ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 322 (1999) 87–94

CARBOHYDRATE RESEARCH

Dynamics of carbohydrate residues of α_1-acid glycoprotein (orosomucoid) followed by red-edge excitation spectra and emission anisotropy studies of Calcofluor White

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

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

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

[c] *Laboratoire de Chimie Biologique (UMR no. 111 du CNRS), Université des Sciences et Technologies de Lille, F-59655 Villeneuve d'Ascq, France*

Received 25 January 1999; accepted 8 July 1999

Abstract

Dynamics studies on Calcofluor White bound to the carbohydrate residues of sialylated and asialylated α_1-acid glycoprotein (orosomucoid) have been performed. The interaction between the fluorophore and the protein was found to occur preferentially with the glycan residues with a dependence on their spatial conformation. In the presence of sialylated α_1-acid glycoprotein, excitation at the red edge of the absorption spectrum of calcofluor does not lead to a shift in the fluorescence emission maximum (440 nm) of the fluorophore. Thus, the emission of calcofluor occurs from a relaxed state. This is confirmed by anisotropy studies as a function of temperature (Perrin plot). In the presence of asialylated α_1-acid glycoprotein, red-edge excitation spectra show an important shift (8 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 Calcofluor molecules are bound tightly to the carbohydrate residues, a result confirmed by anisotropy studies.

Keywords: Calcofluor White; α_1-Acid glycoprotein; Carbohydrate dynamics; Sialic acids; Steady-state fluorescence anisotropy; Red-edge excitation shift

1. Introduction

The human α_1-acid glycoprotein (orosomucoid), a plasma glycoprotein of molecular weight of 41,000 Da, consists of a chain of 181 amino acids. It contains 40% carbohydrate by weight and has up to 16 sialic acid residues (10–14% by weight) [1]. Five-heteropolysaccharide groups are linked via an *N*-glycosylic bond to the asparaginyl residues of the protein [2]. This high degree of sialylation and the presence of acidic amino acid residues give rise to a very low pI of 2.8–3.8 [3,4]. The

* Corresponding author. Tel.: +33-3-2033-7770; fax: +33-3-2045-9218.

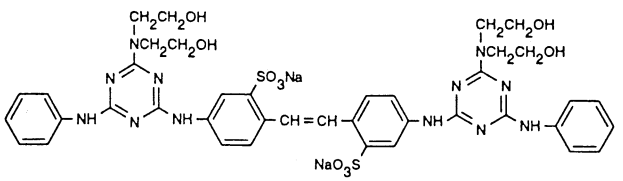
E-mail address: jihad-rene.albani@univ-lille1.fr (J.R. Albani)

0008-6215/99/$ - see front matter
PII: S0008-6215(99)00205-0

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

α_1-Acid glycoprotein contains three Trp residues, one at the surface and two embedded in the protein matrix [1,2]. The protein also binds the fluorescent probe, 2-(*p*-toluidinyl)naphthalene-6-sulfonate (TNS) [5]. We have used the fluorescence properties of the TNS and the Trp residues of the protein to study the local and global dynamics of the protein and to show the relationship that exists between the structure and the dynamics of the protein part of α_1-acid glycoprotein and its ability to bind ligands such as the propranolol, a β-adrenergic blocker, and steroid hormones such as progesterone [6,7].

Calcofluor White is a fluorescent probe capable of making hydrogen bonds with β-(1→4)- and β-(1→3)-linked polysaccharides [8]. The fluorophore shows a high affinity for chitin, cellulose and succinoglycan, forming hydrogen bonds with free hydroxyl groups [9].



Calcofluor White

Calcofluor 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 [10,11]. Recently, we reported for the first time experiments in vitro characterizing the nature of the interaction between calcofluor and α_1-acid glycoprotein. The interaction between the fluorophore and the protein occurs preferentially with the glycan residues, and it depends 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. We also found that binding of the fluorophore to the protein induces a red shift of the emission of the fluorophore [12].

