KEYNOTE ADDRESS

PICOSECOND FLUOROMETRY IN PRIMARY EVENTS OF PHOTOSYNTHESIS

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ABSTRACT Many laboratories in different countries are involved in the study of the mechanism of conversion of light energy into chemical energy, namely photosynthesis. As is evident from the literature, the initial phases of photosynthesis, which determine the character of this process, proceed at time intervals of 10^{-8} and 10^{-13} s. They are associated with absorption of light quanta and energy transfer from the molecules of light-harvesting antenna (LHA) chlorophyll and accessory pigments to the reaction centers (RC), where the key reaction of photosynthesis occurs: photo-induced charge separation. Evidently it is of importance to study experimentally the process that occurs within the 10^{-8}–10^{-13} s time domain.

In my lecture, I will try to discuss how picosecond fluorometry can help us to understand the mechanism and nature of primary events in photosynthesis. But before presenting any results, I want to acquaint you with our group in Moscow University working in the picosecond fluorometry region: Professor Andrew Rubin, Dr. V. Paschenko, and Dr. A. Kononenko. Our group consists of biologists and physicists who help us do experimental work with a wide variety of biological systems. Chloroplasts, isolated photosystems I and II (PI and PII), as well as reaction centers (RC) from photosynthetic bacteria were studied with a picosecond spectroscopy setup with high sensitivity and time resolution.[1]

One of the problems that can be solved by picosecond fluorometry techniques is the energy migration between light harvesting antennae (LHA) and RC. We can describe this process in terms of energy transfer from donor to acceptor, where the RC plays the role of acceptor of energy or quencher of electronic excitation of LHA. If one excites the LHA by a picosecond light pulse, the measurements of the fluorescence decay of the LHA will give information about the energy migration from LHA to the

[1] Dr. Rubin did not supply references with the text of his talk.—*Editor.*

RC. It is well known that the maximum of fluorescence of the LHA of PI is at 735 nm and that of PII is at 690 nm. This makes it possible to study separately the processes of energy migration in both photosystems of the green plants.

Fig. 1 presents the principal scheme of our picosecond fluorometry apparatus. It consists of a picosecond ruby or Nd^{3+} laser, a Pockels cell selecting a single picosecond pulse, and a streak camera with a time resolution of 10^{-11} s and an amplification of 5×10^6. In all of our experiments the energy of the picosecond pulse did not exceed 5.10^{12} photons cm^{-2}; much higher energies damage the chloroplast.

The fluorescence decay of pea chloroplasts has three components with a lifetime (τ) of 80, 300, and 4,500 ps, ascribed to PI, PII, and monomeric chlorophyll not involved in photosynthesis, respectively. The only reason that the lifetimes of PI and PII are much shorter than the lifetimes in solutions of chlorophyll is the very efficient energy migration between the LHA and RC. This fast transfer makes it possible to evaluate the constant of energy migration: $K_M \approx 1/\tau$.

Our measurements resulted in the following values of $K_{M\text{PSI}} = 1.25 \times 10^{10}\ s^{-1}$, $K_{M\text{PSII}} = 3.3 \times 10^9\ s^{-1}$. It is not difficult to show clearly that if one knows the value of the constant of energy migration K_M and spectral properties of LHA and RC, one can apply the formula that describes the process of inductive-resonance energy migration and evaluate the distance R between the donor (LHA) and acceptor (RC) of energy. Our calculations indicate that $R \approx 15$ Å.

According to the well-known hypothesis suggested by Duysens, the RC can quench the electronic excitation of the LHA only in the reduced form; in the oxidized form it is "closed," and as a consequence there should be an increase in the fluorescence lifetime τ and quantum yield. Taking into account the high quantum yield of primary steps of photosynthesis, ~100%, one would expect that when the RC is oxidized, the molecules of chlorophyll in LHA would emit fluorescence with a lifetime and quantum yield close to those observed in pigment solution.

In another series of experiments we studied the dependence of fluorescence lifetimes of PI and PII on the degree of oxidiation of the RCs induced by potassium ferricyanide

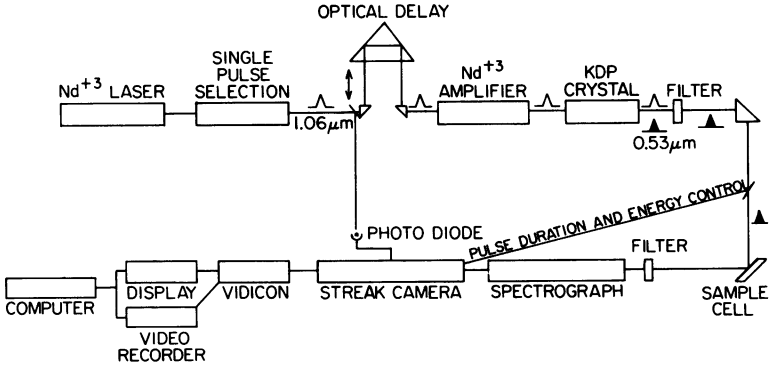

FIGURE 1 Picosecond fluorometry apparatus.

