Eur Biophys J (2012) 41:969–977
DOI 10.1007/s00249-012-0856-y

ORIGINAL PAPER

EBSA
Biophysics in Europe

Influence of stearic acids on resveratrol-HSA interaction

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Received: 26 July 2012 / Revised: 22 August 2012 / Accepted: 3 September 2012 / Published online: 18 September 2012

Abstract The interaction between the natural polyphenol resveratrol and human serum albumin (HSA), the most abundant transport protein in plasma, has been studied in the absence and in the presence of up to six molecules of stearic acids (SA) pre-complexed with the protein. The study has been carried out by using the intrinsic fluorescence of both HSA and resveratrol. Protein and polyphenol fluorescence data indicate that resveratrol binds to HSA with an association constant $k_a = (1.10 \pm 0.14) \times 10^5\ M^{-1}$ and $(1.09 \pm 0.02) \times 10^5\ M^{-1}$, respectively, whereas Job plot evidences the formation of an equimolar protein/drug complex. Low SA content associated with HSA does not affect significantly the structural conformation of the protein and its interaction with resveratrol, whereas high SA content induces conformational changes in the protein, and reduces resveratrol binding affinity. The photostability of resveratrol in the different samples changes in the order: buffer < (high [SA]/HSA) < HSA < (low [SA]/HSA). The results on (SA/HSA)-resveratrol samples highlight the ability of the protein to bind hydrophobic and amphiphilic ligands and to protect from degradation an important antioxidant molecule under biologically relevant conditions.

Keywords Human serum albumin · Resveratrol · Stearic acid · Fluorescence · Ligand binding

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Introduction

Resveratrol is a polyphenolic compound produced by some plants, such as grapes, peanuts and mulberries, to protect against infection or environmental stress. It is also found in food and beverages, particularly red wine (Langcake and Pryce 1976; Celotti et al. 1996). The biological activity of resveratrol is associated with health benefits that make it particularly interesting for applications in pharmaceutical and nutraceutical fields (Frémont 2000). Structurally, resveratrol is present in the form of two isomers, *trans*- and *cis*-resveratrol (Fig. 1a).

The *trans* isomer is the natural form and converts to the *cis* isomer as a consequence of either aging, or exposure to sunlight or to UV radiation (Camont et al. 2009). Most of the beneficial health properties of resveratrol are ascribed to the biologically active *trans* isomer. These include antioxidant and antiatherosclerotic effects (Orallo 2006), inhibition of blood platelet aggregation (Olas and Wachowicz 2005), cardioprotective activity (Bradamante et al. 2004), and chemoprevention action against cancer proliferation (Jang et al. 1997; Asensi et al. 2002). The activity and bioavailability of resveratrol, however, are limited by its low solubility in water (0.023 mg/ml) (Asensi et al. 2002; King et al. 2006). These limitations are overcome via binding to proteins or conjugation, so that the molecule can remain at high concentration in the circulatory system. Resveratrol was shown to have an affinity for various proteins such as breast cancer resistance protein and human cyclin-dependent kinase (Feng et al. 2006) and to interact with plasmatic lipoproteins (Belguendouz et al. 1998) and proteins such as hemoglobin (Lu et al. 2007) and albumins (Jannin et al. 2004; N'soukpoé-Kossi et al. 2006; Lu et al. 2007; Bourassa et al. 2010).

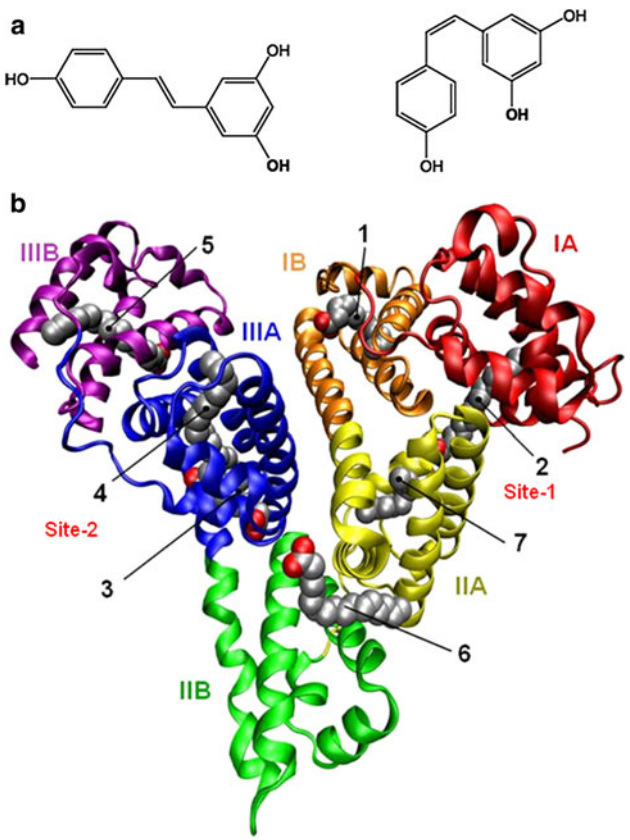

Fig. 1 **a** Molecular structures of (*left*) *trans*- and (*right*) *cis*-resveratrol. **b** Crystal structure of HSA (PDB entry 1E7I) (Bhattacharya et al. 2000) indicating the protein domains and subdomains, the location of the binding sites (1–7) for stearic acids and site-1 and site-2 for drugs

