ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 327 (2000) 333–340

CARBOHYDRATE RESEARCH

Interaction between carbohydrate residues of α_1-acid glycoprotein (orosomucoid) and saturating concentrations of Calcofluor White. A fluorescence study

Jihad R. Albani [a,*], Alain Sillen [b], Yves D. Plancke [c], Bernadette Coddeville [c], Yves Engelborghs [b]

[a] *Laboratoire de Biophysique Moléculaire, Université des Sciences et Technologies de Lille, B.P. 649, F-59656 Villeneuve d'Ascq, France*

[b] *Laboratory of Biomolecular Dynamics, Katholieke Universiteit Leuven, Celestijnenlaan 200 D, B-3001 Leuven, Belgium*

[c] *Laboratoire de Chimie Biologique, Université des Sciences et Technologies de Lille, F-59655 Villeneuve d'Ascq, France*

Received 12 October 1999; accepted 7 February 2000

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 followed by fluorescence titration of the Trp residues of the protein. A stoichiometry of one Calcofluor for one protein has been found [J.R. Albani and Y.D. Plancke, *Carbohydr. Res.*, 318 (1999) 193–200]. α_1-Acid glycoprotein contains 40% carbohydrate by weight and has up to 16 sialic acid residues. Since binding of Calcofluor to α_1-acid glycoprotein occurs mainly on the carbohydrate residues, we studied in the present work the interaction between Calcofluor and the protein by following the fluorescence change of the fluorophore. In order to establish the role of the sialic acid residues in the interaction, the experiments were performed with the sialylated and asialylated protein. Interaction of Calcofluor with sialylated α_1-acid glycoprotein induces a red shift of the emission maximum of the fluorophore from 438 to 450 nm at saturation (one Calcofluor for one sialic acid) and an increase in the fluorescence intensity. At saturation the fluorescence intensity increase levels off. Binding of Calcofluor to asialylated acid glycoprotein does not change the position of the emission maximum of the fluorophore and induces a decrease in its fluorescence intensity. Saturation occurs when 10 molecules of Calcofluor are bound to 1 mol of α_1-acid glycoprotein. Since the protein contains five heteropolysaccharide groups, we have 2 mol of Calcofluor for each group. Addition of free sialic acid to Calcofluor induces a continuous decrease in the fluorescence intensity of the fluorophore but does not change the position of the emission maximum. Our results confirm the presence of a defined spatial conformation of the sialic acid residues, a conformation that disappears when they are free in solution. Dynamics studies on Calcofluor White and the carbohydrate residues of α_1-acid glycoprotein are also performed at saturating concentrations of Calcofluor using the red-edge excitation spectra and steady-state anisotropy studies. The red-edge excitation spectra experiments show an important shift (13 nm) of the fluorescence emission maximum of the probe. This reveals that emission of Calcofluor occurs before relaxation of the surrounding carbohydrate residues occurs. Emission from a non-relaxed state means that the microenvironment of bound Calcofluor is rigid, inducing in this way the rigidity of the fluorophore itself, a result confirmed by anisotropy studies.

Keywords: Calcofluor White; α1-Acid glycoprotein; Carbohydrates; Sialic acids; Fluorescence titration; Red-edge excitation spectra; Fluorescence anisotropy

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

0008-6215/00/$ - see front matter
PII: S0008-6215(00)00053-7

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]. The protein contains tetra-antennary as well as di- and tri-antennary glycans.

Calcofluor White is a fluorescent probe capable of making hydrogen bonds with β-(1→4)- and β-(1→3)-linked polysaccharides [4]. 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 [5,6].

Recently, we established that Calcofluor interacts preferentially with the glycan residues of α_1-acid glycoprotein, the interaction being dependent on their spatial conformation. The stoichiometry and the association constant of the Calcofluor α_1-acid glycoprotein complex were found to be equal to 1:1 and 0.15 μM^{-1}, respectively [1]. Also, we found that at high concentrations of Calcofluor compared with the protein (200 μM of probe in the presence of 7 μM of protein), the emission maximum of the fluorophore is located at 445 instead of 438 nm when free in water, while at equimolar concentrations of Calcofluor and α_1-acid glycoprotein, the red shift of the emission maximum of the fluorophore is small (between 2 and 5 nm) [1].