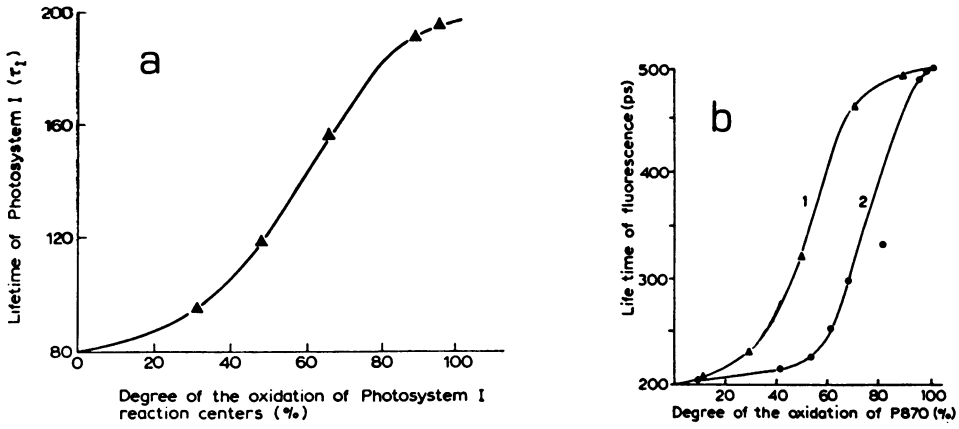

FIGURE 2 *a*. Dependence of the fluorescence lifetime (in picoseconds) of PI light-absorbing pigments in sub-chloroplast particles on the degree of RC oxidation. *b*. Lifetime of the fluorescence emitted by chromatophores from *R. sphaeroides*, 1760-I, as a function of a redox state of BChl P870 of photosynthetic RCs. λ_{exc} = 694.3 nm; λ_{meas} = 730–1,000 nm. The redox state of P870 was monitored by electron spin resonance measurements. P870 was oxidized either chemically by adding potassium ferricyanide (1) or photochemically with background illuminations from a He-Ne laser (2).

and by exposing chloroplasts to saturating intensities of actinic light and blocking PII by DEMU treatment. In all cases, we found only a two- to threefold increase in the maximum values as a result of any of the above-mentioned treatments (Fig. 2 *a, b*). Thus the lifetime of LHA of PI increased from 80 to 20 ps, PII from 300 to 600 ps, and in bacterial chromophores from 300 to 550 ps.

The relatively small observed rise of τ for PSI and LHA indicates that when the RC's are in a closed state and cannot donate electrons to the primary acceptor, the quenching of the singlet excited electron state of LHA still occurs considerably efficiently. The conversion of electron excitation into heat seems improbable, for under "normal" conditions this would lead to considerably lower quantum yields of the primary events of photosynthesis.

It is well known that the oxidation of RCs causes a strong red shift of its absorption band from 700 to 830 nm in the case of RC of PSI and from 830 nm to 1,250 nm in the bacterium *Rhodopseudomonas sphaeroides*. These changes in the absorption spectra must decrease the value of integral overlapping at least by 20–30 times and accordingly increase the τ of LHA by 20–30-fold. But our experiments indicate that the efficiency of energy migration from LHA to RC^+ does not decrease by 20–30 times, but only by a factor of 2 to 3. To explain this result, we propose that oxidation of the RC causes the conformational changes in photosynthetic apparatus, decreasing the distance between the RC^+ and LHA by a factor of 1.3 to 1.4. This is evident if one considers the sixth-power dependence of the K_M on the distance between energy donor and acceptor. It can compensate by the decrease in the value of the overlap integral.

