FLASH-INDUCED OXYGEN EVOLUTION MEASUREMENTS IN PHOTOSYSTEM II SAMPLES

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Report passed:
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Overview

1. Introduction

Photosynthetic water oxidation in higher plants, algae and cyanobacteria is one of the most important biological processes on our planet due to the formation of molecular oxygen and carbohydrates ($C_nH_{2n}O_n$), which are required for the survival of all oxygen-consuming organisms. The overall reaction of the photosynthetic process can be represented briefly as follows:

$$nH_2O + nCO_2 \xrightarrow{sunlight} C_nH_{2n}O_n + nO_2$$

The reaction is performed in thylakoid membrane by a continuous serial process of electron transfer through three different protein complexes (figure 1.1). These complexes, which are different in function and structure, include photosystem II (PSII) (water oxidation and oxygen formation), Cytochrome b_6f (Cyt b_6f) and photosystem I (PSI) (NADPH production). One further complex is the ATP synthase (ATP synthesis). There are two different kinds of photosynthetic reactions. These are light-dependent (the production of oxygen, NADPH and release of proton directly; ATP synthesis indirectly) and light-independent reactions (reduction of carbon dioxide to generate carbohydrate). [15]

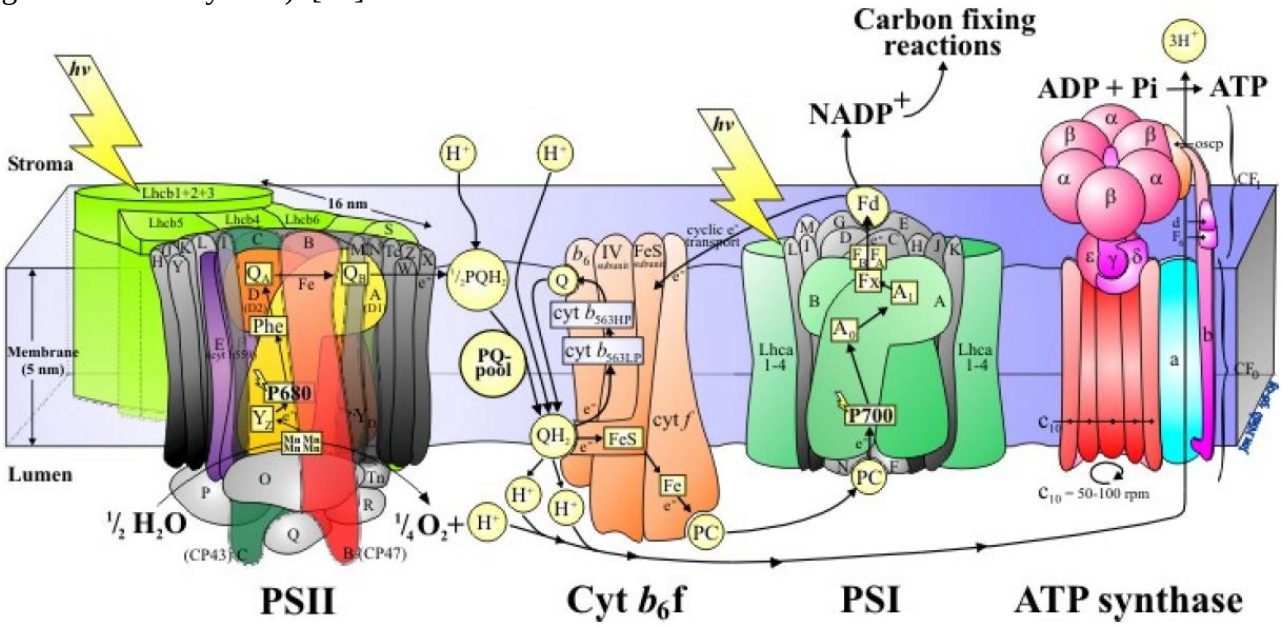

Figure 1.1: Working model (including transfer of protons, electrons and ATP's synthesis) of four major protein complexes in thylakoid membrane from a plant cell [15]

In particular, PSII is pivotally important to life on the earth because it is the principle means by which molecular oxygen is generated. Based on modern spectroscopic techniques (e.g. EXAFS - extended X-ray absorption fine structure, EPR – electron paramagnetic resonance, NMR – nuclear magnetic resonance, XANES – X-ray absorption near edge structure, etc), the structure of PSII (and the information of function) has been assigned more and more clearly, precisely. Structurally, PSII is a multi-subunit Chl/protein complex that includes approximately 30 polypeptide components (e.g. PsbE, PsbF, PsbG, PsbO, etc) [2, 3].

The light dependent reaction is activated by the absorption of photons (activation energy must be higher than 1.8 eV) by Chlorophyll (Chl) molecules in the antenna complexes (LHC II - light-harvesting complexes II or CP43 or CP47). The photon energy is leading to an electrically excited state for the pigment P680, a special Chl that is the primary electron donor. The excited P680* molecule is going to become $P680^{\bullet+}$ after releasing an electron to pheophytin (Pheo). Thereafter, this electron is transferred from Pheo to plastoquinone Q_A and to plastoquinone Q_B. The transferring process makes Q_B become negatively charged. Q_B^{2-} is formed when a second electron migrates successfully to Q_B^{-}. In order to neutralize the negative charge of Q_B^{2-}, two protons from the stroma bind to Q_B^{2-} and as a result the neutral charged molecule PQH_2 is formed that can leave the PSII protein complex and move to the lipid membrane. The vacant binding site is filled by a free plastoquinone of the lipid membrane.

In addition, the $P680^{\bullet+}$ molecule must be neutralized with the aim to prepare for the next excitation. Thus, an electron from tyrosine Z (Y_Z) is dislocated to the $P680^{\bullet+}$ molecule and reconverts it to normal P680 through the oxidation process of Y_Z to $Y_Z^{\bullet}$. Then, the neutral radical $Y_Z^{\bullet}$ is reduced by accepting one electron from the manganese cluster to return to ordinary Y_Z side chain. The electron donation of the manganese cluster is performed by the oxidation of four manganese atoms in the core of PSII. After the manganese cluster has lost four electrons (corresponding to S_4) for the charge neutralization of $P680^{\bullet+}$, the water oxidation is executed (with the release of oxygen and protons to the lumen) by splitting two water molecules with the target to return four electrons for manganese cluster and convert the S_4 state to the S_0 state.

With the target to measure the oxygen evolution of photosynthetic systems, many techniques and approaches were developed that are able to analyze the trace oxygen concentrations. One of these modern methods that is still being used is the Joliot electrode (and polarograply in general) [1].This electrode type was developed by Pierre Joliot and his co-workers in order to measure oxygen evolution with single-turnover light flashes. In addition, they realized that the oxygen yield shows a periodicity of four as a function of flash number (fig. 1.2)

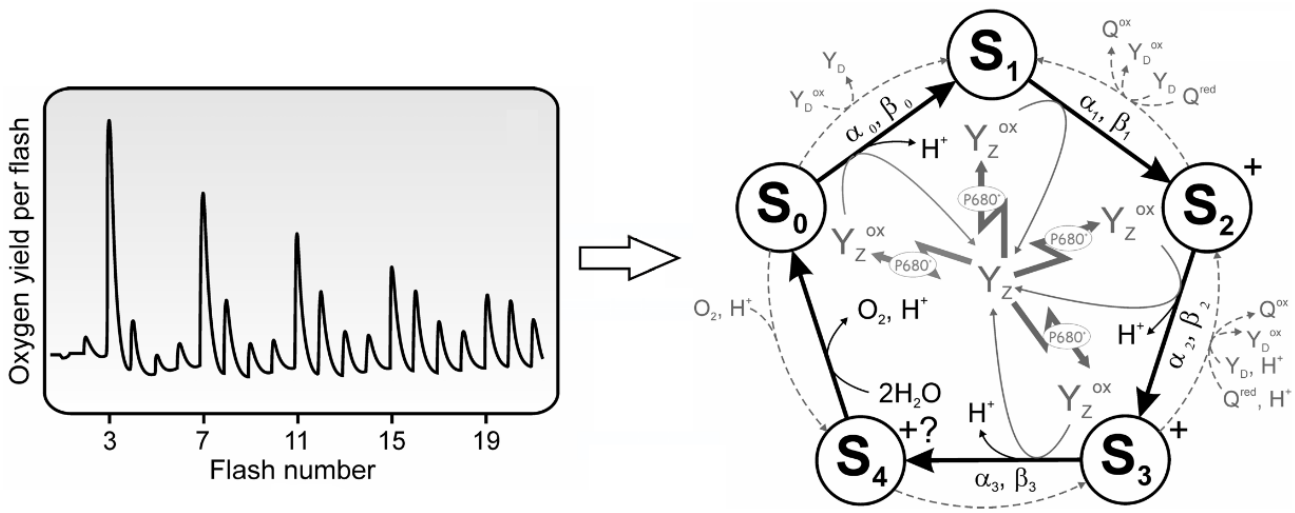

Figure 1.2: Oxygen yield of PSII as a function of flash number (left figure) and a current version of the Kok model with five oxidation states (right figure) [14, 17]

The periodicity of four readily disappeared after several cycles and the maximum oxygen yield occurred on the third flash rather than fourth flash. These observations indicated an interesting complexity in the mechanism of water oxidation that was not easy to explain. After one more year (1970), Kok and his co-workers suggested an elegant model (the "Kok model") to interpret Joliot's findings. In the Kok model, the oxygen-evolving complex (PSII) can exist in one of the five oxygen oxidation states, labeled S_0, S_1, S_2, S_3 and S_4 (see figure 1.2). Moreover, each photochemical reaction removes a single electron from PSII to advance the system to the next higher S state until four electrons have been released from PSII and the oxidation of two water molecules to one molecule of dioxygen is initiated. Of course, the mechanisms of breaking the covalent O-H bonds and connecting the two oxygen atoms are really challenging problems and are not explained by this kinetic scheme.

The aim of this project is to perform flash-induced oxygen evolution measurements using a Joliot-type electrode and to optimize some of the experimental conditions. The specific goals are to identify the influences of polarization time and voltage and of catalase, determine the lifetime of S_2 in spinach thylakoids sample (with or without the addition of catalase, variation of polarization time), and the oxygen evolution activity of PSII protein complexes at different conditions (pH, temperature, etc). The results of this project could supply hopefully some hints and orientations for the next investigations in the photosynthetic field.

2. Experiments, results and discussions

2.1. Introduction of polarography and the Joliot electrode

The three most popular methods for determination of the oxygen evolution activity of photosynthetic samples are the Clark electrode, the Joliot electrode and membrane inlet mass spectrometry (MIMS). The other methods are for example EPR, ZrO_2 electrode and gas chromatography (GC) [1]. The Joliot electrode was designed and constructed according to the basic principles of polarography and is a specific approach for exploring the oxygen evolution activity of dark-adapted photosynthetic systems under illumination with single turn-over flashes. Based on such data, the kinetics and mechanism of the water oxidation process can be investigated. However, the working mechanism of the artificial photosynthetic systems that do not work according to the Kok-cycle cannot be studied by Joliot electrode experiments. Besides, Joliot electrode can be utilized to detect and measure oxygen evolution at the trace concentration. The interior structure of the home-built Joliot electrode is described in the diagram below.

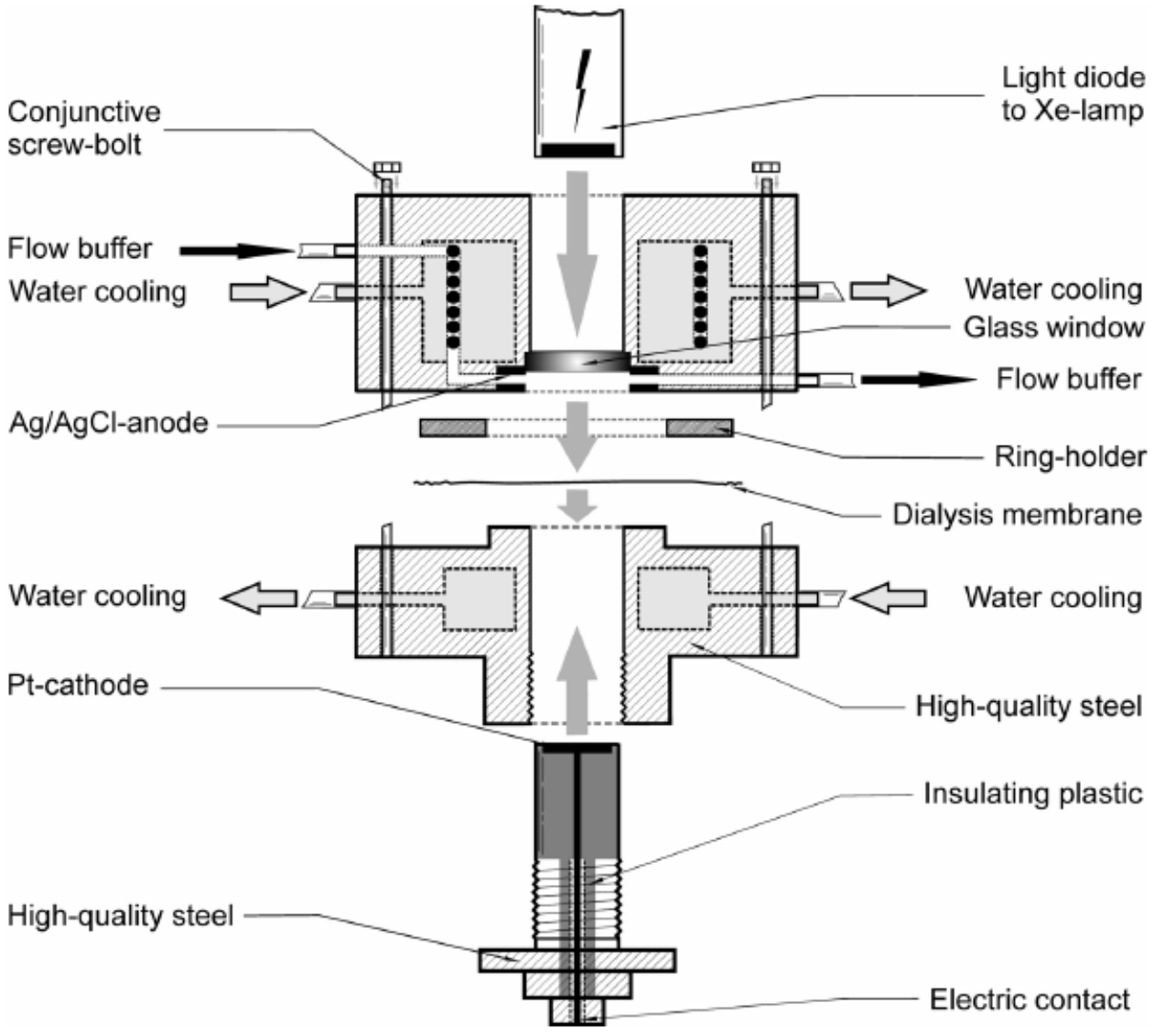

Figure 2.1: The interior structure of the home-built Joliot electrode [16, 18]