In this previous study [1] the binding parameters of Calcofluor–α_1-acid glycoprotein complex were determined by following the decrease in the fluorescence intensity of the Trp residues of the protein in the presence of increasing concentrations of Calcofluor. Since the fluorophore binds preferentially to the carbohydrate residues, it is interesting to know whether the data obtained when the fluorescence parameters of Calcofluor itself are followed, are identical or different to those obtained with the fluorescence intensity decrease of the Trp residues. Therefore, in the present work, we carried out titration experiments of sialylated and asialylated α_1-acid glycoprotein with Calcofluor by following the fluorescence intensity variation and the change of the wavelength of maximum intensity of the probe. The results reported here clearly show that it is possible to titrate the sialic acid residues of α_1-acid glycoprotein and that the method used is sensitive to the spatial conformation of the sialic acid residues.

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 [7]. We investigated the dynamic 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 the flexibility of the microenvironment to be studied, while anisotropy allows the dynamics of the fluorophore itself [8,9] to be monitored.

Since in vivo experiments are usually carried out at excess concentration of Calcofluor, we investigated in the present work 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 show that at saturating concentrations of Calcofluor, the emission maximum of the fluorophore is shifted to longer wavelengths upon changing the excitation wavelength. This means that the microenvironment of Calcofluor is rigid.

Anisotropy measurements as a function of temperature confirm the results of the red-edge excitation spectra experiments, i.e., Calcofluor is bound tightly to the sialic acid and to the other carbohydrate residues of α_1-acid glycoprotein.

2. Materials and methods

α_1-Acid glycoprotein was purified from serum by a successive combination of ion-displacement chromatography, gel-filtration chromatography and ion-exchange chromatography as already described [10]. 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 [11].

Calcofluor White was obtained from Sigma. Its concentration was determined spectrophotometrically using an extinction coefficient of 4388 M^{-1} cm^{-1} at 352.7 nm [1].

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 a λ_{ex} of 300 nm. The bandwidths used for the excitation and the emission were 2.5 nm. For the red-edge excitation spectra experiments, the bandwidths used for excitation and 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 as described in Refs. [12,13]. Finally, fluorescence spectra were corrected for the background intensities of the buffer solution.

The Perrin plot was obtained from anisotropy data measured with the same instrument, (λ_{ex}, 300 nm and λ_{em}, 445 nm). 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 [7,14].

The fluorescence intensity decay of Calcofluor, whether free in solution or bound to the carbohydrates of α_1-acid glycoprotein, is nonexponential [7]. When the fluorophore is free in solution, the intensity average fluorescence lifetime τ [15] is 0.85 ns. It increases to 4.8 ns when the fluorophore is bound to the protein [7]. This average lifetime 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.

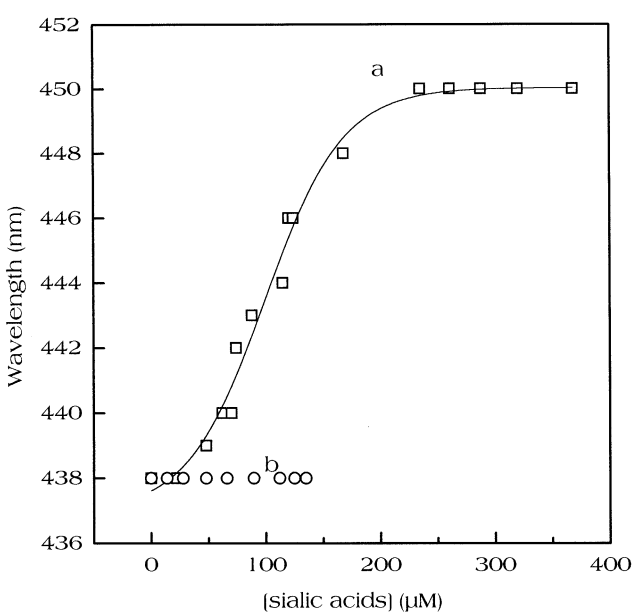

Fig. 1. Variation of the emission maximum of 220 μM of Calcofluor as a function of increasing concentrations of sialylated (a) and of asialylated α_1-acid glycoprotein (b). λ_{ex} 300 nm and temperature = 20 °C. The buffer is 10 mM phosphate and 0.143 M NaCl buffer, pH 7. The protein concentrations are expressed in sialic acid residues. 1 mol of sialylated α_1-acid glycoprotein contains 16 sialic acid residues. The concentration of the asialylated protein is also expressed in equivalent sialic acid residues.