Human serum albumin (HSA) is the principal extracellular protein with a high concentration (40 mg/ml, ~0.6 mM) in blood plasma where it performs different functions (Peters 1997). It is a globular protein (585 residues, 66 kDa, 67 % α-helix) composed of three structurally similar domains (I, II, and III), each of them further divided into two subdomains (A and B), and stabilized by 17 disulphide bridges (Fig. 1b) (He and Carter 1992; Sugio et al. 1999). Combining diverse affinities and wide specificity, it binds, stores and transports a wide variety of exogenous and endogenous molecules, drugs and metals. Aromatic and heterocyclic drugs were found to bind to two hydrophobic binding sites, named site-1 and site-2 for drugs, located in the subdomains IIA and IIIA, respectively (Sudlow et al. 1975; Sudlow et al. 1976) (Fig. 1b). Moreover, HSA shows a strong affinity to bind reversibly and non-covalently fatty acids (Peters 1997; Curry et al. 1998), which are then transferred to membranes and cell compartments (Peters 1997; Hamilton 1998; Abreu et al. 2003; Estronca et al. 2005; Pantusa et al. 2005, 2010; Pantusa and Bartucci 2010). By this molecular mechanism, the fatty acid homeostasis is controlled and the supply to mammalian cells assured. For medium (C8:0–C14:0) and long-chain (C16:0–C18:0) non-esterified fatty acids, seven binding sites (1–7) have been identified (Curry et al. 1998; Bhattacharya et al. 2000; Simard et al. 2005). They are long and narrow hydrophobic pockets localized in subdomains IB, IIIA, IIIB, and on the subdomain interfaces (Fig. 1b). "In vivo", under normal physiological conditions, between 0.1 and 2 mol of fatty acid are invariably complexed per mole of protein. Moreover, a higher amount of fatty acids can bind to the protein in some pathological conditions characterized by an elevated lipid level in the blood. The presence of fatty acids associated with HSA can influence the binding with drugs and other ligands. Indeed, cooperative and competitive interactions between fatty acids and different classes of ligands have been observed in a number of HSA studies (Curry et al. 1998; Vorum and Honore 1996; Petitpas et al. 2001, 2003; Bojko et al. 2008).

In the present paper we have studied the interaction between resveratrol and HSA in the absence and in the presence of stearic acids (SA) pre-complexed with the protein. The concentration of SA was varied between 0 and 6 mol per mole of protein, to include both physiological and pathological conditions. The study has been carried out by using the intrinsic fluorescence of both HSA and resveratrol. The aim is to investigate the influence of SA on the binding affinity of the polyphenol to the protein, on the stoichiometry of (SA/HSA)-resveratrol samples and on resveratrol stability in the protein complexes upon UV irradiation.

Materials and methods

Materials

Essentially fatty acid-free and globulin-free human serum albumin (purity approximately 99 %), stearic acid (grade I, purity approximately 99 %) and resveratrol (3,4′,5′-trihydroxy-*trans*-stilbene) were from Sigma-Aldrich (St. Luis, MO, USA). The reagent grade salts for the 10 mM phosphate buffer solution (PBS) at pH 7.4 were from Merck (Darmstadt, Germany). All materials were used as purchased with no further purification. Bidistilled water was used throughout.

Sample preparation

All the samples are freshly prepared (SA/HSA)-resveratrol buffered complexes. HSA stock solution was made in PBS and the concentration determined by spectrophotometry using a molar extinction coefficient $\varepsilon_{280} = 35{,}219\ M^{-1}\ cm^{-1}$ (Pace et al. 1995). Resveratrol stock solution was prepared in

water by sonication and the concentration determined by using $\varepsilon_{304} = 31{,}335\ M^{-1}\ cm^{-1}$ (Camont et al. 2009). To prepare SA/HSA complexes, a volume of ethanol/chloroform containing SA was first evaporated under a flow of nitrogen gas and any residual solvent removed under vacuum; the fatty acid film was then hydrated with PBS by heating at 60 °C and periodically vortexing for 30 min. The SA solution was cooled to room temperature and, finally, mixed with albumin solution in buffer to obtain the desired SA/HSA molar ratio. From electron spin resonance measurements by using stearic acid spin-labelled at the 16th carbon atom along the acyl chain, there is no evidence of free, unbound fatty acids in solution. This confirms that the SA are bound to the protein.

