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Interaction between carbohydrate residues of α_1-acid glycoprotein (orosomucoid) and progesterone. A fluorescence study

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Abstract

Interaction between progesterone and the carbohydrate residues of α_1-acid glycoprotein was followed by fluorescence studies using calcofluor white. The fluorophore interacts with polysaccharides and is commonly used in clinical studies. Binding of progesterone to the protein induces a decrease in the fluorescence intensity of calcofluor white, accompanied by a shift to the short wavelengths of its emission maximum. The dissociation constant of the complex was found equal to 8.62 μM. Interaction between progesterone and free calcofluor in solution induces a low decrease in the fluorescence intensity of the fluorophore without any shift of the emission maximum. These results show that in α_1-acid glycoprotein, the binding site of progesterone is very close to the carbohydrate residues. Fluorescence intensity quenching of free calcofluor in solution with cesium ion gives a bimolecular diffusion constant (k_q) of 2.23×10^9 M^{-1} s^{-1}. This value decreases to 0.19×10^9 M^{-1} s^{-1} when calcofluor white is bound to α_1-acid glycoprotein. Binding of progesterone does not modify the value of k_q of the cesium. Previous studies have shown that the terminal sialic acid residue is mobile, while the other glycannes are rigid [Albani, J. R.; Sillen, A.; Coddeville, B.; Plancke, Y. D.; Engelborghs, Y. *Carbohydr. Res.* **1999**, *322*, 87–94]. Red-edge excitation spectra and Perrin plot experiments performed on sialylated and asialylated α_1-acid glycoprotein show that binding of progesterone to α_1-acid glycoprotein does not modify the local dynamics of the carbohydrate residues of the protein.

Keywords: Calcofluor white; α_1-Acid glycoprotein; Carbohydrate residues; Fluorescence titration; Fluorescence intensity quenching; Red-edge excitation spectra; Fluorescence anisotropy

1. Introduction

α_1-Acid glycoprotein (orosomucoid) is a small acute-phase glycoprotein ($M_r = 41{,}000$) that is negatively charged at physiological pH. It consists of a chain of 181 amino acids, contains 40% carbohydrate by weight and has up to 16 sialic acid residues (10–14% by weight).[2] Five heteropolysaccharide groups are linked via an N-glycosylic bond to the asparaginyl residues of the protein.[3]

Although the biological function of α_1-acid glycoprotein is still obscure, a number of activities of possible physiological significance have been described such as the ability to bind drugs like warfarin,[4] heparin[5] and a steroid hormone such as progesterone.[6] Many of these activities have been shown to be pathophysiologically dependent.[7–9]

α_1-Acid glycoprotein contains three Trp residues. One residue, Trp-166, is at the surface of the protein, and two are located in the protein matrix.[2,3,10] The three Trp residues contribute to the fluorescence of the protein.[11,12] Binding of progesterone to α_1-acid glycoprotein occurs near the hydrophobic Trp-25 residue[2] that belongs to the N-terminal side chain. The five carbohydrate units are linked to this portion of the polypeptide moiety,[13] i.e., the N-terminal fragment is in contact with the solvent. Recently, we showed that the N-terminal fragment would adopt a spatial conformation so that a pocket in contact with the buffer is induced, and to which Trp-25 residue belongs. The five carbohydrate units are linked to the pocket. Thus, progesterone can bind directly to this pocket since it diffuses from the buffer immediately to its binding site within or at the surface of the pocket.[14]

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Calcofluor white is a fluorescent probe capable of making hydrogen bonds with β-(1→4)- and β-(1→3)-polysaccharides.[15] It interacts preferentially with the glycan residues of α_1-acid glycoprotein, the interaction being dependent on their spatial conformation.[16]

In the present work, we studied the interaction (directly and/or indirectly) between progesterone and the carbohydrate units of α_1-acid glycoprotein, using calcofluor white as a probe. Since the carbohydrate residues are hydrophilic and the progesterone is hydrophobic, they should not interact together. However, in the case of these two entities, are located within a definite space such as a pocket, the interaction between them would be possible.

Our results have shown that binding of progesterone to α_1-acid glycoprotein induces a decrease in the fluorescence intensity of calcofluor white accompanied by a shift to the short wavelengths of its emission maximum. Interaction between progesterone and free calcofluor in solution induces a decrease in the fluorescence intensity of the fluorophore without any shift of the emission maximum. These results show that in α_1-acid glycoprotein, the binding site of progesterone is very close to the carbohydrate residues.