The dynamics of proteins are currently investigated by fluorescence anisotropy studies and by the red-edge excitation spectra method [13,14]. Excitation at the red edge of the absorption spectrum of the fluorophore molecules allows one to study the flexibility of their microenvironment. In the presence of local motion, the position of the maximum of the fluorescence spectrum of the fluorophore does not vary with the excitation wavelength. However, in a viscous or rigid medium, the fluorescence maximum position shifts to higher wavelengths upon red-edge excitation [15,16].

Anisotropy studies allow one to monitor the dynamics of the fluorophore itself. Thus, both techniques are complementary, but different types of information are collected.

Fluorescence spectroscopy studies performed on the Trp residues of the asialylated α_1-acid glycoprotein have indicated that the sialic acid residues do not affect the internal dynamics of the protein [17].

In the present work, we studied the dynamics of Calcofluor bound to sialylated and asialylated α_1-acid glycoprotein. In the presence of sialylated protein, we found that the emission maximum of the fluorophore does not vary with the excitation wavelength. This result reveals that the carbohydrate residues surrounding the Calcofluor exhibit free motion.

When calcofluor is bound to asialylated α_1-acid glycoprotein, the emission maximum of the fluorophore is shifted to longer wavelengths upon changing the excitation wavelength. This means that the calcofluor microenvironment is rigid.

Anisotropy measurements as a function of temperature confirm the red-edge excitation spectra experiments, i.e., in the presence of sialylated α_1-acid glycoprotein, Calcofluor does have residual motion, while in the presence of asialylated protein, it is bound tightly to the carbohydrate residues of the α_1-acid glycoprotein. Therefore, our results indicate that the highly mobile sialic acid residues induce the mobility of Calcofluor. Also, they indicate that most of the other glycan residues are tightly bound to the protein core.

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 previously described [18]. 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 [5].

The asialylation of α_1-acid glycoprotein was performed with *Clostridium perfringens* neuraminidase, as described in Ref. [19].

Calcofluor White was purchased from Sigma Chemical Co. Its concentration was determined spectrophotometrically using an extinction coefficient of 4388 M^{-1} cm^{-1} at 352.7 nm [12].

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 bandwidth used for excitation and emission was 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. [20,21]. 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}, 435 or 450 in the presence of sialylated or asialylated α_1-acid glycoprotein, respectively). The bandwidth used for the excitation and the emission was 10 nm.

Fluorescence lifetime data of Calcofluor free in water or when bound to α_1-acid glycoprotein were determined at 20 °C by phase fluorometry using the multifrequency and the cross-correlation methods [22].

In this work, lifetime measurements were obtained with an automated multifrequency phase fluorometer. The instrument and the analysis techniques are as described previously [23,24], except for the excitation source which now consists of a mode-locked titanium sapphire laser (Spectra Physics Tsunami) pumped by a Beamlock 2080 Ar^+-ion laser (Spectra Physics 2080). The excitation wavelength of 295 nm was obtained after frequency tripling in a Spectra Physics GWU unit, and the lowest modulation frequency was obtained from a pulse picker (Spectra Physics). Emission was collected through a cut-off filter at 390 nm. Therefore, although at 295 nm both Trp residues and calcofluor were excited, at the emission wavelengths, we observed only the fluorescence of the calcofluor.

Phase measurements were carried out at 50 frequencies between 1 MHz and 1 GHz. Data analysis is described in Ref. [23]. Assignment of the minimum number of lifetimes necessary to obtain a good fit is judged on the basis of the least-squares sum and the autocorrelation function of the residuals. As a reference, P-terphenyl dissolved in cyclohexane was used with a lifetime of 1 ns [25]. All experiments were performed at 20 °C.

The fluorescence intensity of Calcofluor, whether free in solution or bound to the carbohydrates of α_1-acid glycoprotein, decays as a sum of four exponentials. When the fluorophore is free in solution, the intensity average fluorescence lifetime $\langle\tau\rangle$ [26] is 0.85 ns. It increases to 4.8 and 3.9 ns when the fluorophore is bound to the sialylated and to the asialylated protein, respectively.

The values of $\langle\tau\rangle$ were used to calculate the rotational correlation time from the Perrin plots.

