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FLUORESCENCE OF BACTERIOCHLOROPHYLL AS RELATED TO THE PHOTOCHEMISTRY OF CHROMATOPHORES OF PHOTOSYNTHETIC BACTERIA

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SUMMARY

Time courses and the emission spectra of fluorescence and light-induced absorption changes of P890 in chromatophores of the photosynthetic bacteria *Chromatium* D, *Rhodopseudomonas spheroides* and *Rhodospirillum rubrum* were investigated.

The time course of fluorescence in chromatophores was separated into two phases, *i.e.* an initial rapid rise (f_i) and a subsequent slow increase towards a steady level of emission (f_v). The f_i and the f_v components showed different emission spectra having different peak position. The f_v component was emitted from the longest wavelength-absorbing form of bulk bacteriochlorophyll (B890), the f_i component from both B890 and B850.

The magnitude of the f_v component depended on experimental conditions controlling the states of the cyclic electron transport in chromatophores, including changes in levels of redox potential of the medium, additions of electron donors and inhibitors. The magnitude of the f_i component was not affected by these experimental conditions. It was, therefore, concluded that only the f_v component is related to the cyclic electron transport, and that the magnitude of f_v is controlled by the oxidation–reduction state of the primary electron acceptor for the photochemical reaction center in chromatophores.

INTRODUCTION

The time course of fluorescence in cells or chromatophores of photosynthetic bacteria is divided into two phases, namely, a rapid rise of fluorescence at the onset of illumination and a subsequent slow increase towards a steady level of fluorescence. This effect was first noticed by Vredenberg and Duysens[1] in cells of

Abbreviations: PMS, phenazine methosulfate; DCIP, 2,6-dichlorophenolindophenol.

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Rhodospirillum rubrum. Clayton[2] observed in cells of a green photosynthetic bacterium *Chloropseudomonas ethylicum* that the emission from the major pigment, *Chlorobium* chlorophyll, predominated in the initial rapid component of fluorescence, whereas the emission from the minor pigment predominated in the variable part of the fluorescence. Zankel and Clayton[3], in a study in which the intensity of the excitation light was changed 10-fold, showed that there occurred two components of fluorescence emission, F860 and F890, in chromatophores of a green mutant of *Rhodopseudomonas spheroides* strain Ga, and that the relative magnitude of the longer wavelength emission, F890, was higher at higher intensity of excitation light.

A generally accepted view is that the variable part of the fluorescence is intimately related to the state of electron transport in photosynthetic bacteria, as well as in green plants[1,4-6]. Vredenberg and Duysens[1] inferred that the total fluorescence yield depended on the oxidation–reduction state of the reaction center P890, and pointed out a linear relationship between the reciprocals of the fluorescence yield and the amounts of the light-induced change of P890 in cells of *Rsp. rubrum*. Clayton[4] concluded that the relation between fluorescence and the reaction center was best expressed in terms of an "open" or "closed" state of the trap. On the other hand, Cramer[7], working with chromatophores of *Chromatium*, *Rps. spheroides* and *Rsp. rubrum*, suggested the contribution of the primary electron acceptor for the fluorescence yields. The aim of the present study is to elucidate the mechanism by which the fluorescence yields of bacteriochlorophyll in chromatophores are controlled. Comparative studies were made, using chromatophores isolated from *Chromatium* D, *Rps. spheroides* and *Rsp. rubrum*.

MATERIALS AND METHODS

The photosynthetic bacteria used were *Chromatium* D, *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*. Cells from 3 to 5 days of growth (inorganic medium of Newton and Kamen[8] for *Chromatium*; organic medium of Ormerod *et al.*[9] for *Rsp. rubrum* and *Rps. spheroides*) were disrupted in a French Pressure Cell (Ohtake Co., Japan). Chromatophores were collected by fractional centrifugation of the disrupted cell suspension between 10 000 × *g*, 20 min and 80 000 × *g*, 60 min.

