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Insight into the interaction between α-lapachone and bovine serum albumin employing a spectroscopic and computational approach

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Abstract: Serum albumin is the most abundant protein in blood plasma; among its functions is the transport of a high variety of drugs in the body. Quinones show several biological and pharmacological activities, such as anti-malarial, antitumor, anti-microbial, anti-inflammatory and anti-parasitic. We report fluorescence and circular dichroism (CD) spectroscopic studies to try to understand the interaction process between α-lapachone (α-LAP) and bovine serum albumin (BSA). Studies using computational methods, such as molecular docking, were performed to identify the main cavity in which this interaction occurs as well as the type of intermolecular interactions between the amino acid residues from albumin and the ligand. The BSA fluorescence quenching by added α-LAP is a static process, indicating an initial association BSA: α-LAP. The K_a and K_b values for the interaction BSA: α-LAP are in the range 10^5-10^4 L·mol^{-1}, indicating a strong binding between these two species. CD data show that there is no significant perturbation on the secondary structure of the protein with binding. The negative ΔG^o values are consistent with spontaneous binding occurring endothermically (ΔH^o = 127 kJ·mol^{-1}), and possibly driven by hydrophobic factors (ΔS^o = 0.526 kJ·mol^{-1}·s^{-1}). The number of binding sites (*n*) indicates the existence of just one main binding site in BSA for α-LAP, with molecular docking results showing that it binds preferentially to the albumin in the domain IIA, where the Trp-212 residue is located. The ligand interacts *via* hydrogen bond with Arg-259 and Tyr-149 residues and *via* T-stacking with the fluorophore Trp-212 residue.

Keywords: Bovine Serum Albumin, α-Lapachone, spectroscopy, molecular docking.

Introduction

Quinones form a large and diverse group of secondary metabolites, being present in several families of higher plants, fungi, lichens, bacteria, arthropods and echinoderms[1,2]. α-Lapachone (α-LAP, 2,2-dimethyl-3,4-dihydro-2H-benzo[g]chromene-5,10-dione, Fig. 1A) is a natural naphthoquinone present in small amounts in trees of the *Tabebuia* species (family *Bignoniaceae* family), which occurs in most of Central and South America. They are commonly called "*ipê*" or "*pau d'arco*" in Brazil and "*lapacho*" in Argentina and other Spanish-speaking countries[3]. In addition, it can be obtained from the isomerization of lapachol (2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone), a quinone that is more abundant and readily extracted from the same sources[4]. The biological activities of these compounds and simple derivatives have been investigated from 1940 (anti-malarial) up to the present (anti-tumor, anti-microbial, anti-inflammatory and anti-parasitic)[5-8]**.** It is worth noting that "*ipê-roxo*" has been traditionally used in folk medicine[6].

Serum albumin is a protein present in the circulatory system with a variety of physiological functions: maintenance of osmotic pressure; transport, distribution and participation in the metabolism of several endogenous and exogenous ligands (such as, drugs, metabolites, fatty acids, amino acids and hormones). The interaction between drugs and serum albumin is a major and important factor for understanding the interaction of the organism with drugs, since it influences their distribution and excretion[9,10].

The Bovine Serum Albumin (BSA) structure consists of three structurally similar domains (I, II and III), each containing two subdomains, A and B[11,12]. Each of these subdomains has a major binding pocket.

Fluorescence spectroscopy, especially quenching of fluorescence from tryptophan residues – Trp, is

