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Photooxidation of lysozyme or serum albumin bound to *meso*-tetra-arylporphyrins

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ABSTRACT

The photoprocesses of *meso*-tetra(4-sulfonatophenyl)porphyrin and *meso*-tetra(4-hydroxyphenyl) porphyrin were studied in the presence of lysozyme. Light-induced damage of the dyes and proteins were measured in aqueous solution at pH 4 and 8 under the exclusion of oxygen. The results were compared with those of bovine serum albumin, where virtually no photooxidation takes place. Non-covalent binding to the proteins is strongest for the diprotonated porphyrins. The porphyrin triplet state is longer lived due to shielding by binding to a protein but such binding essentially inhibits further reactions. Electron transfer to the excited porphyrin singlet state is indicated by a lower triplet yield for loading to lysozyme, in contrast to serum albumin. The quantum yields of protein damage are comparable and rather low, they depend on the protonation state and are largest (1.4×10^{-3}) for the *meso*-tetra (4-sulfonatophenyl)porphyrin/lysozyme system. The effects of protein concentration and protonation state on the quantum yields of protein and dye damage were studied in detail.

1. Introduction

Porphyrins are the subject of many photochemical studies due to their efficiency as sensitizers in the photodynamic therapy of cancer [1–5]. The spectroscopic properties and photoredox features of synthetic porphyrins are well-known [6–14]. The reactive state of porphyrins towards intermolecular processes is generally the triplet, which is efficiently populated. For *meso*-tetra(4-sulfonatophenyl)porphyrin (TSPP) in neutral aqueous solution the fluorescence is weak and relatively large quantum yields of intersystem crossing (Φ_{isc}) and singlet molecular oxygen production (Φ_{Δ}) of 0.64–0.66 have been reported [14]. Porphyrins have been employed for the photooxidation of bovine (BSA) or human (HSA) serum albumin [15–21]. In particular, TSPP is a frequently applied sensitizer for the photooxidation of serum albumins [22–37]. However, proteins as well as porphyrins may be likely candidates for aggregation [12,22–25]. Even in the absence of proteins, TSPP forms J-aggregates at low pH and high ionic strength [11,12].

It has long been known that serum proteins bind porphyrins and protect them from reactivity by self-quenching or quenching by oxygen. Various other molecules have been applied for the photocleavage of proteins, e.g., methylene blue [16,17], chain-substituted pyrenyl peptides [38–40], xanthene [17,41] and cyanine [42] dyes. The latter have attractive spectroscopic properties, but their Φ_{isc} and Φ_{Δ} values are rather low.

In this paper we have studied the porphyrin-sensitized cleavage of either lysozyme or BSA under oxygen-free conditions. Aggregation was avoided. TSPP and *meso*-tetra(4-hydroxyphenyl)porphyrin (THPP) were chosen since they are both well characterized, but their photochemical behaviour in aqueous solution is strikingly different [1–14]. The measurements were carried out in two characteristic protonation states, at pH 3.9 for diprotonated and at pH 7.8 for non-protonated porphyrins. Most porphyrin-sensitized interactions with proteins have been carried out under air [25–37] and involve singlet molecular oxygen as key-intermediate.

The two proteins were chosen since they have often been applied and their properties differ significantly. Binding of THPP to BSA has recently been studied at several pH values [21]. Lysozyme is one of the best understood small enzymes, its activity can be readily monitored and it has specific binding sites, one of which is surrounded by four tryptophan residues [37–40]. BSA is a plasma protein with many physiological functions which binds rather unspecifically. The unique properties of both proteins allow us to draw mechanistic conclusions concerning the photocleavage of proteins [38–40].

We aimed at a better understanding of the photoprocesses which were induced by binding of both dyes to the two proteins.

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Thereby, we elucidated the binding features, photochemical pathways and some mechanistic aspects of the photosensitized protein cleavage under conditions not involving singlet molecular oxygen. The complexation with BSA is much higher than with lysozyme, but this has no consequences on the protein cleavage which takes place preferentially for lysozyme. Moreover, a comparison with results from photoreduction of the dyes and photooxidation of the proteins was carried out under argon and under air.