Effects of the binding of progesterone on the dynamics of the carbohydrate residues were also studied. Fluorescence intensity quenching of free calcofluor in solution with cesium ion compared to that observed when the fluorophore is bound to α_1-acid glycoprotein indicates that the calcofluor is partly shielded from the cesium. Binding of progesterone does not modify the value of the diffusion constant of the cesium. Previous studies have shown that the terminal sialic acid residue is mobile while the other glycannes are rigid.[1]

In the present work, red-edge excitation spectra and Perrin plot experiments performed on sialylated and asialylated α_1-acid glycoprotein show that binding of progesterone to α_1-acid glycoprotein does not modify the local dynamics of the carbohydrate residues of the protein.

2. Materials and methods.

α_1-Acid glycoprotein was purified from serum by a successive combination of ion-displacement chromatography, gel-filtration and ion-exchange chromatography as already described.[17] The lyophilized protein was dissolved in a 10 mM phosphate–0.143 M NaCl buffer, pH 7. Its concentration was determined spectrophotometrically using an extinction coefficient of 29.7 mM^{-1} cm^{-1} at 278 nm.[18] Asialylation of α_1-acid glycoprotein was performed with *Clostridium perfringens* neuraminidase, as already described.[19] Progesterone (from Sigma) was dissolved in methanol. The concentration of the stock solution was 2.5 mM. The stock solution of CsCl (from Sigma) was 2 M. Calcofluor white was from Sigma. Its concentration was determined spectrophotometrically using an extinction coefficient of 4388 M^{-1} cm^{-1} at 352.7 nm.[16] 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. Titration experiments were performed at λ_{ex} of 310 nm. Binding of progesterone to α_1-acid glycoprotein was obtained by adding aliquots of progesterone (4.8 μM) to a solution of 8 μM of α_1-acid glycoprotein and 42 μM calcofluor. In parallel, a control experiment was performed by titrating 28 μM of calcofluor with aliquots of progesterone (4.8 μM each). The bandwidths used for the excitation and the emission were 2.5 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. Corrections for the inner filter effect were not necessary since the absorption of progesterone and cesium was not significant at the excitation and emission wavelengths.

The Perrin plot was obtained from anisotropy data measured with the same instrument. The bandwidths used for the excitation and the emission were 5 nm.

Fluorescence lifetime data of calcofluor free in water or when bound to α_1-acid glycoprotein were previously obtained at 20 °C using multifrequency phase fluorometry with the cross-correlation method as described.[1,20]

The fluorescence intensity decay of calcofluor, whether free in solution or bound to the carbohydrates of α_1-acid glycoprotein, is nonexponential.[21] When the fluorophore is free in solution, the intensity average fluorescence lifetime $\langle\tau\rangle$ is 0.85 ns. It increases to 4.8 and 3.9 ns when the fluorophore is bound to the sialylated and asialylated α_1-acid glycoprotein, respectively. This average lifetime was used to calculate the bimolecular diffusion constants and the rotational correlation times from the Perrin plot.

All experiments were performed in a 10 mM phosphate–0.143 M NaCl buffer, pH 7.

3. Results

Titration experiments.—Binding of progesterone to sialylated α_1-acid glycoprotein induces a decrease in the fluorescence intensity of calcofluor, accompanied by a shift to shorter wavelengths of the emission maximum. The maximum is located at 438 nm in absence of progesterone and shifts to 431 nm in presence of 62 μM of progesterone (data not shown).

The intensity decrease is the result of the binding of progesterone to the protein. Plotting the fluorescence intensity at a fixed wavelength (440 nm) as a function

of progesterone concentration yields a monophasic curve, suggesting one binding site (Fig. 1(a)). The fluorescence intensity decrease can be described by the following balance of fluorescence:

$$Flu = \frac{Flu_0 \times (L_0 - L_b) + Flu_1 \times L_b}{L_0} \quad (1)$$

where Flu is the observed fluorescence, Flu_0 and Flu_1 are the fluorescence of free and bound calcofluor, respectively, and L_0 and L_b are the concentrations of total and bound calcofluor.[21]

