

Research Paper

Femtosecond Time-resolved Spectroscopy for the Study of Photosystem I Reaction Centers at 77 K

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Abstract

Femtosecond time-resolved spectroscopy has been used to study the light-induced bioenergetics in isolated cyanobacterial photosystem I (PSI) reaction centers from *Synechocystis sp.* PCC 6803 at 77 K. A method was developed to produce optically clear samples at 77 K without the use of cryoprotectants, which considerably simplifies the experiments. Relatively intense laser pulses were used for sample excitation. Following 400 nm excitation, predominantly chlorophyll *a* pigments in PSI with Q_y absorption maxima in the 670–685 nm region are excited. This initially excited distribution of pigments transfers energy in ~400 fs to pigments absorbing near 690 nm, and to pigments absorbing near 715 nm. Further equilibration processes occur and are characterized by a 2.8 ps time constant. Following equilibration, energy trapping and formation of the secondary radical pair state, P700⁺A1⁻, occurs with a time constant of 32.5 ps.

Keywords

Photosynthesis, Electron transfer, Photosystem I, Time-resolved spectroscopy.

Introduction

Photosystem I (PSI) uses light energy to produce a highly reducing species that will eventually reduce carbon dioxide, ultimately resulting in glucose production.¹ PSI is a membrane embedded protein complex where light energy is used to transfer of electrons, via a series of protein-bound pigments, across this biological membrane (the thylakoid membrane).²

Isolated PSI complexes from cyanobacterial PSI contain ~100 chlorophyll *a* (Chl *a*) molecules,² organized around a reaction center core where electron transfer (ET) takes place. Light energy impinging on PSI is absorbed by these antenna Chl *a* pigments, raising them to an excited singlet state. Energy transfer between pigments in the antenna occur until this excited state energy is transferred to a centralized electron donor species called P700, so-called because it absorbs at 700 nm. P700 is dimeric

Chl *a* species that when excited rapidly transfers an electron to an acceptor molecule called A_0 . A_0 is a monomeric Chl *a* molecule. From A_0^- , an electron is transferred to A_1 , which is a phylloquinone (PhQ) molecule (also called vitamin K_1). An alternative light-induced primary ET mechanism has been proposed, but this mechanism also results in ET from A_0^- to A_1 .³ Beyond A_1 , the two ET branches converged on a [4Fe-4S] cluster called F_X . Two peripherally bound iron-sulfur clusters, F_A and F_B , serve as terminal electron acceptors.

In the so-called *menB* deletion mutant from the cyanobacterium *Synechocystis* sp. PCC 6803 (*S6803*), the cells lack the ability to synthesize PhQ, and plastoquinone-9 was found to occupy the A_1 binding site instead of PhQ.⁴ In isolated PSI complexes from the *menB* deletion mutant cells, PhQ can be reincorporated back into PSI by simple incubation.^{5,6} *MenB* PSI with PhQ reincorporated displays identical kinetics at both 298 and 77 K, to that obtained using native PSI from *S6803*.^{5,7}

In PSI, the protein-bound pigments involved in ET are organized into two nearly-symmetric branches,⁸⁻¹⁰ called the A- and B-branches. At room temperature (RT, ~298 K) ET occurs down both branches, with a branching ratio that is species dependant.¹¹ At low temperature (77 K), however, ET occurs almost entirely down the A-branch.¹² Probing the initial light induced bioenergetics in PSI might therefore be simplified at 77 K.

To date, only one group has undertaken a study of the ultrafast excitation dynamics and ET in PSI at low temperature (77 K), and no work has been undertaken to investigate further, or even to assess the validity of the previous study. The work presented here is a first attempt to address this problem.

Our main goal is to study the ultrafast light-induced bioenergetics in PSI with different quinones incorporated into the A_1 binding site. To do this requires the use of *menB* PSI. Given the identical nature of the ultrafast light-induced bioenergetics in native PSI, and in *menB* PSI with PhQ incorporated, at RT,⁷ we deem it appropriate to initiate our studies directly using *menB* PSI complexes.