2. Materials and methods

TSPP dihydrochloride and THPP (Porphyrin Products) were used as received. BSA (Sigma) and lysozyme (Fluka) were the same as used previously [41]. Water was from a millipore (milli Q) system. THPP was first dissolved in methanol and then mixed with 95–98% water. The pH was typically 7.6–8.0 and shifted by addition of $HClO_4$. The molar absorption coefficient of TSPP at pH 7–8 is $\varepsilon_{410} = 1.6 \times 10^5\ M^{-1}\ cm^{-1}$ [4]. The steady-state spectral measurements were used to determine the concentrations of free dye and dye bound to protein, as well as to analyze the concentration and pH dependences. The UV–vis absorption spectra were recorded on a diode array spectrophotometer (HP, 8453). A spectrofluorimeter (Varian, Cary eclipse) was employed to measure the corrected fluorescence spectra. Irradiation was performed with a 1000-W Xe–Hg lamp and a monochromator or a 250-W Hg lamp (Schöffel) and suitable interference filters (Schott). The relative quantum yield of decomposition of dyes in an argon-saturated aqueous medium was obtained from the changes in absorption at λ_a^0 upon continuous irradiation at 400–700 nm. The changes were measured by $(A_0 - A_t)/(A_0 - A_e)$, where A_t is the absorption at a given time, A_e is that at 1000 s or longer times and A_0 at the beginning. The choice of one wavelength is justified by the large signal to noise ratio. The relative quantum yield of the photooxidation of the proteins was obtained by the fluorescence intensity of the tryptophyl residues around 350 nm which decreases *vs.* the irradiation time [34–37]. The dyes were freshly prepared and mixed with proteins without buffer unless indicated otherwise. Essentially the same fluorescence results were obtained in either air- or argon-saturated aqueous solution, in contrast to steady-state and time-resolved photolysis. We assumed examination of intact proteins since no significant change of the secondary protein structure has been reported upon purging by argon or nitrogen [18,22]. The flash photolysis data refer to an excimer laser (Lambda Physik, EMG200) operating at 308 nm. Alternatively, a Nd-YAG laser and an optical parametric oscillator system with $\lambda_{exc} = 410$–450 nm was applied (GWU-Lasertechnik) and the absorption signals were measured by a Luzchem system; the results were similar and only those with $\lambda_{exc} = 308$ nm are presented here. For this excitation wavelength the number of absorbed photons in samples of either free or complexed porphyrins did not require correction for the calculation of the quantum yield of triplet states. The measurements were carried out at 24 °C.

3. Results and discussion

3.1. UV–vis absorption

Major changes of the UV–vis absorption and fluorescence spectra are due to the formation of ground state complexes between porphyrins and proteins. Several spectral and kinetic features change with increasing the protein concentration ([P]). The dye concentration ([D]) was kept constant for each method. The results refer to pH 3.9 for diprotonated and to pH 7.8 for non-protonated porphyrins and may be roughly distinguished as high loading with many porphyrins bound to one protein ($[P]/[D] \leq 0.1$) and low loading ($[P]/[D] > 1$) with only one dye or less per protein. Note that the major pK_a of TSPP and THPP is at pH 5–6 [5,10].

The spectrum of TSPP in aqueous solution at pH 3.9 has maxima (λ_a) at 434 and 645 nm. Examples for binding of TSPP to BSA and lysozyme are shown in Figs. 1 and 2, respectively. The Soret peak is red-shifted from λ_a^0 (no protein) to λ_a^P upon increasing the protein concentration at pH 7.8 and contrastingly blue-shifted at pH 3.9, e.g. $\lambda_a^P = 418$–420 nm. The maxima of both Soret and Q-bands of the two dyes are compiled in Tables 1 and 2. Either one or two isosbestic points indicate the presence of only two species, free and bound porphyrin. Examples are shown in Fig. 1 for [BSA]/[TSPP] = 0.015 and 0.04. The signal at $\lambda_a^0 = 434$ or 645 nm decreases with increasing the [P]/[D] ratio and A_{420} increases correspondingly (inset of Fig. 1). The effect is described by the characteristic protein/dye concentration ratio for 50% change, $([P]/[D])_{1/2}$, which varies between 0.04 for BSA and 0.25 for lysozyme (Table 3).

Another characteristic value is the absorption ratio with and without protein A^P/A^0, measuring the change in absorbance at low loading, which is <0.1 for BSA/TSPP. One major effect is the shift of $([P]/[D])_{1/2}$ from 0.1 in neutral solution to 0.01 at pH 3.9. $A^P/A^0 = 0.4$ at pH 7.8 but negligible in the acidic range. Thirdly, at pH 3.9 we registered a lysozyme-induced peak at 490 nm (Fig. 2), which appears at $([P]/[D])_{1/2} = 0.05$. This is assigned to J-aggregation in agreement with the literature of the induced spectra at pH around 2 with albumin [25–27] and lysozyme [25]. The inset in Fig. 2 illustrates the mirror image effect of protein concentration on the J-aggregate (A_{488}) and the Soret band (A_{448}), keeping the TSPP concentration constant. It is noteworthy to mention that the J-aggregate also appears in the presence of BSA.

