Spectroscopic Studies on the Interaction of a Water Soluble Porphyrin and Two Drug Carrier Proteins

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ABSTRACT The interaction of meso-tetrakis(p-sulfonatophenyl)porphyrin (TSPP) sodium salt to human serum albumin and β-lactoglobulin was studied by steady-state and dynamic fluorescence at different pH of aqueous solutions. The formation of TSPP J-aggregates and a noncovalent TSPP-protein complex was monitored by fluorescence titrations, which depend on pH and on the protein nature and concentration. The complex between TSPP and protein displays a heterogeneous equilibrium with large changes in the binding strength versus pH. The large reduction of the effective binding constant from pH 2 to 7 suggests that electrostatic interactions are a major contribution to the binding of TSPP to the aforementioned proteins. TSPP aggregates and TSPP-protein complex exhibit circular dichroism induced by the presence of the protein. Circular dichroism spectra in the ultraviolet region show that the secondary structure of both proteins is not extensively affected by the TSPP presence. Protein-TSPP interaction was also examined by following the intrinsic fluorescence of the tryptophan residues of the proteins. Fluorescence quenching by acrylamide and TSPP itself also point to small changes on the protein tertiary structure and a critical distance R0 ~56 Å, between tryptophan and bound porphyrin, was estimated using the long distance Förster-type energy transfer formalism.

INTRODUCTION

In recent years there has been a growing interest in the use of porphyrins and related compounds as therapeutic drugs. They are applied in medicine on important areas as cancer detection and as photosensitizers in photodynamic therapy of cancer (Bonnett, 1995). Potential applications of porphyrins have recently appeared in the treatment of nonmalignant conditions such as psoriasis, blocked arteries, and viral and bacterial infections, including HIV (Ben-Hur and Horowitz, 1995).

Biological effects of porphyrins largely depend on their physicochemical properties, which in turn lead to important changes in their photophysical behavior. In particular, aggregation and axial ligation induce alterations on the porphyrin absorption spectra, quantum yield, fluorescence lifetime, and triplet state lifetime (Uehara et al., 1993; Tominaga et al., 1997; Togashi and Costa, 2000). Porphyrins are usually introduced in the blood as relatively concentrated solutions, which may diminish its action or even cause adverse effects. Moreover, interactions with macromolecules may control the efficacy and biodistribution of porphyrins, which are known to locate preferentially in the cytoplasm and bind poorly to cell membranes. Therefore, the interaction of these molecules with proteins, especially those that provide carriage through the blood stream, is of utmost importance to formulate safe drugs and effective dosages. In fact, the affinity of some porphyrins for human serum albumin (HSA) binding site II depends on their chemical structure and correlates with the photodynamic efficacy in vivo (Tsuhida et al., 1997).

Water-soluble synthetic porphyrins are structurally simpler than native porphyrin derivatives in the physiological state making easier the interpretation of the structure-function relationship. The aggregation of the anionic porphyrin meso-tetrakis(p-sulfonatophenyl)porphyrin sodium salt (TSPP) has been studied extensively. It has been shown that under appropriate conditions, which involve very acidic media and high ionic strength, TSPP forms highly ordered molecular J and H aggregates (Ohno et al., 1993; Akins et al., 1994; Maiti et al., 1995). Very few porphyrins are known to form J-aggregates, the main requisite being the zwitterionic character with the protonation of the pyrrole nitrogen in the macrocycle. More recently, it was reported that these aggregates could also be promoted by interaction with surfactants (Maiti et al., 1998) and proteins (Borissievitch et al., 1996; Huang et al., 1998).

In the present work, we aim to investigate further the interaction of TSPP with carrier proteins, the aforementioned HSA, a transport protein in the blood plasma, which binds to a wide variety of therapeutic drugs, and β-lactoglobulin (βLG). The proper functionality has not been clarified yet, but being also a transport protein interacts with a variety of ligands such as retinol (Dufour et al., 1990) and fatty acids (Frapi et al., 1993) especially through its hydrophobic binding sites even in acid environments. Thus, the proteins sensitivity to pH and the porphyrin aggregation make the study at pH = 2 of physiological relevance.

The binding characteristics were followed by absorption, steady-state, and transient-state fluorescence of TSPP over a large pH range. Because TSPP is a symmetrical molecule, no circular dichroism (CD) signals are obtained. However, they can be induced by the presence of an asymmetric
peptide environment bound to the macromolecule as normally occurs in hemoglobins (Ogoshi and Mizutani, 1998). It is then expected that TSPP-protein complexes present CD spectra, which are useful to understand better the binding process.

The fluorescence of these proteins that arise mainly from the tryptophan (Trp) residues (Trp$^{214}$ in HSA, Trp$^{19}$ and Trp$^{61}$ in βLG) show how the relative exposure of the probe varies and may be used to gain information concerning changes in protein conformation upon ligand interaction. Quenching studies involving acrylamide and TSPP itself as quenchers were carried out, and the efficiency of the latter was used to evaluate their location on the protein’s environment.

**MATERIALS AND METHODS**

**Sample preparation**

NATA (catalog no. A-6501), HSA fraction V, 96 to 99% purity (catalogue no. A-1653), and bovine TSPP chromatographically purified and lyophilized >90% purity (catalogue no. L-3908) were purchased from Sigma (St. Louis, MO) and used without further purification. TSPP was obtained from Fuka ≥98% purity (catalogue no. 88074) and acrylamide 99% electrophoresis grade (catalogue no. 14,866-0) from Aldrich (Milwaukee, WI), and both were used as received. The probe concentrations were determined spectrophotometrically considering the molar extinction coefficient $e^{HSA}_{280nm} = 42,864$ M$^{-1}$ cm$^{-1}$ (Esposito et al., 1999); $e^{βLG}_{280nm} = 17,600$ M$^{-1}$ cm$^{-1}$ (Barteri et al., 2000); $e^{TSPPf}_{413nm} = 5.1 \times 10^4$ M$^{-1}$ cm$^{-1}$ at pH 6 (Huang et al., 1998).