Materials and Methods

Sample Preparation

Trimeric PSI particles from *menB*⁻ mutant cells from *S6803* were isolated and stored until use, as described previously.¹³ PSI thin films for femtosecond time-resolved (TR) spectroscopy were prepared in the same manner as that described previously for TR FTIR spectroscopy.^{5,14} The absorbance of the thin film sample (at 298 K) at 680 nm, the peak of the Q_y absorption band, was ~0.8.

Measurements at 77 K were undertaken as described previously.⁵ Ascorbate was added to aid in the re-reduction of $P700^+$. Incorporation of the native PhQ into the A_1 binding site was performed as described previously.¹⁵ For measurements at 77 K, the sample was loaded into a Model ND 110H liquid nitrogen cooled cryostat (Cryo Industries of America, Manchester, NH).

Transient Absorption Spectrometer

The home-built transient absorption spectrometer is based around a 1kHz repetition-rate regenerative amplifier (Coherent Legend Elite seeded by a Mira Optima 900) producing, 130 fs pulses at 800 nm, with a power of ~600 mW. Part of the 800 nm fundamental is frequency doubled in a BBO crystal to generate 400 nm excitation pulses (bandwidth 3.11 nm) which are then attenuated to 0.5 μ J per pulse and focused with a 750 mm focal length lens onto the sample, after passing through an optical chopper (Thorlabs MC2000) operating at 500 Hz. The remainder of the 800 nm fundamental is passed through a neutral density filter and focused into a 3 mm sapphire crystal to generate ~60 nJ white-light continuum (WLC) probe pulses covering a wavelength range from 446 to 767 nm. The spectrum of the WLC is recorded in transmission mode with a fiber spectrometer (AvaSpec-1650F-USB2). Pump/probe pulses are polarized vertically/horizontally, respectively. The differential absorbance is computed as the difference in optical density with the pump blocked and unblocked. The time evolution of the absorption spectrum is obtained using a variable optical delay between the pump and probe pulses, employing a retroreflector mounted on a computer-controlled linear translation stage (Newport, ILS150PP with an ESP 301 controller) within the probe beam path. Transient spectra were collected at pump-probe delays ranging from -1.5 ps to 720 ps. Data were collected in 100 fs increments in the 0.0–2.3 ps range, 1 ps increments in the 2.3–11.3 ps range, 10 ps

increments in the 11–171 ps range, and 50 ps increments in the 170–671 ps range. The sample cryostat was mounted on a translation stage (Newport, ILS150PP with an ESP 300 controller) oscillating at 0.5 Hz over a range of ~8 mm.

