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DECLINE IN BACTERIOCHLOROPHYLL FLUORESCENCE INDUCED BY CAROTENOID ABSORPTION

J. C. GOEDHEER AND A. K. VAN DER TUIN
Biophysical Research Group, Physics Institute, State University, Utrecht (The Netherlands)
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SUMMARY

A marked decline in the fluorescence of bacteriochlorophyll from chromatophores of purple bacteria occurs when high intensity incident light of a wavelength absorbed by carotenoids is used for excitation. Intact bacteria show the same effect at temperatures below −20°. The fluorescence capacity is restored in the dark at temperatures higher than about −40°. At liquid N_2 temperature the decline in fluorescence is not reversible in the dark.

Absorption in the weak 800-nm bacteriochlorophyll band of *Rhodospirillum rubrum* was also found to be effective in producing the above effect.

INTRODUCTION

During investigations on the fluorescence of bacteriochlorophyll at the temperature of liquid N_2 it was found that strong illumination results in a decline in fluorescence intensity to about 10 % of its initial value. Not all wavelengths of excitation were equally effective. Neither were all wavelengths in the fluorescence emission spectrum equally affected. Similar, though less marked, effects were found with chlorophyll-*a* fluorescence of green plants at −196°.

In a preliminary paper it was reported that this decline in fluorescence with chromatophores of *Rhodospirillum rubrum* and *Rh. molischianum* results from illumination with light absorbed by carotenoids, but not by bacteriochlorophyll[1]. At room temperature and at temperatures down to −20°, such 'quenching' was reversed in the dark. At temperatures below −40° down to the temperature of liquid N_2 the effect was usually irreversible. With bacteria the decline in fluorescence induced by carotenoids was weak or absent at room temperature, but bacteria behaved as chromatophores for this effect at temperatures below −10°.

Fluorescence was also strongly quenched by addition of oxidants such as $K_3Fe(CN)_6$ or H_2O_2, and restored by subsequent addition of excess reductants. It might be assumed that a photo-oxidation of some compound, resulting from carotenoid absorption, is responsible for the fluorescence quenching effect.

A decline in fluorescence of bacterial chromatophores upon illumination with blue light was also found by MAYNE[2]. He measured the fluorescence intensity as

a function of the redox potential of the sample after addition of ferri-ferrocyanide mixtures.

The decline in fluorescence induced by carotenoids was much less marked with chromatophores of *Rhodopseudomonas spheroides*, in which energy absorbed by carotenoids is transferred to bacteriochlorophyll with a high efficiency[3,4]. Such lack of effect may indicate that at least a fraction of the carotenoids in purple bacteria does not belong to the pigment system responsible for the fluorescence of bacteriochlorophyll. As at the temperature of liquid N_2 all diffusion-limited reactions are inhibited, further investigation of the fluorescence quenching phenomenon can yield information about the molecular structure in the immediate environment of the light-absorbing pigment molecules.

METHODS

Fluorescence was measured with an apparatus described earlier[5]. For fluorescence excitation a Philips 100-W projection lamp (type 13 116C/04) was used. 'Monochromatic' light was isolated by interference filters ($\Delta\lambda = 8$–12 nm). In the low temperature experiments the bacteria were absorbed on filter paper and lightly pressed against the inner wall of a Dewar vessel. For the room temperature experiments bacteria and chromatophores were suspended in a 1-cm cuvette (max. absorbance 0.3), bacteria in their culture medium, and in some experiments in phosphate buffer (pH 7.5), chromatophores in 0.01 M phosphate buffer (pH 7.5). Parallel experiments with intact bacteria on filter paper and in a cuvette yielded the same results. Bacteria on filter paper were dried in a stream of dry air.

For the measurements of the action spectra the relative fluorescence decline occurring in the first 30 sec of illumination was taken. The measurement of the effect in the near infrared was made as follows. First the sample was illuminated for 2 min with light transmitted by an interference filter of chosen wavelength. A black screen was placed in front of the monochromator to protect the photomultiplier against scattered light. Then the filter was replaced by an interference filter of 590 nm ($\Delta\lambda = 10$ nm), combined with a neutral reflection filter (0.6) and a $CuSO_4$ solution (6 %, 3 cm). The intensity and wavelength of this combination was such that the light did not produce a decline in fluorescence. When the near infrared illumination resulted in a reversible fluorescence quenching—as occurs at 800 and 782 nm with *Rh. rubrum*—the dark restoration was measured with the dim 590-nm light.

