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Effects of photoproducts on the binding properties of protoporphyrin IX to proteins

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Abstract

Photosensitisers are the photoactive molecules used in photodynamic therapy (PDT) of cancer. Despite the importance of their interaction with polypeptides, only the binding to plasma proteins has been investigated in some detail. In our study we compared the binding of Protoporphyrin IX (a clinically useful photosensitiser) to an immunoglobulin G, with the binding to albumins. Binding to IgG is relevant because a possible method of increasing tumour specificity of photosensitisers is to bind them to tumour-specific antibodies. Binding constants to albumins and the immunoglobulin were comparable ($\cong 6\times10^{-6}$ M^{-1}). The apparent number of PPIX molecules bound to each protein was also within a similar range (from 4 to 7). The absence of a shift in the emission spectrum of PPIX bound to IgG, however, indicates that either larger aggregates of PPIX bind to the immunoglobulin or that the binding site leaves PPIX exposed to the buffer. We observed that PPIX photoproducts compete with PPIX for the same binding sites. The number of PPIX molecules bound to each protein in the presence of photoproducts decreased by 50–80%. Due to the spectral overlap between PPIX and its photoproducts, the binding in the presence of photoproducts was investigated using Derivative Synchronous Fluorescence Spectroscopy (DSFS) to improve the spectral separation between chromophores in solution. We also concluded that fluorescence measurements underestimate the number of PPIX molecules binding each protein. In fact, non-linear Scatchard plots (in the case of albumin binding) by definition yield a minimum number of molecules attached to a protein. Moreover, the binding of large aggregates, formed by an unknown number of PPIX molecules, to IgG results in the underestimate of the number of molecules bound. The number of PPIX molecules bound to these proteins is also much larger than the number of sites estimated by protein fluorescence quenching.

Keywords: Drug-protein binding; Photodynamic therapy; Synchronous fluorescence spectroscopy; Protoporphyrin IX

1. Introduction

Protoporphyrin IX is a clinically useful Photosensitiser (PS). Photosensitisers are molecules used in photodynamic therapy (PDT) of cancer and pre-cancerous lesions [1]. Upon illumination with light of proper wavelength, PS initiate photochemical reactions that lead to the formation of cytotoxic compounds such as radicals and singlet oxygen [1]. When the PS are localised in cells, radicals and singlet oxygen can trigger necrosis or

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apoptosis of the illuminated cells. In vivo fluorescence spectra suggest that at the time of PDT, PPIX is bound to either proteins and/or membranes [2]. Investigations of the interaction between PS and proteins [3,4] have been, however, directed exclusively to the binding to plasma proteins [5–8]. Recent advances show that other PS-protein interactions are important for PDT [9]. Among these is the interaction with small immunoglobulins. They can in fact be used in the synthesis of photoimmunoconjugates (PIC). These macromolecules are formed by covalently linking several PS molecules to a tumour-specific antibody [10,11]. The synthesis of PIC may be affected by the non-covalent binding of PS to the antibody. Our manuscript reports the results of the investigation of non-covalent binding of Protoporphyrin IX (PPIX) to an IgG immunoglobulin (Antibody to the α-chain of human IgA). The binding of PPIX to IgG is characterised by comparison with the well-known binding to albumins (human and bovine). Although not all porphyrin derivatives behave equally upon binding to proteins [12], binding of PPIX can be investigated spectroscopically as it is normally accompanied by a large increase in fluorescence intensity and a red-shift in the absorption and emission maxima [8,13].

We also investigated the effects of PPIX photoproducts on the binding properties of PPIX. Photoproducts are formed upon irradiation of PPIX through the back-reaction of radicals and singlet oxygen with PPIX itself [14,15]. Once formed, the photoproducts will likely interact with proteins and their presence in solution may affect the binding properties of PPIX. The extent of the influence of photoproducts on binding has not been quantified before. To characterise the effect of photoproducts we did not use traditional emission spectroscopy. Because of the spectral overlap between PPIX and its photoproducts [15,16] we used DSFS [17,18]. DSFS yields a better separation between the two contributions and is normally used in analytical and clinical chemistry to separate the fluorescence contribution of overlapping chromophores [18–20]. SFS is based on the simultaneous scanning of excitation and emission monochromator that keeps a constant interval ($\Delta\lambda$) between excitation and emission wavelengths. When $\Delta\lambda$ is set to match the excitation/emission maxima of a specific chromophore, SFS enhances the contribution of this chromophore against all the others in the mixture [17]. Derivative spectroscopy provides additional separation of the chromophores during data analysis and it has been used for the analysis of mixtures of porphyrins in urine [18].