Light-induced changes of P890 were measured at 788, 810 and 890 nm, using an Aminco–Chance dual wavelength spectrophotometer. Actinic light was obtained by passing the light from a 650-W tungsten–iodine lamp (Ushio Electronic Co.) through a combination of a 590-nm interference filter and a band-pass filter that transmitted the light between 500 and 700 nm.

Fluorescence was analyzed using a Bausch and Lomb grating monochromator or an interference filter (930 nm). Cut-off filters (Wratten 88-A and a Toshiba VR-69) were inserted between the sample and the monochromator to exclude stray light. The excitation light was supplied with the same combination of the light source and filters as described for the measurements of the P890 change. The signals of fluorescence thus obtained were amplified and recorded on a strip chart servorecorder or on a memory oscilloscope (Hitachi V-108). The magnitude of fluorescence at each wavelength measured was corrected for the sensitivities of the photomultiplier (7102, Hamamatsu Television Co.) and the monochromator.

Concentrations of chromatophores used in the determination of emission spectra were adjusted to give an absorbance of 0.1 at the position of maximal peak in the near infrared region in each photosynthetic bacterium. The chromatophore concentrations used in the measurements of time courses of fluorescence and the light-induced change of P890 were 0.2 to 0.3 at the absorbance maximum.

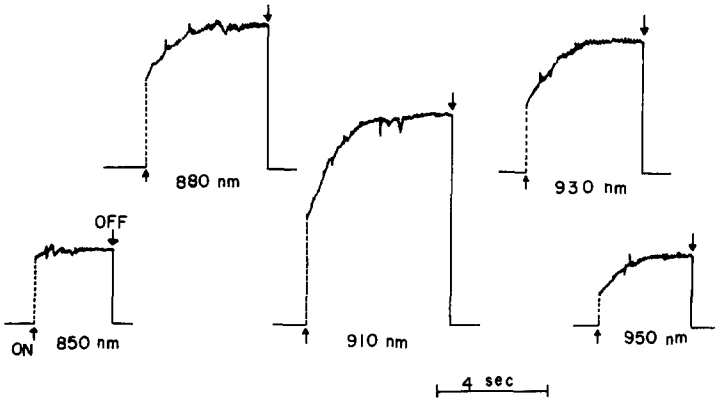

Fig. 1. Time course of fluorescence in *Chromatium* chromatophores in the presence of 1 mM ascorbate. Excitation light, 590 nm, 10^4 ergs/cm^2 per s. Fluorescence was measured at 850, 880, 910, 930 and 950 nm (bandwidth 10 nm). Absorbance of the chromatophores at 810 nm, 0.1.

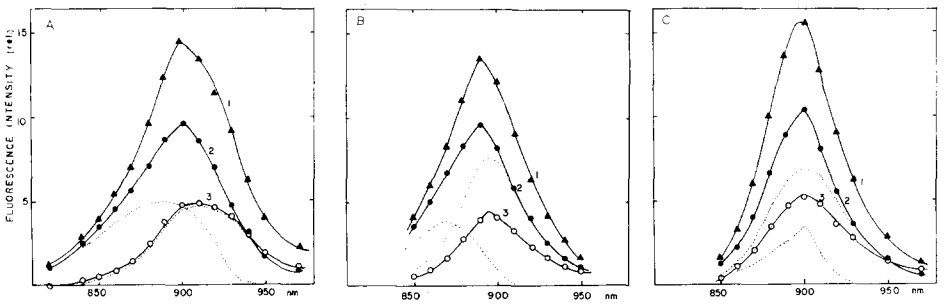

Fig. 2. Fluorescence emission spectra of the chromatophores in the presence of 1 mM ascorbate. Experimental conditions are the same as for Fig. 1. (A) *Chromatium*. (B) *Rps. spheroides*. (C) *Rsp. rubrum*. 1, total fluorescence; 2, f_i component; 3, f_v component. Dotted lines, two constituent parts of f_i component (see text).

