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Interaction of Human Serum Albumin with Novel 3,9-Disubstituted Perylenes

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Abstract

Human serum albumin (HSA) has been used as a model for the binding of a number of different ligands, including polyaromatic hydrocarbons, to proteins. In this case we have investigated the interaction of HSA with a novel set of perylene derivatives. Di-substituted perylene analogues have been synthesized as potentially useful organic photovoltaic materials. Their photophysical properties may make them viable for fuel cell applications too. However, these molecules are poorly soluble especially in aqueous solvents. Binding to water-soluble proteins may provide a way to solubilize them. At the same time one can study whether the photophysical processes initiated by the irradiation of a perylene ligand can cause conformational changes to the host protein. With the present study we demonstrated that of the three perylene derivatives investigated only one, the dimethoxy analogue, has a significant affinity for HSA at a binding site near the bottom of the central cleft (in proximity of the Trp214 residue). The small affinity prevents any significant photoinduced changes to occur in the protein.

Keywords

Perylene fluorescence; Protein binding; Albumin; Docking simulations

1 Introduction

Perylene and its analogues are an important class of polyaromatic hydrocarbons (PAH) investigated for diverse applications that include organic solar cells [45], fluorescence labeling [24], molecular sensors [13], and molecular electronics [15]. Perylene analogues, such as diimides, have been extensively studied because of the electron accepting properties of the π -system [4, 5, 49]. The π - π^* transition in perylene and its substituted analogues, in

fact, has been used as a model for many photophysical studies [19, 23]. The Negrete's group has recently reported the synthesis of novel 3,9-disubstituted perylenes [37] and their photophysical characterization [16]. One of the possibilities offered by these molecules is the substitution with electron accepting or electron donating groups that could modulate the photophysical properties of the compounds and in particular the photoinduced electron transfer (PET) mechanism. The potential to prompt PET would provide photoactive ligands capable of modifying the local environment at the dye binding sites. The ability to control PET would be beneficial as a trigger of localized conformational changes in polypeptides and could be used to modulate the structure and function of otherwise non-photosensitive proteins. Our focus on PET stems from the fact that electron transfer (whether photoinduced or not) is a fundamental event for living systems. It is the pathway adopted by plants and photosynthetic bacteria to convert solar energy into chemically stored energy, but it also regulates many other activities that depend on redox reactions (e.g. apoptosis, anti-oxidation, etc.) in both lower and higher organisms. In addition, this capability would be paramount since we have demonstrated that photophysical events initiated by docked photoactive ligands can be used to prompt conformational and functional changes in proteins [14]. Thus, besides mimicking native mechanisms, PET could be used to prompt charge-induced conformational changes as a way to produce artificial properties of otherwise non-photosensitive proteins. The binding of perylenes to proteins could also be exploited as a vehicle to solubilize these molecules in devices designed to promote solar energy conversion in aqueous environment.

We have investigated the interaction of human serum albumin (HSA) with three novel 3,9-disubstituted perylenes: (3,9-dimethoxyperylene (DMOP), 3,9-bis(1-octyloxy)-perylene (DOOP) and 3,9-bis(1-octanoyloxy)-perylene (PDO) [16]) that have been synthesized as potential organic photovoltaic materials. Compared to previous models of photoactive dyes/protein complexes, perylenes offer three desirable photophysical and structural properties: (1) the possibility to synthesize a large array of analogues with different photophysical properties is relatively straightforward [37], (2) some analogues have efficient PET quantum yields [15, 30, 50] and (3) their structure can be quite compact and could adapt well to the docking of proteins and peptides of different size.

Despite the body of work on perylenes and its analogues, very little is known about the interaction of these dyes with proteins. Because of the variety of docking sites [38], HSA is often used as a model to study the binding of hydrophobic ligands (including PAH) and offer the potential for applications that extend beyond the purely biological or biomedical fields [27]. HSA is comprised of 585 amino acids and has a molecular mass of 66 kDa (Fig. 1). A very desirable feature of HSA is its well-established structure [18] which reveals three homologous domains (I–III), each divided into two subdomains (A and B) that contain six and four α -helices, respectively [8, 17]. The main drug docking locations on HSA, known as warfarin- (or Sudlow's site I) and benzodiazepine- (or Sudlow's site II) binding sites [44], are placed in subdomains IIA and IIIA [8], respectively. However, a number of additional binding locations have been resolved including several sites for fatty acids [42] and even a location for the binding of heme [3, 52].

This manuscript reports the first attempt to study the interaction of these ligands with proteins. The investigation is focused on (1) the interaction of perylenes with HSA and (2) the effects (if any) that the irradiation of these perylene analogues produces on the structure of the protein. Absorption and fluorescence spectroscopy, as well as fluorescence lifetime and circular dichroism spectroscopy (CD) were used to quantitatively investigate the binding of perylenes to HSA and investigate whether the irradiation of the perylenes produces modifications of the structure of the protein.

