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Primary photoprocesses in oxyblepharismin interacting with its native protein partner

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Abstract

The primary photoprocesses in the photoreceptor for the step-up photophobic response of the light-adapted cells of *Blepharisma japonicum* (OBIP, OxyBlepharismin bInding Protein) have been studied by ultrafast UV–vis transient spectroscopy. The results are rationalized in terms of heterogeneity of the OBIP sample. Two independent classes of chromoprotein are proposed: a "reactive" species, which presents a specific 680-nm band decaying in 4 and 56 ps and a "non-reactive" one, which behaves like the free chromophore (OxyBP) in solution. A bimolecular photooxidation of OxyBP in the presence of 1,4-benzoquinone was performed to record the absorption spectrum of the OxyBP radical cation. Comparison with reactive OBIP suggests that an electron transfer could be involved in the primary photoprocesses of OBIP and possibly trigger the sensory transduction chain of *B. japonicum*. In addition, the specificity of the chromophore–protein interaction was investigated by studying the artificial complex that OxyBP forms with human serum albumin (HSA). OxyBP–HSA happens to be spectroscopically much closer to free OxyBP than to OBIP. This highlights the specific nature of the interaction between OxyBP and its native protein partner and further supports the proposal that OBIP is the actual photoreceptor for the photophobic response of *B. japonicum*.

Keywords: *Blepharisma japonicum*; Oxyblepharismin; Oxyblepharismin binding protein (OBIP); Human serum albumin (HSA); Photoinduced electron transfer; Ultrafast transient absorption spectroscopy

1. Introduction

Oxyblepharismin (OxyBP) is the photoreceptor molecule of the light-adapted (blue-colored) form of *Blepharisma japonicum,* a ciliated protozoan that exhibits a strong step-up photophobic response [1,2]. Oxyblepharismin is a phenanthroperylene quinone derivative, closely related to hypericin. It is the photooxidized form of blepharismin (BP), which is the photoreceptor molecule of the dark-adapted (red-colored) form of *B. japonicum* and belongs to the class of benzoanthrapleiadenedione compounds [3–5] (Fig. 1). A readily extractable and very stable, non-covalently bound [6] oxyblepharismin–protein complex (200 kDa), which we will name OBIP (OxyBlepharismin bInding Protein), has been proposed to mediate the photophobic response of the blue form of *B. japonicum* [6–8].

Photoinduced electron transfer from the first excited singlet state of OxyBP to a suitable electron acceptor was shown to be an efficient quenching process of the fluorescence in organic solution [9]. It was suggested that such an electron transfer could also occur *in vivo*, a disulphide bridge playing the role of the electron acceptor [10]. The fluorescence quantum yield of OBIP in a phosphate buffer is indeed smaller than that of free OxyBP in the same medium [11].

We recently undertook a study of the primary photochemical processes taking place in OBIP, by means of optical subpicosecond transient absorption spectroscopy [12,13]. We showed that free OxyBP in organic solution and OBIP exhibit distinctive spectro-temporal behaviors. We found that up to 50% of the excited-state decay of OBIP occurs in the picosecond regime. Two exponential decay components were actually reported, with lifetimes of 4 and 56 ps and roughly comparable weights. Based on spectral observations, the OBIP sample was suggested to be heterogeneous and the fast photoinduced behavior was assigned

Abbreviation: OBIP, OxyBlepharismin bInding Protein

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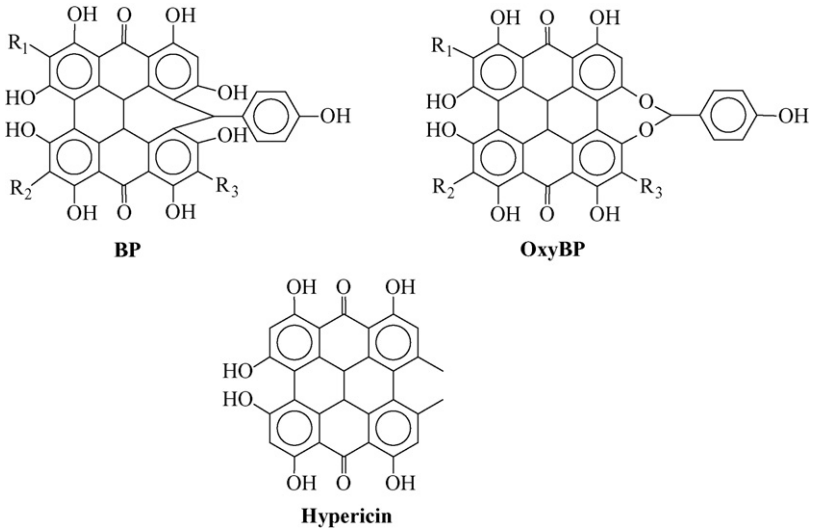

Fig. 1. Structures of blepharismin (BP), oxyblepharismin (OxyBP) and hypericin.

to a particular reactive form of the chromoprotein, possibly yielding an electron transfer.

In this paper we revisit our previous analysis of the primary photoreactivity of OBIP, confirming our hypothesis on heterogeneity and discarding a photodissociation mechanism. On the other hand, we bring new experimental evidences in favor of an excited-state intermolecular electron transfer reaction as the primary photoprocess in the reactive form of OBIP. A bimolecular photooxidation experiment was performed on OxyBP in ethanol in order to record the spectral signature of the OxyBP radical cation. A direct comparison was done with the transient absorption spectra of OBIP at short times.

In addition, we propose a first approach to the question of the specificity of the interaction between OxyBP and its native protein partner in OBIP. No final evidence has been given so far that OBIP is beyond any doubt the actual photoreceptor for the step-up photophobic response of *B. japonicum*. One can thus wonder if the observed transient absorption features reveal a specific photoreactivity of OxyBP with its native apoprotein, or if it is somehow "easily" reproducible within other OxyBP–protein complexes. Such eventuality would of course be incoherent with the idea that OBIP is a specialized photoreceptor. For this purpose, we have examined the transient absorption behavior of an artificial complex between OxyBP and a well-known protein, human serum albumin (HSA).

2. Materials and methods

2.1. Isolation of OxyBP and OBIP

Red *B. japonicum* cells were grown in the dark, at 23 °C, in the presence of the *Enterobacter aerogenes* bacterium as the food supply, as previously described [14]. Blue (light-adapted) cells were produced by *in vivo* photoconversion of blepharismin (BP) into oxyblepharismin (OxyBP) under a low intensity cold white lamp (less than 10 W m^{-2}) for 36 h. Blue cells were washed, collected by low speed centrifugation and resuspended in a 20-mM sodium cholate (NaCh) solution.