RESULTS

Two components of fluorescence emission in chromatophores of photosynthetic bacteria

Time courses and emission spectra of fluorescence

The time course of fluorescence of bacteriochlorophyll in chromatophores consisted of two distinct phases as reported by other investigators[1,2]. We will denote the magnitudes of these changes as f_i (initial fluorescence)and f_v (variable fluorescence), respectively. Fig. 1 shows the time courses of fluorescence in chromatophores of *Chromatium* in the presence of 1 mM ascorbate (added as electron donor). The

magnitudes of f_i and f_v and the ratio of f_i to f_v varied according to the emission wavelengths. Fig. 2A shows the emission spectra in chromatophores from *Chromatium* in the presence of 1 mM ascorbate. The peak position in the steady state of fluorescence appeared at about 900–905 nm, whereas, the peaks of the f_i and f_v components were at 900 and 910 nm, respectively. Similar results with respect to the differences of the peak positions and the shapes of the f_i and f_v spectra were obtained in chromatophores of *Rsp. rubrum* and *Rps. spheroides*. The emission peaks were located at 890 nm (f_i) and 895–900 nm (f_v) in both species (Figs 2B and 2C).

Effects of oxidation–reduction reagents on the emission spectra

The addition of a strong reductant such as $Na_2S_2O_4$ has been known to increase the fluorescence yields in chromatophores[10,11]. In view of the above-observed difference in the nature of f_i and f_v, the effects of dithionite on the fluorescence of chromatophores were investigated in more detail. The initial rise of fluorescence was greatly accelerated by the presence of dithionite (10 mM), so that the final steady-state level was attained without any detectable period of induction (Fig. 3).

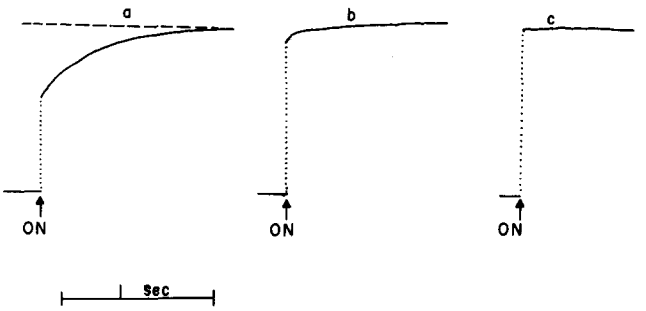

Fig. 3. Time course of fluorescence in *Chromatium* chromatophores. Excitation light, 590 nm, 10^4 ergs/cm^2 per s. Fluorescence was measured at 930 nm. Absorbance of chromatophores at 810 nm was 0.1. Additions: (a) 1 mM ascorbate; (b) 0.1 mM $Na_2S_2O_4$ was added to (a); (c) 10 mM $Na_2S_2O_4$ was added to (a).

The following experiment was carried out with the purpose of deciding whether the addition of dithionite caused an increase in magnitude of the f_i component, or an accelerated rise of the second component, f_v.

Fig. 4 shows the emission spectra in the presence and absence of dithionite in chromatophores of *Chromatium*. Curve 1 represents the emission spectrum for the steady state of fluorescence in the presence of 1 mM dithionite. This curve is similar in shape and peak position to that of the emission spectrum in the absence of dithionite shown in Fig. 2A-1. The emission spectrum for f_i in the absence of dithionite (Fig. 2A-2) is reproduced as Curve 2 and a difference spectrum (Curve 3) was drawn by subtracting Curve 2 from Curve 1. The pattern of the difference curve was reminiscent of that of the emission spectrum for f_v observed in the absence of dithionite (Fig. 4, Curve 4), both showing a peak at around 910 nm. This was taken as indicating that the change caused by the presence of dithionite consisted in an acceleration of the f_v component. Similar results were also obtained with chromatophores of *Rsp. rubrum* and *Rps. spheroides*.