2 Experimental Methods

2.1 Chemicals

Pyridine (anhydrous 99.8 %) and globulin-free Human serum albumin (HSA), were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. DMOP, DOOP and PDO were synthesized by tandem Friedel–Crafts annulation of the corresponding tetrahydronaphthol as reported previously [16, 37]. The ligands used for the competitive binding study, warfarin and ibuprofen sodium salt (98 %), were also purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification.

2.2 Sample Preparation

Except for the Circular Dichroism (CD) experiments all samples were studied in aqueous phosphate buffers prepared using deionized water. One tablet of phosphate buffered saline (Sigma-Aldrich) was diluted in 200 ml of water to produce a 10 mM buffer at pH ~ 7.5 (as directed by the manufacturer). Protein concentrations were determined spectroscopically from the absorption value. Concentration of perylene in each sample was calculated using the absorption spectrum of the original stock solution using the values of ϵ for perylenes in pyridine obtained previously ($\sim 30,000 \text{ M}^{-1} \text{ cm}^{-1}$) [16]. The measurements allowed us to establish the concentration of the perylene stock. The concentration in aqueous solution was then obtained from the dilution of this stock since the value of ϵ is not available in buffer and none of the perylenes used in this study show significant optical absorption under these conditions. The concentration of HSA was also determined spectroscopically using $\epsilon_{280} = 3.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ [21, 40]. A stock solution of HSA was prepared by dissolving the protein directly in buffer. The preparation of solutions containing perylenes was different depending on the type of experiment (as described below).

2.2.1 Perylene-Fluorescence—Interaction between perylenes and HSA was investigated by recording the fluorescence of the dyes as a function of the concentration of HSA. Since DMOP, DOOP and PDO are hydrophobic, an aliquot of the solid perylene was first dissolved in a small volume of pyridine where all the compounds are readily soluble. This yielded stock solutions in the range of $\sim 1\text{--}18 \mu\text{M}$. A constant volume ($70 \mu\text{l}$) of the stock solution of perylene in pyridine was placed in a vial that was left open under a fumehood in the dark until the pyridine had completely evaporated. Once evaporation was complete, 1 ml of buffer was added to the vial and the mixture was sonicated for 30 min. This procedure enabled the partial solubilization of the perylenes (as monomer, dimer, or oligomer) and eliminated any contribution of pyridine to the absorption or emission spectra. The sample was then placed in a ($1 \times 1 \text{ cm}$) quartz cell for the emission experiments. Aliquots of $100 \mu\text{l}$ of HSA stock solution ($\sim 30 \mu\text{M}$) were consecutively added to the sample and the emission spectra of the perylene were recorded after each addition. The samples were excited at 422 nm and spectra recorded in the 435–600 nm range. Since the absorption spectra of the perylenes in the samples were almost negligible, they could not be used for the normalization of the recorded fluorescence spectra.

2.2.2 HSA-Fluorescence Quenching—Perylene/HSA interaction was also investigated by recording the quenching of the intrinsic fluorescence of the protein produced by the addition of perylene to the solution. Increasing volume (from 20 to $500 \mu\text{l}$) of a stock solution of DOOP, DMOP or PDO in pyridine was placed in 10 vials and left to evaporate overnight in the dark under a fumehood. Concentrations of DMOP, DOOP or PDO in each sample were calculated using the absorption spectra of the original stock solution as described earlier. After complete evaporation, a constant volume of (2 ml) of a stock solution of HSA in buffer was added to the dried perylene vial. The vials were then placed on a shaker for 2 h to facilitate the release of perylene in solution and favor the binding to

the protein. After the procedure, each sample contained different amounts of perylene and the same concentration of HSA. The samples were then transferred to a (1 × 1 cm) quartz cell. The fluorescence spectra of HSA were recorded, with excitation at 295 nm, in the 300–550 nm range. The absorption at 280 nm of the same solution was also recorded and used to determine the concentration of HSA.

The samples prepared for CD experiments were the same as described for the perylene fluorescence experiments, however, due to the sensitivity of the CD in the UV range, the samples were diluted to reduce the HSA concentration. Depending on the HSA initial concentration, the samples were diluted until the final HSA concentration was ~5 μM. For these samples absorption spectra in the 1 mm cuvette were recorded until 190 nm (lower limit of the UV–Vis spectrometer) in order to detect large far-UV absorption which would prevent the detection of CD signal due to saturation of the detectors. All samples for these experiments were kept at an optical density <1 at 220 nm.