Buffer solutions were made up with bidistilled water, following the recommended procedures. In the pH 2 to 7 range, a citrate-phosphate buffer (25 mM) was used. All solvents were spectroscopic grade. In all experiments we used fresh stock solutions of proteins and porphyrin in water.

**Apparatus**

The pH was measured at 24.0°C with a Crison microchip 2002 and the adjustment to the desired pH values accomplished by the addition of HCl or NaOH. A Jasco V-560 spectrophotometer was used in ultraviolet (UV)-Vis absorption measurements. Fluorescence measurements were recorded with a Perkin-Elmer Applied Biosystems (Foster City, CA) LS 50B spectrophotometer. Band-pass slits of 7.5 were used for both fluorescence excitation and emission monitoring TSPP, whereas 5.0 slits were used for Trp residues and derivative. The instrumental response at each wavelength was corrected by means of a curve obtained using appropriate fluorescence standards (until 400 nm) together with the one provided with the instrument. Fluorescence quantum yields of aerated solutions of TSPP were determined relative to that of TPP in toluene ($φ \approx 0.11$) (Figueiredo et al., 1999) with appropriate corrections for the refractive index of the solvent. The quantum yields of Trp in proteins or NATA were determined relative to Trp molecule alone at pH 7 aerated aqueous solution ($φ = 0.13$) (Andrade and Costa, 2000). Fluorescence decay profiles were obtained by using the time-correlated single-photon counting method (O’Connor and Phillips, 1984) with a Photon Technology International instrument. Excitation was made with the use of a lamp filled with Hg ($λ_{exc}^{Hg} = 245$ nm and $λ_{em}^{Hg} = 295$ nm) and sample emission measurements were performed until a maximum of 10$^6$ counts. Data analysis was performed by a deconvolution method using a nonlinear least squares fitting program, based on the Marquardt algorithm. The goodness of the fit was evaluated by statistical parameters (reduced chi-square ($χ^2$) and Durbin-Watson) and graphical methods (autocorrelation function and weighted residuals).

CD spectra were obtained with a Jasco spectropolarimeter J-720 with spectral averaging and baseline correction. The studies have been performed at a constant TSPP concentration of 2 μM in the visible range and protein concentration was varied (0–7.5 μM for βLG and 0–1 μM for HSA). In the far-UV region the protein concentration was kept constant at 5 μM for both proteins and TSPP concentration was varied (0–20 μM).

All cells used were quartz with 1 cm path lengths, and temperature was maintained at 24.0 ± 0.2°C in a thermostated chamber by using a circulating water bath.

**Data analysis**

**Titration of TSPP with HSA and with βLG**

A titration procedure using a fixed amount of TSPP (2 μM) and varying the protein concentration (HSA or βLG) was carried out in buffered solutions at different pH (2 to 7). This titration can be monitored by following the absorbance (Davila and Harriman, 1990a,b) or fluorescence changes (Avdulov et al., 1996) on the TSPP spectra. The binding parameters can be calculated using the Scatchard’s equation. This formalism assumes the existence of n ligands binding with equal affinities (for n = 1 this equation is similar to the Langmuir isotherm). When there is some cooperativity in the binding process, an equation derived from the Freundlich isotherm (and analogous to the Hill formalism) may be used

$$[\text{TSPP}]_b = \frac{[\text{TSPP}]_{max} K [\text{TSPP}]_f^n}{1 + (K [\text{TSPP}]_f^n)_{10^{-(OD_{total} - OD_{prot})}}},$$

where $b$ and $f$ subscripts refer to the bound and free TSPP, $K$ is the equilibrium binding constant and if there is cooperativity in the binding process n expresses the system’s heterogeneity (if n = 1 we have the usual Scatchard’s equation). The errors of the calculated parameters were assessed using the error propagation theory and the distribution F of Snedcor by Figueiredo et al., 1999 with appropriate corrections for the refractive index of the solvent. A correction factor ($φ$),

$$φ = \frac{OD_{total}}{OD_{prot}} 1 - 10^{-OD_{abs}},$$

was applied to $F$ to account for the fact that the quencher absorbs at the excitation wavelength ($λ_{exc}^{HSA} = 0.27 \pm 0.03$ M$^{-1}$ cm$^{-1}$) (Andrade and Costa, 2000). Quenching data were analyzed using Stern-Volmer equation (Eftink and Ghiron, 1981), $F_0/F = (1 + K_s Q) e^{-θ}$ in which $F_0$ and F are the fluorescence in the absence and in the presence of the quencher $Q$. $K_s$ and $θ$ are related, respectively, to the fluorescence extinction rate constant for the dynamic ($K_s = k \tau_p$). $k$ and $τ_p$ is the fluorescence lifetime in the absence of the quencher and static processes.
TABLE 1 Absorption and fluorescence emission maxima of TSPP (2 \(\mu\)M) in aqueous solution at different pH in the presence of proteins (HSA and \(\beta\)LG) and in pure solvents ([TSPP] = 4 \(\mu\)M), (\(\lambda_{\text{exc}} = 425 \text{ nm or } 518 \text{ nm}, T = 24.0^\circ\text{C}\))