The Joliot electrode is an electrochemical system that consists of two electrodes, which are connected to each other by low voltage direct current (usually 750mV) and a salt bridge. The anode of the system is often made of Ag metal, this electrode is immersed by a solution of buffer (salt bridge) which contains the electrolytes as NaCl (or $CaCl_2$, $MgCl_2$) to give rise to a constant potential with insoluble AgCl. Due to the structural differences between Joliot electrode and Clark electrode, the source of chloride ion that was employed to supply for our Joliot electrode came from

the buffer flow, instead of using KCl standard solution (1M) as in the Clark electrode. Hence, the electrochemical reactions that take place on the surface of Ag-anode are as follows [1, 4]

Ag-anode: $4Ag \rightarrow 4Ag^{+} + 4e^{-}$

$$4\mathrm{Ag}^{+} + 4Cl^{-} \rightarrow 4AgCl$$

Totally: $4\mathrm{Ag} + 4Cl^{-} \rightarrow 4AgCl \downarrow + 4e^{-}$

The four electrons, which are released from Ag-anode, are delivered to the Pt-cathode in order to reduce dissolved oxygen molecules. In addition, the electrochemical reaction on the surface of the Ag-anode can be the combination of Ag^{+} and OH^{-} (instead of Cl^{-}) to produce AgOH in some special situations (measurements at high pH or electrolyte standard solution for Clark electrode from manufacturer can be KOH instead of KCl).

The surface of the cathode makes contact with dissolved oxygen, which diffuses from the flow buffer through the dialysis membrane (or molecular weight cut-off membrane, size of pore 14kDa) or is produced by PSII protein complexes directly above the Pt-surface. Consequently, a noble metal must be used to manufacture this cathode with the aim to avoid oxidizing this metal by oxygen molecules and form metal oxide (basic oxide). In addition, this metal is only intermediate place to transfer electrons that were supplied from Ag-anode and it must not exchange any of its own ions or electrons with the above electrolytic environment (buffer solution in this case). This metal must also promote the oxygenic reduction reaction on its surface to show exactly the change of dissolved oxygen concentration. Therefore, there are only few different kinds of metal that can satisfy all of these strict conditions, and Platinum is the most optimal choice. Moreover, the reactions that occur on surface of Pt metal are represented below. [1,4]

Pt-cathode: $\mathrm{O}_2 + 2H_2O + 2e^{-} \rightarrow H_2O_2 + 2OH^{-}$

$$\mathrm{H}_2O_2 + 2e^{-} \rightarrow 2OH^{-}$$

Totally: $\mathrm{O}_2 + 2H_2O + 4e^{-} \rightarrow 4OH^{-}$

The small amount of hydroxide ions that is formed on the surface of Pt-cathode does not affect so much the pH value of the sample due to the presence of buffer (usually MES or HEPES). The Joliot electrode, which was used in this project, was designed and manufactured at the TU-Berlin, Germany. The Joliot electrode experiments usually use a Xenon discharge lamp. For the experiments described in this report, a Perkin Elmer optoelectronics flash lamp, product number LS-1130-4, serial number 1952 was employed. This lamp can be set to a certain frequency (often 2Hz) to release single-turnover light flashes to sample. That is why the results of Joliot electrode experiments are often referred to as be flash-induced oxygen evolution patterns (FIOPs).

The Joliot electrode cannot be calibrated like the Clark electrode. Therefore, one cannot know accurately how many PSII protein complexes participated in to the formation of molecular oxygen and how many mol of oxygen was produced. That is why the peak's amplitude of oxygen flash yield does not have a certain unit. Moreover, this could be a disadvantage and shortcoming of this electrode in order to compare exactly the measurable results of the same sample at the different times. Besides, it is not possible to add artificial electron acceptors due to the interference with the bare platinum electrode. [1]

In Joliot electrode measurements, the samples are applied at the position between Pt-cathode surface and dialysis membrane. This means the sensitivity and time-resolution of Joliot electrode is much greater than Clark electrode. The dialysis membrane plays an important role as an intermediate partition between flow buffer and sample with the purpose to avoid the dissolution of sample to the huge amount of buffer solution. Beside this, this membrane also has an essential role as an intermediate conductor in order to maintain a stable voltage between Ag-anode and Pt-cathode. This allows the reversible migrations of charged molecules that have molecular weight smaller than pore's size of dialysis membrane (14kDa in this case) such as chloride, protons or the other ions coming to cathode and anode.

The data that are collected with the Joliot electrode experiments usually display many peaks of oxygen evolution flash yield. However, there are always the appearances of artifacts (often 5-6

milliseconds wide) before the real signals. Therefore, with the purpose of analyzing the data accurately, any artifacts on the raw data had to be removed manually. In addition, the removal of artifacts also needs the aid of Origin software (version 8) to identify where the artifacts are and where the real signals are. All of the data that were saved and supplied for data analysis were passed through the process of artifact removal. Moreover, the filter function of data receiver can be employed to remove automatically most of the artifacts. That is why the highest level of filter function (number 4) was usually used for the experiments of this project. The below figures are illustrating the difference between the raw data (with artifacts) and processed data (without artifacts).

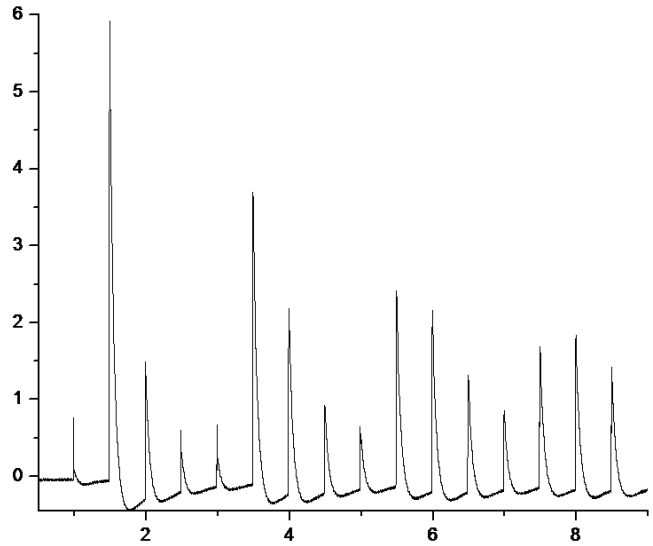

Figure 2.2: Raw FIOPs of thylakoid sample with artifacts

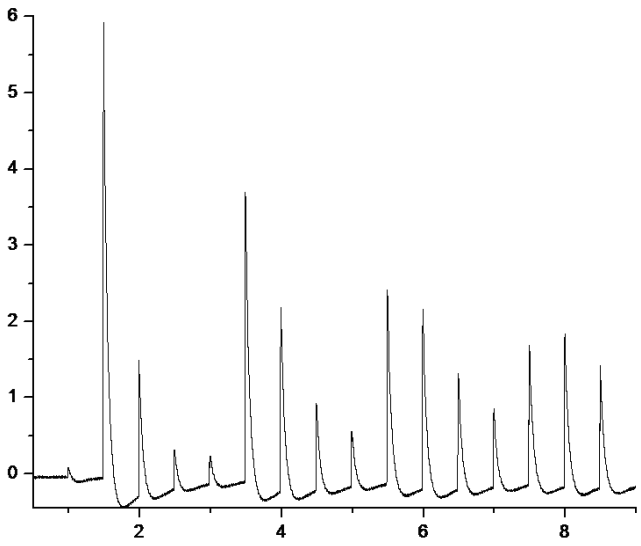

Figure 2.3: Processed FIOPs of thylakoid sample without artifacts

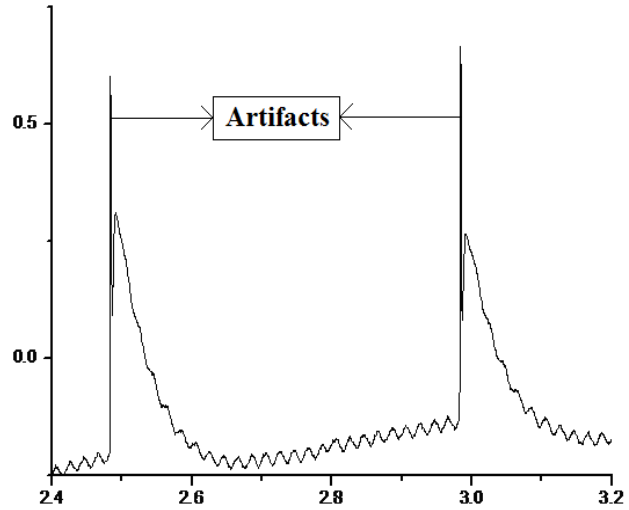

Figure 2.4: Zoom-in diagram of raw FIOPs showing the fourth and fifth peaks (with artifacts) of figure 2.2

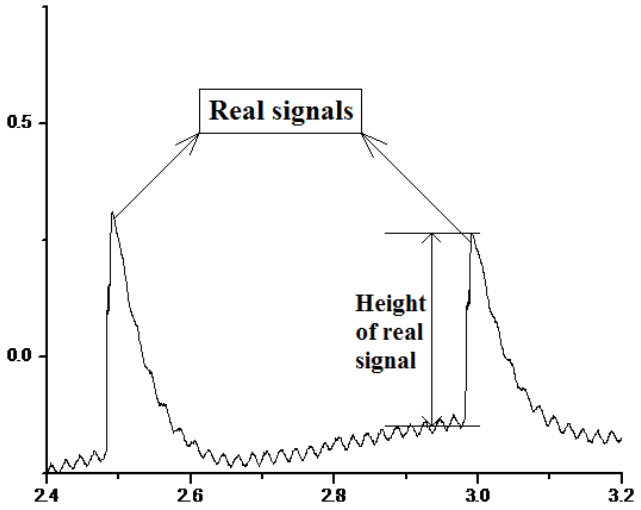

Figure 2.5: Zoom-in diagram of processed FIOPs at showing the fourth and fifth peaks of figure 2.3 after artifacts removal

There is the difference in the sign of peak in this project compared with the data of my previous project (Bachelor degree project) in last year. This means the peak's direction of all data in this project is positive while previously they were negative. This is due to the change in the electronics that collect the data (now with computer running a LabView program modified by Sergey Koroidov). The collected data were further processed as described above by Origin software (version 8). In this process also they were also labeled with important information (e.g. pH value, measurable temperature, polarization voltage, polarization time, sample, sediment time, etc). After finishing the process of artifact removal, the peak's amplitudes of oxygen flash yield were measured by ruler for each of the peak. Thus, the variation of peak's dimension does not affect so

much to the data analysis process and the unit of oxygen flash yield can be presented by millimeter (mm) or centimeter (cm). Relied on the collected data and the specific Excel sheets that were designed and programmed by my supervisor (Prof. Johannes Messinger), the parameters of misses, double hits, damping and percents of S_i states were calculated.

