

RESEARCH NOTE

PHYCOBILISOMES AND ISOLATED PHYCOBILIPROTEINS. EFFECT OF GLUTARDIALDEHYDE AND BENZOQUINONE ON FLUORESCENCE

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Abstract—The fluorescence of the biliproteins C-phycoerythrin from *Spirulina platensis*, B-phycoerythrin from *Porphyridium cruentum* and of isolated whole *P. cruentum* phycobilisomes is quenched in the presence of glutardialdehyde (GA) or benzoquinone (BQ). The kinetics of fluorescence decrease thus induced is biphasic. If GA is used as a quencher, the fluorescence can be recovered at 77 K. Contrary to the GA-effect, only a minor recovery takes place with BQ at 77 K, thus demonstrating a different mechanism of action of GA and BQ on biliproteins.

INTRODUCTION

Phycocyanins and phycoerythrins are biliproteins with covalently linked chromophores (Rüdiger, 1979; Rüdiger and O'Carra, 1969). They constitute a special class of light harvesting pigments. In the blue-green and red algae, they are aggregated to particles of distinct appearance and fine-structure called phycobilisomes (Gantt and Lipschultz, 1972; Gantt, 1975; Wanner and Köst, 1980). One of the best characterized phycobilisomes are those of *Porphyridium cruentum*, a unicellular red alga (e.g. Gantt, 1975; Mörschel and Wehrmeyer, 1979; Wanner and Köst, 1980). Isolated phycobiliproteins and phycobilisomes are highly fluorescent, which is essential for their biological function. Any factors influencing fluorescence are interesting for the understanding of these interactions, and of energy transfer both within the phycobilisomes and from the latter to the chlorophylls in the photosynthetic membrane.

The investigations described below started from the observation that the fluorescence is quenched with higher concentrations of glutardialdehyde (GA)* while the pigments derived from *P. cruentum* were fixed for electron microscopy and could be reconstituted at 77 K. Another reagent known to be a fluorescence quencher is *p*-benzoquinone (BQ)* which has been studied on several C-phycoerythrins (C-PE) (Zickendraht-Wendelstadt, 1979). Fluorescence is also lost reversibly upon denaturation, e.g. with urea. In the latter case, it is due to the uncoupling of non-covalent chromophore-protein interactions, which is evident from pronounced absorption changes (Scheer and Kufer, 1977). By contrast, the fluorescence quenching

by BQ and GA does not lead to pronounced color changes of biliprotein solutions, which indicates different quenching mechanism(s). Here we wish to report results on the effects of GA and BQ on the absorption and fluorescence of isolated biliproteins and intact phycobilisomes at room temperature and at 77 K.

MATERIALS AND METHODS

C-Phycoerythrin was isolated from *Spirulina platensis* (Kufer and Scheer, 1979), B-phycoerythrin (B-PE) (Köst-Reyes and Köst, 1979) and phycobilisomes (PBS) from *Porphyridium cruentum* (Gantt and Lipschultz, 1972). Stock solutions of the lyophilized biliproteins were prepared in tris buffer (0.01 M, pH 7.7) and diluted immediately before measurements. Phycobilisomes were freshly prepared according to Gantt and Lipschultz (1972). Their functional integrity was checked by their fluorescence properties (excitation spectrum of the fluorescence at 680 nm). Glutardialdehyde (Interchem, München) was used from 25% stock solutions of glutardialdehyde in water. Benzoquinone (Merck, Darmstadt, analytical reagent) was sublimed prior to use. All other solvents and reagents were of analytical grade.

Typical experiments were carried out as follows: in a quartz fluorescence cuvette, tris/HCl-buffer (1.3 m^l, pH 7.7, 0.01 M) was pipetted; 200 m^l of a biliprotein stock solution were added, then 200 m^l of GA or BQ stock solution.

For the studies at room temperature, two parallel samples were usually prepared. One sample was used to record a UV-Vis absorption spectrum, the other sample was taken to record fluorescence emission spectra. Within times of up to 6 h, repeated spectra were taken. At low temperatures, only fluorescence spectra were recorded. OD_{max} of samples (long wavelength maxima) was kept <0.3 to avoid self-absorption effects. The fluorescence spectra shown were not corrected.

UV-Vis absorption spectra were recorded at an ambient temperature on a model 320 (Perkin-Elmer, Überlingen); fluorescence spectra were recorded on a model DMR 22 (Zeiss, Oberkochen) recording spectrophotometer

*Abbreviations: APC, allophycocyanin; BQ, benzoquinone; C-PE, C-phycoerythrin; GA, glutardialdehyde; PBS, phycobilisome; PC, phycocyanin.

equipped with a fluorescence attachment (M 4 Q II monochromator and 450 W Xe lamp). Low temperature fluorescence spectra were recorded at 77 K on a home-built fluorimeter (Friedrich and Dörr, to be published). We thank Professor S. Schneider (Garching) for making the instrument available to us, and Mrs. E. Kudler (Garching) for skilful operation.

