progress of the irradiance treatments as already mentioned in Chapter 1, Section 1.1. Thus mimicking effect of lowering of thylakoid luminal pH and continuous irradiance treatments has been observed.

This observation was consistent with the earlier report of Singh-Rawal, (Singh-Rawal et al, 2010). However a slight decrease in \(F_{\text{PSI}}/F_{\text{PSII}}\) ratios at certain pH levels was also noticed. Previously the overall oscillation in the kinetics of \(F_{\text{PSI}}/F_{\text{PSII}}\) was too observed, when thylakoids were treated with continuous irradiance (Chapter 1, Figure 1.1). This observation of lowering of thylakoid luminal pH on the kinetics of \(F_{\text{PSI}}/F_{\text{PSII}}\) might also be one of the determinants for the oscillation of \(F_{\text{PSI}}/F_{\text{PSII}}\) under continuous irradiance, at least to some extent.

b. Effect of acidification of thylakoid lumen pH on kinetics of \(A_{580}/A_{678}\)

Furthermore, changes in pH are known to alter the membrane rearrangements within thylakoid membrane architecture (Chow et al, 2005; Chow et al, 1980). To monitor the variations in extent of stacking as a function of varying luminal pH, absorption spectra (400-750 nm) of thylakoids were recorded in the pH range 5.0-8.0. In order to quantify the increase in absorbance the signals at 580 nm were normalized to those at 678 nm (\(A_{580}/A_{678}\)) and plotted as a function of varying pH (Figure 4.3). There was overall increased amplitude in the plot of \(A_{580}/A_{678}\) with decrease in thylakoid luminal pH. The increase in extent of stacking with decrease in thylakoid luminal pH has been reported earlier by Singh-Rawal et al. (Singh-Rawal et al, 2010).

The overall increase in the amplitude of \(A_{580}/A_{678}\) ratio at later time-interval was also noticed as a function of prolonged irradiance at later time-intervals (Chapter 2, Figure 2.2). Thus the increased amplitude of \(A_{580}/A_{678}\) at later time-intervals following continuous irradiance might also be a consequence of acidification of thylakoid lumen apart from decrease in \(A_{678}\) noticed for \(A_{580}/A_{678}\) analysis.

Summary

Lowering of pH in absence of irradiance mimicked the effect of prolonged exposure to continuous irradiance for the progress of \(F_{\text{PSI}}/F_{\text{PSII}}\) and \(A_{580}/A_{678}\).
Figure 4.2: Kinetics of $F_{PSI}/F_{PSII}$ as a function of pH. Thylakoid membranes isolated from dark adapted leaflets were subjected to varying pH treatments at 25°C. Low temperature fluorescence (77K) emission spectra of the treated thylakoids were recorded at an excitation wavelength of 480 nm and normalized at 650 nm. $F_{PSI}/F_{PSII}$ ratios ($F_{722}/F_{687}$) were recorded from the corresponding spectra and plotted as a function of pH (upper left panel). The corresponding spectra are shown in upper right panel. Progress of $F_{PSI}/F_{PSII}$ under continuous irradiance of 200µE m$^{-2}$ S$^{-1}$ intensity (data taken from Chapter 1, Figure 1.1) has been shown for comparison (lower panel). Error bars indicate SDs ($n = 2–3$).
Figure 4.3: Kinetics of $A_{580}/A_{678}$ as a function of pH. Thylakoid membranes isolated from dark adapted leaflets were subjected to varying pH treatments and absorption spectra were recorded from 400-750 nm. Normalization of absorbance at 580 nm to the 678 nm signal ($A_{580}/A_{678}$) were calculated from the absorption spectra and plotted as a function of pH (upper left panel). The corresponding spectra are shown in (upper right panel). Progress of $A_{580}/A_{678}$ under continuous irradiance of 200µE m$^{-2}$ S$^{-1}$ intensity (data taken from Chapter 2, Figure 2.2) has been shown for comparison (lower panel). Error bars indicate SDs ($n = 2-3$).
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Conclusions

i. Reciprocal relationship exists between the variations in extent of stacking and $F_{\text{PSI}}/F_{\text{PSII}}$ as a function of continuous irradiance (for 30 minutes).

ii. Mimicking effect of lowering of thylakoid luminal pH, in absence of irradiance and prolonged exposure to continuous irradiance on progress of $F_{\text{PSI}}/F_{\text{PSII}}$ and $A_{580}/A_{678}$ has been observed.

Discussions

It is known that variable partitioning of the proton motive force into $\Delta p\text{H}$ and $\Delta \psi$ can modulate down-regulatory sensitivity. Thus thylakoid membranes were treated with buffers at different pH values in absence of light so that reduction of the electron transport chain components (quinone pool) can be evaded (Avenson et al, 2004; Singh-Rawal et al, 2010).

Low pH was also shown to cause reversible structural reorganizations in the thylakoid membrane; affecting the long range order of the protein complexes (Jajoo et al, 2012) thereby providing an explanation for pH induced redistribution of excitation energy. Changes in the PSII-LHCII macro-structure are a crucial element in the regulation of light harvest and have been shown to depend on the formation of $\Delta \text{pH}$ (Johnson et al, 2011). These reports are consistent with our observations.

Also the luminal pH, and as a result the trans-thylakoid $\Delta \text{pH}$, has a complex and important function in the general control of photosynthesis. The $\Delta \text{pH}$ is not only the driving force for ATP synthesis, but also regulates electron transport through PSII and the cytochrome b$_6$f complex.

In other words, formation of a proton gradient across the thylakoid membranes by light-induced acidification of thylakoid lumen is perhaps inducing, changes in the $F_{\text{PSI}}/F_{\text{PSII}}$ and in stacking arrangements of thylakoids.

However, no studies have been undertaken to correlate light-induced acidification with progress of $F_{\text{PSI}}/F_{\text{PSII}}$ and $A_{580}/A_{678}$.

Thus it is clear that the observations may promote further research to quantitate the relative contributions of acidification of luminal pH on chromatic adaptation and structural rearrangements under continuous white light.

Statistical analysis

Statistical significance was ensured by analyzing the data using MATLAB R2013b software. We compared each level of time of exposure to irradiance with conditions of no irradiance (control). Group means were compared by student t test for paired comparisons and most differences were significant with $P < 0.05$. 

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