The binding of THPP to BSA at pH 3.9 (Fig. 3) is similar to that observed for the TSPP case shown in Fig. 1 for the spectra and in the Fig. 1 inset for the effect of protein concentrations. At pH 7.8, however, the change in absorbance *vs.* BSA concentration is decreasing (inset of Fig. 3). The A^P/A^0 ratio for BSA/THPP is likewise small, whereas for lysozyme it is 1.2. The $([P]/[D])_{1/2}$ ratio is smallest, 0.02–0.03 for both proteins in the acidic range (Table 3).

3.2. Fluorescence

The emission maximum of TSPP in aqueous solution is positioned at $\lambda^{em}_f = 669$ and 643 nm and the excitation peak is at $\lambda^{ex}_f = 650$ and 634 nm at pH 3.9 and 7.8, respectively. The peak values of free and bound dyes are compiled in Tables 1 and 2. The

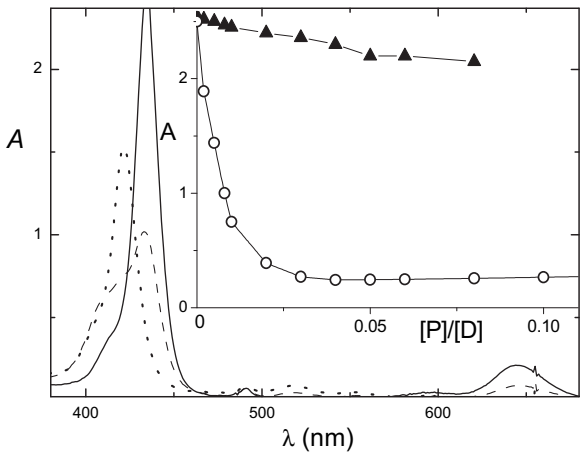

Fig. 1. Absorption spectra of TSPP (15 μM) in air-saturated aqueous solution at pH 3.9 in the absence (full) and presence of BSA at [P]/[D] 0.015 (dashed) and 0.04 (dotted line); inset: plots of A_{430} as a function of the BSA concentration at pH 3.9 (○) and 7.8 (▲).

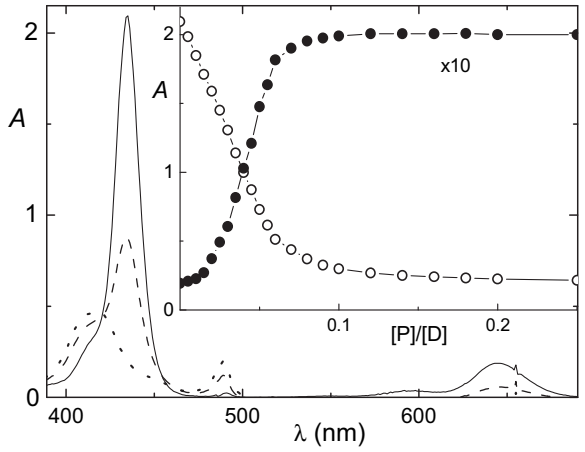

Fig. 2. Absorption spectra of TSPP (14 μM) in aqueous solution at pH 3.9 in the absence (full line) and presence of lysozyme at [P]/[D] = 0.06 (dashed) and 0.1 (dotted line); inset: plot of A_{448} (open) and A_{488} (full) as a function of the lysozyme concentration.

maximum is slightly red-shifted with increasing BSA concentration at pH 7.8. Examples of the emission spectra of TSPP at pH 7.8 in the absence and presence of BSA are shown in Fig. 4. This is due to low loading, in contrast to most effects with porphyrins reflecting high loading ([P]/[D] ≤ 0.1). Virtually no shift of λ^{em}_f occurs for THPP.

Lysozyme and BSA quench the quantum yield Φ_f of TSPP at pH 3.9, but enhance Φ_f at pH 7.8 (Table 3). Plots of the changes in Φ_f with increasing protein concentration are shown in the inset of Fig. 4. The ratio of quantum yield values for [P]/[D] = 1 *vs.* 0 (high *vs.* no protein concentration) is $\Phi^P_f/\Phi^0_f = 2.8$ for both proteins at pH 7.8. BSA also enhances Φ_f (1.6–3.7 fold) of THPP at pH 4 or 8, whereas fluorescence quenching by lysozyme is negligible.