We also studied the temperature dependence of the lifetime of the LHA of PI and

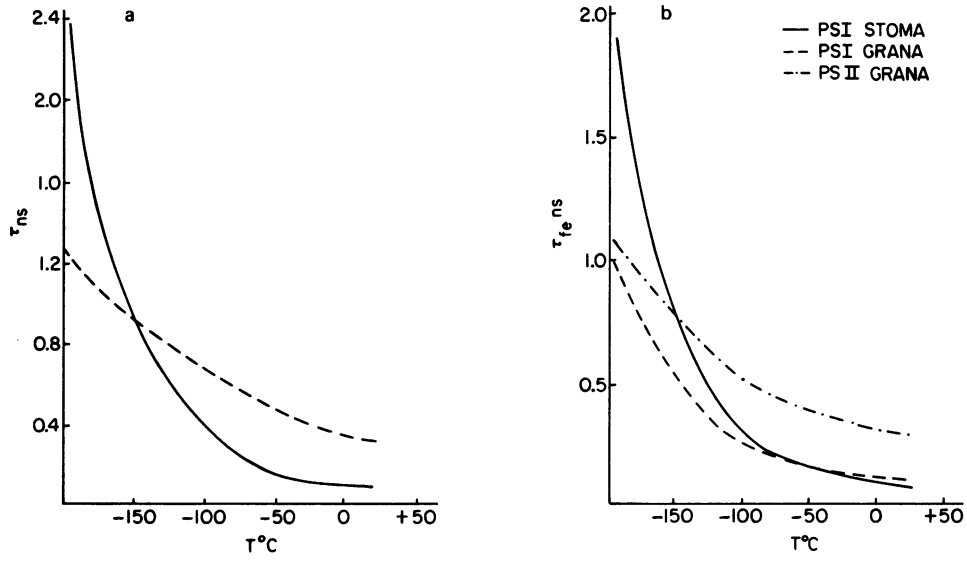

FIGURE 3 *a*. Dependence of the lifetime (τ) of the fluorescence of pea chloroplasts on temperature. Spectral region of the fluorescence measurement: λ = 730 nm (——); λ = 680 nm (- - -). *b*. Dependence of the lifetime (τ) of the fluorescence of PI (stroma), PI (grana), and PII (grana) of pea chloroplasts on temperature.

PII. These results are shown in Fig. 3 *a* and *b*. It was strange for us to find a dependence of τ on temperature because in a pure solution of chlorophyll the dependence of τ on temperature is very slight. But according to our results, the τ of PSI from the stroma part of pea chloroplasts increased from 80 to 2,000 ps and the change in the τ value occurs only below −70°C. These experiments certainly indicate that at temperatures below −70°C the efficiency of energy migration between LHA and RC decreases, although the fluorescence and absorption spectra of LHA and RC do not show any change. We propose that the main reason for the strong increase in the value of the fluorescence lifetime is that the temperature-dependent conformational changes increase the distance between LHA and RC. I want to draw your attention to the fact that the dependence of τ of PI from stroma and grana parts of the pea chloroplasts are different. This means that the structural organization of these photosystems cannot be identical. Therefore, if one studies the nature of PI, one must know whether the sample contains PI particles from the stroma or from the grana part of the chloroplasts.

According to our experimental results, the energy migration from LHA to the RC is provided by inductive-resonance mechanism and it takes about 100–300 ps for the energy of light quanta absorbed by the LHA to be trapped by the RC. It is clear that measurements of the LHA fluorescence lifetime cannot give information as to what happens to the energy of the electron excitation in the RC. To study this problem by picosecond fluorometry, one must provide the direct excitation of the RC's pigments

so that its fluorescence would not be masked by a more intense fluorescence from LHA. These experiments must be done with isolated RC without any antenna chlorophyll or carotenoid molecules.

Unfortunately, because I came to the USA in January 1978, I cannot present the most recent results of B. Gulayev on the primary step of energy conversion in the RC of PI isolated from pea chloroplasts. The details of the electron transfer reactions in the RC, isolated from purple bacteria, have recently been revealed by Rentzepis and co-workers, using picosecond absorption spectroscopy. It was shown that within 10 ps after light absorption the first step of charge separation occurs: electron transfer from the bacteriochlorophyll dimer $(BChl)_2$ to the bacteriopheophytin (BPh); the second step

$$\text{light quanta} \rightarrow (BChl)_2\, BPh\, X \xrightarrow[I]{10\ ps} (BChl)_2^+ BPh^-\, X \xrightarrow[II]{150\ ps} (BChl)_2^+ BPh\, X^-,$$

includes the process of electron transfer from BPh^- to the primary acceptor quinone X.

