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Photoinduced electron transfer in crystal violet (CV^+)–bovine serum albumin (BSA) system: evaluation of reaction paths and radical intermediates

A.C. Bhasikuttan, A.V. Sapre*, L.V. Shastri

Radiation Chemistry and Chemical Dynamics Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085, India

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Abstract

Photoinduced electron transfer (PET) from excited probes attached to proteins is of considerable current interest. Photochemical processes following 532 nm excitation of triphenyl methane dye, crystal violet (CV^+) bound to a protein, bovine serum albumin (BSA), have been investigated in picosecond (ps) to microseconds (μs) time scales by flash photolysis technique. The excited singlet state lifetime of CV^+ is found to be increased to ∼130 ps as compared to ∼1–5 ps for the unbound dye in low viscosity solvents. From flash photolysis studies in microsecond region, transient absorption in the region ∼650 nm is observed which is attributed to the dication radical $CV^{\bullet 2+}$ formed by electron transfer from $^3CV^{+*}$ to BSA, contrary to electron transfer from BSA to the excited dye as proposed in a recent report. Supporting spectral evidence for the electron transfer from $^3CV^{+*}$ to BSA is obtained from pulse radiolysis studies.

Keywords: Photoinduced electron transfer; Bovine serum albumin; Crystal violet

1. Introduction

Study of photoinduced damage to cellular components by free radical species generated from excited state precursors of chromophoric attachments is of considerable recent interest [1–4]. Photodynamic therapy (PDT), which is based on the photochemical generation of highly reactive cytotoxic species using photosensitizing agents that cause damage to the undesired components like tumor cells, has become a topic of contemporary research in medical sciences [5]. The mechanisms leading to tumor destruction by PDT are not yet fully understood and several questions remain unanswered regarding the primary cytotoxic events occurring on the cellular level as well as possible secondary effects related to the tumor environment [5,6]. The combined involvement of a photosensitizer, light and dissolved molecular oxygen has been considered as the requirements for the phototoxic effects to develop. Photosensitized reactions are generally mediated by the triplet excited state of the sensitizer. The excited state of the sensitizer, singlet or triplet ($^1S^*/^3S^*$), may interact with the substrate by electron transfer (reductive/oxidative) and/or hydrogen atom abstraction process (reactions I and II). Another key reaction route is energy transfer between the triplet excited state of the sensitizer and oxygen to give singlet oxygen (reaction III).

$$^1S^*/^3S^* + RH \rightarrow S^{\bullet -}/S^{\bullet +} + RH^{\bullet +}/RH^{\bullet -} \quad \text{Electron transfer} \quad \text{I}$$

$$^1S^*/^3S^* + RH \rightarrow SH^{\bullet} + R^{\bullet} \quad \text{H atom abstraction} \quad \text{II}$$

$$^3S^* + O_2 \rightarrow S + {}^1O_2 \quad \text{Singlet oxygen generation} \quad \text{III}$$

In the reactions I and II, both singlet and triplet state of the sensitizer may be involved. These reactions become more competitive to reaction III when the dye chromophore is directly bound to the substrate.

Among many classes of compounds that have been tested as photosensitizers, cationic dyes of the triphenyl methane (TPM) class seem to be especially promising. TPM dyes are well-known class of organic dye molecules having wide ranging commercial, technological and medical applications [7–15]. In chromophore-assisted laser inactivation (CALI) technique, laser energy is targeted to damage single protein species by binding it with a specific antibody that has been labeled with a chromophore. Liao et al. [16] have used a TPM dye, malachite green isothiocyanate, to study selective photoinactivation of enzyme β-galactosidase. They concluded that a sequential two-photon process results in the generation of hydroxyl radicals that act as reactive species

* Corresponding author. Fax: +91-22-5505151.
E-mail address: cdscd@magnum.barc.ernet.in (A.V. Sapre).

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for inducing photodamage. Victoria Blue BO (VBBO), another TPM dye, has been examined as a new photosensitizer for PDT [17,18]. In their work, Viola et al. [17] showed that VBBO is unable to generate singlet oxygen by visible light irradiation of the oxygenated cellular matrices, however, the photocytotoxicity proceeds by a mechanism involving $O_2^{\bullet -}$ and $OH^{\bullet}$ radicals. In a recent paper, Baptista and Indig [19] have reported photophysical and photochemical properties of three TPM dyes, ethyl violet (EV), crystal violet and malachite green on a model protein, bovine serum albumin (BSA). They found that on binding to BSA, fast non-radiative relaxation processes in the excited singlet state that occur via rotational motion of the aromatic rings of TPM dyes are decelerated, thus increasing fluorescence quantum yields, lifetimes of singlet states, intersystem crossing efficiencies and photoreactivity towards protein substrate.