Results

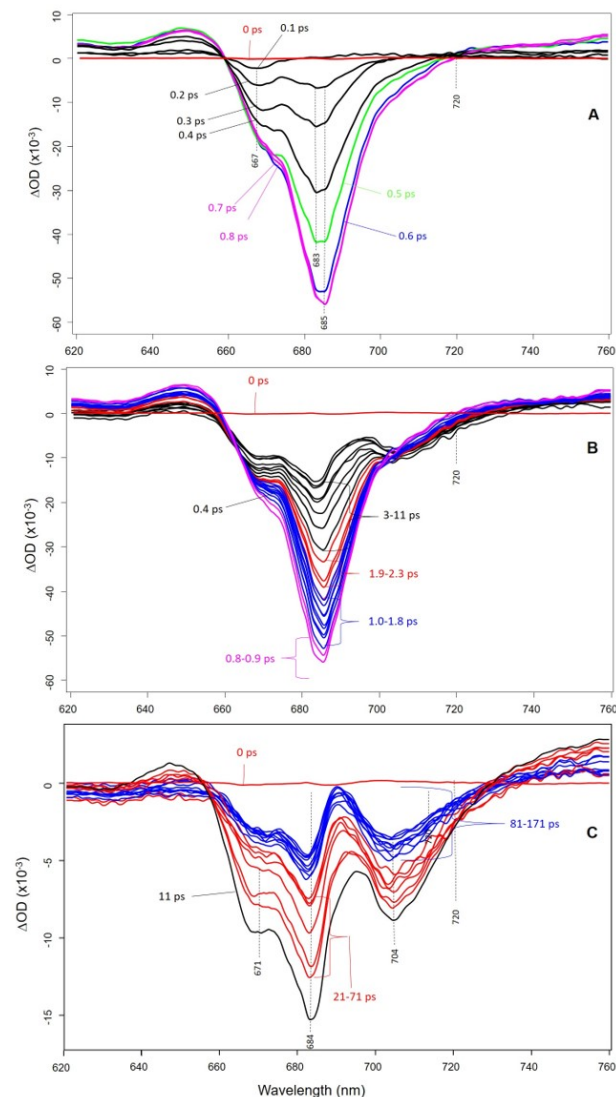


Figure 1. Transient spectra collected within the ~0–1 ps (A), ~1–11 ps (B) and ~21–171 ps (C) time ranges. The averaged of all of the spectra collected before excitation (labeled as 0 ps) is also shown.

The TR data obtained at all wavelengths was fitted to a sum of exponential functions using Glotaran.¹⁶ Global analysis within Glotaran included an instrumental response function and a dispersion correction procedure.

TR data was collected in the 480–760 nm spectral region, although here we focus only on data collected in the ~630–750 nm region. Data were collected at ~100 time points, on ever increasing time scales, over a ~720 ps time interval. Figure 1 shows experimental spectra collected over three different time ranges, from 0–1 ps (A), 1–11 ps (B) and 11–171 ps (C).

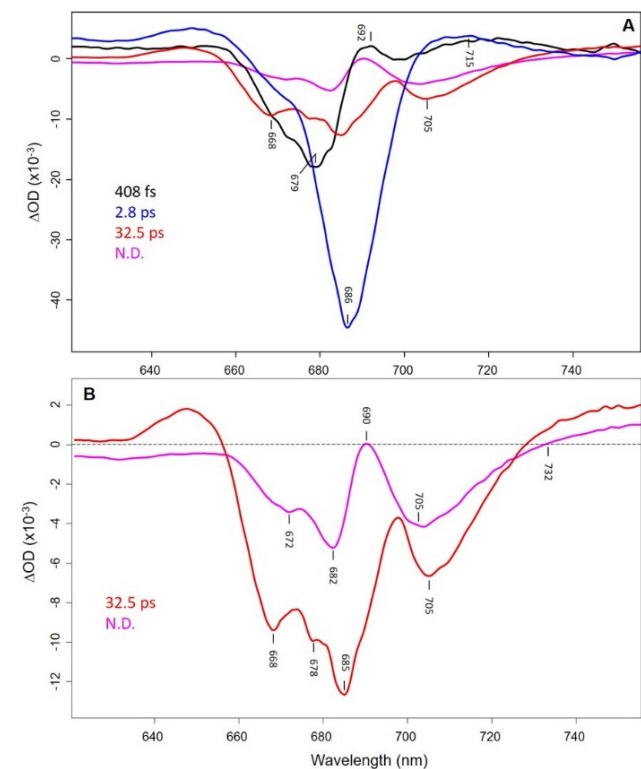


Figure 2. (A) DAS obtained from global analysis of the transient spectra. (B) Expanded view of the 32.5 ps and ND DAS.

All of the data, in the 620–760 nm region over the 0–720 ps timescale, was fitted simultaneously to a sum of four exponential components. The four decay associated spectra (DAS) are shown in Figure 2. One of the components has a lifetime in the microsecond regime and is essentially non-decaying (ND) on the timescales considered here. For repetitive flash excitation the ND state is due to the $P700^+A_1^-$ state,

which decays in ~ 350 μs at 77 K.⁵ The A_1^- state does not absorb in the 620–760 nm region, and thus the ND DAS is due mainly to $P700^+$. The ND DAS is due to absorption changes associated with probably the two chlorophyll (Chl) pigments of $P700$, and thus the area (or band intensity near 700 nm) under the ND DAS, compared to the area (or band intensity near 685 nm) under the 2.8 ps DAS can be used to provide a rough estimate of the number of photons delivered to each PSI complex (see below).