Two important parameters of the data analysis process that are usually mentioned in FIOPs experiments are "miss" and "double hit" probabilities. The miss parameter gives the percentage of the PSII protein complexes that are in a certain S_i state before and after flash excitation [15, 16]. The occurrence of miss probability is dependent on the redox equilibria on the donor ($Y_Z P680^{\bullet+} \leftrightarrow Y_Z^{OX} P680$) and the electron acceptor side ($Q_A^- Q_B \leftrightarrow Q_A Q_B$) of PSII complexes [19, 20, 21]. Based on the results of some biochemical investigations [15, 16, 20, 22], the miss parameter is considered to depend on the S_i state of the water oxidizing complexes (WOCs). Nevertheless, the accurate dependence of miss parameter on the S_i state of PSII complexes is very difficult to measure and could not be concluded from Joliot electrode measurements [12, 13]. Consequently, it is often assumed that the miss probability is independent on the S_i state of PSII protein complexes in the sample. In addition, with the target to simplify the calculation of miss parameter, it is usually assumed that the miss probabilities of all transformations from S_i to S_{i+1} have the same value. This means there is only one value of miss parameter for all of the advancements from S_i to S_{i+1} and this is called the "equal miss parameter" approach.

The double hit probability is identified by the percentage of PSII protein complexes that are advanced twice in a single flash. The double hit parameter depends on the rate of the $Q_A^{\bullet-}$ re-oxidation [16, 23] and the flash profile in case of Xenon discharge lamp [24, 12, 16]. The flashes of Xenon discharge lamp usually consists of a "tail" of residual intensity that extends over a time range of more than 100μs [14, 24, 25]. The double hits are absent if the illumination of sample is performed by laser flashes [15]. For the sample of spinach thylakoids, the typical values of miss and double hit probabilities with Xenon flashes are 6-12% and 3-5%, respectively. In addition, all of experiments in this project were performed with thylakoid membranes that were extracted from spinach. Moreover, with the aim to keep constantly (and change easily) the experimental temperature in measurable period of time, the thermostat was used for all of the experiments in this project.

2.2. Optimizations of the measuring conditions for FIOPs experiments

2.2.1. *Optimization of chlorophyll concentration*

Many factors can affect the results of the Joliot electrode measurements. Therefore, the first task was to establish the optimal conditions, so that these conditions can apply for the rest experiments. The tested conditions include the chlorophyll concentration ([Chl]), composition of buffer, pH, temperature, voltage of polarization, polarization time, sample treatment (preflash or non-preflash), the value of amplification and filter in data receiver, etc.

All of the experiments that were performed in this project were treated by giving one strong flash outside the Joliot electrode. For this original thylakoid sample that has been stored in the freezer at -80^{0}C was defrosted completely in an ice bath. After that, it was washed with buffer pH=7.0 and was centrifuged down at the spinning velocity 14000 rpm in 2 minutes. The supernatant was then removed, more buffer pH=7.0 was added with the calculated volume in order to dilute the original sample to desired chlorophyll concentration. The sample then re-suspended and transfer to a glass vial before giving one strong light-flash by a Xenon lamp. After giving a strong flash to glass vial, the sample was got out of Xenon lamp (Perkin Elmer optoelectronics flash lamp, product number LS-1130-4, serial number 1952). Subsequently, the sample was kept in the dark for at least 10 minutes at room temperature before performing the measurement.

The purpose of taking pre-flash procedure was to oxidize tyrosine D (Y_D^{OX} or $S_1Y_D^{OX}$) in most of the PSII center. Normally, the thylakoid samples that were kept in the dark for a long time contain a dominated amount of S_1 population (manganese cluster) and of reduced tyrosine D ($S_1Y_D^{RED}$) and a small amount of S_1 population with oxidized tyrosine D ($S_1Y_D^{OX}$). When the sample absorbs a strong flash, this makes the $S_1Y_D^{RED}$ advance to the next higher S state, i.e. $S_2Y_D^{RED}$ is formed. Then, $S_2Y_D^{RED}$ becomes $S_1Y_D^{OX}$ when the sample was put in the dark for 10 minutes at room temperature due to the transfer of one electron from Y_D^{RED} (as an electron donor) to the manganese cluster (reduce S_2 state to S_1 state). Moreover, because of the advancement of $S_1Y_D^{OX}$ by a strong flash, the next higher level state $S_2Y_D^{OX}$ was produced. Then, the storage of sample for 10 minutes at room temperature is long enough for the manganese cluster to accept one electron from the acceptor side Q_AQ_B of PSII and to return to $S_1Y_D^{OX}$. This process can be briefly summarized as follows

$$S_1Y_D^{RED} \xrightarrow{Flash} S_2Y_D^{RED} \xrightarrow[\text{Room temperature}]{\text{Dark, 10 minutes}} S_1Y_D^{OX}\ (Stable)$$

$$S_1Y_D^{OX} \xrightarrow{Flash} S_2Y_D^{OX} \xrightarrow[\text{Room temperature}]{\text{Dark, 10 minutes}} S_1Y_D^{OX}$$

As first step of the optimization procedure, the chlorophyll concentration was varied in order to find out the maximum chlorophyll concentration that can be light-saturated with the current Xenon discharge lamp. Based on some biochemical articles [5, 6, 7, 8, 9] that describe FIOPs measurements, the other conditions were selected such as Joliot buffer (20mM NaCl, 5mM $MgCl_2$, 50mM MES), pH=6.5, 20^{0}C, polarization voltage as -750mV in 40 seconds, give a strong flash at the outside of electrode (also called as $S_1Y_D^{OX}$).

Table 2.1: The calculated results of measurements between the spinach thylakoid samples that have the different chlorophyll concentration at 20^{0}C, -750mV in 40 seconds, sediment time (t_{sed}) = 3 minutes, $S_1Y_D^{OX}$, pH=6.5 (Joliot buffer), repeat 3 times for each of chlorophyll concentration

	miss (α)	double hit (β)	damping	S_1	S_{-1}
[Chl]=0.25 mg/ml	8.85 ± 0.33	1.27 ± 0.04	98.57 ± 0.21	93.60 ± 0.76	6.40 ± 0.76
[Chl]=0.5 mg/ml	8.89 ± 0.10	1.27 ± 0.01	98.51 ± 0.10	93.43 ± 0.60	6.57 ± 0.60
[Chl]=1 mg/ml	9.51 ± 0.60	1.24 ± 0.01	98.49 ± 0.10	93.03 ± 1.35	6.97 ± 1.35

FIOPs were measured under the above conditions at three different chlorophyll concentration and subsequently analyzed to extract the values of the following five parameters: miss, double hit, damping parameter, percent of S_1 and S_{-1}. The results are displayed in Table 2.1. In fact, we can recognize the significant differences of miss, S_1, S_{-1} values between [Chl] = 1mg/ml and two other concentration while the results obtained at [Chl] = 0.25mg/ml and [Chl] = 0.5mg/ml

are identical within the error margines. Thus, [Chl] = 1mg/ml is not a good choice for next experiments and the best chlorophyll concentration is 0.5 mg/ml, because [Chl] = 0.25mg/ml is too diluted and results in small signal intensities (large errors).

2.2.2. *Optimization of measuring temperature*

Beside chlorophyll concentration, the pH value and the temperature also play the crucial role in FIOPs measurements, because they affect the chemical kinetics of the oxygen evolution process and the stability of the PSII protein complexes. Relying on some scientific investigations [7, 8, 9, 15, 16] that were also performed with FIOPs methods, the experimental temperature that is suitable for spinach thylakoid sample is smaller than 20^0C. According to the literatures, the lowest value of miss probability was achieved at measuring temperatures of 5-10^0C. Moreover, our exploration at various temperatures and different pH values is not only to find out the best temperature for FIOPs experiments, but also to make extension of the Kok model [14]. The series of these experiments were performed by varying the measuring temperature and the buffers that are different with regard to pH values and buffer composition. In addition, the thylakoid sample was treated by giving a strong flash to enrich the amount of $S_1Y_D^{OX}$. The two kinds of buffer that were employed in the experiment consisted of

- Succinic acid buffer (pH=4.0, 4.5 and 5.0): 0.4M mannitol, 10mM $MgCl_2$, 20mM $CaCl_2$, 30mM succinic acid.
- MES buffer (pH=5.0, 5.5 and 6.0): 0.4M mannitol, 10mM $MgCl_2$, 20mM $CaCl_2$, 50mM MES.

Table 2.2: The values of miss parameter in the buffers at different pH and two different temperatures with the spinach thylakoid sample, [Chl]=0.5mg/ml, -750mV in 40 seconds, sediment time = 3 minutes, repeat 3 times for each of conditions, $S_1Y_D^{OX}$

	10^0C	25^0C
Succinic acid buffer (pH=4.0)	46.17 ± 3.72	38.06 ± 5.49
Succinic acid buffer (pH=4.5)	34.57 ± 1.19	33.21 ± 1.71
Succinic acid buffer (pH=5.0)	27.38 ± 2.62	25.09 ± 0.05
MES buffer (pH=5.0)	24.75 ± 2.92	20.40 ± 1.02
MES buffer (pH=5.5)	23.77 ± 0.03	17.38 ± 0.95
MES buffer (pH=6.0)	13.14 ± 0.25	10.59 ± 0.25

The data of these experiments were collected to calculate the values of miss parameter that were used to evaluate the effect of these conditions (temperature, pH values and buffer composition in this case) on PSII protein complexes. Based on the results of the experiments in above table (table 2.2), we can evaluate the differences of miss parameter between the different temperatures (in the same row) and different pH values (in the same column). In general, the miss parameters are found to be smaller at the higher temperature (25^0C). With the comparison of miss parameters column by column, the reducing trend of miss parameter is also achieved when pH value is so closer to neutral pH value (pH=7.0). In brief, the extreme conditions as low pH and low temperature is not good for the occurrence of oxygen evolution process and that is why the peak's amplitude of oxygen flash yield in this case is very short (even overlap with the be base line or noises). Vice versa, the appropriate temperatures (around 20^0C-25^0C) and neutral pH values (6.5-7.0) are the good conditions for the kinetics and yield of oxygen evolution process. Beside, there are the great differences of miss parameter on the experiments that were performed at the same pH value (pH=5.0 is this case), the same measuring temperature and different buffer composition (MES buffer and succinic acid buffer in this situation). It can be realized that the smaller values of miss probability were achieved by using MES buffer at the same measuring temperatures (both of 10^0C and of 25^0C). That is the reason why let us make the other doubt about the dependence of miss parameter, oxygen evolution activity on the buffer composition. Consequently, the

optimization of buffer composition was performed by varying the buffer components with the target to find out the best buffer composition for FIOPs measurements of thylakoid membrane samples (**see table 2.3**).

However, based on the comparison about the values of miss parameter between these results and literature, we can realize the similarities and great differences between them. The first similarity is the strong dependence of miss probability on pH values and the experimental temperature. Secondly, the best pH values for spinach thylakoid samples are around the pH range of neutral pH (6.8-7.0). Nevertheless, the smallest values of miss parameter (6%-7%) are achieved at the temperature range 5^0C-10^0C according to the literature, not in the range 20^0C-25^0C as the above results. Therefore, we suspect the collected data that were used for process of data analysis are not completely reliable due to some occurred problems in process of data collection. Indeed, it is very difficult to recognize and measure the real signals at the extreme conditions as low temperature and low pH value due to the overlap of real signals with base line and noises. On the other hand, the experiments at the low temperature had a problem about the big amplitude of noises. The reason of this problem can come from the condensation of water vapor in the air, which depends on the air humidity in these days, at the inside and outside of Joliot electrode. Therefore, the values of calculated miss parameter and the evaluation based on them are relatively correct, not perfectly reliable.

On the other hands, the optimizations of experimental conditions in Joliot electrode measurement (based on the values of miss parameter) and the dependence of miss, double hit probabilities on measurable conditions (e.g. pH values, temperature, etc) were explored by many previous biochemical investigations and literatures [6, 10, 23, 26, 27]. Correspondingly, although the next series of experiments were performed by the same approaches with these previous literatures, their results could be discussed with the different purposes as follow

- Could the values of miss parameter be stable at extreme pH values over long incubation time (t_{incub}) or are there slow protein defoldings or similar that change slowly the miss parameters ? It means that is the pH effect thermodynamic on water oxidation reaction or on protein level ?
- How does the total oxygen yield (addition of oxygen flashes yield 3-12) change with pH and incubation time ?