<table>
<thead>
<tr>
<th>Sample</th>
<th>Soret (B)</th>
<th>(\bar{Q}_Y)</th>
<th>(\bar{Q}_X)</th>
<th>Emission maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{H}_2\text{O (pH 2)})</td>
<td>434</td>
<td>593</td>
<td>644.5</td>
<td>674</td>
</tr>
<tr>
<td>(\text{H}_2\text{O} + \text{HSA (0.2 (\mu)M) (J-aggregate)})</td>
<td>420</td>
<td>591</td>
<td>648</td>
<td>704</td>
</tr>
<tr>
<td>(\text{H}_2\text{O} + \beta\text{LG (4 (\mu)M) (J-aggregate)})</td>
<td>432.5</td>
<td>593</td>
<td>648</td>
<td>704</td>
</tr>
<tr>
<td>(\text{H}_2\text{O (pH 7)})</td>
<td>412.5</td>
<td>579</td>
<td>633</td>
<td>645</td>
</tr>
<tr>
<td>(\text{H}_2\text{O} + \text{HSA (10 (\mu)M)})</td>
<td>420</td>
<td>592</td>
<td>646.5</td>
<td>653</td>
</tr>
<tr>
<td>(\text{H}_2\text{O} + \beta\text{LG (10 (\mu)M)})</td>
<td>412.5</td>
<td>589</td>
<td>646</td>
<td>654</td>
</tr>
<tr>
<td>Dimethylsulfoxide</td>
<td>419</td>
<td>591</td>
<td>645.5</td>
<td>652</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>414</td>
<td>591</td>
<td>645.5</td>
<td>652</td>
</tr>
<tr>
<td>Methanol</td>
<td>413</td>
<td>586</td>
<td>642.5</td>
<td>649.5</td>
</tr>
</tbody>
</table>

*Excitation at 490 nm.

**TSPP quenching**

Similar procedure to that described for acrylamide was carried out using TSPP as quencher. The experimental data were computer-fitted according to the following equation

\[
\frac{\Delta F}{F_0} = \left(\frac{\Delta F_{\text{max}}/F_0}{K_d + [\text{TSPP}]^n}\right)
\]

in which \(\Delta F\) is the change in fluorescence caused upon addition of TSPP, \(K_d\) is the dissociation constant, and \(n\) is the Hill coefficient (Nelson et al., 2000).

**(Förster) Long distance energy transfer**

Förster resonance energy transfer is usually expressed in terms of a critical radius, \(R_0\), the distance at which the rate of energy transfer is equal to the reciprocal of the donor fluorescence lifetime and was calculated by the following expression (Lakowicz, 1985)

\[
R_0 = 0.2108 \left(\frac{k^2 \phi_0 n^4}{2 \int F(\lambda)\epsilon(\lambda)\lambda^4 d\lambda}\right)^{1/6}
\]

with \(R_0\) in Å, in which \(k^2\) is the orientation factor (two-thirds assumed) describing the relative orientation in space of the transition dipoles of the donor (Trp residues) and acceptor (TSPP), \(\phi_0\) is the donor quantum yield in the absence of the acceptor, \(n\) is the refractive index of the solution, \(F(\lambda)\) is the normalized fluorescence spectrum of the donor, \(\epsilon(\lambda)\) is the molar extinction coefficient (M\(^{-1}\) cm\(^{-1}\)), and \(\lambda\) is in nanometers.

**RESULTS**

**Solution properties of TSPP**

TSPP is water-soluble and exists as a monomer in aqueous solution below 30 \(\mu\)M shown by the absorption studies. Two chemical forms can be in equilibrium, Scheme 1, due to protonation of the two pyrrolic nitrogen atoms in the porphinato macrocycle (pK\(_a\) \(\sim 4.8\) at 25°C and \(\mu = 0.1\)) (Huang et al., 1998).

The absorption spectra of the deprotonated species, TSPP\(^{4-}\), exhibits features of D\(_{2h}\) symmetry. However, at pH 2 symmetry increases to D\(_{4h}\) featuring a shift of the Soret band from 413 to 434 nm and the nonsplitting of the Q-bands (Table 1). In these acidic conditions, two new absorption bands may appear simultaneously at \(\approx 490\) and \(\approx 705\) nm. These two new bands have been assigned to the formation of J-aggregates in solution and are enhanced by increasing the porphyrin concentration, the ionic strength, and/or the acidity.

At pH 7, \(\phi = 0.10 \pm 0.01\) is close to the values found for other free base porphyrins in solvents. Such invariance, found for porphyrins with distinct aryl groups almost orthogonal to the macrocycle plan, must derive from the fact that the \(\pi\) system of the macrocycle is not distorted significantly by changes in the ring peripherical structure. The fluorescence lifetimes agree well with literature data (Table 2). A global analysis treatment of the TSPP singlet state decays was possible and leads to the confirmation of the pK\(_a\) for this acid-base equilibrium.

**Association of TSPP with proteins in aqueous solution**

Titrations of TSPP solutions at a fixed dye concentration (2 \(\mu\)M) and variable protein amounts, HSA or \(\beta\)LG were performed at several pH in the range 2 to 7. The course of the reaction was followed by absorption and fluorescence
spectroscopy. Several changes are detected in the TSPP absorption spectra and are more significant at pH below the porphyrin pKₐ. At pH 2, two new bands appear, at [Protein]/[TSPP], with exactly the same maxima as those assigned to the J-aggregate (Fig. 1). These bands (490 and 704 nm) increase with protein concentration at the expenses of the Soret band for the first one and of the Qₓ(0,0) band for the second one. It seems that the amount of J-aggregate depends on the protein nature. In fact, at [HSA]/[TSPP] = 0.01, but the same ratio is only achieved at [BLG]/[TSPP] = 0.1 M.

Noteworthy is the fact that only in the case of HSA, at [HSA]/[TSPP] = 0.08, there is a decrease in the ODₜₜₑᵣₜₑ/ODₐₗₜₑ of 2.5, but the same ratio is only achieved at [BLG]/[TSPP] > 0.1 μM. Curiously, the prominent absorption band at 705 nm is not visible in the excitation spectra, which points to a small