Free BP was extracted in acetone from dark-adapted cells, dried and photooxidized *in vitro* into OxyBP by irradiation for a few hours under a dim white light (less than 10 W m^{-2}). Finally the sample was purified by HPLC as previously described [15]. OxyBP was then dissolved in organic solvents such as ethanol (EtOH; Merck, Uvasol) or dimethylsulphoxide (DMSO; Aldrich, spectrophotometric grade).

OBIP was obtained following Matsuoka's protocol [6,11,16], that is, by liquid chromatography of blue cell extracts on a hydroxyapatite column. The applied eluent was a phosphate buffer (pH 7.4, containing 10 mM NaCh and 100 mM sodium chloride), first 0.05 M and then 0.2 M. In the first step free OxyBP and residual BP are eluted while OBIP is retained by the hydroxyapatite phase. Upon ionic strength jump the affinity of the biomolecules with the hydroxyapatite phase is reduced and OBIP detaches. The sample was then reconcentrated by means of Pall (East Hills, USA) centrifugable membrane filters (30 kDa cutoff), in order to obtain an optical density (OD) of about 1 per mm at the redmost absorption maximum (605 nm).

2.2. Preparation of the OxyBP–HSA complex

Since the stoichiometry and stability of the artificial OxyBP–HSA complex were unknown, the following empirical method was employed to prepare it. In the first place, the hypericin–HSA complex has been used as a reference system. Hypericin is known to form a 1:1 complex with HSA (in the II-A [17,18] or III-A [19,20] subdomain depending on authors) with an association constant of 7.5×10^5 M^{-1} [21]. Transposing this values to OxyBP, a solution containing 4.5×10^{-4} M OxyBP and 9×10^{-4} M HSA, expected to lead to a complex fraction superior to 99%, was prepared.

Such solution cannot be prepared directly because OxyBP is not soluble in water and must previously be dissolved in an adequate solvent. The following protocol was devised to achieve this goal. OxyBP solubilized in 1 mL ethanol (4.5×10^{-4} M) was mixed to a 19-mL aqueous HSA (Fluka, ~99%, essen-

tially fatty acid and globulin free) solution (4.7×10^{-5} M, pH 7.4 adjusted with a 10^{-3} M phosphate buffer). The 5% ethanol content of this mixture is not large enough to modify the hydration of the protein or to induce its denaturation [22]. The solution was then freeze-dried and the lyophilized sample redissolved in 1 mL pure water. It is important to note that the validity of this protocol relies on the fact that free OxyBP is not soluble in water. This ensures that the OxyBP molecules present in solution are complexed with HSA.

The absorbance of the final solution at 595 nm (maximum of absorption) was measured to be 0.82 per mm. Supposing that the molar absorption coefficients at the maximum of absorption the OxyBP–HSA system and free OxyBP in ethanol are identical ($22{,}000\ M^{-1}\ cm^{-1}$), one would deduce that about 18% of OxyBP has not been solubilized. Alternatively, supposing that 99% of OxyBP molecules are complexed to HSA, this result would imply that the absorption coefficient at the maximum of absorption of OxyBP–HSA is 83% that of free OxyBP.

2.3. Steady-state spectroscopy

Steady-state absorption spectra were measured using a Safas UV-mc2 dual-beam spectrophotometer. Fluorescence emission and excitation spectra were recorded by means of a Jobin-Yvon Fluoromax-3 spectrofluorometer. Spectra were corrected for the spectral distribution of the lamp intensity and the response of the excitation monochromator unit on the one hand and for the spectral response of the emission monochromator and of the photomultiplier on the other hand.

2.4. Subpicosecond transient absorption spectroscopy

Transient absorption experiments were performed by the pump–probe technique with a white-light continuum probe. The subpicosecond light source is a homemade dye laser driven by a seeded 10-Hz Q-switched Nd:YAG laser [23]. It delivers two intense synchronous 600-fs pulses at different visible wavelengths. One was used as the pump in the vicinity of 600 nm and the other, at 568 nm, was used to generate a white-light continuum used as the probe. Pump and probe beams crossed on the sample, held in a 1-mm cuvette, at an angle of $\sim 10^{\circ}$ and their polarizations were set at the magic angle. The pump beam was tuned at the redmost absorption maximum of each sample and focused onto a 1-mm^2 section, with an energy of about 60 μJ. The probe beam was delayed with respect to the pump beam in an optical delay line allowing a maximum time delay of 1.6 ns. Photolysis was avoided by continuously moving the sample cuvette up and down with a translation stage. Spectra formed by an imaging spectrograph (Jobin-Yvon 270 M, 100 grooves/mm) were recorded on a CCD detector (Roper Scientific, RTE/CCD-128-H, 100 × 1024 pixels) and accumulated over 500 to 25,000 pump shots, depending on the experiment. The time-resolved differential absorbance spectra ($\Delta A(t)$ = difference between the absorbances of the excited sample, at time t, and the unexcited sample) were corrected for group velocity dispersion of the probe beam. The experimental error was better than ±2 mOD (rms) and the time resolution of the experiments was found to be around 1.3 ps. After having checked that no time-resolved spectral shifts were present, a global kinetic analysis of the data was undertaken. The temporal profiles at multiple wavelengths were simultaneously fitted to a sum of exponentials convoluted by a Gaussian function representing the response function of the experimental set-up.

2.5. Nanosecond transient absorption spectroscopy

In order to reach nanosecond time delays between the pump and the probe beams, two independent subpicosecond lasers (such as the one described above) were electronically synchronized [24]. One of the lasers was used as the pump and was tuned at the redmost absorption maximum of the sample while the other laser, tuned at 610 nm, was used to generate the white-light continuum probe. The time resolution of this experiment, limited by the jitter of the homemade synchronization device, was around 2 ns.