Figure 3A shows kinetic data at six wavelengths, over a 0–3 ps time window (*dotted*). The fitted data is also shown (*solid red line*). The kinetic data aids in assessing the DAS in Figure 2. For example, the 670 nm kinetic shows the rise of a bleaching on a 400 fs time scale but the decay of a bleaching on a 3 ps timescale. Similarly, the 710 nm kinetic shows the rise of a bleaching on both 400 fs and 3 ps time scales, suggesting energy transfer to 710 nm absorbing pigments on both timescales. Spectra collected at 13.6 and 225 ps after excitation are shown in Figure 3B, along with the fitted data. The quality of the fitting procedure can be assessed from the data in Figure 3.

Discussion

PSI samples were excited into the Soret absorption bands using 400 nm excitation. The spectra in Figure 1A shows the start of a bleaching centered near 670 nm within ~ 100 fs. This bleaching grows within the next ~ 300 fs, in addition to a more rapid growth of a bleaching near 683 nm. This 683 nm bleaching shifts slightly to 685 nm, and has a maximum peak intensity at ~ 800 fs. The spectral evolution over the 0–800 fs time window suggest some energy transfer from pigments absorbing near 670 nm to pigments absorbing near 683 nm. The “early” spectral evolution demonstrated in Figure 1A is similar to that observed previously for cyanobacterial PSI complexes at 77 K following excitation with low energy (1–3 $\mu\text{J}/\text{pulse}$), 100 fs, 670 nm laser pulses.¹⁷ The spectral evolution over the 0–800 fs time window can be assessed via a consideration of the 408 fs DAS in Figure 2A, which shows a negative peak near 680 nm and positive peaks near 692 and 715 nm. This shape suggests the transfer of energy from pigments absorbing at ~ 680 nm to pigments absorbing near 690 and 715 nm. This conclusion can be easily seen from a consideration of the kinetic data in Figure 3A. Pigments that absorb

beyond 700 nm are usually called “long-wavelength absorbing pigments” and this naming convention will be used below. Previously, from data collected over a 0–2 ps time window, two time constants of 350 and 470 fs were obtained.¹⁷ Given the similarity in the time constants we would think that such a level of discrimination is difficult to achieve. The shape of the 408 fs DAS in Figure 2A could be somewhat similar in shape to a combination of the 350 and 470 fs DAS observed previously.

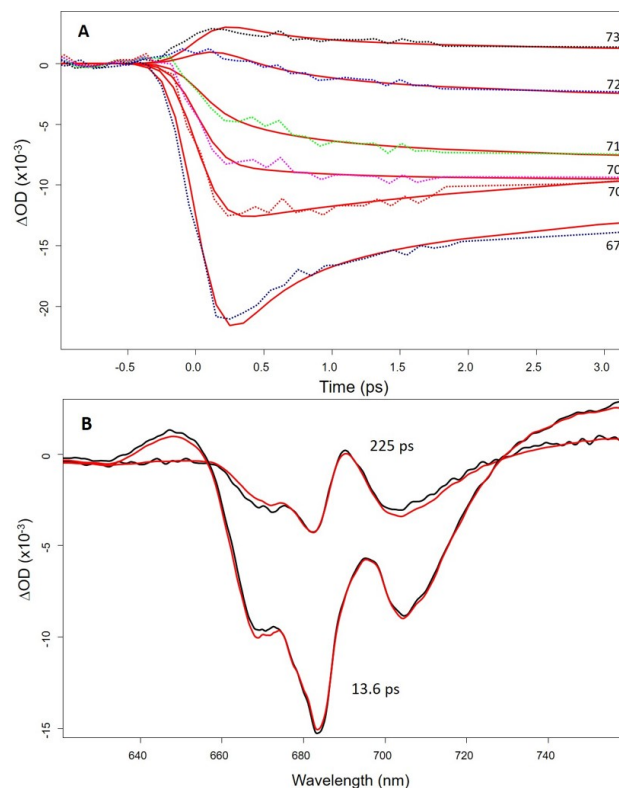


Figure 3. (A) Kinetic data at various wavelengths and (B) spectra at 13.6 and 225 ps. Experimental (*black*) and fitted (*red*) data are shown.