We have also studied the fluorescence kinetic decay from RCs isolated from *Rhodopseudomonas sphaeroides,* strain 1760-I, after excitation with a picosecond laser pulse. It appeared that the fluorescence decay consists of two components with τ_1 = 15 ± 7 ps and τ_2 = 150 ps (Fig. 4). When the redox potential of the medium is increased to a point that $(BChl)_2$ becomes fully oxidized, the amplitude of the short-lived component diminished, while the lifetime of the long-lived one increased up to 700 ps. A detailed analysis shows that the τ_1 of 15 ± 7 ps corresponds to the fluorescence of $(BChl)_2^+$ and a τ of 250 ps to the BPh.

FIGURE 4 Decay kinetics of the fluorescence from photosynthetic RC perparations made from *R. sphaeroides,* strain 1760-I. λ_{exc} = 694 nm; λ_{meas} = 850–1,000 nm. 1, 100% reduction of P870; 2, 40–50% oxidation of P870, 3, 100% oxidation of P870. The redox state of P870 was posed by adding sodium ascorbate and potassium ferricyanide and monitored by electron spin resonance measurements (after V. Paschenko, 1977).

These results strongly indicate that picosecond fluorometry as well as absorption spectroscopy can give information about the energy distribution and electron transfer in the RC.

According to our measurements, the electronic excitation of $(BChl)_2$ is released within 15 ps in the electron transfer to the BPh. In that case BPh cannot emit fluorescence, since all its π-bonding orbitals are occupied. But the ruby laser radiation 6,943 Å can be directly absorbed by BPh molecules and the excited molecules of BPh emit fluorescence with $\tau_2 = 250$ ps, much shorter than the 2,000-ps lifetime of the fluorescence of BPh in solution. It is reasonable to ascribe such a strong decrease in τ to the electron transfer from BPh to the primary acceptor X. This means that BPh can be the donor of the electron to the primary acceptor X.

Summarizing our results, we come to the conclusion that picosecond fluorometry gives us approximately the same information about the primary steps of electron transfer in the RC as picosecond absorption spectroscopy. I want to draw your attention to the fact that the electron transfer from (BChl) to BPh occurs between the excited electron levels in both molecules and only on the next step of electron transfer from BPh to primary acceptor X will an electron be localized on the ground level X.

Among the problems regarding the picosecond events occurring in the RC, so far little is known about the mechanisms governing the fast electron transfer between components of the RC. To answer this question, we studied the dependence of the fluorescence lifetime of $(BChl)_2$ and BPh on temperature that gives us information about the dependence of the electron transfer from $(BChl)_2$ to BPh and to X (Fig. 5*a, b*). It is clear that the τ of $(BChl)_2$ and BPh fluorescence depends very little on the temperature. That means that the constants of electron transfer within the RC's components are also independent of temperature in the region from 0°C to −186°C. It is interesting to mention that at temperatures of about −70 to −90°C, the τ of $(BChl)_2$ and BPh fluorescence doubled.

These results lead us to the conclusion that the electron transfer within the RC is due to a tunneling mechanism. Without going into details beyond the scope of my lecture, I want to stress that all the proposed tunneling mechanisms invoke the coupling of the electron-nuclear interaction, which dissipates enough energy to prevent back electron tunneling, providing a high efficiency of charge separation.

I want to draw your attention to the fact that electron transfer between the cytochrome and RC also proceeds by a tunneling mechanism, but the dependence on the temperature in the +300 to −70°C region of that step of photosynthesis is quite different from those occurring in the RC itself. That means that the mechanism and nature of processes preventing the back electron transfer reaction may not be identical.

We hypothesize that the charge separation with the RC is identical to the appearance of the dipoles within a very strong electric field. According to measurements provided recently by J. Lee and P. M. Rentzepis, the value of the electric field can exceed 10^5 V · cm^{-1} in the 20 Å area. Such a strong electric field will induce the polariza-