The $([P]/[D])_{1/2}$ ratios from fluorescence measurements follows the trend derived from absorption, i.e. a small value in the acidic range and a ca. 30-fold larger value in neutral aqueous solution. It is worth mentioning that the presence of 10% glycerol leads to a greater fluorescence enhancement and larger $([P]/[D])_{1/2}$ ratios, but causes no principle differences (Table 3).

3.3. Triplet absorption

The triplet state of TSPP has a strong bleaching peak at $\lambda_T = 420$ nm and the decay follows first-order kinetics. The lifetime (τ_T) in argon-saturated aqueous solution at pH 7.8 is $\tau^0_T = 200$ μs. The rate constant of TSPP triplet quenching by oxygen is $k_{ox} = 1.0 \times 10^9\ M^{-1}\ s^{-1}$. The spectrum is red-shifted at pH 3.9 with $\tau^0_T = 0.25$ ms (Table 4). For THPP no triplet was observed in 95% water in contrast to methanol–water (1:1) and glycerol–water (9:1) at pH 3.9, where the difference spectra and kinetics (not shown) are very similar to those of TSPP in aqueous solution.

Table 1
Absorption and fluorescence maxima of free porphyrins and quantum yield of decomposition[a].

Dye	pH	λ_a/nm	λ^{ex}_f/nm	λ^{em}_f/nm	$\Phi_d \times 0.001$
TSPP	7.8	413, 516	634	643, 704	(0.1)[b,c]
	3.9	434, 645	650	669	(0.16)
THPP	7.8	450, 612		662	0.04 (0.25)
	3.9	450, 684[b]		725[b]	(0.6)

[a] In air-saturated aqueous solution.
[b] In parentheses: argon saturation.
[c] $\Phi_d = 4 \times 10^{-5}$ in the presence of 5 mM phorphate buffer.

Table 2
Absorption and fluorescence maxima of bound porphyrins[a].

Dye	Protein	pH	λ_a/nm	λ^{ex}_f/nm	λ^{em}_f/nm
TSPP	BSA	7.8	421, 518	647	654, 719
	Lysozyme		414, 520	647	660, 719
	BSA	3.9	420, 518		662, 722
	Lysozyme		418, 489		666
THPP	BSA	7.8	444, 600		660, 720
	Lysozyme		427	619, 677	662
	BSA	3.9	426, 655[b]		662, 725[b]
	Lysozyme		445, 680	684	725

[a] In air-saturated aqueous solution at [P]/[D] = 0.8–1.2.
[b] Same values in the presence of 10% glycerol.

The transient difference spectra reveal a small shift of λ^0_T to λ^P_T, when a protein is added. As examples, the spectra and kinetics are shown for TSPP in the presence of BSA at pH 7.8 and 3.9 (Fig. 5). The triplet yield and decay kinetics are strongly dependent on the protein concentration. Plots of $1/\tau_T$ and relative quantum yields $\Phi^P_{isc}/\Phi^0_{isc}$ (high *vs.* no protein concentration) as a function of the [P]/[D] ratio at pH 3.9 and 7.8 are shown in Fig. 6a and b. The results at pH 3.9 are similar in the respect that no protein-induced triplet quenching is observable (Table 4). Upon addition of BSA Φ_{isc} is only slightly smaller, whereas for [lysozyme]/[TSPP] = 1 the triplet yield is strongly reduced: $\Phi^P_{isc}/\Phi^0_{isc} = 0.4$.

3.4. Photodamage of porphyrins and proteins

Photochemical decomposition, often denoted as photobleaching, of TSPP or THPP in argon-saturated aqueous solution was measured from the changes in absorption at $\lambda^0_a = 420$–450 nm upon continuous irradiation at 400–500 nm. Plots of the decrease of the normalized absorbance as a function of time are initially linear. The absorption of the porphyrins at λ^P_a in the presence of proteins each change in a similar manner with the irradiation time. Examples for TSPP are shown in Fig. 7. The slope of the linear part is proportional to $1/\Phi_d$. The Φ_d values are relatively small for free porphyrins (Table 1), much stronger in the presence of lysozyme and inefficient when bound to BSA (Table 5). The most significant photodamage was found for the lysozyme/TSPP case at pH 7.8.

Photodamage also takes place in proteins. Photocleavage of proteins in argon-saturated aqueous solution was observed by fluorescence in the 300–350 nm range. I_f has been reported to be a reliable measure for sensitized inactivation of proteins and defines the quantum yield of oxidation, Φ_{ox} [18–20]. The slope of the linear part *vs.* irradiation time is proportional to $1/\Phi_{ox}$. Results from the fluorescence method are compiled in Table 5 and shown in Fig. 8 for several [P]/[D] ratios. In argon-saturated solution Φ_{ox} is largest (0.0014) for lysozyme/TSPP at pH 7.8, where Φ_d is also largest.