Spectra of whole phycobilisomes at room temperature were taken in 0.75 M phosphate buffer in order to avoid disaggregation. At 77 K, glycerol (20% to up to 50%) was added to obtain a glass-like structure of the frozen sample, which results in some dissociation (see text).

RESULTS AND DISCUSSION

Glutardialdehyde effects

The fluorescence of isolated biliproteins is quenched by nearly one order of magnitude within a few minutes after the addition of GA (Table 1). The shape and positions of the emission bands remain unchanged during this process. The decrease of fluorescence is accompanied only by small changes in the absorption spectra.

Phycobilisomes have two fluorescence bands, a major one around 680 nm, corresponding to APC and a minor one around 580 nm corresponding to PE. A fluorescence emission due to the third pigment R-PC has not been observed in the samples used. Glutardialdehyde reduces the intensity of both fluorescence

bands, but to a different degree (Fig. 1). The major band due to APC decreases more strongly than the one due to PE. Again, the absorption changes are much less pronounced. The maximum absorption due to PE is decreased by about 30%. Noteworthy is a change in the absorption band shape; the maxima situated at first at 545 and 565 nm are shifted to a common maximum at 555 nm.

Kinetics of fluorescence decrease

Increasing amounts of GA accelerate fluorescence decrease in the case of isolated phycobiliproteins [see Fig. 2, $t_{1/2} = f(c)$] as well as phycobilisomes (not shown). The decays can be fitted by two first order reactions, assuming a final fluorescence value of zero (Fig. 2). However, due to the instability of the pigments, this final value is reached only at higher concentrations of GA within the measuring time (< 14 h). In Fig. 3, only the first reaction constants of this biphasic decay have been used. The biphasic decrease can be due to either two consecutive reactions, or to a differential effect on the different types of the chromophores (Zickendraht-Wendelstadt *et al.*, 1980; Grabowski and Gantt, 1978; Teale and Dale, 1970; Verotte, 1971; Langer *et al.*, 1980). Such a differential reactivity has been observed, for example, in C-PC, during denaturation (Scheer and Kufer, 1977) and

Table 1. Maximum wavelengths of fluorescence and fluorescence intensity values (arbitrary values; the fluorescence spectra are not corrected) of isolated phycobiliproteins (B-phycoerythrin, B-PE and C-phyococyanin, C-PC) and *Porphyridium cruentum* phycobilisomes, PBS

Material investigated	Temp K	Fluorescence		Fluorescence	
		max λ_1 nm	Intensity	Max λ_2 nm	Intensity
PE	RT	583	36.8	—	—
PE + GA	RT	588	7.5	—	—
PE + GA	77	583	47.0	—	—
PE + BQ†	RT	580	2.7	—	—
PE + BQ†	77	561	20.0	—	—
PE + BQ‡	77	583	6.0	—	—
PC	RT	—	—	661	8.5
PC + GA	RT	—	—	666	2.8
PC + GA	77	—	—	651	7.5
PC + BQ†	RT	—	—	653	1.9
PC + BQ†	77	—	—	668	3.0
PBS	RT	582	34.5	644*	3.3
PBS + GA	RT	578	17.5	640*	1.5
PBS + GA	77	584	13.0	685*	10.0
PBS + BQ†	RT	579	5.0	659*	2.0
PBS + BQ†	77	590	8.5	645*	1.5

The data are taken at ambient temperature (298 K) and at 77 K without and after glutardialdehyde (GA) and benzoquinone (BQ) treatment, respectively. In each case, immediately following the measurement at room temperature, the sample was cooled down to 77 K (time needed: 30 min). Spectra of PBS were taken after addition of 50% of glycerol.

*Fluorescence emission of PC. The emission maximum of PBS is usually from APC ($\lambda_{max} = 675-680$ nm, Grabowski and Gantt, 1978). The emission from PC is due to the uncoupling of APC in the glycerol buffer. The energy transfer from PC to APC in the presence of GA (but not BQ) is restored at 77 K (see text and the respective lines in this Table).

†Freshly prepared solution of BQ.

‡'Aged' solution of BQ.

