Biochimica et Biophysica Acta, 1100 (1992) 278–284

BBABIO 43615

Fluorescence of P700 and antenna chlorophylls in Photosystem I particles that contain 11 chlorophylls/P700

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(Received 5 November 1991)
(Revised manuscript received 19 February 1992)

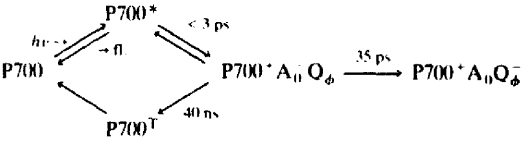
Key words: Photosystem I; Chlorophyll; Fluorescence; Reaction center; Energy transfer; Photosynthesis

The organization of chlorophylls in ether-extracted Photosystem I particles from spinach, which contain 11 chlorophylls per reaction center chlorophyll P700, was studied by analysis of fluorescence at temperatures between 273 and 77 K. P700 appears to emit both prompt and delayed fluorescence, with absorption and emission peaks of P700 at 696 nm and 701 nm, respectively, at 77 K. Both peaks shifted by 4 nm to the blue on warming from 77 K to 273 K. Chlorophylls with absorption peaks at 662, 670, 682, and 686 nm at 77 K transfer excitation energy to P700. Chlorophylls with an absorption peak at 676 nm emit fluorescence with a peak at 679 nm at 77 K and do not transfer excitation energy to P700.

Introduction

The Photosystem I reaction center (PS I RC) complex contains 50–100 chlorophylls per primary donor chlorophyll (P700), as well as carotenoids and electron transfer components [1,2], embedded in two polypeptides of about 80 kDa each [3]. The large number of antenna chlorophyll contrasts with the low chlorophyll content of RCs of PS II [4] or purple bacteria [5,6], which have only four to six chlorophylls and two pheophytins per primary donor chlorophyll, embedded on two polypeptides of about 30 kDa each [4–6]. Detergent treatments of chloroplasts have been used to isolate PS I complexes that contain 20–150 Chl/P700 [2], which have low fluorescence yields at room temperature but high yields at around 735 nm at cryogenic temperatures [7,8]. Ikegami and Katoh [9] developed an ether-extraction method, providing a PS I particle that contains 10–11 total Chl/P700 with a high fluorescence yield [10].

Upon light excitation of PS I RC, an electron is transferred sequentially from P700 to A0, to the secondary electron acceptor phylloquinone Q_ϕ (A_1), to the iron-sulfur center F_x and then iron-sulfur centers F_A/F_B [1,2].



Scheme I

Treatment with diethyl ether extracts the phylloquinone as well as 50–95% of chlorophylls [11]. The removal of Q_ϕ suppresses the rapid (35 ps) oxidation of A_0^- (Scheme I) and enhances the charge recombination between $P700^+$ and A_0^-. The reaction produces P700 either in a triplet state ($P700^T$) [12,13] or in a singlet excited state ($P700^*$) which gives rise to delayed fluorescence [14]. The enhanced fluorescence in this preparation provides a useful tool with which we can study the mechanism of transfer of excitation energy. Emission and excitation spectra of fluorescence from the 11 Chl/P700 PS I particles were analyzed to study the organization of chlorophylls in the PS I RC complex.

Materials and Methods

PS I particles prepared from spinach chloroplasts were freeze-dried and extracted twice with a 2:8 (v/v) mixture of water-free and water-saturated diethyl ether,

This paper is dedicated to Prof. Yoshihiko Fujita on the occasion of his 60th birthday.

Abbreviations: PS, photosystem; Chl, chlorophyll; P700, Photosystem I primary electron donor chlorophyll; A_0^-, primary electron acceptor chlorophyll; Q_ϕ (A_1), secondary electron acceptor phylloquinone; RC, reaction center.

