Biochimica et Biophysica Acta, 767 (1984) 501–506
Elsevier

BBA 41620

DIFFERENCE PICOSECOND SPECTROSCOPY OF PIGMENT-PROTEIN COMPLEXES OF PHOTOSYSTEM I FROM HIGHER PLANTS

MARINA D. IL'INA [a,*], VITAUTAS V. KRASAUSKAS [b], RICHARDAS J. ROTOMSKIS [b]
and ALEXANDER YU. BORISOV [a]

[a] *A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow W-234, 119899 and* [b] *Department of Astronomy and Quantum Electronics, Vilnius State University, Vilnius 232043 (U.S.S.R.)*

(Received April 6th, 1984)

Key words: Energy transfer; Charge separation; Photosystem I; Picosecond spectroscopy; Pigment-protein complex; (Bean chloroplast)

Using a difference picosecond spectrophotometer with a time resolution of 10 ps, we investigated excitation energy transfer and charge separation in pigment-protein complexes of Photosystem I from bean leaves (chlorophyll/P-700 = 60). Under 20 ps excitation at 650 or 667 nm, the difference absorption spectra in the spectral region 600–720 nm were measured. They are associated with transition of antenna chlorophylls into singlet excited states and P-700 photooxidation. It was shown that the excited states in the whole inhomogeneous antenna were generated within 10 ps and deactivated with three-component kinetics, the $t_{1/e}$ values being 20–45, 100–300 and over 500 ps. Formation of P-700$^+$ has a rise time of 15–30 ps. The fast component of the depletion of the antenna excited states is suggested to be due to transfer of excitation energy from antenna pigments to reaction centers and its trapping. The kinetics of the fast component is independent of excitation energy and a redox state of P-700.

Introduction

The mechanism of energy coupling of the photosynthetic light-harvesting antenna with the reaction center is of major interest both in theoretical and practical terms, specifically, for designing light-converting model systems. Earlier studies of plant photosynthesis aimed at the elucidation of this mechanism were carried out using mainly the pulse fluorescence method (for reviews see Refs.1 and 2). Chlorophyll (Chl) fluorescence decay served to indicate deactivation of excitations in antenna pigments, and thus tentative conclusions about trapping of excitations by reaction center molecules could be drawn (see, e.g., Ref. 3). Much more conclusive data were obained through the use of absorption spectroscopy with a high time resolution. The kinetics and spectra of absorption changes recorded were connected with generation of the excited states of antenna molecules and charge separation in reaction centers [4–7]. The absorption study of reaction center-enriched Photosystem I particles (Chl/reaction center = 8–10) revealed that the disappearance of excitations in the antenna and appearance of a cation-radical of P-700 (the primary electron donor of Photosystem I) have the same kinetics with a time constant of 25 ps [7]. The fluorescence of the particles decayed similarly to excitations in the antenna [8].

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[*] To whom all correspondence should be addressed.

Abbreviations: Chl, chlorophyll; bChl, bacteriochlorophyll; P-700, primary electron donor of Photosystem I; PPC-I, pigment-protein complex of Photosystem I; $h\nu$/RC, a quantity of photons absorbed per one reaction center; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine.

A picosecond difference spectrophotometer [9] with sensitivity 50-times higher than that used by Kamogawa et al. [7] made it possible to investigate photosynthetic objects having large light-harvesting antennae. This instrument was used for the measurements with intact chromatophores of purple bacterium *Rhodospirillum rubrum* (bChl/reaction center ≈ 40). The antenna was found to contain a minor bChl spectral form which is in a long-wavelength side of a major, near infrared absorption band of chromatophores [10]. The authors assumed that this form was involved in the coupling of antenna pigments with reaction centers during the excitation energy transfer [11].

In this paper, we present the spectra and kinetics of absorption changes reflecting the dynamic behavior of antenna pigments and P-700 induced by selective picosecond excitation. The object under study is a photoactive preparation of pigment-protein complexes of Photosystem I (PPC-I).

Materials and Methods

The PPC-I preparation was obtained by treatment of bean chloroplasts with 3% Triton X-100 and subsequent column chromatography of Cellulose DEAE-52 and Sephadex A-25, as described previously [12].

The absorption spectrum was measured with a SF-10 (LOMO, U.S.S.R.) spectrophotometer, the spectra of fluorescence emission excited by the 436 nm mercury line were measured with an Aminco Bowman spectrofluorimeter. P-700 absorption changes induced by continuous light were determined with a modified SF-10 instrument, as reported previously [12].