2.2.3. *Optimization of pH and buffer composition*

The next series of experiments were performed at a longer pH range (4.0-9.0) and buffer compositions. The continuous condition that was chosen to explore is buffer with the different pH value and dissimilar composition. A good buffer solution is very important to most of the biological experiments in order to keep stable for pH value, investigated target (thylakoid membrane in this case) inside the experimental system. In addition, the good buffer compound have to satisfy most of the conditions as midrange pKa, good solubility in water, minimum solubility in the other solvents, minimal salt effects, minimal change of pKa with variation of temperature, chemically stable, minimal absorption in UV-Vis range, easily synthesized, etc. The most important condition is this buffer compound does not have any bad effects to investigated target (e.g. buffer components can bind strongly to investigated target, resulting this target to become unstable or losing its biological activity, etc). The buffer compounds that can satisfy most of these strict conditions and appropriate with most of biological samples, biological systems are about ten compounds. Two of those buffer compounds that were usually used in biochemical laboratory are 2-(N-morpholino)ethanesulfonic acid (MES) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) with the buffer range as 4.5-6.5 and 6.5-8.5, respectively. Thus, two buffer compounds MES and HEPES were employed for buffer preparation of the experiments that buffer solutions were prepared at the different pH values and different buffer compositions. The list of buffers with pH values and buffer composition were presented as below:

Table 2.3: The total oxygen flash yields ΣY_{Oxygen} (addition of peak's amplitudes 3-12) and composition of the buffers that used in the measurements, 20^0C, [Chl]=0.5mg/ml, -750mV in 40 seconds, sediment time = 3 minutes, $S_1Y_D^{OX}$

Name of buffer	Components		pH	ΣY_{Oxygen} (mm - %)	
				(t_{incub} =5min)	(t_{incub} =60min)
Buffer 1	20mM NaCl 5mM $MgCl_2$	50mM MES	4.0	121 – 24.59%	118 - 23.98%
Buffer 2	20mM NaCl 5mM $MgCl_2$	50mM MES	5.0	205 – 41.67%	156 – 31.71%
Buffer 3	20mM NaCl 5mM $MgCl_2$	50mM MES	6.0	398 – 80.89%	330 - 67.07%
Buffer 4	20mM NaCl 5mM $MgCl_2$	50mM HEPES	7.0	492 – 100.0%	464 – 94.31%
Buffer 5	20mM NaCl 5mM $MgCl_2$	50mM HEPES	8.0	289 – 58.74%	326 – 66.26%
Buffer 6	20mM NaCl 5mM $MgCl_2$	50mM HEPES	9.0	36 – 7.32%	68 – 13.82%
Buffer 7	20mM NaCl 5mM $MgCl_2$	50mM MES 400mM mannitol	4.0	171 – 34.76%	180 – 36.59%
Buffer 8	20mM CaCl2 10mM MgCl2	400mM mannitol 30mM succinic acid	4.0	72 – 14.63%	58 – 11.79%

The targets of the experiments are to investigate the difference about the oxygen evolution activity when thylakoid sample was stored in the buffers that have different pH values in the pH range (4.0-9.0) and buffer compositions. Based on the peak's amplitudes of oxygen flash yields, the calculated values of miss parameter, we can evaluate how good of the oxygen evolution activity and how effective of buffer capacity with the dissimilar buffer solutions. In addition, the series of the experiments were performed by an approach that has many steps. The thylakoid sample is given a strong flash to enrich the amount of $S_1Y_D^{OX}$ in the buffer pH=7.0 because the $S_1Y_D^{OX}$ is very stable at neutral pH. After that, the samples were centrifuged to remove buffer pH=7.0 (supernatant) and exchanged with a new buffer at desired pH value. The pellet of thylakoid membrane was re-suspended in the new buffer and was measured at the different incubation time (5, 10, 20, 40 and 60 minutes). Relied on process of data analysis, the figures of miss parameters as a function of incubation time and pH value were present below.

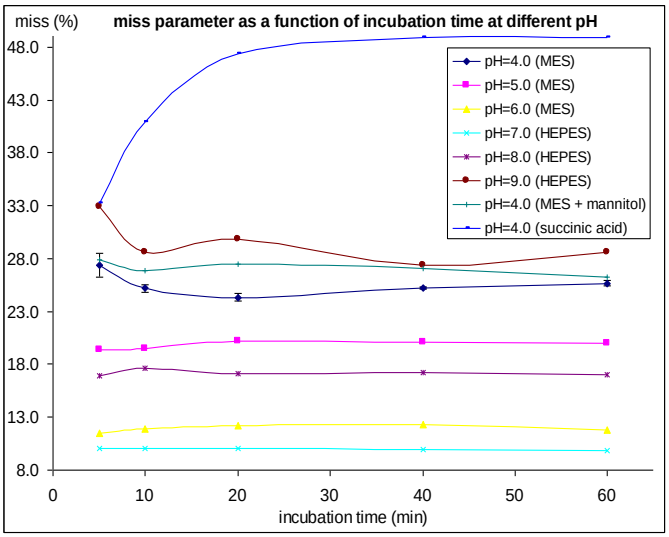

Figure 2.3: the miss parameter as a function of incubation time at different pH values, 20^0C, spinach thylakoid sample, [Chl]=0.5g/l, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$

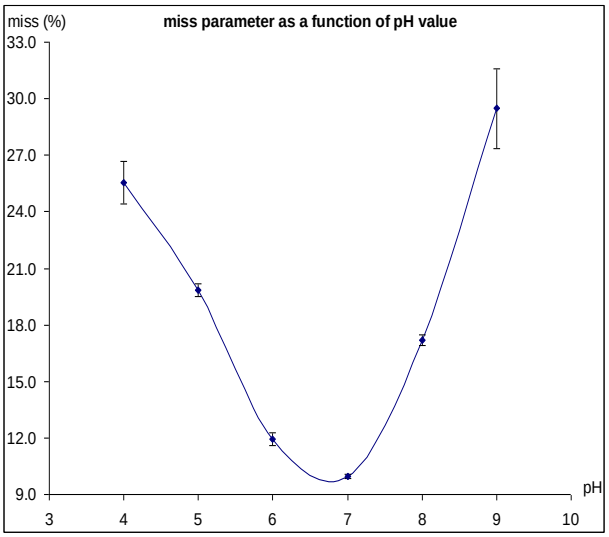

Figure 2.4: the miss parameter as a function of pH value at 20^0C, spinach thylakoid, repeat 5 times for each of pH values (at different incubation times, 5-60 minutes), [Chl]=0.5g/l, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$

Based on the plot of miss parameter as a function of incubation time at the different pH value (figure 2.3), it can be noticed that the miss parameters of almost all measurements are independent on the incubation time at a certain pH value. Although the basic buffer (pH=9.0) and acidic buffer (pH=4.0) are not good conditions for PSII protein complex, the oxygen evolution process leads to the production of smaller amount of oxygen (compare with neutral pH) at a constant miss parameter. Quantitatively, based on the values of total oxygen flash yields that were calculated by the addition of peak's amplitude 3-12 (see table 2.3), the amount of produced oxygen at low pH (pH=4.0) and high pH (pH=9.0) are 121mm (24.59%) and 36mm (7.32%), respectively. This means the efficiency of oxygen evolution process can achieve approximately 10%-25% compared with neutral pH values (pH=7.0; 492mm–100%) in case of spinach thylakoid sample at the extreme pH values. In addition, there are some differences about the total oxygen flash yields (see table 2.3) between basic buffers (pH=8.0 and 9.0) and acidic buffers (pH=4.0, 5.0 and 6.0) in a longer incubation time (60 minutes). When the incubation time was elongated to 60 minutes, the decrease of total oxygen flash yields that is approximately 5%-15% is indicated at the low pH values from pH=4.0 to pH=6.0. Vice versa, the slight increase (about 5%) of total oxygen flash yields can be considered at the high pH values (pH=8.0 and 9.0). It is so surprised that the total oxygen flash yields is still decreased slightly at neutral pH value (pH=7.0). This mean the oxygen evolution activity of PSII protein complexes can be slightly decreased in a long incubation time although the buffer at neutral pH value is the best condition for sample preservation. Nevertheless, this quantitative results of total oxygen flash yields that were employed to make discussions are only approximately correct since the Joliot electrode cannot be calibrated.

Besides, the difference of miss parameter and total oxygen flash yields can be realized clearly between the buffers that do not have the similar buffer compositions. With the comparison of miss parameters and total oxygen flash yields between buffer 1 (pH=4.0, MES) and buffer 8 (pH=4.0, succinic acid), the buffer system of succinic acid and succinate sodium could not be considered a good buffer for experiments of thylakoid sample due to the highest miss parameter (35%, especially at incubation time longer than 20 minutes – 48%) and the smallest amount of formed oxygen (72mm-14.63%). Moreover, the small differences of miss parameter and total oxygen flash yields between buffer 1 and buffer 7 can proclaim that presence of mannitol does not brings about any bad effects to oxygen evolution of PSII complex. In addition, the diagram of miss parameter as a function of pH value (figure 2.4) indicates the significant dependence of miss parameter on pH value and this conclusion is overlap with the view point of some scientific articles

[10, 11, 12, 13]. The neutral pH from 6.5 to 7.0 is the best condition for oxygen evolution process of thylakoid membrane sample and storage (incubation) in a long time due to the smallest miss parameter. Moreover, the stability of oxidized tyrosine D ($S_1Y_D^{OX}$) cannot be evaluated relatively relied on the oxygen evolution activity of PSII complex, total oxygen flash yields and miss parameter.

As above mentioned, the oxygen evolution process in PSII protein complexes is strongly suppressed at extreme pH values (pH=4.0 or 9.0). This may indicate that buffers at the extreme pH values just block temporarily the oxygen evolution center instead of destroying them. With the aim to check this aspect, another series of experiments were performed. The approach of these experiments consisted of the method of above experiments (investigations of buffer composition and pH value) and some additional steps. After re-suspending the pellet of spinach thylakoid membrane in the buffer at the desired pH, the suspension was divided into several aliquots, incubated for the various times. At a certain incubation time, an aliquot of sample was diluted by buffer pH=7.0 with volumetric ratio 1:20 to neutralize the sample so that the pH of new sample suspension is closed to 7.0. The new suspension was centrifuged again to remove both of the old buffer and buffer pH=7.0 at supernatant. The ample was added more buffer pH=7.0 with the desired volume to guarantee that [Chl] of new suspension must be equal to [Chl] of original sample (0.5 mg/ml).

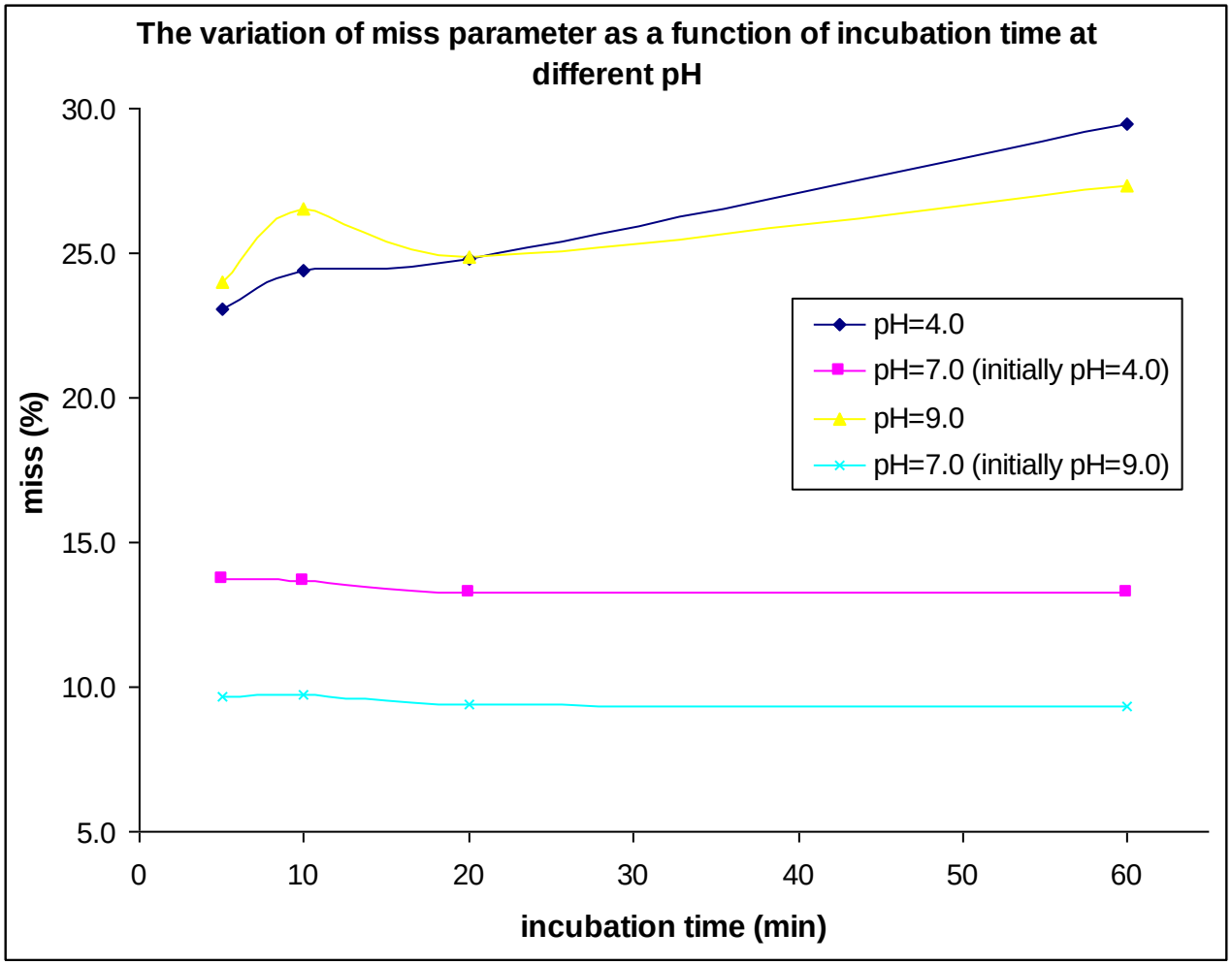

Figure 2.5: the miss parameter as a function of incubation time (min) at pH=4.0 and pH=9.0; after exchanging with neutral pH (pH=7.0), spinach thylakoid sample, 20^0C, [Chl]=0.5mg/ml, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$