RESULTS

The time course of fluorescence decline of chromatophores of *Rh. molischianum* excited by light of 510 nm of different intensities is given in Fig. 1. The figure shows that a marked decline in fluorescence occurs with this species with intensities as low as 10^3 ergs/cm^2·sec (corresponding to about 10^{14} absorbed light quanta/cm^2·sec in a sample absorbing 50 %). With chromatophores of *Rh. rubrum* the required intensity is about 3 times higher, while with chromatophores of *Rh. spheroides* and *Rh. gelatinosum* an intensity at least 10 times higher is needed to obtain a measurable reversible decline in fluorescence.

In Fig. 2 the dark regeneration curve, measured either by short light flashes or

by light not absorbed by carotenoids (592 nm), is plotted for different temperatures. The half time of restoration ranged from 5 sec at 45° to 45 sec at −25°. Below about −40° no reversibility of the phenomenon was found. After the material had been warmed to temperatures above this point reversibility re-occurred. At about −30° the reversibility was about 30 %.

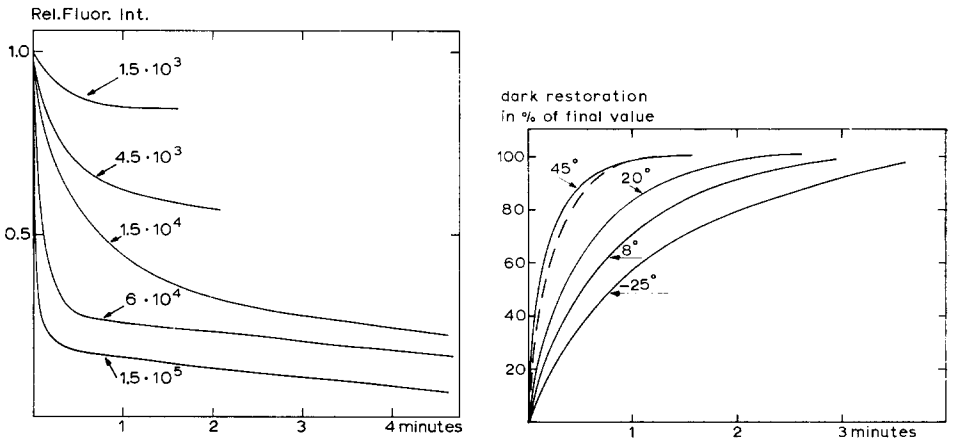

Fig. 1. Time course of fluorescence decline of chromatophores of *Rh. molischianum* at different intensities of blue incident light (in ergs/cm²·sec).

Fig. 2. Dark restoration of fluorescence capacity of chromatophores of *Rh. molischianum* irradiated at 590 nm at different temperatures, after 30 sec illumination with strong blue light. – – – –, restoration curve of intact bacteria at −25°.

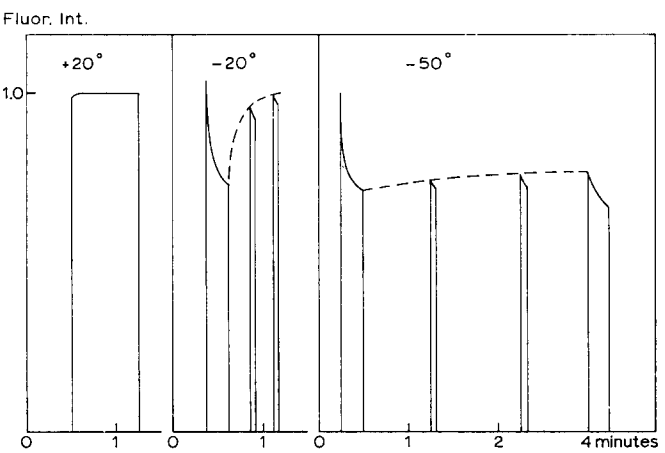

Fig. 3. Fluorescence intensity *versus* time of illumination for intact *Rh. rubrum*, illuminated with blue light of 2.10⁴ ergs/cm²·sec, and at different temperatures.

