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Effect of binding of Calcofluor White on the carbohydrate residues of α_1-acid glycoprotein (orosomucoid) on the structure and dynamics of the protein moiety. A fluorescence study

Jihad R. Albani*

Laboratoire de Biophysique Moléculaire, Université des Sciences et Technologies de Lille, Bâtiment C6, 59655 Villeneuve d'Ascq, France

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Abstract

Calcofluor White is a fluorescent probe that interacts with polysaccharides and is commonly used in clinical studies. Interaction between Calcofluor White and carbohydrate residues of α_1-acid glycoprotein (orosomucoid) was previously studied at low and high concentrations of Calcofluor compared to that of the protein. α_1-Acid glycoprotein contains 40% carbohydrate by weight and has up to 16 sialic acid residues. At equimolar concentrations of Calcofluor and α_1-acid glycoprotein, the fluorophore displays free motions [Albani, J. R.; Sillen, A.; Coddeville, B.; Plancke, Y. D.; Engelborghs, Y. *Carbohydr. Res.* **1999**, *322*, 87–94], while at high concentration of Calcofluor, its surrounding microenvironment is rigid, inducing the rigidity of the fluorophore itself [Albani, J. R.; Sillen, A.; Plancke, Y. D.; Coddeville, B.; Engelborghs, Y. *Carbohydr. Res.* **2000**, *327*, 333–340]. In the present work, red-edge excitation spectra and steady-state anisotropy studies performed on Trp residues in the presence of Calcofluor, showed that the apparent dynamics of Trp residues are not modified. However, deconvoluting the emission spectra with two different methods into different components, reveals that the structure of the protein matrix has been disrupted in the presence of high Calcofluor concentrations. © 2001 Published by Elsevier Science Ltd.

Keywords: Calcofluor White; α_1-Acid glycoprotein; Carbohydrate residues; Trp residues; 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).[3] Five heteropolysaccharide groups are linked via an *N*-glycosylic bond to the asparaginyl residues of the protein.[4] The protein contains tetra-antennary as well as di- and tri-antennary glycans.

Although the biological function of α_1-acid glycoprotein is still obscure, a number of activities of possible significance have been described such as the ability to bind the β-drug adrenergic blocker, propranolol,[5] basic drugs like warfarin,[6] and certain steroid hormones such as progesterone.[7] Many of these activities have been shown to be dependent on the

* Tel.: +33-320-337770; fax: 33-320-459218.
E-mail address: jihad-rene.albani@univ.lille1.fr (J.R. Albani).

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glycoform of α_1-acid glycoprotein.[8] As the serum concentration of specific glycoforms of α_1-acid glycoprotein changes markedly under acute or chronic inflammatory conditions, as well as in pregnancy and tumor growth, a pathophysiological dependence change in the carbohydrate-dependent activities of the protein would occur.

α_1-Acid glycoprotein contains three Trp residues. One residue, Trp-160, is at the surface of the protein, and two are located in the protein matrix.[3,4,9] One of these two Trp residues, Trp-25, lies in a hydrophobic environment in a pocket formed by the *N*-terminal side chain of the protein.[10]

The dynamic behavior of each class of tryptophanyl residues was investigated by performing steady-state measurements of emission anisotropy and lifetime between -40 and $+20$ °C[11] and quenching resolved emission anisotropy at 20 °C with cesium.[10] Our results showed that both classes of Trp residues exhibit residual motions.

Calcofluor White is a fluorescent probe capable of making hydrogen bonds with β-(1→4) and β-(1→3) polysaccharides.[12] It is commonly used to study the mechanism by which cellulose and other carbohydrate structures are formed in vivo and is also widely used in clinical studies.[13,14]

Recently, we presented evidence that Calcofluor interacts preferentially with the glycan residues of α_1-acid glycoprotein, the interaction being dependent on their spatial conformation.[15] In a second study performed at equimolar concentrations of Calcofluor and α_1-acid glycoprotein, we showed that the sialic acid residues are highly mobile, while the other glycan residues exhibit restricted motions.[1] Since in vivo experiments are usually carried out at excess concentration of Calcofluor, we then investigated the effect of the interaction between Calcofluor and α_1-acid glycoprotein on the dynamics of the carbohydrate residues at high concentrations of fluorophore. Our results showed that at saturating concentrations of Calcofluor, its microenvironment is rigid.[2]

In the present work, we studied the effect of Calcofluor binding on α_1-acid glycoprotein on the structure and the dynamics of the protein moiety. The work was performed by fluorescence anisotropy studies and by the red-edge excitation spectra method. The two techniques are complementary since the red-edge excitation spectra allows one to study the flexibility of the microenvironment, while anisotropy studies allow one to monitor the dynamics of the fluorophore itself.[16,17]

Our results showed that the apparent rotation of the Trp residues and their microenvironment are not modified upon binding of Calcofluor to α_1-acid glycoprotein. However, the structure of the protein in the presence of saturated concentrations of Calcofluor is altered. Deconvoluting the spectrum recorded in the presence of high Calcofluor concentrations reveals the presence of three emission spectra located at 330, 337 and 355 nm. These peaks characterize the emission from Trp residues located in three different regions of the protein with different hydrophobicity.