3. Results and discussion

3.1. Steady-state optical spectroscopy

Fig. 2 displays the steady-state absorption (hairlines) and fluorescence (dotted lines) spectra of OxyBP in DMSO (part A), OBIP (B) and the OxyBP–HSA complex (C). In the three cases

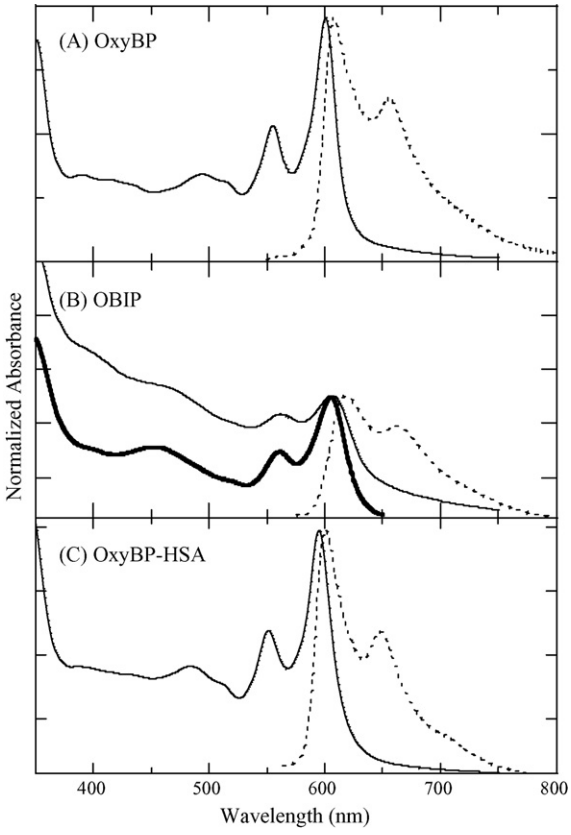

Fig. 2. Steady-state absorption (hairline), fluorescence (dashed line) and excitation (bold line) spectra of OxyBP in DMSO (A), OBIP (B) and of the OxyBP–HSA complex (C) in a phosphate buffer solution.

Table 1
Absorption and fluorescence wavelength maxima of OxyBP, OBIP and OxyBP–HSA

	λ_{abs} (nm)	λ_{em} (nm)
OxyBP	601	608
OBIP	605	618
OxyBP–HSA	595	601

the absorption and fluorescence spectra exhibit clear mirror-image symmetry and a small Stokes shift (190 cm^{-1} for free OxyBP, 350 cm^{-1} for the chromoprotein and 170 cm^{-1} for the OxyBP–HSA complex). This suggests that the geometrical and electronic structure of the fluorescent state is close to that of the Frank–Condon state reached by the lower absorption transition. The fact that the spectra of OBIP are noticeably broader than those of the other two systems might be explained assuming the sample is not completely homogeneous. One can also observe that the absorption spectrum of OBIP displays an important scattering background, probably due to impurities co-separated with the chromoprotein during the chromatography and reconcentration process. A fluorescence excitation spectrum of OBIP is given in Fig. 2B (bold line) in order to, at least partially, avoid distortion due to that effect. Only little differences are observed between the absorption or fluorescence maxima of OxyBP in the three environments (see Table 1). This is not very surprising because OxyBP displays small solvatochromic effects (data not reported here) just as hypericin, the (weak) solvatochromic shifts of which were however rationalized in terms of polarity ($E_T(30)$) and hydrogen-bond donating character (α) of the solvent [25,26].

The fluorescence quantum yields of free OxyBP and of OBIP were measured with rhodamine 101 as a reference compound [27]. The value obtained for OxyBP in DMSO is 0.07, which is considerably smaller than that reported for hypericin (0.3 [25,28]). Since the triplet yield of OxyBP is even smaller (0.12 in ethanol [29]) than that of hypericin (0.5–0.7 [30–32]), it can be concluded that the reduced fluorescence quantum yield of OxyBP is due to a strong non-radiative process, the rate of which dominates the excited-state lifetime of the molecule (1.3 ns in DMSO [9]). The fluorescence quantum yield of OBIP is 0.03, indicating that the fluorescence of OxyBP in its protein environment is further quenched. This shows either that the above radiationless deactivation of OxyBP is favored by the protein environment or that an additional non-radiative pathway is facilitated by the interaction with the protein. One can think that such a channel is the actual trigger of the phototransduction chain in the living cell.

3.2. Transient absorption of OBIP: description

Fig. 3 shows the transient absorption spectra of the OBIP chromoprotein in a phosphate buffer (0.2 M, pH 7.4), accumulated over 5000 laser shots. Their overall shape reminds that of free OxyBP and hypericin in organic solution [13,33]. In brief, a large negative peak is observed around 610 nm, which arises from the merged contributions of the main bleaching and stimulated-emission bands. Since scattering of the pump beam (605 nm) was large in this experiment, a small spectral region around the maximum of this band was removed from the figure. On both sides of the central peak, transient absorption (positive) bands are observed, the short-wavelength one bearing a clear bleaching (negative) contribution at 560 nm. However, substantial differences with the cases of OxyBP or hypericin are observed in the 640–740 nm spectral range. During the first ca. hundred picoseconds this region is dominated by a new absorption band located around 680 nm (Fig. 3, inset). As previously described [13], this spectral region displays a highly non-homothetic fast evolution. An initial positive signal rapidly transforms into a negative signal around 660 nm. This negative contribution arises from stimulated emission as expected from the steady-state fluorescence shoulder seen at the same wavelength (Fig. 2B). In the case of free OxyBP or hypericin this contribution is dominant at all times [13,33].

It is interesting to note that the band at 680 nm is already present at the end of our excitation pulse and is seen to be quite

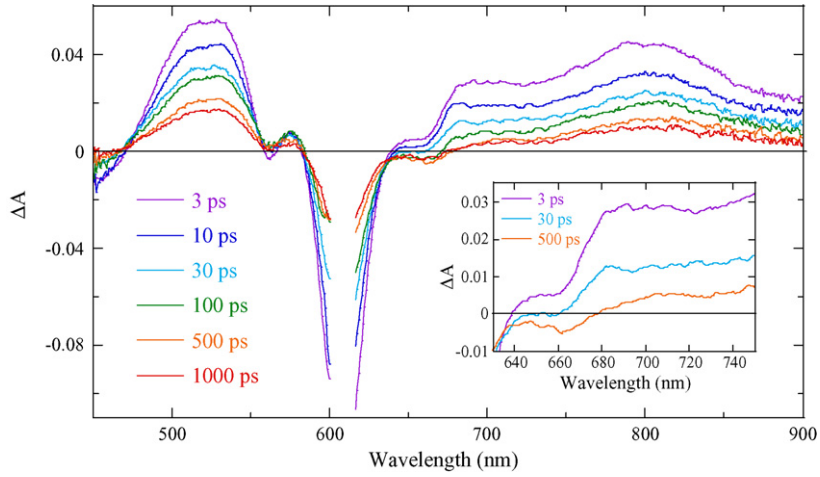

Fig. 3. Transient absorption spectra of the OBIP chromoprotein for different pump–probe delays after subpicosecond excitation at 605 nm. Both the reactive and non-reactive OBIP contribute to the observed spectra (see text). Inset: blowup of the spectra in the 630–750 nm region where the photoproduct of the reactive protein is dominant.