Fig. 3 gives fluorescence intensity as a function of time of illumination with blue incident light (3 cm 6 % $CuSO_4$ and Schott BG 72 mm) for intact bacteria (*Rh. rubrum*) measured at various temperatures. The curves below −10° are similar to those obtained with chromatophores.

The action spectrum for the decline in fluorescence of chromatophores of *Rh.*

molischianum is given, together with the absorption spectrum, in Fig. 4a. No measurable effect occurred in the region of any of the far-red bands of bacteriochlorophyll or of the 590-nm bacteriochlorophyll band. The activity in the Soret band (around 370 nm) was slight and probably due to overlapping carotenoid absorption. In Fig. 4b the action spectrum of the decline in fluorescence of chromatophores of *Rh. rubrum* is given with the absorption spectrum. In this species a slight activity is also seen at

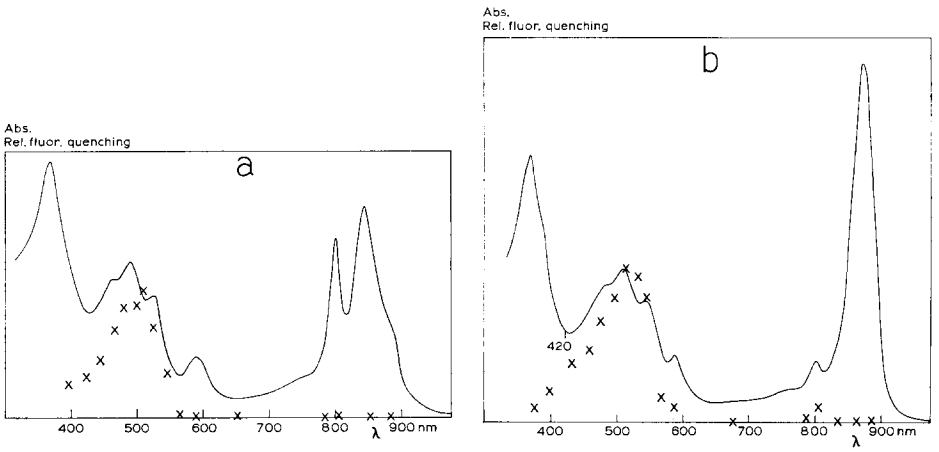

Fig. 4a. Action spectrum of fluorescence decline (×—×) and absorption spectrum of chromatophores of *Rh. molischianum* measured at 20°. 4b. Action spectrum of fluorescence decline and absorption spectrum of chromatophores of *Rh. rubrum*, measured at 20°.

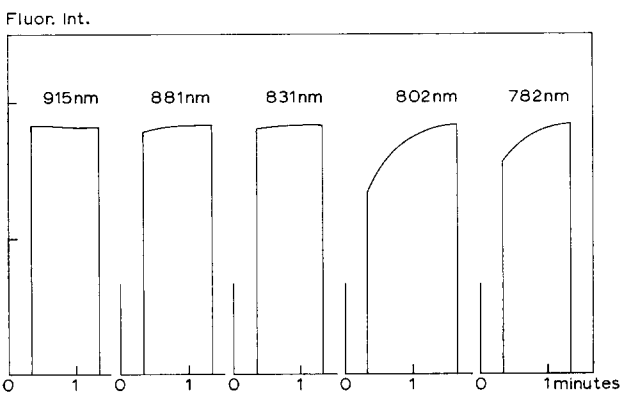

Fig. 5. Dark restoration curve for fluorescence capacity of chromatophores of *Rh. rubrum* (measured with low-intensity 592-nm light) after 1 min irradiation with light of indicated wavelength.

780 and 800 nm, but not in the main band at 880 nm. Fig. 5 shows the increase in fluorescence measured with 592-nm light —which occurred after illumination with different far-red wavelengths. In the carotenoid region the effect of 566-nm light in *Rh. rubrum* is much higher than in *Rh. molischianum*. This indicates that the carotenoid spirilloxanthin, which absorbs at longer wavelengths than the other carotenoids and which is usually abundant in *Rh. rubrum*, is also active in the quenching effect.