The fluorescence yields in bacterial chromatophores were altered by various experimental conditions, such as changes in the redox potential of the medium, additions of electron donors (*e.g.* ascorbate and diphenylcarbazide[12]) and an electron carrier for the cyclic flow of electrons (phenazine methosulfate (PMS) [13,14]). However, it was always the magnitude of f_v that was altered by these experimental factors, whereas the band position, as well as the shape of the emission spectrum for this component of fluorescence, remained unchanged in chromatophores of all the photosynthetic bacteria tested. The magnitude of f_i was not altered, nor was the position or shape of its emission spectrum changed, provided the treatments were not so drastic as to cause a destruction or bleaching of bulk bacteriochlorophyll in chromatophores as reported by Goedheer and Van der Tuin[15].

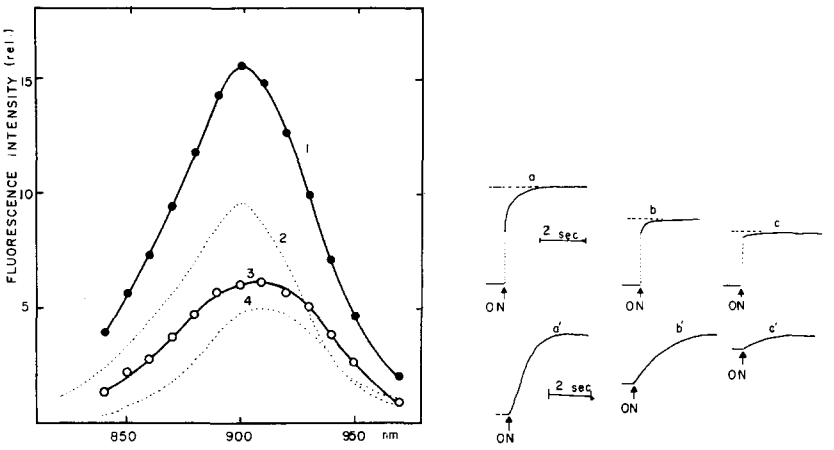

Fig. 4. Fluorescence emission spectra of *Chromatium* chromatophores. Excitation light, 590 nm, 10^4 ergs/cm^2 per s. Absorbance at 810 nm, 0.1. 1, total fluorescence (1 mM ascorbate and 10 mM $Na_2S_2O_4$); 2, f_i component, the same as in Fig. 2; 3, difference emission spectrum obtained by subtracting 2 from 1; 4, f_v component, the same as 3 in Fig. 2.

Fig. 5. Time course of fluorescence and light-induced change of P890 in *Chromatium* chromatophores. Excitation and actinic light, 590 nm, 10^4 ergs/cm^2 per s. Absorbance at 810 nm, 0.2. Fluorescence (> 720 mm) was measured using a Corning 2600 and a Toshiba-VR-69 filter (see Methods). The P890 change was measured at 788 nm. (a), (b) and (c) fluorescence; (a'), (b') and (c') P890 change. Additions: (a) and (a') 1 mM ferricyanide and 3 mM ferrocyanide; (b) and (b') 1 mM ferricyanide and 0.1 mM ferrocyanide; (c) and (c') 1 mM ferricyanide and 0.01 mM ferrocyanide.

Behavior of f_i and f_v as related to the photochemistry of bacterial photosynthesis

The experimental results described in the preceding section indicate a difference of nature between f_i and f_v. There arises a need of re-investigating the behavior in these two components as separate entities. With this in view, the following experiments were carried out with the aim of elucidating the mechanisms by which the change of these components of fluorescence are related to the photochemistry of the bacterial chromatophores. The behavior of P890, which has been shown to have some correlation with the fluorescence changes[1,4], was also investigated.