Photo and radiation chemical studies of some TPM dye molecules in aqueous and organic media have been actively pursued by our group in the past few years [20–25]. We have reported the spectral and kinetic characteristics of the transient intermediates derived from some of the TPM dyes, especially from the cationic dye crystal violet (CV^+), in detail. The dication $CV^{\bullet 2+}$ has been characterized through photoionization process as well as through reaction with oxidizing agents [22,23]. Reduced CV^+ radicals generated by the reaction with hydrated electron in a pulse radiolysis experiments have been characterized [24]. Properties of triplet state of CV^+ produced by the interaction of CV^+ with aromatic triplet states employing energy transfer methods in a radiolysis experiment have been studied [21,25]. It has been established that all these intermediates and precursors have considerable optical absorption in the window region (360–460 nm) of the parent dye absorption. Further, from literature reports it is known that the excited singlet–singlet absorption spectrum of CV^+ and EV in alcohols, also display strong absorption bands centered at ~410 nm [26–29]. Hence, spectral identification of these intermediates becomes inconvenient when more than one reaction channel is operative in the reaction system. From the spectral studies, Baptista and Indig [19] have proposed a mechanism involving an electron transfer from the BSA moiety to the excited dye as a major reaction channel in the photoexcited CV^+–BSA system. Owing to the difficulties in the identification of the transients, we have undertaken a detailed study of the system. Results from flash photolysis experiments are augmented using a 7 MeV electron linear accelerator (LINAC) based pulse radiolysis, for arriving at the direction of electron transfer after photoexcitation of the above system.

2. Experimental

Crystal violet in its chloride form was obtained from Sigma Chem., and was used as received. BSA (fraction V powder, Mol. Wt. 67,000) was purchased from SISCO, India and was used as received. All aqueous solutions were prepared using nanopure water obtained from a Barnstead system (resistivity 18.3 MΩ cm). Oxygen-free N_2 (Iolar-2, purity >99.9%, Indian Oxygen) or N_2O (Indian Oxygen, Iolar grade, purity >99.8%) was used for deaerating the solutions, depending upon the experimental conditions. All the solutions were buffered with 5 mM phosphate buffer (sodium salts, BDH AR) to get the pH of the solution in the range 7.0–7.2.

Picosecond laser flash photolysis set up has been described in detail elsewhere [30,31]. Briefly, the second harmonic (532 nm, 10 mJ) output pulses (35 ps) from an active–passive mode locked Nd:YAG laser (Continuum, model 501-C-10) were used for excitation and the continuum (400–900 nm) probe pulses were generated by focusing the residual fundamental in H_2O/D_2O mixture. The probe pulses were delayed with respect to pump using 1 m long linear translation stage. The transient absorptions were recorded at different probe delays (up to 6 ns) by an optical multichannel analyzer (Spectroscopic Instruments, Germany), interfaced to an IBM-PC. Transient absorption signals in the microsecond time scale were studied by monitoring the optical absorption (rise time 100 ns) using the same picosecond Nd:YAG laser for excitation and a cw tungsten lamp in combination with a Bausch and Lomb monochromator (350–800 nm), Hamamtsu R928 PMT, 500 MHz digital oscilloscope (Tektronix, TDS-540A) connected to a PC. Detailed experimental setups for the electron pulse radiolysis and the kinetic spectrophotometric techniques have been described elsewhere [24,32]. Sample solutions taken in a Suprasil cuvette of 1 cm path length were irradiated by electron pulses of 50 ns duration (FWHM) from a 7 MeV linear electron accelerator (Ray Technology, UK) at a radiation dose of 10–12 Gy per pulse as measured by an air saturated 0.05 mol dm^{-3} KCNS dosimeter, taking $G\varepsilon$ for $(CNS)_2^{\bullet -}$ as 21,522 $dm^3\,mol^{-1}\,cm^{-1}$ at 500 nm (G being defined as number of molecules formed per 100 eV energy absorbed) [33]. The transient absorption profiles were monitored by a kinetic spectrophotometric arrangement consisting of a 450 W pulsed xenon lamp in conjunction with a monochromator (CVI Model 110) and a photomultiplier tube (Hamamatsu R 928), connected to a digital oscilloscope (L&T Gould 4072). The data were transferred to an IBM PC and analyzed [24]. Steady-state absorption and fluorescence measurements have been carried out using Shimadzu-160A UV visible spectrophotometer and Hitachi model F-4010 spectrophotofluorimeter, respectively.

3. Results and discussion

Ground state complexation and the corresponding spectral changes in the absorption spectrum on binding CV^+ to BSA were readily seen as reported by Baptista and Indig [19]. The spectrum of the bound dye is narrower in the 500–630 nm region than that of free dye in aqueous solution