At high intensities of illumination of chromatophores the curves of the decline in fluorescence have a clear biphasic character. Irradiation at different wavelengths

indicates that the fast decline is probably due to carotenoid absorption and is reversible at room temperatures, whereas the slow decline is due to absorption by bacteriochlorophyll as well as carotenoids and is not reversible.

With bacteria cooled to about —20° the percentage of reversibility approaches 100 %. Thus the slow component appears to be much less important than in chromatophores at room temperature.

As far as could be observed, the action spectrum for the decline in fluorescence of intact bacteria at low temperature does not differ from that of chromatophores at room temperature. Except with *Chromatium* (Fig. 6), no reversible decline in fluorescence was found to occur at room temperature with intact bacteria. Either a slight increase, or an irreversible decrease caused primarily by bacteriochlorophyll absorption at high-light intensities, was seen.

After about 10 min drying in a stream of air, intact bacteria suspended on filter paper also showed the carotenoid-induced reversible quenching of fluorescence at room temperature. However, an appreciable fraction of the total quenching effect

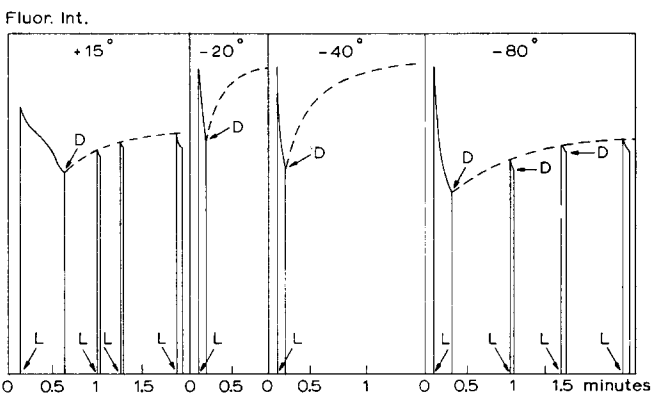

Fig. 6. Fluorescence-time curve of *Chromatium* irradiated with blue light at different temperatures.

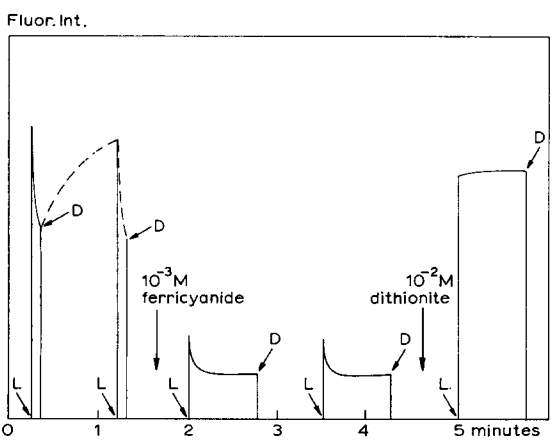

Fig. 7. Fluorescence-time curve of chromatophores of *Rh. rubrum* in the presence of 1 mM $K_3Fe(CN)_6$, 0.01 M sodium dithionite and without addition of reagents. For each curve a fresh sample of equal absorbance was used.

appeared to be irreversible and due to bacteriochlorophyll absorption. The decline in fluorescence was absent or much reduced in magnitude when reductants, such as $K_4Fe(CN)_6$, ascorbate, sodium dithionite or NADPH, were added. Addition of oxidants, such as $K_3Fe(CN)_6$ or H_2O_2, resulted in a strong decrease in fluorescence intensity, but the decline in fluorescence induced by carotenoids did not disappear. Fig. 7 shows the decline in fluorescence of chromatophores of *Rh. rubrum* with and without 0.01 M dithionite or 0.001 M ferricyanide. The same intensity of incident light (isolated with a 545-nm interference filter) was used for all three samples. The dependence of the decline and intensity of fluorescence on redox potential is illustrated by the addition of ferricyanide, resulting in a marked drop in fluorescence, and subsequent addition of ferrocyanide, which restored the fluorescence nearly to its initial intensity and annihilated the quenching effect.