Dependence of fluorescence emission and the P890 change on redox potential of the medium

Fig. 5 shows the time course of the light-induced change of fluorescence and that of P890 in chromatophores of *Chromatium* in reaction media of varied levels of redox potential regulated by changing the ratio of ferri- to ferrocyanide. An increased ratio of ferri- to ferrocyanide decreased the magnitude of f_v. Such relationship was also found with respect to the magnitude of the light-induced change of P890, as reported by other investigators[22,23]. Fig. 6 shows the dependence of f_v and the P890 change on the redox potential of the medium. Both changes were measured at saturating light intensity (10^4 ergs/cm^2 per s). Both the changes of f_v and P890 are in accord with the respective theoretical curves drawn for a one-electron change. The experimental results were not altered by changing the concentration of the redox buffer without changing the ratio of ferri- to ferrocyanide. The values for the midpoint potentials for f_v differed from those for the P890 change. The values were 415 mV and 460 mV for f_v and the P890 change, respectively, in *Chromatium* (Fig. 6A), and 415 and 430 mV in *Rps. spheroides* (Fig. 6B), assuming a value of 409 mV for the normal redox potential of the ferri- to ferrocyanide system[16].

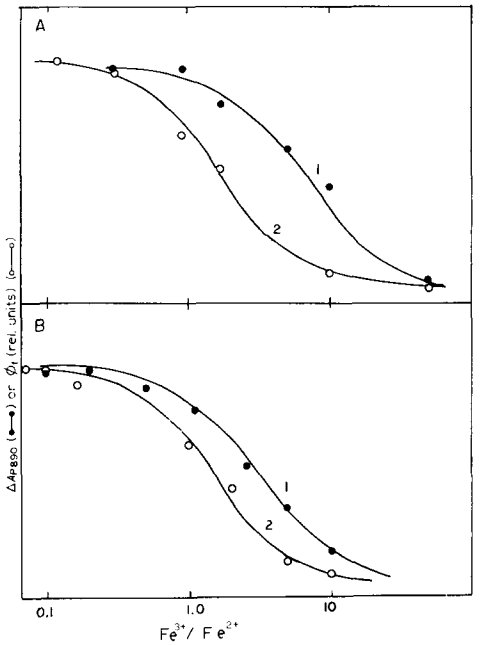

Fig. 6. Dependencies of the light-induced change of P890 (1) and the yield of f_v (2) on the redox potential in chromatophores of *Chromatium* (A) and *Rps. spheroides* (B). Concentration of ferricyanide, 1 mM. Other conditions are the same as for Fig. 5. The curves were normalized to coincide at the maximal levels of change.

Effects of electron donors on f_v and the P890 change

Ascorbate serves as an electron donor for the electron transport system in photosynthetic bacteria. At low concentrations of ascorbate (10–100 μM), the magnitudes of both the f_v and the light-induced P890 change were increased by the

addition of ascorbate (Fig. 7); f_i was not affected. At higher concentrations of ascorbate (1 to 10 mM), the light response of P890 took a complicated time course as shown in Fig. 7, Curve c. In contrast, the magnitude of f_v was not further altered by increasing the concentration of ascorbate.

Diphenylcarbazide, which is in current used as an electron donor for System II of photosynthesis in chloroplasts[12], was also effective as a donor in the bacterial electron transport, for instance, for the reduction of cytochrome c_{555} in *Chromatium* chromatophores (unpublished data not shown in the present paper). This electron donor, although not shown in Fig. 7, had similar effects on the P890 change and on the f_v to those with ascorbate.

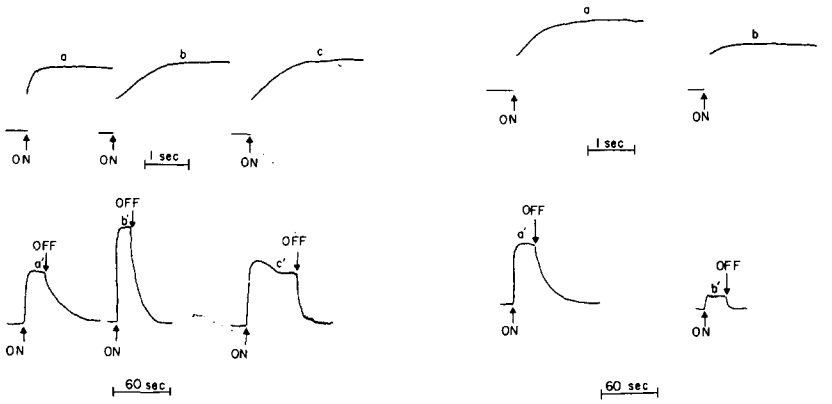

Fig. 7. Time courses of fluorescence and the light-induced change of P890 in *Chromatium* chromatophores. (a)–(c) fluorescence; (a')–(c') P890 change. Additions: (a) and (a') none; (b) and (b') 0.1 mM ascorbate; (c) and (c') 1 mM ascorbate. Other conditions are the same as for Fig. 5.