To prepare (SA/HSA)-resveratrol complexes, an amount of protein solution (with or without SA) and resveratrol solution in PBS are mixed to obtain the needed molar ratios for the various experiments.

Fluorescence measurements

All fluorescence data were collected at RT on a LS 50B spectrofluorometer (Perkin-Elmer, Beaconsfield, UK). The excitation wavelength was chosen according to the type of experiment, whereas the slit widths of excitation and emission are 6 and 4 nm, respectively. The fluorescence measurements were repeated to test their reproducibility.

HSA fluorescence

The intrinsic protein fluorescence is used to study SA/HSA and (SA/HSA)-resveratrol samples.

To the intrinsic HSA fluorescence there is a contribution by the single tryptophan residue Trp214 located in the domain II, 18 tyrosine and 31 phenylalanine residues (which are of very low quantum yield). The maximum of absorption of Trp and Tyr in water occurs at 280 and 275 nm, respectively.

To study SA/HSA complexes, the fluorescence spectra were registered between 300 and 450 nm whereas two wavelengths of excitation are used, namely, $\lambda_{ex} = 295$ nm, where only Trp214 is involved, and $\lambda_{ex} = 280$ nm, where also Tyr residues are excited. In the SA/HSA samples, protein concentration was fixed to 3 μM and the stearic acid concentration was varied from 0 to 18 μM.

To study (SA/HSA)-resveratrol complexes, HSA concentration was fixed to 3 μM and the resveratrol concentration was varied from 0 to 10 μM. The protein fluorescence spectra were recorded in the range 280–450 nm and, to minimize resveratrol absorption, λ_{ex} was set at 265 nm. Nevertheless, as resveratrol shows a broad absorption band between 260 and 360 nm (data not shown), the spectra were corrected for inner-filter effect, i.e., an effect that refers to the absorption of light at the excitation and/or emission wavelength by a compound present in a solution. The following equation has been used for correction (van de Weert and Stella 2011):

$$F = F_{obs} \times 10^{\frac{A_{ex} \times d_{ex} + A_{em} \times d_{em}}{2}}, \quad (1)$$

where F_{obs} is the measured fluorescence, F the correct fluorescence intensity that would be measured in absence of inner-filter effect, $d_{ex} = 1$ cm and $d_{em} = 0.4$ cm the cuvette pathlength in the excitation and emission direction, respectively, and A_{ex} and A_{em} the measured change in absorbance value at the excitation and emission wavelength, respectively, caused by ligand addition.

Resveratrol fluorescence

When resveratrol fluorescence is used to study (SA/HSA)-resveratrol samples, the resveratrol concentration is 3 μM and the concentration of protein (containing various amount of stearic acid) varies between 0 and 33 μM. The samples were excited at 315 nm (i.e., a wavelength were HSA does not absorb light) and the emission registered between 320 and 550 nm.

Resveratrol fluorescence is also used to determine the binding stoichiometry of (SA/HSA)-resveratrol complexes by means of the continuous variations method (Ward 1985). In such an approach, the mole fraction of resveratrol and HSA were varied while keeping the total concentration ([resveratrol] + [HSA]) constant at 50 μM. To obtain Job plots, the normalized ΔF, i.e., the difference between resveratrol fluorescence intensity in the presence of albumin at different concentration with respect to resveratrol in buffer, is plotted versus resveratrol mole fraction, $X_{resveratrol}$. The maximum point of the curve provides the amount of resveratrol for the binding stoichiometry of the complex.

Results and discussion

To study the influence of stearic acids on the interaction between HSA and resveratrol, the fluorescence data on SA/HSA complexes will be presented first and then the results on the (SA/HSA)-resveratrol complexes will be given.

Stearic acid/HSA interaction

The intrinsic fluorescence spectra of SA/HSA samples in PBS at different molar ratio for $\lambda_{ex} = 295$ nm and $\lambda_{ex} = 280$ nm are shown in Fig. 2a and b, respectively.

For $\lambda_{ex} = 295$ nm, the fluorescence intensity is only due to Trp214 and it is first slightly enhanced at SA/HSA molar ratio ≤2, then it is progressively reduced up to the molar

ratio of 4 and a more marked attenuation is obtained when the molar ratio varies between 5 and 6 (see Fig. 2a).

Similar results are obtained for $\lambda_{ex} = 280$ nm (see Fig. 2b) at which both Trp214 and Tyr residues emit fluorescence. However, also at 280 nm, in spite of the relative large number of tyrosine residues, the protein fluorescence is dominated by Trp214 that shows a quantum yield higher than that at 295 nm. The low emission of tyrosines depends on the three-dimensional structure of HSA and is due to energy transfer to Trp214 and to quenching by nearby groups on the peptide chain (Lakowicz 1983). As it can be seen in Fig. 2b, a tyrosine-specific fluorescence shoulder at 308 nm (upshifted by about 5 nm relative to the position at 303 nm detected for the residue in water) is more clearly evident at 5 and 6 molcules of SA per molecule of protein.