broad (see time 3 ps in Fig. 3). It then develops into a much narrower peak, centered at 680 nm, in a few ps (see times 10 or 30 ps in Fig. 3) and then disappears completely in a couple hundred picoseconds (see time 500 ps in Fig. 3). One can propose that the initial (3 ps in Fig. 3) broad transient absorption band in the 680-nm region is a characteristic feature of the primary excited state of OBIP, due to specific interactions between OxyBP and the apoprotein. It could for instance be assigned to a "chromophore-to-protein" charge transfer band (informal suggestion from Prof. H. Masuhara, Department of Applied Physics, Osaka University), somehow analogous to the well-known metal-to-ligand charge transfer bands in organometallic complexes. Alternatively, the high similarity between the steady-state optical spectra of OBIP and free OxyBP (Fig. 2) suggests that the primary excited state of the chromoprotein may also be spectrally similar to that of free OxyBP. Such an argument holds quite well for the comparison of OxyBP and hypericin in organic solution [13,33]. Accordingly, the initial band should be assigned to a photoproduct rapidly formed during the excitation pulse, that is, in the femtosecond regime. In both alternatives, the narrower peak observed at 680 nm at 10 ps appears as a further evolution of the initial broad band.

A kinetic global analysis of a different and previously reported set of data [13] showed that the decay traces at 15 significant wavelengths across the spectrum are well described by a sum of three exponentials. The fast evolution, in particular detected in the 640–740 nm range, is accounted for by two largely weighted exponentials, the lifetimes of which are 4 and 56 ps. No such decays were detected for free OxyBP or hypericin in organic solution. The third exponential has a lifetime of about 1.8 ns which is comparable to the component that dominates the excited-state decay of free OxyBP.

It is very important to note that for long pump–probe delays (after 150 ps), i.e. when the 4 and 56 ps components have completely vanished, the transient absorption spectra of the chromoprotein are very similar to those of free OxyBP. Fig. 4 illustrates this far-reaching similarity. It should be kept in mind that the steady-state absorption and fluorescence spectra of those two systems are slightly shifted from each other, by a few nanometers. Consequently the negative bleaching and stimulated contributions of the spectra shown in Fig. 4 are also expected to be shifted. Once this phenomenon has been taken into account, the similarity of their spectral distribution is quite striking. This observation strongly suggests that the excited-state species remaining at long pump–probe delays in the case of the chromoprotein is, spectroscopically, an excited free-like OxyBP species. On the other hand, as pointed out above, its lifetime was found to be of the order of 1.8 ns, which is in fair agreement with the lifetime of free OxyBP in organic solution (1.3 ns in DMSO, 1.6 ns in ethanol [9]).

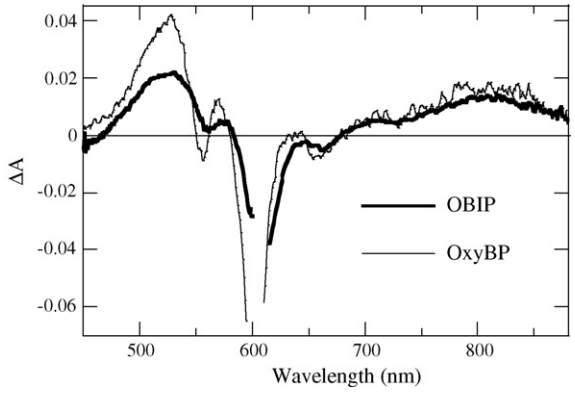

Fig. 4. Transient absorption spectra of OBIP (bold line) and free OxyBP in DMSO (hairline), for a pump–probe delay of 500 ps after subpicosecond excitation at 605 and 601 nm, respectively (the region of scattered light has been masked). This comparison shows that the transient spectroscopy of the non-reactive chromoprotein (see Section 3.3) is similar to that of the free chromophore.

3.3. Transient absorption of OBIP: interpretation

The two most salient transient absorption features of the OBIP chromoprotein are, on the one hand, an initial specific spectro-temporal feature at 680 nm which decays in few hundred picoseconds and, on the other hand, a free OxyBP-like species that dominates in the longer nanosecond time scale.

A possible explanation for this observation would be to invoke an excited-state photodissociation process, occurring with characteristic lifetimes of 4 and 56 ps. This would mean that the initial excited chromophore–protein complex, characterized by the 680-nm band, would dissociate into a free-like species. It could either be a complete dissociation of the chromophore–protein complex, leading to the release of the chromophore in the solution or an "intra-cavity" dissociation due to a disruption of the interaction between the protein and the chromophore, within its protein pocket. Since we observed that the OBIP sample is very stable and keeps the same spectro-temporal features after long periods of irradiation, it would be reasonable to assume that re-association takes place once OxyBP returns to its ground state. We can show that such a hypothesis can be discarded on a step-wise energy argument. We have seen above that the (steady-state) Stokes shift of the OBIP chromoprotein has a value of about 350 cm^{-1}. Considering the photoinduced decomplexation scenario, this energy must be considered as the sum of three contributions: the excited-state dissociation energy of the complex, the ground-state re-association energy of OxyBP with the protein and the sum of the relaxation energies of the Frank–Condon states, respectively, reached by the absorption and the fluorescence transitions. This last relaxation term can be approximated by the value of the Stokes shift in the case of free OxyBP (~250 cm^{-1}), that is, when no dissociation is present. It follows that the sum of the dissociation and re-association energies can be estimated to be about 100 cm^{-1} (0.29 kcal mol^{-1}). A first conclusion to be drawn is that the ground-state re-association energy of the complex should clearly be inferior to kT at room temperature (0.58 kcal mol^{-1}). This in turn implies that the two forms of the chromophore, associated and dissociated, would be in equilibrium (with nearly equal concentration) in the ground state because no high potential energy barrier between them is expected (no covalent bond is involved in the binding). At this point, it must be recalled that the OBIP samples were prepared by filtration over 30-kDa-cutoff mem-

branes, which initially eliminates free OxyBP from the solution. We further checked by filtrating again the sample several weeks after preparation that no free OxyBP molecules had dissociated from their protein partner with time. We can safely conclude that no free OxyBP ever gets in equilibrium with OBIP and thus that complete photodissociation of the complex must be ruled out. The only photodissociation possibility left would be an "intra-cavity" one. If this were true, OxyBP would stay close to its initial binding site and an excited-state equilibrium would necessarily be reached between the associated and dissociated forms because: (i) the dissociation energy is inferior to kT and (ii) the OxyBP excited-state lifetime is much longer than the times speculated here to characterize the dissociation process (4 and 56 ps). As a consequence, the transient spectral features that have been shown to be characteristic of the OBIP complex should at least partly remain during all the nanosecond lifetime of the system. This prediction is inconsistent with our experimental observation that the 680-nm band disappears completely after 200 ps and finally allows us to completely reject the photodissociation hypothesis.