2. Materials and methods

α_1-Acid glycoprotein was purified as described.[7] 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]

Calcofluor White was obtained from Sigma Chemical Co. Its concentration was determined spectrophotometrically using an extinction coefficient of 4388 M^{-1} cm^{-1} at 352.7 nm.[15] 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 5 nm. The quartz cuvettes had optical pathlengths equal to 1 and 0.4 cm for the emission and excitation wavelengths, respectively. Observed fluorescence intensities were corrected for the absorption at the excitation and emission wavelengths as described.[19,20] Finally, fluorescence spectra were corrected for the background intensities of the buffer solution.

At low Calcofluor concentration, the optical densities at three excitation wavelengths was less than 0.09, while it varied from 0.014 to

0.044 along the emission spectra. Therefore, the highest corrections of the fluorescence intensity for the inner filter effect did not exceed 6%. Also, the corrections did not change the position (334 nm) of the recorded emission spectra. At high Calcofluor concentration, the correction for the inner filter effect was more important than at low concentrations. In fact, the optical densities varied from 0.117 to 0.7. Upon correction for the inner filter effect, the intensity increase was one- to threefold depending on the emission wavelength. Also, corrections for the inner filter effect were shown to induce a shift in the emission maximum from 325 to 335 nm.

A Perrin plot was obtained from anisotropy data measured with the same instrument, ($\lambda_{ex} = 300$ nm and $\lambda_{em} = 330$ nm). The bandwidths used for the excitation and the emission were 10 nm. Values of A were obtained from parallel and perpendicular fluorescence intensities after subtraction of the Raman signal of the buffer solution.

Fluorescence lifetime data of the Trp residues in α_1-acid glycoprotein were previously obtained at 20 °C using multifrequency phase fluorometry with the cross-correlation method as already described.[21]

The fluorescence intensity decay of the Trp residues of α_1-acid glycoprotein is nonexponential. The mean fluorescence lifetime is the second order mean $<\tau> = 2.23$ ns and was used to calculate the rotational correlation time from the Perrin plot.

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

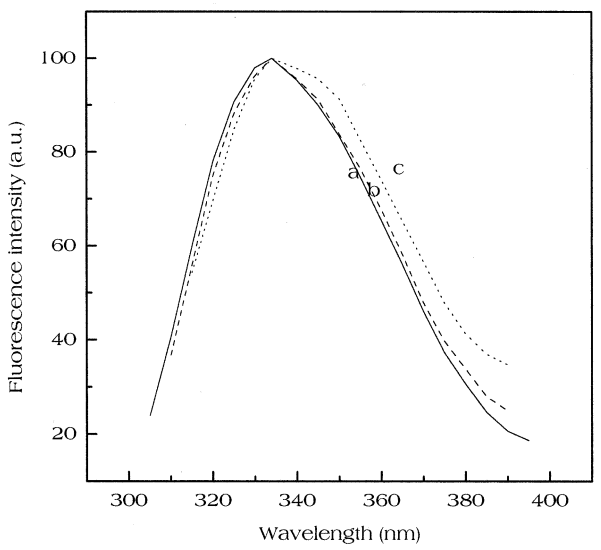

Fig. 1. Normalized steady-state fluorescence spectra of 15 μM α_1-acid glycoprotein in the presence of 10 μM Calcofluor recorded at three excitation wavelengths, 295 (a), 300 (b) and 305 nm (c). The fluorescence maximum is equal to 334 nm at the three excitation wavelengths.

3. Results

Dynamics of Trp residues of α_1-acid glycoprotein at low concentration of Calcofluor

Red-edge excitation spectra. Red-edge excitation spectra are used to monitor motions around the fluorophores.[22,23] 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 fluorophore results in a redistribution of its electronic charge inducing a significant change in both 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 motion may induce that of the Trp residues. 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. 1 displays the normalized fluorescence spectra of 15 μM α_1-acid glycoprotein in the presence of 10 μM Calcofluor obtained at three excitation wavelengths (295, 300 and 305 nm). The maximum (334 nm) of the emission of the Trp residues does not change with the excitation wavelength. This result indicates that the microenvironments of the Trp residues have a residual motion independent of the global rotation of the protein. This motion may induce a local motion of the Trp residues that can be detected with a Perrin plot.

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

$$\Phi_p(T) = 3.8\eta(T) \times 10^{-4} M \quad (1)$$

where M is the protein molecular weight and η the viscosity of the medium.[24] α_1-Acid glyco-