2.2.3 Competitive Binding Study—The samples with warfarin and ibuprofen were prepared in buffer solution as mentioned above. The concentration of warfarin ($\epsilon_{308} = 1.2 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$) and ibuprofen ($\epsilon_{222} = 9.9 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$) were determined using absorption spectrometry. A solution containing HSA and warfarin (or ibuprofen) was prepared at a molar ratio of 2:1 (ligand:HSA) in order to maintain an excess of competitive ligand. For quenching experiments this solution was then adjusted to yield an absorption of the protein of ~0.1 at 295 nm to which increasing aliquots of perylene was added (as described in Sect. 2.2.2). For perylene fluorescence, the solution containing HSA and warfarin or ibuprofen was added to the one containing aqueous perylene (as described in Sect. 2.2.1). The control experiment was carried out using the same protocol but no competitive ligand (i.e., in the absence of warfarin/ibuprofen).

2.3 Instrumentation

2.3.1 Absorption Spectroscopy—Absorption spectra were recorded with a dual beam Evolution 300 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Spectra were recorded with 2 nm resolution and 120 nm/min speed. HSA spectra were recorded between 250 and 350 nm, while spectra of the perylenes were recorded between 250 and 550 nm. Appropriate baseline and reference cells were used for each scan. The samples prepared for CD experiments were recorded from 190 to 450 nm in 1 mm path length quartz cuvettes in order to include the optical density in the region of the amide absorption [25].

2.3.2 Fluorescence Spectroscopy—Steady state fluorescence spectra were recorded using a double-monochromator AB2 fluorimeter (ThermoElectron, Waltham, MA) with a 4 nm bandpass in excitation and emission, and acquisition speed at 2 nm/s. The high voltage to the photomultiplier was set at 650 V and was kept constant during the experiment. The excitation wavelength for HSA was set at 295 nm in order to excite the lone tryptophan residue (Trp214) while the excitation wavelength for the perylenes was 422 nm which excites the middle vibronic band of these compounds [16]. Fluorescence spectra were corrected for the instrumental response (file provided by manufacturer). Fluorescence intensities were calculated as the integral of the emission band and normalized for the absorbed energy by dividing the value of the integrated emission peak for the value of the optical density at the excitation wavelength [29, 31].

2.3.3 Fluorescence Lifetime—Fluorescence decay lifetimes were measured using a time-correlated single photon counting instrument (Fluorocube, Horiba JobinYvon, Edison, NJ) with a 293 nm pulsed LED (NanoLED-293, Horiba JobinYvon, Edison NJ) light source

having 1 ns pulse width and 1 MHz repetition rate. Decays were recorded at the emission maximum of HSA with a 32 nm bandwidth.

2.3.4 Irradiation—Perylene-HSA complexes were irradiated at 405 nm, using a 185 mW CW Model LDCU8/7840 laser (Power Technology Inc. Alexander, AR). The beam was attenuated with a series of neutral density filters and diffused in order to achieve an irradiance of $\sim 15 \text{ mW/cm}^2$ at the sample. Exposure was for 60 min with a total radiant exposure of $\sim 56 \text{ Joules/cm}^2$.

2.3.5 Circular Dichroism—CD was carried out using a DSM 14 Spectropolarimeter (Olis Inc., Bogart, GA). The signal of HSA was recorded in the far-UV region between 190 nm and 240 nm. This is the region of the $\pi \rightarrow \pi^*$ transition band of the amide (peptide bond) and is known to produce a CD signal that is sensitive to the secondary structure of the polypeptide [10]. All spectra were recorded using a 1 mm path length quartz cuvette and constant integration time (2 s). The final spectra were averaged over three consecutive scans. The value of the voltage at the two detectors was also recorded in order to determine whether they saturated during the scan.

2.4 Data Analysis

2.4.1 Molecular Docking—Docking of the perylenes to HSA was performed with AutoDock 4.0 using the Lamarckian genetic algorithm [34]. The structures of DMOP, DOOP and PDO were created as pdb files using Spartan 5.0 (Wavefunction Inc., Irvine, CA). In the same software suite we also carried out an initial structure optimization of the molecules using the semi-empirical MNDO method. The crystal structure of HSA bound to heme (PDB code 1O9X) was used as the starting model. The heme was removed and the structure of the HSA was saved as a separate file. For the docking, three grid maps were chosen which included: domain I and subdomain IIA (Grid 1), domain II and subdomain IIIA (Grid 2) and domain III of HSA (Grid 3) respectively. These grids were chosen in order to successively scan the entire protein since the max grid size in Autodock would not allow for the scanning of the entire molecule at once. Grid maps were calculated using a $126 \times 126 \times 126$ point grid size with $0.375 \text{ \AA}$ grid spacing. HSA was held rigid while all the torsional bonds of the ligand were left free to rotate during the docking calculations. Polar hydrogens for HSA were then added using AutoDock Tools, and Kollman united atom partial charges were assigned [22]. In docking calculations, the preferred conformation depends on selection of the appropriate algorithm and scoring function (based on their energies). Moreover, the protein is usually set to be rigid, and there is no consideration of the effect of solvent molecules on docking. In our calculations, 15 docking runs were carried out, each retrieving up to 10 docked structures. Based on grid scores and root mean squares cluster tolerance ($2.0 \text{ \AA}$ used in this work) between structures [36], these complexes were sorted into clusters, i.e., separate clusters with different HSA binding modes. The docking showed that a site located at the bottom of the central cleft, as illustrated in Fig. 1 was the most favorable for binding the perylene analogues investigated. In some cases, i.e., the HSA–PDO docking calculation in Grid 3, no multimember clusters were found, thus the structure with the lowest binding energy ($-5.05 \text{ kcal mol}^{-1}$) was chosen as the preferred binding mode.