<table>
<thead>
<tr>
<th>Sample</th>
<th>λₛₑᵣₑ (nm)</th>
<th>τ₁ (ns)</th>
<th>τ₂ (ns)</th>
<th>τ₃ (ns)</th>
<th>χ²</th>
<th>DW</th>
<th>Singlet quantum yield*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O (pH 2)</td>
<td>–</td>
<td>3.83</td>
<td></td>
<td></td>
<td>1.10</td>
<td>1.65</td>
<td>0.14</td>
</tr>
<tr>
<td>H₂O + HSA (1 μM)</td>
<td>0.98</td>
<td>3.83</td>
<td>12.9</td>
<td></td>
<td>1.10</td>
<td>1.65</td>
<td>0.08</td>
</tr>
<tr>
<td>H₂O + BLG (4 μM)</td>
<td>0.10</td>
<td>3.76</td>
<td>12.9</td>
<td></td>
<td>1.11</td>
<td>1.89</td>
<td>0.08</td>
</tr>
<tr>
<td>H₂O (pH 7)</td>
<td>–</td>
<td></td>
<td>9.8</td>
<td>1.20</td>
<td>1.75</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>H₂O + HSA (10 μM)</td>
<td>0.96</td>
<td>12.9</td>
<td>9.8</td>
<td>1.25</td>
<td>1.55</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>H₂O + BLG (10 μM)</td>
<td>0.50</td>
<td>12.8</td>
<td>9.8</td>
<td>1.25</td>
<td>1.50</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>Dimethylsulfoxide</td>
<td>0.83</td>
<td>12.7</td>
<td>7.0</td>
<td>1.05</td>
<td>1.70</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>0.74</td>
<td>11.1</td>
<td>6.60</td>
<td>0.94</td>
<td>2.10</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>0.90</td>
<td>11.2</td>
<td>5.63</td>
<td>1.04</td>
<td>1.99</td>
<td>0.22</td>
<td></td>
</tr>
</tbody>
</table>

*Using φᵣ = 0.1 for TPP in cyclohexane.

The absorption spectra of different species of TSPP present in solution during titration with a given protein are shown in Fig. 2. The conversion of the di-acid monomer (λₛₑᵣₑ = 434 nm) to J-aggregates (λₛₑᵣₑ = 490 nm) is almost completed when [TSPP] is ~40 times that of HSA and is twice the concentration of [BLG]. J-aggregates become unstable at higher protein concentrations and vanishes when [TSPP]/[HSA] ~ 4:1 and [TSPP]/[BLG] ~ 1:1. At this stage the species with a Soret absorption peak at ~420 nm are stabilized and dominate absorption. So, a similar pattern is followed by the interaction of TSPP with both proteins, except for the fact that higher concentrations of BLG are needed to obtain the same effects as HSA on TSPP.
Stokes shift for J-aggregate emission. Together with a short fluorescence lifetime (50 ps, Maiti et al., 1995) this suggests that these transitions occur without detectable intramolecular relaxation. At pH 2, in the absence of protein the excitation spectral peaks are in agreement with absorption peaks of the monomer TSPP and independent of emission wavelength. The fluorescence obtained upon excitation either at 434 or 580 nm shows a single band with a peak at 674 nm, which is quenched in the presence of protein until [HSA] ≤ 0.05 μM and [βLG] ≤ 0.5 μM (Fig. 1). When the 490-nm band is dominant, the fluorescence spectra depend upon the excitation wavelength (insert Fig. 1b), which clearly puts into evidence the presence of multiple species in solution. The excitation spectra obtained (data not shown) from emission at 640 nm give peaks at 436 and 595 nm, whereas those from emission at 720 nm show also the peak at 500 nm. In the case of HSA, at concentrations where the shifted Soret band (peak = 420 nm) predominates, fluorescence spectra no longer depend on the excitation wavelength and show two intense bands with maxima at 655 and 720 nm (insert Fig. 1a). These maxima resemble those obtained for TSPP in dimethylsulfoxide, which at this stage seem to confirm a less hydrophilic location for the interacting dye in the protein matrix.

Fluorescence decays of TSPP in the presence of protein were obtained upon excitation at 425 nm and emission at 650 nm and were best analyzed with a sum of two exponentials. A global analysis fitting was attempted, and the results are shown in Fig. 3. The shorter lifetime may be assigned to the dianionic monomer because it is the lifetime value obtained without protein. At these excitation/emission conditions, in the case of HSA, one is clearly monitoring the changes in the population of TSPP monomers and in the population of the porphyrin-protein complex. So, the long-lived component τ₁ ≈ 13 ns may be assigned to that complex. The J-aggregate lifetime is reported to lie in the picosecond domain as mentioned earlier, which is outside the equipment resolution.

The amount of complexed porphyrin is much less in the case of βLG, where J-aggregation itself is promoted, also at higher protein concentrations comparatively to those of HSA, as mentioned above.

Similar studies performed at pH ≤ pKₐ show a common pattern in the results. In general, J-aggregation promoted by the presence of the protein occurs at higher protein/porphyrin ratios with increasing pH but in a more restricted range. At pH > pKᵥ, no J-aggregation is detected and the effect of the protein is noticed by the spectral bathochromic shifts, more notorious in the case of HSA, where the maxima are similar to those found at pH ≤ pKᵥ (Fig. 4). The isosbestic point found although not so well defined seems to rule out the presence of other species-like dimers that were detected in the presence of potassium ion and crown ether at pH 9.0 at higher TSPP concentration (~10 μM) (Maiti et al. 1995). In the present case, checking fluorescence emission, a plot of the area under the spectra versus [HSA] yields a straight line and the decays obtained (λexc = 425 nm and λem = 650 nm) as will be presented below, do not indicate the presence of other entities apart from the deprotonated monomer and the complex protein porphyrin.

It seems that when porphyrin deprotonation occurs, aggregation is not favored even at high protein concentrations. On the other hand, porphyrin-protein complexation still occurs but at increasing protein amounts with higher pH: e.g., ≥0.08 μM (pH 2); ≥0.2 μM (pH 3.3); ≥0.75 μM (pH 5); ≥4 μM (pH 7), in the case of HSA.

Time resolved data also seem to support this type of behavior. Without protein the decays are monoexponentials...
at pH 7 ($\tau_f \approx 9.8$ ns for the monomeric tetra-anion). However, the presence of the protein induces the appearance of another component ($\tau_f \approx 13$ ns), which is identical to that found at acidic pH and assigned to the complex porphyrin protein. In summary, over the pH range covered there may be three different species detected by fluorescence decays: di-acidic and tetra-anionic monomers of free porphyrin in solution and porphyrin (irrespective of the protonation state) “bound” to the protein (HSA or $\beta$LG).