3. Results

Titration experiments.—Fig. 1 displays the variation of the emission maximum of 220 μM of Calcofluor in presence of increasing amounts of sialylated (a) and asialylated α_1-acid glycoprotein (b). The concentrations of the proteins are expressed in sialic acid residues (α_1-acid glycoprotein contains 16 sialic acid residues). We notice that only the binding of Calcofluor to the sialylated protein induces a shift in the emission maximum of the fluorophore. In the absence of protein, the fluorescence maximum is at 438 nm and it shifts progressively to 450 nm with increasing concentrations of sialylated α_1-acid glycoprotein (a). The shift stops when the stoichiometry of 1 Calcofluor for 1 sialic acid residue is reached. Addition of asialylated α_1-acid glycoprotein (b) or of free sialic acids (not shown) does not modify the position of the emission maximum of Calcofluor.

Fig. 2 shows the fluorescence emission spectra of 194 μM of Calcofluor free in solution (a) and in presence of 3 (b) 11(c) and 18 (d)

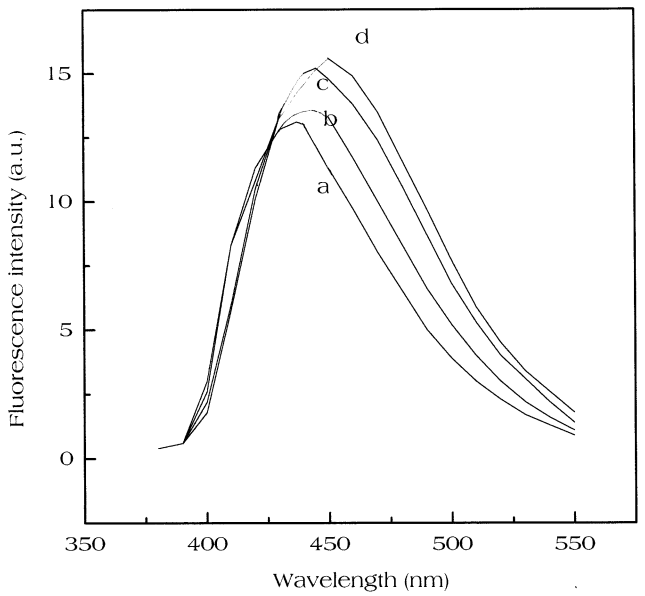

Fig. 2. Observed fluorescence emission spectra of 194 μM of Calcofluor free in solution (a) and in presence of 3 (b) 11 (c) and 18 (d) μM of α_1-acid glycoprotein. The concentrations expressed in sialic acid are 48, 176 and 288 μM, respectively. λ_{ex} 300 nm, $t = 20$ °C.

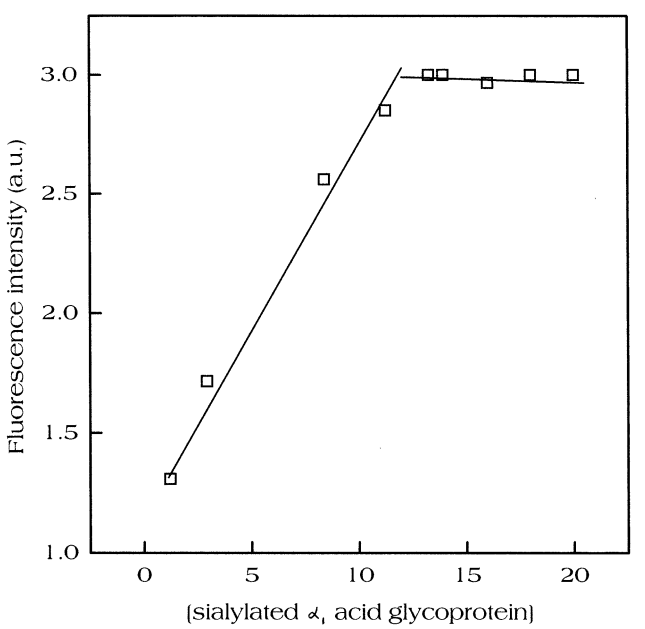

Fig. 3. Titration curve of 194 μM of Calcofluor with sialylated α_1-acid glycoprotein. Saturation occurs for 12.7 μM of protein (200 μM of sialic acids), thus indicative of a stoichiometry of one Calcofluor for one sialic acid residue. The dissociation constant could not be estimated from these experiments, but it must be smaller than the protein concentration at stoichiometry (< 12 μM). $\lambda_{ex} = 300$ nm and $\lambda_{em} = 440$ nm. The fluorescence intensities are corrected for dilution and for the inner filter effect.