2. Experimental

2.1. Chemicals

Protoporphyrin IX, Human Serum Albumin (HSA), Bovine Serum Albumin (BSA) and IgG immunoglobulin (secondary antibody specific for the α-chain of human IgA) were purchased from Sigma Chemical Co. (Poole, UK) and used as received. Dimethylsulfoxide (DMSO) (Sigma Chem. Co., Poole, UK) was also used without purification. Fresh 10 mM Sodium Phosphate buffer solution (pH 7.5) was used throughout the study. During sample preparation PPIX was first dissolved in DMSO and subsequently diluted in buffer to give the final concentration of PPIX (0.5 to 2 μM). The maximum final concentration of DMSO in buffer was 2%. Concentration of PPIX was determined from its absorption using a value of $\varepsilon = 2.42 \times 10^5$ at 406 nm [21]. Protein concentration was also determined spectroscopically from the absorption value at 280 nm. Values of ε_{280} for HSA and BSA were obtained from the literature [6,22] while for IgG we used the value offered by the manufacturer ($\varepsilon_{280} = 1400$).

2.2. Fluorescence experiments

A Hitachi F-2500 spectrofluorimeter (Hitachi Instrument Inc., Wokingham Berkshire, UK) was used for all the measurements. All spectra were recorded with 2.5 nm resolution at a speed of 300 nm/min. PPIX-protein interaction was investigated by recording either the increase of PPIX fluorescence or the decrease of intrinsic protein fluorescence upon binding.

2.3. PPIX fluorescence

Fluorescence increase of PPIX in the presence of proteins was recorded using two methods: (1)

varying the concentration of protein while keeping a constant PPIX concentration or (2) varying the concentration of PPIX while keeping a constant concentration of protein. The first method was performed using PPIX concentrations between 0.5 and 2 μM and protein concentration between 0.1 and 20 μM. The second method was carried out with protein concentration in the 2–5 μM range while PPIX concentration was increased from 0.01 to 1 μM. The experiments were carried out also in the presence of photoproducts. Emission spectra were recorded with excitation at 405 nm. Dissociation constants and the number of bound PPIX molecules were obtained using Scatchard plots [23]. This method retrieves the binding parameters by plotting the ratio bound/free ligand (ν/c) vs. the bound ligand (ν). Values of ν and c were retrieved combining the methods described by Beltramini et al. [8] and Azzi [24]. PPIX fluorescence intensity was calculated as the area under the emission spectrum using the mathematical options offered by the Hitachi F-2500 software.

2.4. Protein fluorescence

All proteins used for our investigations show the typical intrinsic fluorescence due to aromatic amino acids (tryptophan in particular) when excited at 280 nm. The addition of PPIX quenches the intrinsic fluorescence. This quenching can be used to retrieve the binding parameters by using the plot of $\mathrm{Log}[(F_0-F)/(F-F_\infty)]$ against Log [P] [13], where F_0 is the fluorescence of the protein in the absence of PPIX, F is the fluorescence of the protein at a concentration [P] of PPIX and F_∞ is the fluorescence of the protein in solutions saturated with PPIX. The slope of the plot yields the number of binding sites, whereas the intercept at $\mathrm{Log}[(F_0-F)/(F-F_\infty)]=0$ represents the dissociation constant K_d [13].