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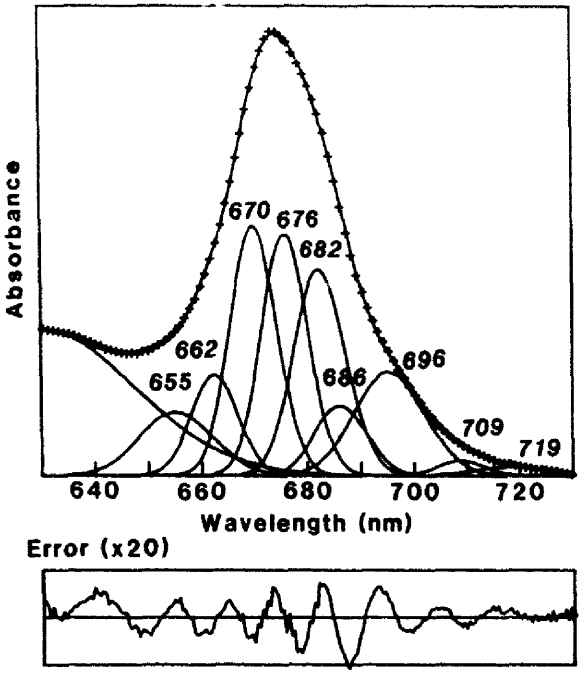
as described previously [9,11,15]. The extracted particles were dispersed in a 50 mM glycine/NaOH buffer (pH 10.0) that contained 0.1% Triton X-100. After removal of undissolved materials, the supernatant was diluted 20–50-times with a medium containing 50 mM Tris-HCl buffer (pH 7.5) and 20% poly(ethylene glycol)-4000 for measurements under continuous illumination, or with a medium containing the same buffer and 50% glycerol for time-resolved measurements. About 95% of chlorophylls, as well as all the phylloquinones and carotenoids, were extracted with almost no change in polypeptide composition. The preparation contained active iron-sulfur centers, F_x, F_A/F_B, and 11.3 molecules of chlorophylls (including P700) per P700 when P700 was determined from the difference (reduced-minus-oxidized) spectrum [16]. P700 was reduced by 2 mM dithionite or oxidized by 0.2 mM ferricyanide for the measurements.

Absorption spectra were measured as described elsewhere [16]. Fluorescence under continuous illumination was measured with a spectrofluorometer (Hitachi-850) with a home-built cryostat and a 2 mm pathlength cuvette. Time-resolved fluorescence emission spectra after laser excitation (532 nm, 10 ns FWHM at 2.5 mJ/cm^2 pulse) were measured as described elsewhere [14].



Gaussian curve analysis of the absorption spectrum of the ether-extracted PS I particles with reduced P700 at 77 K. +, data points; (———) calculated absorption bands. Numbers indicate the peak wavelength of each band. The lower figure represents the difference between data and the simulated spectra.

Results

Absorption spectrum at 77 K

Fig. 1 shows the Gaussian curve analysis of the absorption spectrum (2 at 77 K of the 11.3 Chl/P700 PS I preparation, as performed previously at room temperature [15] and at 90 K [16]. From the area under each curve, the ratios of amounts of chlorophyll forms with peaks at 655, 662, 670, 676, 682, 686, and 696 nm was estimated to be 1.0:1.0:2.4:2.4:2.4:0.8:1.5. Chl *a*-696 is probably responsible for the wide absorption band of P700 (Fig. 1). This band disappears with the concomitant appearance of a new, narrower band at 687 nm upon oxidation by light or chemicals (not shown), as reported elsewhere [16]. Chl *a*-686 is postulated to be the primary acceptor A_0 [16]. However, the roles of the other molecules of chlorophyll *a* and chlorophyll *b* (Chl *b*-655) are unclear. The exact ratio of chlorophyll per P700 depends on the extinction coefficient of P700, which was not determined precisely for this preparation.

Fluorescence emission spectrum

Fig. 2A shows the dependence of the fluorescence emission spectrum of the ether-extracted PS I particles on temperature. When P700 was oxidized the fluorescence had a peak at 680 nm. The peak height increased slightly on cooling (see also Fig. 5A), mainly because of sharpening of the emission band. On reduction of P700 by dithionite (or ascorbate), the fluorescence intensity increased, in particular at about 700 nm, as reported previously [10,14]. The increase was more apparent in the reduced-minus-oxidized difference spectra (Fig. 2B). The peak wavelength of the difference spectrum shifted from 695 nm at 261 K to 702 nm at 92 K. The 695–701 nm component increased significantly on cooling from 270 K to 160 K and remained almost constant below 160 K (also see Fig. 5A).