μM of sialylated α_1-acid glycoprotein. The concentrations expressed in sialic acid are 48, 176 and 288 μM, respectively. In addition to the shift in the emission maximum, we observe an increase in the fluorescence intensity. Fig. 3 displays the fluorescence intensity increase of Calcofluor (194 μM) in the presence of increasing concentrations of sialylated α_1-acid glycoprotein observed at 440 nm. Between protein concentrations 1.2 and 10 μM the fluorescence intensity increase linearly and reaches a plateau in presence of 12.7 ± 1.2 μM of protein (200 μM of sialic acids) and thus at a stoichiometry close to 1:1. The reason why the first point deviates from the straight line is not clear. It could be due to a change in specific fluorescence upon increasing the number of bound Calcofluor molecules per protein. The linear increase of the fluorescence upon protein addition (between 1.2 and 12 μM) indicates that the protein and the Calcofluor concentrations must be much higher than the dissociation constant which cannot be estimated from these experiments.

Addition of increasing amounts of free sialic acids to 212 μM of Calcofluor induces a limited decrease in the fluorescence intensity of Calcofluor (Fig. 4(a and b)). This decrease indicates that interactions occur in solution between free sialic acid and Calcofluor.

Fig. 5 exhibits the fluorescence intensity titration of 57 μM of Calcofluor with asialylated α_1-acid glycoprotein. The intensity decrease is the result of the binding of Calcofluor to the protein and can therefore 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, L_0 and L_b are the concentrations of total and bound Calcofluor.

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 (assuming

all binding sites have the same dissociation constant K_d):

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

where n is the number of binding sites (assumed to be identical), and P_0 is the protein concentration. The parameter Flu_1 was obtained by extrapolation from a reciprocal plot