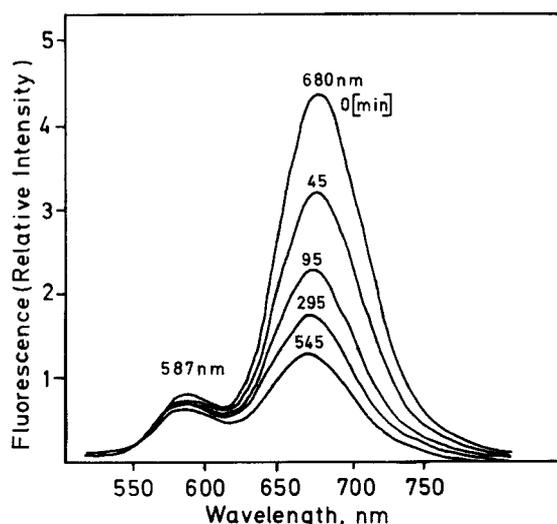


Figure 1. Fluorescence spectra of *Porphyridium cruentum* phycobilisomes (PBS) in 1.9% glutardialdehyde (GA) solution. The fluorescence at the maxima 680 and 587 nm is decreasing as a function of the time passed. The half-life of fluorescence decrease is about one order of magnitude higher than that of isolated biliproteins. $\lambda_{\text{ex}} = 380 \text{ nm}$; spectra not corrected.

chemical modification (Kufer and Scheer, 1979). Since the shapes of the fluorescence emission spectra remain unchanged in the case of isolated phycobiliproteins, the fast decrease could only correspond to 's'-type chromophores. In the phycobilisomes, the two fluorescence bands due to APC and PE both show a biphasic decay. The former is more rapid, however, probably due to concomitant uncoupling of energy transfer between PE and APC.

Fluorescence recovery

The fluorescence is recovered at 77 K. (Table 1). In PE, the fluorescence intensity is increased from 20%

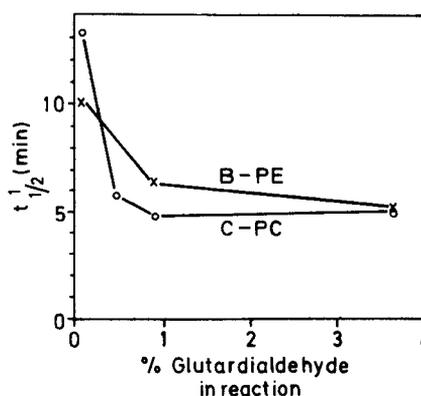


Figure 3. Half-life time of fluorescence decrease of isolated phycobiliproteins (B-phycoerythrin and C-phycoyanin) upon glutardialdehyde treatment. The first half-life constant $t_{1/2}^1$ (in min) has been plotted against the concentration of glutardialdehyde (in %) in the reaction. $\lambda_{\text{ex}} = 380 \text{ nm}$; fluorescence of B-PE determined at 583 nm; fluorescence of C-PC determined at 660 nm.

at ambient temperature (with respect to untreated PE) to 127% at 77 K. In PC, there is an increase from 33 to 88%. In PBS, the two fluorescence bands change in a different way. While the fluorescence intensity due to PE ($\approx 582 \text{ nm}$) shows even a further decay at 77 K (from 51 to 38%), the intensity of the APC fluorescence ($\approx 680 \text{ nm}$) is greatly enhanced (from 45 to 303%). At the same time, the emission maximum is shifted $\sim 40 \text{ nm}$ to a longer wavelength. This clearly shows a reconstitution of energy transfer within the GA treated phycobilisomes.

Mechanism

If judged from its chemical properties, GA is not likely to act directly as a quencher of chromophore fluorescence, but rather by an indirect mechanism through its action on the protein moiety. One of the

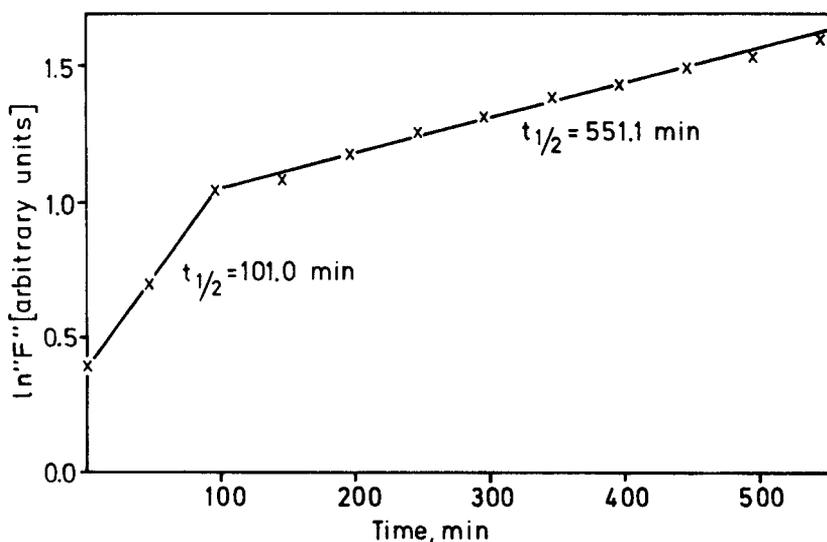


Figure 2. Fluorescence decrease of phycobilisomes in 1.9% glutardialdehyde (GA) solution. The natural logarithm of the fluorescence intensity has been plotted against the time passed. A biphasic reaction can clearly be seen [$t_{1/2}^1$ (fluorescence at 680 nm) = 101 min; $t_{1/2}^2$ (fluorescence at 680 nm) = 551 min].