Fluorescence excitation spectra

Fig. 3A shows the excitation spectra for fluorescence detected at 760 nm under the oxidized and reduced conditions at 77 K. At 760 nm, both the shorter- and longer-wavelength components obtained in Fig. 2 contributed to the emission. Under oxidized conditions, the excitation spectrum had a peak at 675 nm. Upon the reduction of P700, an extended shoulder on the longer-wavelength side was observed, as reported by Ikegami [10]. The difference between the reduced and oxidized excitation spectra (Fig. 3A and B) indicates that the portion of the fluorescence that is increased upon the reduction of P700 is associated with multiple chlorophyll bands with peaks at 670–700 nm. The difference excitation spectra at lower temperatures had a more distinct shoulder on the longer-wavelength side, as shown typically by the spectrum at

105 K in Fig. 3B. This shoulder is explained by the temperature-dependent red-shift from 695 to 701 nm of the longest-wavelength excitation band. This shoulder seems to be correlated with the red-shift of the peak wavelength of the difference emission spectrum on cooling, seen in Fig. 2B. A plausible candidate for the chlorophyll component responsible for these emission and excitation bands is P700, since no other major chlorophyll component in this preparation absorbs and emits at these wavelengths.

Analysis of fluorescence emission and excitation spectra

Fluorescence emission and excitation spectra similar to those in Figs. 2 and 3 were corrected for the sensitivity of the detector system and further analyzed by a Gaussian curve-fitting program, as described by Mimuro et al. [17] (Fig. 4). Initial peak positions for starting the simulation were estimated from the second-derivative spectrum.

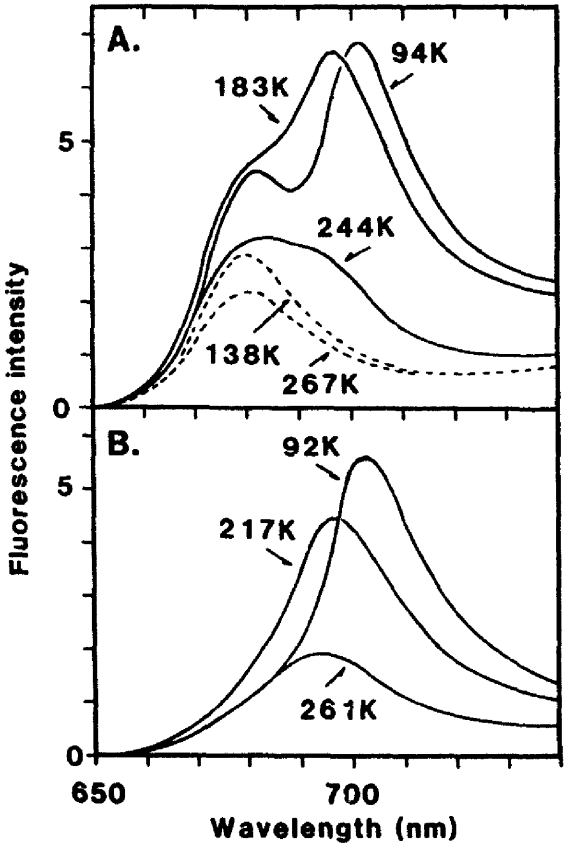


Fig. 2. Dependence on temperature of the fluorescence emission spectrum under continuous illumination. A, fluorescence emission spectra under the oxidized (- - - - - -) and reduced (———) conditions. Spectra were measured every 5 min during warming of the sample from 77 K to 273 K at a rate of 0.5–2.0 degree/min. B, difference between the fluorescence emission spectra measured under reduced and oxidized conditions at similar temperatures (±5 degrees). The concentration of chlorophyll was 5.6 μg/ml. Emission spectra were measured with a slit-width of 2 nm. Excitation: 440 nm with a slit-width of 5 nm.