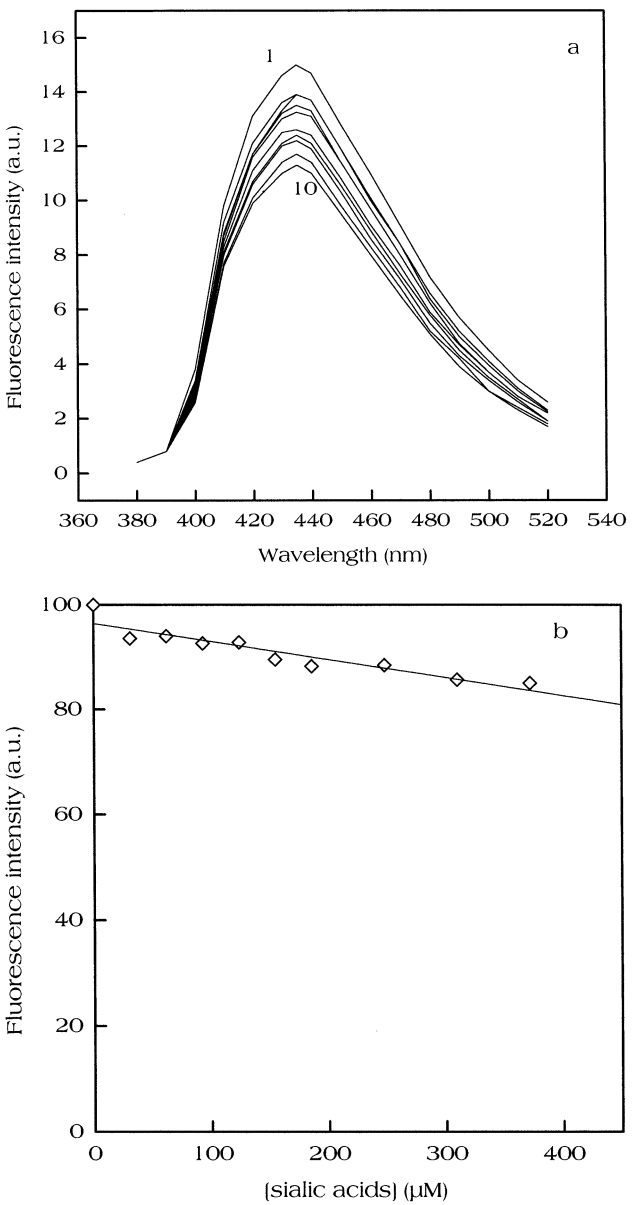

Fig. 4. Titration of 212 μM of Calcofluor with sialic acid residues. Aliquots of 31 μM of sialic acid residues solution were added to the Calcofluor. (a) Observed fluorescence spectra of Calcofluor in solution (1) and in the presence of the increased concentration of sialic acid residues $\lambda_{ex} = 300$ nm. (b) Normalized fluorescence intensities at $\lambda_{em} = 435$ nm as a function of the sialic acids concentration. The intensities were corrected for the dilution and for the inner filter effect.

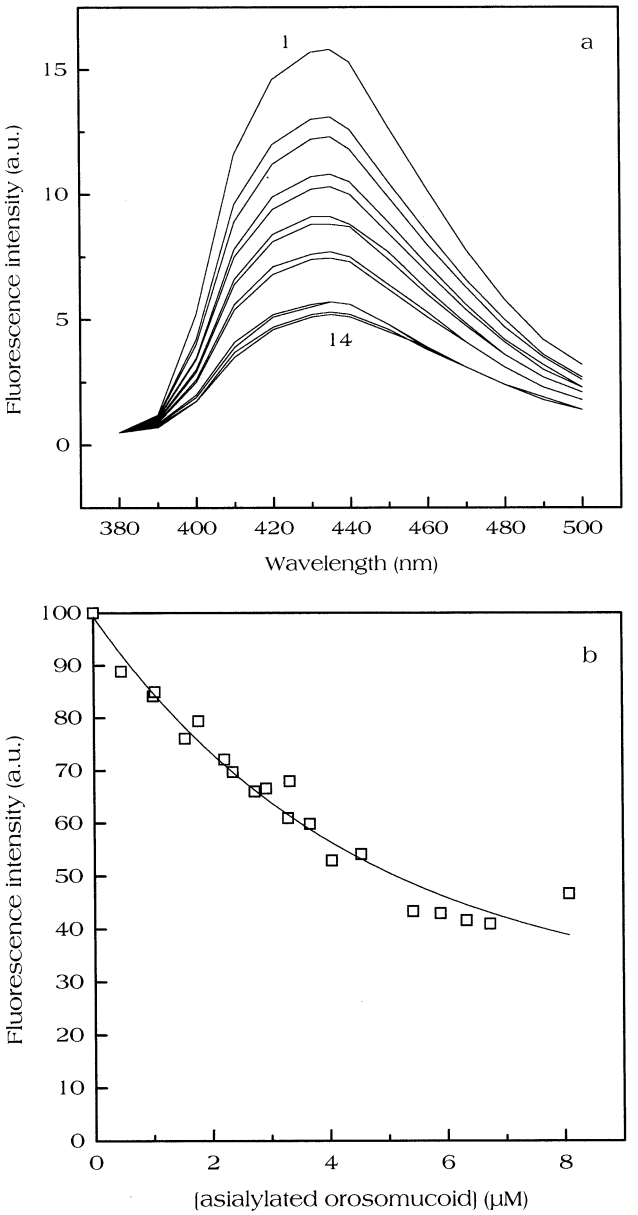

Fig. 5. Titration curve of Calcofluor (57 μM) with asialylated α_1-acid glycoprotein. (a) Observed fluorescence spectra of free Calcofluor in solution (spectrum 1) and in the presence of increased concentrations of protein (spectra 2–14). $\lambda_{ex} = 300$ nm. (b) Normalized fluorescence intensities observed at 435 nm as a function of asialylated protein. The number n of binding sites is equal to 12.7 ± 1.2 mol of Calcofluor for 1 mol of protein. Since α_1-acid glycoprotein contains five heteropolysaccharide groups, we would have about two Calcofluor molecules for each group. The fluorescence intensities were normalized for the dilution and for the inner filter effect.

and found to be 32.5. Using this parameter, nonlinear least-squares fitting gives the continuous line as the best fit (Fig. 5(b)) with $n = 12.7 \pm 1.2$ and $K_d = 6.8 \pm 2.6$ μM. This should be considered as an average dissociation constant per site.