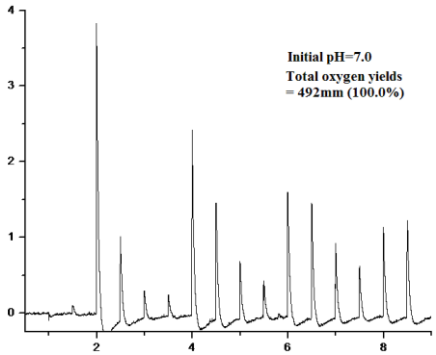

Figure 2.6: The data of spinach thylakoid sample at pH=7.0, 20^0C, [Chl]=0.5g/l, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$

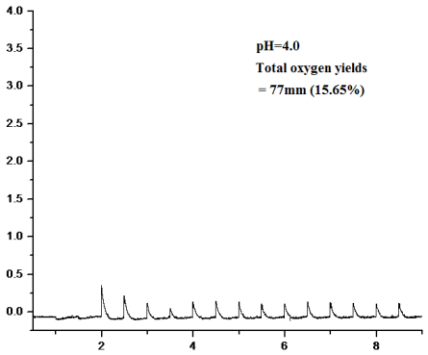

Figure 2.7: The data of spinach thylakoid after exchanging with buffer pH=4.0, 20^0C, [Chl]=0.5g/l, -750mV in 40sec, t_{sed} = 3min, t_{incub}=5min, $S_1Y_D^{OX}$

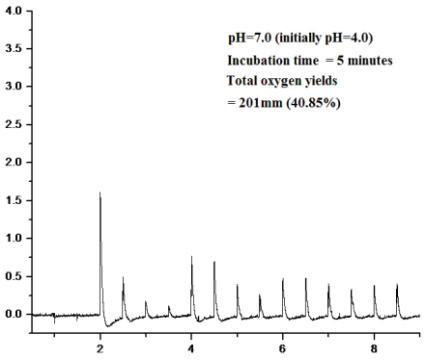

Figure 2.8: The data of the spinach thylakoid sample after returning to buffer pH=7.0, 20^0C, [Chl]=0.5g/l, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$

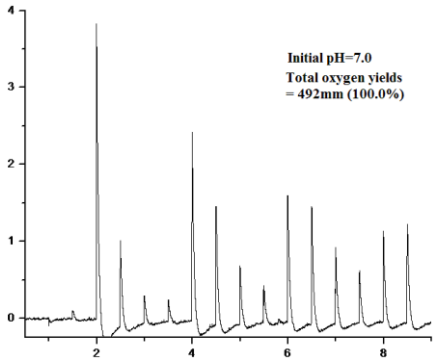

Figure 2.6: The data of spinach thylakoid sample at pH=7.0, 20^0C, [Chl]=0.5g/l, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$

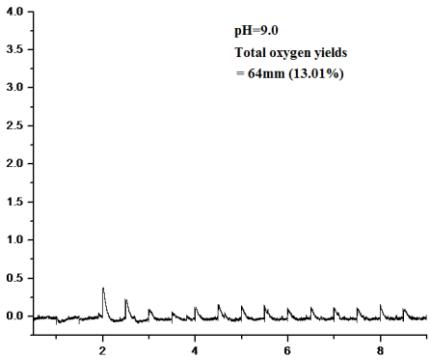

Figure 2.9: The data of spinach thylakoid after exchanging with buffer pH=9.0, 20^0C, [Chl]=0.5g/l, -750mV in 40sec, t_{sed} = 3min, t_{incub}=5min, $S_1Y_D^{OX}$

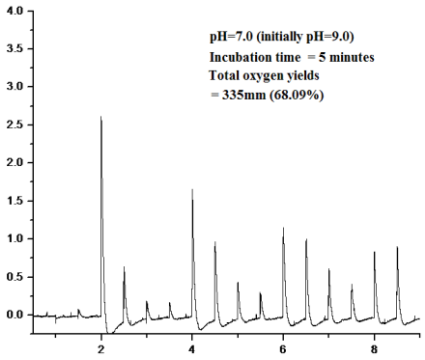

Figure 2.10: The data of spinach thylakoid sample after returning to buffer pH=7.0, 20^0C, [Chl]=0.5g/l, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$

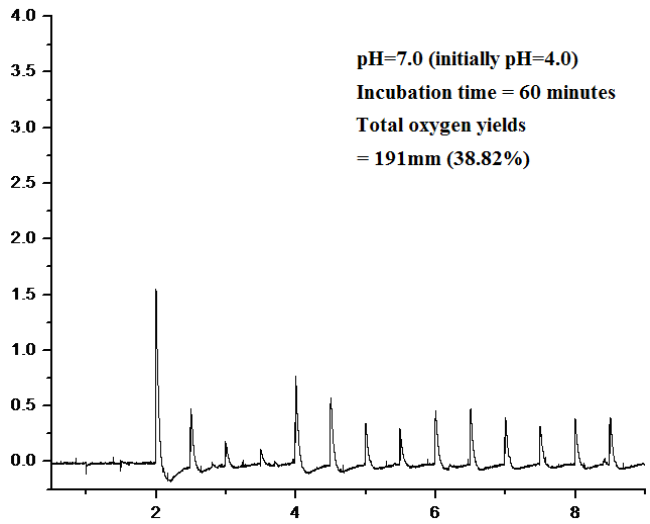

Figure 2.11: The data of the spinach thylakoid sample after returning to buffer pH=7.0, 20^0C, [Chl]=0.5g/l, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$, t_{incub}=60min

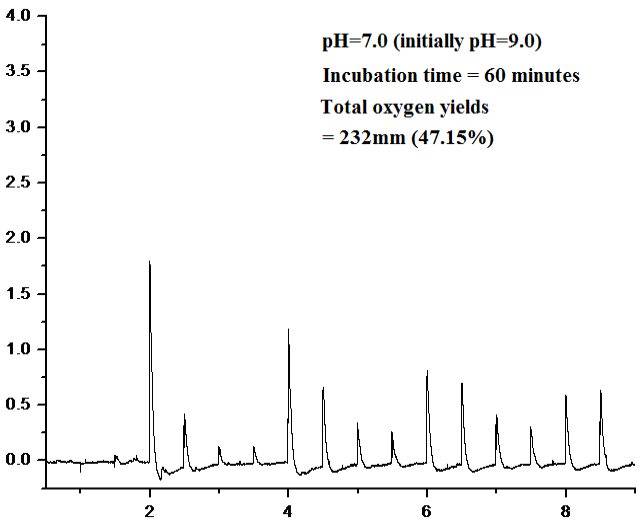

Figure 2.12: The data of spinach thylakoid sample after returning to buffer pH=7.0, 20^0C, [Chl]=0.5g/l, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$, t_{incub}=60min

The difficult occurrence of oxygen evolution process at the extreme pH values (pH=4.0 or 9.0) brought out the big values of miss parameters. The miss parameters at acidic and basic pH values that are very similar compared to results of the previous experiments (figure 2.3 and 2.4)

approximately 25% and 28%, respectively. In addition, the miss parameter slightly increases with incubation time. After the samples were neutralized with buffer pH=7.0, the miss parameter of the samples that were in buffer pH=4.0 and buffer pH=9.0 decreased from 25% to 14% and from 28% to 10%, respectively. Firstly, we can expose an affirmation that buffers at the extreme pH values just block provisionally the PSII protein complex and inactivate temporarily the oxygen evolution process. That is why the oxygen evolution process can be occurred ordinarily when the sample was neutralized by buffer at neutral pH value (pH=7.0).

Nevertheless, we can be realized the significant decrease of peak's amplitude when the peak's amplitude of oxygen flash yield was compared between the sample in buffer pH=7.0 and the sample that is exchanged from extreme pH values to neutral pH. The reduction of peak's amplitude is approximately 35%-60%, depending on the pH values and incubation time. Based on the calculated results of total oxygen flash yields, we can evaluate quantitatively the percentage of PSII protein complexes that were destroyed or lost the oxygen evolution activity. With the comparison of total oxygen flash yields between three figures 2.6, 2.8 and 2.10, the loss of oxygen evolution activity is about 35% at pH=9.0, 60% at pH=4.0 in 5 minutes of incubation. In addition, the total oxygen flash yields continuously decreased to from 201mm-40.85% 191mm-38.82% at pH=4.0 and from 335mm-68.09% to 232mm-47.15% at pH=9.0 in 60 minutes of incubation. This means the loss of oxygen evolution activity or the destruction of PSII protein complexes occurred much more rapidly, violently in the buffer pH=4.0 compared with buffer pH=9.0 at the same incubation time. Moreover, the destruction occurred more slowly at buffer pH=4.0 compared with buffer pH=9.0 when the incubation time was elongated from 5 to 60 minutes.

Although we cannot determine accurately the percentage of the PSII protein complexes that were destroyed, because Joliot electrode cannot be calibrated, the affirmation of the dead of PSII complexes in buffers at extreme pH values is quantitatively correct due to the dramatic decrease of peak's amplitude. This mean the PSII protein complexes were destroyed partially at the extreme pH values (both of pH=4.0 and 9.0), resulting in the loss of oxygen evolution activity and the "dead" of PSII complexes. Actually, the buffer at high or low pH values are the bad condition for the existence of PSII complexes (and thylakoid membrane also). The dead of PSII complexes at the extreme pH values can be explained by many reasons as the structural changes of electron acceptor sides, the release of manganese cluster out of the reaction center, the degradation of PSII complexes to smaller protein subunits, etc. Besides, the miss parameter between two samples in buffers pH=4.0 and 9.0 that were exchanged with buffer pH=7.0 are 14% and 10%, respectively. The difference of miss parameter between these two samples may be due to the partial reduction of Y_D^{OX} (the conversion of Y_D^{OX} to become Y_D^{RED}).

The difference of miss parameter at the extreme pH values (pH=4.0 or 9.0) reveals that amount of $S_1Y_D^{OX}$ was decreased by accepting one electron from any electron donors (maybe water) to become $S_1Y_D^{RED}$. That is reason why the amount of $S_1Y_D^{RED}$ is increased and brought about the increase of miss parameter. Actually, the radical (Y_D^{OX} in this case) that has very high thermodynamic energy cannot be possibly stable in the normal environments unless it is protected around by a special environment (hydrophobic protein barrel in this case). Therefore, we doubted that the hydrophobic protein barrel around the location of Y_D^{OX} was structurally changed, Y_D^{OX} can get exposure more easily with the external media and received one electron become Y_D^{RED}. However, these are our explanations and discussions based on the difference of miss parameter at the extreme pH values (pH=4.0 or 9.0). With the aim to check for these explanation and discussions, the experiments of S_2 life-time were performed to evaluate the amount of $S_1Y_D^{OX}$ and $S_1Y_D^{RED}$ in these samples based on the percentage of fast phase and slow phase **(see the result at part 2.4, page 25)**.

2.3. Polarization time and catalase effects

2.3.1. *Optimization of catalase concentration*

Based on some biochemical explorations [15, 16] about the reduction of S_i state of the WOCs by artificial reductants (e.g. NH_2OH, NH_2NH_2, H_2O_2), we suspect that polarization between cathode and anode of Joliot electrode has some effect to S_i state of manganese cluster in PSII complexes. As we know, the polarization of cathode and anode by a direct current will make the Pt-cathode to become negative charged due to the accumulation of electron at the surface of Pt-cathode. Thus, the reaction on Pt surface (*) between dissolved oxygen (from sample or flow buffer) and water molecules can produce a significant amount of hydrogen peroxide (H_2O_2) that can reduce the S_1 state to the S_{-1} state (or S_2 state to S_0 state). However, the formation of H_2O_2 and the effect of H_2O_2 as an electron donor for the reduction of S_1 state to S_{-1} state, S_2 state to S_0 state are our hypothesis (assumptions). The hypothesis is supported by evidence presented belows.

Pt-cathode: $O_2 + 2H_2O + 2e^- \rightarrow H_2O_2 + 2OH^- (*)$

The reductions of $S_i + 2e^- \xrightarrow{H_2O_2} S_{i-2}$:

$S_1 + 2e^- \xrightarrow{H_2O_2} S_{-1}$ and $S_2 + 2e^- \xrightarrow{H_2O_2} S_0$

With the purpose to find the evidences about the formation of H_2O_2 on the surface of Pt-cathode, the next experiments were performed to deal with our doubts. In fact, these experiments that were performed by varying the polarization time showed the positive results with our predictions. The percent of S_{-1} population increased from 6.5% to 14.5% when the polarization time were increased from 40 seconds to 20 minutes. After that, with the target to prevent the reduction effect of H_2O_2, the certain amounts of catalase suspension (included 30% of glycerol, 10% of ethanol) were added into thylakoid sample to remove H_2O_2 on the surface of Pt-cathode. Nevertheless, the calculated percent of S_{-1} population almost changes randomly when the concentration of catalase was increased from 10.000U/ml to 50.000U/ml. The reason of random variation of S_{-1} population can come from the large amount of glycerol and ethanol (30% and 10%, respectively) in catalase suspension. In addition, the best catalase concentration that was found out in this raw investigation is 10.000U/ml due to its smallest change of S_{-1} population.

