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A 160-KILODALTON PHOTOSYSTEM-I REACTION-CENTER COMPLEX

LOW-TEMPERATURE FLUORESCENCE SPECTROSCOPY *

ISAMU IKEGAMI ** and BACON KE

Charles F. Kettering Research Laboratory, Yellow Springs, OH 45387 (U.S.A.)

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Fluorescence changes following sequential reduction of the electron acceptors in the 160 kDa Photosystem-I reaction-center complex (Ikegami, I. and Ke, B. (1984) Biochim. Biophys. Acta 764, 70–79, accompanying paper) were studied. Three major fluorescence bands, designated as F_{675}, F_{655} and F_{705} according to their peak wavelength positions, were observed depending on the redox conditions. F_{675} is essentially constant under all redox conditions, suggesting that it originates from the remaining antenna chlorophyll molecules in the complex. In the oxidized sample containing 0.5 mM ferricyanide, little F_{705} appeared, but it became prominent after the addition of 10 mM dithionite to the complex at pH 10 under anaerobic condition. Maximum F_{705} developed upon a brief intense illumination of the dithionite-treated sample, prior to freezing. On the other hand, F_{705} almost disappeared after extended illumination (20 min) of the sample at a lower temperature (approx. −40°C), in which A_1, the primary electron acceptor of Photosystem I, is assumed to be reduced. F_{705} could be completely restored when the above sample was briefly thawed and refrozen. The excitation spectrum of F_{705} indicates that its emitter had an absorption peak near 455 nm, which changed little in its shape through the above-mentioned changes of F_{705}. We have interpreted these results by proposing the charge recombination between P700$^+$ and A_1^- as the origin of F_{705}. In the oxidized complex containing 0.5 mM ferricyanide, 470 nm excitation brought out an additional emission band at 655 nm (F_{655}), which almost disappeared after intense illumination of the complex in the presence of 10 mM dithionite at pH 10 under anaerobic condition, either for a brief period near 0°C or for an extended period at approx. −40°C. F_{655} was partially restored after extended thawing in the dark of the complex in which A_2 was reduced. The excitation spectrum of F_{655} has a major band at 468 nm, which is attributed to the chlorophyll *b* molecule remaining in the complex. Both emission and excitation spectra showed that the chlorophyll *b* molecule is still capable of transferring its excitation energy to the emitters of both F_{675} and F_{705}.

Introduction

In green plants, the photosynthetic chlorophyll pigments absorb the visible-light quanta, which bring the chlorophyll molecules into the electronically excited singlet state. Under physiological conditions, the bulk of absorbed light energy is

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* Contribution No. 810 from the Charles F. Kettering Research Laboratory.

** Permanent address: Laboratory of Chemistry, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan. Please send reprint requests to this address.

Abbreviations: PS I, Photosystem I; PS II, Photosystem II; Chl, chlorophyll.

used for photochemistry and photosynthesis. A small fraction, however, is either lost as heat or reemitted as fluorescence. At room temperature, fluorescence is emitted mostly near 685 nm by chlorophyll molecules of Photosystem II (PS II). At low temperatures, the fluorescence spectrum usually has bands at 685, 695 and 720–740 nm, presumably originated from both PS I and II [1,2].

With the advent of detergent fractionation of thylakoid membranes of higher plants into fragments representing the two photosystems, much work on their fluorescence followed, mostly in an attempt to identify the various fluorescent species with the two photosystems [3–8]. At room temperature, the emission spectra of both photosystems were similar, although that from PS I had a much lower yield. At 77 K, the F_{725} amplitude is relatively lower than that of F_{685} in PS II fragments, while it is much higher in the PS I fragments, compared with intact chloroplasts. However, the chemical origin of the various fluorescent species remained unknown.

Since the mid-1970's, highly enriched PS I chlorophyll-protein complexes have been prepared. Ikegami and Katoh obtained by diethyl ether extraction a 'P700-enriched' particle containing one P700 per 6–9 Chl molecules [9]. This and other similarly enriched particles were soon utilized for examining the effect of the redox state on fluorescence yield [10–13]. The P700-enriched particles obtained by ether extraction showed fluorescence yield depending on the redox state of both the primary donor and primary acceptors [10]. The variable portion of the fluorescence has a peak at 694 nm at room temperature and 697 nm at 77 K. This emission is quenched by $P700^+$ as well as the oxidized primary acceptor. Maximum fluorescence at 697 nm was observed in samples in which the primary acceptor (as well as the donor) was reduced. Similar findings were subsequently reported by others, variably ascribed either to a photooxidized P700 dimer ($P700^+ \cdot P700$) [12], or to a light-harvesting chlorophyll closely associated with P700 [13].