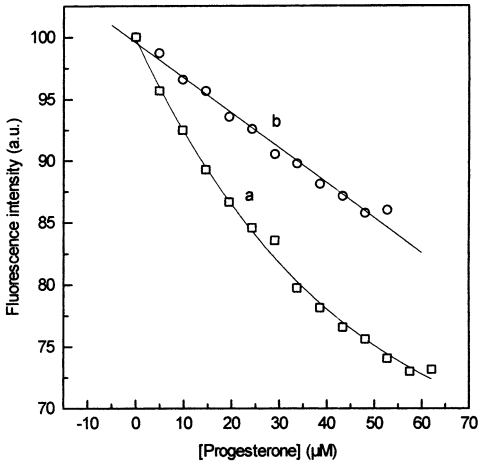

Fig. 1. (a) Fluorescence intensity variation of 42 μM of calcofluor bound to 8 μM of α_1-acid glycoprotein as a function of progesterone. $\lambda_{ex} = 310$ nm and $\lambda_{em} = 440$ nm. The fluorescence intensities are corrected for the dilution. (b) Fluorescence intensity variation of free calcofluor in presence of increasing concentrations of progesterone. Conditions: Temperature = 20 °C, 10 mM phosphate–0.143 M NaCl buffer, pH 7.

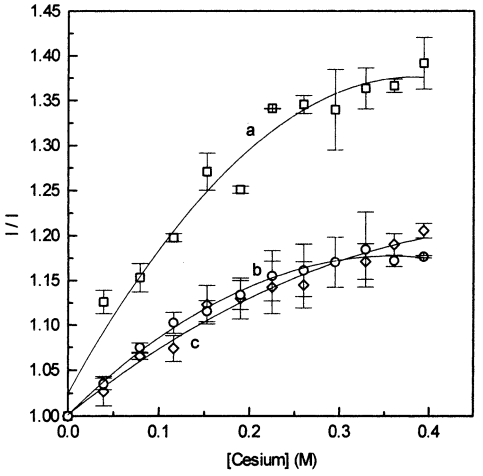

Fig. 2. Fluorescence intensity quenching with cesium of calcofluor free in solution (a), the calcofluor–α_1-acid glycoprotein complex (b) and the calcofluor–α_1-acid glycoprotein–progesterone complex (c). $\lambda_{ex} = 330$ nm and $\lambda_{em} = 430$ nm. [calcofluor] = [α_1-acid glycoprotein] = 5 μM. [Progesterone] = 12.5 μM.

The intensity decrease is clearly hyperbolic, and therefore a mathematical binding analysis can be performed using the following quadratic equation obtained from the definition of the equilibrium constant:

$$L_b = 0.5[(nP_0 + L_0 + K_d) - \{(P_0 + L_0 + K_d)^2 - 4nP_0L_0\}^{1/2}] \quad (2)$$

where P_0 is the protein concentration. The parameter Flu_1 was obtained by extrapolation from a reciprocal plot and was found to be 10. The dissociation constant K_d of the α_1-acid glycoprotein–progesterone complex was found equal to 60 ± 1 μM.

Addition of progesterone to a solution of calcofluor induces a decrease in the fluorescence intensity of the fluorophore without any shift in the emission maximum (data not shown). The decrease of the fluorescence intensity is linear (Fig. 1(b)), indicating the difficulty in obtaining a complex between free calcofluor and progesterone. Therefore, the shift observed in Fig. 1 indicates that progesterone binds to α_1-acid glycoprotein in proximity of calocofluor, i.e., near the glycosysation site.

Fluorescence intensity quenching with cesium.— Quenching of the fluorescence intensity of calcofluor by cesium is the result of the dynamic interaction between the two molecules. Dynamic fluorescence quenching was analyzed by the Stern–Volmer equation:[22]

$$I_0/I = 1 + K_{SV}[Q] = 1 + k_q\langle\tau\rangle[Q] \quad (3)$$

where I_0 and I are the fluorescence intensities in the absence and presence of quencher respectively, k_q the bimolecular diffusion constant, $\langle\tau\rangle$ the mean fluorescence lifetime and $[Q]$ the concentration of cesium added. Fig. 2 shows the Stern–Volmer plots of the intensity quenching of free calcofluor in water (a) and of calcofluor bound to sialylated α_1-acid glycoprotein in absence (b) and in presence (c) of progesterone. The Stern–Volmer plot of the free fluorophore intensity quenching is not linear as it would be expected. However, when we performed the experiments in pure water, we obtained linear plots (data not shown), indicating that the curvature obtained could be the result of the presence of the salt (NaCl) of the buffer. This salt would aggregate some of the calcofluor in absence and in presence of protein. The initial slope of the Stern–Volmer plot obtained in presence of buffer is equal to the slope of the linear plot obtained in water and thus will give the value of the dynamic quenching constants.