The red absorption band of PPC-I peaks at 676 nm at room temperature, the fluorescence maximum is near 685 nm at room temperature and near 720 nm at 77 K. The ratio of fluorescence intensities at 720 and 680 nm at 77 K is 2.5. At least one minor Chl *a* spectral form with absorption maximum wavelength longer than 697 nm is present, as revealed by a second derivative of the absorption spectrum. The Chl/P-700 ratio estimated as previously [12] is about 60. As previously [13], the measurements of a quantum yield of P-700 photooxidation allowed us to discover a fraction of the pigments (Chl *a*, pheophytin *a*), inactive in the process of energy transfer to reaction centers. The presence of this fraction results from the use of the detergent for obtaining of PPC-I. The amount of the photoinactive pigments was estimated to be about 40% of the bulk pigments. The quantum yield of P-700 photooxidation is at least 70% if the fraction of the photoinactive pigments is taken into account.

Picosecond absorption studies were carried out at room temperature with a difference spectrophotometer described previously [9]. This instrument made it possible to detect absorption changes down to $2 \cdot 10^{-4}$ units with a time resolution of 10 ps. The wavelengths of exciting and probing pulses were tuned independently in a 600–800 nm spectral range. The pulse width at a half-maximum was 20 ps. The pulse repetition frequency was about 1 Hz. The energy density of the exciting pulse determined with a 2-fold error could be varied from 10^{12} to 10^{16} photons $\cdot$ cm^{-2}. Each experimental point presented in the figures is an average of 120 measurements.

The ratio between the number of photons absorbed and overall quantity of reaction centers in an illuminated volume of the sample ($h\nu$/RC) was calculated as previously [14]. The molar extinction coefficient in a red spectral maximum of PPC-I, $7 \cdot 10^4$ M$^{-1} \cdot$ cm^{-1}, was used.

Results

Difference spectra of absorption changes of the PPC-I preparation excited with single picosecond pulses or continuous light are shown in Fig. 1. The bleaching of the band with the maximum at 676 nm is observed when the probing pulse coincides in time with the exciting pulse (Fig. 1A). The position of the maximum and the width at a half-height (approx. 28 nm) of this band are nearly the same as those of the main red band in the absorption spectrum of PPC-I. However, this difference spectrum changes significantly when the probing pulse has a time delay about some hundred picoseconds. Besides, the shape of the spectrum measured is highly dependent on the excitation energy density. Despite the large error bars, two separate bands with maxima near 676 and 697 nm and with widths at half-heights about 12 and 15

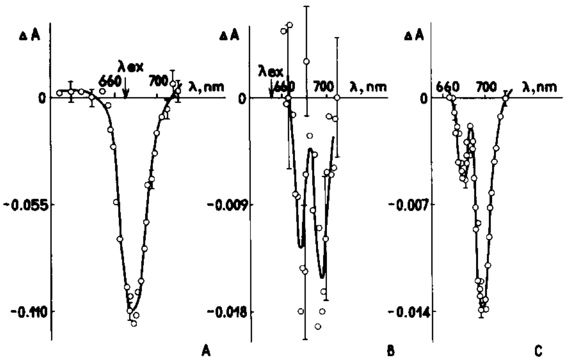

Fig. 1. The difference (light minus dark) absorption spectra of the PPC-I preparation recorded upon excitation and probing with single picosecond pulses (A, B) and continuous light (C). (A) The energy density of the 667 nm exciting pulse was $5\cdot10^{15}$ photons·cm^{-2}. The probing and exciting pulses passed through the sample simultaneously (zero time delay). Measurements were made in a 2 mm cuvette. The reaction mixture contained 50 μM TMPD, 10 mM Tris-HCl buffer (pH 8) and 100 μg/ml Chl. $A_{667} = 1.3$, $h\nu$/RC = 24. (B) The energy density of the 650 nm exciting pulse was $3\cdot10^{14}$ photons·cm^{-2}. The probing pulse had a 350 ps time delay vs. the exciting pulse. Measurements were made in a 1 mm cuvette. The reaction mixture contained 50 μM TMPD, 10 mM Tris-HCl buffer (pH 8) and 300 μg/ml Chl. $A_{650} = 0.75$, $h\nu$/RC = 0.8. The solid curve is the result of a 5-point smoothing procedure. (C) Measurements were carried out with the sample containing 5 μM TMPD, 10 mM Tris-HCl buffer (pH 8) and 5 μg/ml Chl (curvette path-length 1 cm).

nm, correspondingly, may be resolved in the spectrum in the case of the low energy density ($h\nu$/RC $\leqslant 1$) (see Fig. 1B). The amplitudes of both bands have close values. The 7-fold increase in the energy density of the exciting pulse results in a doubling of the amplitude of a short-wavelength band and in its broadening. Upon further energy density increasing, the spectral resolution of a long-wavelength band diminishes much. The spectrum of the absorption decrease shown in Fig. 1B is similar to the well-known difference (P-700$^+$ − P-700) spectrum obtained during PPC-I exposure to continuous light (Fig. 1C). However, the height-to-height ratio of the long-wavelength and short-wavelength bands in the last spectrum is 2-times as much.