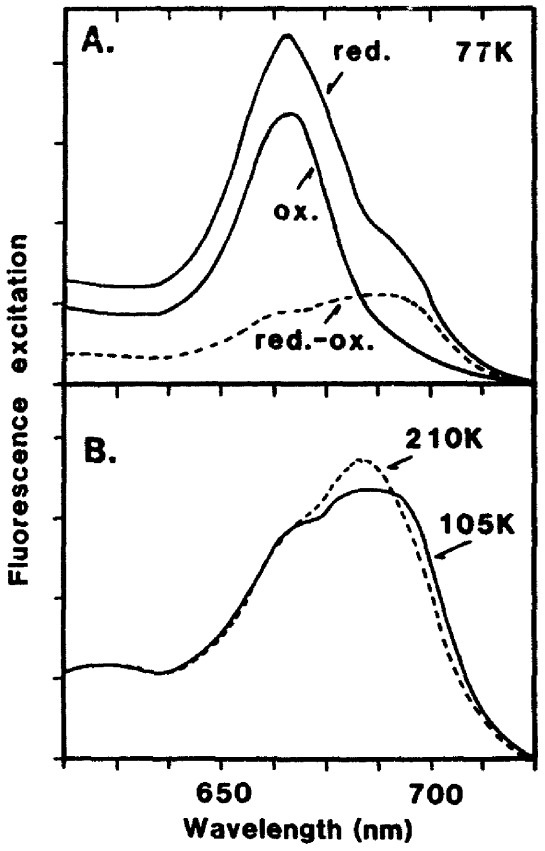


Fig. 3. Fluorescence excitation spectra under continuous illumination. (A) excitation spectra at 77 K under the oxidized (ox.) and reduced (red.) conditions. (- - - - - -) the difference between the spectra under reduced and oxidized conditions. (B) reduced-minus-oxidized excitation spectra at 210 and 105 K. Excitation slit-width, 3 nm. Fluorescence emission was monitored at 760 nm with a slit-width of 5 nm through three layers of Kodak-Wratten-88A filters.

In the oxidized state, the excitation spectrum for the 760 nm fluorescence (Fig. 4A) had a major band with a peak at 677 nm, and minor bands with peaks at 662, 670, 682, 687, 695, 706 and 712 nm. The emission spectrum at 77 K was found to be composed of bands due to chlorophylls with peaks at 670, 679, 685, 691, 700, 708 and 713 nm. Among them the band at 679 nm was predominant and accounted for 43% of the sum of the seven main bands (Fig. 4B and Table I). A chlorophyll with an excitation peak at 677 nm appeared to emit fluorescence at 679 nm by itself and to be inactive in the transfer of excitation energy to other chlorophylls.

The reduced-minus-oxidized difference excitation spectra (Fig. 4C) were deconvoluted into major bands with peaks at 671, 682, 687 and 696 nm, and into minor bands with peaks at 661, 677, 703 and 713 nm. The reduced-minus-oxidized difference emission spectra (Fig. 4D) are composed of major bands at 701, 708 and

715 nm. The 701 nm band made the largest contribution (52%) to this spectrum. Thus, chlorophylls with excitation bands at 671, 682 and 687 nm transfer excitation energy to the chlorophyll that absorbs at 696 nm and emits at 701 nm. The peak positions of the excitation bands almost correspond to those of the absorption bands (see Fig. 1). The Chl *a*-696 that emits at 701 nm is postulated to be P700.