The binding of TSSP to HSA was followed by absorption and fluorescence (steady state and time resolved). A titration procedure using a fixed amount of TSSP and varying the protein concentration was carried out in buffered solutions at different pH. Equilibrium is established between free TSSP molecules and bound to the protein. Using absorption data, it was possible to decompose TSSP Soret band in a sum of three Gaussian functions over the entire pH range covered (Fig. 5 a, an example of those fittings is presented in Fig. 5 b) at pH 2 and 7. The OD of each component plotted versus the protein concentration shows the growth (at 420 nm) and the decrease (at 413 nm) of the components (insert Fig. 5). These values were used to calculate the associated binding constants.

An attempt to analyze the entire titration curves using the Scatchard formalism failed. A plot of the above equation ($n = 1$) with our data does not hold because it becomes nonlinear at all pH studied, indicating either that there is more than one class of binding sites or that the binding of each successive molecule alters the association constant of the next molecule (i.e., cooperativity). A similar behavior has been observed previously for binding of hematoporphyrin...
rhamine derivative (Grossweiner and Goyal, 1984) and a zinc porphyrin (Davila and Harriman, 1990a) with HSA. In some cases a concavity has been detected in such representations and interpreted as indicating the existence of two sets of binding sites where electrostatic and hydrophobic interactions are differently important (Saboury et al., 1996).

To determine the cooperativity in the binding process, Eq. 1 was used. The best fits are shown in Fig. 6a, and Table 3 summarizes the binding parameters obtained and the errors calculated. The value of \( n \) is close to 1.4 at \( pH \leq pK_a \) of TSPP and below unity (\( \approx 0.85 \)) above that \( pH \). The value of \( K \) is quite high at all \( pHs \), although it seems to decrease with this parameter, pointing to important electrostatic interactions in the complex.

Time-resolved fluorescence lead also to the same conclusion. The fractional intensity associated to the longest component (\( r \approx 13 \) ns), \( f_{bl} \), should be proportional to the amount of bound TSPP. Using Eq. 4,

\[
\frac{1}{f_b} = 1 + \epsilon f \phi_b \frac{1}{\epsilon_b \phi_b K [P]}
\]

in which \( \epsilon f \) and \( \epsilon_b \) are the extinction coefficients of free and bound TSPP, respectively, and \( \phi_b \) and \( \phi_b \) correspond to the fluorescence quantum yields of those species, a plot of \( f_b \) versus the protein concentration allows the estimation of a binding constant at each \( pH \) studied, which are consistent with those obtained using absorption data (Fig. 6b and Table 3). Taking \( \Delta G = -RT \ln K \), a plot against \( pH \) (insert Fig. 7) shows a sigmoidal behavior with a turning point at 5.2 \( \pm 0.2 \) for HSA, which is similar to the \( pI \) of the protein.

**CD Spectra**

TSPP is a symmetrical molecule and does not yield signals in CD spectra at the conditions above studied of \( pH \) and probe concentration. However, it tends to show a significant CD signal upon formation of the aggregate in the presence of the protein (Fig. 7) indicating an asymmetrical perturbation in the fluorophore. Heme proteins exhibit characteristic induced CD in the Soret band, reflecting the interaction between achiral porphyrins and chiral proteins. These studies showed that the carbonyl and the \( OH^- \) group can induce CD in a heme if they are fixed near the porphyrin plane (Ogoshi and Mitzutani, 1998).

**FIGURE 6** (A) Plot of the absorption spectral data to Eq. 1 at \( pH \) 2 ( ), \( pH \) 3.3 ( ); \( pH \) 5 ( ); \( pH \) 6.9 ( ). (B) Fractional intensities of TSPP fluorescence decays associated with the longest lifetime (\( r \approx 13 \) ns) versus protein concentration at different \( pH \) ( * \( pH \) 11, the remaining symbols have the same correspondence as in A). The full lines represent best fits with Eq. 4. (Insert) Plot of free energies versus \( pH \). The “turning point” of the sigmoidal fit gives a \( pH 5.2 \pm 0.2 \).

**TABLE 3** Binding parameters for the interaction TSPP-protein in aqueous solution at different \( pH \) obtained by fit of experimental data using Eq. 1

<table>
<thead>
<tr>
<th>pH</th>
<th>( n )</th>
<th>( K \times 10^{-6} ) (M(^{-1}))</th>
<th>( \beta LG )</th>
<th>( K \times 10^{-6} ) (M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>1.5 ± 0.1</td>
<td>5 ± 2; 5.1 ± 0.4* (0.2 ± 0.1)*</td>
<td>1.2 ± 0.2</td>
<td>3 ± 2 (0.1 ± 0.1)*</td>
</tr>
<tr>
<td>3.3</td>
<td>1.2 ± 0.2</td>
<td>2 ± 1; 4.5 ± 0.5*</td>
<td>1.1 ± 0.1</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>5.0</td>
<td>1.1 ± 0.1</td>
<td>1 ± 1; 1.1 ± 0.4*</td>
<td>0.9 ± 0.1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>6.9</td>
<td>0.80 ± 0.05</td>
<td>1 ± 1; 0.6 ± 0.1*</td>
<td>0.80 ± 0.05</td>
<td>1 ± 1</td>
</tr>
</tbody>
</table>

*Value obtained using Eq. 4.
†Values obtained from fluorescence quenching of the protein by TSPP using Eq. 2.
The CD signal appears initially near the 490-nm absorption maximum of J-aggregate but for higher [HSA] that signal diminishes and a CD dispersion is observed around 425 nm. Such behavior is similar to the findings observed on absorption but different from that reported (Ohno et al., 1993). Increasing pH, the CD signal at 490 nm disappears but the CD dispersion around 425 nm remains suggesting that it does not belong to the J-aggregate but rather to the monomer in a different chiral environment or to a different chiral species.