Figs. 2 and 3 demonstrate the kinetics of PPC-I absorption changes in a picosecond time range. The kinetic curve at 678 nm (Fig. 2A) is biphasic. At first the bleaching of the sample is observed. The rise of the bleaching coincides with the front profile of the exciting pulse. Then relaxation of the bleaching takes place. It consists of at least two fast components ($t_{1/e} \approx$ 20–45 ps and 100–300 ps) and a slow ($t_{1/e} >$ 500 ps) one. Their kinetics practically do not change: (i) upon decreasing of exciting pulse energy (e.g., when the $h\nu$/RC ratio diminishes from 13 to 2), (ii) upon oxidation of P-700 by saturating continuous light (Fig. 2B), (iii) after addition of Triton X-100 (Fig. 2C). In the first and third cases, the contribution of the slow component to the overall amplitude of the bleaching increases.

The kinetic curve of absorption change at 704 nm is shown in Fig. 3A. As in Fig. 2, the initial

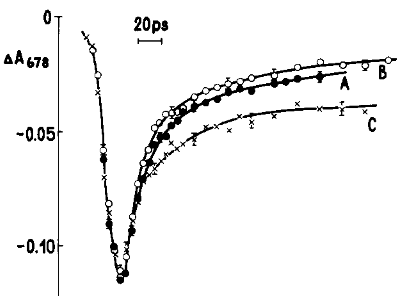

Fig. 2. The kinetics of PPC-I absorption changes at 678 nm induced by the single 650 nm picosecond pulses. The energy density of the exciting pulse was $5\cdot10^{15}$ photons·cm^{-2}. Measurements were made in a 1 mm cuvette. The reaction mixture contained 50 μM TMPD, 10 mM Tris-HCl buffer (pH 8) and 300 μg/ml Chl. $A_{650} = 0.75$, $h\nu$/RC = 13. (A) The sample was dark-adapted. (B) The sample was exposed to continuous saturating light from He-Ne-laser (λ = 630 nm). (C) Same as in (A) plus the addition of 1.5% Triton X-100.

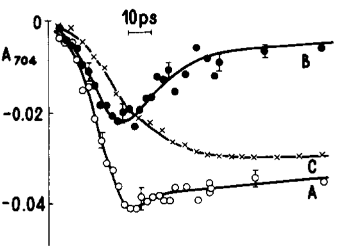

Fig. 3. The kinetics of PPC-I absorption changes at 704 nm induced by the single 650 nm picosecond pulses. The energy density of the exciting pulse was $1\cdot10^{15}$ photon·cm^{-2}. Measurements were made in a 2 mm cuvette. The reaction mixture contained 50 μM TMPD, 10 mM Tris-HCl buffer (pH 8) and 300 μg/ml Chl. $A_{650} = 1.5$, $h\nu$/RC = 1.7 (A) and (B) as in Fig. 2. The results of subtraction of (B) from (A) point by point.

decrease in absorption appears simultaneously with the passing of the exciting pulse through the sample. But contrary to the kinetic curves in Fig. 2, the bleaching observed does not relax practically in a picosecond time range. Photooxidation of P-700 by saturating continuous light results in the 2-fold drop of the amplitude of the bleaching at 704 nm and acceleration of its decay. The latter becomes similar to the kinetics at 678 nm in Fig. 2. Apparently, when P-700 is oxidized, the remaining absorption changes near 700 nm belong to the absorption band centered at 676 nm, whose bleaching spectrum is given in Fig. 1A. As shown above, the relaxation kinetics of the bleaching of this band is independent of P-700 photooxidation (Fig. 2A and B). Then the net kinetics of the long-lived absorption change associated with the photoactivity of P-700 can be obtained by subtraction of curve B from curve A in Fig. 3. The result of this operation is presented in Fig. 3C. The kinetics has a time constant about 15–30 ps in the monoexponential approximation. The value of $t_{1/e}$ remains unchanged when the energy density of the exciting pulse is diminished by a factor of 4 (the $h\nu$/RC ratio drops from 1.7 to 0.4).