Table 1
Fluorescence lifetimes (ns) and corresponding fractional intensities of Calcofluor free in solution and bound to sialylated and asialylated α_1-acid glycoprotein obtained by global analysis

τ_1	τ_2	τ_3	τ_4	f_1	f_2	f_3	f_4	$\langle\tau\rangle$
Calcofluor free in solution (3.2 μM). $\chi^2 = 1.4$								
0.3 ± 0.03	0.6 ± 0.3	2.28 ± 0.5	14.05 ± 4	0.34 ± 0.1	0.6 ± 0.1	0.03 ± 0.01	0.02	0.85
Calcofluor (6.6 μM) in the presence of 4.7 μM sialylated α_1-acid glycoprotein. $\chi^2 = 2.3$								
0.41 ± 0.01	1.07 ± 0.03	4.01 ± 0.2	12.13 ± 0.14	0.27 ± 0.008	0.25 ± 0.005	0.15 ± 0.04	0.32	4.8
Calcofluor (4.55 μM) in the presence of 5.5 μM asialylated α_1-acid glycoprotein. $\chi^2 = 3$								
0.35 ± 0.007	0.80 ± 0.021	3.3 ± 0.12	11.42 ± 0.12	0.22 ± 0.02	0.35 ± 0.01	0.16 ± 0.03	0.26	3.9

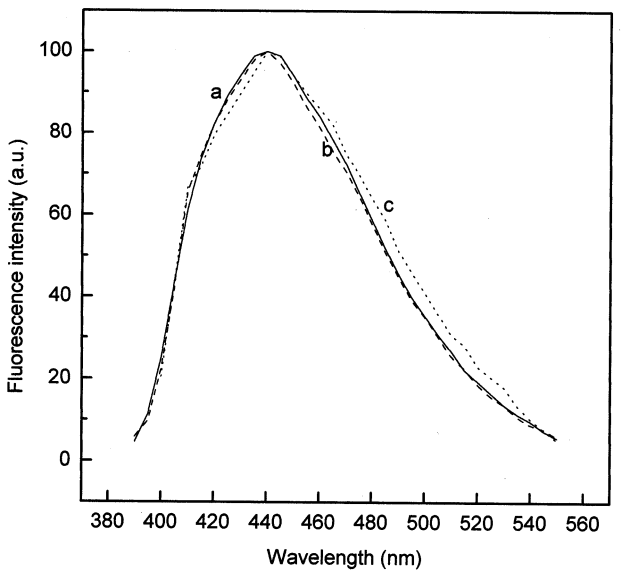


Fig. 1. Normalized steady-state fluorescence spectra of 10 μM Calcofluor in the presence of 15 μM sialylated α_1-acid glycoprotein recorded at three excitation wavelengths, 385 nm (a), 395 nm (b) and 405 nm (c). The fluorescence maximum is equal to 440 nm at the three excitation wavelengths.

Table 1 summarizes the lifetimes obtained for Calcofluor free in solution, in the presence of sialylatyed and asialylated α_1-acid glycoprotein.

3. Theory of red-edge excitation shift

Red-edge excitation spectra are used to monitor motions around the fluorophores [13–16]. 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 solution results in a redistribution of electronic charge on the fluorophore, inducing a significant change in both direction and strength of its dipole moment. Thus, dipole–dipole interaction in the ground state is different from that in the excited state. This new interaction is unstable. To reach stability, fluorophore molecules need to use some of their energy to reorient the dipole of the binding site. The dipole reorientation is called the relaxation phenomenon. After relaxation, fluorescence emission occurs. This is the case when relaxation is faster than fluorescence, i.e., the relaxation lifetime τ_r is shorter than the fluorescence lifetime $\langle\tau\rangle$. This happens when the binding site is flexible. This motion may induce that of calcofluor. Emission from a relaxed state does not change with the excitation wavelength.

