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# Progesterone binding to the tryptophan residues of human $\alpha_1$-acid glycoprotein

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**Abstract**—Binding studies between progesterone and $\alpha_1$-acid glycoprotein allowed us to demonstrate that the binding site of progesterone contains one hydrophobic tryptophan residue and that the structure of the protein is not altered upon binding. The data obtained at saturated concentrations of progesterone clearly reveal the type of interaction at physiological levels.


*Keywords:* $\alpha_1$-Acid glycoprotein; Tryptophan; Progesterone; Protein dynamics and structure; Red-edge excitation spectra; Fluorescence anisotropy

## 1. Introduction

Human $\alpha_1$-acid glycoprotein (orosomucoid), a plasma glycoprotein with a molecular weight of 41,000, consists of a chain of 183 amino acids.[1,2] It contains 40% carbohydrate by weight and has up to 16 sialic acid residues (10–14% by weight).[3] Five glycan chains are linked via an *N*-glycosidic bond to the asparagine residues of the protein.[1] This high degree of sialylation and the presence of acidic amino acids residues give rise to a very low pI of 2.8–3.8.[4]

Although biological function of $\alpha_1$-acid glycoprotein is still obscure, a number of activities of possible physiological significance have been described such as the ability to bind basic drugs like warfarin,[5] vanilloids,[6] immunoglobulins G3,[7] heparin,[8] and steroid hormones such as progesterone.[9] Many of these activities have been shown to be pathophysiologically dependent.[10–12]

$\alpha_1$-Acid glycoprotein binds a small portion of progesterone, the major part of the circulating ligand is associated with serum albumin and corticosteroid binding globulin.[13] The binding parameters (stoichiometry and association constant) of $\alpha_1$-acid glycoprotein–progesterone complex are 1:1 and $80 \pm 20\ \mathrm{mM}^{-1}$, respectively.[9,14,15]

$\alpha_1$-Acid glycoprotein contains three tryptophan (Trp) residues. Decay associated spectra and experiments performed in the presence of high concentrations of calcofluor white, a fluorophore that binds specifically to carbohydrate residues of $\alpha_1$-acid glycoprotein,[16] show that the three Trp residues contribute to the fluorescence of the $\alpha_1$-acid glycoprotein.[17,18] Emission maxima were at 347 (Trp-160), 337, and 324 nm (Trp-122 and Trp-25). Anisotropy and quenching experiments performed in the absence and presence of cesium and progesterone allowed us to describe the following model for $\alpha_1$-acid glycoprotein: N-terminal fragment should be in contact with the solvent, and would adopt a spatial conformation so that a pocket in contact with the buffer is induced, and to which Trp-25 and/or Trp-122 residues belong. The microenvironment of the two hydrophobic Trp residues is not compact or rigid (low anisotropy and thus high mobility). The five carbohydrate units are linked to the pocket. Thus, progesterone can bind directly to this pocket because it diffuses from the buffer immediately to its binding site within or at the surface of the pocket. This binding site is mainly hydrophobic as progesterone is hydrophobic.[15]

This model is in good agreement with the fact that the binding site of progesterone on $\alpha_1$-acid glycoprotein

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contains both a hydrophobic and a polar surface on the protein. Also, the possible presence of this pocket has been evoked from binding experiments as a function of the pH.[19] In fact, interaction studies between carbohydrate residues of $\alpha_1$-acid glycoprotein and progesterone showed that progesterone interferes with the hydrophilic domain of the pocket.[20]

In all the experiments performed to date by different authors, concentration of progesterone was in range of that of $\alpha_1$-acid glycoprotein, in micromolar range. These concentrations are in the same order of magnitude or are slightly higher than the physiological conditions for both $\alpha_1$-acid glycoprotein and progesterone. In fact, physiological conditions of the protein are around 5–7 μM while concentrations of progesterone in the luteal phase are less than 100 nM and, in the third trimester of pregnancy, less than 1 μM. Results obtained under physiological conditions showed that binding of progesterone to $\alpha_1$-acid glycoprotein modifies the mean emission peaks of both hydrophobic and hydrophilic Trp residues.[21] Also, Raman studies suggest proximity of Trp-122 to hydrophobic site of $\alpha_1$-acid glycoprotein.[22] However, all these experiments were not sufficient to describe what is really happening within the pocket near the Trp residues when progesterone binds to the protein. In fact, we do not know if local structure of protein is affected in the presence of progesterone and we do not know how many Trp residues are involved in the binding of progesterone.

To clarify these problems, we performed interaction experiments between $\alpha_1$-acid glycoprotein and saturating concentrations of progesterone (≈415 μM). Although we are far from physiological conditions, the results allowed us to determine that one hydrophobic Trp residue is clearly involved in the binding of progesterone. The results showed that direct interaction between progesterone and the hydrophilic Trp residue appears not to occur, but if it does, the interaction is very weak. Also, experiments showed that structure of $\alpha_1$-acid glycoprotein is not affected upon progesterone binding. Therefore, these unambiguous results obtained at saturating concentrations of progesterone can be extrapolated to physiological conditions revealing how interaction between $\alpha_1$-acid glycoprotein and progesterone occurs.