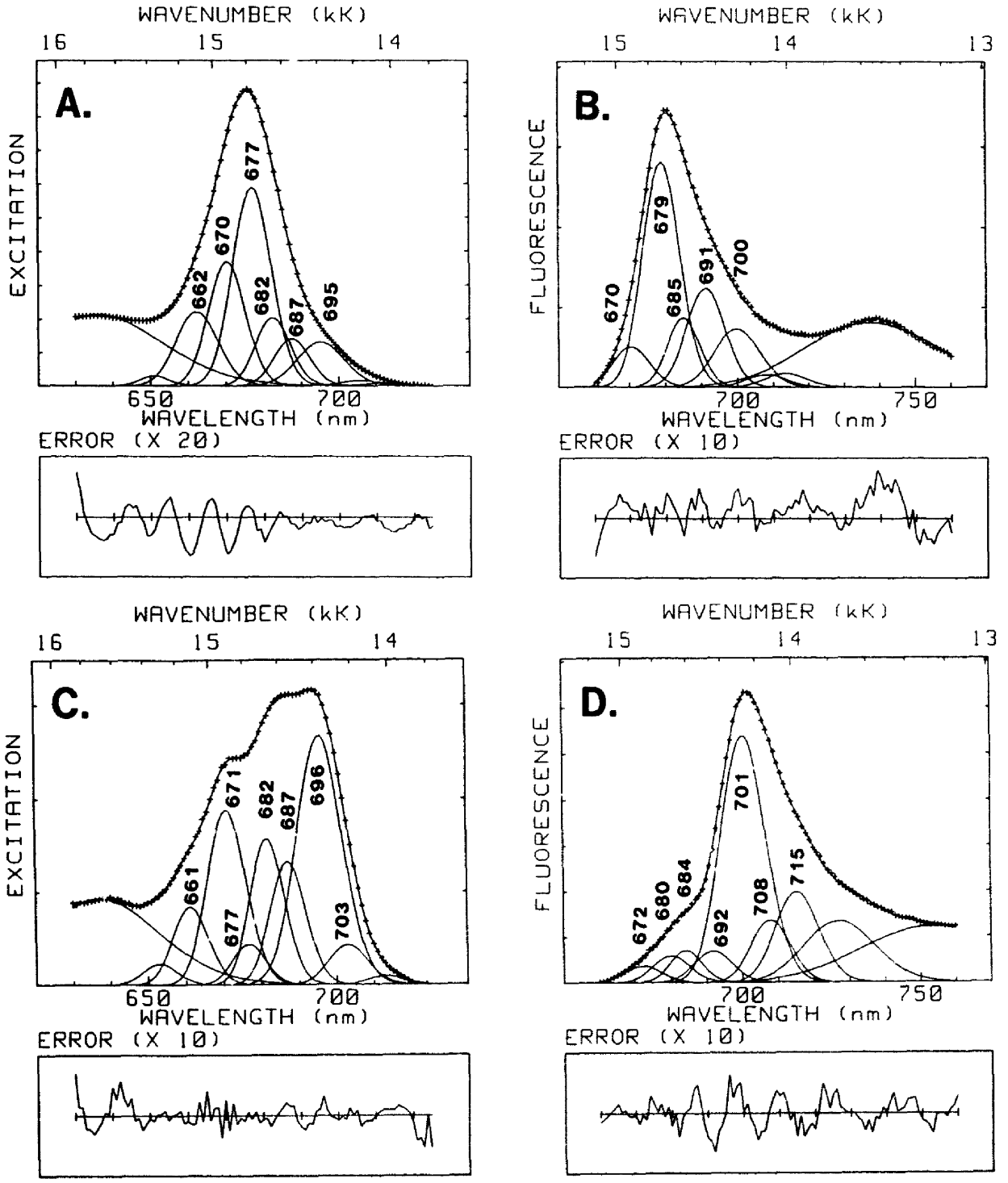


Fig. 4. Curve analysis of the fluorescence emission and excitation spectra at 77 K. A and B, fluorescence excitation and emission spectra, respectively, in the oxidized state. C and D, reduced-oxidized difference fluorescence excitation and emission spectra, respectively. Data points obtained in experiments are shown by plus signs. The errors under each figure represent the difference between data and simulated curves.

The peak wavelengths of the excitation (closed circles in Fig. 5B) and emission (open circles) bands of P700, estimated from the second-derivative spectra, shift by about 4–5 nm towards longer wavelengths on cooling. The peak positions estimated from the absorption spectra showed similar shifts (squares). The shifts are not correlated with changes in fluorescence intensity on cooling (Fig. 5A).