When the binding site is rigid, fluorescence emission occurs before relaxation. In this case excitation at the longer-wavelength edge of the absorption band photoselects a population of fluorophores energetically different from those photoselected when the excitation wavelength is shorter. When the red-edge excitation is performed, the energy $h\nu^{edge}$ of the electronic transition is equal to $E_e^{edge} - E_g^{edge}$, where $E_e - E_g$ refers to the difference of energy between excited and ground states; $h\nu^{edge}$ is lower than $h\nu^{sw}$, the energy of the electronic transition that occurs at short wavelengths. Thus, excitation at the red edge gives a fluorescence spectrum with a maximum located at higher wavelengths than that obtained when excitation is performed at short wavelengths.

Thus, the observation of a red shift of $\lambda_{em,max}$ upon a red shift in λ_{em}, indicates that the system meets with the $\langle\tau\rangle < \tau_r$ condition. This means a decreased mobility of the fluorophore on its binding site with respect to the dipolar matrix of the site.

4. Results

Dynamics of Calcofluor bound to sialylated α_1-acid glycoprotein

Red-edge excitation spectra. Fig. 1 displays the normalized fluorescence emission spectra of 10 μM of calcofluor in the presence of 15 μM of α_1-acid glycoprotein obtained at three excitation wavelengths. The maximum (440 nm) of the Calcofluor fluorescence does not change with the excitation wavelength (λ_{ex}, 385, 395 and 405 nm). The results obtained clearly indicate that the microenvironment of the Calcofluor has residual motions independent of the global rotation of the protein, and this may induce the local motion of Calcofluor.

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 mass and η the viscosity of the medium [27]. α_1-Acid glycoprotein can be considered to be spherical [5,6], thus using Eq. (1), a rotational correlation time of 16 ns is calculated for the protein.

Steady-state fluorescence anisotropy of 10 μM of Calcofluor in the presence of 5 μM of α_1-acid glycoprotein ($\lambda_{em} = 435$ nm and $\lambda_{ex} = 300$ nm) was performed at different temperatures. A Perrin plot representation (Fig. 2(a), Eq. (2)) [28] is drawn:

$$1/A = 1/A_0 + \langle\tau\rangle/\Phi_R A_0 = 1/A_0 + (1/A_0)(1 + RT\langle\tau\rangle/\eta V) \quad (2)$$

where A and A_0 are the anisotropies in the presence and the absence of rotational diffusion, respectively, $\langle\tau\rangle = 4.8$ 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. (2) will be equal to that obtained at −45 °C. When the fluorophore exhibits significant motions when bound to the protein, Φ_R will be lower than Φ_P and will represent an apparent rotational correlation time Φ_A. Thus, Φ_A will be the result of two motions, that of the protein and that 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.

The rotational correlation time Φ_A (7.5 ns at 20 °C) is lower than that (16 ns) expected for α_1-acid glycoprotein and thus indicates that calcofluor displays segmental motions independent of the global rotation of the protein. Thus, two motions contribute to the depolarization process, the local motion of the carbohydrate residues and the global rotation of the protein, i.e., a fraction of the total depolarization is lost due to the segmental motion, and the remaining polarization decays as a result of the rotational diffusion of the protein.

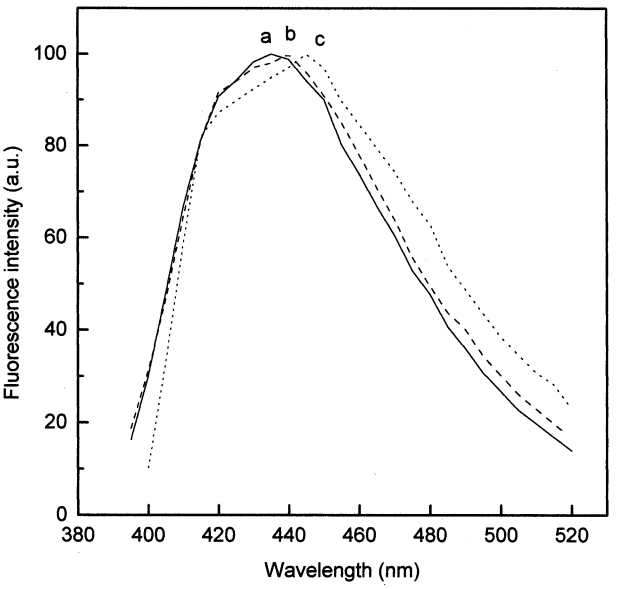


Fig. 3. Normalized steady-state fluorescence spectra of 7.5 μM Calcofluor in the presence of 5 μM asialylated α_1-acid glycoprotein recorded at three excitation wavelengths, 385 nm (a), 395 nm (b) and 415 nm (c). The fluorescence maximum is equal to 437, 440 and 445 nm at λ_{ex}, 385, 395 and 405 nm, respectively.