The low-temperature far-red fluorescence was observed in vivo more than two decades ago in *Euglena gracilis* and *Ochromonas danica* [14], and later in bean leaves [15] and greening bean leaves [16]. From measurements of fluorescence and fluorescence excitation as well as absorption spectra, Butler [15] suggested that F_{725} at 77 K in vivo was due to a component absorbing at 705–710 nm. Furthermore, energy transfer from Chl *a* to the F_{725} species (absorbing at 705 nm) was also demonstrated. From the greening bean leaf work, Goedheer [16] also concluded the F_{725} species to be an essential component in photosynthesis.

While the digitonin-fractionated subchloroplast particles have a high fluorescence yield near 735 nm, the ether-extracted, P700-enriched particles prepared by Ikegami and Katoh has very little of it [9]. It was found recently that reincorporation of chlorophyll together with certain lipids into the P700-enriched particles restored a long-wavelength-absorbing form near 700 nm and a prominent fluorescence emission band near 735 nm at 77 K [17]. It was inferred from these results that the chlorophyll molecules reincorporated into the P700-enriched particles can serve effectively as light-harvesting pigments of PS I.

In the accompanying paper, we have reported the preparation of a 160 kDa PS I reaction-center complex with a Chl/P700 of 10–15. Chemical and photochemical treatments followed by absorption and EPR-spectroscopic analysis showed the complex to be photochemically active and to retain an intact donor-acceptor complex. Because of its simple pigment composition, we have further examined its low-temperature fluorescence properties, in the hope that some light might be shed on the nature of the various fluorescent species.

Experimental

The PS I reaction-center particle was prepared as described in the accompanying paper [18]. The spectrofluorometer used in this work has also been described previously [6], except that two Jobin-Yvon model H10 monochromators were used instead, one for isolating the excitation light from a 150-watt xenon lamp and one for detecting fluorescence emission. Appropriate cut-on and cut-off filters were used so that no excitation light leaks through the detection monochromator. A quartz cuvette with 1 mm pathlength and cradled in a metal holder [19] was used for the low-temperature fluorescence measurements. The cuvette was placed in a dewar and oriented at 45° with respect to the excitation and emission monochromator – both excitation and emission at the front surface of the sample. The total chlorophyll content in the 1 mm pathlength was 4–5 μg Chl/cm^2.

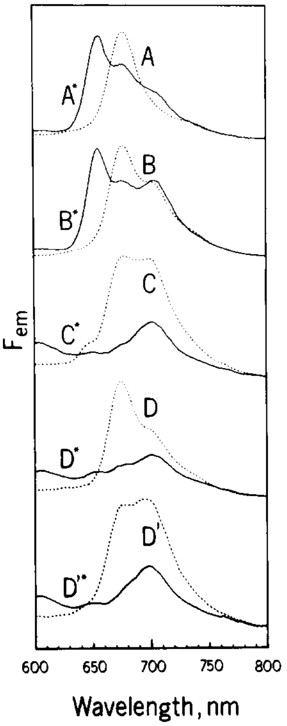
Results

Dependence of the fluorescence emission spectra of the PS-I reaction-center particles on the extent of chemical and photochemical reduction

When 0.5 mM ferricyanide was added to the PS-I reaction-center particle and then illuminated at room temperature for 1 min, all its P700 was converted to the oxidized state. When excited by 430 nm light, the oxidized particle shows a relatively simple fluorescence spectrum, with a single peak at 678 nm (Fig. 1, spectrum A (dashed)). When the sample containing 10 mM dithionite was kept in the dark, a new emission band developed at 702–705 nm (Fig. 1, spectrum B). Under increasingly reducing condition C (see the accompanying paper [18] for explanation of various reducing conditions), spectrum C, with a maximum development of the 702 nm emission band, resulted. When the sample was subject to still more reducing conditions, i.e., condition D with variable periods of illumination, the 702 nm emission gradually disappeared again. For instance, when the sample represented by spectrum C was illuminated for 30 min at −40°C, the 702 nm emission band eventually almost all disappeared (see Fig. 1, spectrum D). Interestingly, when this last sample (spectrum D) was thawed briefly in the cuvette and then refrozen, the 702 nm emission was completely restored (Fig.1, spectrum D′).