2.4.2 Stern–Volmer (S–V) Analysis—We assume that quenching of HSA fluorescence by perylenes is due to the binding between the two molecules. Collisional quenching, in fact, would likely occur on time scale longer than the average lifetime of the Trp214 excited state due to the relatively small diffusion coefficient of the perylenes and the relatively buried location of the Trp residue (Fig. 1). The fluorescence lifetime experiments (see below) support this assumption. Thus, we assume (see the Sect. 4) that static quenching [29] is

responsible for the decrease of protein fluorescence upon addition of the perylenes. S–V analysis for static quenching predicts that the ratio (F_o/F) between the fluorescence of HSA in the absence of perylenes (F_o) and the fluorescence of HSA in the presence of perylene (F) is, for small amounts of quencher, a linear function of the concentration of the ligand [29].

$$\frac{F_o}{F} = 1 + K_s [Q] \quad (1)$$

where in this case [Q] represents the concentration of the perylene ligand in solution. The slope obtained from the linear regression provides the static quenching constant, which is one of the relevant binding parameters.

2.4.3 Benesi–Hildebrand (B–H) Analysis—The assumption used to include the B–H approach is that when a perylene molecule forms a non-covalent complex with the protein, $[P] + [HSA] \rightleftharpoons [P \cdots HSA]$ its fluorescence is enhanced compared to the intensity in buffer. If one holds the concentration of the perylene ligand constant while increasing the concentration of HSA, the following equations can be assumed for a 1:1 stoichiometry:

$$K_a = \frac{[HSA * P]}{[P][HSA]} \quad (2a)$$

$$[P]_o = [HSA * P] + [P] \quad (2b)$$

$$[HSA * P] = \frac{[P]_o K_a [HSA]}{1 + K_a [HSA]} \quad (2c)$$

Since the complex is formed in an inert solution, i.e. buffer, the value in the left-hand side of Eq. 2c can be calculated using fluorescence changes from the emission of the perylene analogues [28] as shown by

$$[HSA * P] = \Delta F = F - F_o = \frac{F_o K_a [HSA]}{1 + K_a [HSA]} \quad (3)$$

where F is the fluorescence intensity of perylene in the presence of HSA, and F_o is the fluorescence intensity of perylene in the absence of HSA. A reciprocal plot in the form

$$\frac{1}{\Delta F} = \frac{1}{K_a [HSA] F_o} + \frac{1}{F_o} \quad (4)$$

yields a linear relationship from which the values of K_a can be determined through linear regression of the data.

2.4.4 Fluorescence Lifetime—The fluorescence decay lifetime of the protein was analyzed using the deconvolution software DAS6 (Horiba Scientific). The software uses an iterative re-convolution of the source time profile of the instrument, $G(t)$ or prompt, with the theoretical fluorescence decay which is modeled as a sum of exponentials.

$$F(t) = \sum_i A_i e^{-t/\tau_i} \quad (5)$$

where A_i is the amplitude and τ_i is the lifetime of the i th component. $G(t)$ is recorded from a scattering sample that does not fluoresce (in this case a 1 mg/ml suspension of glycogen in DI water). Both $F(t)$ and $G(t)$ are recorded at a sampling rate below 2.5 % of the maximum repetition rate of the pulsed source in order to avoid non-linear effects in the acquisition [21]. The convolution of $F(t)$ and $G(t)$ is fitted, using the least-squares method, to the experimental decay curve, $I(t)$, by iterative change of the amplitude and lifetime parameters. The quality of the fit is determined by ensuring that the χ^2 statistical parameter is between 1 and 1.5 and the Durbin-Watson parameter is between 1.8 and 2.0 [34, 40]. From the components of Eq. 2 the average lifetime can then be expressed as:

$$\tau = \sum_i A_i \tau_i \quad (6)$$

3 Results

3.1 Molecular Docking

According to our simulations the lowest energy binding configurations for all three compounds occurs at a site located at the bottom of the central cleft of HSA between subdomains IIA and IIIA (Fig. 1). This site does not overlap with any established site, although it is located between Sudlow's sites I and II [7, 39]. The salient feature of this binding site, which we named site-T, is the presence of two aromatic residues (Phe211 and Trp214) whose rings, as given by 1O9X.pdb, are nearly parallel to each other and located ~8 Å apart (Fig. 2).