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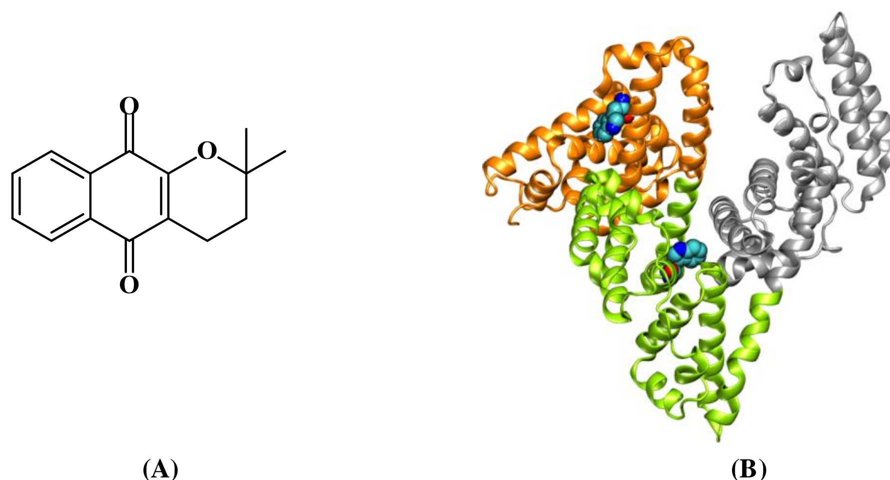
often applied to the binding study in biomolecules. The BSA structure has two tryptophan residues, one in the region IB (Trp-134) and another in the region IIA (Trp-212)[13] (Fig. 1B). Human Serum Albumin (HSA) is commonly replaced by BSA in laboratory experiments due to the higher availability and lower cost of the later. On a first approach, much of binding capacity of BSA may be inferred by its similarity with the human counterpart, since the BSA structure shares 76% identity and 88% similarity in protein sequences with HSA[14].



(A) **(B)**

Figure 1: **(A)** Chemical structure of α-LAP. **(B)** Crystallographic structure of BSA (pdb: 4F5S) showing the domain I (**brown**), II (**green**) and III (**gray**). Trp-134 is located in domain I (**brown**) and Trp-212 in domain II (**green**).

Results and Discussion

Fluorescence Spectroscopy and Fluorescence Quenching

Fluorescence quenching can be employed to evaluate the binding affinities between macromolecules and ligands (quenchers)[15]. Fig. 2 shows quenching of the tryptophan residue of BSA ($Conc._{BSA}$ = 1.0 x 10^{-5} mol·L^{-1}) by addition of aliquots of known concentration of α-LAP solution at 298K, one of the three temperatures employed in this experiment (288K; 293K and 298K). This quenching process indicates that the naphthoquinone is located inside the protein and next to a tryptophan residue[16]. The absence of significant changes in the maximum of the fluorescence emission for BSA is clear evidence that the presence of α-LAP does not exert any influence on the polarity of the microenvironment inside the cavity containing the tryptophan residue[17].

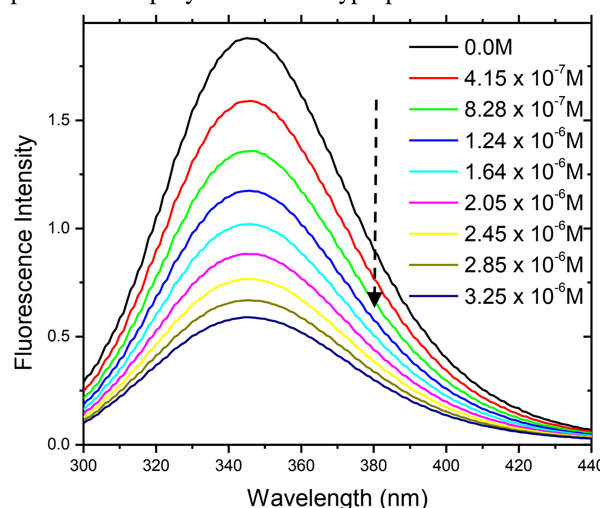


Figure 2. Fluorescence spectra of BSA (conc.: 1.00 x 10^{-5} mol·L^{-1} in PBS buffer solution – pH 7.4) and fluorescence quenching by incremental addition of α-LAP (conc.: 4.15 x 10^{-7} to 3.25 x 10^{-6} mol·L^{-1}) T = 298K, λ_{exc} = 280 nm.

Applying equation 1 to each of the quenching experiments performed at different temperatures (288K; 293K and 298K), the Stern-Volmer quenching constant (K_{sv}) and the quenching rate constant (k_q) for the interaction BSA: α-LAP at each temperature can be obtained (Figure 3 and Table 1)[18].

$$\textbf{(A)} \quad \frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV}[Q] \qquad \textbf{(B)} \quad k_q = \frac{K_{SV}}{\tau_0} \qquad \textbf{(1)}$$

(Where, F_0 and F are the fluorescence intensities of BSA without and with the quencher (α-LAP), respectively; K_{sv} is the Stern-Volmer quenching constant, k_q is the quenching rate constant of BSA fluorescence, [Q] the quencher concentration and τ_o is the lifetime of BSA without the quencher (10^{-8} s)[17].)