The Stern–Volmer constants K_{SV} calculated from the fluorescence intensity quenching of free calcofluor (Fig. 2(a)) and from that of the calcofluor bound to α_1-acid glycoprotein in absence (Fig. 2(b)) and in the presence (Fig. 2(c)) of progesterone are 1.9 ± 0.2, 0.9 ± 0.15 and 0.7 ± 0.2 M^{-1}, respectively. Thus, cesium is accessible to the bound calcofluor on α_1-acid glycoprotein. The

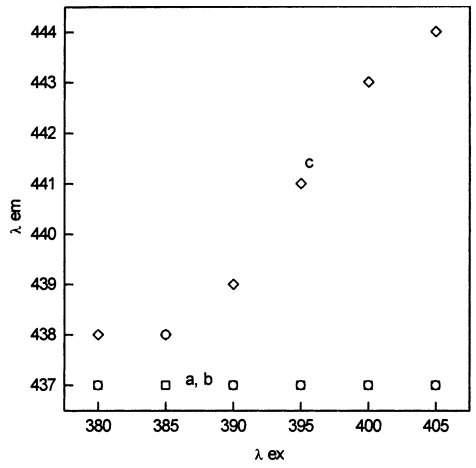

Fig. 3. Position of the emission maximum as a function of the excitation wavelength of 5 μM of calcofluor free in solution (a), in presence of 5 μM of sialylated α_1-acid glycoprotein and 12.5 μM of progesterone (b), and of 5 μM of asialylated α_1-acid glycoprotein and 12.5 μM of progesterone (c) as a function of the excitation wavelength. Note that, only in presence of asialylated α_1-acid glycoprotein, a shift of 6 nm is observed as the function of the excitation wavelength.

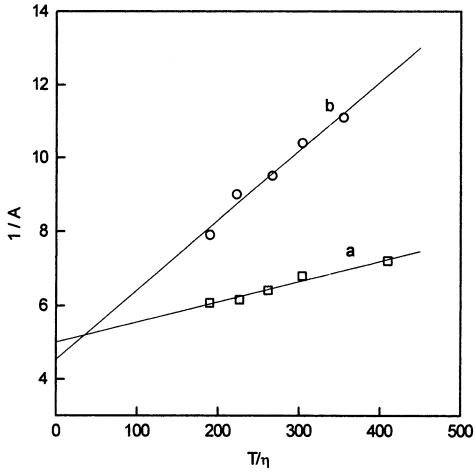

Fig. 4. Steady-state fluorescence anisotropy vs. temperature over viscosity for 8 μM of calcofluor in presence of 5 μM of asialylated α_1-acid glycoprotein and 10 μM of progesterone (λ_{ex}, 300 nm and λ_{em}, 440 nm) (plot 6.a) and of equimolar (10 μM) concentrations of calcofluor, sialylated α_1-acid glycoprotein and progesterone (plot 6.b). The data are obtained by thermal variation in the range of 5–38 °C. The ratio T/η is expressed in Kelvins over centipoise.

presence of progesterone does not affect significantly this accessibility. However, this accessibility is lower than that observed for free calcofluor in solution, since the probe is surrounded by carbohydrate residues that decrease the frequency of the collisions with the quencher. The bimolecular diffusion constant of cesium is equal to $2.23 \pm 0.24 \times 10^9$, $0.19 \pm 0.03 \times 10^9$ and $0.14 \pm 0.04 \times 10^9$ M^{-1} s^{-1} when the interaction occurs with free calcofluor and calcofluor bound to α_1-acid glycoprotein in absence and in presence of progesterone, respectively. Thus, diffusion of cesium in the vicinity of calcofluor when bound to α_1-acid glycoprotein is hindered by the surrounding carbohydrate residues and by the protein matrix. Binding of progesterone does not significantly modify the diffusion of cesium in the protein.

Red-edge excitation spectra.—Red-edge excitation spectra are used to monitor local motions (flexibility) around the fluorophores.[23,24] 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 calcofluor. 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.