2.5. Formation of photoproducts

Photoproducts were formed by irradiating stock solutions (10–100 μM) of PPIX in DMSO at 630 nm with a total dose of 300 J/mols. The presence of photoproducts was periodically monitored during irradiation by recording emission spectra (excitation at 405 nm). The concentration of PPIX and photoproducts after irradiation was calculated by recording the decrease of the intensity of the fluorescence peak at 634 nm. The final concentrations of PPIX and photoproducts were obtained by multiplying the initial concentration of PPIX for the ratio between the 634 nm intensity after irradiation and the initial intensity. We established that 300 J/mols is the irradiation where, under our experimental conditions, we reached saturation of photoproduct formation. These concentrations were used for Scatchard plots of solutions containing simultaneously PPIX and photoproducts. Irradiation was carried out using the output of a 630 Diomed Laser (Diomed Ltd., Cambridge, UK).

2.6. Derivative synchronous fluorescence spectroscopy (DSFS)

Because of the spectral overlap between PPIX and its main photoproducts we used DSFS to study the binding to protein of irradiated solutions. DSFS has been used in analytical studies to quantify the individual contribution in mixtures of organic chromophores [19] but also, for instance, in clinical investigations of mixtures of porphyrins in urine samples [18]. In SFS emission and excitation monochromator are simultaneously scanned maintaining a fixed wavelength increment ($\Delta\lambda$) [17]. The resulting spectrum is the product between two spectra (excitation and emission) shifted from one another by an amount equal to $\Delta\lambda$ [17]. The value of $\Delta\lambda$ can be chosen to match the difference between emission and excitation maxima of a particular chromophore. This combination accentuates the emission of a specific chromophore over all the others in the mixture [17]. Band narrowing, typical of SFS, also improves the spectral resolution [17]. Derivative spectroscopy further improves the separation obtained with SFS [18]. We used DSFS to study the binding of PPIX to proteins in the presence of photoproducts. DSFS improves the separation between the two contributions and maintains proportionality to the fluorescence changes of the two species upon protein binding [17]. For the investigation of PPIX binding to protein in the presence of photoproducts we used SFS spectra recorded with a value of $\Delta\lambda$ corre-

sponding to the separation between the excitation and emission maxima of the main photoproducts ($\Delta\lambda_2$). At this $\Delta\lambda$ two peaks appear when PPIX and photoproducts are simultaneously present in solution. The peak at shorter wavelength corresponds to PPIX and the one at longer wavelength corresponds to the main photoproduct (Fig. 1a, dotted curve). In the same solution, SFS recorded at a value of $\Delta\lambda$ corresponding to the excitation/emission maxima of PPIX ($\Delta\lambda_1$) yields a single peak due to PPIX (Fig. 1a, solid line). The shift of the SFS maximum of PPIX with $\Delta\lambda$ is an intrinsic property of SFS and can be verified by investigating the SFS at $\Delta\lambda_1$ and $\Delta\lambda_2$ of solutions containing PPIX alone (with or without proteins) (Fig. 1b). In the presence of PPIX and photoproducts the appearance of one peak at $\Delta\lambda_1$ and two peaks at $\Delta\lambda_2$ is maintained with or without proteins. When PPIX alone is in solution SFS show only one peak with or without proteins. In solutions containing both PPIX and photoproducts the second derivative of the SFS spectra at $\Delta\lambda_2$ yields two pairs of maxima and minima (Fig. 2, solid line) which are proportional to the fluorescence intensity of PPIX (I_1^d) and its photoproducts (I_2^d), respectively. Without photoproducts the feature corresponding to (I_2^d) disappears (Fig. 2, dotted line).

The peak-to-peak values I_1^d and I_2^d were used to construct Scatchard plots for the binding of PPIX to proteins in the presence of photoproducts. This method was validated by studying the binding of PPIX to protein in the absence of photoproducts, and comparing the Scatchard plots obtained from DSFS with those obtained from traditional emission spectra under the same experimental conditions.