From Fig. 2a and b, it is also evident that the wavelength of maximum emission, λ_{max}, of Trp214 for the protein alone is centred at 347 nm, i.e., close to the position of the maximum reported for free tryptophan in aqueous solution ($\lambda_{max} = 348$ nm) (Lakowicz 1983). When increasing amount of stearic acid is added to the protein, λ_{max} remains almost unchanged up to the molar ratio of 4 and then it is blue shifted by about 6 nm.

Fatty acids do not have elements that can quench protein fluorescence and they are not themselves fluorescent; therefore, the variations in HSA fluorescence are related to conformational changes that occur in the protein upon complexation with stearic acids. The spectral variations in Fig. 2 are more evident at high SA content (4–6 mole per mole of protein). The intensity of fluorescence remains constant at $\lambda_{ex} = 295$ nm whereas it decreases at $\lambda_{ex} = 280$ nm where the contribution due to Tyr residues becomes more evident. The energy transfer from tyrosines to Trp214 is less effective, pointing to an increase in the distance between Trp214 and the nearby Tyr residues. At the same time, the decrease in λ_{max} suggests that the solvent exposed fluorescent residue Trp214 is forced in a more hydrophobic protein environment in the presence of stearic acids. Taken together these results reflect a conformational change in HSA upon binding SA at high content. This effect could involve rotations of domains I and III relative to the central domain II, as it has been evidenced by X-ray crystallography on HSA complexed with fatty acids (Curry et al. 1998; Bhattacharya et al. 2000).

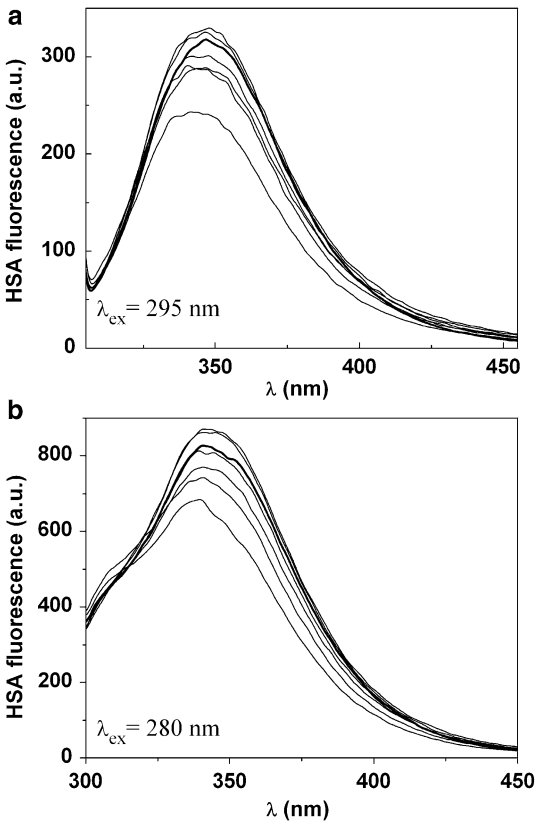

Fig. 2 **a** Fluorescence spectra of HSA at different SA/HSA molar ratios in PBS. *Bold line* refers to HSA alone in buffer and the other lines from *top* to *bottom* to SA/HSA molar ratio of 1, 2, 3, 4, 5, and 6. Protein concentration is 3 μM and $\lambda_{ex} = 295$ nm. **b** As in **a** but at $\lambda_{ex} = 280$ nm

(SA/HSA)-resveratrol complexes: HSA fluorescence

The fluorescence spectra for $\lambda_{ex} = 265$ nm of 3 μM HSA complexed with different concentration of resveratrol in PBS are shown in Fig. 3.

The fluorescence intensity decreases and λ_{max} is red shifted when resveratrol concentration is increased from 0 to 10 μM. At the maximum amount of resveratrol, the fluorescence intensity halves and λ_{max} is shifted of ca.