## 2. Materials and methods

$\alpha_1$-Acid glycoprotein was purified from serum by a successive combination of ion-displacement chromatography, gel filtration, and ion-exchange chromatography as previously described.[23] The lyophilized protein was dissolved in a 10 mM phosphate and 0.143 M NaCl buffer (PBS buffer), pH 7. Its concentration was determined spectrophotometrically using an extinction coefficient of 29.7 $mM^{-1}\,cm^{-1}$ at 278 nm.[24]

Progesterone (from Sigma) was dissolved in pure distilled EtOH. Two stock solutions were prepared, 2.5 mM (used for the quenching and red-edge experiments) and 6 mM (used to perform the anisotropy experiments). The stock volumes added to the protein solutions were 200 and 100 μL, respectively, inducing final concentrations in the cuvette around 415 and 545 μM, respectively. L-Trp was from Sigma and was used as received.

Absorbance data were obtained with a Shimadzu MPS-2000 spectrophotometer using 1-cm pathlength cuvettes. Fluorescence spectra were recorded with a Perkin–Elmer LS-5B spectrofluorometer. The bandwidths used for the excitation and the emission were 2.5 or 5 nm. The quartz cuvettes had optical pathlengths equal to 1 and 0.4 cm for the emission and excitation wavelengths, respectively. Fluorescence spectra were corrected for the background intensities of the buffer solution. Observed fluorescence intensities were first corrected for the dilution, then corrections were made for the absorption as described.[25,26] Anisotropy data were measured with the same Perkin–Elmer fluorometer. The bandwidths used for the excitation and the emission were 5 nm. All experiments were performed at 20 °C in 10 mM phosphate buffer and 0.143 M NaCl buffer (PBS buffer), pH 7.

## 3. Results

The fluorescence emission spectrum of the $\alpha_1$-acid glycoprotein with excitation at 295 nm is shown in Figure 1a. The position of the maximum (331 nm) and the value of the bandwidth (53 nm) are typical for proteins containing Trp residues at the surface and surrounded by a hydrophobic environment.[27] Binding of 415 μM progesterone induces a shift in the emission to 340 nm, but does not change the value of the bandwidth of the spectrum (53 nm) (Fig. 1b). Thus, in the presence of high concentrations of progesterone, emission still occurs from both classes of Trp residues. The shift observed could be the result of local structural reorganization around the binding site or the fluorescence quenching of one of the hydrophobic Trp residues, a phenomenon that would lead to a shift of the fluorescence emission peak to the red (higher wavelengths).

Subtracting spectrum (b) from (a) yields spectrum (c) with an emission peak at 325 nm and a bandwidth of 49 nm. These features are characteristic of emission from Trp residues surrounded by a hydrophobic environment. Also, the fluorescence properties of spectrum c are identical to those already obtained when we performed fluorescence intensity quenching with cesium[21] or when we studied the effect of high concen-

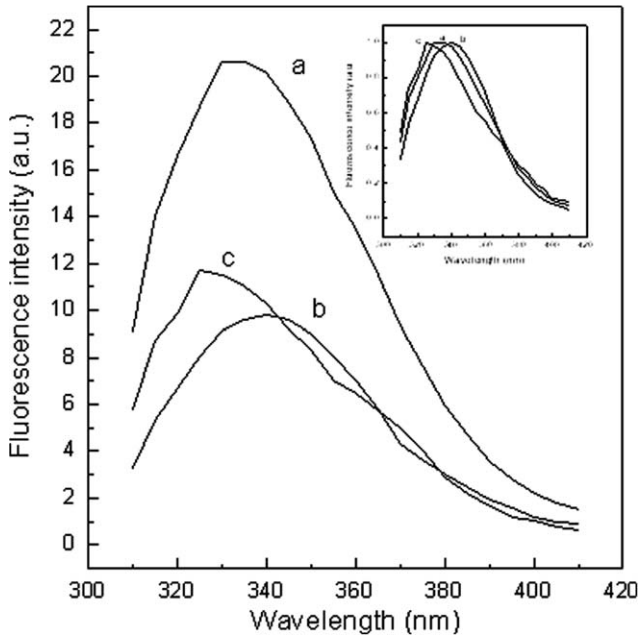


**Figure 1.** Fluorescence emission spectra of $\alpha_1$-acid glycoprotein in the absence (a) and presence (b) of 415 μM progesterone; $\lambda_{ex} = 295$ nm. The emission peaks are at 333 and 340 nm, respectively and the bandwidths are 53 nm for both spectra. Emission in the absence of progesterone occurs from the three Trp residues of $\alpha_1$-acid glycoprotein, while emission in the presence of progesterone occurs from the surface Trp residue (Trp-160) and the hydrophobic Trp residue (Trp-25 or 122), which does not belong to the binding site of progesterone. Spectrum c is obtained by subtracting spectrum b from a. The emission peak of spectrum c is equal to 325 nm and its bandwidth is equal to 49 nm, features that characterize an emission from Trp residue surrounded by a hydrophobic domain. Spectrum c is that of the Trp residue that makes part of the binding site of progesterone on $\alpha_1$-acid glycoprotein. Inset: The three normalized spectra.