The most energetically favourable configuration for DMOP is shown in Fig. 2, whereas the configurations of docked DOOP and PDO are shown in the Supporting Information (Figure S1A and SA2). The binding energies are very different among the ligands (Table 1). DMOP appears to have the lowest binding energy of all the ligands (−7.3 kcal/mol). This is more than 4 kcal/mol lower than the binding energy for DOOP at the same site. PDO shows a binding energy which is intermediate between the other two (Table 1). The docked conformation of DMOP and PDO at this site places the perylene ring stacked between the aromatic rings of Phe211 and Trp214 (Fig. 2 and S1B). The conformation for DOOP instead shows staggering of the perylene ring with respect to the two residues and shows that one of the two octyloxyl substituent is interposed between the perylene ring and the Phe211 residue. This could explain the less favorable binding energy (Table 1) of DOOP compared to the other two ligands at this site.

3.2 Quenching of HSA by Perylene

Addition of the perylenes to solutions containing HSA produces quenching of the protein intrinsic fluorescence (Supporting Information, Figure S2A through C). DOOP and PDO produce a modest quenching of protein fluorescence, whereas DMOP causes the largest quenching of Trp214 among the perylenes investigated. This effect is best summarized by the S–V plots (Fig. 3) from which a linear regression yields the quenching constants (K_s) summarized in Table 2. The emission spectra (Figure 2S) show an overall decrease of emission intensity without a change in the position of the emission maximum which indicates that the effect of the ligand is not accompanied by a significant conformational change of the protein in the region of Trp214 or the significant residual contribution of Tyr emission which would result in the blue-shift of the fluorescence maximum [47].

3.3 Fluorescence of Perylene Bound to HSA

As is the case for other poorly soluble aromatic compounds [46, 47] the binding of perylene to HSA in aqueous solution produces an increase of perylene fluorescence intensity (Fig. 4). In the case of DMOP the emission spectrum resembles the one expected for the binding of the monomeric dye. There are three vibronic emission bands centered at 460, 487 and 529 nm which are comparable to the ones recorded for the same chromophore in more apolar solvents such as CH_2Cl_2 and CHCl_3 . The monomerization is particularly evident from the inversion in the intensity between the vibronic peaks at 457, 487 and 530 nm (Fig. 4a) whereby the spectrum in the presence of HSA shows the typical feature of the DMOP monomer in organic solvents. On the other hand the emission spectra of DOOP do not show monomerization in the presence of HSA. The emission spectrum remains broad, nearly featureless and small to the point that the main raman band of water (~ 517 nm [35]) remains visible throughout the experiment. Broad emission spectra have been associated in the past with the presence of aggregates [32] or with spectra of water soluble perylenes in polar solvents [51]. PDO yields an emission spectrum that has the vibronic features expected for a monodispersed perylene, however the addition of HSA only produces a very small increase of emission. In a similar range of HSA concentration, DMOP shows the largest fluorescence increase ($\sim 89\%$) while DOOP and PDO show smaller increases in intensity after the addition of HSA (~ 66 and 22%) respectively. Despite the small changes the data still produced linear B–H plots (Fig. 5). Even though from the B–H plot PDO and DOOP produce a larger slope, they also yield a negative intercept extrapolated at $\frac{1}{[\text{HSA}]}=0$ which in turn yields values of $K_d < 0$ (Table 2).

3.4 Binding Competition

As mentioned above, HSA provides well-characterized binding sites to various ligands (fatty acids, heme, etc.) at different locations. In order to better identify whether the sites where perylenes bind are the same (or overlap) as those already established for other ligands we carried out competition binding studies with two well-established HSA ligands: warfarin and ibuprofen. Warfarin binds to subdomain IIA [39] whereas ibuprofen possesses both a high and a low-affinity binding site in subdomain IIIA and IIB respectively [11]. Since the results discussed in the previous section strongly suggest that only DMOP shows significant monomeric affinity for HSA (whereas binding of DOOP and PDO may be in oligomeric form) we investigated the competitive binding of only this perylene analogue with warfarin and ibuprofen. The B–H plots (Fig. 6) and the related binding constant (Table 2) show that warfarin has no effect on the binding of DMOP. On the other hand ibuprofen seem to help the binding of the perylene to albumin as shown by the increased linearity of the plot as well as the change in K_a .

The S–V plots (Fig. 3a) and the related quenching constant (Table 2) show that warfarin lowers ($\sim 40\%$) the quenching efficiency of Trp214 by DMOP and that ibuprofen increases it by $\sim 13\%$ in agreement with the effects seen in the B–H plot.

3.5 Fluorescence Lifetime Decay

The information given by fluorescence spectroscopy can be enhanced by the inclusion of fluorescence lifetime decay. An increase or decrease in fluorescence lifetime can again indicate local environmental factors surrounding the Trp214 residue [48]. The emission decay of HSA fluorescence is shown in Figure S3 and the results of lifetime decay fitting are summarized in Table 3. The intrinsic Trp214 fluorescence decay required a multi-exponential model with three components (Eq. 2): one component that has sub-nanosecond to approximately 1.5 ns lifetime, a second component with ~ 3 –4 ns decay and a longer component with lifetime > 7 ns. These are within the typical range observed for HSA [1].