In the presence of βLG, more intense CD signals are obtained around 490 nm and 425 nm at pH 2 and 3.3. The differences found between the two proteins reveal different chiral environments (different orientations of the peptide chain relatively to the porphyrin plane). Also, a change in the pH of the solution from 2 to 3.3 caused a tremendous effect on CD spectra of TSPP with HSA in contrast to those with βLG. HSA is known to undergo a conformational transition producing two isomeric forms, the F form around pH 4 and the E form below pH 3.0 (Muzammil et al., 1999). Thus, it seems that the aggregation process takes place in the protein framework rather than in free aqueous solution because it depends on the protein nature and concentration.

The CD signal on far-UV reflects largely the secondary structure of the protein and arises from the inherent chirality of the polypeptide chain. Native HSA is a globular protein that contains ≈66% α-helix conformation at pH 7, which seems to be kept at pH 2 (Muzammil et al., 1999). The presence of TSPP induces spectral changes (data not shown) namely a red shift of 4 nm and a decrease in the CD signal, indicating a certain loss of helicity probably caused by electrostatic interactions (Davis et al., 1996). In turn, βLG native conformation is essentially β-sheet (52 and 10% α-helix) (Papiz et al., 1986) characterized by an intense negative band centered at 216 nm. The presence of TSPP does not induce important changes apart from a small gradual broadening of that band reflecting some loss of β-sheet content.

Because the secondary structure of the protein seems to play a crucial role on the TSPP aggregation process, it is

![Diagram](image-url)

**TABLE 4** Bimolecular rate constants for dynamic and static quenching of HSA and βLG by acrylamide in aqueous solutions with different concentrations of at pH 2, using Stern-Volmer equation and a modified one, Eq. 5 (for βLG). (λ<sub>ex</sub> = 295 nm and λ<sub>em</sub> = 338 nm, T = 24.0°C)

<table>
<thead>
<tr>
<th>[TSPP] μM</th>
<th>HSA</th>
<th>βLG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k&lt;sub&gt;q&lt;/sub&gt; = 0.03 × 10&lt;sup&gt;-9&lt;/sup&gt;s&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>k&lt;sub&gt;q&lt;/sub&gt; = 0.05 × 10&lt;sup&gt;-9&lt;/sup&gt;s&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>V = 0.01</td>
<td>V = 0.05</td>
</tr>
<tr>
<td>0</td>
<td>0.92</td>
<td>0.58</td>
</tr>
<tr>
<td>2</td>
<td>0.78</td>
<td>0.84</td>
</tr>
<tr>
<td>5</td>
<td>0.78</td>
<td>0.75</td>
</tr>
<tr>
<td>10</td>
<td>0.79</td>
<td>0.62</td>
</tr>
<tr>
<td>15</td>
<td>0.87</td>
<td>0.61</td>
</tr>
<tr>
<td>20</td>
<td>0.95</td>
<td>0.54</td>
</tr>
<tr>
<td>30</td>
<td>1.10</td>
<td>0.55</td>
</tr>
</tbody>
</table>

<sup>*</sup>k<sub>q</sub> = K<sub>q</sub>/⟨t<sub>q</sub>⟩ where ⟨t<sub>q</sub>⟩ is an average value.
interesting to follow the protein fluorescence in the presence of the porphyrin.

**Protein quenching studies**

Acrylamide is an excellent neutral quencher that is sensitive to exposure of Trp residues. The fluorescence quenching of Trp residues in HSA (Eftink and Ghiron, 1976; Chadborn et al., 1999) and in βLG (Eftink and Ghiron, 1984; Palazolo et al., 2000) have already been studied in free aqueous solution. An upward curvature in the Stern-Volmer plot using fluorescence intensity data has been identified with the existence of static contributions, similar to that of free tryptophan (Eftink and Ghiron, 1984) or N-acetyl-L-tryp tophan (Eftink and Ghiron, 1984; Andrade and Costa, 2000) in an aqueous solution.

**HSA**

The slope of the Stern-Volmer plot for HSA at pH 2 was 3.26 M⁻¹ in agreement to that reported in literature of 3.3 M⁻¹ at pH 2.5, which compared with 17.0 M⁻¹ for NATA (N-acetyl-l-tryptophanamide) or even for l-tryptophan alone, at pH 7 shows an ~sixfold lower value indicating that the Trp residue is not fully accessible to the quencher. A quenching sphere volume (V) of 0.70 M⁻¹ points to a location of the Trp residue within the protein matrix, because values of V = 1.0 M⁻¹ have been reported for a fully exposed residue (Eftink and Ghiron, 1976).

The excited state decay was measured using a time-correlated single photon counting, which was best described by a double-exponential function at pH 2, τ₁ = 2.20 ns (60%), and τ₂ = 5.19 ns (40%). Because both lifetimes and preexponential factors are affected by acrylamide, an average lifetime was used to calculate the bimolecular quenching rate, kₚ, which can only have a qualitative meaning. A value of 0.90 × 10⁷ M⁻¹ s⁻¹ again indicates a relatively inaccessible Trp residue.

The presence of TSPP, at pH 2, yields an upward curvature, which were well fitted using the Stern-Volmer equation presented in the Data analysis section, over the range of 0 to 0.25 M of acrylamide (Fig. 8 a). These relatively high quencher concentrations do not seem to have significant effects on HSA conformation because no fluorescence shift was detected.

Both dynamic and static quenching processes of HSA by acrylamide are affected by the presence of TSPP. In fact, for [TSPP] < 10 μM, the dynamic process seems to be less effective than without TSPP the opposite occurring for the static process. Above that concentration, there is no apparent change of the static contribution and the dynamic quenching efficiency increases and is even greater than without TSPP.

The TSPP presence leads to a red-shift of the fluorescence spectra from 334 nm (HSA without TSPP, pH 2) to 338 nm in the presence of 30 μM TSPP.