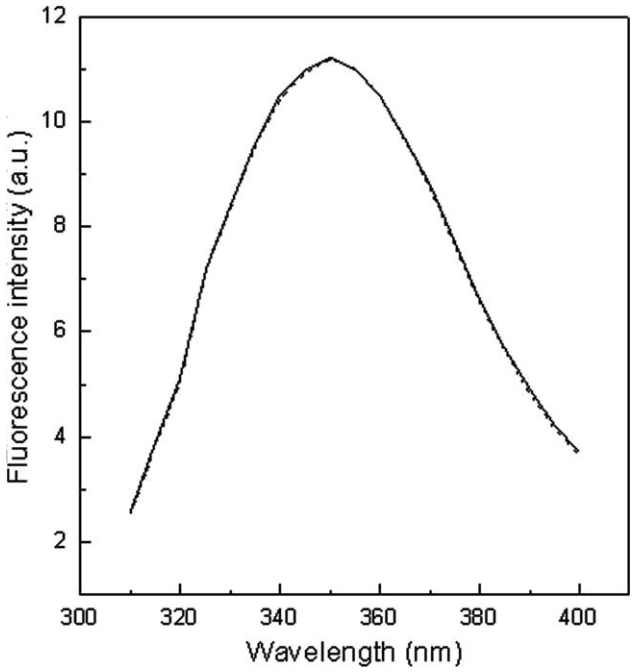


**Figure 2.** Fluorescence emission spectra of L-Trp free in solution in the absence (continuous line) and in the presence (dotted line) of 417 μM progesterone ($\lambda_{ex} = 295$ nm). The absence of any significant modification in the emission spectrum in the presence of progesterone means that interaction between progesterone and Trp in solution is not occurring.

trations of calcofluor white on fluorescence emission of $\alpha_1$-acid glycoprotein Trp residues.[18] Nevertheless, it is not possible to assign these spectral parameters to Trp-25 or Trp-122 residue.

To find out whether the shift to the red is clearly the result of the fluorescence emission quenching of a hydrophobic Trp residue, we performed titration of free L-Trp in PBS buffer with progesterone. Figure 2 displays the recorded emission spectra of 50 μM L-Trp in the absence (continuous line) and in the presence (dotted line) of 415 μM progesterone ($\lambda_{ex} = 295$ nm). At all progesterone concentrations used, we did not observe any decrease in the fluorescence intensity or a shift to the red of the emission peak of L-Trp. Therefore, interaction nature between L-Trp free in solution and progesterone differs from that observed between Trp residues in the $\alpha_1$-acid glycoprotein and progesterone (see also Discussion below).

Based upon fact that $\alpha_1$-acid glycoprotein contains a pocket that interacts with the solvent, it was important to determine if the addition of ethanol alone, without progesterone, affects protein fluorescence. Figure 3

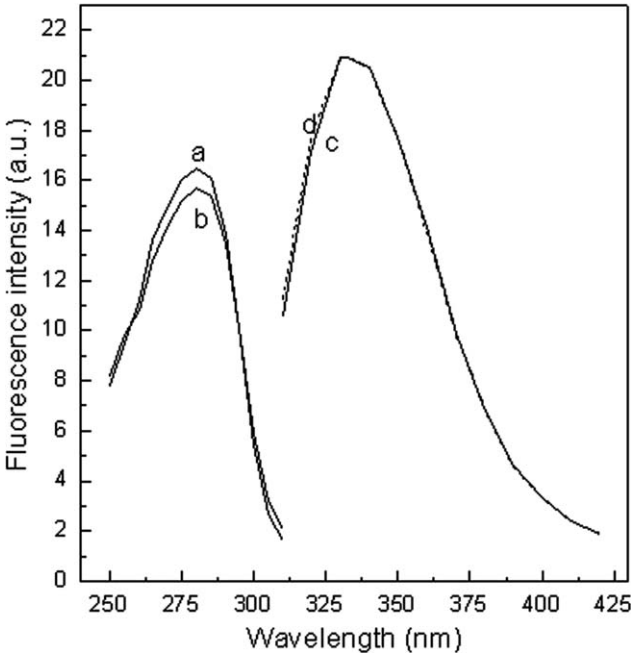


**Figure 3.** Fluorescence excitation ($\lambda_{em} = 360$ nm) (left side) and emission ($\lambda_{ex} = 295$ nm) (right side) spectra of 6.8 μM $\alpha_1$-acid glycoprotein in the absence (a and c) and in the presence (b and d) of 200 μL ethanol. The solvent does not quench the Trp residues fluorescence of $\alpha_1$-acid glycoprotein and does not disrupt the protein structure.

displays the fluorescence excitation (left side) and emission (right side) spectra of 6.8 μM $\alpha_1$-acid glycoprotein in the absence and presence of 200 μL ethanol. We can notice that the spectra are not affected by the presence of ethanol. Therefore, the decrease in the fluorescence intensity of $\alpha_1$-acid glycoprotein Trp residues observed in Figure 1 is the result of the binding of progesterone to the protein.