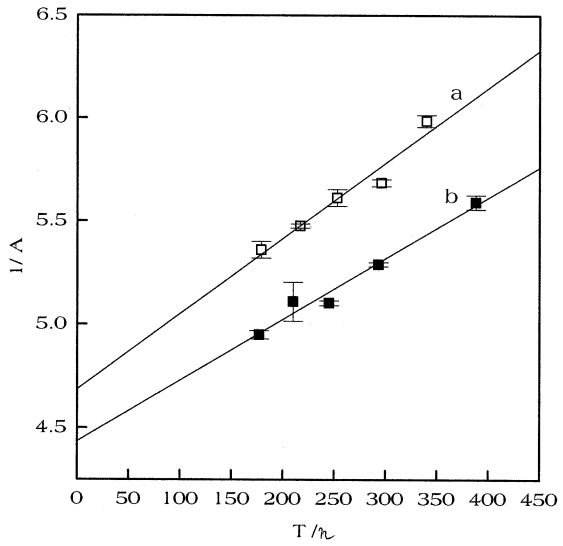

Fig. 2. Perrin plots for 15 μM α_1-acid glycoprotein in the presence of 10 μM Calcofluor (plot a) and in the presence of 150 μM Calcofluor (plot b) ($\lambda_{ex} = 300$ nm and $\lambda_{em} = 330$ nm). The data are obtained by thermal variation in the range of 5–35 °C. The ratio T/η is expressed in Kelvins over centipoise.

protein can be considered to be spherical.[25,26] Thus, Eq. (1) yields a calculated rotational correlation time of 16 ns for the protein.

Steady-state fluorescence anisotropy of 15 μM α_1-acid glycoprotein in the presence of 10 μM Calcofluor ($\lambda_{em} = 330$ nm and $\lambda_{ex} = 300$ nm) was performed at different temperatures. A Perrin plot representation (Fig. 2, plot a) based on Eq. (2)[27] is drawn:

$$1/A = 1/A_o + <\tau>/\Phi_R A_o$$
$$= 1/A_o + (1/A_o)(1 + RT<\tau>/\eta V) \qquad (2)$$

where A and A_o are the anisotropies in the presence and the absence of rotational diffusion, respectively, $<\tau> = 2.23$ 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_o obtained experimentally with Eq. (2) will be equal to that obtained at −45 °C. At this temperature, α_1-acid glycoprotein does not show any residual motion.[11] 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, $A(o)$ will be lower than the A_o value obtained at −45 °C. However, since at λ_{ex} of 300 nm, both Calcofluor and Trp residues absorb, the value of $A(o)$ at extrapolation cannot be used to calculate the amplitude, if any, of the residual motions.

The rotational correlation time Φ_A (8.5 ns at 20 °C) (Fig. 2, plot a) lower than that (16 ns) expected for α_1-acid glycoprotein as a whole is an apparent one, and indicates that Trp residues display segmental motions independent of the global rotation of the protein.

The red-edge excitation spectra and Perrin plot, as a function of temperature, give information on the mean motion of the Trp residues. Both experiments indicate that binding of Calcofluor at low concentration does not hinder the global motions of the Trp residues and their microenvironments.

Dynamics of Trp residues of α_1-acid glycoprotein in the presence of high concentration of Calcofluor

Red-edge excitation spectra. Fluorescence emission spectra of 14 μM α_1-acid glycoprotein in the presence of 160 μM Calcofluor were obtained at three excitation wavelengths (295, 300 and 305 nm). The maximum (335 nm) of the Trp residues emission did not vary with the excitation wavelength (data not shown). Thus, the microenvironments of the Trp residues display motions independent of the global rotation of the protein.

We noticed that the fluorescence spectra were disrupted compared to the spectra obtained in the absence of Calcofluor or to those obtained in the presence of low concentrations of Calcofluor (Fig. 1). Therefore, addition of Calcofluor at high concentrations affects the structure of the surrounding microenvironments of the Trp residues. We did not verify up to which concentration of Calcofluor, the local structure around the Trp residues is not modified.

Fig. 3 displays the fluorescence emission spectra of α_1-acid glycoprotein in the presence of 10 μM (a) and 160 μM of Calcofluor (b) both obtained at λ_{ex} of 295 nm. We notice that

in the presence of high concentrations of Calcofluor, the bandwidth of the spectrum increases by 6 nm and a shoulder at 350 nm appears. These spectrum disruptions indicate that a possible structural modification of the protein has occurred in the presence of high concentrations of Calcofluor, i.e., addition of Calcofluor at high concentrations would affect the structure of the surrounding microenvironments of the Trp residues.

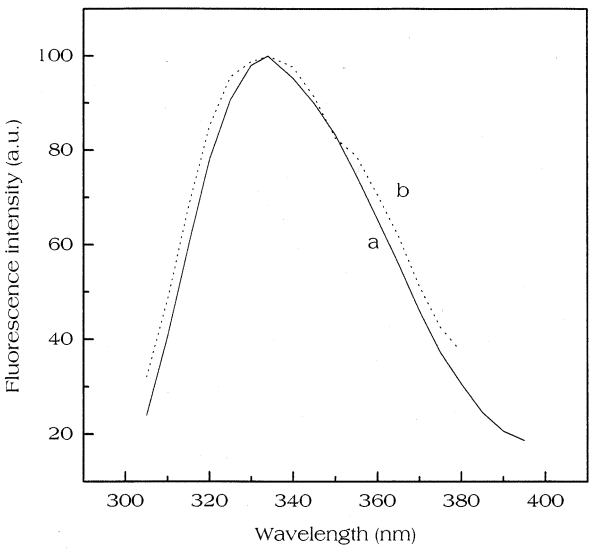

Fig. 3. Fluorescence emission spectra of α_1-acid glycoprotein in the presence of 10 (a) and 160 μM Calcofluor (b) both obtained at λ_{ex} of 295 nm. The bandwidth of the spectrum increases by 6 nm in the presence of high concentrations of Calcofluor.

