RESEARCH NOTE

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

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Abstract—The fluorescence of the biliproteins C-phycocyanin 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/, pH 7.7, 0.01 M) was pipetted; 200 m/ of a biliprotein stock solution were added, then 200 m/ 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

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

equipped with a fluorescence attachment (M 4 Q II mono-chromator 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, $t^1_{1/2} = f(c)$] 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; Vernotte, 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-phycocyanin, C-PC) and *Porphyridium cruentum* phycobilisomes, PBS

Material investigated	Temp K	Fluorescence max λ_1 nm	Intensity	Fluorescence 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 (λ_{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).

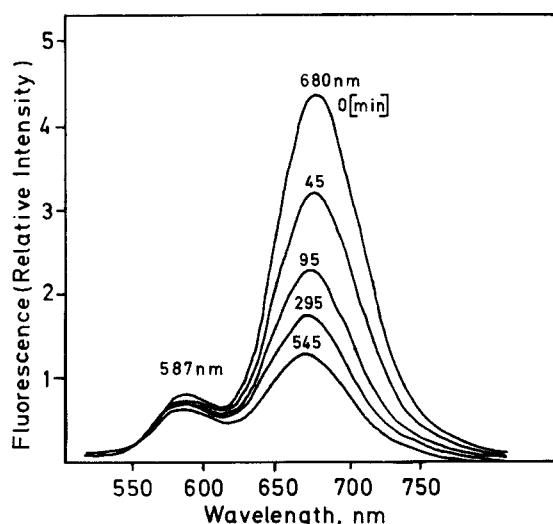†Freshly prepared solution of BQ.

‡'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. $\lambda_{ex} = 380$ 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

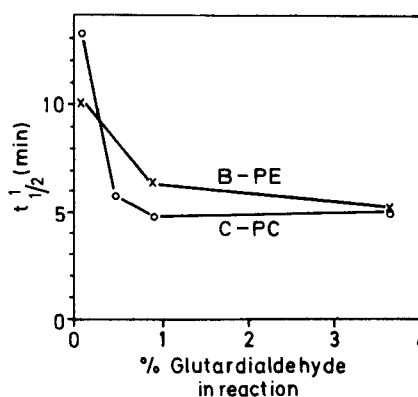The fluorescence is recovered at 77 K. (Table 1). In PE, the fluorescence intensity is increased from 20%

Figure 3. Half-life time of fluorescence decrease of isolated phycobiliproteins (B-phycoerythrin and C-phycocyanin) upon glutardialdehyde treatment. The first half-life constant $t^1_{1/2}$ (in min) has been plotted against the concentration of glutardialdehyde (in %) in the reaction. $\lambda_{ex} = 380$ 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 (≈ 582 nm) shows even a further decay at 77 K (from 51 to 38%), the intensity of the APC fluorescence (≈ 680 nm) is greatly enhanced (from 45 to 303%). At the same time, the emission maximum is shifted ~ 40 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
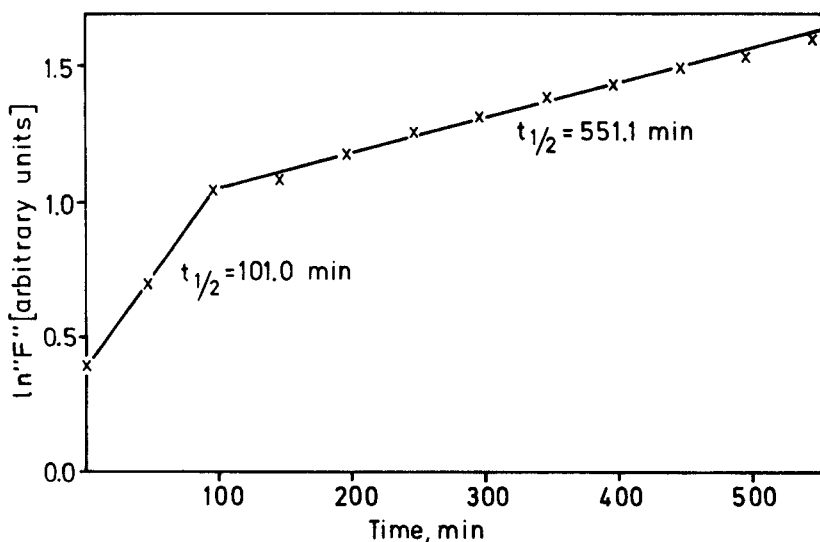

Figure 2. Fluorescence decrease of phycobilisomes in 1.9% glutardialdehyde (GA) solution. The natural logarithm of the fluorescence intensity has been plotted against the time passed. A biphasic reaction can clearly be seen [$t^1_{1/2}$ (fluorescence at 680 nm) = 101 min; $t^2_{1/2}$ (fluorescence at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

major pathways of radiationless decay in bile pig-
ments 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 appearence of strong fluorescence
[see Scheer (1980) for leading references]. One con-
ceivable 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 some-
what increased motional freedom of the chromo-
phores. 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 basi-
cally preserved, if judged from the activity of GA-
treated enzymes (Fahimi, 1980). These cross-links may
induce, however, local deviations from the native con-
formation.

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 de-
naturation 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 chromo-
phores.

The effect on phycobilisomes can be rationalized by
the same mechanism. Lowering the temperature in-
creases 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 phycobili-
somes, 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 flu-
orescence than GA. Within a very short time

($\leqslant 12$ 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 phycobili-
somes is broken rapidly. Contrary to GA, the fluor-
escence of the PE molecules, as well as of the PC
molecules, decreases rapidly.

In contrast to the GA-effect, too, only a minor re-
covery of fluorescence takes place at 77 K, thus dem-
onstrating 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 chromo-
phores 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 sol-
ution, 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 sol-
utions which itself is an oxidant. However, semi-
quinone 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 treat-
ment or digestion with enzymes.

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