Fig. 3 displays the position of the emission maximum of 5 μM of calcofluor free in solution (a) in presence of 5 μM of sialylated α_1-acid glycoprotein and 12.5 μM of progesterone (b) and of 5 μM of asialylated α_1-acid glycoprotein and 12.5 μM of progesterone (c) as a function of the excitation wavelength. We notice that only in presence of asialylated α_1-acid glycoprotein, a shift of 6 nm is observed as the function of the excitation wavelength. This is taken as direct evidence that the carbohydrate residues of asialylated α_1-acid glycoprotein in the microenvironment of calcofluor exhibit restricted flexibility, i.e., the fluorophore would follow the motion of the protein, suggesting a rotational correlation time equal to that of the protein. These results are similar to those obtained in absence of progesterone.[1,21] Thus, binding of the hormone to its site on α_1-acid glycoprotein does not induce any apparent or significant modification in the dynamics of the carbohydrate residues.

Steady-state anisotropy as a function of temperature.—The rotational correlation time Φ_p of a hydrated sphere is obtained from the equation

$$\Phi_p(T) = 3.8\eta(T) \times 10^{-4} M \quad (4)$$

where M is the protein molecular weight and η the viscosity of the medium.[25]

α_1-Acid glycoprotein has 16 sialic acid residues of molecular weight of 314 each. Thus, molecular weight of asialylated α_1-acid glycoprotein is near 36,000. As α_1-acid glycoprotein (sialylated and asialylated forms) can be considered as spherical,[26] Eq. (4) yields a rotational correlation time of 16 and 14 ns for the sialylated and asialylated protein, respectively.

Steady-state fluorescence anisotropy of 10 μM of calcofluor in presence of 5 μM of asialylated α_1-acid glycoprotein ($\lambda_{em} = 440$ nm and $\lambda_{ex} = 300$ nm) was performed at different temperatures. A Perrin plot representation (Fig. 4(a)) based on Eq. (5):[27]

$$1/A = 1/A_0 + \langle\tau\rangle/\Phi_R A_0 = 1/A_0 + (1/A_0)(1 + RT\langle\tau\rangle/\eta V) \quad (5)$$

where A and A_0 are the anisotropies in the presence and the absence of rotational diffusion respectively, $\langle\tau\rangle =$ 3.9 ns, η, V and Φ_R are the mean fluorescence lifetime, the viscosity, the fluorophore rotational volume and its rotational correlation time, respectively. This plot enables us to obtain information concerning the motion of the fluorophore. When the fluorophore is tightly bound to the protein, its motion will correspond to that of the protein. In this case, Φ_R will be equal to Φ_p and A_0 obtained experimentally with Eq. (5) will be equal to that obtained at -45 °C. At this temperature, α_1-acid glycoprotein does not show any residual motion.[28] When the fluorophore exhibits significant motions when bound to the protein, Φ_R will represent an apparent rotational correlation time Φ_A. Φ_A will be the result of two motions, that of the protein and that of the segmental motion of the fluorophore. Also, in the presence of a segmental motion, the extrapolated value of A, $A(0)$ will be lower than the A_0 value obtained at -45 °C. However, since at λ_{ex} of 300 nm, both calcofluor and Trp residues absorb, the value of $A(0)$ at extrapolation cannot be used to calculate the amplitude, if any, of the residual motions.

The rotational correlation time of the fluorophore Φ_p using 3.9 ns as average lifetime is found to be 12 ± 1 ns at 20 °C, which is very close to the theoretical value (14 ns) expected for asialylated α_1-acid glycoprotein revealing the absence of a segmental motion. Anisotropy results are in good agreement with those obtained by red-edge excitation spectra experiments, i.e., calcofluor is bound tightly to the carbohydrate residues and follows the global motion of the protein.

The Perrin plot obtained for 10 μM of calcofluor in presence of equimolar concentrations of sialylated α_1-acid glycoprotein and progesterone (Fig. 4(b)) yields a rotational correlation time of 4 ns. This value lower than that (16 ns) expected for sialylated α_1-acid glycoprotein is an apparent one and indicates that calcofluor displays segmental motions independent of the global rotation of the protein. The result confirms that obtained with red-edge excitation spectra experiment. Also, these results are identical to those obtained in the absence of progesterone.[1] Thus, binding of progesterone to α_1-acid glycoprotein does not affect the rigidity of the glycosylation site.

4. Discussion

This work reports for the first time a study concerning the interaction between progesterone and the carbohydrate residues of α_1-acid glycoprotein. In fact, calcofluor binds to the carbohydrate residues, and the variation in the fluorescence parameters observed upon binding of progesterone to the protein is the result of the interaction between the ligand and the carbohydrate residues.