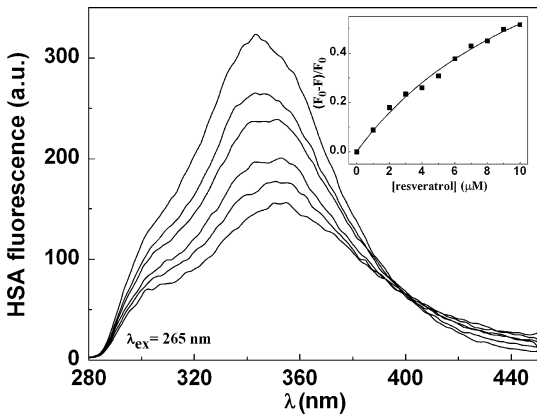

Fig. 3 Fluorescence spectra of HSA at $\lambda_{ex} = 265$ nm for HSA-resveratrol complexes in PBS. From *top* to *bottom*: 3 μM HSA with 0, 2, 4, 6, 8 and 10 μM resveratrol. *Inset*. $(F_0 - F)/F_0$ as a function of resveratrol concentration in HSA-resveratrol complexes in PBS. The data points are fitted according to Eq. 2

12 nm towards a longer wavelength. These results indicate that the polyphenolic molecule binds to the protein and makes the tryptophan environment more polar. Fluorescence quenching in HSA-resveratrol complexes can be mainly ascribed to protein conformational changes and to the energy transfer from albumin to resveratrol. It should be mentioned that the absorption spectrum of the polyphenol overlaps the emission spectrum of HSA (data not shown). The formation of a non fluorescent ground-state complex, instead, can be excluded because the absorption spectrum of HSA-resveratrol complex is simply the superposition of the spectra of the two components (data not shown). Evidences of conformational changes affecting the secondary structure of HSA upon resveratrol binding have been also shown by circular dicroism and infrared spectroscopy (N'soukpoé-Kossi et al. 2006; Lu et al. 2007).

The quenching in protein fluorescence is used to determine the association constant, K_a, of resveratrol to HSA in the absence and in the presence of SA. To this end, the fluorescence intensity data have been corrected for inner-filter effect as described in "Materials and methods" section by using Eq. 1, plotted as $(F_0 - F)/F_0$ versus resveratrol concentration and analyzed according to the following equation (van de Weert and Stella 2011):

$$\frac{F_0 - F}{F_0 - F_c} = \frac{[P]_t + [L]_a + K_d - \sqrt{([P]_t + [L]_a + K_d)^2 - 4[P]_t[L]_a}}{2[P]_t}, \quad (2)$$

where F is the measured fluorescence at different resveratrol concentration, F_0 the fluorescence intensity in the absence of resveratrol, F_c the fluorescence of the fully complexed protein, $K_d = 1/K_a$ the dissociation constant, $[P]_t$ and $[L]_a$ the analytical concentrations of HSA and of added resveratrol, respectively.

The use of Eq. 2 is appropriate for our experimental conditions, in which the maximum concentration of resveratrol is ca. 3×HSA concentration. More simplified versions of Eq. 2 present in literature (such as the Stern–Volmer equation and its modifications) may be obtained when the amount of added ligand is comparable to the amount of unbound ligand (a condition fulfilled when the ligand concentration is at least above a factor ×10 of the protein concentration) and the complexed protein is not fluorescent. In our case, the first condition cannot be realized because at high resveratrol concentration both the absorption and the fluorescence spectrum would be dominated by resveratrol bound to HSA and the intrinsic protein fluorescence would not be correctly appreciated. For the same reason, it should not be possible to verify whether HSA-resveratrol complex is fluorescent.

In the inset to Fig. 3 are reported the data for HSA-resveratrol complexes in PBS and the non-linear regression fit based on Eq. 2, whereas the values of the association constant, K_a, for the different (SA/HSA)-resveratrol samples are listed in Table 1.

For HSA-resveratrol complex, i.e., in the absence of stearic acids, K_a is $(1.09 \pm 0.02) \times 10^5\ M^{-1}$, which is close to literature values (N'soukpoé-Kossi et al. 2006; Lu et al. 2007; Jiang 2008).

From the binding constant values in Table 1, is evident that fatty acids modulate the binding affinity of the protein to resveratrol. K_a does not change significantly up to 3–4 molecules of stearic acid per HSA molecule, but it markedly decreases (i.e., the binding affinity reduces) at the molar ratio SA/HSA = 5 and 6. Reasonably, the reduced affinity of the protein towards the polyphenol is due to the conformational changes induced in HSA by high amounts of SA, as we found in the previous subsection "Stearic acid/HSA interaction" and Fig. 2. The result is also in keeping with a number of previous studies which have shown that fatty acids in HSA, beside inducing conformational alterations, can either increase or decrease the binding of other ligands. Palmitate at high concentration reduces the affinity of the protein to valproate (Brodersen et al. 1990). A concentration dependent influence of fatty acids on the binding of warfarin and aspirin to HSA has been detected (Vorum and Honore 1996; Petitpas et al. 2001; Bojko et al. 2008). It has been found that HSA retains a high-affinity site for thyroxine in the presence of an excess of fatty acids, and even creates an additional binding site for this hormone (Petitpas et al. 2003).

(SA/HSA)-resveratrol complexes: resveratrol fluorescence

The fluorescence spectra of resveratrol dissolved in PBS and in ethanol, and of resveratrol-HSA complexes in PBS at increasing amount of albumin are shown in Fig. 4.