Table 3
Binding of porphyrin to protein[a].

Dye	Protein	pH	$([P]/[D])_{1/2}$	A^P/A^0	Φ^P_f/Φ^0_f
TSPP	BSA	7.8	0.1 [0.3][b]	0.4	2.8
	Lysozyme		0.2 [0.3]	0.4	2.8
	BSA	3.9	0.008 [0.01]	0.06	0.2
	Lysozyme		0.04	0.05	0.05
THPP	BSA	7.8	0.5 [1]	1.6	3.7
	Lysozyme		0.1 [2]	0.8	0.8
	BSA	3.9	0.02 [0.3]	0.3	4.5
	Lysozyme		0.03 [0.2]	1.2	0.5
	BSA[c]	7.8	3 [3]	1.2	≥10
	BSA[c]	3.9	0.2 [0.5]	0.1	3

[a] In air-saturated aqueous solution.
[b] In brackets: obtained by fluorescence titration.
[c] 10% glycerol.

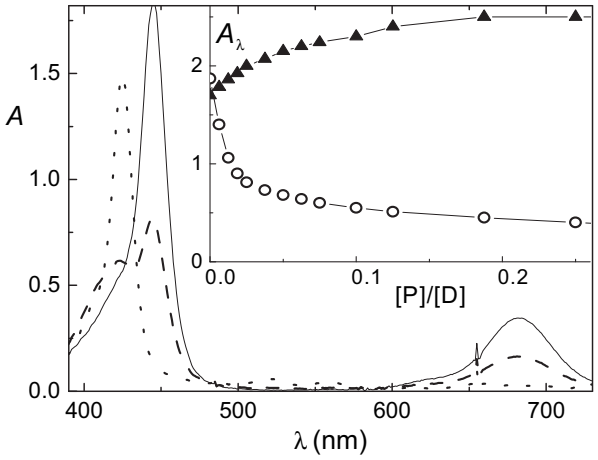

Fig. 3. Absorption spectra of THPP (8 μM) in aqueous solution at pH 3.9 in the absence (full line) and presence of BSA at [P]/[D] = 0.02 (dashed) and 0.1 (dotted); inset: plots as a function of the BSA concentration of A_{426} at pH 3.9 (○) and of A_{444} 7.8 (▲).

3.5. Effects of porphyrin protonation on binding to proteins

Four forms have been considered for porphyrin dyes in the presence of BSA: the free monomeric dye, dye aggregates and bound monomeric dyes, whereby the dye is either bound at one protein molecule or inside the BSA aggregates [22]. Free TSPP at low concentrations of <0.1 mM in aqueous solution is present as a monomer [23]. BSA is characterized by three domains and a rigid structure due to seventeen disulfide bonds compared to 6–8 in other proteins; it has 65% α-helices whereas β-motives, i.e. a stretch of polypeptide chain with backbone in an almost fully extended conformation, are absent. The main driving force for the binding of THPP to HSA has been ascribed to hydrophobic domains and the induced conformational change reduces the number of α-helices in the albumin [21].

Generally, the diprotonated porphyrin is much more sensitive to protein binding than the tetraanion in neutral solution. This is confirmed in the present work, see $([P]/[D])_{1/2}$ in Table 3. The $([BSA]/[TSPP])_{1/2}$ values are 0.1–0.2 at pH 7.8 and 5–10 times smaller at pH 3.9. The new band at 488 nm for TSPP at pH 3.9 in the presence of proteins has been attributed to J-aggregation. This protein-induced spectrum is in agreement with the literature

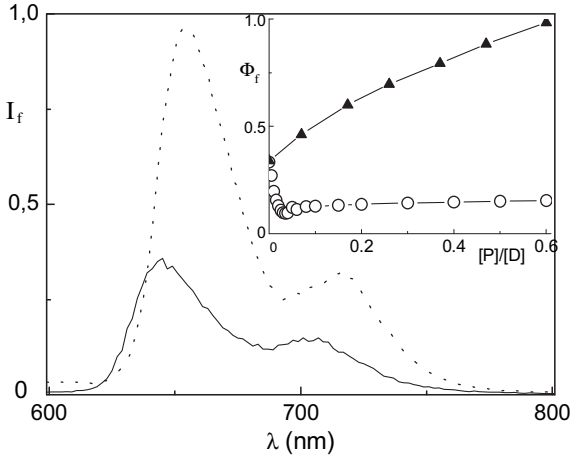

Fig. 4. Fluorescence emission spectra of TSPP (5 μM, λ_f^{ex} = 620 nm) in aqueous solution at pH 7.8 in the absence (full line) and presence of BSA [P]/[D] = 0.5 (dotted); inset: plots of Φ_f (normalized) as a function of the BSA concentration pH 3.9 (○) and 7.8 (▲).