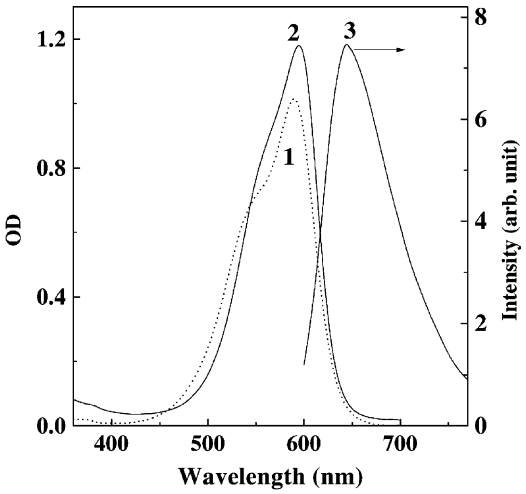

Fig. 1. Ground state absorption spectrum of CV^+ (1) and CV^+–BSA complex (2). Spectrum (3) represents the emission spectrum recorded on excitation of CV^+–BSA at 532 nm. CV^+ concentrations—for spectrum 1 and 2: 1.3×10^{-5} mol dm^{-3}, and 2×10^{-4} mol dm^{-3} for spectrum 3, pH 7.0, BSA: 1.2×10^{-5} mol dm^{-3} for spectrum 2 and 1.6×10^{-3} mol dm^{-3} for spectrum 3.

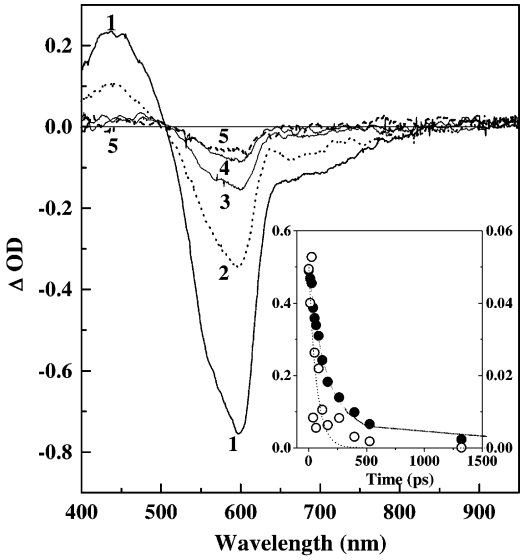

Fig. 2. Transient absorption spectra recorded on 532 nm excitation of CV^+–BSA in aqueous solutions buffered at pH 7: (1) 0, (2) 130, (3) 330, (4) 530 ps and (5) 1 ns. Inset: recovery of bleaching and stimulated emission recorded in the above solution at 600 nm (●) and 680 nm (○), respectively. The dotted lines, respectively, represent the bi-exponential and single exponential fit. Buffered at pH 7.0, CV^+ concentration: 1.5×10^{-5} mol dm^{-3}, BSA: 1.2×10^{-4} mol dm^{-3}.

and both are shown in Fig. 1. The absorption band for the former shows a red shift of ~8–10 nm with an increase in the absorption coefficient at the λ_{max}. The figure also shows the fluorescence spectrum recorded for CV^+–BSA complex in solution excited at 532 nm. The emission has a maximum at 630 nm with tailing beyond 750 nm. Though CV^+ in aqueous solution is almost non-fluorescent ($\phi_f < 10^{-4}$, [13]), on binding to BSA there is a remarkable enhancement in the emission yield [19]. In the present work the dye:BSA ratio has been maintained in the 1:8 composition for total binding of the dye to the BSA moiety. The ratio was estimated from the saturation in the fluorescence intensity on increasing the BSA concentration at a given dye concentration.

3.1. Studies with picosecond time resolution

Fig. 2 shows the time resolved transient absorption spectra observed on 532 nm, 35 ps excitation of non-covalently bound CV^+–BSA complex in aqueous solution buffered at pH 7. The spectrum recorded immediately after the pulse (Fig. 2, spectrum 1) displays a broad absorption band centered at ~450 nm and a negative absorption band in the 520–800 nm region. Following the assignments in the literature [26–29], the transient absorption ~450 nm is attributed to singlet–singlet absorption ($S_n \leftarrow S_1$) of the dye. The negative absorptions at ~600 and ~650 nm are attributed to the dye bleaching and stimulated emission, respectively.

The excited singlet state absorption band at ~450 nm is found to decay fast with simultaneous bleach recovery in the 500–640 nm region and also as the decrease in the emission intensities above 650 nm (Fig. 2, spectra 1–5). Though the transients generated from CV^+ exhibit overlapping absorptions in the spectral region below 650 nm (vide infra), the absorbance changes recorded in the picosecond time domain would mainly represent the S_1 state characteristics. It has been found that the transient decay at 450 nm and the bleach recovery at 600 nm conformed to similar bi-exponential kinetics whereas the stimulated emission decay as recorded at 680 nm showed a faster decay. The representative traces at 600 and 680 nm are shown in the inset of Fig. 2. The former fits to a good bi-exponential decay with 130 ps and 2.5 ns decay constants whereas the latter one shows a single exponential fit with 60 ps lifetime. Though, in principle, the decay of stimulated emission would represent the fluorescence lifetime, Ben-Amotz and Harris [26] have found that the lifetimes obtained from stimulated emission for CV^+ in alcoholic solvents are 2–3 times shorter than those obtained from either transient absorption ($S_n \leftarrow S_1$) decay or bleach recovery. In accordance with this point, we attribute 130 ps decay constant obtained at 450 nm as well as at 600 nm to the S_1 state lifetime of CV^+ bound to BSA. It is to be noted here that the S_1 state lifetime is about 50 times longer in BSA environment as compared to CV^+ in methanol [26]. Though TPM dyes are known to undergo very fast (<1–2 ps) non-radiative deactivation through phenyl ring rotation [34–36], the structural rigidity imposed on binding to BSA retards such fast processes and makes it possible to study singlet state properties in picosecond time domain. This is reflected in the observation of fluorescence too (Fig. 1). The viscosity of the binding site seems to be quite high (~68 cP) on comparing with the results of Ben-Amotz et al. [28] on pressure dependent decay times and reflects the rigidity of molecular environment of the dye as stated above.