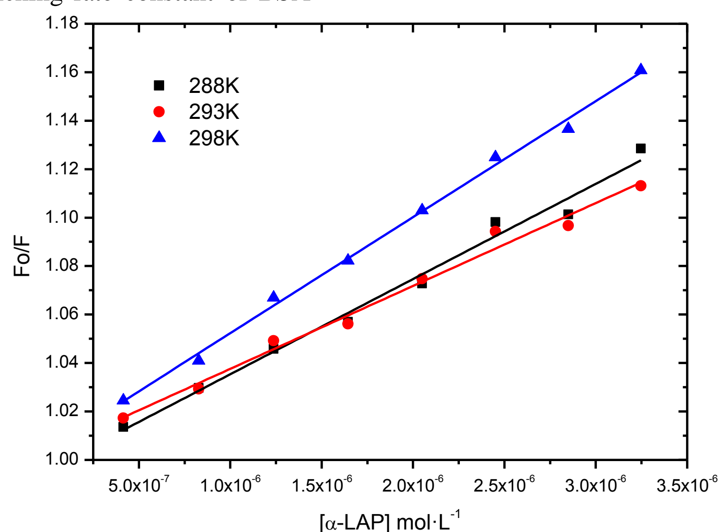


Figure 3. Stern-Volmer plots for the fluorescence quenching of BSA by α-LAP at different temperatures.

Table 1. Stern-Volmer quenching constant (K_{sv}) and quenching rate constant (k_q) values for the association BSA: α-LAP at 288K, 293K and 298K.

T (K)	K_{SV} ($L\cdot mol^{-1}$)	k_q ($L\cdot mol^{-1}\cdot s^{-1}$)	r^2
288	(3.93±0.18) x 10^4	3.93 x 10^{12}	0.9855
293	(3.42±0.16) x 10^4	3.42 x 10^{12}	0.9854
298	(4.79±0.11) x 10^4	4.79 x 10^{12}	0.9962

The fluorescence quenching can be induced at a certain distance between the fluorophore and the quencher and does not require contact between them (Förster's theory). Quenching of a fluorophore can also occur as a result of the formation of a non fluorescent complex between the fluorophore and a non-fluorescent molecule in the ground state. This mechanism is known as "ground-state complex formation"[15]. The quenching rate constants ($k_q \approx 10^{12}$ $L\cdot mol^{-1}\cdot s^{-1}$) have higher values than the diffusion rate constant ($k_d \approx 5 \times 10^9$ $L\cdot mol^{-1}\cdot s^{-1}$ in water at 25°C), indicating that the probable mechanism of fluorescence quenching is static[17-19]. In this case, the initial formation of a non-fluorescent association in the ground-state between the fluorophore (tryptophan residue in BSA) and the quencher can be expected. The small and irregular variation in K_{SV} values with temperature does not show a significant trend.

Binding Constant (K_a) from a Stern-Volmer Modified Plot

To obtain further information about the interaction between BSA and α-LAP, if it can be considered strong, moderate or weak, we calculated the modified Stern-Volmer binding constant (K_a)[18,20] (Figure 4 and Table 2). This constant can be obtained according to equation 2:

$$\frac{F_0}{F_0 - F} = \frac{1}{fK_a}\frac{1}{[Q]} + \frac{1}{f} \qquad \textbf{(2)}$$

(Where, F_o and F are the fluorescence intensities of BSA without and with the quencher (α-lapachone) at 350 nm, respectively; K_a is the modified Stern-Volmer binding constant; f the fraction of the initial fluorescence that is accessible to the quencher and [Q] the quencher concentration.)