Table 4
Triplet properties of the porphyrins[a].

Dye	Protein	τ_T/ms		$\Phi_{isc}^P/\Phi_{isc}^0$	
		pH 3.9	pH 7.8	pH 3.9	pH 7.8
TSPP	None	0.20	0.25		
	BSA	0.5	0.6	0.8	0.8
	Lysozyme	0.3	0.2	0.4	0.3
THPP[b]	None	0.1 (0.1)[c]	0.1		
	BSA	0.3 (0.3)	0.4	0.8 (0.4)	0.8
	Lysozyme	(0.1)	0.3	(0.4)	0.8

[a] In argon-saturated aqueous solution at [P]/[D] = 0.8–1.2.
[b] Using 50% methanol.
[c] In parentheses: 10% glycerol, without methanol.

[11,12,23–25]. Apparently, lysozyme is only loosely bound, as indicated by the much larger $([P]/[TSPP])_{1/2}$ values at pH 3.9 and 7.8 (Table 3).

The interaction of dyes and proteins is commonly described by the apparent macroscopic dissociation constant (K_d, the inverse binding association constant) and the number (n) of binding sites. For binding of TSPP to HSA at pH 7 K_d = 1 μM has been reported [23]. THPP, with respect to TSPP, has only scarcely been studied. The available data concerning THPP refer to HSA, where n = 1 and K_{d1} = 0.7 μM and K_{d2} = 4 μM at pH 7 and K_d is ca. 7-fold lower at pH 3 [21]. Much smaller $([BSA]/[THPP])_{1/2}$ values in Table 3 are in agreement with the better binding of the diprotonated dye. Biphasic Scatchard plots with BSA have also been presented for the binding of xanthene and thiacarbocyanine dyes [41,42].

3.6. Excited singlet and triplet state properties

TSPP in neutral aqueous solution has a low Φ_f of 0.05 [14], whereas the biprotonated TSPP at pH 3 has a much larger Φ_f of 0.3; a consequence of the latter is a lower Φ_{isc} of 0.3 at pH 4 [11]. However, Φ_f of the *m*-isomer of THPP is larger in neutral methanol than in the acidic range [1]. BSA quenches the fluorescence of TSPP at pH 3.9, but enhances Φ_f twofold at pH 7.8 (Fig. 4 and Table 3). This effect of BSA has already been observed and analyzed [35]. An enhancement of Φ_f with increasing albumin concentration has been reported previously [31]. The enhanced fluorescence upon low loading to BSA is tentatively attributed to bound monomers which have in most cases a much higher Φ_f than bound dimers. The sensitivity is relatively low at pH 7.8, where the characteristic protein/dye concentration ratio for 50% change is 0.1, when compared with a much smaller value of $([P]/[D])_{1/2}$ = 0.008 at pH 3.9. Similar values were obtained using fluorescence spectroscopy, see Table 3.

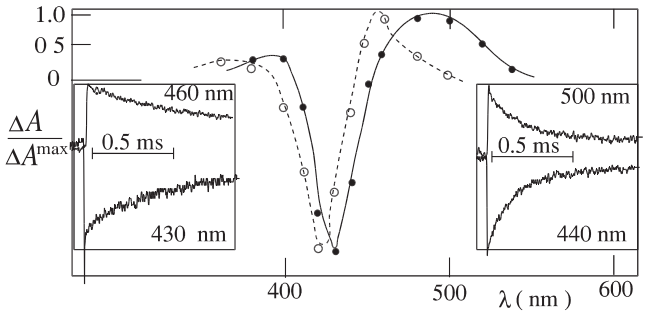

Fig. 5. Transient difference spectra of TSPP (8 μM) in argon-saturated solution in presence of BSA ([P]/[D] = 0.5) at pH 7.8 (open symbols) and 3.9 (full, right) at 1 μs after the 308 nm pulse; inset: kinetics at pH 7.8 (left) and 3.9 (right) as indicated.