Structural modifications, if any, within the protein can be monitored by recording the fluorescence excitation spectrum of Trp residues in absence and in the presence of low and high concentrations of progesterone. In fact, fluorescence excitation spectrum characterizes electron distribution of molecule in the ground state. Because progesterone does not emit, any modification of the fluorescence excitation spectrum in the presence of progesterone would be the result of a structural modification of the protein in the ground state.

Figure 4 displays the fluorescence excitation spectrum of Trp residues of $\alpha_1$-acid glycoprotein in the absence (a) and in the presence of 415 μM progesterone (b) ($\lambda_{em} = 350$ nm). We observe a significant decrease in the intensity of the excitation spectrum and the recorded spectrum displays a peak at 290 nm. Subtracting spectrum (b) from (a) yields spectrum (c) with a peak around 265–270 nm. Spectra (b) and (c) are similar to

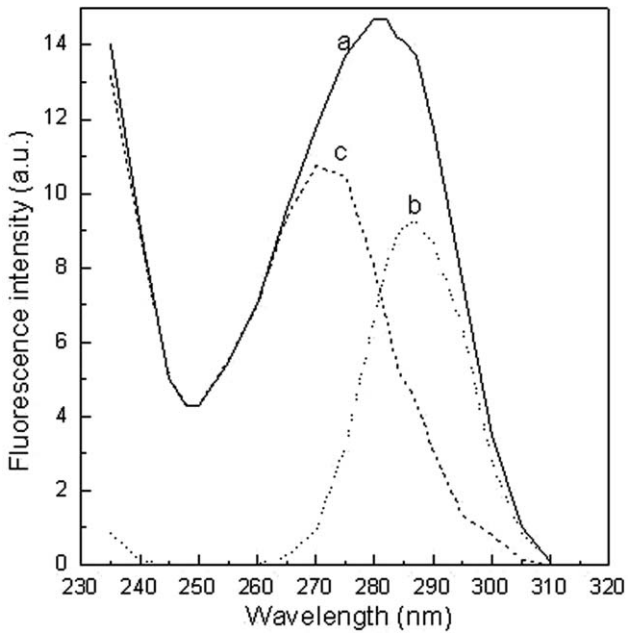


**Figure 4.** Excitation spectra of $\alpha_1$-acid glycoprotein in PBS buffer in the absence (a) and in the presence (b) of 415 μM progesterone ($\lambda_{em} = 350$ nm). Excitation and emission slits are 5 nm. Spectrum c is the difference between (a) and (b). Spectra b and c correspond to the $^1L_b$ and the $^1L_a$ transitions, respectively. High progesterone concentrations act as a filter of the $^1L_a$ transition. The shape of the $^1L_b$ transition and the position of its peak (290 nm) in the presence of progesterone are identical to that observed for this transition in the whole spectrum without progesterone. Binding of progesterone at high concentrations does not affect the local structure of the protein.

$^1L_b$ and $^1L_a$ transitions that characterize the two singlet states of Trp residues.[28] The transition dipoles of these two states are oriented almost perpendicular to each other. Addition of progesterone to $\alpha_1$-acid glycoprotein inhibits completely $^1L_a$ state. Thus, $^1L_b$ state would be primary contributor to Trp emission, in presence of high progesterone concentrations and when excitation is performed, for example, at 295 nm. In fact, at this wavelength, the $^1L_b$ state is preferentially selected and the shape of the corresponding excitation spectrum matches that of the excitation spectrum recorded in the absence of progesterone. Also, its peak at 290 nm, a value characteristic of the $^1L_b$ state, is observed as a shoulder in the excitation spectrum of $\alpha_1$-acid glycoprotein recorded in the absence of progesterone. Thus, because the spectrum shape of the $^1L_b$ state and its peak position are not modified in the presence of progesterone, binding of progesterone to $\alpha_1$-acid glycoprotein does not modify the local structure around Trp residues. This means that the shift to 340 nm of emission spectrum of $\alpha_1$-acid glycoprotein Trp residues observed in the presence of progesterone cannot be the consequence of a structural modification of the Trp residues of the protein but rather the result of an almost complete fluorescence quenching of the Trp residue that forms part of the hydrophobic binding site of progesterone on $\alpha_1$-acid glycoprotein.

The data of Figure 4 indicate that high progesterone concentrations act as a filter of $^1L_a$ state. If this is the case for Trp residues in proteins, this should be the same for a Trp in solution. In fact, addition of 415 μM progesterone to 50 μM Trp free in PBS buffer induces complete abolishment of a part of excitation spectrum of the Trp (Fig. 5, $\lambda_{em} = 360$ nm). Subtracting the resulting spectrum (b) from the total spectrum (a) yields spectrum (c). Spectra (b) and (c) can be assigned to $^1L_b$ and $^1L_a$ states, respectively. This filter effect of $^1L_a$ state is simply result of the high absorbance of progesterone between 230 and 280 nm (Fig. 6).