When internal conversion from the first excited singlet state to the ground state is suppressed by increasing molecular rigidity, the probability of other photophysical and photochemical processes increases and this has been verified in a number of studies. The observed time dependent changes in the S_1* state absorption represent the photophysical/photochemical reaction channels in the excited singlet state of the complex. In other words, in the case of overlapping absorptions of more than one transient, the kinetic profile would represent the combined contribution of the transients at that wavelength. Such indications are clearly seen as unrecovered bleaching at longer time delay (see Fig. 2, spectrum 5). These processes could be an ISC process giving $^3CV^{+*}$ or a chemical channel like an electron transfer process within the excited CV^+–BSA complex. Photoinduced energy and electron transfer reactions have been demonstrated in various molecular systems bound to model proteins. In a recent work, Scaiano and co-workers [36] presented the characterization of electron transfer reactions of α-tertheinyl triplet in α-tertheinyl–BSA conjugates and also in freely associated complex. Though singlet oxygen generation has been widely accepted as the key process in the dye-based applications to the PDT, the importance of electron transfer from the excited singlet or triplet states has also been recognized in the photochemical destruction of cellular components. Apart from providing structural rigidity to the dye molecule, the non-covalently bound protein–dye complex can undergo electron transfer reactions within the complex since BSA contains amino acid units susceptible to redox reactions [19,36]. Due to the close contact of donor and acceptor units in the bound complex, diffusion of reactants would not be a limiting factor and hence electron transfer reaction from the singlet state of the dye might compete with the ISC process. However, due to instrumental limitations, low absorbances could not be measured in our picosecond flash photolysis setup. Hence, absorption changes arising from long-lived species were measured with a kinetic spectrophotometric arrangement described earlier [24,32].

3.2. Studies with microsecond time resolution

Fig. 3 represents the time-resolved transient absorption spectra recorded in the microsecond time domain on 532 nm excitation of the CV^+–BSA complex in aqueous solutions. The spectrum recorded at 1 μs after the laser pulse displays absorption bands in the 380–480 and 630–700 nm region with dye bleaching at 600 nm. The absorption region of the spectrum is quite broad in the 400 nm region and extends up to 510 nm and differs from the usual bell-shaped spectral feature displayed by the reduced $CV^{\bullet}$ radical reported in aqueous solution [24]. Both the transient absorption in ∼440 nm region and bleach recovery in the 600 nm region decreases with time from 1 to 30 μs as shown in the spectra 1–3 of Fig. 3. Complete recovery is not seen at both the wavelengths up to 50 μs. Since the singlet state of CV^+ decays in <1 ns, the

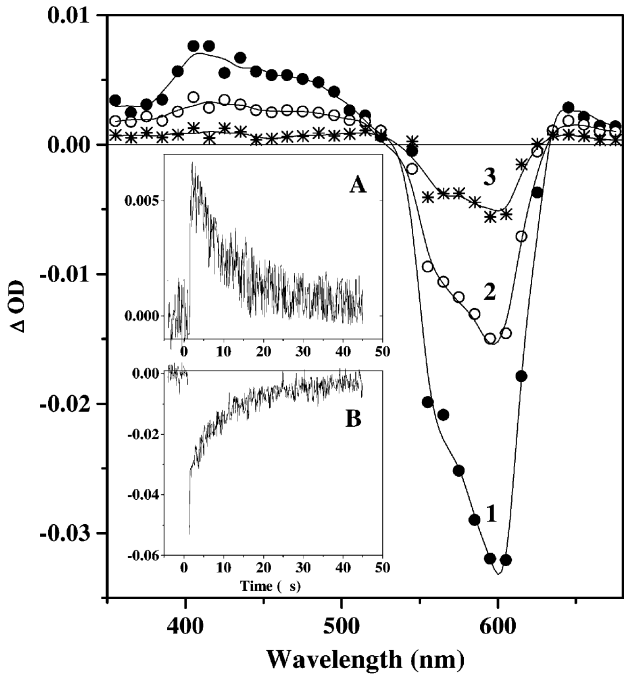

Fig. 3. Transient absorption spectra recorded on 532 nm excitation of CV^+–BSA in aqueous solution buffered at pH 7: (1) 1, (2) 15, (3) 30 μs. Inset: kinetic traces recorded in the above solution at 400 (A) and 600 nm (B). Concentrations: CV^+, 1.4×10^{-5} mol dm^{-3} and BSA, 1.2×10^{-4} mol dm^{-3}, buffered at pH 7.

transient absorption recorded in the microsecond time domain would represent a long-lived intermediate which could be the dye triplet or a transient generated from the excited singlet/triplet state by electron transfer. Hence, to establish the identity of the transient species, some of the probable reaction channels within the CV^+–BSA complex have been considered and are discussed in the following paragraphs.