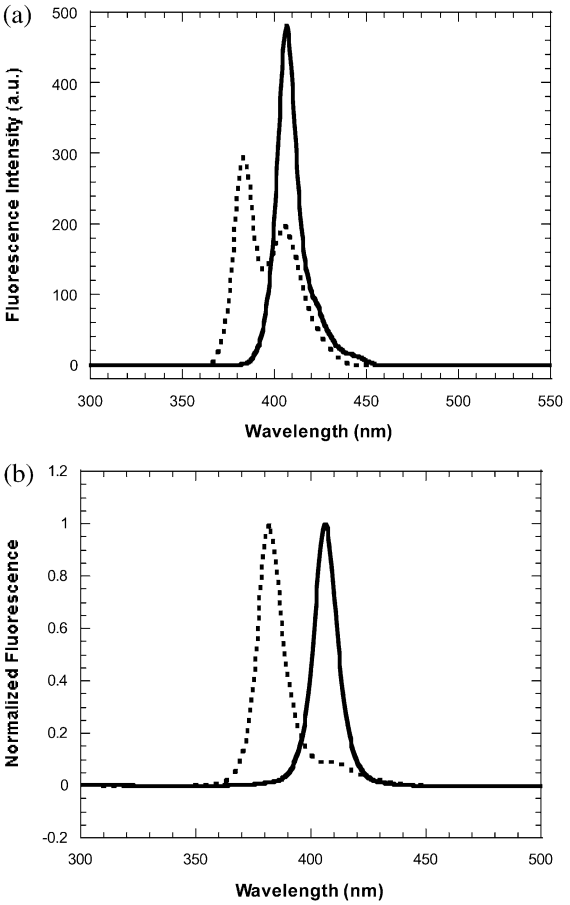

Fig. 1. Synchronous spectra. (a) Representative synchronous spectra of a solution containing PPIX (0.5 μM) and its photoproduct (0.4 μM) in the presence of HSA (1.2 μM). $\Delta\lambda = 227$ nm (solid line), $\Delta\lambda = 253$ nm (dotted line). The only peak at $\Delta\lambda = 227$ nm (located at 406 nm) is due to PPIX. In the spectrum recorded at $\Delta\lambda = 253$ nm the peak at 383 nm is due to PPIX while the peak at 405 nm is due to the photoproducts. (b) Representative synchronous spectra of a solution containing PPIX only (0.7 μM) in the presence of HSA (1.2 μM). $\Delta\lambda = 227$ nm (solid line), $\Delta\lambda = 253$ nm (dotted line). The shift from 406 nm to 383 nm of the PPIX peak is an intrinsic property of the $\Delta\lambda$ dependence of synchronous spectra.

3. Results

3.1. PPIX binding to proteins at pH 7.5

3.1.1. PPIX fluorescence in the presence of human serum albumin (HSA) and bovine serum albumin (BSA)

In agreement with previous reports [8,13], binding of porphyrins to albumins produces a large increase in PPIX fluorescence accompanied by a red shift of the emission (Table 1) and excitation maxima (in comparison with the emission in buffer). Scatchard plots (Fig. 3) can be constructed from the change in fluorescence intensity [8]. Based on the calculations of Dahlquist [23] the initial linear portion of the plot yields the minimum number of bound PPIX molecules and the extrap-

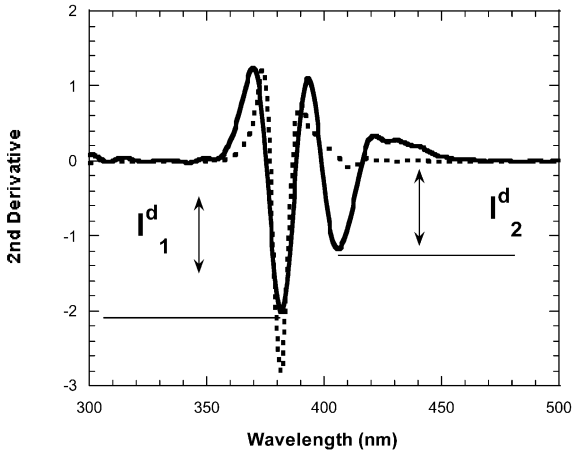

Fig. 2. Representative 2nd derivative synchronous spectra recorded at $\Delta\lambda = 253$ nm. Solid line: solution containing PPIX (0.5 μM) and its photoproduct (0.4 μM) in the presence of HSA (1.2 μM). Dotted Line: solution containing PPIX (0.7 μM) and its HSA (1.2 μM). To investigate protein binding of PPIX and photoproducts co-existing in solution we used the intensity I_1^d (PPIX) and I_2^d (photoproducts). I_1^d and I_2^d were employed in the contruction of Scatchard plots.