Figure 1B demonstrates that the broad bleaching centered near 685 nm at 0.8–0.9 ps decays rapidly over the next 10 ps. This is also indicated in the 2.8 ps DAS (Figure 2A) which shows a large decay of a bleaching band centered near 686 nm. The 2.8 ps DAS also shows positive absorption near 715 nm, indicating further energy transfer to long wavelength absorbing pigments. The 2.8 ps DAS has a very broad negative band extending from ~ 665 nm to beyond ~ 700 nm, and is very intense. Such a large intensity suggests several photons from the excitation beam absorbed per PSI

complex. The number of photons can be estimated from the intensity of the bands in the DAS. For example, the intensity near 700 nm in the ND DAS is $\sim 5 \times 10^{-3}$ in OD units, which could correspond to one or two Chl *a* pigments bleached upon P700⁺ formation. The 2.8 ps DAS has an intensity of $\sim 44 \times 10^{-3}$ in OD units, which after accounting for stimulated emission, would correspond to excitation of roughly 4–8 Chl *a* molecules per PSI complex. Given such a high excitation intensity, it is likely that much of the 2.8 ps decay occurring in the 665–700 nm region is due to singlet-singlet annihilation, and the associated loss of stimulated emission. Such annihilation processes, with ~ 4 ps lifetime, were observed in previous studies of PSI at RT where high excitation intensities were also used.¹⁸ Such annihilation processes may also contribute to spectral features in the 408 fs DAS.

In previous studies of PSI at 77 K, following 670–695 nm excitation, under annihilation free conditions, energy transfer from pigments absorbing in the 680–690 nm region to pigments absorbing near 710 nm was observed to be characterized by a time constant of 4–6 ps.^{17,19} As mentioned above, such processes do contribute to our 2.8 ps DAS, but spectral changes associated with this equilibration process are obscured by the spectral changes associated with singlet-singlet annihilation.

Within 5–10 ps the singlet-singlet annihilation process and the equilibration of excitations between different Chl *a* spectral forms, is for the most part complete. From ~ 11 –70 ps, bleaching bands at 671, 684 and 704 nm decay further, along with the decay of excited state absorption bands at wavelengths above ~ 730 nm (Figure 1C). These spectral changes are associated with the 32.5 ps DAS, which is shown in Figure 2A, and enlarged in Figure 2B. The 32.5 ps DAS in Figure 2B, broadly speaking, indicates the ground state recovery of Chl *a* molecules with predominantly two spectral types with peaks near 705 and 680 nm. The broad bleaching band near 680 nm displays peaks at 668, 678 and 685 nm, again indicating the decay of the excited states of multiple spectral forms. The recovery of the ground state absorption of Chl *a* pigments with a 32.5 ps time constant is due to mainly energy transfer to the reaction center and subsequent radical pair formation (commonly referred to as “trapping”), which results in a long lived state with spectrum indicated by the ND DAS in Figure 2B. Energy transfer and trapping occur on similar timescales, and are associated with

pigments that absorb at similar wavelengths, and the processes therefore cannot easily be distinguished.

In previous studies of cyanobacterial PSI, the overall decay of an equilibrated distribution of pigments, due to trapping, was found to be characterized by a time constant of 40–50 ps, very similar to the 32.5 ps time constant observed here. As mentioned, the 32.5 ps DAS in Figure 2B displays broad peaks at 705 and 680 nm, which is markedly different from the 40–50 ps DAS found previously, where a broad peak was found at 703 nm and only a weak shoulder at 685–690 nm.¹⁷ Given the relative intensities of the 32.5 ps and ND DAS in Figure 3B it seems reasonable that there is still more than 1–2 photons per PSI complex, and therefore that singlet-singlet annihilation processes may contribute to some degree to the 32.5 ps DAS. In any case, we associate the 32.5 ps time constant with trapping, noting that such a time constant probably represent a lower limit (as contributing annihilation processes are probably faster, leading to a shorter time constant).