In a recent study on VBBO, another TPM dye derivative, Viola et al. [17] have demonstrated that VBBO, which exhibits potential as a photosensitizer for the PDT of leukemic cells, is unable to generate singlet oxygen in oxygen saturated aqueous solutions and synthetic model membranes such as liposomes. However, they proposed that the phototoxicity proceeds through a mechanism involving $O_2^{\bullet -}$ and $OH^{\bullet}$ radicals. Liao et al. [16] in their CALI experiments using another TPM dye derivative, malachite green isothiocyanate, suggested that the active free radical species which causes the protein inactivation is the $OH^{\bullet}$ radical generated in a sequential two photon process involving the chromophoric attachment. Reactivity of $OH^{\bullet}$ radicals towards some of the TPM dyes in aqueous solutions has been well established and the spectral and kinetic parameters have been reported in the case of CV^+ and MG^+ [23]. The presence of $OH^{\bullet}$ radicals in the present system has been examined by introducing thiocynate ions (SCN^-) as $OH^{\bullet}$ radical scavenger in the CV^+–BSA system. However, the kinetic traces recorded at different wavelengths did not show any change even up to 0.01 M SCN^- concentration, ruling out

the involvement of $OH^{\bullet}$ radicals in the photo-processes of CV^{+}–BSA system.

The contribution of triplet state absorption in the signals obtained in the microsecond time domain has been examined. Fig. 4 shows the effect of oxygen on the transient signals recorded at selected wavelengths on excitation of CV^{+}–BSA complex. The transient decay became faster on moving from N_2 saturated solution to aerated and oxygenated solutions. The changes are well pronounced at the bleaching wavelength, 600 nm (Fig. 4B) and the dye recovery is complete in oxygenated solutions (Fig. 4B, trace 3). It is to be noted that no appreciable absorbance changes are