Time-resolved fluorescence emission spectra and decay kinetics

Time-resolved fluorescence spectra at 0 and 50 ns after laser excitation had a dominant band of fluorescence at 701 nm under reduced conditions as previously reported [14] (Fig. 6A and B). Under oxidized conditions, the 701 nm band was eliminated and the emission band at around 680 nm, which decayed with a half time of 6 ns, was detected. These results confirm that the chlorophyll that fluoresces at about 680 nm does not transfer excitation energy to P700.

As previously reported [14], fluorescence decay at 700 nm was biphasic at 110 K (Fig. 6C). The fast and slow decay phases had decay times of less than the response time (3.5 ns) and 100 ns, respectively. Ferricyanide significantly suppressed both phases. The decay time of the slow phase is almost identical to the reported decay time of the $P700^+A_0^-$ biradical [12,18], and is estimated to represent the delayed fluorescence induced by the charge recombination. The yield of delayed fluorescence contributes 17% of the total fluorescence at 110 K (Fig. 6C) with a small increase at 283 K [14]. The increase in the fluorescence intensity at 695–701 nm on cooling under continuous illumination seen in Fig. 5A is, thus, due mainly to the increase in the fast decay phase.

Discussion

Optical properties of P700: temperature-dependent shifts of absorption and emission peaks

P700 seems to be excited at 696 nm and emits at 701 nm at 77 K, as judged from the peak positions and the band widths of difference excitation and emission spectrum (Table I). The fluorescence band changed its peak location from 696 nm to 701 nm on cooling from 273 to 77 K. Peak positions in the second-derivative absorption and fluorescence excitation spectra (Fig. 5B) also indicated a shift from 692 nm to 696 nm on cooling. This result supports a previous suggestion of a shift of the peak wavelength of P700 from analysis of reduced-minus-oxidized difference absorption spectra [16]. The peak shift represents a unique feature of P700, since other chlorophylls in this preparation showed peak shifts of less than 2 nm on cooling (not shown). P700 has been assumed to be a dimer of chlorophyll *a* [1,2] with a wide absorption band [15,16] and a dimer-like circular dichroism signal [15,19,20]. The peak shift and the narrowing of band-width on cooling could be due to a change in the structure of P700. This feature may be similar to that associated with the bactriochlorophyll dimer in the RC of purple bacteria, which shows a more significant peak shift and narrowing of band-width on cooling [21].

TABLE I

Excitation and emission bands of fluorescence at 77 K for the ether-extracted particles, estimated by the deconvolution program

Data were obtained from the analysis for which results are shown in Fig. 5. Band width represents the full width at $1/e$ of the maximum peak height. Area under each Gaussian curve is expressed as a percentage of the sum of the major bands listed in the Table. Minor excitation bands with peak at 651–53 nm and emission bands at 730–760 nm, which cannot be well resolved because of the overlap of vibrational bands, are not indicated.

(1) Oxidized spectra								
Excitation bands								
Peak wavelength (nm)	662.3	670.3	677.0	682.4	687.4	695.0	705.5	711.8
Band width (cm^{-1})	358	307	316	279	280	369	281	296
Area (%)	14.9	20.8	35.7	10.7	7.5	9.0	0.9	0.5
Emission bands								
Peak wavelength (nm)	670.3	679.0	685.1	691.2	699.9	707.8	713.3	
Band width (cm^{-1})	316	314	268	313	359	312	321	
Area (%)	7.9	43.3	11.5	19.0	13.0	2.5	2.8	
(2) Reduced-minus-oxidized difference spectra								
Excitation bands								
Peak wavelength (nm)	661.3	671.0	676.5	681.7	687.1	695.7	703.0	712.8
Band width (cm^{-1})	302	317	269	275	280	353	298	255
Area (%)	8.8	20.9	4.1	15.1	13.0	32.8	4.5	0.8
Emission bands								
Peak wavelength (nm)	672.1	680.1	684.1	691.7	700.5	707.8	715.3	
Band width (cm^{-1})	321	315	264	313	360	305	332	
Area (%)	3.2	4.9	5.0	5.8	51.7	11.1	18.3	