Red-edge excitation spectra are used to monitor local motions (flexibility) around the fluorophores.[29,30] Trp residues and their direct microenvironment (which consists of the dipole of both surrounding amino acids and solvent molecules) are associated by their dipoles. The dipoles referred to here are the result of the charge distribution in the molecular plane. The excitation of the solution results in a redistribution of electronic charge on the fluorophore inducing a significant change in both the direction and strength of its dipole moment. If the dipole of the fluorophore microenvironment is able to relax before fluorophore emission, then this environment is considered to be fluid. This flexibility may induce that of the Trp. The emission maximum from a relaxed state does not change with the excitation wavelength, while an emission maximum from a non-relaxed state will depend on it. Emission maxima are compared

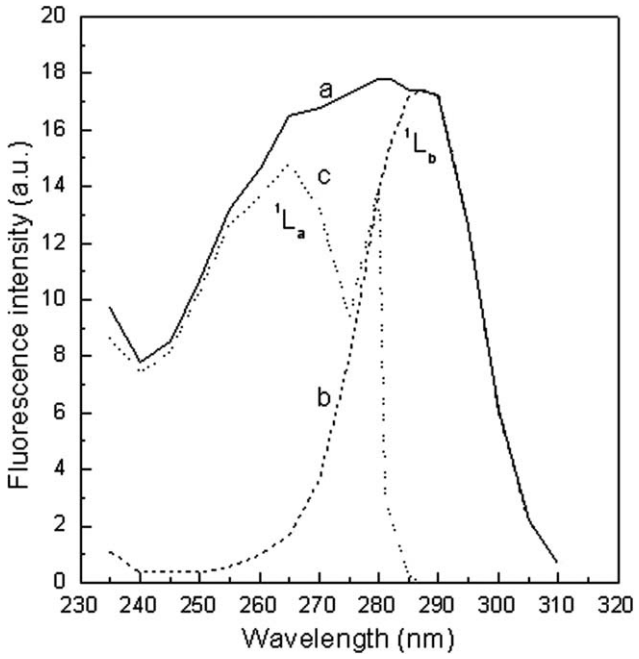


**Figure 5.** Fluorescence excitation spectra of 25 μM L-Trp in PBS buffer in the absence (a) and in the presence (b) of 416 μM progesterone ($\lambda_{em} = 360$ nm). Excitation and emission slits are 2.5 nm. Spectrum (c) is the difference between (a) and (b). Spectra (b) and (c) correspond to the $^1L_b$ and the $^1L_a$ transitions, respectively.

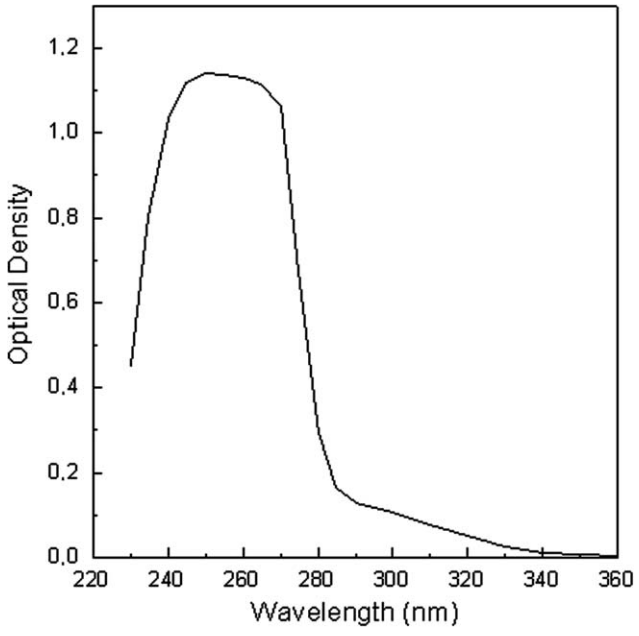


**Figure 6.** Absorption spectrum of 416 μM of progesterone bound to $\alpha_1$-acid glycoprotein. The high optical density from 220 to 280 allows the progesterone to totally abolish the $^1L_a$ transition.

only if the spectra are symmetric. Otherwise, the centers of gravity should be compared.

The maximum (340 nm) of the Trp fluorescence of the $\alpha_1$-acid glycoprotein in the presence of 415 μM progesterone does not change with the excitation wavelength (Fig. 7, $\lambda_{ex}$, 295, 300, and 305 nm), that is, microenvironments of the Trp residues are mobile in the presence of the ligand. As the emission spectra are asymmetric, we converted them to a wavenumber scale:

$$v = 1/\lambda \quad (1)$$

then, we compared the positions of their centers of gravity or mean wavenumber $\bar{v}$

$$\bar{v} = \frac{\sum I_i v_i}{\sum I_i} \quad (2)$$

where $I_i$ is the fluorescence intensity at the wavenumber $v_i$. The values of the centers of gravity are $2.924 \times 10^4$ cm$^{-1}$ (342 nm), $2.915 \times 10^4$ cm$^{-1}$ (343 nm), and $2.907 \times 10^4$ cm$^{-1}$ (344 nm) at $\lambda_{ex}$, 295, 300, and 305 nm, respectively.