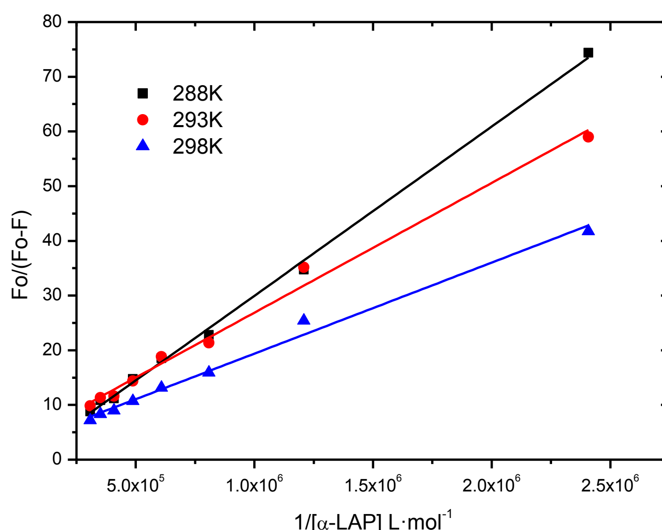


Figure 4. Modified Stern-Volmer plots for the quenching of BSA fluorescence by α-LAP at different temperatures.

Table 2. Modified Stern-Volmer binding constant (K_a) values for the association BSA: α-LAP at 288K, 293K and 298K.

T (K)	K_a (L·mol^{-1})	r^2
288	(3.1±0.1) x 10^4	0.9975
293	(1.3±0.1) x 10^5	0.9896
298	(1.6±0.1) x 10^5	0.9896

The K_a values are in the range 10^4-10^5 L.mol^{-1}, indicating a strong interaction between albumin and α-LAP [21,22]. The binding constant values depend on the charge and structure of α-LAP, as well as on the conformation and charge of BSA[23].

Thermodynamic Parameters (ΔG°, ΔH°, ΔS°)

To get some insight on the thermodynamic parameters ΔG°, ΔH°, ΔS° which control the interaction BSA: α-LAP, data from Table 2 were plotted according to the van´t Hoff equation (3A) (Figure 5) and ΔG° was calculated employing the Gibbs free energy equation[24] (3B) (Table 3):

$$\textbf{(A)} \quad \ln K_a = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \qquad \textbf{(B)} \quad \Delta G^0 = \Delta H^0 - T\Delta S^0 \qquad \textbf{(3)}$$

(Where, ΔH°, ΔS°, ΔG° are the enthalpy, entropy and Gibbs free energy, respectively; R is the gas constant (R = 8.314 x 10^{-3} kJ·mol^{-1}·K^{-1}), T is the temperature (288K, 293K and 298K) and K_a the binding constant.)

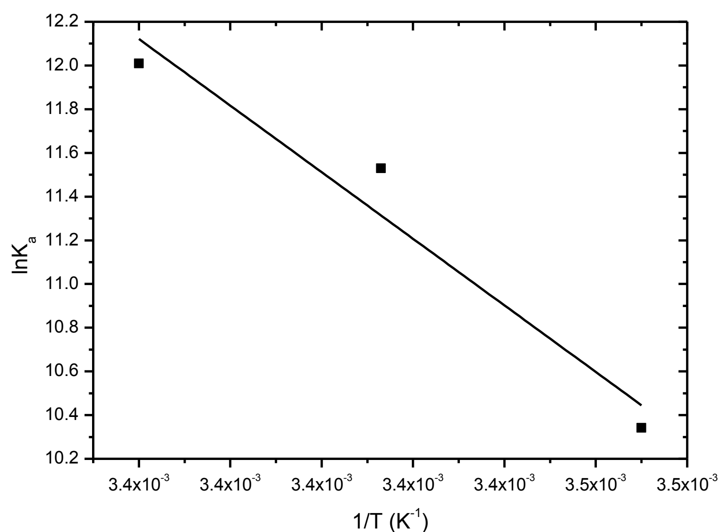


Figure 5. Van´t Hoff plot employing K_a values obtained from modified Stern-Volmer plots at 288K, 293K and 298K.

Table 3. Thermodynamic parameters (ΔH^o, ΔS^o, ΔG^o) for the interaction between BSA and α-LAP at 288K, 293K and 298K.

T (K)	ΔH^o ($kJ\cdot mol^{-1}$)	ΔS^o ($kJ\cdot mol^{-1}\cdot K^{-1}$)	ΔG^o ($kJ\cdot mol^{-1}$)	r^2
288			-24.9	
293	127	0.526	-27.6	0.9056
298			-30.2	

The negative values of ΔG° shown in Table 3 are consistent with a spontaneous binding between BSA and α-LAP, whereas the positive value of ΔH° indicates that the binding process is endothermic. Finally, the positive value of ΔS° shows that the interaction is mainly due to hydrophobic factors[25], which can be related to the influence of hydration molecules. There are two possible contributions that may explain the increase in entropy: hydration molecules can be expelled from the protein cavity as a consequence α-LAP entry into it, or the desolvation of the α-LAP, as it enters the cavity, can cause an increase in the number of micro-states of the system[23-25].