As an alternative, we propose that the chromoprotein sample is actually heterogeneous and can be divided into two independent classes of population. A first one, named "reactive", would be at the origin of the specific spectral and dynamical features we observed at short times. The second one would be "non-reactive" and behave like free OxyBP in organic solution, explaining thus the long-time behavior of the system. As we previously suggested, this putative heterogeneity could arise from different configurations of the chromophore within the protein, such as different binding sites, different interactions between the protein and the five spectroscopically indistinguishable forms of OxyBP [34,35] or different orientations of OxyBP inside the protein pocket [13]. The heterogeneity of the OBIP sample could also explain the significant width of its steady-state absorption spectrum. Moreover, on the basis of the relative weights of the 4- and 56-ps components (assigned to the reactive population) measured in the bleaching region of the spectrum, namely around 25% for each, it can be argued that around 50% of the OBIP molecules are in a reactive configuration while the other half are in a non-reactive one.

At this point it is crucial to identify the nature of the photochemical reaction through which the reactive species comes across. It is especially important to assign the specific transient absorption band at 680 nm which occurs together with a background due to the non-reactive chromoprotein (Fig. 3). Since the latter is observed in a pure form in the transient absorption spectrum of OBIP at 500 ps (Fig. 4) and decays in 1.8 ns, its expected amplitude can be estimated at anytime. Let us focus here on the spectrum of OBIP at 10 ps. Its non-reactive background can be approximated by dividing the spectrum at 500 ps by the decay factor $e^{-490/1800}$. The result can then be subtracted from the spectrum of OBIP at 10 ps in order to obtain the pure transient absorption spectrum of reactive OBIP at 10 ps. Fig. 5 displays this difference spectrum, which exhibits a well-defined peak at 680 nm. As we mentioned in the Introduction it had been suggested that intermolecular electron transfer could be the primary mechanism involved in the photophobic response of *B. japonicum* [9,10]. Indeed, the 680-nm spectral feature of Fig. 5 reminds the spectrum of the radical cation of blepharismin (BP) obtained both by radiolysis and flash-photolysis experiments [36]. In the following, we recorded the spectral signature of the radical cation of OxyBP ($OxyBP^{\bullet+}$) with a photooxidation experiment and compared it directly to the pure reactive OBIP transient spectrum at 10 ps (Fig. 5).

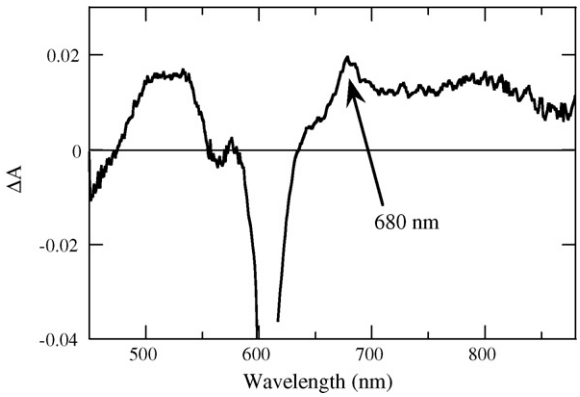

Fig. 5. Pure transient absorption spectrum of reactive OBIP at 10 ps, obtained by subtracting the non-reactive background from the spectrum of OBIP at 10 ps. The non-reactive background has been estimated by dividing the spectrum at 500 ps by $e^{-490/1800}$.

3.4. Photooxidation of OxyBP

Photooxidation of free OxyBP in ethanol was achieved in the presence of a suitable electron acceptor, 1,4-benzoquinone (BZQ), known to quench the fluorescence of OxyBP with a nearly diffusion-limited quenching constant of $8 \times 10^9\ M^{-1}\ s^{-1}$ [9]. Considering this value, experimental conditions ($[OxyBP] = 4.5 \times 10^{-4}$ M, $[BZQ] = 8 \times 10^{-2}$ M) were chosen in order to ensure that 50% of the first excited singlet population of OxyBP transfers an electron to BZQ. It was checked that the absorption of OxyBP is not changed by the presence of BZQ, i.e. no ground-state complexes are formed. The fluorescence spectrum is not changed either, indicating that no fluorescent species is formed during the excited-state lifetime. The fluorescence quantum yield of OxyBP in the presence of BZQ has been calculated to be four times lower than that of free OxyBP in ethanol (i.e. quenching yield = 3/4), that is, twice lower than the expected value. This phenomenon is likely due to the so-called "transient effect" [37], i.e. the non-diffusion-limited electron transfer reaction between an excited OxyBP molecule and a BZQ molecule initially localized in its solvation sphere. Such an effect is only found in the case of high quencher concentrations, as it is the case here.

Fig. 6A displays the transient absorption spectra, averaged over 25,000 pump pulses, of free OxyBP in ethanol with and without BZQ, upon subpicosecond excitation at 592 nm, for pump–probe delays of 10 and 20 ns, respectively. These delays allow to record transient absorption spectra free from any contribution of the excited singlet state of OxyBP, the (unquenched) lifetime of which is 1.6 ns [9]. In the absence of BZQ, we obtain the differential absorption spectrum of the triplet state (T_1) of OxyBP, the shape of which is in good agreement with

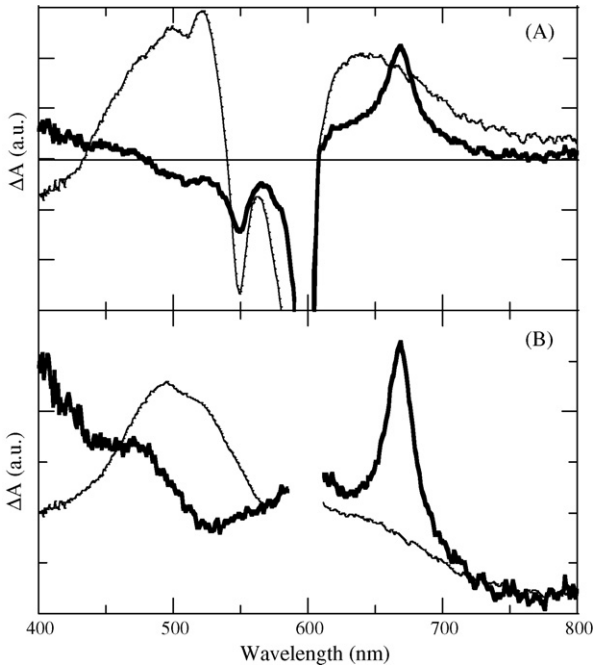

Fig. 6. (A) Transient absorption spectra of OxyBP in ethanol (hairlines) and of OxyBP in the presence of BZQ (bold lines) for pump–probe delays of 20 and 10 ns, respectively, after subpicosecond excitation at 592 nm. (B) The same spectra corrected from the bleaching contribution.