**HSA-TSPP**

Binding of “substrates” to proteins can often cause quenching of the intrinsic Trp fluorescence. The addition of TSPP (2–30 μM) to HSA resulted in concentration-dependent quenching of Trp214 fluorescence, which reaches a plateau (Fig. 8 b). The intrinsic dissociation constant for binding, Kₜ, and the maximum quenching reached at saturation,
\( \Delta F_{\text{max}} \) can be estimated by fitting of the equation representing different binding sites with some degree of interaction (Eq. 2) to the quenching data giving good fittings. The errors and statistical significance were determined as described in the Data analysis section. The value of \( K_d \approx 5.5 \) \( \mu \text{M} \) is higher than that obtained previously by TSPP absorption after binding to HSA, \( K_d = 1/K_a \approx 0.5 \) \( \mu \text{M} \). Although the Trp residue is sensitive to TSPP binding it does not quantitatively report the binding affinity. Fluorescence lifetime measurements were also performed at different TSPP concentrations showing a decrease in both components of the biexponential fitting below saturating concentration of the porphyrin.

\( \beta \text{LG} \)

Quenching by acrylamide leads to linear Stern-Volmer plots even in the presence of TSPP \( \leq 15 \) \( \mu \text{M} \). Above this concentration a downward curvature appears in the \( F_0/F \) plots (insert Fig. 8a). In such a situation in which proteins contain more than one Trp residue each emitting independently, the proper equation is:

\[
F_0/F = \left[ \sum_{i=1}^{n} (1 + K_i) \frac{f_i}{V_i} \right]^{-1}
\]

in which \( K_i \) and \( V_i \) are the dynamic and the static quenching constants for each fluorescent component \( i \), and \( f_i \) is its respective fractional contribution to the total fluorescence (Eftink and Ghiron, 1981).

The results obtained applying the former equation to both steady-state and transient-state data show that there are two type of Trp residues, one which is moderately accessible to acrylamide \( k_q1(\text{eff}) \approx 0.62 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \) \( k_q1(\text{eff}) = K_{q1} \times f_1/\tau_1 \) and another that is almost inaccessible to collisions with the quencher \( k_q < 1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \). The value of \( k_q1 \) is comparable with that reported by Eftink and Ghiron (1984) at pH 4.5, 0.53 \( \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \). The presence of TSPP seems to initially facilitate quenching by acrylamide, although for [TSPP] \( > 15 \) \( \mu \text{M} \), a decrease in \( k_q1 \) is obtained. Static quenching has also an important contribution especially for the less fluorescent contributing residues at higher [TSPP]. Nevertheless, the small changes found point to the inexistence of major conformational changes of \( \beta \text{LG} \) by the presence of TSPP.

\( \beta \text{LG-TSPP} \)

Similarly to HSA, the addition of TSPP (2–30 \( \mu \text{M} \)) to \( \beta \text{LG} \) also resulted in saturable concentration-dependent quenching of Trp fluorescence (Fig. 8b), and a binding constant of 7.3 \( \mu \text{M} \) was found.

\section*{DISCUSSION}

As mentioned before, TSPP is one of the few examples of molecules not belonging to the carbocyanine family that form pure J aggregates. Previous data concerning TSPP aggregation was achieved in the presence of high concentrations of cations including \( \text{H}^+ \) (Maiti et al., 1995, 1998), and in the presence of counter-charged surfactants, which were able, at low concentrations (below c.m.c.), to induce aggregation at a higher rate than cations (Maiti et al., 1998). Aggregation was also reported in confined media like aluminosilicate mesostructure (Xu et al., 2001) and adsorbed into polycation films (Van Patten et al., 2000) and TiO\(_2\) nanoparticles (Yang et al., 2001). A common link may be established among them: all took place at low pH (\( \leq 3.5 \)) when TSPP is a zwitseron and point to important electrostatic interactions. The strong Coulombic attraction between the positively charged macrocycles and the negatively charged sulfonate groups of neighboring molecules contribute to stabilize these J aggregates. Foremost, upon protonation TSPP is converted from a configuration in which the aryl moiety is twisted relative to the macrocycle plane to one in which it is nearly coplanar, in a fashion with a displacement between the next nearest neighbor such that oppositely charged sites are positioned closed to one another (head-to-tail alignment of the transition dipole moments).

The presence of HSA and \( \beta \text{LG} \) introduces remarkable changes in the spectroscopic features of TSPP. Both proteins possess positive global charge (\( p_{\text{HSA}} \approx 5.4 \) (Dockal et al., 1999) and \( p_{\beta \text{LG}} \approx 5.2 \) (Collini et al., 2000)) in acidic conditions. For instance, \( \beta \text{LG} \) has \( +20 \) charges at pH 2.0. So they may provide a positive microphase, distinct from the aqueous bulk, which induce TSPP aggregation in the presence of a submicromolar concentration of protein (a little higher for \( \beta \text{LG} \) whose positive charged residues may be less accessible to the porphyrin).

As more protein is added to the solution the balance between electrostatic and hydrophobic mutual interactions is such that J-aggregates are no longer stabilized and a complex TSPP-protein prevails. The Soret band of this complex shifts to the blue (for TSPP\(^2-\)) and to the red (for TSPP\(^4-\)) to a maximum around 420 nm in the presence of HSA, resembling that of TSPP in dimethylsulfoxide. This spectral shift is much less notorious in the presence of \( \beta \text{LG} \) (\( \leq 3 \) nm), which seems to point to a more hydrophobic binding region on the HSA matrix while keeping an almost aqueous environment in the \( \beta \text{LG} \) binding location. This band at 420 nm has generated some controversy in the literature regarding its origin and was first assigned to an H aggregate-band, based on Raman data (Akins et al., 1994). This was ruled out due to Coulombic repulsion between the positively charged sulfonate groups (Maiti et al., 1995) and to CD spectroscopic features, which seemed to point to a common origin to the signals at 420 nm and 490 nm (Ohno et al., 1993). These authors supported the idea that H-dimers may be formed as intermediates in the J-aggregate process and in a more recent study, involving the interaction of TSPP with CTAB, they have only detected a weak CD.
signal in the 490-nm region (Maiti et al., 1998). The latter findings are in agreement with our CD data reinforcing the idea that this maximum (at 420 nm) does not belong to the J-aggregate. Moreover, at pH 7 where no J-aggregates are detected, only a very weak CD signal is obtained exactly in the 420-nm region.