Number of Binding Sites (*n*)

The binding site number (*n*) and the binding constant (K_b) can be obtained from the binding site model which assumes that there are similar and independent binding sites for a quencher in the biomolecule[26], as expressed by equation 4[27]. These results are shown in Figure 6 and Table 4.

$$\log \frac{F_0 - F}{F} = \log K_b + n \log[Q] \quad \textbf{(4)}$$

(Where F_0 and F are the fluorescence intensities in the absence and presence of α-LAP, K_b is the binding constant with BSA, and *n* is the number of binding sites).

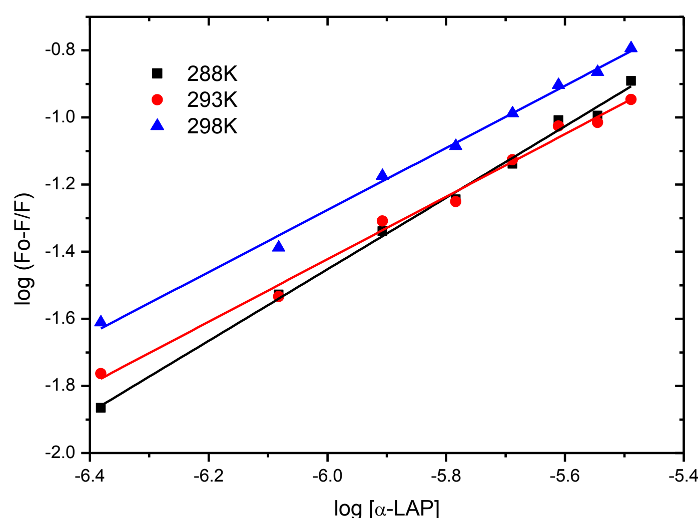


Figure 6. Linear fit for the values of log [(F_0–F)/F] *vs*. log [Q] for BSA: α-LAP at 288K, 293K and 298K.

Table 4. Number of binding sites (*n*) and binding constant (K_b) values for the association BSA: α-LAP at 288K, 293K and 298K.

T (K)	*n*	K_b ($L\cdot mol^{-1}$)	r^2
288	1.07±0.03	(8.8±0.2) x 10^4	0.9952
293	0.93±0.03	(1.5±0.2) x 10^4	0.9905
298	0.93±0.02	(1.9±0.1) x 10^4	0.9951

As can be seen in Table 4, K_b values are in the range 10^4 $L\cdot mol^{-1}$, comparable to the range of the modified Stern-Volmer binding constant (K_a) values (Table 2). In both cases there is no consistent trend in the variation with temperature. The *n* values were approximately 1 at different temperatures (288K, 293K and 298K), which indicates the existence of just one main binding site in BSA for α-LAP [17,26].

Circular Dichroism (CD)

Circular dichroism spectra were obtained to evaluate the changes in the secondary structure of

BSA induced by the addition of α-LAP. The CD spectra of BSA exhibit two minus signs (negative Cotton effects) at 208 nm and 222 nm, which are characteristic of the α-helix protein structure. These bands originate from n-π* protein transitions[28-30].

Initially the molar residual ellipticity (MRE) was calculated applying equation 5[27], and used to obtain the α-helix % (equation 6).

$$MRE = \frac{\theta}{(10.n.l.C_P)} \quad (5)$$

(Where, θ is the observed ellipticity (mdeg); n is the number of amino acid residues (582 for BSA)[31]; l is the optical length of the optical cuvette (1 cm) and C_p is the molar concentration of BSA (1,00 x 10^{-6} mol·L^{-1})).

$$\text{(A)}\ \%\ \alpha-helix = \left[\frac{(-MRE_{208}-4000)}{(33000-4000)}\right] x\,100 \quad \text{(B)}\ \%\ \alpha-helix = \left[\frac{(-MRE_{222}-2340)}{30300}\right] x\,100 \quad (6)$$

(Where, MRE_{208} and MRE_{222} are the significant molar residual ellipticity at 208 nm and 222 nm (deg.cm^2.$dmol^{-1}$), respectively.)