The three individual components do not substantially change with the type of perylene added and the average lifetime (Eq. 3) remains virtually constant.

3.6 Circular Dichroism Spectroscopy

CD was carried out with the goal of investigating whether the binding (and the subsequent irradiation) of the perylenes produced an effect on the secondary conformation of the protein in solution. As it can be seen from the spectra in Fig. 7, the presence of the perylene ligands does not substantially affect the secondary conformation of the protein in solution. The CD spectra are the ones expected from a mostly helical protein such as HSA, with the double trough at 222 and 208 nm [17]. Spectra in the region of the aromatic absorption (250–300 nm) were recorded but due to the concentration of our samples the CD signal was nearly undetectable and could not be used to retrieve information on the tertiary structure (as far as it affects the aromatic amino acids) of the protein upon addition of the perylenes. The same low concentration prevented us from detecting possible dichroic effects induced by docking on the perylene molecules. As expected for planar symmetric molecules, spectra of the monodispersed perylene in pyridine between 400 and 500 nm did not show any dichroic activity of the docked ligands.

3.7 Irradiation

As mentioned earlier one of the motivations for studying the interaction between this new class of perylenes and HSA is the potential of these compounds to be photoactive ligands, capable of prompting conformational changes in proteins upon laser irradiation. As fluorescence spectroscopy (Figure S2), fluorescence decay (Supporting Information, Figure S3A through C) and CD spectroscopy (Fig. 7) show, however, the three perylene derivatives studied so far are not viable choices as mediator of photoinduced conformational changes. Irradiation of the complex formed by HSA with DMOP and DOOP produces a small (~5 %) decrease in the emission intensity of Trp214, whereas irradiation of PDO does not produce any significant effect (Figure S2 in Supporting Information). The insignificant changes in steady state are confirmed by fluorescence decay measurements. As seen in Figure S3 and Table 3, the fluorescence decay of Trp, which is often sensitive to changes in tertiary structure [12, 43, 48], is not affected by the irradiation of any of the three perylene ligands and the average lifetime of the protein remains virtually constant before and after irradiation.

In agreement with the lifetime measurements, irradiation (up to 56 J/cm²) of the complex of HSA with DOOP and DMOP does not show any change in the dichroic spectrum of the protein (Fig. 7a, c). Conversely, irradiation of docked PDO appears to produce a small effect on the CD spectrum which is within the instrumental error for the detection of secondary structural changes (Fig. 7b).

4 Discussion

All spectroscopic data indicate that of the three perylene analogues, only DMOP shows significant monomeric binding to HSA. The increase of fluorescence of DMOP with increasing HSA concentration is an indication that the molecule, which is insoluble and does not fluoresce efficiently in aqueous solution (Fig. 4), bind to HSA. The linearity of the B–H plots obtained from DMOP fluorescence as a function of protein concentration (Fig. 5) not only indicates binding but it suggests that the association has a 1:1 stoichiometry [6]. The small value of K_a (Table 2) retrieved for DMOP indicates that the association between this ligand and HSA is not an efficient process. Conversely, in the case of DOOP and PDO spectroscopic signs of binding are negligible (Fig. 4a, c); in the case of DOOP the fluorescence spectra remain very small and broad even upon addition of an excess of protein (Fig. 4c). The small affinity between protein and these perylene analogues could be

explained by the presence of aggregates of the dyes that do not bind the protein or, if they do, remain aggregated and do not change their contribution to the absorption or the emission spectra. The linearity of the B–H plots even for these two compounds may point to either an inadequacy of the B–H method when the affinity is very low, or the fact that a very small fraction of monomeric DOOP and PDO bind (as indicated by the fluorescence quenching data of Fig. 3 and Figure S2) but remains nearly undetectable through the fluorescence of the ligands. Low affinity and binding of aggregated ligands would introduce uncertainty on the values of the y-axis in Fig. 5 sufficient to produce a negative intercept [26]. Nevertheless, even in the case of DOOP and PDO, the association between the protein and the perylenes is confirmed by quenching of the only Trp residue in HSA.