Time resolved fluorescence decays also indicate the existence of a common lifetime at the pH range studied ($\tau_c \approx 13$ ns), which appears in the presence of any of two proteins and whose population grows with the increase of the protein concentration, therefore being associated with the TSPP-protein complex.

The dissociating constants obtained from absorption and/or fluorescence are more than 10 times higher than those reported for hemin binding at pH 7 to HSA ($K_d \approx 2 \times 10^{-8}$ M) and to $\beta$LG ($K_d \approx 2.5 \times 10^{-7}$ M) (Dufour et al., 1990). The metal presence in hemin could promote specific interactions with a disulphide bridge of the protein justifying the differences found. However, the same group reported a $K_d \approx 4 \times 10^{-7}$ M for the interaction between $\beta$LG and protoporphyrin IX, which is also a free-base porphyrin. These dissociating constants reflect a balance of a series of possible interactions ligand protein. In our study the $K_d$ dependence on pH puts into evidence the existence of electrostatic interactions. Although studies indicate that for some ligands hydrophobic interactions may be stronger than the electrostatic forces in albums, the latter must be significant because uncharged hydrocarbon chains have low binding affinity to the protein (Gelamo and Tabak, 2000).

At this stage, it is clearly necessary to look at the complex from the protein side. As it has been thoroughly emphasized, Trp fluorescence is highly sensitive to the polarity of its environment (Lakowicz, 1983), usually presenting a red shift in a more polar surrounding. Regarding HSA-TSPP interaction at pH 2, a double exponential fitting showed nearly invariance of preexponentials and a decrease in each lifetime, which reflects different solvent exposure of Trp$^{214}$ location of Trp$^{214}$ residue was assumed to be in a hydrophobic and whose population grows with the increase of the protein complex.

The three-dimensional structure of HSA is well characterized by x-ray crystallography with a resolution of 2.8 Å (He and Carter, 1992) and more recently with a resolution of 2.5 Å (Diaz et al., 2001). There are two binding sites of high affinity for small heterocyclic or aromatic compounds like warfarin or aspirin (located on subdomains IIA and IIIA) and another two dominant sites for long-chain fatty acids (IB and IIIB) and two distinct metal-binding sites. The location of Trp$^{214}$ residue was assumed to be in a hydrophobic pocket of subdomain IIA, therefore its emission may be used to examine this domain. The other binding site in domain IIA lies within 10 Å distance from Trp$^{214}$ (He and Carter, 1992). Both binding sites (IIA and IIIA) are structurally characterized by the presence of a buried hydrophobic cavity capped by charged and polar residues. Parr and Pasternack (1977) found no evidence that positively charged porphyrins like TMpyP and CoTMpyP bind to HSA. Nevertheless, TSPP binding is favorable at both ionic states (di-anion and tetra-anion) and a hydrophobic environment must be involved because the spectral shift is significant. All data obtained converge to a probable location of TSPP within such binding subdomains (IIA and/or IIIA) where the hydrophobic cavity would justify the spectral shifts and less accessibility to acrylamide sensed in the presence of the porphyrin, but close to Trp$^{214}$ considering the effective energy transfer from that residue. Because
charged residues surround the binding site, conformational changes are expected upon pH increase and could account for the changes in the binding constants and on the CD spectra in both the far UV and visible region.

βLG appears to lack specificity for particular ligands and some studies suggest the existence of at least three independent binding sites (Sawyer et al., 1998). One is an internal site localized in the β-barrel of the protein, which can readily accommodate retinol and some fatty acids. The others are “surface” sites. From a recent review (Sawyer et al., 1998) it is possible to conclude that elongated ligands are favored in the barrel site.

The small changes found in TSPP spectra point to a preferential location on the “surface” sites. Moreover, recent studies suggest that there is a pH control of access to the interior of the calyx of βLG: the closed calyx characteristic of βLG at low pH, protects the ligand in the acidic stomach while the opened calyx, characteristic of βLG at high pH permits release of the ligand in the small intestine for absorption (Qin et al., 1998; Uhrínová et al., 2000). Because at pH 2 TSPP clearly senses the acidic environment, one is lead to assume that the porphyrin is not deep inside the calyx of the protein structure. On the other hand, the need of higher βLG concentration to achieve the same effect as that found with HSA could mean that TSPP has affinity for both binding sites populating also the more hydrophobic one. Thus, one would expect to find a spectral shift due to these interactions, which however do not occur.

The binding constants at increasing pH follow a sigmoidal trend with a “turning point” at pH 4.0. In a recent study (Collini et al., 2000), the pK value for the titration of the surface site was reported to be 4.0, whereas that for the internal site was 4.6, suggesting that Glu (glutamate) and Asp (aspartate) were the residues involved. At pH < pK, Glu and Asp residues are neutral and global positive charge prevail. On the other hand, above pK, these residues become negative, and so the global charge on the protein turns negative. Several of these residues lie next to both the surface site and the β-barrel entrance. So, once again the most probable location for TSPP on βLG seems to be the surface site.

By contrast with HSA, βLG possess two tryptophan residues, Trp19 in an apolar environment lying only 3.0 to 4.0 Å from a guanidine group of Arg124 and Trp61 near the molecular surface but close to a disulfide bridge. Because both Arg and the disulfide bridge can be efficient quenchers of Trp fluorescence, some discrepancy has been found in literature as to which residue the quenching is. Andrade and Costa (2000) and Collins and colleagues (2000) have concluded that elongated ligands are favored in the barrel site.

Fluorescence quenching of Trp also confirms such “binding” locations and clearly puts into evidence a mechanism of energy transfer from Trp residue(s) to TSPP at pH 2.

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