Dynamics experiments

Red-edge excitation spectra. Red-edge excitation spectra are used to monitor motions around the fluorophores [16,17]. Calcofluor molecules and their direct microenvironment (which consists of the dipole of both surrounding glycan residues 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 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.

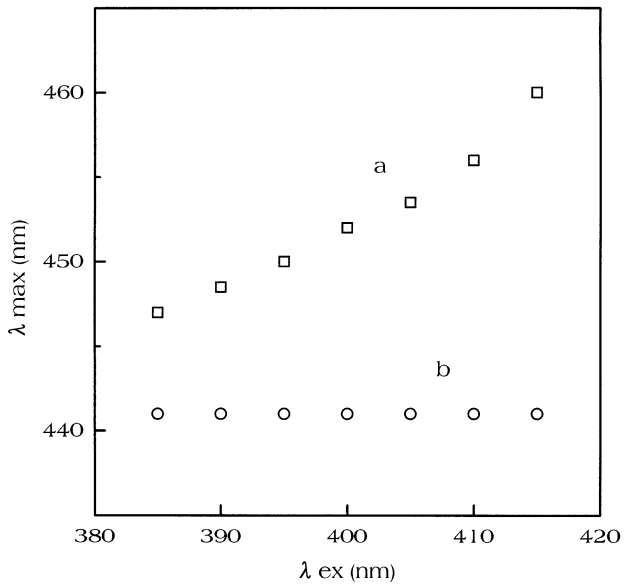

Fig. 6. (a) The dependence of the fluorescence emission maximum of 300 μM of Calcofluor in the presence of 5 μM of sialylated α_1-acid glycoprotein on the excitation wavelengths. (b) shows this dependence for 10 μM of Calcofluor in presence of an equivalent amount of protein.

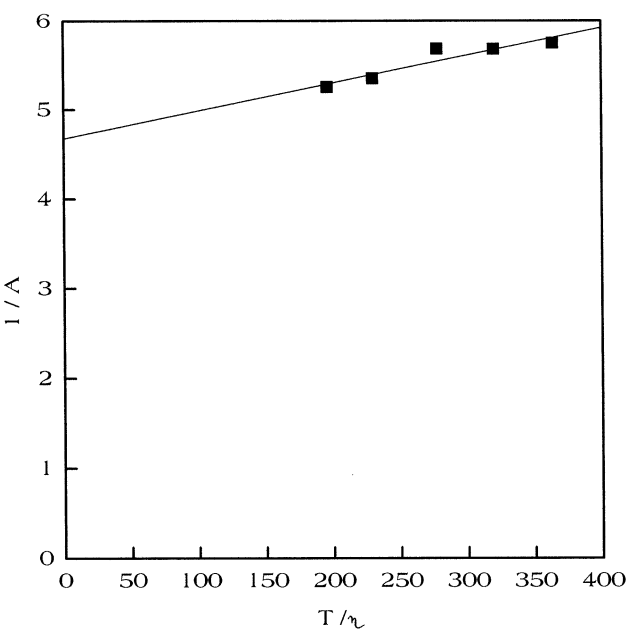

Fig. 7. Steady-state fluorescence anisotropy vs. temperature over viscosity for 250 μM of Calcofluor in presence of 5 μM of sialylated α_1-acid glycoprotein (λ_{ex}, 300 nm and λ_{em}, 445 nm). The data are obtained by thermal variation in the range 5–35 °C. The ratio T/η is expressed in Kelvins over centipoise.

Fig. 6(a) shows the variation of the emission maximum of 300 μM of Calcofluor in the presence of 5 μM of sialylated α_1-acid glycoprotein as a function of the excitation wavelength. At 385 nm the emission maximum is located at 447 nm. It shifts to higher wavelengths (452 and 458 nm) when the excitation wavelengths are 400 and 415 nm, respectively. This is taken as direct evidence that the carbohydrate residues in the microenvironment of Calcofluor exhibit restricted motions. For comparison, the emission maximum of 9.8 μM of Calcofluor in the presence of 10 μM of α_1-acid glycoprotein recorded as a function of the excitation wavelength is equal to 441 nm at all wavelengths (Fig. 6(b)).