The difference in the lifetime values of absorption changes is manifested in the dependences of their amplitudes on the absorbed energy density of the exciting pulse (Fig. 4). The amplitude of the short-lived absorption change at 675 nm does not reach its maximal value under the excitation used (Fig. 4B), unlike the amplitude of the long-lived absorption change at 700 nm (Fig. 4A). However, the courses of both dependences are the same in their initial parts within the energy density range up to $2 \cdot 10^{14}$ photons $\cdot$ cm^{-2} ($h\nu$/RC $\leqslant 1$).

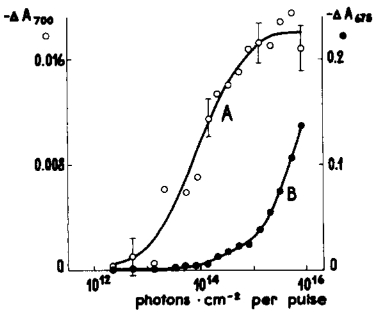

Fig. 4. Absorbed energy density dependence on PPC-I absorption changes at 700 (A) and 675 (B) nm. The time delay between the 667 nm exciting and the probing pulses was zero (B) and 420 ps (A). Measurements were carried out in a 2 mm cuvette with the sample containing 50 μM TMPD, 10 mM Tris-HCl buffer (pH 8) and 100 μg/ml Chl. $A_{667} = 1.3$.

Discussion

Three types of absorption changes in the red absorption region of Chl *a* have been recorded under excitation of the Photosystem I preparation with 650 or 667 nm single picosecond pulses.

The changes of the first type have a difference bleaching spectrum (Fig. 1A) resembling the absorption band of PPC-I with the maximum at 676 nm. They appear in the time interval not longer than 10 ps and disappear mainly in some tens and hundreds of picoseconds (Fig. 2). The amplitude of these changes is not saturated even at the highest energy density of the excitation used (Fig. 4B). The emergence of absorption changes of the first type apparently reflects the transition of antenna pigments into the singlet excited state [2]. The same interpretation was given for a 25 ps component [7] and a 45 ps component [5] of absorption changes observed in the preparations of Photosystem I with the Chl/P-700 ratio of 8–10 and 25, correspondingly.

It is known that the red absorption band of PPC-I is inhomogeneously broadened: it includes some populations of Chl *a* molecules [13,15]. Similarity between the difference spectrum in fig. 1A and the red absorption band of PPC-I allows us conclude that (i) the 20 ps exciting pulse creates excitons which are spread over molecules belonging to various pigment populations or (ii) the molecules exchange excitations in a time interval considerably shorter than the pulse duration.

The second type of absorption changes has a lifetime longer than 500 ps. Its maximal amplitude is approx. 1/60 of the overall absorbance in the PPC-I red maximum. The difference spectrum of these changes is very similar to that of (P-700^{+} – P-700) induced by continous light (Fig. 1C). Therefore, these changes may be attributed to photooxidation of the primary electron donor of Photosystem I, P-700. We estimated that the proportion of directly excited molecules of P-700 under excitation with 650 or 667 nm pulses was at least two orders below that of antenna molecules. However, the amplitude of the $P700^+$ signal was observed to be much greater than the one expected from diirect excitation of P-700. Consequently, the appearance of the P-700^+ signal is mainly due to energy transfer from antenna pigments to P-700 molecules. Indeed, the kinetics of $P700^+$ formation (Fig. 3C) is somewhat delayed as compared to the kinetics of the antenna excitation and is characaterized by the rise time, 15–30 ps, close to the shortest decay time of antenna excited states (Figs. 2, 3B).

The third type of absorption changes, appearing simultaneously with the exciting pulse, remains stable up to 500 ps (as the second type) and, similarly to the first type, does not dpeend on the redox state of P-700 (Fig. 2B). These changes are maximal at 676–678 nm and, therefore, obscure to some extent the red satellite band due to P-700 photooxidation (see spectra B and C in Fig. 1). The amplitude of these background changes depends on excitation energy. For example, the changes amount to 55% of the total bleaching at 676 nm, when the $h\nu$/RC ratio is about 1 (Fig. 1B), and increased up to 80% of that, when the ratio is raised to 6. These calculations have been made using the ratio of the band amplitudes in the difference spectrum of (P-700^+ – P-700) under continuous illumination (Fig. 1C).