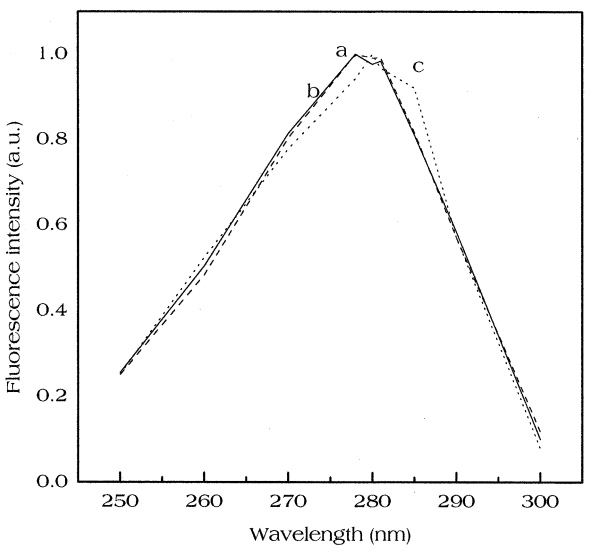

Fig. 4. Fluorescence excitation spectra of 3 μM α_1-acid glycoprotein in the absence (a) and in the presence of 12 μM Calcofluor (b) and of 120 μM Calcofluor (c). $\lambda_{em} = 330$ nm.

Steady-state anisotropy as a function of temperature. Steady-state fluorescence anisotropy of Trp residues in α_1-acid glycoprotein (15 μM) in the presence of 150 μM Calcofluor ($\lambda_{em} = 330$ nm and $\lambda_{ex} = 300$ nm) was performed at different temperatures. The Perrin plot representation (Fig. 2, plot b) based on Eq. (2)[27] allowed one to measure a rotational correlation time of 5.4 ns. This value is lower than the rotational correlation time of α_1-acid glycoprotein (16 ns) and indicates that Trp residues display free motions.

This result is in good agreement with that obtained with the red-edge excitation spectra experiment obtained at high Calcofluor concentrations (not shown), i.e., binding of high concentrations of Calcofluor to α_1-acid glycoprotein does not hinder the motion of the Trp residues of the protein.

Effect of high Calcofluor concentration on the local structure of α_1-acid glycoprotein.— The disruptions of the emission spectrum of the Trp residues of α_1-acid glycoprotein in the presence of high Calcofluor concentrations (Fig. 3) would originate from modification of the local structure of the protein and/or from a high-energy transfer from the Trp residues to the Calcofluor. Although a weak energy transfer occurs (data not shown), it is improbable that this energy transfer induces the important disruptions of the emission spectra. A structural modification within α_1-acid glycoprotein can be monitored by recording the fluorescence excitation spectrum of the Trp residues in the absence and in the presence of low and high Calcofluor concentrations. In fact, the fluorescence excitation spectrum characterizes the electron distribution of the molecule in the ground state. At $\lambda_{em} = 330$ nm, Calcofluor does not emit and thus, only the excitation spectrum of the Trp residues would be recorded. Therefore, any modification of the fluorescence excitation spectrum in the presence of Calcofluor would be the result of a structural modification of the protein in the ground state.

Fig. 4 displays the fluorescence excitation spectrum of the Trp residues of α_1-acid glycoprotein in the absence (a) and presence of 10 μM (b) and 120 μM (c) Calcofluor. We notice

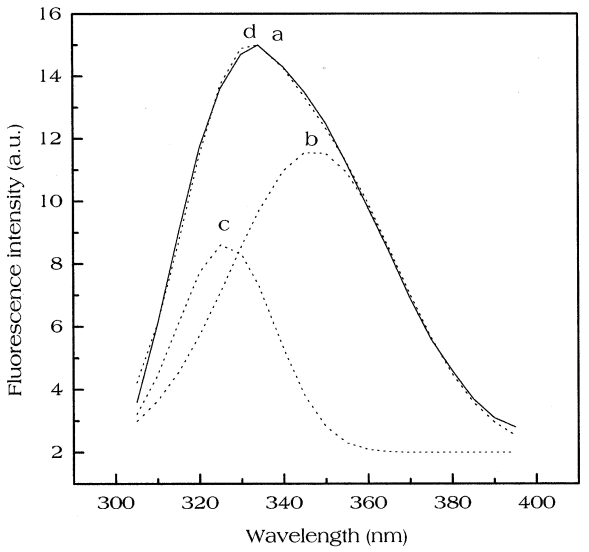

Fig. 5. Fluorescence emission spectrum of 15 μM α_1-acid glycoprotein in the presence of 10 μM Calcofluor recorded at excitation wavelength of 295 nm. The figure displays also (dotted lines) the two Gaussian components of the fluorescence spectrum. The maximum of the bands are 347 (b) and 326 nm (c). These positions correspond to the emission from surface and hydrophobic Trp residues, respectively. However, the bandwidths of the two bands (40 and 23 nm, respectively), are lower than those known for Trp residues, i.e., α_1-acid glycoprotein has kept its folded structure. Spectrum (d) shows the sum of spectra (c) and (b).