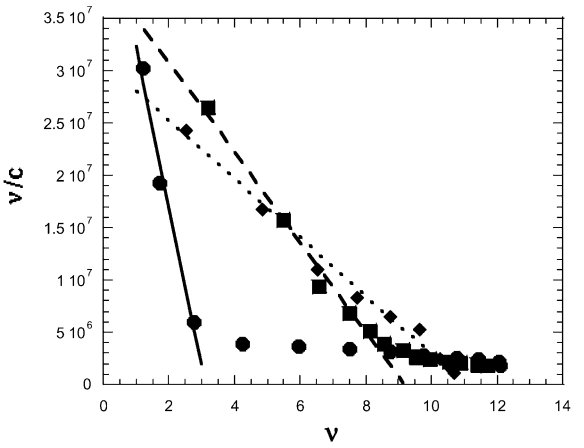

Fig. 3. Binding of PPIX to proteins measured from the emission of PPIX. Scatchard plots obtained by following the changes of PPIX fluorescence (excitation at 410 nm). The fluorescence was calculated as the area of the emission spectrum from 550 to 750 nm. Circles: binding to BSA; Squares: binding to HSA; Diamonds: binding to IgA.

olated binding constant [Table 2 (I and II)]. The minimum number of PPIX molecules bound to HSA ($\cong 7$) is larger than that bound to BSA ($\cong 4$), however, the dissociation constant [Table 2 (I)] is similar for the two proteins. Similar parameters were obtained when using values of I_1^d (Fig. 2) to construct the Scatchard plots [Table 2 (III and IV)] in solution where PPIX alone (without photoproducts) is binding to proteins. In this case SFS were recorded at Δλ values of 227 nm in the presence of HSA, and 223 nm in the presence of BSA.

3.1.2. HSA and BSA fluorescence in the presence of PPIX

The addition of PPIX to solutions containing BSA or HSA causes the quenching of protein fluorescence. Like the increase of PPIX emission intensity, protein fluorescence quenching can also be used to retrieve PPIX-protein binding parameters [13] (Fig. 4). The parameters retrieved from plots like the ones of Fig. 5 are consistent with the presence of 2 binding sites [Table 2 (VI)] and dissociation constants in agreement with the ones obtained from PPIX fluorescence [Table 2 (V)].

Table 1
Emission maxima of PPIX (free and protein bound) and its photoproduct with excitation at 410 nm

Solution	Emission maximum of PPIX (nm)	Emission maximum of photoproducts (nm)
DMSO	634	654
Buffer pH 7.5	622	651
Buffer pH 7.5 + HSA	636	661
Buffer pH 7.5 + BSA	630	658
Buffer pH 7.5 + IgA	622	651
Buffer pH 5.5	635	
Buffer pH 9.5	622	

Table 2
Parameters for PPIX binding to albumins and IgG at pH 7.5

Protein	I Dissociation constant[a] (μM)	II no. of bound PPIX molecules[a]	III Dissociation constant[b] (μM)	IV no. of bound PPIX molecules[b]	V Dissociation constant[c] (μM)	VI Avg. n. protein sites[c]
HSA	6.8 (±5)	7 (±1)[d]	6.1 (±5)	7 (±1)[d]	4.8 (±5)	1.7 (≅2) (±1)
BSA	5.7 (±1)	4 (±1)[d]	5.4 (±1)	4 (±1)[d]	4.4 (±5)	1.6 (≅2) (±1)
IgG	4.1 (±2)	6 (±1)	4.1 (±1)	6 (±1)[e]	5.2 (±5)	1.1 (≅1) (± 1)

[a] Retrieved from emission spectra of PPIX (excitation at 410 nm).
[b] Retrieved from SFS spectra (HSA $\Delta\lambda = 227$ nm, BSA $\Delta\lambda = 223$ nm).
[c] Retrieved from protein intrinsic fluorescence quenching.
[d] Minimum number of bound PPIX molecules according to the approximation of Dahlquist [20] for non-linear Scatchard plots.
[e] Actual number of bound PPIX molecules (linear Scatchard Plot).