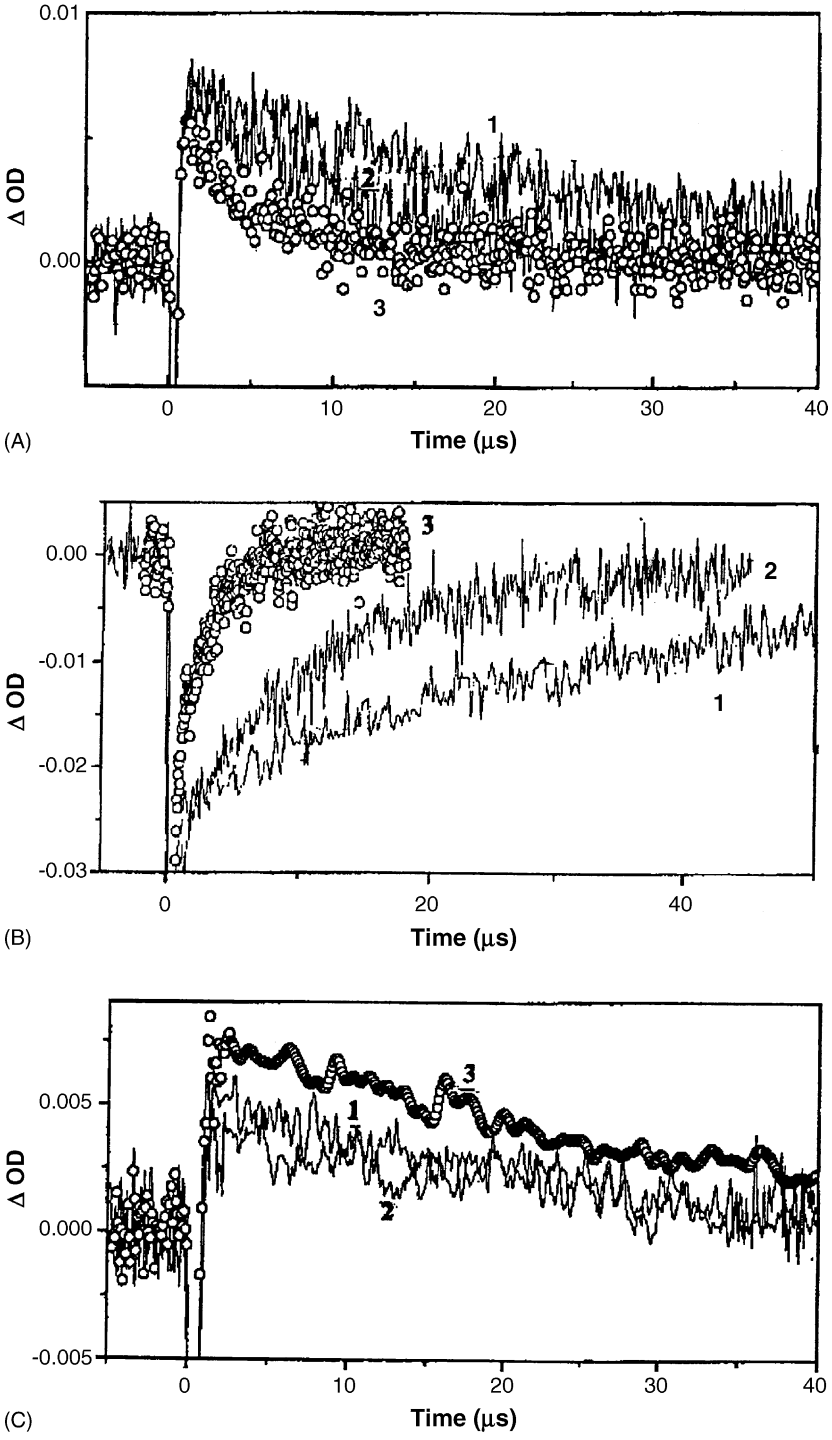

Fig. 4. Effect of oxygen on the kinetic traces recorded on 532 nm excitation of CV^{+}–BSA in aqueous solution at pH 7. (A) Decay of the transient absorption at 400 nm; (B) bleach recovery seen at 600 nm; (C) decay of transient absorption at 650 nm. For all the figures, trace numbers 1, 2 and 3 refer to N_2, air and O_2 saturated solutions, respectively. Concentrations: CV^{+}, 1.4×10^{-5} mol dm^{-3} and BSA, 1.2×10^{-5} mol dm^{-3}, buffered at pH 7.

seen on the traces recorded at 650 nm at different oxygen concentrations (Fig. 4C). To ascertain the presence of $CV^{\bullet}$ in the system, changes in the transient absorption have been examined in the presence of an electron acceptor, methyl viologen, MV^{2+}. If $CV^{\bullet}$ is present in the system one would expect the formation of MV^{+}, generated through the reaction of $CV^{\bullet}$ with MV^{2+}, which has a strong absorption at ~605 and 395 nm. However, the signals at 400 and 600 nm were unaltered even up to 10 mM concentration of MV^{2+} indicating the absence of $CV^{\bullet}$ in the system. Thus, the results on O_2 scavenging and the effect of MV^{2+} on the transient absorbance suggest that the transient species detected in the microsecond time domain must be either the triplet state or the oxidized form of the dye bound to BSA.

The fate of the transient species on BSA moiety generated by photoexcitation was followed at a longer time delays. The decay of absorbance at 400 nm and the bleach recovery at 600 nm are shown in the insets of Fig. 3. It may be recalled that the initial fast bleach recovery in the picosecond time domain mainly represents the decay of the excited singlet state and would not contribute to the absorbance decay recorded in microsecond time domain. It seems clear that $^1CV^+$ does not transfer electron to BSA, rather it undergoes ISC to $^3CV^{+*}$ which in turn undergo electron transfer reaction with BSA. However, due to instrumental limitations the time domain between 6 ns and 1 μs could not be probed. Thus, the observed transient absorption changes have contribution from $^3CV^+$ as well as CV^{2+}.

The transient spectral characteristics displayed in Fig. 3 agrees with that reported by Baptista and Indig [19] in the case of another TPM dye, EV on BSA. The authors have attributed the transient absorption to the reduced dye radical and the spectral broadening has been attributed to some spectral contribution from the triplet state absorption. However, it may be recalled that the spectral features of the transient $CV^{\bullet}$ in aqueous solutions have extensively characterized by our group and by others in the recent past [21–25]. These reports show that the excited singlet state, triplet state, oxidized and reduced radicals $CV^{\bullet}$ have considerable absorption in the window region (360–460 nm) of the parent dye spectrum. Further, the present flash photolysis results suggest that the transient species could be due to triplet state of the dye or its oxidized radical $CV^{\bullet 2+}$. To compliment the above results, we have employed pulse radiolysis method to selectively generate reduced and oxidized dye radicals in the protein environment to ascertain the spectral and kinetic behavior of CV^+ radicals bound to BSA.