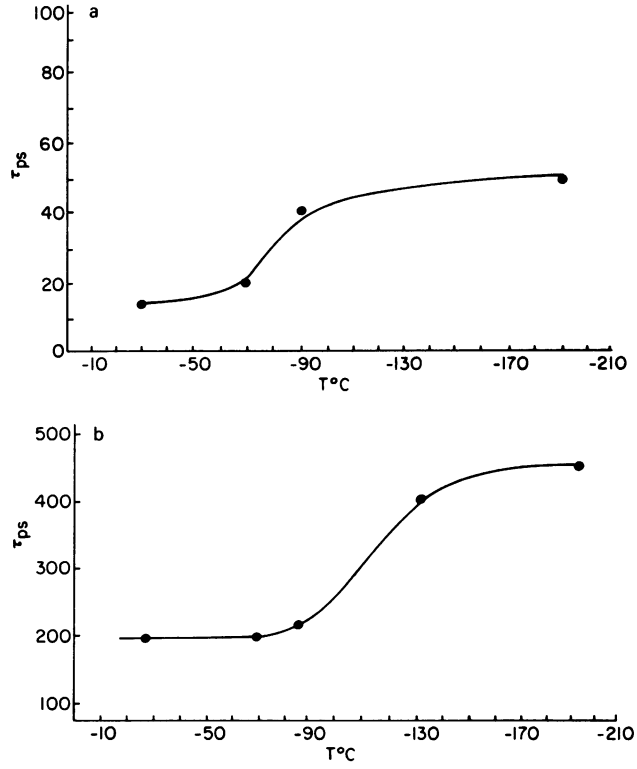

FIGURE 5 Dependence of the lifetime (τ) of the fluorescence of *R. sphaeroides* RCs on the temperature. *a*, fast component; *b*, slow component.

tion of the surrounding media-protein matrix. In its turn, the polarization of protein can change the efficiency of the back electron tunneling.

According to this hypothesis, the local electric fields within the RC may provide excellent feedback that regulates the efficiency of charge separation and stabilization. That means that the changes in the redox state of the components in the RC must influence the constant of the electron transfer. In this case I want to remind you of results that show that oxidation of the $(BChl)_2$ increases the time of the electron transfer from BPh to primary acceptor *X* from 250 ps to up to 700 ps. The paper by M. J. Pellin, C. A. Wraight, and K. J. Kaufmann on page 361 of this Discussion also gives evidence that the reduction of the secondary acceptor changes the constant of the electron transfer between BPh and the primary acceptor.

From this point of view the RC is a unique photoactive enzyme complex, not merely a set of a separate redox reactions. Light-induced electron-conformational changes proceed in a definite sequence and lead eventually to the release of an electron, while the whole complex relaxes through several steps to the original state, thus completing its working cycle. It is possible that the extra energy of ~0.5 eV released for such a

cycle is not lost as heat dissipation, but is stored in some form of membrane-protein conformation.

DISCUSSION

GEACINTOV: You have reported a series of lifetime measurements and it is now known that the intensity of the excitation pulses plays a very important role in the measured decay times. What was the intensity of your pulses in your measurements, and are you sure that nonlinear effects were unimportant in your fluorescence decay measurements?

RUBIN: It is an important question. About two or three years ago it was shown that the lifetimes of PI and PII depend on the light intensity. Light intensities of more than 10^{13} or 10^{14} quanta/cm^2 generate nonlinear effects. In our experiment we use the light energy of a single pulse, 5.10^{12} photons/cm^2.

About two years ago Shapiro published some results saying that everybody who used the high intensity pulse got incorrect results—everybody who used picosecond for fluorescence or photosynthesis, PI as well as PII. Now I know that maybe even you or Shapiro, or both together, have proved that the lifetime of PI does not depend on the light intensity, but it is not so.

GEACINTOV: At low temperatures only! At 77°K, the decay time of the PI emission does not depend on the intensity of the picosecond pulses (at least up to 10^{15} quanta/cm^{-2} $pulse^{-1}$), while that of PII varies strongly with intensity.

RUBIN: But we measured the dependence of the lifetime of the intensity up to the 10^{16} quanta/cm^2. We measured the decrease in lifetime in the region where there is no linear response of the decreasing of lifetime.

GEACINTOV: If your lifetimes were indeed measured with low-intensity pulses, then there seems to be a difference between your measurements and those of other workers using more conventional phase fluorometry and single-photon counting techniques, which show that the decay is longer than 30–200 ps. In fact the values given for the decay times are ~400–800 ps.

RUBIN: 400 ps. You are talking about which lifetime?

GEACINTOV: Lifetime of PI at room temperature.

RUBIN: No. The lifetime of PI at room temperature is 80 ps. But when you lower the temperature the lifetime of PI can be increased up to 2,500 ps.

GEACINTOV: In fact it is difficult to resolve photosystem I emission at room temperature because of its intense fluorescence tail above 700 nm.

RUBIN: We can separate the fluorescence of PI and PII because of their different region of fluorescence. And I tell you that one cannot compare the results of the streak camera measurements of fluorescence decay and the data obtained by phase fluorometry. They are quite different. Phase fluorometry gives you some average lifetime; if there is nonexponential decay it is difficult to get a direct answer.