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

$$\Phi_p(T) = 3.8\eta(T) \times 10^{-4}\ \mathrm{M} \qquad (3)$$

where M is the protein molecular weight and η the viscosity of the medium [18]. α_1-Acid glycoprotein can be considered to be spherical [19,20]. Thus, Eq. (3) yields a calculated rotational correlation time of 16 ns for the protein.

Steady-state fluorescence anisotropy of 250 μM of Calcofluor in presence of 5 μM of α_1-acid glycoprotein ($\lambda_{em} = 445$ nm and $\lambda_{ex} = 300$ nm) was performed at different temperatures. A Perrin plot representation (Fig. 7) based on Eq. (4) [21]:

$$1/A = 1/A_o + \langle\tau\rangle/\Phi_p A_o$$
$$= 1/A_o + (1/A_o)(1 + RT\langle\tau\rangle/\eta V) \quad (4)$$

should enable us to obtain information concerning the motion of the fluorophore. The rotational correlation time of the fluorophore Φ_p using 4.8 as average lifetime is found to be 16 ± 1 ns at 20 °C, which equals the theoretical value (16 ns) expected for α_1-acid glycoprotein revealing the absence of a segmental motion (despite the presence of a substantial fraction of unbound Calcofluor). 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.

4. Discussion

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 [22,23]. Titration experiments are usually carried out by following the intensity variation. In the present work, we show that it is possible to perform titration experiments by following not only the fluorescence intensity variation, but also the shift in the position of the emission maximum of Calcofluor. This shift is sensitive to sialic acid residues only when they are bound to α_1-acid glycoprotein (Fig. 1). Therefore, sialic acids of α_1-acid glycoprotein possess a spatial conformation that can be detected by Calcofluor.

Binding of Calcofluor to sialylated α_1-acid glycoprotein also induces an increase in the fluorescence intensity of the fluorophore (Figs. 2 and 3). The titration curve gives a stoichiometry of one Calcofluor for one sialic acid residue. This result is in good agreement with that obtained when the shift of the emission maximum of Calcofluor was followed (Fig. 1). The dissociation constant could not be determined from the titration curve, but must be smaller than the protein concentration at stoichiometry (< 12 μM), which is in agreement with that calculated when binding experiments were performed by following fluorescence intensity decrease of Trp residues [1].

Addition of free sialic acid to Calcofluor decreases the fluorescence intensity of the fluorophore (Fig. 4). Thus, the type of interaction between free sialic acid residues and Calcofluor is different from that observed when sialic acids are bound to α_1-acid glycoprotein.

Interaction of Calcofluor with the other carbohydrate residues occurs also, since we observe a decrease in its fluorescence intensity upon addition of asialylated α_1-acid glycoprotein (Fig. 5). The dissociation constant of the Calcofluor-asialylated α_1-acid glycoprotein complex is 6.8 μM, a value close to that measured for the Calcofluor–sialylated protein (Fig. 1 and Ref. [1]). Thus, we suggest that this binding constant may characterize the interaction between the carbohydrate residues and Calcofluor. However, an interaction even weak with the protein matrix cannot be excluded. 12 binding sites were found for the Calcofluor–asialylated α_1-acid glycoprotein complex. Since the protein contains five heteropolysaccharide groups, we would have about two Calcofluor molecules for each group. In the presence of sialic acids the type of interaction of Calcofluor with the carbohydrate residues is modified. The dissociation constants measured in the absence and the presence of sialic acids do not show that the affinity of Calcofluor to sialic acid residues is higher than that for the other carbohydrate residues. However, the titration curves clearly indicate that Calcofluor fluorescence is sensitive to the presence or to the absence of sialic acids of α_1-acid glycoprotein.

The fact that the interactions between Calcofluor and sialic acids differ whether the latter are on the protein or free in solution, indicates that the sialic acids on α_1-acid glycoprotein possesses a defined spatial conformation. Also, one may suppose that the presence of the sialic acid gives the carbohydrate residues backbone a spatial conformation that may change or disappear upon desialylation of the protein. In the absence of any crystallographic data, we are not able for the moment to confirm the last hypothesis.

The red-edge excitation spectra and anisotropy measurements yield information on the dynamics of the fluorophore and of its environment. At high concentration of Cal-

cofluor compared with that of the α_1-acid glycoprotein, a red-edge excitation shift is observed (Fig. 6(a)) (even if a substantial fraction of the Calcofluor remains unbound). This shift indicates that the microenvironment of the fluorophore exhibits restricted motions. Therefore, the carbohydrate residues in the vicinity of Calcofluor are rigid and do not show any segmental motions.