3.3. Pulse radiolysis studies

Pulse radiolysis technique has been another choice to generate and study the above dye radicals in solution in free or bound conditions. Pulse radiolysis studies on some of these TPM dyes have been reported by us in detail elsewhere [21,23–25]. In brief, radiolysis of aqueous solution gives rise to various reducing and oxidizing reactive species (e_{aq}^-, $H^{\bullet}$, $OH^{\bullet}$) in known concentrations. Among them e_{aq}^- and $H^{\bullet}$ are strong reducing agents and $OH^{\bullet}$ radical is a strong oxidizing agent. By introducing suitable scavengers into the solution one can conveniently carry out the oxidation and reduction reaction with the molecule of interest. In order to set the reducing conditions, OH radicals were scavenged with *t*-butanol in a deaerated aqueous solution (reactions 1 and 2):

$$H_2O \rightsquigarrow e_{aq}^-, H^{\bullet}, OH^{\bullet} \quad (1)$$

$$OH^{\bullet} + (CH_3)_3COH \rightarrow H_2O + {}^{\bullet}CH_2(CH_3)_2COH \quad (2)$$

The reduction reaction can be followed either as the growth of the transient dye radical or by following the decay of hydrated electron.

$$e_{aq}^- + D^+ \rightarrow D^{\bullet} \quad (3)$$

For maintaining oxidizing conditions, e_{aq}^- is scavenged by saturating the solution containing appropriate concentrations of the dye and NaN_3, with well-known electron scavenger, N_2O. Here, e_{aq}^- reacts with N_2O to generate $OH^{\bullet}$ radicals, which subsequently react with N_3^- to give the secondary oxidizing radical $N_3^{\bullet}$ (reactions 4 and 5):

$$e_{aq}^- + N_2O \rightarrow N_2 + OH^{\bullet} + OH^- \quad (4)$$

$$OH^{\bullet} + N_3^- \rightarrow OH^- + N_3^{\bullet} \quad (5)$$

$N_3^{\bullet}$ radical is a strong oxidizing agent and reacts with CV^+ to give $CV^{\bullet 2+}$ (reaction 6) [23]

$$N_3^{\bullet} + CV^+ \rightarrow N_3^- + CV^{\bullet 2+} \quad (6)$$

Triplet state of CV^+ has been conveniently generated through energy transfer route [21,25]. On radiolysis of deaerated benzene solution, various excitation and charge recombination mechanisms give rise to the triplet state of benzene ($E_T \sim 82$ kcal/mol) in good yield. Triplet energy transfer reactions can be followed in such system by suitably adjusting the scavenger concentration to effect the transfer of triplet energy (reactions 7 and 8)

$$Bz \rightsquigarrow Bz^*, (Bz^+ + e^-) \rightarrow {}^3Bz^*, {}^1Bz^* \quad (7)$$

$$^1Bz^* \rightarrow {}^3Bz^* \quad (8)$$

$$^3Bz^* + CV^+ \rightarrow {}^3CV^{*+} + Bz \quad (9)$$

Our earlier communications report the details for the above transient intermediates of CV^+ in the aqueous and organic solutions [21–25]. Similar experiments have been carried out in the case of CV^+–BSA complex to selectively generate and to obtain the spectral features of the reduced and the oxidized radical ($CV^{\bullet 2+}$) bound to BSA. Under these conditions, though the primary radiolytic radical can have competitive reactions with BSA moiety also, blank experiments containing BSA without CV^+ indicate that in any of the cases mentioned above, transients from BSA do not absorb above 400 nm. The reactivity of primary radiolytic radicals

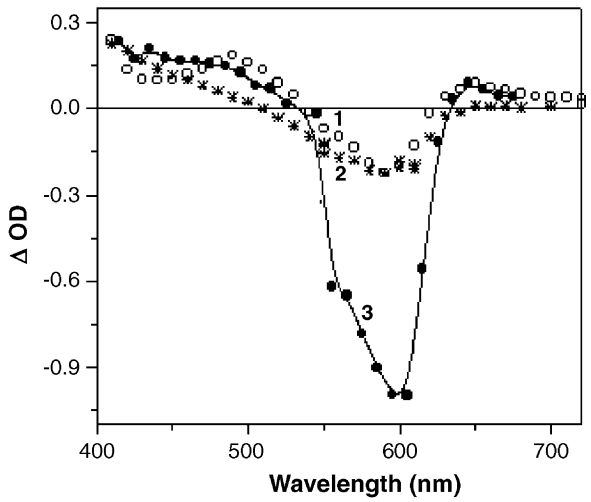

Fig. 5. Transient absorption spectra obtained on pulse radiolysis of CV^+–BSA solution under oxidation (1) and reduction (2) conditions (see text for details). The OD values given in the spectra were taken at ~2 μs after the 50 ns electron pulse and refer to the maximum in the growth/bleaching curves at different wavelengths. Concentrations: CV^+, 2.0×10^{-5} mol dm^{-3} and BSA, 1.6×10^{-5} mol dm^{-3}, buffered at pH 7. Spectrum (3) represents the transient spectrum of Fig. 3 (curve 1) given for comparison. Spectrum 1 and 3 are normalized at 650 nm.

towards the dye bound to BSA and related spectral information can be obtained from bleaching of the dye absorption.