major pathways of radiationless decay in bile pigments is by the coupling to highly excited vibrational states by internal motions of the molecule. In native biliproteins, the flexibility of the chromophore is reduced by non-covalent interaction with the protein, which leads to the appearance of strong fluorescence [see Scheer (1980) for leading references]. One conceivable way for the fluorescence quenching of GA is then a partial uncoupling of the interactions by the strain put onto the bilipeptide chain by the GA induced crosslinking, which would lead to a somewhat increased motional freedom of the chromophores. Glutardialdehyde links protein regions together via Schiff-base formation with pairs of amino groups which are close to each other in space, but might be even a long distance away from each other in the primary sequence. The tertiary structure is basically preserved, if judged from the activity of GA-treated enzymes (Fahimi, 1980). These cross-links may induce, however, local deviations from the native conformation.

Fluorescence is known to be sensitive to partial denaturation (Langer *et al.*, 1980; Kufer and Scheer, 1979), and the effect of GA would then correspond to such a partial denaturation. This is supported by the absorption changes. The creation of 'strained regions' in the case of biliproteins does have an effect on the visible spectrum. The two maxima of B-PE at 545 and 565 nm, for example, are shifted to one single, broad maximum, at 555 nm, while the absorbance is only slightly diminished. This is comparable to the denaturation effect of low concentrations of urea (Langer *et al.*, 1980; Kufer and Scheer, 1979). The recovery at low temperatures would then be due to an increased rigidity ('resolidification') of the chromophores.

The effect on phycobilisomes can be rationalized by the same mechanism. Lowering the temperature increases the excited state lifetime in all three pigments. In APC, this leads to an increased fluorescence. In PC and PE still contained within the phycobilisomes, the same fact would lead to an increased rate of the Förster type energy transfer, which would increase the fluorescence of the acceptor, APC, even more. Since this can occur only in structurally intact phycobilisomes, the pronounced increase of APC fluorescence at low temperatures would argue for an uncoupling of the chromophores from the protein influence upon dissolution in glycerol-water mixtures, rather than to a dissociation of the phycobilisome.

Effect of benzoquinone

Benzoquinone has a more pronounced effect on fluorescence than GA. Within a very short time

(≤ 12 min), the fluorescence is diminished to about one-half of its former value. This figure varies widely, however, depending on the quality ('age') of the BQ solution. A freshly prepared solution will give a much longer half-life of fluorescence decrease than an old solution. Also, an old BQ solution does fluoresce by itself, probably due to the formation of condensation products during aging. Energy transfer in phycobilisomes is broken rapidly. Contrary to GA, the fluorescence of the PE molecules, as well as of the PC molecules, decreases rapidly.

In contrast to the GA-effect, too, only a minor recovery of fluorescence takes place at 77 K, thus demonstrating a different mechanism of action of GA and BQ. The quenching mechanism of BQ has not yet been studied in detail. One possibility is a reversible electron transfer from the chromophore(s) to BQ. Free bile pigments structurally related to BQ chromophores are readily photooxidized with oxidants of moderate redox potentials (Scheer *et al.*, 1977; Krauss *et al.*, 1979) and biliproteins are known to aggregate with and in the presence of aromatic compounds (MacColl and Berns, 1973). Electron transfer in native biliproteins has been observed earlier (Chen and Berns, 1975; Frackowiak and Skowron, 1978; Kufer and Scheer, 1979). Since aged BQ is a more effective fluorescence quencher than a freshly prepared solution, it appears that not BQ by itself but rather a decomposition product is the true quencher. Aging is accompanied by a darkening of the originally light yellow solution. Quenching due to reabsorption can be ruled out, since the eventual absorbance of aged BQ solutions is 0.1 above 500 nm. Semiquinone is one of the products expected in aged quinone solutions which itself is an oxidant. However, semiquinone itself is ineffective as a fluorescence quencher. A quenching process by electron transfer within a chromophore-BQ complex would be compatible with the finding that fluorescence is not recovered at low temperatures.

CONCLUSIONS

Fluorescence is a very sensitive probe of the state of the biliproteins. The present study indicates that there are different ways of fluorescence quenching for the two reagents, GA and BQ. The former may act by local changes of the native conformation, while for the latter, a charge-transfer process appears possible. If modifications of the protein take place, fluorescence will go down first, the absorption will go down later. Similar phenomena can be observed upon urea treatment or digestion with enzymes.

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