Fluorescence parameters such as the intensity and the position of the emission maximum are sensitive to the modifications occurring in the microenvironment of the fluorophore. In fact, the energy of the emission is important when the environment of the fluorophore shows a low polarity, inducing by that a spectrum with a maximum located at low wavelength. Fluorescence intensity is proportional to the number of emitted photons, and thus relates to the polarity of the fluorophore. A low polarity will increase the number of emitted photons, and by the same way will increase the intensity of the fluorescence.[29,30]

Binding of progesterone to α_1-acid glycoprotein induces a decrease in the emission intensity and a shift in the position of the maximum, a shift absent when progesterone was added to calcofluor free in solution. Therefore, the result obtained in presence of α_1-acid glycoprotein clearly indicates that progesterone and calcofluor are very close, i.e., progesterone interacts with the carbohydrate residues of α_1-acid glycoprotein. This interaction is occurring in a defined region of the protein, where the presence of progesterone renders the region more and more hydrophobic, affecting by that the position of the maximum of the fluorescence spectrum of calcofluor. Since progesterone is a hydrophobic molecule, its interaction with the carbohydrate residues does not take place at the surface of the protein (a hydrophilic zone), but in a well-defined area of the protein that is the pocket formed by the N-terminal segment.

However, the changes in the fluorescence (blue shift and intensity quenching) do not necessarily indicate a direct (physical) interaction between progesterone and calcofluor. Progesterone could potentially bind at a separate site within the pocket inducing a protein conformational change within the pocket altering by that the fluorescence properties of the probe. Although this indirect mechanism cannot be ruled out, since the progesterone binds to the pocket where the five carbohydrate units are linked, a direct (physical) interaction between them cannot be excluded.

Fluorescence quenching with cesium experiments shows that calcofluor is still accessible to cesium when it is bound to α_1-acid glycoprotein. However, this accessibility is two times lower than that observed for calcofluor free in solution. More interesting is the value of the bimolecular diffusion constant that is 12 times higher when calcofluor is free in solution compared to that observed for calcofluor bound to α_1-acid glycoprotein. Thus, the diffusion of a cesium ion within the protein is reduced as the result of the presence of the carbohydrate residues that surround the protein including the pocket making by that a shield around calcofluor.

Also, carbohydrate residues of α_1-acid glycoprotein adopt a spatial conformation[1] that makes the diffusion

of cesium even more difficult than in the absence of any spatial conformation.

In recent work we found that binding of progesterone to α_1-acid glycoprotein increases the dynamics of the protein matrix.[14,28] In the present work, experiments with cesium ion indicate that binding of progesterone does not modify the local dynamics of the carbohydrate residues, since the bimolecular diffusion constant is identical in the absence and in the presence of progesterone. This could be explained by the fact that the glycosylation site and all of the carbohydrate residues but the terminal sialic acid are rigid.[1]

We also investigated the dynamics properties of the carbohydrate residues by fluorescence anisotropy studies and by the red-edge excitation spectral method. The two techniques are complementary since the red-edge excitation shift allows to study the flexibility of the microenvironment while anisotropy studies allow one to monitor the dynamics of the fluorophore itself.[29–31]

The results obtained in this work in presence of progesterone are identical to those observed in its absence.[1] Therefore, binding of progesterone to or in the vicinity of the carbohydrate residues of α_1-acid glycoprotein does not affect the local dynamics of the carbohydrate residues. This result is in good agreement with that obtained with quenching experiment with cesium in absence and in presence of progesterone.

In conclusion, our work confirms that the interaction between progesterone and the carbohydrate residues of α_1-acid glycoprotein is occurring within a pocket. This region is formed by the N-terminal fragment of the protein and contains two zones, one hydrophilic and the second hydrophobic.

Our results also show the importance of the carbohydrate residues in the interaction between α_1-acid glycoprotein and progesterone. Since the glycosylation site belongs to the pocket, our results demonstrate the physiological importance of this pocket in the activity of α_1-acid glycoprotein, especially when we know that many ligands of the protein share the same binding site.

The fact that the glycosylation site of α_1-acid glycoprotein is within the pocket would explain why the carbohydrate residues adopt a spatial conformation.[1,16,21] Such a conformation could be the key factor in determining the different physiological roles of the protein.

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