As Figure S2 shows all perylene analogues quench HSA intrinsic fluorescence. Since fluorescence was recorded upon excitation at 295 nm one can assume that the only excited chromophore in HSA is Trp214. This residue is located near the bottom of the central cleft of the protein (Fig. 1) and, although accessible, it is unlikely that is collisionally quenched by PAH such as perylenes. Unsubstituted perylene has been shown to have a diffusion coefficient $<1 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ [20]. If one assumes that bimolecular quenching constant, k_q , can be calculated as [9]

$$k_q = 4\pi\nu R_{FQ} D_{FQ} \quad (7)$$

where ν is the number of molecules in solution, R_{FQ} is the combined radius of HSA [2] and the perylene (quencher), and D_{FQ} is the combined diffusion coefficient of the protein and the perylene ($D_{\text{perylene}} = 1 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$, $D_{\text{HSA}} = 1 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ [33]), then $k_q = 2.2 \times 10^3$ for the perylene/HSA system. This value is several orders of magnitude smaller than a typical value for diffusional quenching [29], thus ruling out quenching generated by collisions between HSA and perylenes. Therefore, we interpret the quenching as static in nature [29] and due to the binding of the perylene analogues to the protein. The data of Fig. 3 and Table 2 show that DMOP despite having smaller substituents, is a more efficient quencher of the fluorescence of HSA than DOOP or PDO. The first indication of this evidence is that the smaller methoxy substituent allows DMOP to stack efficiently between the Trp214 and the Phe211, thus positioning at a location that is very close ($\sim 4.5 \text{ \AA}$) to the indole ring which prompts quenching through proximity between π electrons in the two rings [41]. The other conclusion that the quenching data appear to suggest is that docking of DOOP and PDO does not occur with the configuration suggested by the simulations of Figures S1A and S1B. In fact the simulated configurations also place these analogues stacked in proximity of Trp214 which would lead to an equally efficient quenching. The docking simulations (Figure S1) of DOOP and PDO, places these molecules stacked between Trp214 and Phe211. DOOP is located $5.1 \text{ \AA}$ away from Trp214, whereas PDO is $4.9 \text{ \AA}$ from the indole ring. The fact that the quenching constant is instead smaller, suggest that DOOP and PDO do not bind as monomers or that the number of monomers is very small thus producing an underestimated quenching constant due to the overestimation of PDO and DOOP monomeric concentration in the S–V plots. All the data shown here suggest that DMOP binds much more efficiently to HSA, and stacks in proximity of Trp214 whereas DOOP and DMOP do not bind as monomers or, if they do, the number of bound monomers is very small.

The competition experiments with warfarin and ibuprofen validate the interpretation of the location of the binding of DMOP. In fact docking of the perylene is not prevented by the presence of an excess of either of the two drugs. The binding affinity (Fig. 6 and Table 2) is not affected by the presence of warfarin and is even slightly increased by the presence of ibuprofen. This suggests that DMOP does not bind at either of the two Sudlow's sites and

the site we have identified by computational simulation is the likely location for the docking of this PAH ligand. The decrease of quenching efficiency in the presence of warfarin is not due to any real structural effects but is instead the result of the overlap of warfarin absorption and emission to the one of the protein.

The irradiation results indicate that none of the perylenes in this study prompts conformational changes of the protein upon laser irradiation in the visible. The lack of effect is likely due to the absence of PET or other excited state events capable of prompting conformational changes of the protein. Nevertheless, we cannot entirely rule out the possibility that conformational effects remain below the detection limit due to the small affinity of the dye for the protein. Small affinity between the photoactive ligand and the protein would translate into an exceedingly small molar ratio between the dye/protein complex and the dye-free protein. Because in the irradiation experiments we have measured the effects by probing spectral changes of the protein, the presence of a very small fraction of dye/protein complex would render any contribution from the irradiated complexes negligible compared to the one of the dye-free HSA, which would not be affected by irradiation.

In summary, we have demonstrated for the first time that 3,9-perylene derivatives dock to proteins such as HSA and that their affinity depends on their solubility and on the type of substituents at the 3,9-position. The analogue with the methoxy substituents appears to be much more efficient in docking the protein than the analogues containing dioctyloxy or dioctanoate residues, however in all cases the affinity of these dyes for a large globular protein such as HSA remains small. The dyes used in this study do not prompt conformational changes in proteins, however they open the opportunity to investigate other perylene analogues and study their effects on protein structure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HSA	Human serum albumin
PAH	Polyaromatic hydrocarbons
PET	Photoinduced electron transfer
DMOP	3,9-Dimethoxyperylene
DOOP	3,9-Bis(1-octyloxy)-perylene
PDO	3,9-Bis(1-octanoyloxy)-perylene
CD	Circular dichroism
S-V	Stern-Volmer
B-H	Benesi-Hildebrand

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Fig. 1. Secondary structure of human serum albumin (PDB code: 109X). Domains: *I* (Blue), *II* (green), *A* and *B* subdomains are depicted in *dark* and *light shades*, respectively. Domain *IIIA* (yellow), *IIIB* (red). Tryptophan (orange) is located at Site-T which is indicated approximately by the region in the *square*. Marked are also the sites for ibuprofen (red) and warfarin (purple) (Color figure online)

Fig. 2. Computational model of the location of the DMOP (*cpk color scheme*) binding sites on HSA and the close up view of the binding configurations. The conformation of Phe 211 and Trp214 are also shown. This configuration represents the lowest energy among the clusters at this site as determined by the Lamarckian algorithm adopted with Autodock 4.0

Fig. 3.