Pure resveratrol in PBS has a maximum of fluorescence at $\lambda_{max} = 402$ nm and in ethanol shows an intensity of fluorescence sixfold higher and λ_{max} down shifted to 380 nm. The spectra at increasing HSA concentration in PBS show enhanced fluorescence intensity, are blue

Table 1 Association constant, K_a, of resveratrol to HSA complexed with stearic acids at different molar ratio in PBS at pH = 7.4

SA/HSA molar ratio	$K_a \times 10^5$ (M^{-1})
0	1.09 ± 0.02
1	1.00 ± 0.08
2	1.08 ± 0.07
3	1.54 ± 0.07
4	1.05 ± 0.05
5	0.330 ± 0.004
6	0.23 ± 0.01

Data derived from the non-linear fit according to Eq. 2

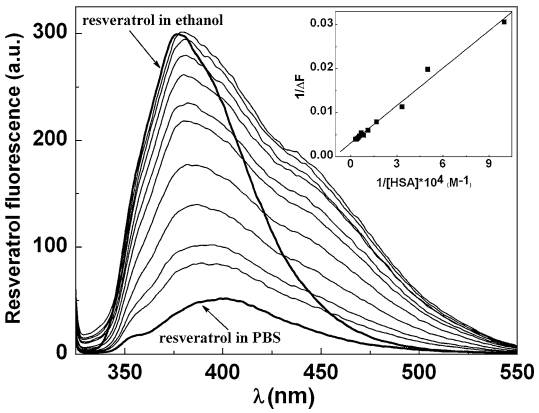

Fig. 4 Fluorescence spectra at $\lambda_{ex} = 315$ nm of 3 μM resveratrol in PBS (*lower bold line*), in ethanol (*upper bold line*), and, in the presence of 1, 2, 3, 6, 9, 15, 18, 21, 24 and 33 μM HSA in PBS (from *bottom* to *top*). *Inset*: Double reciprocal linear plot of 1/ΔF versus 1/[HSA]. The data points are fitted according to Eq. 3

shifted, and progressively, at the highest albumin content, their maximum intensity become comparable to that of resveratrol in ethanol. This suggests that resveratrol transfers from the hydrophilic environment of the aqueous solution to an hydrophobic protein environment with polarity similar to that of ethanol. A plausible explanation of this result is that the binding site for resveratrol in HSA is an hydrophobic pocket, most likely the site-1 for drug (see Fig. 1b) as previously proposed by using molecular docking (Lu et al. 2007). For comparison, the fluorescence intensity of resveratrol bound to β-lactoglobulin is lower compared to resveratrol in 75 % ethanol solution, suggesting that the polyphenol binds on the surface and not in the hydrophobic internal calyx of the protein (Liang et al. 2008). A further indication supporting site-1 as resveratrol binding site in HSA is from the results on HSA-resveratrol samples given in the previous paragraph (see, e.g., Fig. 3). The ref shift of λ_{max} is related to changes in the environment around Trp214, which, after the interaction with the polyphenol molecule, becomes more exposed to the solvent. These data might indicate the proximity of resveratrol to Trp214 which is close to site-1 for drugs.

The fluorescence enhancement of resveratrol upon incubation with increasing amount of HSA in Fig. 4 can also be used to calculate the binding constant, K_a, of the drug. This is done by fitting the 1/ΔF versus 1/[HSA] data with the following equation (Guharay et al. 2001; Liang et al. 2008):

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_{max}} + \frac{1}{K_a \times \Delta F_{max} \times [\mathrm{HSA}]}, \quad (3)$$

where $\Delta F = F_x - F_0$, F_x and F_0 representing the fluorescence intensity of resveratrol in the presence and absence of HSA, ΔF_{max} the maximal change of fluorescence intensity, and [HSA] the protein concentration.

Table 2 F_{shift} and λ_{shift} in (SA/HSA)-resveratrol samples at different SA/HSA molar ratio in PBS at pH = 7.4 ([resveratrol = 3 μM and [HSA] = 33 μM)

SA/HSA molar ratio	F_{shift} (a.u.)	λ_{shift} (nm)
0	249.4	22
1	211.7	19
2	207.5	19
3	220.0	21
4	209.3	21
5	208.3	18
6	197.5	15
Resveratrol in Ethanol	247.5	25

The entries in the last line refer to resveratrol in PBS and in ethanol

From the slopes of the plots of 1/ΔF versus 1/[HSA] (the example given in the inset to Fig. 4 refers to HSA-resveratrol complexes), it comes out that the resveratrol binding constant, K_a, reduces from $(1.10 \pm 0.14) \times 10^5$ M^{-1} in the absence of stearic acids to $(0.369 \pm 0.008) \times 10^5$ M^{-1} in the presence of 5 mol of SA per mole of HSA. These results are in agreement with the previous ones determined by using Eq. 2 (see Table 1), and further evidence that stearic acids at high concentration reduce the affinity of HSA towards resveratrol.