The addition of oxidants to some bacteria may also affect the shape of the fluorescence spectrum. With *Rh. rubrum* the shape of the spectrum is not affected by the addition of ferricyanide or by illumination. The spectrum consists of a single band with maximum at 893 nm at room temperature, and at 908 nm at the temperature of liquid N_2. With *Rh. molischianum* or *Rh. spheroides* the fluorescence spectrum showed a maximum at 893 and a weak shoulder at about 875 nm at room temperature. Addition of 1 mM ferricyanide resulted in a strong decrease in the intensity of fluorescence and a shift of the maximum from 893 to 875 nm (Fig. 8a).

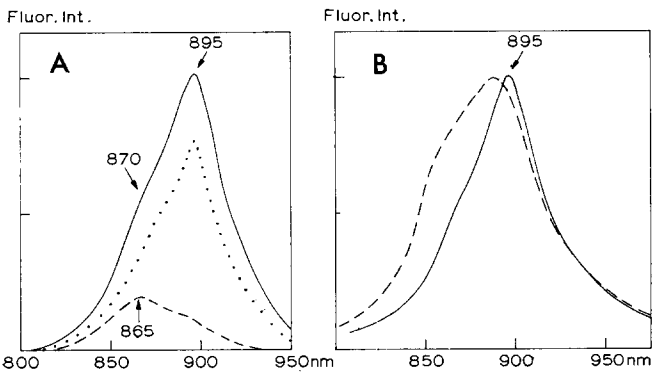

Fig. 8a. Fluorescence spectrum of a suspension of chromatophores of *Rh. spheroides* (———); fluorescence spectrum after addition of 0.01 M ferricyanide (– – –); fluorescence spectrum after subsequent addition of 0.03 M ferrocyanide (·····). 8b. Fluorescence spectrum of *Rh. molischianum* after 5 min illumination with light of 590 nm. – – – –, fluorescence spectrum after 5 min illumination with light of 510 nm (approx. 8×10^4 ergs/cm^2·sec).

The light-induced reversible quenching of fluorescence also mainly affected the long wavelength emission. Fig. 8b shows the fluorescence spectrum of *Rh. molischianum* with incident light of 590 nm, in which no decline in fluorescence occurred, and after 5 min illumination with strong blue light (510 nm). The latter spectra become equal at the maximum. The 875-nm emission is, proportionally, much more pronounced in the experiment with blue light than in that with yellow light.

DISCUSSION

The action spectra of the decline in reversible fluorescence indicate that this effect is produced primarily by light absorbed by carotenoids.

The phenomenon is less marked with those chromatophores in which a high efficiency of energy transfer from carotenoids to bacteriochlorophyll occurs (*e.g.* in *Rh. spheroides* and *Rh. gelatinosum*). This may mean that two carotenoid systems occur in these purple bacteria. (a) A system from which the absorbed light quanta are transferred to bacteriochlorophyll with nearly 100 % efficiency. (b) A system from which this energy transfer to fluorescing bacteriochlorophyll is low. Light absorbed by this system is used in reactions that result in a decline in bacteriochlorophyll fluorescence. The latter is excited either by bacteriochlorophyll or by carotenoids (a).

Whether or not the decline in fluorescence occurs *via* the weak 800-nm band of bacteriochlorophyll (probably in its 'oxidized position', *cf.* GOEDHEER[6]) is not clear, as this band is also visible in the fluorescence action spectra (*cf.* AMESZ AND VREDENBERG[7]).

However, such a band was not observed in the action spectra of the decline in fluorescence with chromatophores of other bacterial species, while its presence in the absorption spectra of these bacteria is likely in view of absorption difference spectra (DUYSENS *et al.*[8], CLAYTON[9]). It could be that the high percentage of absorption at 800 nm of a bacteriochlorophyll type, which does not shift upon illumination at high intensities, obscures the effect with these bacteria.