a previously published spectrum [29]. In the presence of BZQ a very different spectrum is obtained, exhibiting a distinctive narrow band at 670 nm, quite similar to a structure previously reported around the same wavelength for the radical cation of BP [36]. We assign this spectrum to the radical cation of OxyBP ($OxyBP^{\bullet+}$). The 670-nm band strongly reminds the transient band at 680 nm of reactive OBIP at 10 ps (Fig. 5). The latter however appears somewhat broader, possibly due to the interaction of the cation radical with the protein, just as the protein is known to broaden the steady-state absorption spectrum of OxyBP (compare Fig. 2A and B). The ∼10-nm spectral shift can be understood to simply arise from the difference between the absorption maxima of OxyBP in ethanol (592 nm) and OBIP in the phosphate buffer (605 nm). Consequently, one may propose that photoinduced electron transfer indeed occurs within the reactive population of OBIP, with a very fast characteristic time since no delayed rise of the 680-nm band is actually detected. The remaining transient absorption band that appears around 800 nm in Fig. 5 after subtraction of the non-reactive protein transient spectrum may however indicate the presence of a residual contribution of the initially excited state of the reactive OBIP. A detailed kinetic analysis [38] (to be submitted in a future article) confirms the qualitative observations of Section 3.2, namely the fact that a rather broad transient absorption band is initially produced by the excitation pulse in this spectral region, which develops in 4 ps into a narrower 680-nm peak, tentatively identified with the $OxyBP^{\bullet+}$ radical. The decay of this radical species occurs with a characteristic time of 56 ps.

Fig. 6B displays bleaching-corrected spectra of the photooxidation experiment, obtained by adding to the original differential absorption spectra a fraction of the ground-state absorption spectrum. When the characteristic dips of the bleaching (in particular at 550 nm) are being completely compensated, the fraction (q) of the initial ground-state population that remains in any transient state is then obtained (see Ref. [39] for details). In a standard picosecond experiment (without any quencher) and under the same experimental conditions, the q coefficient is found to be around 20% immediately after the pump pulse. In the case of OxyBP in the presence of BZQ, since the observed quenching ratio is 3/4, the expected value of q at 10 ns is of the order of 15%. Surprisingly, the actual value is found to be around 2%, meaning that the effective photooxidation yield is only of the order of 10%. This effect is likely due to an important geminate recombination, restoring the ground state of OxyBP, which occurs before the two reaction partners have moved away. In fact the above calculation neglects the formation of triplet state but its contribution to the q value at 10 ns is expected to be small. Since the triplet yield in the absence of BZQ is 0.12 [29], it should be reduced to 0.03 in the presence of BZQ (same quenching factor as the fluorescence). As a matter of fact no trace of triplet state is seen in the transient spectrum of OxyBP in the presence of BZQ, which means that the triplet state of OxyBP also has the ability to transfer an electron to BZQ, as was shown to be the case for BP [36].

It is worth mentioning that a contribution of the radical anion of BZQ ($BZQ^{\bullet-}$) is expected in the 400–500 nm region [40,41] of the OxyBP transient spectrum in the presence of BZQ. This contribution may however not be clearly recognized because the molar extinction coefficient of $BZQ^{\bullet-}$ ($9400\ M^{-1}\ cm^{-1}$ at 458 nm in methyltetrahydrofuran at 77 K [42]) is not high enough. Similarly if we assume that, in reactive OBIP, OxyBP rapidly transfers an electron to the protein, the spectrum of the acceptor's radical anion is also expected to contribute to the transient spectra at short times. As previously suggested [10], the electron acceptor could well be a disulphide bridge, the radical anion of which absorbs between 350 and 550 nm at pH 7 [43]. Such a contribution could explain the rapid evolution of the transient spectra of OBIP below 550 nm (Fig. 3).

To conclude this section, it is important to recall that the transient absorption spectrum of the OxyBP/BZQ system was recorded for a 10-ns delay. Consequently one cannot exclude that the rapidly produced $OxyBP^{\bullet+}$ species could react on this rather long-time scale. One can in particular imagine that a proton could be released by the $OxyBP^{\bullet+}$ radical cation, as was formerly suggested [10]. According to this hypothesis, the specific 680-nm band would be the spectral signature of the deprotonated radical cation of OxyBP, produced in at most 4 ps and decaying in 56 ps. A very interesting matter would then be accessing to the detailed rates of the precursor steps, that is, electron transfer and deprotonation. It is probable that the fastest reaction would then occur on a femtosecond scale, beyond the time resolution of our present experiment. Further studies are under preparation in order to unravel this particular issue.

3.5. OxyBP–human serum albumin (HSA) complex

The steady-state absorption and fluorescence spectra of the artificial complex OxyBP–HSA in a phosphate buffer (Fig. 2C)

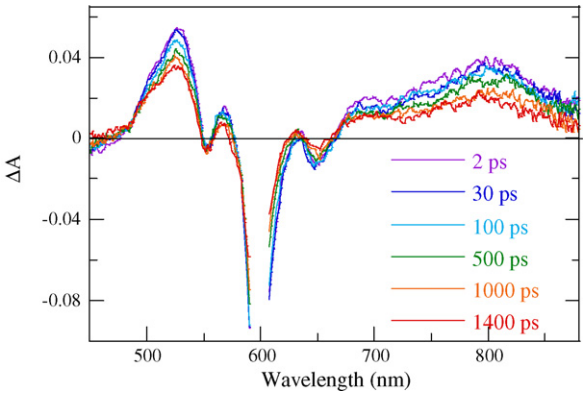

Fig. 7. Transient absorption spectra of the OxyBP–HSA complex in a phosphate buffer (0.2 M, pH 7.4, no NaCh) for different pump–probe delays after subpicosecond excitation at 595 nm (the scattered excitation light has been masked).

are extremely similar to those of the free OxyBP in organic solution (Fig. 2A). The same strong mirror-image symmetry and small Stokes shift are observed. However, as for the OBIP chromoprotein, the fluorescence quantum yield of this system is lower than for free OxyBP (0.03 versus 0.07), indicating the presence of additional non-radiative pathways.