Fig. 5 shows the transient absorption spectrum of the CV^+ radicals bound to BSA obtained on oxidation and reduction reactions of the CV^+–BSA complex. Spectrum 1 of Fig. 5 represents the oxidized CV^+ on BSA generated by the reaction of $N_3^\bullet$ radical (reactions 4–6) and spectrum 2 represents the reduction of CV^+ on BSA carried out by e_{aq}^- reaction (reactions 2 and 3). For comparison, the transient spectrum recorded at 1 μs on 532 nm photoexcitation (see Fig. 3 also) is also shown, after normalization with spectrum 1 of Fig. 5 at 650 nm. It is seen that the spectrum of the oxidized dye radical, in the region >620 nm, generated through pulse radiolysis agree well with the spectrum obtained from flash photolysis experiment. The mismatch in the region <620 nm is understandable as the transient spectrum pertaining to flash photolysis represents the photooxidation as well as other processes responsible for the dye bleaching, and a quantitative evaluation of their spectral contribution at a given time become difficult. On the other hand, the radiolysis experiment represents the quantitative reaction of CV^+–BSA with the radiolytically generated oxidizing species and would provide the true spectrum of CV^{2+}. It is to be noted that considerable spectral differences were found with the reduced CV^+ on BSA (spectrum 2) as compared to spectra 1 and 3, especially in the 650 nm region. Moreover, the O_2 effect on the transient absorption at 650 nm (Fig. 4C) gives clear proof that triplet state of CV^+–BSA does not have any absorption ~650 nm.

Jones et al. [37] have investigated the photochemistry of CV^+ and EV^+ bound to polymer units, PMMA and PAA as monomer/dimer units. The broad absorption band with a maximum at 460 nm accompanied by ground state bleaching and weaker absorption in the 640 nm region was attributed to the bound monomer triplet. This assignment was done in parallel with their work on photosensitization of CV^+ by triplet benzophenone in acetonitrile solutions [14]. However, the cited reference does not give enough information on the characterization of the CV^+ triplet. It is well established that the major channel in the interaction of benzophenone triplet with CV^+ is an electron transfer process. On the other hand, Jones et al. [37] have demonstrated that on oxidation by using strong electron acceptors like tetranitromethane (TNM), the polymer-bound EV^+ shows absorption band in the 630–700 nm region in addition to the absorbance seen in the 400 nm region. Baptista and Indig [19] have postulated an electron transfer or H atom transfer from the protein to the dye moiety as the first step of the bleaching process. Analogous reaction mechanism was also proposed for protein-bound CV^+. However, our present study, employing the flash photolysis and pulse radiolysis approach, leads us to propose an intramolecular electron transfer from the dye to the protein moiety as a net chemical consequence, upon photoexcitation of aqueous CV^+–BSA complex.

Accounting the above observations, we believe that the singlet state of CV^+–BSA generated on photoexcitation of CV^+–BSA undergo ISC to the triplet state of CV^+. The triplet state of CV^+ then transfers an electron to the BSA moiety to give the oxidized radical of $CV^{\bullet 2+}$. The signatures of $CV^{\bullet 2+}$ have been clearly seen in microsecond time region:

$$CV^+ + h\nu \rightarrow {}^1CV^{+*} \rightarrow {}^3CV^{+*} \quad (10)$$

$${}^3CV^{+*} + BSA \rightarrow CV^{\bullet 2+} + BSA^{\bullet -} \quad (11)$$

Baptista and Indig [19] focused their studies mainly on the photodegradation channel of the protein–dye system. They estimated the product formation as 10% of the amount of the photobleached CV^+. The low yield is explained as due to the primary photoproducts, both leuco-CV and Michler's ketone being removed from the solution through secondary reactions or as the consequences of the existence of competitive photodecomposition pathways leading to the formation of dye–protein hetero-adducts. It may be noted that many workers have identified amino-substituted benzophenones as the photooxidation products of some TPM dyes. However, the major reaction channel appears to be the regeneration of the CV^+–BSA complex, as indicated by bleach recovery profiles, by a back electron transfer process. The decay profiles were not very much different at different BSA concentration indicating that the back electron transfer takes place within the bound system. This may not involve the same receptor site as for the forward electron transfer from CV^+, but another group like the terminal carboxyl in the folded protein substrate situated within the vicinity for an electron hop. However, we feel that although the direction of the electron transfer in the first step is different than proposed by

Baptista and Indig, the mechanism of minor channels producing the final products like aminobenzophenone need not change.

4. Conclusions

Photochemical processes following 532 nm excitation of CV^+–BSA complex have been studied in detail using flash photolysis technique in picoseconds to microseconds time scale. The singlet state life time of CV^+ bound on BSA is increased to ∼130 ps, as compared to the value for the unbound dye and increases from ∼1 to 5 ps in typical low viscosity solvents like methanol. The singlet state lifetime obtained from stimulated emission decay is nearly 2–3 times shorter than those obtained from transient absorption and bleach recovery measurements. Electron transfer is seen to occur from the triplet state of the dye to BSA generating $CV^{\bullet 2+}$ radical, contrary to a recent report suggesting electron transfer from BSA to excited dye molecule. Oxygen effect on the transient absorption confirms the involvement of triplet state of the dye in the ET process. Supporting spectral evidence for the oxidized radical in BSA environment was generated through pulse radiolysis studies of CV^+–BSA system in aqueous solutions.

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