Fig. 8. Time course of fluorescence and the light-induced change of P890 in *Chromatium* chromatophores. (a) and (b) fluorescence; (a') and (b') P890 change. Additions: (a) and (a') 1 mM ascorbate; (b) and (b') DCIP(0.01 mM) and 1 mM ascorbate. Other conditions are the same as for Fig. 5.

The 2,6-dichlorophenolindophenol(DCIP)–ascorbate couple is also effective as an electron donor in the electron transport in photosynthetic bacteria[17,18]. Addition of this donor couple (10 μM DCIP and 1 mM ascorbate) caused a decrease of about 75 % in magnitude of f_v as compared with its level obtained on the addition of 1 mM ascorbate alone. The level of f_i was not changed. The effects of the DCIP–ascorbate couple on the fluorescence and the P890 change were similar to those described below for PMS, but were less effective when compared at the same concentration of the dye (*cf.* Fig. 9). Fig. 8 shows the effects of the DCIP–ascorbate couple on *Chromatium* chromatophores.

The reduced form of PMS opens up a short circuit between the pool near the primary electron acceptor and cytochrome c_2 or c_{555} in the chromatophores[13,14]. When PMS was added to *Chromatium* chromatophores, the light-induced change of P890 was greatly suppressed, and disappeared at a still higher concentration of PMS (10 μM). The magnidude of f_v was also suppressed in a similar way, whereas f_i remained unaffected. As will be discussed later, the observed suppression of f_v may be related to an increased rate of the dark recovery of P890 as indicated by the steep decay of P890 in the dark, presumably through the short circuit (Fig. 9, Curve

b). Similar behavior of the P890 change and the fluorescence towards the addition of PMS was also observed in chromatophores of *Rsp. rubrum* and *Rps. spheroides*.

Effects of antimycin A on f_v and the P890 change

Antimycin A is known to inhibit the cyclic electron transport in photosynthetic bacteria[19,20]. In the presence of ascorbate (1 mM), antimycin A caused an increase in magnitude of f_v. In a medium containing the DCIP–ascorbate couple, addition of antimycin A also caused an increase in magnitude of f_v, but the magnitude of the increase was smaller than that produced by the addition of antimycin A in the presence of ascorbate alone. In the presence of PMS, antimycin A had no effect on the magnitude of f_v. Under all these conditions, the magnitude of f_i was not affected by the inhibitor.