The polarity of resveratrol binding site in HSA is influenced by SA bound to the protein. This can be seen in Table 2 where the values of F_{shift} and λ_{shift} are reported. $F_{shift} = F_{highest} - F_0$ and $\lambda_{shift} = |\lambda_{highest} - \lambda_0|$ are the differences in the fluorescence intensity and in λ_{max}, respectively, between resveratrol in the protein at the highest concentration (33 μM) and the corresponding values in buffer. For comparison, the data relative to resveratrol in ethanol are also shown.

At any fatty acid concentration, F_{shift} and λ_{shift} decrease, pointing to a resveratrol binding site more accessible to the solvent, especially at the highest SA concentration.

Resveratrol fluorescence is also used to determine the binding stoichiometry between resveratrol and HSA in the absence and in the presence of SA by means of Job plot analysis (see paragraph *resveratrol fluorescence* under "Materials and methods" section).

Job plots for (SA/HSA)-resveratrol complexes at three different SA/HSA molar ratios are reported in Fig. 5.

For the HSA-resveratrol complex (squares in Fig. 5), the maximum change in the fluorescence occurs at the mole fraction $X_{resveratrol} = 0.5$ which corresponds to a stoichiometry of binding of one molecule of resveratrol per molecule of HSA, in agreement with literature data (N'soukpoé-Kossi et al. 2006; Lu et al. 2007; Jiang 2008). The stoichiometry of binding between HSA and resveratrol is not modified by the presence of 3 mol of SA per mole of

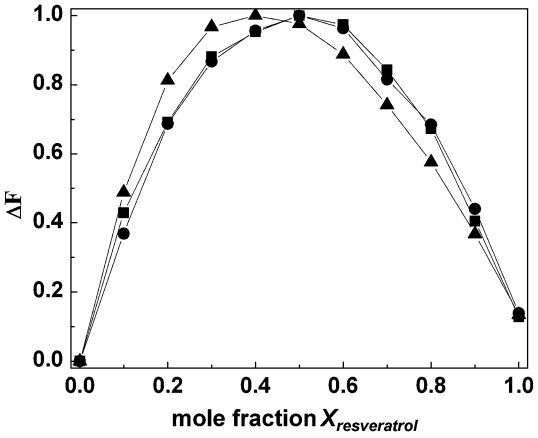

Fig. 5 Job plots for HSA-resveratrol (*squares*), (3SA/HSA)-resveratrol (*circles*), and (5SA/HSA)-resveratrol (*triangles*) complexes in PBS

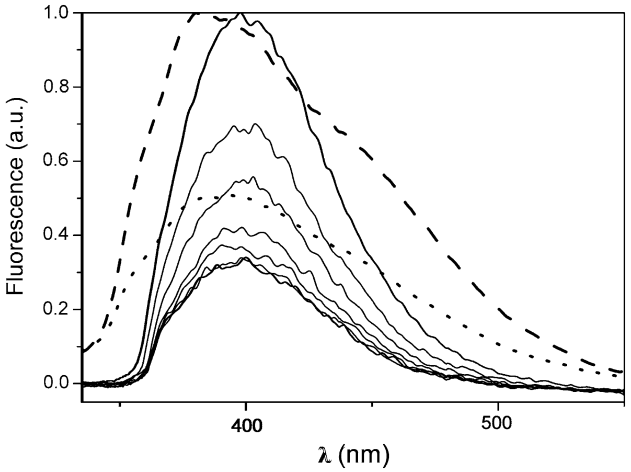

Fig. 6 Fluorescence spectra at $\lambda_{ex} = 315$ nm of resveratrol in PBS displayed from $n = 1$ to $n = 7$ (*solid lines* from *top* to *bottom*), and in HSA for $n = 1$ (*dashed line*) and $n = 7$ (*dotted line*). The spectra are normalized with respect to the first recorded spectrum for $n = 1$

HSA (circles in Fig. 5), whereas it is down shifted at about $X_{\text{resveratrol}} = 0.4$, when 5 molecules of SA are bound to HSA (triangles in Fig. 5). From Job plots is therefore evident once again a lower affinity of the protein for the polyphenol in the presence of high SA content.

Photostability of *trans*-resveratrol

Trans-resveratrol converts to *cis*-resveratrol upon exposure to sunlight or to UV radiation. The optical properties of resveratrol are different in the two isomeric forms. In particular, the maximum absorption of light decreases and shifts to shorter wavelength on going from *trans* to *cis* conformation (Camont et al. 2009; Liang et al. 2008). Exploiting the optical properties of resveratrol and considering that they lead to a decrease of the fluorescence intensity, we have studied the resveratrol *trans–cis* transition in the different (SA/HSA)-resveratrol complexes exposed to UV light at the fixed wavelength of $\lambda_{ex} = 315$ nm (i.e., a wavelength where the protein does not absorb and does not influence the fluorescence spectra of resveratrol). This is done by repeating the fluorescence measurements at $\lambda_{ex} = 315$ nm on the same sample left in the spectrofluorimeter and monitoring the decrease in the fluorescence intensity as a function of the number of measurements, n.