Dependence of fluorescence emission spectra of the PS-I reaction-center particles on excitation wavelength

The fluorescence emission spectra for the PS I reaction-center particle at different stages of reduction became dramatically different if the excitation wavelength was changed. Fluorescence spectra of the PS-I reaction-center particle excited at 470 nm for each of the five conditions A to D′ are also shown in Fig. 1. In the sample containing oxidized P700, 470 nm excitation brought out a major new emission band at 655 nm and some noticeable emission at 705 nm (spectrum A* (solid



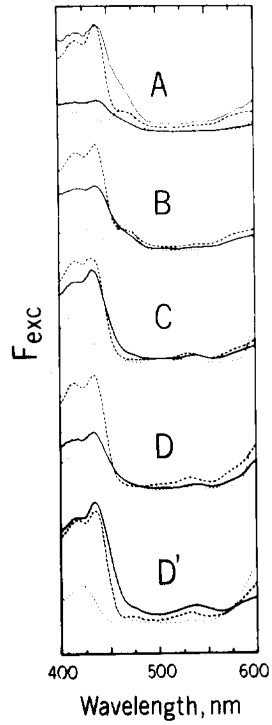


Fig. 1. Fluorescence emission spectra at 77 K of the PS-I reaction-center particles under various redox conditions. Sample was excited with either 430 nm light (dashed line) or 470 nm light (solid line). Sample conditions were: (A) sample containing 0.5 mM ferricyanide and illuminated for 10 s at 0°C, then frozen in liquid nitrogen; (B) sample containing 10 mM dithionite and frozen in liquid nitrogen in the dark; (C) sample containing 15 mM dithionite and illuminated at 0°C for 2 min, then frozen in liquid nitrogen in the dark; (D) the frozen sample in C illuminated further near −40°C for 20 min, then frozen in liquid nitrogen in the dark; (D′) the frozen sample in D briefly thawed in the cuvette and refrozen in liquid nitrogen. The half bandwidths for excitation and emission were 16 and 8 nm, respectively. The emission spectra excited with 470 nm light were recorded at a 3 × gain. The emission spectra were not corrected for the spectral response of the instrument.

Fig. 2. Fluorescence excitation spectra for the emissions at 650 nm (dotted line), 670 nm (dashed line) and 705 nm (solid line) in PS I reaction-center particles under various redox conditions (alphabetical designations same as in Fig. 1). In A, the excitation spectrum for the 705 nm emission was also recorded at a 3 × gain (thinner solid line). The amplitudes of each excitation spectrum at any given wavelength represent the relative fluorescence yield produced by light of the respective excitation wavelength. The half bandwidths for excitation and emission were 8 and 16 nm, respectively. The excitation spectra were automatically normalized with respect to the excitation-light intensities.

curve)). Under condition B, only the 705 nm emission increased further (spectrum B*). Under condition C, the 655 nm emission almost disappeared, whereas some emission remained at 705 nm (spectrum C*). Under condition D, even the 702 nm emission became very weak (spectrum D*). Upon brief thawing followed by refreezing, the 470 nm excited emission spectrum (spectrum D′*) returned to those for state C. Longer thawing (15 min) recovered some 655 nm emission as well (not shown). It is clear that the 702–705 nm emission is at its maximum under condition C, and disappears gradually under condition D with increasing illumination time.

Excitation spectra

Emission bands excited by both 430 and 470 nm light fall in three spectral regions (Fig. 1), hereafter designated as F_{655}, F_{675} and F_{705}. In order to identify the sources of emission, excitation spectra for the three major emission bands were measured (Fig. 2). Since the three major bands vary in amplitude depending on the extent of reduction, the excitation spectra were also measured for the reaction-center particles under different redox conditions.

In the oxidized PS-I reaction-center particle (condition A (light)), the predominant emission band at 678 nm has excitation bands near 440 and 420 nm (dashed spectrum), and a much weaker excitation band at 470 nm. The 705 nm emission was very weak in the oxidized reaction-center particle; its excitation band (solid spectrum) was mainly at 440 nm, with a 450–470 nm shoulder more clearly seen in the same spectrum recorded at a 3 × gain (thin solid spectrum in Fig. 2A). The excitation spectrum of F_{655} has a band near 470 nm (dotted spectrum).

Under condition B, the excitation spectra for F_{675} and F_{655} were essentially the same as those for condition A, the excitation spectrum for F_{705} increased in amplitude without changing the spectral profile.