Fig. 7 shows the circular dichroism spectra at 298K for free BSA (1.00 x 10^{-6} mol·L^{-1}) and in the presence of α-LAP.

The circular dichroism signals are similar, indicating that the naphthoquinone does not make a significant perturbation on the secondary structure of BSA[32,33].

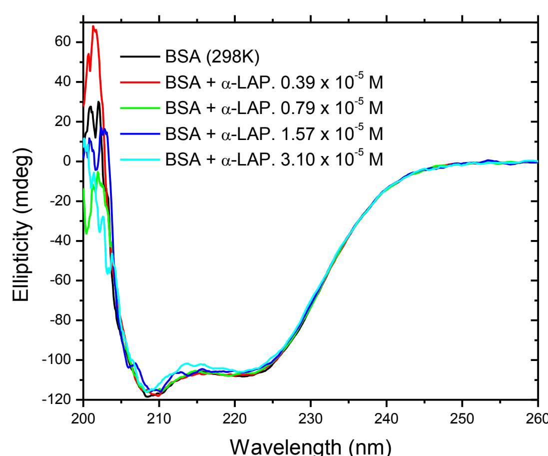


Figure 7. Circular dichroism spectra of BSA and BSA: α-LAP at 298K Conc.$_{BSA}$ = 1.00 x 10^{-6} mol·L^{-1}.

Table 5. Values for α-helix % at 208 nm and 222 nm resulting from the association BSA: α-LAP at 298K.

		T (K)	**298**	
	C_{BSA} (mol·L^{-1})	**$C_{\alpha\text{-lap}}$ (mol·L^{-1})**	***208***	***222***
BSA α-LAP	1.00 x 10^{-6}	-	55.24	53.36
	1.00 x 10^{-6}	0.39 x 10^{-5}	54.56	53.04
	1.00 x 10^{-6}	0.79 x 10^{-5}	54.46	52.73
	1.00 x 10^{-6}	1.57 x 10^{-5}	52.32	52.14
	1.00 x 10^{-6}	3.10 x 10^{-5}	48.37	51.77

Results (at Table 5) show that the addition of α-LAP to BSA results in a change of 1.6% to 6.9% in α-helix % for BSA, demonstrating again that the naphthoquinone does not make a significant perturbation on the secondary structure of the protein.

Molecular Docking Studies

HSA is commonly substituted for BSA in laboratory experiments due to its higher availability and lower cost, and on a first approach much of its binding capacity may be inferred by similarity with its human counterpart. Like HSA, with which it shares a 76% identity and 88% similarity in sequence, the globular protein BSA consists of three structurally similar domains (I, II, and III), each containing two sub-domains (A and B)[34], with the domains I (Sudlow I) and II (Sudlow II) being the most important. Site I, named *warfarin binding site*, is located in the IIA subdomain, while site II, named *indole/benzodiazepine binding site* is located in subdomain IIIA[35]. From the studies of BSA fluorescence quenching described above, it is known that α-LAP can interact with a tryptophan residue, either the Trp-134 or the Trp-212. A molecular docking study was performed to analyze the

interaction sites as well as the nature of the intermolecular interactions between α-LAP and the amino acid residues in each site.

The docking score results suggest that α-LAP has more favorable interactions with the Trp-212 site (docking score 57.4) than in the Trp-134 site (docking score 44.9). The docking results clearly suggested that α-LAP is more probably bound to the Sudlow's site I (subdomain IIA) where the Trp-212 residue is located. Analysis of the interaction between BSA and α-LAP in this site shows that the ligand can also interact via hydrogen bonds with an arginine and a tyrosine amino acid residue (Fig. 8). The Tyr-149 residue is able to form a hydrogen bond with the carbonyl group of α-LAP nearest to the pyran oxgen with a distance between the donor and acceptor atoms of 1.74 Å. The oxygen of the pyranic group of α-LAP can also interact through hydrogen bond with the Arg-259 residue, with a distance of 2.77 Å. Besides these interactions, the ligand interacts with BSA through hydrophobic interactions. The molecular docking results show that the ligand aromatic ring makes a T-stacking interaction with the Trp-212 residue, with a distance of 3.49 Å. This proximity between the quencher and the fluorophore can explain the efficiency of the fluorescence quenching[15].