Stern–Volmer plots of the static quenching caused by DMOP on HSA fluorescence **a** S–V plots for HSA (*filled square*), HSA/ibuprofen complex (*filled triangle*) and HSA/warfarin complex (*filled circle*). **b** S–V plots produced by the addition of DOOP and PDO on free HSA. To a 5 μM solution of HSA at $\sim\text{pH}$ 7.4, increasing amounts of perylene were added. For each addition the absorption spectrum was recorded between 250 and 500 nm and the fluorescence spectrum of the protein was recorded in the 305–450 nm range upon excitation at 295 nm. The fluorescence intensity was calculated as the integral between 305 and 450 nm of the emission spectrum. The integral was then normalized by dividing its value for the optical density at 295 nm. The parameter so calculated yielded the values of F_0 and F represented in the pictures. In the case of HSA/warfarin or HSA/ibuprofen, the concentration of the competing ligand (ibuprofen or warfarin) was incubated for 1 h at a concentration of 10 μM (i.e., double the concentration of albumin)

Fig. 4. Fluorescence emission spectra of perylenes as a function of the concentration of HSA. **a** DMOP, **b** DOOP, **c** PDO. To μM solutions of each perylene, additional aliquots were added from a stock solution containing $30 \mu\text{M}$ of HSA. After each addition the spectra of the perylenes were recorded upon excitation at 422 nm

Fig. 5.

Benesi–Hildebrand plot with linear regression for the addition of HSA to aqueous solutions containing DMOP (*filled triangle*), DOOP (*filled square*) and PDO (*filled circle*). These plots were derived from the spectra shown and described in Fig. 4. The spectra were first corrected for the instrumental response. The fluorescence intensity was retrieved from the integral of the spectra in the 450–600 nm range normalized by dividing the value of the integral by the optical density of the perylenes at the excitation wavelength (422 nm). ΔF was then calculated as the difference between the emission intensity in the absence of HSA and the fluorescence intensity in the presence of a concentration $[HSA]$ of the protein

Fig. 6. Benesi-Hildebrand plot with linear regression obtained from the fluorescence of DMOP upon addition of: HSA (*filled square*), HSA/ibuprofen complex (*filled triangle*), HSA/warfarin complex (*filled circle*). The procedure to obtain the plot is identical to that explained in Fig. 5. In this case to the solution containing the perylene derivatives it was added a stock solution containing a 2:1 concentration ratio of ibuprofen (or warfarin) and albumin. The concentration of albumin in the stock was 30 μM

Fig. 7.

CD Spectra for HSA/perylene complexes. The plots show the overlay between the dichroic signal in the region of the amide absorption of the protein in the protein/perylene complex before irradiation of the ligand (*blue*) and after exposure to 56 W/cm^2 irradiation (*red*). It can be noted that irradiations does not change to the spectra of HSA. **a** HSA/DMOP complex, **b** HSA/DOOP complex, **c** HSA/PDO complex. Spectra were recorded from solutions containing a diluted amount of protein. The original solution containing $5 \mu\text{M}$ HSA and $\sim 5 \mu\text{M}$ perylenes was diluted as to obtain an optical density of ~ 0.9 at 220 nm in 1

mm quartz cells. This is the optimal optical density for CD measurements. This implied a ~10 times dilution of the original solution (Color figure online)

Table 1

Binding energies and binding constant from Stern–Volmer and Benesi–Hildebrand analysis of the Perylene diimides

	$(\Delta G)_T$ kcal/mol
DMOP	−7.3
DOOP	−3.2
PDO	−5.1

Table 2

Binding constant from Stern–Volmer and Benesi–Hildebrand analysis of the perylene analogues

	Stern–Volmer		Benesi–Hildebrand
	$K_s \times 10^{-3} (\mu\text{M}^{-1} \text{ cm}^{-1})$	Adj. R^2	$K_a \times 10^{-2} (\mu\text{M}^{-1} \text{ cm}^{-1})$
DMOP	13.9	0.98	0.06
+ Ibuprofen	16.7	0.96	1.19
+ Warfarin	7.2	0.93	0.07
DOOP	5.0	0.99	< 0
PDO	5.0	0.99	< 0

Lifetime values for Perylene bound HSA. τ_0 and τ_{60} give the weighted average of a 3 exponential fit at irradiation time 0 and 60 min, respectively

Table 3

	Fractional contribution (%)			Individual lifetimes (ns)			Average (ns)
	f_1	f_2	f_3	τ_1	τ_2	τ_3	
HSA	2	40	58	0.6	4.2	7.5	6
HSA-DMOP	2	27	72	0.2	3.5	7.1	6
	3	43	54	0.6	4.2	7.7	6
HSA-DOOP	3	57	40	0.7	4.8	8.3	6.1
	3	60	36	0.8	4.9	8.5	6
HSA-PDO	3	39	58	0.4	4	7.5	5.9
	3	34	63	0.2	3.7	7.3	5.9