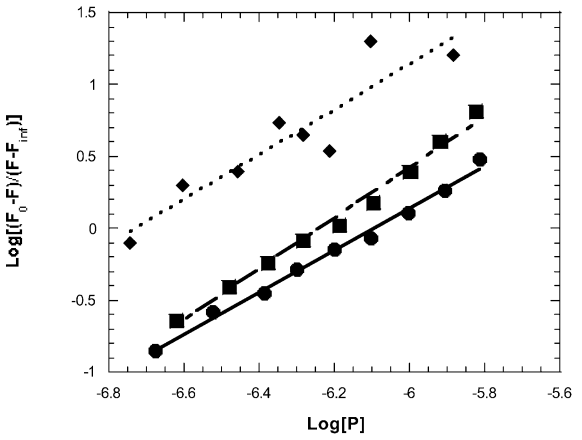

Fig. 4. Binding of PPIX to proteins measured from the emission of the proteins. These plots are obtained by following the changes of protein intrinsic fluorescence (excitation at 280 nm). The fluorescence was calculated as the area of the emission spectrum from 300 to 550 nm. The parameters plotted on the two axis are described in the experimental section. Circles: binding to BSA; Squares: binding to HSA; Diamonds: binding to IgG.

3.1.3. PPIX fluorescence in the presence of IgG

PPIX fluorescence increases in the presence of IgG. The increase of fluorescence is not accompanied by a red shift of the fluorescence maximum (Table 1). Scatchard plots of PPIX-IgG binding (Fig. 3) are linear, as a consequence the actual number of PPIX molecules bound to IgG can be directly retrieved ($\cong 6$). The dissociation constant is only slightly smaller then with albumins [Table 2 (I and II)]. The binding parameters retrieved using DSFS were in total agreement with those obtained using emission intensity [Table 2 (III and IV)].

3.1.4. IgG fluorescence in the presence of PPIX

The addition of PPIX quenches the intrinsic fluorescence of IgG. From the quenching experiment we estimated a single binding site for PPIX [Table 2 (VI)] with a dissociation constant comparable to the one obtained from PPIX fluorescence increase [Table 2 (V)].

4. Effects of photoproducts

Photoproducts were formed prior to protein binding as described earlier. In buffer the peak of PPIX remains near 622 nm while the peak of the main photoproduct occurs near 651 nm.

The addition of albumins produced the red shift of PPIX maximum and a slight shift of the maximum of the photoproduct (Table 1). The intensity of the fluorescence peak of PPIX increases with the addition of proteins while the emission intensity of the peak of photoproducts does not change. The addition of IgG to the PPIX/photoproduct aqueous mixture yields a slight increase of PPIX fluorescence not accompanied by any shift and no effect on the peak of the photoproduct.

Because of the overlap of emission and excitation spectra between PPIX and its photoproduct binding of PPIX to proteins was studied using DSFS. As described earlier (Experimental) the Scatchard plots for the binding of PPIX to proteins were constructed using the intensity of the second derivative spectrum (I_1^d) of SFS recorded at $\Delta\lambda_2$ (249 nm with IgG, 250 nm with BSA and 253 nm with HSA). I_1^d of the PPIX peak-to-peak value of Fig. 2 increases with increasing concentration of protein, while the intensity of the peak-to-peak value relative to the photoproduct (I_2^d) is not affected by the presence of proteins (Fig. 2).

Scatchard plots of PPIX binding in the presence of photoproducts are linear (Fig. 5). From linear

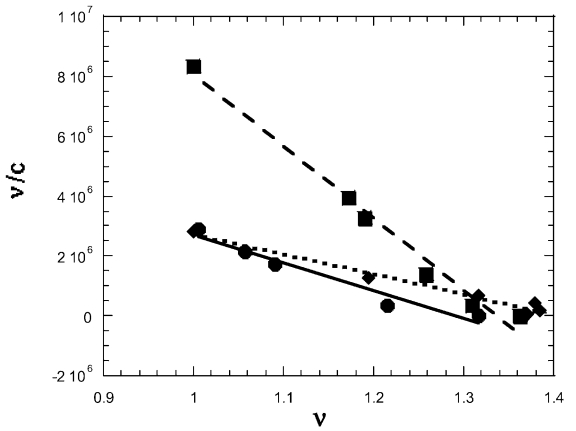

Fig. 5. Binding of PPIX to proteins in the presence of photoproducts measured from DSFS. Scatchard plots obtained by following the changes of the I_1^d (PPIX) of synchrnous spectra recorded in solutions containing PPIX and its photoproducts. Circles: binding to BSA; Squares: binding to HSA; Diamonds: binding to IgG.