that in the presence of high concentrations of Calcofluor, the peak is located at 280 nm instead of 278 nm, and a shoulder is observed at 295 nm. Thus, the global shape of the excitation spectrum is modified in the presence of high Calcofluor concentrations, i.e., binding of Calcofluor at high concentrations has modified the local structure of the Trp residues of α_1-acid glycoprotein. The fact that binding of low concentrations of Calcofluor (10 μM) does not affect this local structure indicates that a minimum concentration of fluorophore is necessary to induce any structural modification of the protein.

Deconvolution of the emission spectra obtained at low and high concentrations of Calcofluor into different components.—In order to find out which of the Trp residues is the one most affected by the presence of Calcofluor, two deconvolution methods were applied. In the first method, we fitted the spectra obtained as a sum of Gaussian bands. In the second one, we used the method described by Siano and Metzler,[28] which has been applied by Burstein and Emelyanenko,[29] using a four-parametric ln–normal function (a skewed Gauss equation). The analyses were done at the three excitation wavelengths (295, 300 and 305 nm), although the results shown are only those obtained at $\lambda_{ex} = 295$ nm.

Analysis as a sum of Gaussian bands. In this analysis, we used the ORIGIN 3.5 program. For each spectrum, we have proposed one, two or three maxima, and we asked the program to draw the best Gaussian curves we can obtain from our spectra.

The best fit was obtained with the sum of two bands. At low concentration of Calcofluor, one band was obtained at a maximum of 326 nm and a bandwidth of 23 nm. The second band had a maximum at 347 nm and a bandwidth of 40 nm (Fig. 5). While the positions of the maxima are significant and correspond to those found for the hydrophobic and surface Trp residues, respectively,[11,23] the values of the bandwidths do not correspond to those known for Trp residues (48 and 59 nm for the hydrophobic and surface residues, respectively).[11,23,30] This means that it is difficult to separate the real spectra one from each other, i.e., in the presence of low Calcofluor concentration, α_1-acid glycoprotein has kept its folded structure.

Spectrum (d) shows the sum of spectra (c) and (b). This spectrum overlaps the original spectrum (a) indicating how well the two components fit the original spectrum.

Performing the fits for the spectrum obtained at high concentrations of Calcofluor, yields also two bands (Fig. 6). One has a maximum at 324 nm and a bandwidth of 28 nm. The second shows a maximum at 349 nm and a bandwidth of 53 nm. We notice that the bandwidth of the second band is close to that of fluorescence spectrum of Trp residue near the protein surface.

The ln–normal analysis. In this analysis, the following equation was used:

$$I_{(\nu_m)} = I_m \exp\{-(\ln 2/\ln^2 \rho) - \ln^2[(a-\nu)/(a-\nu_m)]\} \quad (3)$$

where $I_m = I_{(\nu_m)}$ is the maximal fluorescence intensity. ν_m is the wavenumber of the band maximum, $\rho = (\nu_m - \nu_-)/(\nu_+ - \nu_m)$ is the band asymmetry parameter. ν_+ and ν_- are the wavenumber positions of left and right

half maximal amplitudes. $a = v_m + H\rho/(\rho^2 - 1)$ and H is the bandwidth: $v_+ - v_-$.

Eq. (3) allows us in principle to draw a spectrum that matches entirely the one obtained experimentally. This is correct only if the recorded spectrum originates from one fluorophore (i.e., Trp in solution) or from compact protein within a folded structure. However, when the experimental spectrum originates from two fluorophores (i.e., mixtures of tyrosine and tryptophan in solution) or from a disrupted protein that has two classes of Trp residues, the calculated spectrum using Eq. (3) does not match with the recorded one. In the present work, all the spectra are drawn as the intensity as a function of the wavelength.

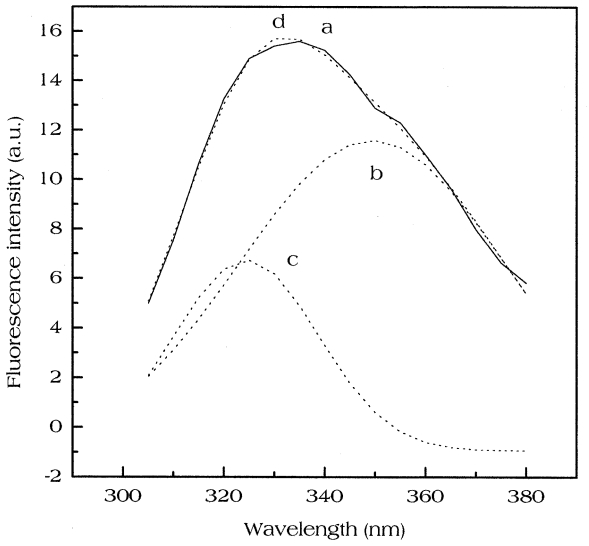

Fig. 6. Fluorescence emission spectrum of 14 μM α_1-acid glycoprotein in the presence of 160 μM Calcofluor recorded at excitation wavelength of 295 nm. The figure displays also (dotted lines) the two Gaussian components of the fluorescence spectrum. The maximum of the bands are 349 (b) and 324 nm (c). These positions correspond to the emission from surface and hydrophobic Trp residues, respectively. The bandwidth of band (b) is equal to 53 nm, indicating that the structure of the protein surface is disrupted. Spectrum (d) shows the sum of spectra (c) and (b).