Based on some first results of this raw investigation, the other experiments were performed with the frozen-dried catalase powder (Product of Sigma-Aldrich, CAS number 9001-05-2, product number C9322-5G) instead of catalase suspension to avoid the influences of glycerol and ethanol at catalase concentration 10.000U/ml. The new experiments were performed by varying the polarization time at -750mV in the samples that have or do not have the addition of catalase (10.000U/ml). Then, the data that were collected after these experiments were analyzed to calculate the S_{-1} population. The below figure (figure 3.1) that was plotted from the results of these experiments is the variation of S_{-1} population as a function of polarization time in the samples with or without the addition of catalase.

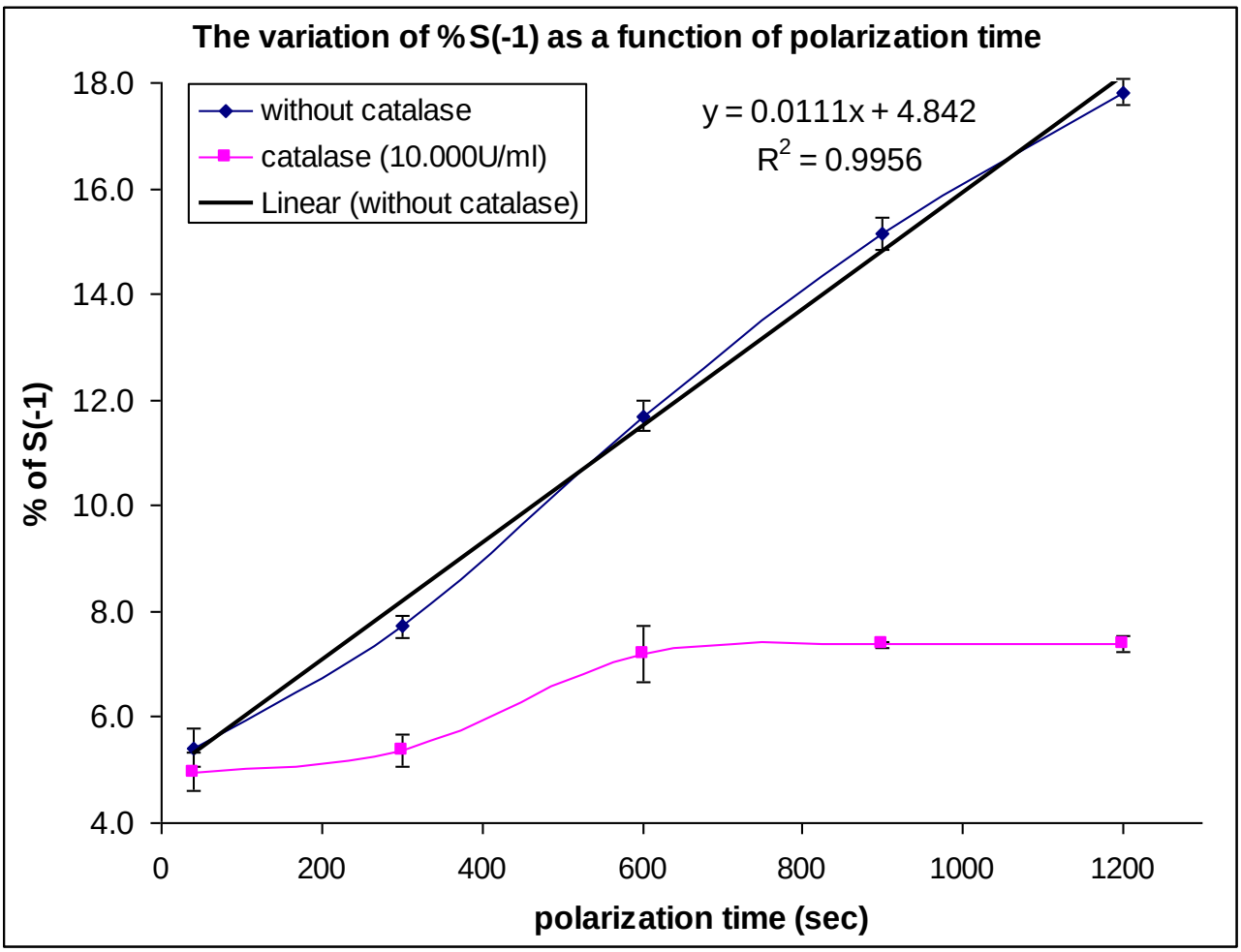

Figure 3.1: the variation of percent of S_{-1} population as a function of polarization time (sec) in the spinach thylakoid samples without or with frozen-dried catalase (10.000U/ml) at 20^0C, pH=6.5, [Chl]=0.5g/l, -750mV in 40sec, t_{sed} = 3min, repeat 3 times for each polarization times, $S_1Y_D^{OX}$

The linear increase of the percentage of S_{-1} population from 5.5% (40sec) to 18% (1200sec) as a function of polarization time (sec) was observed to probe our hypothesis about the formation of hydrogen peroxide on the surface of Pt-cathode that is reducing S_1 to S_{-1}. The catalase solution with concentration 10.000U/ml was added in the second experiments. In presence of catalase, a much smaller increase in S_{-1} population is seen during the same time periods (from 5% to 7%). This strongly supports the idea of hydrogen peroxide being the two-electron reductant. However, there is the second other idea that was released with the purpose to explain for the increase of S_{-1} population. This idea considers when the polarization time is long enough to sediment most of the thylakoid membrane on the cathode's surface, the PSII protein complexes could be reduced directly on the surface of Pt-cathode and there is not the formation of H_2O_2 in the sample. Moreover, the addition of catalase into the thylakoid sample just has the effect to stabilize the PSII protein complexes and prevent the destruction of PSII complexes by proteases from plant cells. Hence, the presence of catalase does not play an expected role as a special protein to remove H_2O_2, just has a good influence in protein crowding effects. Due to these two opposite ideas, the control experiments that were added the same weight of protein BSA (Bovine serum albumin) are required as a final control to exclude the protein crowding effects.

Based on the linear equation y=0.0111x+4.842 of thylakoid sample without addition of catalase, when x value (polarization time) is zero, y value obviously is equal 4.8. This mean the original sample usually contains a small amount of S_{-1} state about 4.5%. Due to the slight increase of S_{-1} population of the sample with addition of catalase, we had made the decision to perform the new series of experiments with the aim to optimize the catalase concentration for spinach thylakoid samples. The new experiments were performed by increasing and decreasing the catalase concentration in the range from 2.500U/ml to 20.000U/ml. The below figure was plotted by the

calculated values of S_{-1} population as a function of polarization time from the series of experiments that catalase concentration was varied by increasing and decreasing.

The small increase of percent of S_{-1} population at catalase concentration 10.000U/ml was actually suspected that the catalase concentration is not strong enough to remove all of formed H_2O_2 compound. Hence, the higher catalase concentration (20.000U/ml in this case) was expected that it could remove completely all of formed H_2O_2 compound and make the curve of S_{-1} population as a function of polarization time to become a straight line. However, the real results of this experiments is not similar to our expectation even worse than the previous one (10.000U/ml). In addition, it is very difficult to give a reasonable explanation for the random changes (come up and down) of the curve of catalase concentration 20.000U/ml (see figure 3.2). The random variation of S_{-1} population in the case of catalase concentration 20.000U/ml is approximately 2%, not too much. Anyways, the concentrated amount of catalase in thylakoid sample is not a perfect condition to eliminate all amount of H_2O_2 compound even having some bad influences for PSII protein complexes.

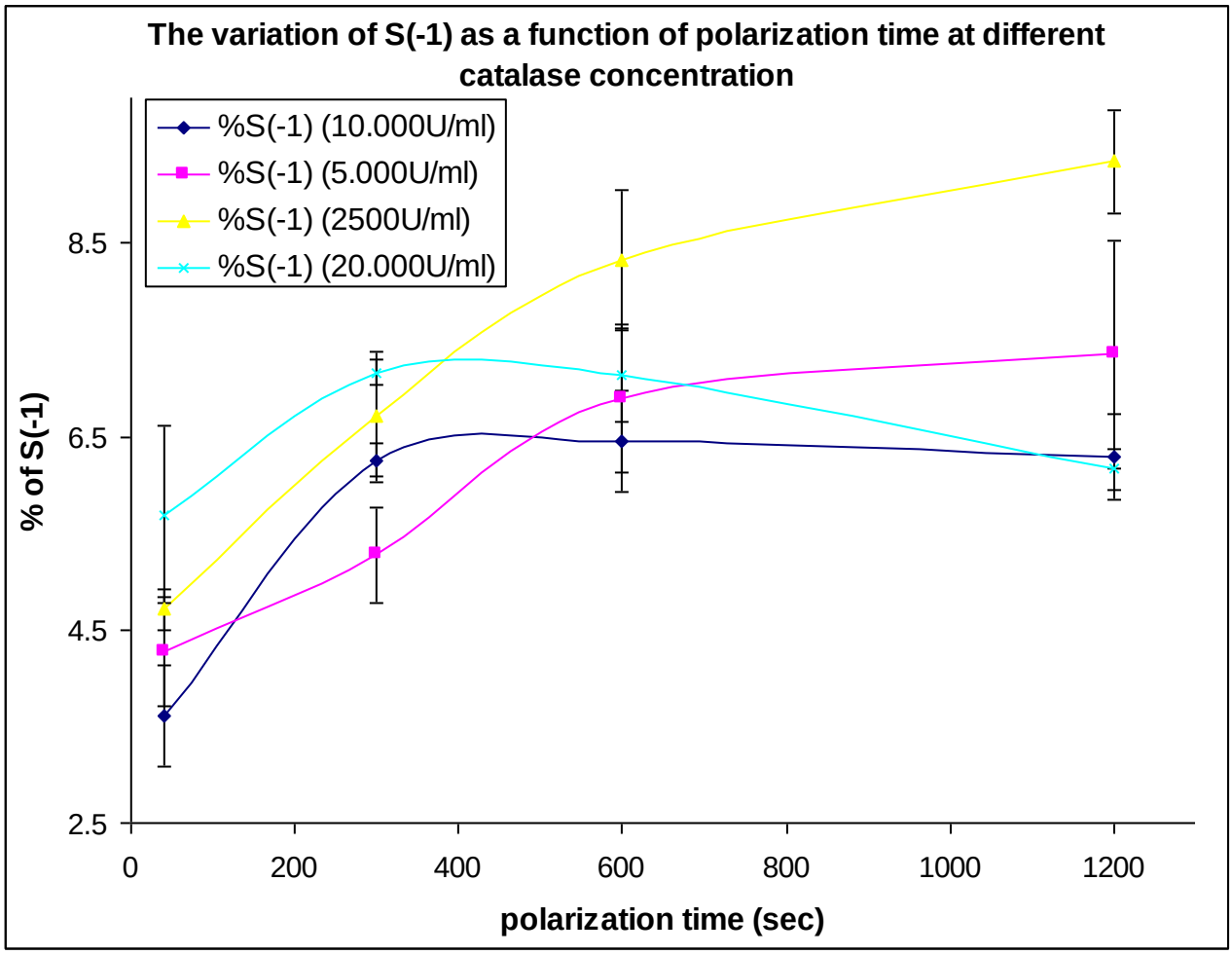

Figure 3.2: The optimization of catalase concentration by varying the catalase concentration in the range from 2.500U/ml to 20.000U/ml at 20^0C, pH=6.5, [Chl]=0.5g/l, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$, repeat 3 times for each polarization times, spinach thylakoid sample.

Consequently, the other explorations that were performed by following the reverse method may be a good solution in this case. It means the experiments with more diluted concentration of catalase could be much better than the concentrated ones. That is why the new experiments were performed by employing a smaller volume of stock solution of catalase with the purpose to achieve the smaller concentration of catalase. The catalase concentrations in the experiments were in a long range from 10.000U/ml to 2.500U/ml. The results of this experiments were collected and plotted on the above figure (figure 3.2). Based on this figure, the lowest catalase concentration 2.500U/ml is not strong enough to capture all produced H_2O_2 compound from the Pt-cathode surface. That is

why the curve of catalase concentration 2.500U/ml that was plotted by the variation of percent of S_{-1} population as a function of polarization still keeps going up. Beside this, the curve of catalase concentration 5.000U/ml is not the positive result compared with the curve of catalase concentration 10.000U/ml. Moreover, the relative similarity between the curves of catalase concentration 5.000U/ml and 10.000U/ml although the curve of catalase concentration 5.000U/ml still has the increasing tendency instead of the stable trend at the polarization time from 5 to 20 minutes of the curve of catalase concentration of 10.000U/ml. Therefore, based on all of the results and discussions in the experiments with the target to find out the optimum catalase concentratioin, the best catalase concentration is finally 10.000U/ml and this catalase concentration was employed for the next experiments.

2.3.2. *Polarization at different voltages*

After finishing the optimization of catalase concentration, the continuous series of experiments were performed with different polarization voltages with the aim to investigate the effect of different voltage on the formation of H_2O_2. The Pt-cathode surface that is polarized at the high voltage obviously becomes more negatively charged. This means the electron density on the Pt-cathode surface were increased at the high voltage. Hence, the production of H_2O_2 can be occurred more easily and effectively, bring about the significant increase of amount of H_2O_2 and the dramatic rise of percent of S_{-1} population. However, this is just our thought about the effect of polarization voltage and every prediction, assumption usually have to be verified by experiments. The series of these experiments were performed by the variation of polarization time at the different voltages (from -600mV to -750mV) in the samples with or without the addition of catalase 10.000U/ml. The data were collected and analyzed to calculate the values of miss parameter and percent of S_{-1} population. All of the calculated results were summarized and plotted at the below figure.