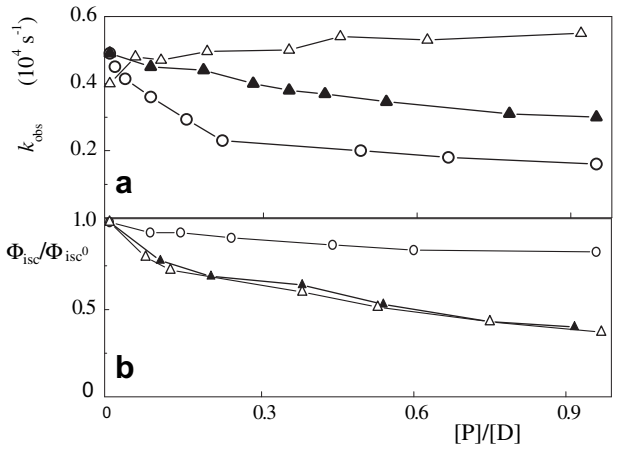

Fig. 6. Plots of (a) $1/\tau_T$ and (b) Φ_{isc} of TSPP (8 μM) in argon-saturated aqueous solution at pH 3.9 (full) and 7.8 (open), $\lambda_{exc} = 308$ nm, as a function of the [P]/[D] ratio for BSA (circles) and lysozyme (triangles).

The triplet of THPP in trifluoro acetic acid and methanol has $\varepsilon_{510} = 5.7 \times 10^5$ M^{-1} cm^{-1} and $\Phi_{isc} = 0.29$ [1]. No value for THPP in aqueous solution has been reported in the literature as yet. Binding to macromolecules may increase or decrease the triplet lifetime. The former could be due to lowering of self-quenching. The latter could be due to electron transfer to a protein component. A value of the rate constant of self-quenching is $k_s = 4 \times 10^7$ M^{-1} s^{-1} for Zn protoporphyrin in ethanol–water [43]. A value for deuteroporphyrin is $k_s = 3 \times 10^6$ M^{-1} s^{-1} [44]. The decreasing dependences of $1/\tau_T$ as a function of the [P]/[D] ratio (Fig. 6a) is suggested to be due to a monomeric triplet bound to the protein and consequently shielding against the quenching processes. Such an effect has already been reported for TSPP/HSA [37]. The long triplet lifetime and the absence of radicals supports the proposal that electron transfer from aromatic amino acids in a protein to the porphyrin triplet state, does not occur in an observable manner. Long-lived components of $\tau_T^P = 0.3$ and 1 ms have been reported for TSPP/BSA at pH 8 and 4, respectively [31]. This is in broad agreement with $\tau_T^P = 0.24$ ms [28] and our data (Table 4).

3.7. Photodamage

The quantum yield of decomposition of the free porphyrin is relatively low. A literature value of TSPP in air-saturated aqueous solution at pH 7.4 is $\Phi_d = 1 \times 10^{-5}$ [2], which is smaller than our value $\Phi_d = 4 \times 10^{-5}$ (Table 1). The photodamage of a dye in the absence of macromolecules is often caused by self-quenching [41]. An enhanced quantum yield for a bound dye in argon-saturated aqueous solution indicates a new pathway via a free radical process. Φ_d of the TSPP-sensitized inactivation of lysozyme at pH 7.4 and [P]/[D] = 4 is $(1.8–2.2) \times 10^{-3}$ [29], which is larger than our value $\Phi_d = 1.6 \times 10^{-4}$ (Table 5). Quantitative results in the presence of lysozyme or BSA could not be found in the literature for the two porphyrins under examination.

Table 5
Quantum yields (× 0.001) of decomposition of bound porphyrin and protein cleavage[a].

Dye	Protein	pH	Φ_d	Φ_{ox}
TSPP	BSA	7.6–8.0	0.08 [0.04][b]	0.3
	Lysozyme		0.5 [0.16]	1.4 [0.16][c]
	BSA	3.8–4.0	0.04	0.06
	Lysozyme		1.4	0.2
THPP	BSA	7.6–8.0	0.04 (<0.02)[d]	0.8
	Lysozyme		0.4 (0.04)	0.7
	BSA	3.8–4.0	0.16	0.6
	Lysozyme		0.4	0.6

[a] In argon-saturated solution for [P]/[D] = 1.
[b] In bracket: air-saturated.
[c] $\Phi_d = 4 \times 10^{-5}$ in the presence of 5 mM phorphate buffer.
[d] In parentheses: 10% glycerol.

The photodecomposition of a dye in the presence of macromolecules is governed by the redox properties. The enhancement of Φ_d is proposed to be due to electron transfer from aromatic amino acid residues of proteins. A reaction to the triplet state of the sensitizer is unlikely on the basis of time-resolved spectroscopy, see above. The triplet yield in the presence of BSA is in line with that of fluorescence (Tables 3 and 4) in as far as the effects are relatively small. This, however, is different for bound lysozyme. Quenching of the excited singlet state of porphyrin by lysozyme is therefore proposed.