The fluorescence intensity of washed chromatophores is also strongly decreased by the addition of oxidants, and it is restored by subsequent addition of reductants. According to MAYNE[2] the intensity of fluorescence decreases markedly at potentials exceeding 480 mV. As shown in Fig. 7, the light-induced decline in fluorescence is absent in chromatophores after the addition of reductants, such as sodium dithionite, ascorbate or ferrocyanide, and the effect is still visible, though less marked, after the addition of ferricyanide. From these results it could be suggested that light absorption by carotenoids results in photo-oxidation of some compound in the chromatophore, which in its oxidized state quenches the fluorescence of bacteriochlorophyll. The redox potential of such an oxidized compound should be higher than the potential reached by addition of ferricyanide (Fig. 7).

A decrease in fluorescence intensity due to the presence of oxidant molecules also occurs with chlorophylls dissolved in organic solvents (*cf.* LIVINGSTON AND KE[10]). It can be brought about either by a decrease in yield of all fluorescing molecules, or by total annihilation of the fluorescence capacity of part of the molecules. In the first event fluorescence intensity is a measure of the mean life of the first excited singlet state of a pigment molecule. In this excited state the pigment molecule is easily oxidizable, *e.g.* by molecular oxygen. A quenching of fluorescence, therefore, which involves a decrease in mean life of the first excited state, can correspond with a stabilization of the pigment molecule against photo-oxidation by oxygen. In this way fraction (b) of carotenoids could function as a protective system for the fluorescing bacteriochlorophyll type.

VREDENBERG AND DUYSENS[11] detected in *Rh. rubrum* a quantitative correlation between the light-induced decrease in absorption around 880 nm and the increase in yield of bacteriochlorophyll fluorescence (up to 50 %). They explain this correlation by a decrease in energy transfer from fluorescent bacteriochlorophyll B890 to a non-fluorescent pigment P890 (presumably also bacteriochlorophyll). The latter pigment is photo-oxidized, and in this state lacks an 880-nm absorption band. As indicated by

absorption difference spectra, all the P890 is oxidized at those light intensities at which fluorescence quenching by carotenoid absorption occurs.

The absorption changes due to photo-oxidation of P890 are reversible at the temperature of liquid N_2, and, with bacterial chromatophores, even at the temperature of liquid helium (ARNOLD AND CLAYTON[12]). This indicates that reduction of some compound with the electron from oxidized P890, as well as restoration of P890 in the dark, is governed by the electronic configuration of neighbouring molecules, and is not influenced by thermal activation energy. Apparently it is also not influenced by structural changes due to cooling. The same holds for the decline in fluorescence induced by carotenoids. The dark restoration of fluorescence capacity, however, ceases to occur at temperatures below about $-30°$, and at $-60°$ with *Chromatium*. The dark restoration is thus temperature dependent, although it is not merely diffusion limited, as but small changes occur in the shape of restoration curves between 20 and $-20°$. It is possibly a structural phenomenon, influenced in some way by changes in configuration of the protein–aqueous phase at various temperatures below zero. The behaviour of restoration of fluorescence at different temperatures below 0° is in some way reminiscent of the light-induced oxidation of cytochromes at these temperatures (VREDENBERG AND DUYSENS[13]).

In living bacteria the phenomenon of decline in reversible fluorescence by carotenoid absorption is seen only when macroscopic diffusion is stopped by freezing or drying. The presence of reduced cell compounds, even in aerobic conditions, may result in a fast reduction of photo-oxidized products produced by carotenoid absorption.

A completely different explanation for the phenomenon of the decline in fluorescence could be the occurrence of a sterioisomerization of carotenoids under the influence of light, analogous to the behaviour in the visual pigment system. In this way the addition of oxidants to chromatophore suspensions might also induce such an effect. BRIL[14] suggests that a *cis–trans* isomerization could explain the protective action of carotenoids in photosynthetic systems by structural changes. An argument against such a stereoisomerization is the presence of the weak bacteriochlorophyll band in the action spectrum of *Rh. rubrum*.

The changes in shape of the fluorescence spectra of *Rh. molischianum* and *Rh. spheroides*, shown in Fig. 8, confirm the observations of BRIL[14] and AMESZ AND VREDENBERG[7] that energy transfer between the infrared bacteriochlorophyll bands does not occur to a full 100 %. The fluorescence band ascribed to emission of B850 is much less affected by addition of oxidant than that of B890. Also the decline in fluorescence, or at least the reversible part induced by carotenoid absorption, mainly affects the latter component.

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