The shapes of the transient absorption spectra of OxyBP–HSA (Fig. 7) and OxyBP [12,13] are quite identical. In particular, the 650-nm region is dominated by the contribution of stimulated emission, contrary to the case of OBIP, no band is observed around 680 nm and no massive evolution of the spectra is seen at short times. These findings allow us to conclude that the specific primary photoprocesses of OBIP do not take place in the case of the OxyBP–HSA complex.

A kinetic analysis of this system gave also comparable results as for free OxyBP in DMSO [13]. A global fit procedure was performed on 20 selected wavelengths across the spectrum. The sum of a biexponential function and a step function, which represents a long-lived species, was found to satisfactorily describe the data (as in the case of OxyBP). Fig. 8 displays four of these kinetic traces chosen in the visible excited-state absorption band (510 and 530 nm), in the stimulated-emission side of the large negative peak (610 nm) and in the far-red absorption band (700 nm).

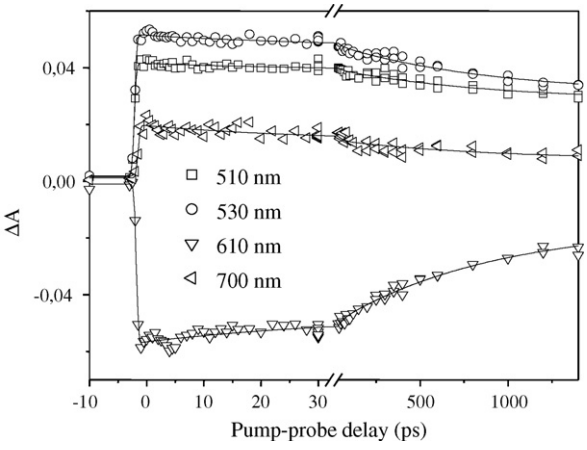

Fig. 8. Global fit of selected kinetic traces of OxyBP–HSA in solution in a phosphate buffer after subpicosecond excitation at 595 nm.

The result highlights a short component of 29 ps, present only as a small-amplitude (less than 15%) decay at all studied wavelengths. The second measured lifetime of 0.9 ns is identified as the fluorescence lifetime of the OxyBP–HSA system.

A similar analysis of a recent experiment on OxyBP in DMSO (data not shown here) produced kinetic components of 16 ps and 1.1 ns, respectively. The significant analogy between both the values and the weights of the short components of OxyBP and OxyBP–HSA suggests that the process at the origin of these short components is probably the same. As we previously published [13,33] and referring to the non-planar structure of OxyBP in the ground state [44,45], such a component could be assigned to a small conformational relaxation of the molecule in its excited state, induced by the π–π^* transition located on the aromatic core of the molecule.

As far as the amplitude of the long-lived species is concerned, a sizeable difference is observed between OxyBP and OxyBP–HSA. While its weight in the bleaching band is around 25% for OxyBP, it is as high as 60% for OxyBP–HSA in the same region. Assuming that the long-lived species in the case of OxyBP–HSA is due to the triplet state of the chromophore, this result indicates that the intersystem crossing (ISC) yield of this artificial complex is more than twice larger than in the case of OxyBP. The surrounding HSA protein seems then to increase the ISC rate and this effect could be due, for instance, to the proximity of OxyBP with a heavy atom, such as the sulphur atom of a cysteine residue of the protein [46].

From all these elements it is finally concluded that the OxyBP–HSA complex provides a definitely poor model of the OBIP chromoprotein. Its transient absorption behavior is very similar to that of free OxyBP in solution and displays no sign of photoinduced electron transfer. Although the results of this particular study can by no way be taken as a general rule, they nevertheless point to the fact that the transient absorption behavior of OBIP has a specific character, which in turn supports the idea that OBIP is indeed a specialized photoreceptor.

4. Conclusion

In the present work we first proposed a detailed and comprehensive analysis of the primary photoreactivity of the chromoprotein responsible of the photophobic response of *B. japonicum*, OBIP, as observed by subpicosecond transient absorption spectroscopy. We ruled out a chromophore–protein photodissociation mechanism by energy arguments based on the small steady-state Stokes shift of the OBIP system and reinforced our previous interpretation [13] that OxyBP is non-covalently bound to its protein pocket in different configurations, leading to the existence of a reactive and a non-reactive population. The reactive population gives rise to specific spectral features, in particular in the 640–740 nm spectral range, and to a fast dynamics described by a biexponential decay of 4 and 56 ps. The non-reactive population mostly behaves like the free chromophore OxyBP in solution.

We performed a bimolecular photooxidation of OxyBP in ethanol, in order to obtain a spectral signature of the OxyBP radical cation, and checked the hypothesis that an excited-state

intermolecular electron transfer within the reactive protein could be the primary step of the sensory transduction chain [9]. This photooxidation experiment evidenced a well-defined peak at 670 nm which, taken into account a small solvatochromic shift between the two systems, strongly reminds the transient absorption spectral feature at 680 nm of reactive OBIP at 10 ps. This is an important element that supports the electron transfer hypothesis. Although a detailed understanding of the kinetics of this reaction is not yet available, speculations can be made over the involvement of a proton release during this primary reaction. It can also be suspected that early steps of it could occur on the femtosecond time scale. Further experiments are presently under preparation in order to check this possibility.

Finally, we approached the question of the specificity of the interaction between OxyBP and its native protein partner by examining the transient absorption behavior of an artificial complex between OxyBP and the well-known protein human serum albumin (HSA). The specific spectro-temporal features of the OBIP chromoprotein could not be reproduced with the artificial OxyBP–HSA complex, which showed that the OxyBP–protein interactions within OBIP bear at least a certain degree of specificity. Such an element favors the hypothesis that OBIP is a specialized macromolecular assembly, most likely playing the role of photoreceptor for the step-up photophobic response of *B. japonicum*.