Table 3.1: The values of miss parameter and total oxygen flash yields ΣY_{Oxygen} (addition of peak's amplitudes 3-12) that used in the measurements at different polarization voltage, [Chl]=0.5mg/ml, polarization time = 40 seconds, sediment time = 3 minutes, $S_1Y_D^{OX}$, spinach thylakoid samples

Voltage (mV)	Catalase (U/ml)	Miss parameter (%)	ΣY_{Oxygen} (mm - %)
600	0	9.42 ± 0.33	202
650	0	9.65 ± 0.28	277
700	0	9.91 ± 0.33	328
750	0	10.32 ± 0.45	395
600	10.000	9.90 ± 0.33	205
650	10.000	10.15 ± 0.23	285
700	10.000	10.64 ± 0.43	293
750	10.000	10.86 ± 0.76	401

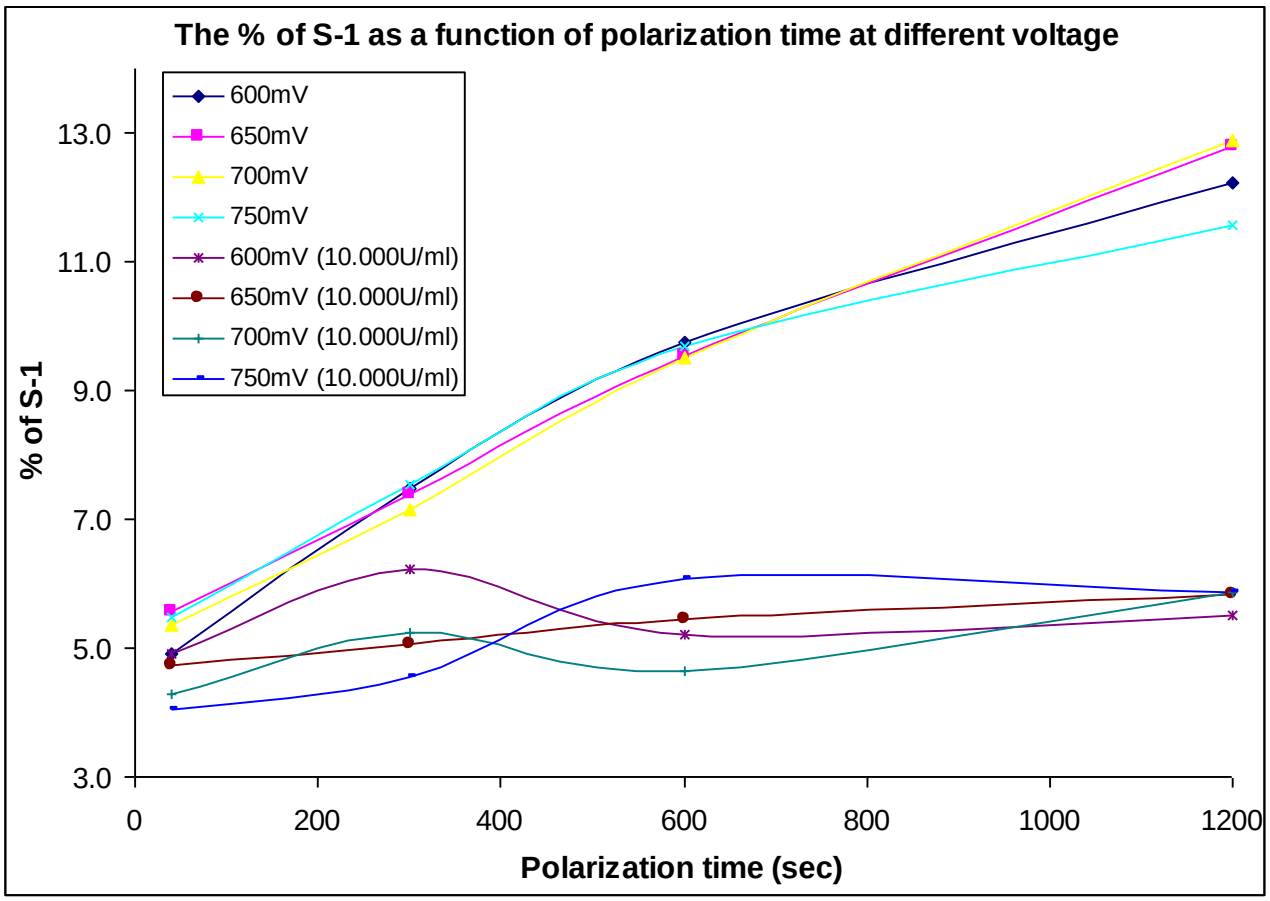

Figure 3.3: Percent of S_{-1} population as a function of polarization time (sec) at the different polarized voltages at 20^0C, pH=6.5, [Chl]=0.5g/l, t_{sed} = 3min, $S_1Y_D^{OX}$, spinach thylakoid sample.

It is more easily for result's discussion when these results are divided to two separated groups as catalase group and non-catalase group. In general, there are not the great dissimilarities about the shape of curves between the samples that were polarized at the different voltage in the same group. It can be easy to observe clearly this phenomenon with these four curves of non-catalase group. Although there are usually the random changes (as wave's shape) of the percent of S_{-1} population in each of the curves of the catalase group, the shape of each curve is relatively similar to a straight line. Totally, we can recognize the large differences of S_{-1} population between two curves belong to two different group at the resemble value of polarization voltage. Moreover, the difference between two samples (with and without addition of catalase) at -750mV is similar to the differences between two curves in the figure 3.1. However, the percent of S_{-1} population at a certain polarization time that belong to two different days is not perfectly in agreement, because two samples of two different days have the dissimilarities about the S_i state (and maybe some other factors) in the original samples. Quantitatively, the percent of S_{-1} population reaches approximately 13% at 20 minutes of polarization time in this case compared with 18% in figure 3.1. Moreover, these four curves of non-catalase group are not completely linear compared with the linear curve (without the addition of catalase) in figure 3.1.

In addition, the increase of polarization voltage also gives rise to the rise of miss parameter (9.4% to 10.3%) in the case of samples without the addition of catalse. Moreover, the values of miss parameter increased from 9.9% to 10.9% in the situation of samples that were added the catalase solution. That is why some investigations [15, 16] that the researchers also performed their experiments with FIOPs approach used the small voltage (-650mV) instead of higher voltage (-750mV). This means the miss parameter does not only depends strongly on the experimental temperature, pH value of buffer, but also slightly on the polarization voltage.

Beside this, because the peak's amplitude of real signals are varied at the different polarization voltages, this results the different values of total oxygen flash yields when the polarization voltage was changed from -600mV to -750mV. Quantitatively, the total oxygen flash

yield is approximately 200mm at -600mV and 400mm at -750mV. These values of total oxygen flash yields do not change significantly in two situations of samples with and without the addition of catalase. Nevertheless, the situation of -700mV had the great difference of total oxygen flash yields in the samples that have or do not have the addition of catalase due to some systematic errors.

2.4. S_2 lifetime decay

As above mentioned (section 2.2), with the purpose to investigate the effect of oxidized tyrosine D (Y_D^{OX}) to the miss parameter of sample that was exchanged from extreme pH value (buffer pH=4.0 in this case) to neutral pH, the S_2 lifetime measurement of this sample were performed. Beside this experiment, one more experiment in the buffer pH=7.0 was also performed to make a comparison between these two experiments. The result of S_2 lifetime measurement had a function as a reference to compare with the result of sample pH=4.0. The S_2 lifetime measurements were performed by the variation of dark time between one pre-flash and the main flash sequence. The data of the experiments were analyzed in order to determine the percentage of S_i populations (S_2, S_1, S_0 and S_{-1}) at the constants of miss, double hit and damping parameters. Three parameters were calculated from the data of first experiment that is corresponding with dark time 0.5 (sec). In addition, the kinetics for the primary photochemical reaction from S_2 to S_1 is usually mentioned by the addition of two smaller processes as fast phase and slow phase. Each of phases was represented as a first order reaction, thus, S_2 lifetime decay is totally considered as the addition of two first order reactions. The following are some introductions about the first order reaction.

The integrated first order-rate law (for first order reaction):

$$A = A_0 * e^{-kt} \Leftrightarrow \ln[A] = -kt + \ln[A_0] (eq.\ 1)$$

Note: A_0 = initial concentration of reagent (t=0)
k = the first order rate constant
t = time
A = the remaining concentration or percent of reagent at a fixed time (t)

The half-life of a first-order reaction: $t_{1/2} = \frac{\ln 2}{k} (eq.\ 2)$

The S_2 lifetime decay could be represented with a kinetic equation, which consists of two first order reactions looks like the below equation:

$$A = A_{01} * e^{-k_1 t} + A_{02} * e^{-k_2 t} \text{ or } S_2 = S_{2.1(t=0)} * e^{-k_1 t} + S_{2.2(t=0)} * e^{-k_2 t}$$

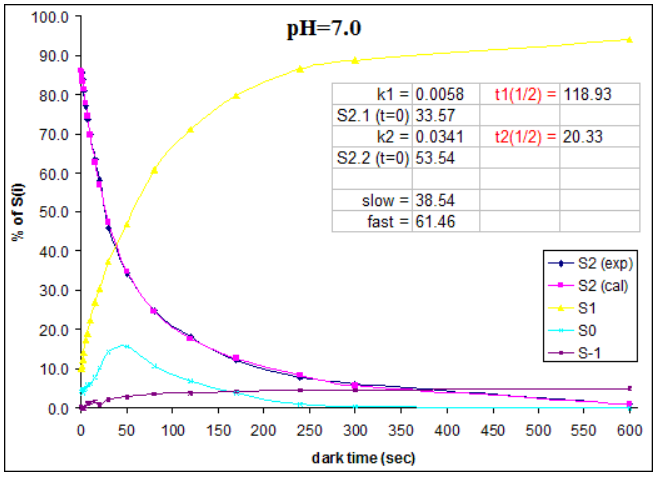

Figure 4.1: the S_i population as a function of dark time (sec) at pH=7.0, 20^0C, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$, spinach thylakoid sample, without catalase, [Chl]=0.5g/l

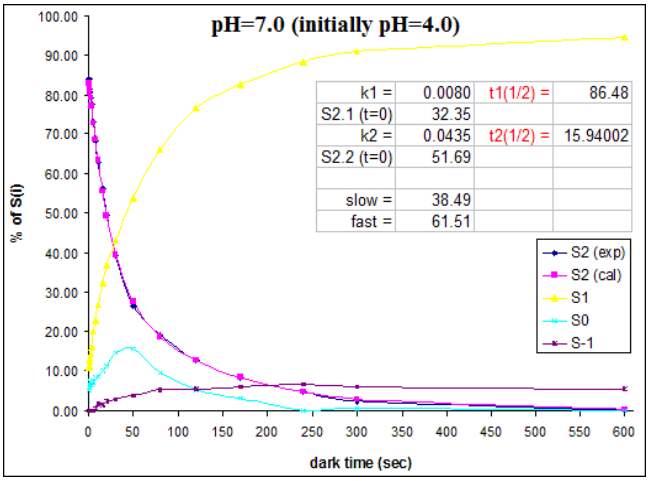

Figure 4.2: the S_i population as a function of dark time (sec) at pH=7.0 that was exchanged with buffer pH=4.0, 20^0C, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$, spinach thylakoid sample, without catalase, [Chl]=0.5g/l

Two of these S_2 lifetime measurements were performed by employing a constant polarization time of 40 seconds to minimize the reduction of S_i to S_{i-2} (S_2 to S_0 and S_1 to S_{-1} in this case) by H_2O_2 compound. Nevertheless, it could be seen that the conversions of S_2 to S_0 and S_1 to S_{-1} were normally occurred in these experiments. The conversion of S_2 to S_0 can be observed clearly at dark times around 50 seconds based on the high percent of S_0 population at these times.

This phenomenon can be explained by the small dissimilarity between dark time (50 sec) and polarization time (40 sec). At the beginning time of polarization process, the H_2O_2 compound is produced so fast and significantly. Hence, if the advancement of S_1 to S_2 is performed at this time by giving a strong flash to thylakoid sample, the S_2 state that has the higher activity compared with S_1 state were reduced remarkably to S_0 by the presence of H_2O_2 compound. In addition, this is the reason why the percent of S_1 population was considerably decreased due to the loss of S_2 population for conversion S_2 to S_0. Nevertheless, the increase of S_0 population disappeared significantly at the longer dark time. This means the percent of S_0 population decreased considerably when the dark time is longer than 50 seconds. When the thylakoid sample was given a strong flash from Xenon discharge lamp, the advancement of S_1 state is occurred violently in order to convert most of the S_1 state to become the next higher level as S_2 state. If the dark time is long enough for the manganese cluster to accept one electron from the electron acceptor side, the reverse conversion is occurred to return to S_2 state to S_1 state. On the other hand, the amount of S_2 population becomes so small at the long dark time. Due to the remarkable decrease of S_2 population at the longer dark time, this brought about the inconsiderable occurrence of conversion of S_2 to S_0. That is the reason why the percent of S_0 population decrease from 15% (at dark time = 50 second) to 0% (at dark time = 10 minutes).