Under condition C, the excitation spectra for F_{675} and F_{705} remained unchanged from those for condition B, except that the excitation band at 420 nm became more prominent for F_{675}. The most dramatic change occurred in the excitation spectrum of the 655 nm emission: the 470 nm excitation band disappeared completely, and the 420 nm excitation band became prominent. At the same time, a weak excitation band near 530 nm became noticeable for all three emission bands.

Under condition D, the general shape of the excitation spectra for the three emissions remained largely unchanged, only the amplitude of the excitation spectra for F_{705} and F_{655} became less. For condition D′, the excitation spectrum of F_{705} was restored to that for condition C, but that of F_{655} changed little. Only after longer period of thawing (15 min) did the 470 nm excitation band began to reappear (data not shown).

Discussion

As discussed in Ref. 18, the 160 kDa aggregate complex of the PS I reaction center, because of its high purity, simple composition and photochemical activity, has provided an experimental material for measuring the difference spectrum of not only P700, but all the early electron acceptors of PS I. As seen below, the highly purified aggregate complex appears to have possibly also provided some new insights about the fluorescing species of PS I.

Under different redox conditions and by using excitation light at two different wavelengths, fluorescence emissions can be grouped into three spectral regions and designated as F_{655}, F_{675} and F_{705} according to their peak wavelength positions. Among the three emissions, F_{675} is essentially constant under all redox conditions examined (Fig. 1). The excitation spectra are also invariable, with major bands near 420 and 440 nm (Fig. 2). This emission most likely originates from the antenna chlorophyll *a* molecules remaining in the 160 kDa reaction-center complex.

F_{655}, with its major excitation bands at 468 nm, is attributed to chlorophyll *b* present in the PS I reaction-center complex. The appearance of $F655$ when excited by 470 nm light under conditions A and B (Figs. 1 and 2) are consistent with the notion of chlorophyll *b* involvement. The disappearance of F_{655} emission and the 468 nm excitation bands starting from condition C lend further support to our previous interpretation that the absorbance decreases at 650 and 468 nm and an increase at 527 nm at the redox level of A_2 or X reduction involved chlorophyll *b* reduction [18]. In agreement with the appearance of the 527 nm absorption band under condition C [18], a weak 530 nm band also appeared in the excitation spectrum under the same conditions.

Chlorophyll *b* in vivo usually does not fluoresce because of its highly efficient transfer of excitation energy to chlorophyll *a*. A weak shoulder near 660–665 nm was reported previously in the emission spectrum of some green algae and attributed to chlorophyll *b* under conditions of decreased efficiency in energy transfer from chlorophyll *b* to *a* when the PS II traps are closed [20]. In our 160 kDa PS-I reaction-center complex, chlorophyll *b* molecules which are usually associated with the light-harvesting complex undoubtedly are detached from the membrane matrix. But it is rather astonishing that one chlorophyll *b* molecule has survived such extended solvent and detergent treatment by which more than 95% of the bulk chlorophyll was removed. Furthermore, this chlorophyll *b* molecule apparently is still capable of transferring its excitation energy to the emitters of both F_{675} and F_{705} (Figs. 1 and 2). All spectral evidence indicates that chlorophyll *b*, though unlikely to be part of the reaction center, may be located in very close proximity to it.

Of further interest is the F_{705} emission and particularly its dependence on the redox state of the PS I reaction center. There is little F_{705} when P700 is oxidized (Fig. 1A). F_{705} begins to develop when P700 is reduced and all the acceptors are oxidized (in the presence of ascorbate, for instance; data not shown) or when Fe-S centers A and B are reduced (Fig. 1B). F_{705} reaches the maximum level when most or all A_2 (or X) is reduced. Under condition D, in which A_1 is assumed to be reduced, F_{705} largely disappeared. In briefly thawed and refrozen reaction-center complex (sample D′), F_{705} was completely restored. The excitation spectrum for F_{705} appears rather similar to that for F_{675}, except for a more prominent shoulder around 460 nm (Fig. 2A). The difference between the excitation spectra for F_{675} and F_{705} results in a 455 nm peak with a slight shoulder around 470 nm. These results indicate that the excitation energy can be transferred from antenna chlorophyll *a* (and *b*) to the emitter of F_{705}, and that the latter has an absorption peak at 455 nm, suggesting that it may be a special form of chlorophyll *a*.