Table 3
PPIX-protein binding parameters in the presence of PPIX photoproducts

Protein	Dissociation constant (μM)	Avg. no. of bound PPIX molecules
HAS	6.7 (±3)	2.3 (±1)
BSA	2.8 (±1)	2.2 (±1)
IgG	1.3 (±2)	1.9 (±1)

regression we retrieve the binding parameters that are shown in Table 3. Dissociation constants are only slightly decreased by the presence of the photoproducts, but the number of PPIX molecules decreases dramatically.

5. Discussion

5.1. Spectroscopic changes

Changes induced by albumins are due to monomerisation or dimerisation of large PPIX aggregates [5,8,12,13] and the difference of PPIX emission maximum between BSA and HSA (Table 1) is due to different microenvironments surrounding PPIX in the binding sites [25].

The increase of PPIX fluorescence not accompanied by a red shift of the emission peak upon binding to IgG is similar to results reported for Photofrin II [26]. Korbelik et al. [26] reported that the immunoglobulin would change the equilibrium between Photofrin aggregates without actually binding any molecule [26]. Since PPIX, unlike Photofrin, is a pure compound we believe it is unlikely that IgG would just induce a change in the equilibrium between aggregates. We suggest instead two alternative scenarios. Small PPIX aggregates may bind directly to the protein as it is the case for other porphyrin derivatives [12]. This could be consistent with the absence of fluorescence red shift (as there would be no monomerisation upon binding) and the fluorescence increase could be explained by local effects of protein residues on the aggregate [12,25]. Since the aggregates in buffer can range from dimers to large micelle-like polymers [27], binding of small oligomers of PPIX could produce the fluorescence increase without necessarily induce an emission red-shift [12]. Recent fluorescence lifetime data (not shown) suggest that the binding of small aggregates is likely and that the presence of protein has a static and not dynamic effect as the lifetimes of the fluorescent species does not change in comparison with PPIX in buffer. Alternatively, since PPIX fluorescence is strongly affected by local environment, the absence of emission red shift upon binding to IgG could be consistent with a binding site that leaves the porphyrin exposed to the aqueous solvent.

5.2. Binding parameters

The non-linearity of the Scatchard plots (Fig. 3) in the presence of albumin suggests the presence of multiple affinity sites [8,28]. In such case binding parameters can still be estimated according to theoretical analysis of non-linear Scatchard plots [23]. The extrapolated values at $\nu \rightarrow 0$ yields the minimum number of PPIX molecules bound to albumin and the average binding constant [23] [Table 2 (I and II)]. Differences in the number of PPIX molecules bound to HSA or BSA could be due to differences in the size of the binding site or in the type of molecular interaction.

The linearity of Scatchard plots for the binding to IgG indicates that, unlike albumins, this immunoglobulin has single affinity sites for PPIX. In this case the slope and the intercept (at $\nu \rightarrow 0$) of the linear regression (Fig. 3) yield the actual binding constant and the actual number of PPIX molecules bound to the immunoglobulin [23,28]. If small PPIX oligomers bind to IgG (according to our first scenario described earlier), the number of PPIX molecules bound to this protein [Table 2 (II)] may be underestimated. In fact if small oligomers of PPIX bind IgG, in the construction of Scatchard plots (Fig. 3) each aggregate will be counted as a single PPIX molecules. This obviously leads to a gross underestimate of the number of PPIX bound to IgG.

It appears that in general fluorescence spectroscopy may underestimate the number of PPIX molecules bound to proteins. Non-linear Scatchard plots (albumins) by definition provide the minimum number of PPIX molecules bound to albumins and as stated above the number of PPIX molecules bound to IgG might be much larger than the one retrieved from Fig. 3.