Moreover, the conversion of S_1 to S_{-1} can be clearly noticed when the percent of S_1 population is significantly increased by complete conversion of S_2 to S_0 at long dark time. Therefore, the percent of S_{-1} population increased from 0% to 6% when the dark time was elongated from 0.5 second to 10 minutes. In addition, the calculated results of half-life time were different between these two options. Both of half-life time of fast phase and slow phase are remarkably dissimilar between sample in buffer pH=7.0 (figure 2.10) and the other one (figure 2.11). The most important thing in the calculated results is the large value of fast phase in both of two options. The thylakoid sample was illuminated with one strong flash before measurement with the target to enrich the amount of $S_1Y_D^{OX}$. It is very unreasonable when the fast phase can get a big percent as the calculated results (approximately 60% instead of smaller than 10%). Due to all of the strange things in these experiments, the new series of other experiments were performed to find out the reason of these problems. The new experiments consist of three options as follows

- Option 1: the experiments are performed by the variation of polarization time and without the addition of catalase.
- Option 2: the experiments are performed by keeping constantly the polarization time and without the addition of catalase.
- Option 3: the experiments are performed by keeping constantly the polarization time and with the addition of catalase 10.000U/ml.

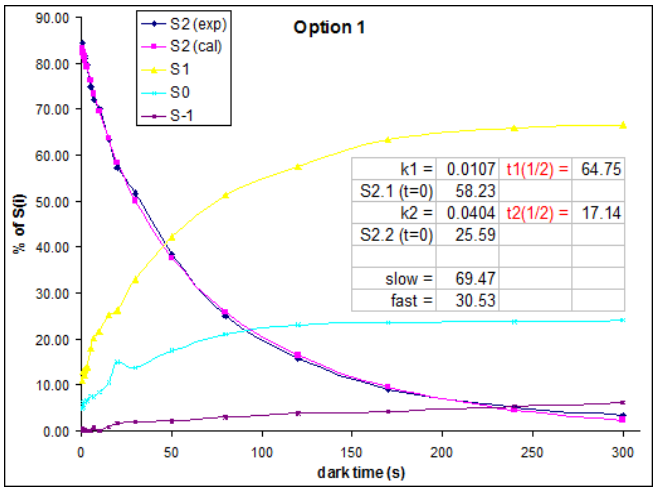

Figure 4.3: the S_i population as a function of dark time (sec) with the variation of polarization time, pH=7.0, 20^0C, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$, spinach thylakoid sample, without catalase, [Chl]=0.5g/l

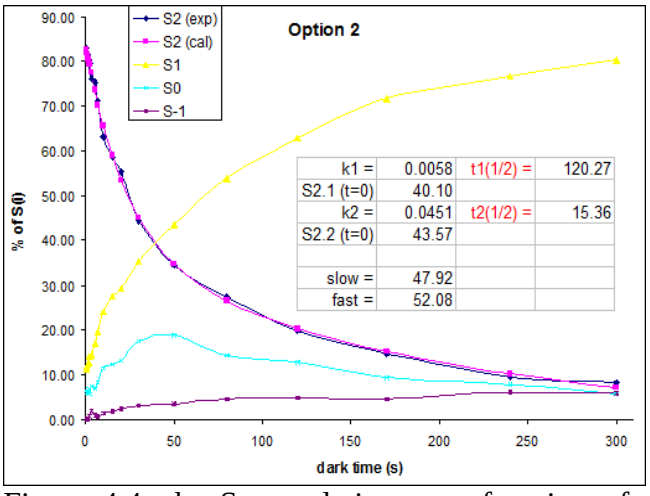

Figure 4.4: the S_i population as a function of dark time (sec) with the constant of polarization time, pH=7.0, 20^0C, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$, spinach thylakoid sample, without catalase, [Chl]=0.5g/l

Firstly, the comparison between these two options 1 and 2 can reveal the significant differences about many factors. The conversion of S_2 to S_1 in the option 1 occurred incompletely due to the competition with the conversion from S_2 to S_0 possibly by H_2O_2 compound. That is why the percent of S_1 population in option 1 just comes up to approximately 65% instead of 80% of option 2. In addition, the percent of S_0 population in option 1 steadily increases from 5% to 25% with the elongation of the dark time. Beside the rise of S_0 population, the percent of S_{-1} population was increased from 0% to 6% in both of these two options. The increase of percent of S_0 population that still has the maximum value at dark time around 40 seconds is so resemble to the previous experiment (figure 2.10 and 2.11). It is so interesting about the half-life time of fast phase and slow phase between these options. There are the small difference about half-life of fast phase and the great dissimilarity about half-life of slow phase. The most essential thing is the percent of fast phase and slow phase; it is so difficult to elucidate the large percent of fast phase in both of two options while the samples were illuminated a strong flash to increase the amount of $S_1Y_D^{OX}$ before performing measurements. The occurred problems let us make assumption that the minor conversions of S_2 to S_0 and S_1 to S_{-1} can possibly affect to our calculation and give rise to the big errors in all of calculated results (percents of S_2, S_1). Consequently, the results of option 3 that the sample of this option was added the catalase solution with catalase concentration 10.000U/ml are expected to be more positive. This means the minor conversions of S_2 to S_0 and S_1 to S_{-1} can hopefully removed or prevented with the addition of catalase.

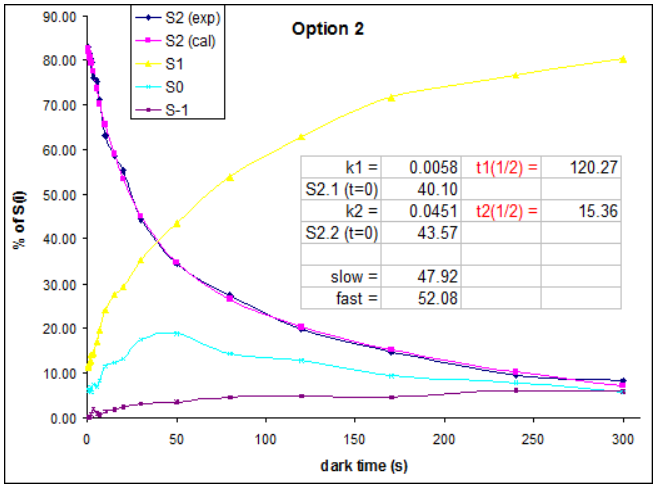

Figure 4.4: the S_i population as a function of dark time (sec) with the constant of polarization time, pH=7.0, 20^0C, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$, spinach thylakoid sample, without catalase, [Chl]=0.5g/l

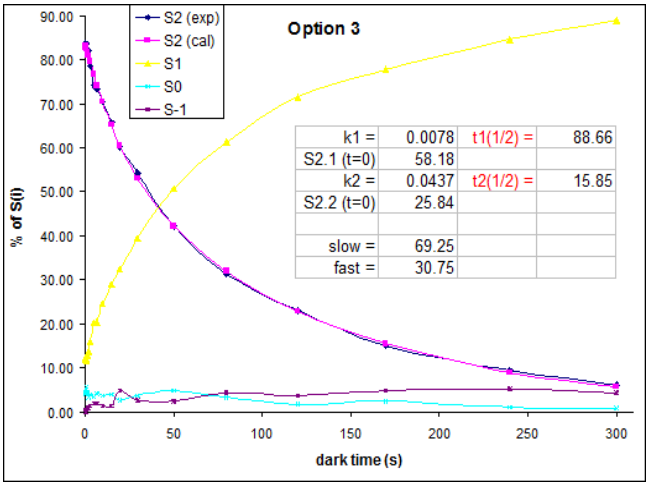

Figure 4.5: the S_i population as a function of dark time (sec) with the constant of polarization time and addition of catalase 10.000U/ml, pH=7.0, 20^0C, -750mV in 40sec, t_{sed} = 3min, $S_1Y_D^{OX}$, spinach thylakoid sample, without catalase, [Chl]=0.5g/l

Based on the right above diagram (figure 4.5), the minor conversions of S_2 to S_0 and S_1 to S_{-1} were considerably impeded as our expectation. The percent of S_0 population seems to become zero at a long dark time (more than 5 minutes). Although the percent of S_{-1} population still increases from 0% to 4% when the dark time is elongated, this value of S_{-1} population (4%) is not really a big number and does not have any bad effects to the other calculated results (percents of S_2, S_1 in this case). Thus, the optimal condition for S_2 lifetime measurement (and S_3 lifetime measurement in future) could be the addition of catalase solution with catalase concentration 10.000U/ml. Beside this positive results, the problem about the big value of fast phase (30%) still occurs. Correspondingly, the other solutions for this problem are the decrease the sample volume in the glass vial before giving a strong flash, exchange the old flash lamp by the other lamp that has a stronger light intensity or taking a second flash to the sample after the first flash. Furthermore, the new other experiments of S_2 lifetime measurement were performed in the condition without pre-flash to identify accurately the percent, the half-life time of fast phase and slow phase.

3. Conclusions

Based on all results of the experiments from the condition's optimizations, the best experimental conditions that give rise to the good miss, double hit and damping parameter for thylakoid sample in Joliot electrode measurements are summarized as follows:

- The best chlorophyll concentration for the current experimental conditions is 0.5mg/ml because this chlorophyll concentration could be completely light-saturated with the light intensity of current flash.
- The extreme conditions (e.g. high and low pH value, measurable temperature) are the bad conditions that can destroy the PSII protein complexes. In addition, the neutral pH (in range 6.5-7.0) and possibly the measuring temperature 20^0C-25^0C are the ideal conditions for FIOPs measurements.
- The buffer composition is also a crucial condition for Joliot electrode measurements. The good buffer compositions could be 20mM NaCl, 5mM $MgCl_2$, 50mM MES (buffer range for pH=4.5-6.5), 20mM NaCl, 5mM $MgCl_2$, 50mM HEPES (buffer range for pH=6.5-8.5). Some different kinds of sugar (mannitol, sucrose, etc) can be added complementarily into buffer at the concentration 400mM that could not bring about any bad effects to the sample and FIOPs measurements.

With the positive results from experiments of polarization time and catalase effects, we can have some useful discussions and conclusions.

- The polarization of thylakoid sample in a certain time can increase a significant amount of S_{-1} population that is performed by the reduction of S_1 to S_{-1} in manganese cluster. The reasonable explanation for this phenomenon is firstly the formation of hydrogen peroxide on the surface of Pt-cathode. Secondly, the reduction of S_1 to S_{-1} is performed directly on the cathode's surface and not through the production of hydrogen peroxide in a long polarization time.
- Besides, the good influence of catalase that was employed to prevent remarkably the formation of hydrogen peroxide or in the protein crowding effect was found out. Through the series of experiments to optimize the catalase concentration, the best catalase concentration is 10.000U/ml.
- The different polarization voltages in the range 600mV-750mV have had the inconsiderable effect to the variation of S_{-1} population (or the conversion of S_1 to S_{-1}) in these two cases of samples with and without the addition of catalase. Relied on these experiments, the miss parameter is not only dependent on pH, experimental temperature, but also depending on the polarization voltage.
- Suggestions for future plan: the frequency of flash sequence can have the remarkable influence to the conversion of S_2 to S_0 compared with the conversion of S_1 to S_{-1} because the S_2 state is more active than S_1. Thus, the new experiments were performed with the variations of flash frequency. With the purpose to conclude for the formation of hydrogen peroxide on the surface of Pt-cathode, the necessary experiments that are performed with the addition of BSA and the inactivation of catalase are required. The experiments that were performed with the variation of polarization time and the addition of catalase can be repeated again with the second spinach thylakoid preparation, with the cyanobacterium Thermosynechococus Elongatus instead of spinach or the other kinds of enzyme peroxidase.

Finally, the figures and calculated results from S_2 lifetime measurements had given us many information about the mechanism of photochemical reaction inside PSII protein complexes:

- The oxygen flash yield of thylakoid samples are according correctly to Kok model [14]. The polarization time that was varied in the range 40-300 seconds or kept in constant had some significant effects to the conversion of S_2 to S_0 and S_1 to S_{-1}. One more time, the addition of catalase into the sample with the aim to impede the competition of conversion S_2 to S_0 population is very effective and useful.

- These two mechanisms (slow and fast mechanisms) are so specific to express for the decay of S_2 to S_1. The half-life time that were concluded from figure 2.14 (option 3) of slow phase is 90 ± 10 (sec) and fast phase is 15 ± 5 (sec) at measurable temperature 20^0C. Nevertheless, the half-time values of fast phase and slow phase are relatively correct and not completely reliable due to the high percent of fast phase (approximately 30%) while the thylakoid sample was illuminated a strong pre-flash to produce a large amount of Y_D^{OX} before measuring.
- Future plans: The lifetime of S_2 could be measured again for the sample without pre-flash. The lifetime of S_3 were measured with the similar approaches (addition of catalase, with or without pre-flash). Most of the S_2 and S_3 lifetime measurements are performed with deuterated media (D_2O instead of H_2O) and the thylakoid sample that is extracted from cyanobacterium Thermosynechococus Elongatus instead of spinach.

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