The calculated emission spectra obtained by assuming the position of the maximum equal to the center of gravity are shown in Figure 7. These results clearly indicate that the microenvironment of the Trp residues has a residual motion independent of the global rotation of the protein, and this may induce a local motion of the Trp residues. Thus, binding of progesterone at saturating concentrations to $\alpha_1$-acid glycoprotein does not hinder residual motion of Trp residues microenvironment.

Shown in Figure 8 is the variation of the steady state anisotropy of Trp residues of $\alpha_1$-acid glycoprotein as a function of emission wavelength in absence (squares) and in the presence of 545 μM progesterone (circles). Experiments were performed at an excitation wavelength equal to 300 nm to avoid excitation of tyrosine residues. Also, by exciting at the red-edge of the absorption spectrum, energy transfer between Trp residues is

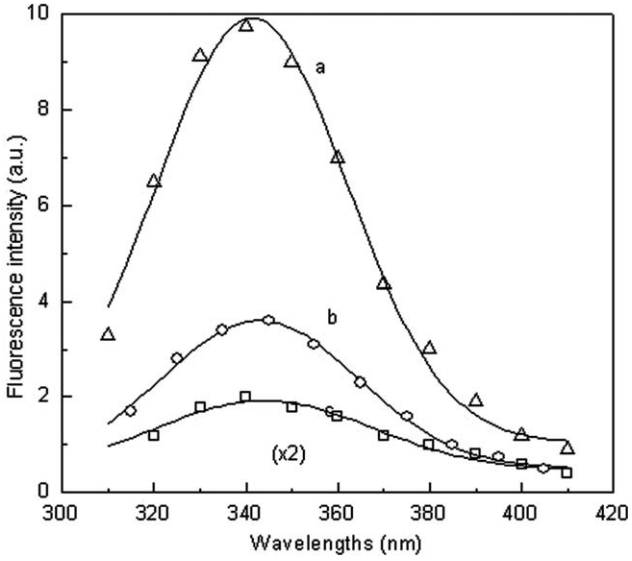


**Figure 7.** Red-edge of Trp residues of $\alpha_1$-acid glycoprotein in the presence of 415 μM progesterone; $\lambda_{ex} = 295$ (a), 300 (b), and 305 nm (c). The emission peak is the same (340 nm) at all excitation wavelengths, that is, the Trp residues environment still displays residual motion in the presence of progesterone.

eliminated as a possible depolarizing process.[31] However, excitation at the red edge of the absorption spectra decreases the amount of light absorbed by the fluorophore and increases the possibility of contributions from scattered light. For this reason, we corrected for the Raman effect of the buffer. In all instances, the Raman effect did not account for more than 5% of the total signal. In the presence of progesterone, the experimental error is more important than in its absence, which makes it difficult to tell in which case the anisotropy is higher. Regardless whether the ligand is present or not, the anisotropy decreases along the emission wavelength.

## 4. Discussion

Fluorescence emission of $\alpha_1$-acid glycoprotein occurs from the two classes of Trp residues: the surface Trp-160 residue and the Trp-25 and 122 residues. In the present work, we showed that the fluorescence of one of the two hydrophobic Trp residues has been completely quenched. This Trp residue is located in the hydrophobic domain of the protein pocket at the binding site of progesterone ($\lambda_{max}$, 325 nm and bandwidth = 49 nm). The remaining fluorescence spectrum observed at high concentrations of progesterone ($\lambda_{max} = 340$ nm and a bandwidth of 53 nm) is the result of the emission of Trp-160 residue and the non-quenched hydrophobic Trp residue and not from a structural modification around the Trp residues. It is clear that one of the two hydrophobic Trp residues is not part of the binding site of progesterone, otherwise the remaining emission spectrum would display a maximum around 350 nm with a bandwidth equal to 59 nm. Our results are in good agreement with those already found by other authors,[3,22] indicating that only one Trp residue seems to be involved in progesterone binding site in $\alpha_1$-acid glycoprotein. However, there is no agreement on which Trp residue is involved, Trp-25[3] or Trp-122.[22]