Considering all the experimental data on F_{705}, we propose that the F_{705} emission originates from A_1^* formed during charge recombination between $P700^+$ and A_1^-. The experimental observations reported thus far appear to be consistent with the proposed mechanism. Thus, little F_{705} should be formed when the reaction partner P700 is already oxidized. When Fe-S centers A and B are oxidized, they would be expected to receive the electron coming down from A_1^-. Under this condition, possibly a small probability of charge recombination still exists, and thus a small F_{705} emission. When centers A and B are reduced, the probability of forward electron transfer is attenuated, thus increasing the probability of charge recombination between $P700^+$ and A_1^- and thus a greater F_{705} emission. When A_2 or X is reduced, maximum charge recombination would lead to maximum F_{705} intensity. Upon phototrapping of A_1^- and restoration of $P700^+$ to P700, F_{705} should decrease in proportion to A_1 phototrapped. In fact, a good parallel correlation has been obtained between the extent of the 14 G EPR signal formed (see Fig. 4, bottom, in Ref. 18) and the extent of photochemical bleaching of the 670 nm absorption band, both of which represent a direct measurement of the extent of reduction of A_1, with the attenuation of

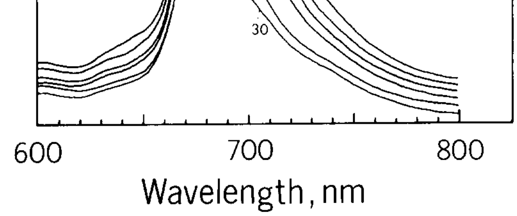


Fig. 3. Decrease in F_{705} intensity when the PS I reaction-center particle containing 10 mM dithionite was illuminated at approx. −40°C for various time periods (in min). Inset shows F_{705} intensity plotted vs. illumination time. The jump (arrow) represents complete restoration of F_{705} after the last sample was briefly thawed and refrozen (D to D′).

F_{705}, all under condition D and as a function of the illumination time (Fig. 3).

It is worth noting that the present proposal is almost the counterpart of one recently proposed by Breton [21] for the F_{695} emission originated from PS II. In PS II, the reaction partners involved in charge recombination are $P680^+$ and $Pheo^-$. Thus, F_{695} in PS II would, according to Breton, originate from Pheo* formed during charge recombination.

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References

1 Goedheer, J.C. (1972) Annu. Rev. Plant. Physiol. 23, 87–112

2 Papageorgiou, G. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 319–371, Academic Press, New York

3 Cederstrand, C.N. and Govindjee (1966) Biochim. Biophys. Acta 120, 177–180

4 Kok, B. and Rurainski, H.J. (1966) Biochim. Biophys. Acta 126, 584–587

5 Boardman, N.K., Thorne, S.W. and Anderson, J.M. (1966) Proc. Natl. Acad. Sci. U.S.A. 56, 586–593

6 Ke, B. and Vernon, L.P. (1967) Biochemistry 6, 2221–2226

7 Vredenberg, W.J. and Slooten, L. (1967) Biochim. Biophys. Acta 143, 583–591

8 Mohanty, P., Braun, B.Z., Govindjee and Thornber, J.P. (1972) Plant and Cell Physiol. 13, 81–91

9 Ikegami, I. and Katoh, S. (1975) Biochim. Biophys. Acta 376, 588–592

10 Ikegami, I. (1976) Biochim. Biophys. Acta 449, 245–248

11 Vacek, K., Wong, D. and Govindjee (1977) Photochem. Photobiol. 26, 269–276

12 Brown, J.S. (1977) Photochem. Photobiol. 26, 519–525

13 Telfer, A., Barber, J., Heathcote, P. and Evans, M.C.W. (1978) Biochim. Biophys. Acta 504, 153–164

14 Brody, M. and Linshitz, H. (1961) Science 133, 705–706

15 Butler, W.L. (1961) Arch. Biochem. Biophys. 93, 413–422

16 Goedheer, J.C. (1961) Biochim. Biophys. Acta 53, 420–422

17 Ikegami, I. (1983) Biochim. Biophys. Acta 53, 492–497

18 Ikegami, I. and Ke, B. (1984) Biochim. Biophys. Acta 764, 70–79 (this issue)

19 Ke, B. (1972) Methods Enzymol. 24, 25–53

20 Govindjee and Briantais, J.-M. (1972) FEBS Lett. 19, 278–280

21 Breton, J. (1982) FEBS Lett. 147, 16–20