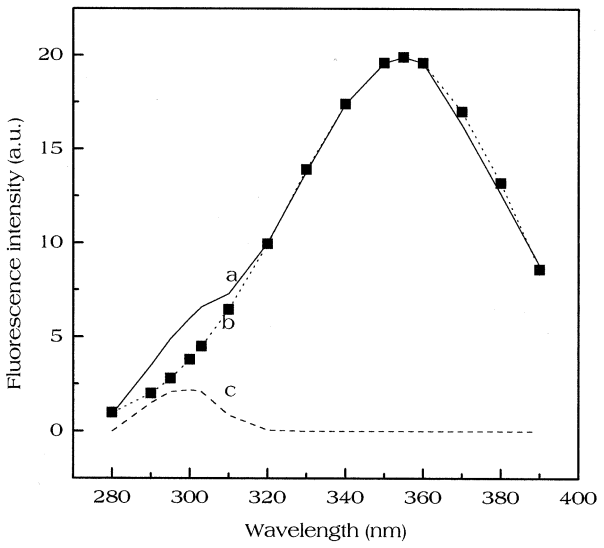

Fig. 7. Experimental fluorescence emission spectrum of a mixture of L-tyrosine and L-tryptophan in water (line, spectrum (a)) and that approximated by Eq. (3) (squares, spectrum (b)). Subtracting spectrum (b) from (a) yields a fluorescence spectrum (c) characteristic of that of the tyrosine. $\lambda_{ex} = 260$ nm.

Fig. 7 shows the experimental fluorescence spectrum of a mixture of L-tyrosine and L-tryptophan in water (line, spectrum (a)). The presence of a small shoulder around 303 nm indicates that the L-tyrosine contributes to the fluorescence emission. Analysis of the data using Eq. (3) yields a fluorescence spectrum (squares, spectrum (b)) that does not match with the experimental one, especially within the region where the tyrosine emits. The calculated spectrum (b) corresponds to that of the tryptophan alone. Subtracting spectrum (b) from (a) yields a spectrum with a maximum at 303 nm corresponding to that of the tyrosine residues.

Fig. 8 shows the experimental fluorescence spectrum of Trp residues of 15 μM α_1-acid glycoprotein ($\lambda_{ex} = 295$ nm) in the presence of 10 μM Calcofluor (line) and the calculated spectrum using Eq. (3) (square). We notice that the calculated spectrum matches the experimental one. Therefore, the binding of low concentrations of Calcofluor to α_1-acid glycoprotein does not perturb the structure of the protein.

Fig. 9 displays the fluorescence emission spectrum of 14 μM α_1-acid glycoprotein in the presence of 160 μM Calcofluor (a). Eq. (3) yields a fluorescence spectrum (square, spectrum (b)) that does not fit the experimental one. Subtracting spectrum (b) from (a) yields two spectra (c and d) with maxima located at 330 and 355 nm, respectively. We notice that the three spectra (b, c and d) participate significantly to the total emission of α_1-acid glycoprotein. The result obtained in the presence of a high concentration of Calcofluor in comparison to that obtained at low concentration

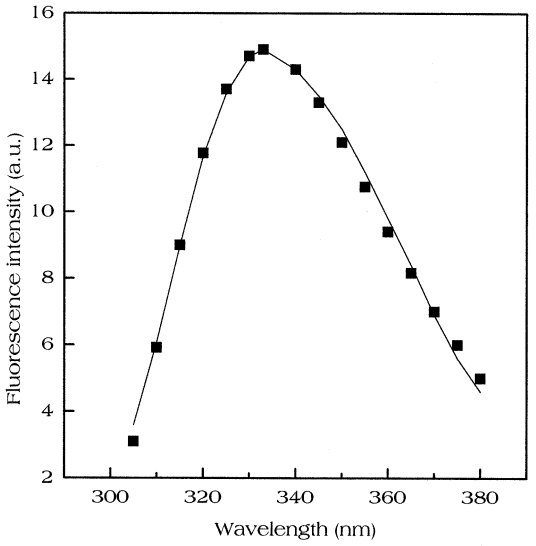

Fig. 8. Experimental fluorescence emission spectrum of the Trp residues of 15 μM α_1-acid glycoprotein in the presence of 10 μM Calcofluor (line) and that approximated by Eq. (3) (squares). $\lambda_{ex} = 295$ nm.