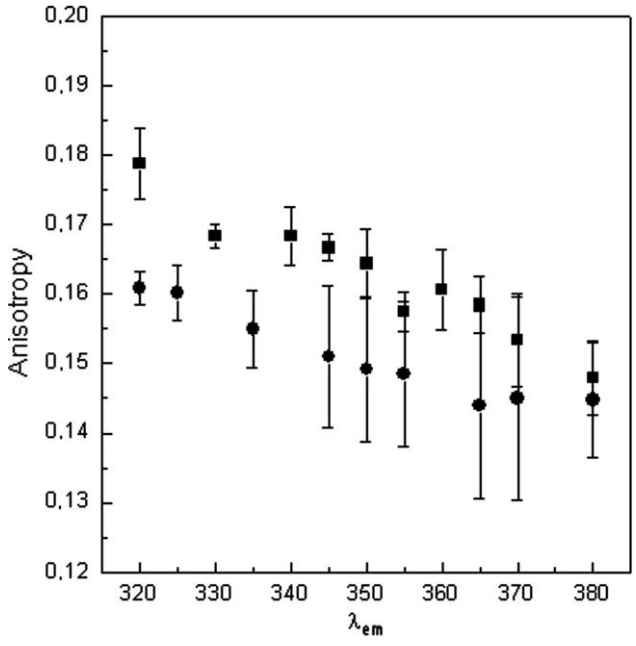


**Figure 8.** Fluorescence anisotropy of Trp residues of $\alpha_1$-acid glycoprotein in the absence (squares) and in the presence (circles) of 545 μM progesterone; $\lambda_{ex} = 300$ nm.

Comparison of the experiments performed with free L-Trp in solution and $\alpha_1$-acid glycoprotein indicates that emission modifications recorded for Trp residues of $\alpha_1$-acid glycoprotein in presence of saturating concentrations of progesterone are the result of the presence in the protein of a definite space (the pocket), where progesterone can bind and where a hydrophobic Trp residue is present. Also, one may not exclude that partial interaction could occur with the second hydrophobic Trp residue whether directly or indirectly. However, if this is the case, we do not understand why at very significant concentrations of progesterone, complete quenching of this second hydrophobic Trp residue did not occur. Therefore, the second hydrophobic Trp residue does not belong to the binding site of progesterone and is probably far from the binding site.

It is clearly indicated in Figure 3 that fluorescence quenching of Trp residues in $\alpha_1$-acid glycoprotein in presence of progesterone dissolved in ethanol (Fig. 1) is not the result of ethanol. The solvent can disrupt proteins structure, however, in $\alpha_1$-acid glycoprotein, the presence of a larger number of carbohydrate residues prevents any partial or total denaturation of the protein by ethanol.

The absence of red-edge excitation shift for Trp residues is in agreement with the fact that emission occurs from a relaxed excited state, that is, fast motions around Trp residues do exist. Binding of progesterone to the protein does not inhibit local motions of two emitting Trp residues. Anisotropy experiments as a function of emission wavelengths indicate that Trp residues display residual motions in absence and in presence of progesterone and that energy transfer between Trp residues is occurring. This energy transfer occurs mainly between the two non-quenched Trp residues by progesterone.

We did not measure fluorescence lifetimes of Trp residues of $\alpha_1$-acid glycoprotein in presence of progesterone because the three Trp residues contribute to all the protein emission spectra. Also, fluorescence lifetimes do not necessarily give the same information as other fluorescence parameters. Although many authors try to attribute the fluorescence origin of Trp to the presence of rotamers, this simplified model does not take into consideration the structure or the dynamics of the environment surrounding the Trp nor does it explain the origin of the multiple fluorescence lifetimes in proteins. Fluorescence of $\alpha_1$-acid glycoprotein is complex and simple lifetime analyses are not sufficient to explain the origin of the fluorescence lifetimes. Com-

paring Figures 1 and 2 from our work shows in fact that the presence of a structure (a pocket) around a Trp residue in $\alpha_1$-acid glycoprotein allows interaction between progesterone and fluorophore. Therefore, the measured fluorescence parameters are dependent on the structure of the fluorophore itself and on its environment.

Another aspect of this work is fundamental and concerns the possibility of using high progesterone concentrations as a filter of the $^1L_a$ state. Trp has two overlapping $S_{0^-} \rightarrow S_1$ transitions ($^1L_a$ and $^1L_b$), which are perpendicular to each other. Both the $S_{0^-} \rightarrow {}^1L_a$ and $S_{0^-} \rightarrow {}^1L_b$ transitions occur in the 260–300 nm range. In polar solvents, $^1L_a$ has a lower energy than $^1L_b$ and emission from this lowest state will be observed. Addition of a hydrophobic molecule such as progesterone that strongly absorbs between 220 and 280 nm abolishes $^1L_a$ state in favor of $^1L_b$ one. Therefore, in presence of saturating concentrations of progesterone and by selecting excitation wavelength, emission of Trp residues occurs only from $^1L_b$ state. However, this does not mean that $S_{0^-} \rightarrow {}^1L_a$ transition is only one responsible for the emission of a specific hydrophobic Trp residue in $\alpha_1$-acid glycoprotein. This conclusion is in good agreement with the fact that the presence of progesterone in solution with free L-Trp abolishes $^1L_a$ transition without modifying fluorescence emission spectrum of the amino acid. Also, it is important to indicate that emission spectra of $\alpha_1$-acid glycoprotein and of free Trp in solution were recorded at $\lambda_{ex} = 295$ nm to avoid absorption of progesterone and to excite mainly $S_{0^-} \rightarrow {}^1L_b$ transition. While intensity of Trp residues in $\alpha_1$-acid glycoprotein decreases as a result of progesterone binding to the protein, the fluorescence intensity of free Trp in solution is not modified due to the lack of interaction with progesterone.