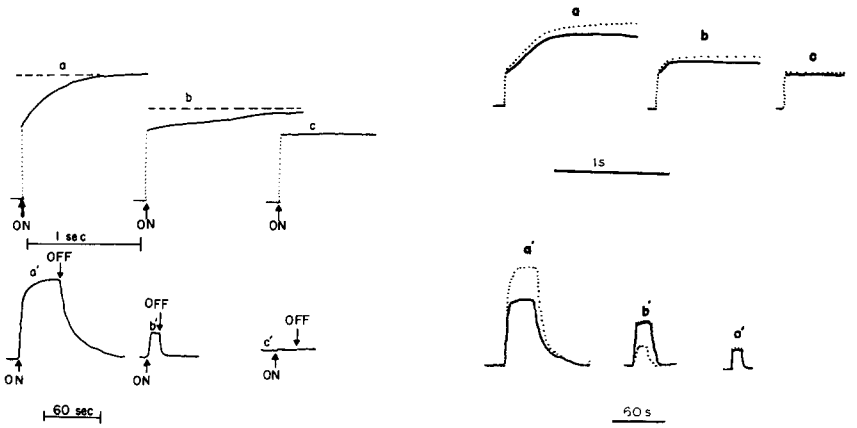

Fig. 9. Time course of fluorescence (a,b,c) and the light-induced change of P890 (a',b',c') in *Chromatium* chromatophores. (a) and (a'), 0.1 mM ascorbate; (b) and (b'), (a) (a') + 1 μM PMS; (c) and (c'), (a) (a') + 0.01 mM PMS. Other conditions are the same as for Fig. 5.

Fig. 10. Effect of antimycin A on the time course of fluorescence and light-induced change of P890 in *Chromatium* chromatophores. (a)–(c) fluorescence; (a')–(c') P890 change. (a) and (a') 1 mM ascorbate; (b) and (b') ascorbate + DCIP (40 μM); (c) and (c'), PMS (5 μM) + ascorbate (1 mM). Dotted lines, antimycin A (10 μM); continuous lines, controls.

The light-induced change of P890 was increased by about 50% when antimycin A (10 μM) was added in the presence of 1 mM ascorbate. On the other hand, antimycin A decreased the P890 change when it was added in the presence of the DCIP–ascorbate couple. In the presence of PMS, antimycin A had no effect. Fig. 10 shows the effects of antimycin A on the P890 change and on f_v in various conditions.

DISCUSSION

The observed differences in emission spectra of the components f_i and f_v (Fig. 2) provide evidence concerning the nature of the emitter of fluorescence in the chromatophores. Graphical analyses of the emission spectra illustrated in Figs 2A and 2B indicate that the f_i component is composed of a superposition of two different emissions, namely F880 and F910 (1:1) in *Chromatium*, and F870

and F900 (1:2) in *Rps. spheroides*. On the other hand, the f_v component seems to be simple in composition, consisting of F910 and F900 in the respective bacteria. The shorter wavelength emissions, F880 and F870, are emitted from B850; and the longer wavelength forms F910 and F900 from B890 and B870 in the respective bacteria[24]. It is noteworthy that for f_i the observed peak of emission is an apparent one and does not represent a true maximum of a particular emission band. In this connection, the finding with *Rsp. rubrum* will be of interest. Here, f_i is apparently simple in composition, the shorter wavelength component being, if present at all, insignificant in amounts as compared with that of F900 (Fig. 2C). This finding seems to reflect the circumstance that B850 is almost lacking in this organism. The f_v component in this case is also the same in nature as in the other two bacteria, *i.e.* F900 emitted from B880.

These conclusions seem to correspond to the finding reported by Zankel and Clayton[3] that there exist two components (peak at 890 nm and shoulder at 860 nm) in a difference spectrum obtained with chromatophores of a green mutant of *Rps. spheroides* (Strain Ga) by varying the actinic light intensity 10-fold. The increase in light intensity must have augmented the contribution of the f_v component of fluorescence having the longer wavelength peak.

In the present study, a close relationship between the magnitude of the variable fluorescence, f_v, and the light-induced change of P890 was observed under various experimental conditions that may cause changes in photochemistry or states of the electron transport system in photosynthetic bacteria.

There are two possible mechanisms accounting for the observations, namely, that f_v is controlled (i) by the conditions of the trap in the reaction center — whether it is in the closed or in the open state — or (ii) by the quenching mechanism — whether the hypothetical quenching substance is in the oxidized or the reduced state. If the former mechanism is valid, the redox potential curve for f_v must coincide with that of the P890 change. However, the result in Fig. 6 shows that the change of P890 and f_v behaved in a different way towards the change in redox potential of the reaction medium, thus excluding the former possibility. The oxidized state of P890 implies that the trap is in the closed state. According to the former mechanism, this must result in an increase in magnitude of the initial rapid phase of fluorescence. The experimental results of this study showed that this was not so.