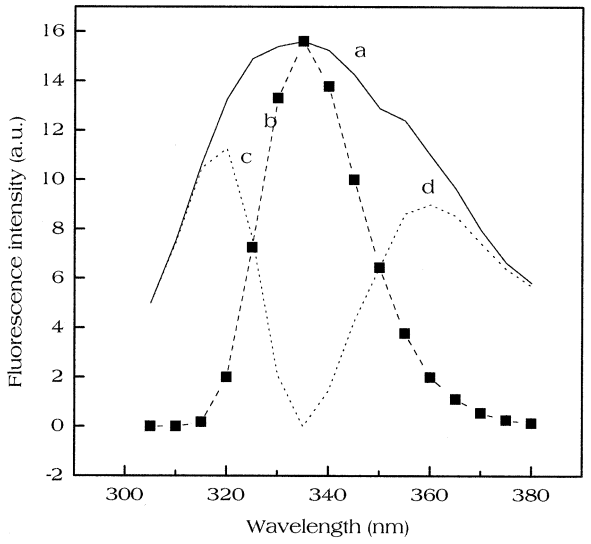

Fig. 9. Experimental fluorescence emission spectrum of the Trp residues of 14 μM α_1-acid glycoprotein in the presence of 160 μM Calcofluor (spectrum (a)) and that approximated by Eq. (3) (spectrum (b)). Subtracting spectrum (b) from (a) yields two spectra (c) and (d) with maxima located at 330 and 355 nm, respectively. $\lambda_{ex} = 295$ nm.

of the external fluorophore, indicates clearly that binding of Calcofluor at high concentration on α_1-acid glycoprotein disrupts the structure of the protein.

The results obtained with the ln–normal analysis shows that this method is more suitable than the one that consists of deconvoluting the recorded emission spectrum into the sum of two spectra. The presence of an emission spectrum with a maximum located at 337 nm indicates that three components can best describe the emission of the Trp residues of α_1-acid glycoprotein. An emission at 337 nm corresponds to that of a Trp residue located in an area between the core of the protein and its surface (see also Section 4).

4. Discussion

The present work provides evidence of the effect of Calcofluor binding to α_1-acid glycoprotein on the structure and the dynamics of the Trp residues microenvironment of the protein. Calcofluor binds mainly to the carbohydrate residues of α_1-acid glycoprotein.[1,2,15] At equimolar concentrations of Calcofluor and α_1-acid glycoprotein, Calcofluor displays free motion,[1] while at saturating concentrations, the fluorophore is rigid within its environment.[2]

Carbohydrate residues are linked to the *N*-terminal fragment of α_1-acid glycoprotein, rendering this portion of the protein hydrophilic.[31] This fragment is in contact with the solvent and adopts a spatial conformation so that a pocket in contact with the buffer is formed.[10] The Trp 25 residue lies in a hydrophobic environment in this pocket.

Carbohydrate residues emerge from the pocket to envelop the surface of α_1-acid glycoprotein. At low concentrations, Calcofluor interacts with sialic acids, the terminal glycan residues, and thus interacts with the carbohydrates at the surface of the protein. The results shown in the present work indicate that binding of Calcofluor at low concentration does not affect the dynamics or the structure of the protein (absence of a red-edge excitation shift (Fig. 1) and a Perrin plot that yields an apparent rotational correlation time (Fig. 2)).

At high Calcofluor concentrations, the dynamics of the protein is maintained, but its structure is altered (Figs. 3 and 4). In fact, the fluorescence excitation and emission spectra of the Trp residues are disrupted at high Calcofluor concentrations, which is not the case when the experiments were performed at low Calcofluor concentrations.

Analysis of the data with a sum of two Gaussian curves or by the ln–normal analysis

indicate clearly that binding of Calcofluor at high concentrations induces a disruption in the structure of α_1-acid glycoprotein. Apparently, Calcofluor induces a pressure at the surface of the protein that partially denatures it. This effect is identical to that observed when hydrostatic pressure is applied on proteins.[32,33]

The ln–normal analysis method shows also that binding of Calcofluor at high concentrations disrupts the protein structure. This method is very sensitive to the modifications occurring around the fluorophore. In fact, in the presence of folded protein structure, the calculated emission spectrum fits perfectly the experimental one (Fig. 8). However, when the structure of the protein is altered, we obtain a calculated spectrum that is different from the experimental one (Fig. 9, spectrum (b), $\lambda_{max} =$ 337 nm). Also, in this case, we can derive two other emission spectra that correspond to the hydrophobic ($\lambda_{max} = 330$ nm) and surface Trp residues ($\lambda_{max} = 355$ nm). This deconvolution is not possible in the presence of a compact protein (Fig. 8). Therefore, the ln–normal analysis allows one to compare disrupted and compact proteins with two or three classes of Trp residues, since in the case of a compact protein, the analysis gives a result identical to that obtained for a free tryptophan in solution.

Since the carbohydrate residues cover almost 90% of the surface of α_1-acid glycoprotein, it is normal that the binding of Calcofluor to the carbohydrate residues affects mainly the surface of the protein. However, this does not mean that the environments of the two other Trp residues could not be affected by the presence of Calcofluor, although the effect would be less important than the one observed at the surface of the protein.