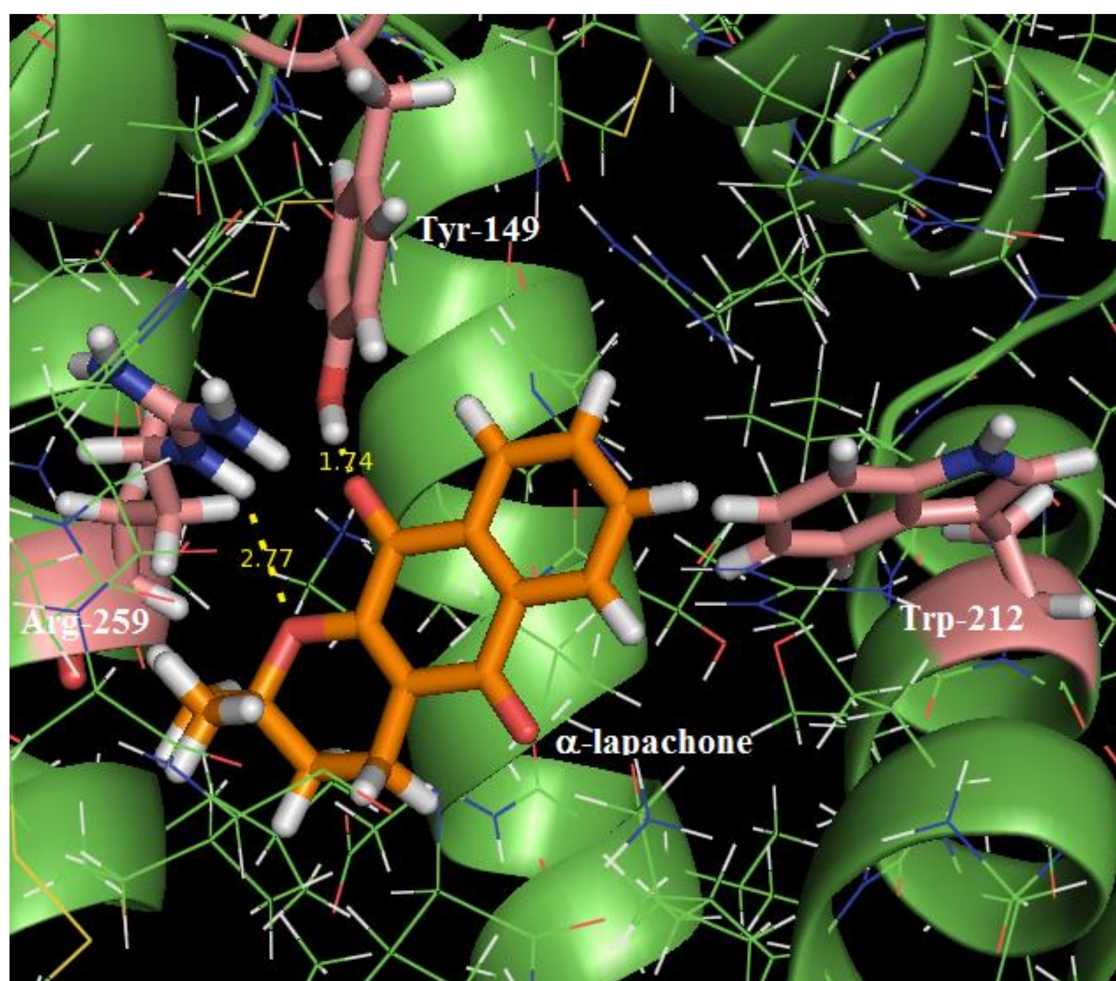


Figure 8. Best score pose for α-LAP in BSA in the cavity containing the Trp-212 residue, obtained by molecular docking (ChemPLP function). Carbon: orange (α-LAP), green (BSA), light beige (selected residues); hydrogen: white; oxygen: red; and nitrogen: blue. (figure generated with the PyMOL software).