3.8. TSPP versus THPP

The two porphyrins examined differ markedly by their aggregation potential in aqueous solution. The possibility of formation of a J-aggregate has been discussed in the literature mainly for TSPP, e.g. albumin-induced at pH 2 [27,28]. Our finding of lysozyme-induced J-aggregation at pH 3.9 (Fig. 2) is new and plausible in view

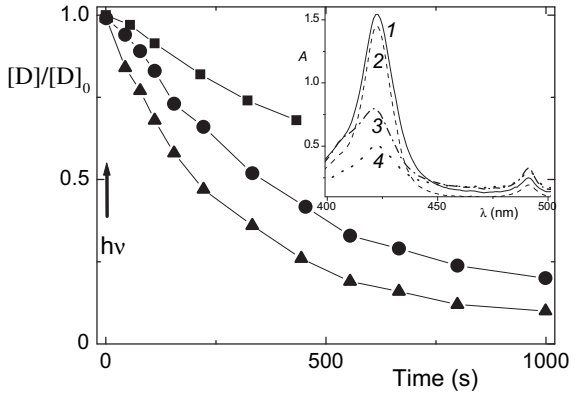

Fig. 7. Photodamage, shown as relative dye concentration *vs.* time of irradiation of TSPP (8 μM) in argon-saturated aqueous solution at pH 7.8 for lysozyme at [P]/[D] = 0.5 (squares), 1 (circles) and 1.5 (triangles); inset: Absorption spectra at [P]/[D] = 1 for BSA (*1*) and lysozyme (*3*) prior to irradiation and after conversion (*2,4*).

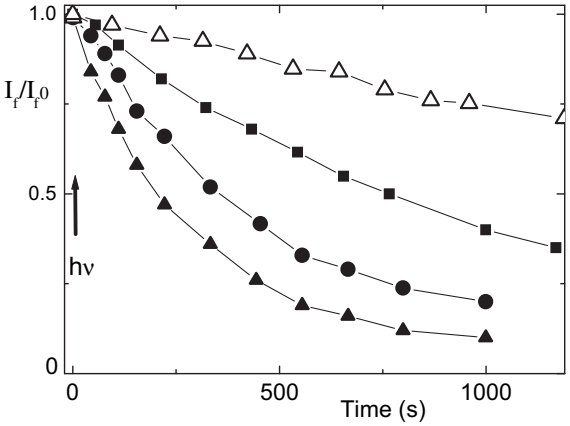

Fig. 8. Photodamage of TSPP (8 μM) in argon-saturated aqueous solution at pH 7.8 for lysozyme (full) and BSA (open), shown as relative I_f ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 350$ nm) *vs.* time of irradiation for [P]/[D] = 3 (squares), 2 (circles) and 1 (triangles).

of the protein effects at pH 1–2. The possibility of formation of higher aggregates for THPP [7,8] is in agreement with results concerning the meta-derivative. Note that most THPP data in the literature refer to the meta-derivative [10,11].

To detect the triplet state of THPP we made use of glycerol or methanol as co-solvents. With 10% glycerol we could detect the triplet at pH 4 but not at pH 8. This approach is rather unusual for biological applications and may result in severe structural changes of the proteins. However, glycerol is not required for protein photocleavage. The largest effect of glycerol is a tenfold reduction in Φ_d for lysozyme to 4×10^{-5} (Table 5). The proposed explanation is that the radicals which cause the damage of both protein and porphyrin are scavenged by glycerol. Interestingly, the enzymatic function of lysozyme is not strongly affected by glycerol as co-solvent [45,46]. The folded structure is stabilized by glycerol more than in water [46].

A major outcome of this work is the previously mentioned enhancement of photodamage by binding to lysozyme. This effect is similar for both porphyrins. On the other hand, binding to BSA shields against photodamage. For example, THPP is 3–8 times and TSPP is 1.2–6 times as stable when bound to BSA as against the free dye (Tables 1 and 5).

4. Conclusion

The spectroscopic and photochemical features of two frequently studied water-soluble porphyrins were examined over a large concentration range of BSA and lysozyme and at characteristic pH values. Loading of a porphyrin to lysozyme quenches the excited singlet state, causing reductive dye damage under argon and oxidative damage of the tryptophan moiety. BSA, however, is virtually unaffected under the same conditions. The 4-sulfonatophenyl or 4-hydroxyphenyl building blocks cause various spectroscopic changes which were described, but no major mechanistic consequences. Whether the porphyrin is doubly- or non-protonated, has specific influences on the dye/protein concentration sensitivity, but no major consequences on the mechanism of photocleavage.

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