Anisotropy data as a function of the temperature (Fig. 7) yield a rotational correlation time (16 ± 1 ns) equal to that (16 ns) calculated theoretically for α_1-acid glycoprotein. Thus, at high concentration, the Calcofluor is bound tightly to the carbohydrate residues and follows the global motion of the protein. This result is in opposition to that found for the Calcofluor–α_1-acid glycoprotein complex when the experiments were performed at equimolar concentrations of protein and probe (5 μM). In fact, we found that the sialic acid residues of the protein are highly mobile while the other glycan residues exhibit restricted motions [7]. At high concentrations of Calcofluor (the present work), a kind of stacking could occur inducing a decrease of the motions of the sialic acid residues.

In conclusion, this work shows that the spatial structure of the carbohydrate residues of α_1-acid glycoprotein induces the binding of the Calcofluor. The absence of this spatial structure (free carbohydrates in solution) modifies the type of their interactions with the fluorophore. Also, this work together with the previous one [7] indicate that the dynamics of the sialic acid residues is dependent on the Calcofluor concentrations. The spatial conformation of the carbohydrate residues and of the sialic acids of α_1-acid glycoprotein could be the key to the different physiological roles of the protein.

References

[1] J.R. Albani, Y.D. Plancke, *Carbohydr. Res.*, 318 (1999) 193–200.
[2] T. Kute, U. Westphal, *Biochim. Biophys. Acta*, 420 (1976) 195–213.
[3] K. Schmid, H. Kaufmann, S. Isemura, F. Bauer, J. Emura, T. Motoyama, M. Ishiguro, S. Nanno, *Biochemistry*, 12 (1973) 2711–2724.
[4] I.D. Rattee, M.M. Greur, *The Physical Chemistry of Dye Absorption*, Academic Press, New York, 1974, pp. 181–182.
[5] J.E. Gonzalez, G.M. York, G.C. Walker, *Gene*, 179 (1996) 141–146.
[6] T.F. Hogan, R.S. Riley, J.G. Thomas, *J. Clin. Lab. Anal.*, 11 (1997) 202–207.
[7] J.R. Albani, A. Sillen, B. Coddeville, Y.D. Plancke, Y. Engelborghs, *Carbohydr. Res.*, 322 (1999) 87–94.
[8] R.F. Chen, *Anal. Biochem.*, 19 (1967) 374–387.
[9] J.R. Lakowicz, H. Cherek, *Biochem. Biophys. Res. Commun.*, 99 (1981) 1173–1178.
[10] Y. Plancke, M. Dautrevaux, G. Biserte, *Biochimie*, 60 (1978) 171–175.
[11] J.R. Albani, *Spectrochim. Acta, Part A.*, 55 (1999) 2353–2360.
[12] J.R. Lakowicz, *Principles of Fluorescence spectroscopy*, Plenum, New York, 1983.
[13] J. Albani, *Arch. Biochem. Biophys.*, 243 (1985) 292–297.
[14] R. Vos, R. Strobbe, Y. Engelborghs, *J. Fluoresc.*, 7 (1996) 33S–35S.
[15] A. Sillen, Y. Engelborghs, *Photochem. Photobiol.*, 67 (1998) 475–486.
[16] J.R. Albani, *J. Fluoresc.*, 8 (1998) 213–224.
[17] A.P. Demchenko, *FEBS Lett.*, 182 (1985) 99–102.
[18] A.J.W.G. Visser, N.H.G. Penners, F. Müller, in H. Sund, C. Veeger (Eds.), *Mobility and Recognition in Cell Biology*, Walter de Gruyter, Berlin, 1983, pp. 137–152.
[19] J.R. Albani, *J. Biochem.*, 116 (1994) 625–630.
[20] J.R. Albani, *Spectrochim. Acta, Part A.*, 54 (1998) 175–182.
[21] G. Weber, *Biochem. J.*, 51 (1952) 145–155.
[22] V. Athès, R. Lange, D. Combes, *Eur. J. Biochem.*, 255 (1998) 206–212.
[23] J.K. Barry, K.S. Matthews, *Biochemistry*, 36 (1997) 15632–15642.