Conclusion

α-Lapachone binds strongly with bovine serum albumin, but this binding does not make a significant perturbation on the secondary structure of BSA. This interaction is spontaneous ($\Delta G° < 0$) and the other thermodynamic parameters suggest that the binding is endothermic ($\Delta H° = 127$ kJ·mol^{-1}) and occur mainly by hydrophobic factors ($\Delta S° = 0.526$ kJ·mol^{-1}·s^{-1}) due to the hydration molecules effect. The quenching rate constant ($k_q \approx 10^{12}$ L·mol^{-1}·s^{-1}) obtained employing fluorescence spectroscopy indicates that the tryptophan fluorescence quenching is a static process, as a consequence of a ground-state interaction BSA: α-LAP. The number of binding sites (*n*) indicates that there is just one main interaction site and the docking results are indicative that α-LAP preferentially binds in the domain IIA, where the Trp-212 residue is located. The naphthoquinone interacts via hydrogen bond with Arg-259 and Tyr-149 residues and via a T-stacking interaction with the fluorophore Trp-212 residue.

Acknowledgements

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Experimental Section

Spectroscopic Experiments:

Chemicals

Commercially available Bovine Serum Albumin (BSA) and PBS buffer (pH = 7.4) were obtained from Sigma-Aldrich Chemical Company. Water used in all experiments was millipore water. Ethanol (spectroscopic grade) was obtained from Vetec. α-LAP was obtained from the Photochemistry Group of the Chemistry Department/Universidade Federal Rural do Rio de Janeiro (Seropédica, RJ, Brazil). Its spectroscopic and spectrometric properties are in full accord with the structure proposed[36].

Instruments

The fluorescence spectra were measured on a Jasco J-815 fluorimeter, in a 1 cm quartz cell and employing a thermostatic cuvette holder Jasco PFD-425S15F. The circular dichroism spectra were measured in a Jasco J-815 spectropolarimeter. All spectra were recorded with appropriate background corrections.

Methodology

In a 3.0 mL solution, containing appropriate concentration of BSA (1.00 x 10^{-5} mol·L^{-1}, in PBS buffer, pH = 7.4), successive aliquots from a stock solution of α-LAP (1.00 x 10^{-3} mol·L^{-1} in ethanol) were added, to obtain concentrations ranging from 0 to 3.25 x 10^{-6} mol·L^{-1}. The addition was done manually by using a micro syringe. Fluorescence spectra were measured in the range 300-440 nm, at 288K, 293K and 298K with excitation wavelength at 280 nm. The circular dichroism spectra were recorded for free BSA (1.00 x 10^{-6} mol·L^{-1}) and α-LAP (0,39; 0,79; 1.57; 3.10 x 10^{-5} mol·L^{-1}), in the range of 200-260 nm, at ≈298K.

In order to compensate for the inner filter effect, the fluorescence intensity values of the samples were corrected for their absorption at excitation and emission wavelengths using the equation 7[15,37].

$$F_{cor} = F_{obs} 10^{[(A_{ex}+A_{em})/2]} \quad (7)$$

(Where F_{cor} and F_{obs} are the corrected and the observed fluorescence intensity values, while A_{ex} and A_{em} represent changes in the absorbance values of the samples at the excitation (λ = 280 nm) and emission wavelengths (λ = 345 nm), respectively.)

Computational Experiments

The crystallographic structure of bovine serum albumin was obtained from the Protein Data Bank (PDB) whose access code is 4F5S[14]. This structure has a resolution of 2.47 Å. The α-LAP structure was built and energy-minimized with the semiempirical method AM1[38], available in the Spartan'14 program (Wavefunction, Inc., Irvine, CA, USA).

The molecular docking was performed with Gold 5.2 program (CCDC). Hydrogen atoms were added to the protein according to the data inferred by the program on the ionization and tautomeric states[39]. A docking interaction cavity in the protein was established with a radius of 10 Å and 15 Å from Trp-134 and Trp-212. The best result was obtained with radius of 10 Å. The number of genetic operations (crossover, migration, mutation) in each docking run used in the searching procedure was set to 100,000. The program optimizes hydrogen-bond geometries by rotating hydroxyl and amino groups of amino acid side chains. The scoring function used was 'ChemPLP'[40], which is the default function of the GOLD program. The score of each pose identified is calculated as the negative of the sum of a series of energy terms involved in the protein-ligand interaction process, so that the more positive the score, the better is the interaction. The figure of the best score was generated by PyMOL 1.leval program (Delano Scientific LLC).

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