Figure 6 shows seven fluorescence spectra of resveratrol in buffer (solid lines) recorded one after the other on the sample.

The spectra are normalized to the maximum fluorescence intensity, F_1, of the first spectrum. On increasing the number of measurements, n, the fluorescence intensity decreases first rapidly ($n = 1$–4), then more slowly ($n = 4$–5) and finally it reaches a plateau in the last two spectra ($n = 6$–7). The confirmation that the fluorescence quenching in Fig. 6 is due to light induced formation of resveratrol *cis*-isomers has been obtained by spectrophotometric measurements (data not shown). The absorption spectrum of resveratrol/PBS sample for $n = 7$ shows a 50 % reduction and a blueshift of the maximum of the absorption relative to the same firstly irradiated sample (i.e., for $n = 1$). These spectral charecteristics are typical of resveratrol in the *cis* configuration and are similar to those obtained by Liang et al. (2008). They have studied the *trans–cis* conversions of resveratrol bound to β-lactoglobulin exposed to light at all visible wavelength by means of spectrophotometry. In that case, it has been necessary to subtract each time the spectrum of β-lactoglobulin alone from the spectrum of the protein–polyphenol mixtures.

(SA/HSA)-resveratrol samples in PBS have also been irradiated with UV light at 315 nm and the fluorescence spectra measured. In any case, depending on sample composition, a fluorescence quenching up to a plateau value has been observed, indicating that the UV light reduces the stability of the polyphenolic compound and favours its *trans*-to-*cis* transition. As an example, in Fig. 6 are given the spectra for HSA-resveratrol for $n = 1$ (dashed line) and $n = 7$ (dotted line).

From the fluorescence intensity data on (SA/HSA)-resveratrol samples, we have defined the parameters $f_c = (F_1 - F_n)/F_1$ and $f_t = 1 - f_c$, which give an estimate of the fraction of resveratrol in the *cis* and *trans* conformation upon UV irradiation, respectively. In turn, both parameters report on resveratrol stability in the different protein environments.

Table 3 lists the values of the fractions, f_t, of resveratrol in the *trans* conformation after $n = 7$ measurements.

Table 3 Fraction, f_t, of *trans*-resveratrol in (SA/HSA)-resveratrol at different SA/HSA molar ratio and in resveratrol/PBS samples after $n = 7$ irradiations at $\lambda_{ex} = 315$

SA/HSA molar ratio	f_t (%)
0	50.5
1	57
2	57
3	50.7
4	52
5	51.5
6	45
Resveratrol in PBS	34

From data inspection, it is evident that, relative to resveratrol/buffer solution, resveratrol bound to the protein is more stable in the *trans* conformation as f_t is increased from 34 to 50 %. When stearic acids are present at low concentration in HSA (i.e., up to 2 mol of SA per mole of protein) the stability of the polyphenol increases further ($f_t = 57$ %). In contrast, compared to protein alone, the stability is unaffected for SA/HSA = 3, 4, 5 mol/mol, and reduced ($f_t = 45$ %) when 6 fatty acid molecules are bound to the protein. As our findings have shown that at high SA content the protein undergoes conformational changes and the resveratrol binding site becomes more accessible to the solvent, it is likely that the *trans–cis* transition of resveratrol is promoted in irradiated HSA samples complexed with high SA concentration. However, HSA is effective in protecting resveratrol from degradation in any samples. For comparison, the interaction of resveratrol with β-lactoglobulin does not prevent the isomerization of *trans*-resveratrol and provides a slight increase in its photostability (Liang et al. 2008).

Conclusions

The fluorescence data on HSA-resveratrol complexes show that resveratrol binds to HSA with an association constant of ca. 1.1×10^5 M^{-1}, forms a 1:1 complex and, relative to solutions in buffer, increases its photostability. Low content of SA pre-complexed with HSA does not modify the binding of resveratrol to HSA but increases resveratrol photostability. SA at high concentration reduces the affinity of the protein towards resveratrol and decreases, relative to HSA-resveratrol samples, the stability of resveratrol in the *trans* conformation.

The results indicate that HSA complexed with stearic acids is able to bind and protect from deterioration an important antioxidant molecule and that these properties are modulated by the content of stearic acid, demonstrating the ability of HSA as a carrier in delivery applications.

Acknowledgments MP thanks EU and Regione Calabria for support by Regional Operative Program Calabria ESF 2007/2013—IV Axis Human Capital—Operative Objective M2- Action D5.

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