Chlorophylls that are active in energy transfer to P700

The chlorophylls with absorption peaks at 670, 682 and 686 nm appeared to fluoresce weakly at 672, 685, and 692 nm, respectively, at 77 K. Fluorescence yields of these chlorophylls increased upon reduction of P700 and were almost independent of temperature. The chlorophylls contributed significantly to the reduced-minus-oxidized difference excitation spectrum of fluorescence, indicating that they transfer excitation energy to P700. Their fluorescence decay times were estimated to be shorter than the resolution of the detection system (< 3.5 ns), since these bands of fluorescence were seen only at the beginning of the laser flash excitation in the time-resolved fluorescence spectrum [14].

Chlorophyll components with absorption peaks at 703–705 nm and 712 nm also seem to be coupled with P700 with respect to energy transfer, since their contributions are detected in the delayed fluorescence (Fig. 6B) and in the difference emission spectrum (Fig. 4D). However they were present at less than 0.1 molecule per P700 in our preparation.

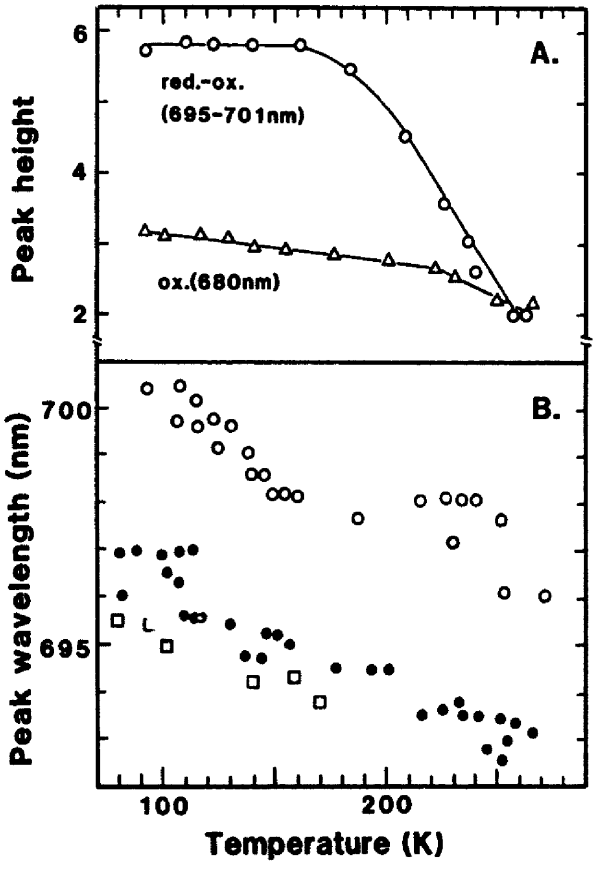


Fig. 5. A, dependence of the peak intensity of fluorescence on temperature. ○, height of the peak at 696–701 nm in the reduced-minus-oxidized difference spectra; △, intensity at 680 nm in the oxidized states B, temperature-dependent changes in peak wavelengths of excitation and emission bands of P700. ●, excitation and ○, emission peak wavelengths. □, peak wavelengths of absorption spectra. The peak wavelengths are estimated from the peaks of the second-derivative spectra.