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References

[1] M. Kraml, W. Marwan, Photochem. Photobiol. 37 (1983) 313–319.
[2] F. Ghetti, in: F. Lenci, F. Ghetti, G. Colombetti, D.P. Häder, P.-S. Song (Eds.), Biophysics of Photoreceptors and Photomovements in Microorganisms, NATO ASI Series, Series A, Life Sciences, vol. 211, Plenum, New York, 1991, pp. 257–265.
[3] P. Scevoli, F. Bisi, G. Colombetti, F. Ghetti, F. Lenci, V. Passarelli, J. Photochem. Photobiol. B: Biol. 1 (1987) 75–84.
[4] T. Matsuoka, S. Matsuoka, Y. Yamaoka, T. Kuriu, Y. Watanabe, M. Takayanagi, Y. Kato, K. Taneda, J. Protozool. 39 (1992) 498–502.
[5] G. Checcucci, G. Damato, F. Ghetti, F. Lenci, Photochem. Photobiol. 57 (1992) 686–689.
[6] T. Matsuoka, D. Tokumori, H. Kotsuki, M. Ishida, M. Matsushita, S. Kimura, T. Itoh, G. Checcucci, Photochem. Photobiol. 72 (2000) 709–713.
[7] T. Matsuoka, M. Sato, M. Maeda, H. Naoki, T. Tanaka, H. Kotsuki, Photochem. Photobiol. 65 (1997) 915–921.
[8] T. Matsuoka, Y. Murakami, Y. Kato, Photochem. Photobiol. 57 (1993) 1042–1047.
[9] N. Angelini, A. Quaranta, G. Checcucci, P.-S. Song, F. Lenci, Photochem. Photobiol. 68 (1998) 864–868.
[10] A. Sgarbossa, G. Checcucci, F. Lenci, Photochem. Photobiol. Sci. 1 (2002) 459–467.
[11] G. Checcucci, Y. Takada, T. Matsuoka, Mem. Fac. Sci. Kochi Univ. Ser. D (Biol.) 22 (2001) 39–44.
[12] M. Mahet, P. Plaza, M.M. Martin, G. Checcucci, F. Lenci, in: M.M. Martin, J.T. Hynes (Eds.), Femtochemistry and Femtobiology. Ultrafast Events in Molecular Science, Elsevier B.V., Amsterdam, 2004, pp. 441–444.
[13] P. Plaza, M. Mahet, M.M. Martin, N. Angelini, M. Malatesta, G. Checcucci, F. Lenci, Photochem. Photobiol. Sci. 4 (2005) 754–761.
[14] D. Gioffré, F. Ghetti, F. Lenci, C. Paradiso, R. Dai, P.-S. Song, Photochem. Photobiol. 58 (1993) 275–279.
[15] G. Checcucci, R.S. Shoemaker, E. Bini, R. Cerny, N. Tao, J.-S. Hyon, D. Gioffré, F. Ghetti, F. Lenci, P.-S. Song, J. Am. Chem. Soc. 119 (1997) 5762–5763.
[16] A. Kida, M. Hase, D. Tokumori, Y. Takada, M. Yasuda, H. Kotsuki, G. Checcucci, T. Matsuoka, Trends Photochem. Photobiol. 10 (2003) 93–101.
[17] P. Miskovsky, J. Hritz, S. Sanchez-Cortes, G. Fabriciova, J. Ulicny, L. Chinsky, Photochem. Photobiol. 74 (2001) 172–183.
[18] P. Miskovsky, Curr. Drug Targets 3 (2002) 55–84.
[19] H. Falk, J. Meyer, Monatsh. Chem. 125 (1994) 753–762.
[20] B. Schwarzinger, H. Falk, Monatsh. Chem. 134 (2003) 1353–1358.
[21] V. Senthil, J.W. Longworth, C.A. Ghiron, L. Grossweiner, Biochim. Biophys. Acta 1115 (1992) 192–200.
[22] J.-P. Sine, Séparation et Analyse des Biomolécules, Ellipses, Paris, 2003, pp. 7–22.
[23] N.D. Hung, P. Plaza, M. Martin, Y.H. Meyer, Appl. Phys. 31 (1992) 7046–7058.
[24] D. Laage, P. Plaza, M. Blanchard-Desce, M.M. Martin, Photochem. Photobiol. Sci. 1 (2002) 526–535.
[25] A. Losi, Photochem. Photobiol. 65 (1997) 791–801.
[26] H. Weitman, M. Roslaniec, A.A. Frimer, M. Afri, D. Freeman, Y. Mazur, B. Ehrenberg, Photochem. Photobiol. 73 (2001) 110–118.
[27] T. Karstens, K. Kobs, J. Phys. Chem. 84 (1980) 1871–1872.
[28] P. Jardon, R. Gautron, J. Chim. Phys. 86 (1989) 2173–2190.
[29] F. Ghetti, G. Checcucci, F. Lenci, P.F. Heelis, J. Photochem. Photobiol. B: Biol. 13 (1992) 315–321.
[30] P. Jardon, N. Lazortchak, R. Gautron, J. Chim. Phys. 83 (1986) 311–315.
[31] H. Racinet, P. Jardon, R. Gautron, J. Chim. Phys. 85 (1988) 971–977.
[32] A. Michaeli, A. Regev, Y. Mazur, J. Feitelson, H. Levanon, J. Phys. Chem. 97 (1993) 9154–9160.
[33] P. Plaza, M. Mahet, O.N. Tchaikovskaya, M.M. Martin, Chem. Phys. Lett. 408 (2005) 96–100.
[34] M. Maeda, H. Naoki, T. Matsuoka, Y. Kato, H. Kotsuki, K. Utsumi, T. Tanaka, Tetrahedron Lett. 38 (1997) 7411–7414.
[35] D. Spitzner, G. Höfle, I. Klein, S. Pohlan, D. Ammermann, L. Jaenicke, Tetrahedron Lett. 39 (1998) 4003–4006.
[36] P.F. Heelis, B.J. Parsons, F. Ghetti, F. Lenci, C.A. Rowley-Williams, S. Naman, S. Navaratnam, J. Photochem. Photobiol. B: Biol. 24 (1994) 41–45.
[37] R.M. Noyes, Progr. React. Kinet. 1 (1961) 129–160.
[38] M. Mahet, Ph.D. Thesis, Université Pierre et Marie Curie, Paris, France, 2005.
[39] H.Y. Meyer, P. Plaza, Chem. Phys. 200 (1995) 235–243.
[40] J.-C. Ronfart-Haret, R. Bensasson, E. Amouyal, J. Chem. Soc., Faraday Trans. I 76 (1980) 2432–2436.
[41] Y. Esaka, N. Okumura, B. Uno, M. Goto, Anal. Sci. 17 (2001) 99–102.
[42] T. Shida, Electronic Absorption Spectra of Radical Ions, Elsevier, Amsterdam, 1988, p. 308.
[43] S.P. Mezyk, Chem. Phys. Lett. 235 (1995) 89–93.
[44] A. Kida, Y. Takada, H. Kotsuki, D. Tokumori, G. Checcucci, T. Matsuoka, Microbios 106 (2001) 189–201.
[45] O. Pieroni, P. Plaza, M. Mahet, N. Angelini, G. Checcucci, M. Malatesta, M. Martin, F. Lenci, Photochem. Photobiol. 81 (2005) 1343–1346.
[46] P. Drössler, W. Holzer, A. Penzkofer, P. Hegemann, Chem. Phys. 286 (2003) 409–420.