According to the generally accepted view of the primary reaction of bacterial photosynthesis, P890 is oxidized by light to its oxidized form $P890^+$ and the primary electron acceptor Q is reduced to its reduced form Q^- [1,21]. If $P890^+$ quenches the fluorescence, the yield of fluorescence must decrease when $P890^+$ is produced by the light, which it did not as shown above (Figs 5 and 7–9). The reduced form of P890 cannot be the quencher since the magnitude of f_v decreased with increasing redox potential of the medium (Fig. 6). Also the result illustrated in Fig. 6 that P890 and f_v showed a different behavior towards changes in redox potential excludes the possibility of P890 as a quencher, either in its oxidized or reduced state. In addition, the fact that the f_v component was increased by light (Figs 1, 3, 5 and 7–10) excludes the possibility that Q^- acts as a quencher. Thus there remains only the possibility that the oxidized form of the primary electron acceptor, Q, may be working as a quencher.

The experimental results obtained will now be re-examined in view of this hypothesis. The oxidized form of the primary electron acceptor, Q, is reduced by light, resulting in a release of the suppressed part of the fluorescence as observed in Figs 1, 5 and 7–9.

Higher levels of redox potential of the medium will accelerate the re-oxidation of Q^- produced by the light; also the light-induced supply of electrons to Q will be suppressed by the oxidation of P890, for instance, by the addition of ferricyanide; both thereby result in a suppression of the level of Q^- and, consequently, of f_v. The discrepancy between the redox potential curves of P890 and f_v illustrated in Figs 5 and 6 can be explained in this manner.

In chromatophores without the addition of a reducing reagent, the amount of electron donor may be limited, as shown by the lower magnitude of the light-induced P890 change, and therefore, the supply of electrons to the primary acceptor Q may be low as actually evidenced by the small magnitude of f_v (Fig. 7, a and a'). The addition of an artificial electron donor such as ascorbate increased the light-induced magnitudes of P890 as well as of f_v (Fig. 7, b and b'). Higher concentrations of ascorbate caused a marked decrease of the P890 change, probably due to the re-reduction of $P890^+$ formed by the light. Since its redox potential is higher than the assumed level of redox potential of the primary electron acceptor[7], ascorbate cannot affect the reaction by reducing the oxidized state of Q in the dark (Fig. 7, c and c'). Similar effects of diphenylcarbazide may be explained in the same way.

Dithionite accelerated the response of f_v to the illumination (Figs 3 and 4), which may be due to a chemical reduction of the primary electron acceptor itself, or its neighboring substance, in the dark before the commencement of illumination.

The observed suppressions of the P890 change and f_v on addition of PMS (Fig. 9), may be a result of an acceleration of the dark recovery rate of P890 and the primary acceptor through the PMS-mediated short circuit in the cyclic electron transport. The effects of the DCIP–ascorbate couple may also be interpreted in a similar way. Antimycin A blocks the cyclic electron transport between the cytochromes in the chain[19,20], so that the dark recovery (re-oxidation) of Q^- is blocked, thus causing an increase in magnitude of f_v. The magnitude of the P890 change showed an increase corresponding to the decrease in supply of electrons to the oxidized form of P890. The presence of high concentrations of reducing reagents and other reagents known to bypass the antimycin A-inhibited site, such as PMS and the DCIP–ascorbate couple, all acted by diminishing the effect of antimycin A. Under such conditions, the addition of antimycin A had no significant effect either on the change of P890 or f_v (Fig. 10). The rather unexpected decrease of f_v observed on addition of antimycin A to the chromatophores in the presence of ferricyanide remains unelucidated.

Most of the experimental results of the present study thus seem to support our tentative postulate that Q, the oxidized form of the primary electron acceptor, works as a quencher in controlling the variable component of fluorescence of bacteriochlorophyll in chromatophores of the photosynthetic bacteria. This conclusion is in accord with the supposition made by Cramer[7] based on the analysis of redox potential dependency of fluorescence of bacteriochlorophyll in chromatophores of the same photosynthetic bacteria as those used in the present study.

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