In previous studies the 40–50 ps time constant was suggested to represent trapping. However the spectra obtained at ~ 200 ps following excitation was not representative of a (P700⁺–P700) difference spectrum (see below),¹⁷ and appeared to contain contributions from long wavelength absorbing pigments (>705 –720 nm) that presumably could not transfer energy uphill to P700 on such a short timescale at 77 K. This conclusion was supported by transient spectra collected ~ 1.5 ns after excitation, which were shown to be more typical of a (P700⁺–P700) difference spectrum (see below). It was therefore concluded that long wavelength absorbing pigments could transfer energy to P700 only on timescales longer than 200 ps, but shorter than ~ 1.5 ns. This conclusion is not supported by our data (see below).

The ND DAS (Figure 2B) is very interesting, with broad bleaching bands centered at 705, 682 and 672 nm, with the peak at 682 nm being more negative than the 705 nm peak. The ND DAS also displays a slight positive peak at 690 nm, and crosses the axis near 732 nm (Figure 2B). The spectral features in the ND DAS observed here are in good agreement with features in previously published (P700⁺–P700) difference spectra obtained using PSI complexes from spinach at 10 K.²⁰ In particular, the (P700⁺–P700) difference spectrum obtained using PSI particles from spinach at 10 K displayed negative peaks at ~ 702 and ~ 684 nm, and a positive peak at 690 nm,²⁰ with the negative band at

684 nm being more negative than the 702 nm band, exactly as is observed in the ND DAS in Figure 2B. No negative band was observed at 672 nm, however. It is possible that the 672 nm band observed in the ND DAS in Figure 2B is due to (damaged) Chl *a* pigments that are uncoupled from the trapping and charge separation process.

A photoaccumulated (P700⁺–P700) difference spectrum has also been obtained using cyanobacterial PSI complexes at 5 K.²¹ Although broadly similar to the ND spectrum in Figure 2B this previously published spectrum contains additional “sharp” features not observed here.

In previous studies of cyanobacterial PSI at 77 K, on a 200 ps timescale,¹⁷ the ND DAS obtained are quite different from the ND DAS presented here. The spectra obtained ~1.5 ns after excitation,^{17,19} however, are more in line with our ND DAS. In particular, the presence of an intense negative band at ~680 nm, that is at least as intense as the negative band near 705 nm. Clearly, in these earlier studies uphill energy transfer from long wavelength absorbing pigments to P700 must occur on timescales greater than 200 ps. This result does not agree with the work presented here, which suggests that mainly trapping occurs with a time constant of 32.5 ps. Of course, there are many differences in experimental procedures used in the current work compared to previous works. In particular, in our work data was collected in a 0–720 ps time window (rather than 0–200 ps) using relatively intense 400 nm excitation pulses. How these differences might yield different results is still not clear, however.

Given the positive absorption at 690 nm in the ND DAS (Figure 2B), it seems likely that this DAS is due to the P700⁺A₁[–] state, and not the P700⁺A₀[–] state, as A₀ is expected to display a bleaching band near 690 nm upon anion formation, similar to that found in RT studies.^{7,18,22} A₁ is a substituted naphthoquinone species (PhQ)²³ that does not absorb in the 660–760 nm region,²⁴ and thus a P700⁺A₁[–] difference spectrum should be similar to a P700⁺ difference spectrum, which is indeed what is observed. The conclusion that trapping results in a P700⁺A₁[–] spectrum implies that ET from P700 to A₀ (or A₁ to A₀) must occur on a timescale that is much faster than ~30 ps. Such a conclusion could be in agreement with previous studies of “highly stripped” PSI particles containing ~10 Chl *a* molecules, which indicated that ET from A₀[–] to A₁ occurred in less than 1 ps.^{25,26}

Conclusions

Following light excitation, energy equilibrates between the different Chl spectral forms in ~400 fs and ~3 ps. The overall decay of these equilibrated excited states occurs in ~32 ps via trapping and radical pair formation.

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