The underestimate becomes even more evident when comparing the number of bound PPIX molecules [Table 2 (II or IV)] with the number of sites on the proteins [Table 2 (VI)]. The number of binding sites retrieved for albumins ($n \cong 2$) is in agreement with previous findings [13], while IgG appears to have only 1 binding site for PPIX. For all proteins the binding sites are less than the number of PPIX molecules bound to them. We believe there are two possible explanations for this discrepancy:

1. Several PPIX molecules may bind a single site, but only one PPIX molecule is sufficient to produce the quenching of the aromatic amino acid that concurs to produce the fluorescence of the protein. In this case additional PPIX molecules that bind a site proximal to an already quenched aromatic amino acid would not produce additional quenching of protein fluorescence. The additional PPIX molecules, however, would be observed when recording the fluorescence increase of PPIX. Hence any additional PPIX molecule binding to a protein would be recorded when monitoring PPIX fluorescence but it would not be observed when recording the fluorescence of the protein.
2. PPIX molecules may bind proteins in sites of similar affinity that do not carry a proximal aromatic amino acid to quench. PPIX molecules that bind proteins distant from aromatic amino acids would be observed when monitoring the increase of PPIX fluorescence but they would not produce any significant quenching of protein fluorescence.

5.3. Influence of photoproducts

The presence of its photoproducts does not affect the spectral features (emission and excitation maxima) of free and bound PPIX in buffer. However, because of the overlap between the absorption and emission spectra of PPIX (free or bound) and its photoproduct, traditional fluorescence spectroscopy may lead to inaccurate estimates of the binding parameters. DSFS dramatically improves the separation between the two components. The shift of the maximum of PPIX from $\cong 400$ nm at $\Delta\lambda_1$ to $\cong 385$ nm at $\Delta\lambda_2$ (Fig. 1b) is an intrinsic characteristic of synchronous spectra and depends on the value of $\Delta\lambda$ [17]. The Scatchard plots obtained from DSFS in the presence of PPIX and its photoproduct show that the presence of photoproducts affects the binding of PPIX to proteins [Table 2 (I and II) and 3]. While the dissociation constant is only marginally decreased, the number of PPIX molecules bound to proteins decreases substantially. This decrease occurs for all proteins investigated and is due to competition between photoproducts and PPIX for the same binding sites. It is likely that photoproducts bind both lower affinity sites [as suggested by the linearity of the Scatchard plots [23] and the small variation of the binding constant [Table 2 (I) and 3] and higher affinity sites, leaving a limited number of them for PPIX binding (as suggested by reduced number of PPIX molecules bound to the protein). Since the fluorescence intensity of the photoproducts does not change upon binding, their binding parameters could not be directly determined.

The use of SFS to construct Scatchard plots was validated in experiments of PPIX-protein binding in the absence of photoproducts. The data obtained from DSFS are in excellent agreement with those obtained from traditional fluorescence spectroscopy [Table 2 (III and IV)].

6. Conclusions

Our most relevant results can be summarised in three points:

1. PPIX appears to bind IgG either as a small aggregate or in a site (probably superficial) that leaves PPIX exposed to the solvent. Moreover, the linearity of the Scatchard plots indicates that IgG has a single affinity-binding site. This is in contrast with the multiple affinity sites presented

by albumins.

2. Fluorescence spectroscopy underestimates the number of PPIX molecules bound to each protein. In albumins this is caused by the presence of multiple sites with different affinities that do not allow for an exact estimate of the number of molecules bound. In IgG it may be caused by the binding of small aggregates whose fluorescence is counted as a single PPIX molecule. We also demonstrated that when only fluorescence spectroscopy is employed there is a discrepancy between the number of PPIX molecules and the number of binding sites. This is basically due to the fact that the increase of PPIX fluorescence and the quenching of protein fluorescence may monitor two separate events.
3. The presence of PPIX photoproducts affects the binding of PPIX to proteins. The photoproducts compete with PPIX for the same binding sites. The linearity of the Scatchard plots also suggest that the efficiency of photoproducts to bind proteins occupies the sites with lower affinity (as demonstrated by the small changes in the binding constant of Table 2 and 4) leaving only a reduced number of high affinity sites available for the binding of PPIX.

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