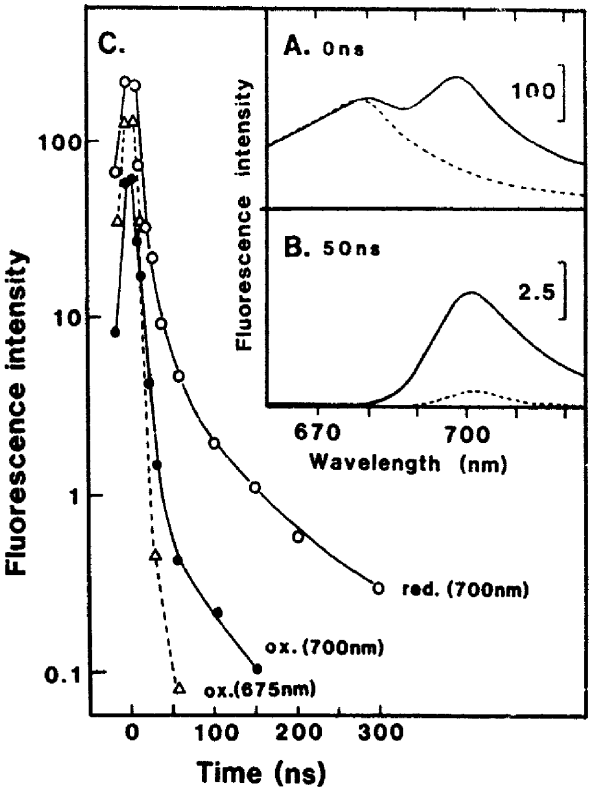


Fig. 6. A and B, time-resolved fluorescence emission spectra at 110 K under the oxidized (· · · · ·) and reduced (———) conditions at 0 and 50 ns after the peak of 10 ns laser flash excitation, respectively. Each spectrum was obtained with the time resolution of 3.5 ns after the accumulation of 16–256 scans at 0.5 Hz. Numbers indicate units of fluorescence intensity, as shown by bars. C, time courses of fluorescence decay at 110 K. Decay at 700 nm in reduced (●) and oxidized (○) conditions. (△) fluorescence decay at 675 nm and 110 K, measured under oxidized conditions. The concentration of chlorophyll was 6.0 μg/ml. The data obtained under reduced conditions were reported previously [14].

Chlorophylls that are inactive in energy transfer

A chlorophyll form with an absorption peak at 676 nm at 77 K was unable to transfer excitation energy to P700. It emitted strong fluorescence with a peak at 679 nm and a half decay time of 6 ns. The fluorescence yield of this band was almost independent of redox conditions and temperature. Chl *a*-676, as well as Chl *b*-655, thus, seems to be uncoupled from P700 as for energy transfer, probably because of the modification of the PS I complex by the treatment with ether or detergent.

Organization of chlorophylls

Chl *a*-676, -662 and -686 appeared to give circular dichroism (CD) signals with a sharp positive 678(+) nm and broader negative 663(−) and 688(−) nm bands,

respectively, at room temperature [15]. Chl *a*-696 (P700) can explain the 694(−) and 683(+) nm pair CD change observed upon oxidation of P700 [15]. The shift of Chl *a*-676 (uncoupled form) seems to explain the 678(−) and 663(+) change in CD induced by oxidation [15]. Chl *a*-670 and 682 do not give corresponding CD bands and, therefore, they appear to be monomeric chlorophylls. Linear dichroism (LD) measurements in squeezed gels at 10 K, on the other hand, indicated that the transitions in the 662–676 nm region are at an angle of about 45° from the orientation of the Q_y transition of P700, which is estimated to lie almost parallel to the membrane surface [22]. The orientation of the Chl *a*-686 (A_0) chromophore is now under investigation by nanosecond dichroic measurements.

Each form of chlorophyll in our preparation, including those effective in the transfer of excitation energy to P700, seems to have a different geometry and function. Two of them constitute a dimeric special pair Chl *a*-696 (P700), and 6–8 molecules (1 × Chl *a*-662, 2–3 × Chl *a*-670, 2–3 × Chl *a*-682, and 1 × Chl *a*-686) transfer energy to P700. Chl *a*-686 also functions as the primary electron acceptor A_0. The rest of the chlorophylls (2–3 × Chl *a*-676 and 1 × Chl *b*-655) are uncoupled from P700 with respect to energy transfer.

Acknowledgements

The authors thank Dr. I. Ikegami of Teikyo University for his valuable advice on the preparation of ether-extracted PS I particles and Mr. A. Murakami for his help in the construction of a nanosecond measurement system. The work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan to S.I. and M.M. and grants from the CIBA-GEIGY Foundation for the Promotion of Science (Japan) to S.I. and the Shimazdu Science Foundation to M.M.

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