Looking closely at Fig. 1, one finds that spectrum (c) obtained at λ_{ex} equal to 305 nm has a shape globally similar to the spectra (a) and (b) obtained at λ_{ex} equal to 295 and 300 nm, but with a shoulder at 352 nm that is more evident. Therefore, it appears in the first instance that the Trp residues of α_1-acid glycoprotein are behaving differently to different excitation wavelengths. However, the bandwidth of the spectrum increases only by 2 nm when excitation occurred at 305 instead of 295 nm, indicating that the emission observed at λ_{ex} of 305 nm still occurs from hydrophobic and surface Trp residues. The shoulder observed is not typical of the presence of Trp residues present at the surface of the protein, since the emission spectrum of the hydrophobic Trp residues of crystals of α_1-acid glycoprotein obtained with λ_{ex} of 305 nm possesses a shoulder at 352 nm that is more evident than when excitation occurs at 295 nm. Also, the bandwidth of the spectrum does not change with the excitation wavelength.[23] Therefore, the results obtained in Fig. 1 do not demonstrate that the Trp residues are behaving differently with the excitation wavelength.

Analyzing the spectra obtained at low concentration of Calcofluor by a sum of two Gaussians indicate that at the three excitation wavelengths, the positions of the maxima of the two spectra are 347 and 326 nm (data not shown for λ_{ex} equal to 300 and 305 nm). The absence of a shift in the position of the maximum with the excitation wavelength indicates that the Trp residues are not behaving differently with the excitation wavelength. The positions of the two maxima (347 and 326 nm) found in our analysis are identical to those (350 and 324 nm) we have already published by performing fluorescence intensity quenching with cesium.[11] However, the ln–normal analysis performed on α_1-acid glycoprotein in the presence of high Calcofluor concentrations is much more accurate than the simple analysis with the sum of different spectra or even with the quenching experiments, since it allows one to determine the presence of a third component with a maximum located at 337 nm.

Decay-associated spectra performed on α_1-acid glycoprotein revealed the presence of a component located at 337 nm. This spectrum was attributed to Trp-122 with a location between the surface of the protein and its core.[34] Although the authors did not obtain a component with a maximum located around 324 nm, the calculation of the degree of hydrophobicity around the Trp residues showed that Trp-25 residue is located in a hydrophobic environment, Trp-160 is the most exposed

to the solvent and Trp-122 is in an intermediate state.[34]

The results shown in our studies also confirm the presence of this third component at 337 nm and thus indicate that the three Trp-residues participate to the fluorescence emission of α_1-acid glycoprotein. This means that the quenching resolved emission spectra with cesium that allowed us to reveal the presence of only two components at 324 and 350 nm[11] is not quite suitable and accurate to obtain a complete and real description of the emission occurring from each Trp residues. We should, however, add that the quenching resolved emission spectra experiments were performed on α_1-acid glycoprotein in the absence of Calcofluor. Therefore, quenching experiments performed in the presence of high concentrations of Calcofluor may allow one to obtain the emission of the third component at 337 nm.

Quenching-resolved emission anisotropy in the presence of cesium[10] and anisotropy and lifetime measurements from -45 to 20 °C[11,35] allowed us to determine the rotation of the Trp residues. The results have shown that both the hydrophobic and the surface Trp residues display residual motions. We were not able to determine a third component characteristic of the intermediate Trp residue, Trp-122. The reason for that could be the very close motions of the Trp residues.[10,11,35]

Fluorescence experiments have recently shown that the carbohydrate residues of α_1-acid glycoprotein possess a secondary structure that is absent in many other glycoproteins or when the carbohydrates are free in solution.[1,2,15] The present work shows the importance of this secondary structure in maintaining the tertiary structure of α_1-acid glycoprotein and thus its physiological properties. Our results are in good agreement with the fact that the physiological role of α_1-acid glycoprotein is dependent on the complementary of the two entities (protein moiety and carbohydrate residues).[36,37]

Also, all the heavily glycosylated proteins are thermally destabilized by carbohydrate removal.[38] The covalently attached carbohydrates confer, to the protein moiety, a conformation with resulting physiological functions that are dependent on the stability of the overall structure of the protein. Thus, our results show that high concentrations of Calcofluor have a destabilizing effect and thus play a role close to that of the carbohydrate residue removal.

α_1-Acid glycoprotein has been studied for more than 50 years. From the results obtained up until now, we note that the protein has not only one specific function, but it acts as a probe that allows the monitoring of physiological changes. For example, it has been reported recently that the concentration of α_1-acid glycoprotein increases with age, particularly in depressed female patients.[39]

Recently, we have demonstrated that binding of progesterone to α_1-acid glycoprotein induces a modification in the structure and dynamics of the protein moiety.[10,11] Progesterone binds to the pocket of α_1-acid glycoprotein,[10] and since the carbohydrate residues are linked to the pocket, binding of the hormone would affect both the protein moiety and the carbohydrate residues. Therefore, our next goal is to study the binding of progesterone and other ligands such as propranolol to α_1-acid glycoprotein by following the fluorescence of Calcofluor present at low concentration. These experiments will allow one to follow the effect of the ligands binding on the structure and dynamics of the carbohydrate residues.

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