Addition of the detergent Triton X-100, used in the procedure of PPC-I isolation, also increases the amplitude of the changes of the third type (Fig. 2C). According to Il'ina and Borisov [13], detergents disrupt the native structure of the light-harvesting antenna of Photosystem I and, as a consequence, energy transfer to P-700. In the extreme case, addition of the detergent results in formation of pigment domains, inactive in P-700 photooxidation, with a fluorescence lifetime as high as 5–6 ns. In our case, the excited states of such pigments are likely to make the major contribution to the absorption changes of the third type. We estimated the proportion of photoinactive pigments in PPC-I to be about one-third, because (i) its bleaching amplitude at 676 nm amounts to half of that of P-700 at 697 nm under excitation with low energy pulses (Fig. 1B), (ii) the difference extinction coefficients of the pigments and P-700 in the above-mentioned maxima are probably identical. The estimation accords well with the one made in the experiments under continuous illumination (see Materials and Methods).

As followed from Fig. 4A, about 70–75% of the P-700 molecules may be photooxidized by the pulses inducing approximately one excited state per reaction center in the photoactive antenna. Taking into account the probability of arrival of an excitation at reaction center, we concluded that the quantum efficiency of the primary charge separation in PPC-I had a rather high value, greater than 50%.

In view of the similarity of corresponding time constants as well as the high efficiency of P-700^+ formation under low energy excitation, we suggest that the fast decay component of the depletion of antenna excited states with $t_{1/e}$ of 20–45 ps reflects the very process of energy transfer to reaction centers and excitation trapping. When the $h\nu$/RC ratio is not more than 1, the maximal proportion of excitations that may be trapped by reaction centers during 20 ps exciting pulse with a time constant of 15–30 ps is about half of excitations formed in the photoactive antenna or one-third of the overall excitations. This proportion must decrease significantly when the $h\nu$/RC ratio is much greater than 1. Therefore, the quantum efficiency of the total bleaching at 676 nm arising within 20 ps is expected to be not more than 70% under a single photon excitation of the reaction center and to approach 100% under a multiple photon excitation. We calculated that the efficiency of the bleaching was about 60–80%, when the $h\nu$/RC ratio was not more than 1, and below 20%, when this ratio exceeded 10. Consequently, under the single photon excitation of the reaction center, the energy transfer to reaction centers in fact is the most efficient way of deactivation of antenna excited states during tens of picoseconds; under the multiple photon excitation, apparently, an appreciable exciton-exciton annihilation [2] in both (active and inactive) pigment fractions takes place in a time shorter than 10 ps.

We have not found a considerable change in the time constant of the above-mentioned fast decay component upon varying exciting pulse energy as well as upon transformation of all reaction centers in the oxidized state with the use of background continuous illumination, the latter also shown previously [5]. These facts may be explained by quenching of excitations (i) by the closed reaction centers and/or (ii) by the long-wavelength Chl *a* forms (present in PPC-I), when reaction centers are closed, as suggested by Paschenko et al. [16]. The independence of the fast component from excitation energy may also be due to the exciton-exciton annihilation under a multiple photon excitation, which efficiently quenches the antenna excited states in a time interval shorter than the reaction center trapping time and thereby simulates the conditions of low energy excitation of the reaction center.

It is interesting to compare our data with those reported by Kamogawa et al. [7]. The authors studied the kinetics and spectra of the bleaching of the Photosystem I preparation, having 8–10 Chl *a* molecules per P-700, under a single photon excitation of the reaction center with 15-ps pulses ($\lambda =$ 532 nm). The nanosecond absorption changes around 677 nm were reported to be twice as high as those at 697 nm attributed to P-700 photooxidation (see Figs. 4 and 6 in Ref. 7). The P-700 absorption changes could hardly be resolved. In the light of our results, these facts may be due to the presence of the significant proportion (about 60%) of photoinactive pigments in the preparation studied. This is confirmed by fluorescence data reported previously [8].

The results presented in this paper indicate that the energy transfer in antenna pigments of PPC-I is rather fast, with the rate constant more than $5 \cdot 10^{10}$ s^{-1}, and does not limit the process of P-700 photooxidation. The similarity of the $P700^+$ rise-time values obtained previously [7] and in this work, despite the significant difference in the sizes of antennae, may be explained in two ways:

(i) The process of P-700 photooxidation in both cases is limited by energy transfer from antenna molecules to reaction centers or by excitation trapping. A limiting stage has identical time parameters for both objects examined.

(ii) Obtaining particles with a high content of P-700 [7] caused a considerable slowing of the antenna energy transfer, thereby making it a limiting stage in P-700 photooxidation.

Acknowledgements

The authors wish to thank A.S. Piskarskas and A.P. Razjivin for helpful discussion and continuous interest, R.V. Danielius for participation in some experiments and E.A. Kotova for correction of the English version of the manuscript.

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