In general, after purification, proteins are crystallized and then studied by X-ray diffraction. After that, results obtained from fluorescence experiments on protein in solution are interpreted by comparison with the X-ray data. However, to date no crystallographic data have been reported for $\alpha_1$-acid glycoprotein, presumably due to the lack of crystals suitable for X-ray diffraction studies. For this reason, we have focused on fluorescence studies because the method can provide information on the structure and dynamics of the macromolecule.

Recently, we demonstrated the presence of a pocket in $\alpha_1$-acid glycoprotein by studying hemin binding to the protein in the presence of TNS.[32] The extrinsic fluorophore binds to $\alpha_1$-acid glycoprotein with a dissociation constant of 60 μM and a stoichiometry of 1:1. The nature of the binding site is hydrophobic although contacts with a polar environment do exist.[33] Addition of hemin, a co-factor that binds to the hydrophobic pocket of hemoproteins, to a solution of a fixed amount of $\alpha_1$-acid glycoprotein–TNS complex induces a complete abolishment of TNS fluorescence by 1 mol hemin/mol of $\alpha_1$-acid glycoprotein. Thus, TNS and hemin bind to same site on $\alpha_1$-acid glycoprotein, a hydrophobic pocket. This straightforward experiment allowed us for the first time to prove the presence of a hydrophobic pocket in $\alpha_1$-acid glycoprotein. Experiments with hemin and others conducted with calcofluor white, a fluorophore that binds to the carbohydrate residues of $\alpha_1$-acid glycoprotein,[16] allowed us to conclude that protein pocket is formed by two domains: a hydrophobic one where ligands such as TNS, progesterone and hemin bind, and a hydrophilic one where carbohydrate residues are linked.

The diameter of hemin is estimated to be 12 Å.[34] Thus, the volume that hemin occupies within the $\alpha_1$-acid glycoprotein pocket is approximately equal to 904 Å$^3$. This volume is very small compared to that of $\alpha_1$-acid glycoprotein (≈81,600 Å$^3$). However, the pocket has a much larger size than the volume occupied by hemin as two domains, hydrophobic and hydrophilic, form it and it is open to the solvent.

Binding studies between $\alpha_1$-acid glycoprotein and progesterone performed at 4 and 50 °C have shown that the affinity decreases drastically when the temperature increases. In contrast, the temperature effect on the progesterone–human serum albumin complex was found to be very slight.[9] The data obtained by these authors can be explained by the fact that binding site of progesterone on $\alpha_1$-acid glycoprotein presents important motions[15,21] and this binding site is very accessible to the solvent.[15]

In two different interaction studies between $\alpha_1$-acid glycoprotein and progesterone[35] or propranolol,[36] it was shown that a sialic acid residue, the terminal carbohydrate on the carbohydrate chains, does not play an essential role in the binding of the two ligands on the $\alpha_1$-acid glycoprotein. This can be explained by fact that binding sites of the two ligands are within the protein pocket far from the sialic acid residues. These sugar residues are highly mobile and are present on the protein surface and not within the pocket.

$\alpha_1$-Acid glycoprotein belongs to lipocalins, a family of ligand binding proteins that appear to display a similarity of function and primary and tertiary structure.[37,38] The term lipocalin is derived from the Greek words 'lipos', meaning fat, and 'calyx', meaning cup.[37] The crystal structures of several lipocalins have been solved and show an eight-stranded anti-parallel beta-barrel fold well conserved within the family. These proteins possess a main cavity delineated by the β-barrel that is not shielded from the solvent although its wall is formed by hydrophobic residues.[39]

Therefore, the model we described using fluorescence spectroscopy in which we showed the presence of a pocket in $\alpha_1$-acid glycoprotein is in good agreement with that already described to other lipocalin family proteins. However, we should be cautious by indicating that in

the absence of any crystallographic data, exact spatial conformation of $\alpha_1$-acid glycoprotein is not yet defined.

In conclusion, this work shows that upon binding of progesterone to $\alpha_1$-acid glycoprotein, structure and dynamics of the protein are not altered. Also, binding of progesterone occurs near a hydrophobic Trp residue within the pocket. These results, which were obtained at saturating concentrations of progesterone, can be extrapolated to physiological concentrations. Binding site of progesterone in $\alpha_1$-acid glycoprotein includes one hydrophobic Trp residue, Trp-122 or Trp-65, and binding of progesterone to the protein does not alter the structure of the protein. It is clear that at physiological conditions, complete quenching of the hydrophobic Trp residue of the binding site does not occur as we observed in the presence of high progesterone concentrations. These results appear to indicate that structural and dynamical conservation of $\alpha_1$-acid glycoprotein are important for and associated with at least one of its functions: binding and regulating the level of progesterone in human plasma.

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