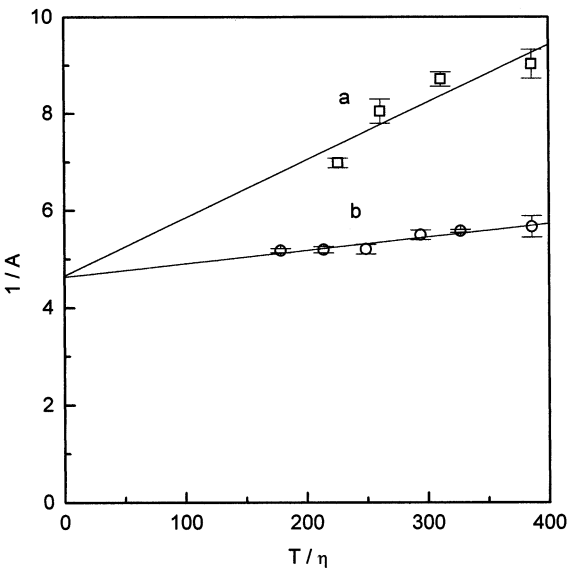


Fig. 2. Steady-state fluorescence anisotropy vs. temperature over viscosity for 5 μM Calcofluor in the presence of 10 μM sialylated α_1-acid glycoprotein (λ_{ex}, 300 nm; λ_{em}, 435 nm) (plot a), and for 8.5 μM Calcofluor in the presence of 5.5 μM asialylated α_1-acid glycoprotein (λ_{ex}, 300 nm; λ_{em}, 445 nm) (plot b). The data shown are the mean values of two measurements, and they are obtained by thermal variation in the range 15–35 °C. The ratio T/η is expressed in Kelvins/centipoise.

Dynamics of Calcofluor bound to asialylated α_1-acid glycoprotein. Fig. 3 exhibits the normalized fluorescence emission spectra of 7.5 μM of Calcofluor bound to 5 μM of asialylated α_1-acid glycoprotein obtained at three excitation wavelengths. The maximum of the fluorescence spectra of Calcofluor is a function of the excitation wavelength. At 385 nm, the emission maximum is located at 437 nm. It shifts to higher wavelengths (440 and 445 nm) when the excitation wavelengths are 395 and 405 nm, respectively. This is taken as direct evidence that carbohydrate residues in the microenvironment of Calcofluor exhibit restricted motions.

Steady-state anisotropy as a function of temperature. α_1-Acid glycoprotein presents 16 sialic acid residues of molecular mass 314. Thus, the molecular mass of asialylated α_1-acid glycoprotein is near 36,000. Eq. (1) yields a rotational correlation time of 14 ns for the asialylated protein.

Steady-state fluorescence anisotropy of 8.5 μM of calcofluor in the presence of 5.5 μM of α_1-acid glycoprotein ($\lambda_{em} = 445$ nm and $\lambda_{ex} = 300$ nm) was performed at different temperatures. The Perrin plot representation (Fig. 2(b)) yields a rotational correlation time Φ_P (19 ns at 20 °C), close to that (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 of the protein.

5. Discussion

The emission of Calcofluor is sensitive to its microenvironment, a characteristic that is shared by many fluorophores such as tryptophan [29], 2-(*p*-toluidinyl)naphthalene-6-sulfonate (TNS) [30], and flavin [31].

α_1-Acid glycoprotein exhibits genetic polymorphism, so that its amino acid backbone and the glycan antennae may vary within a single individual [32]. This heterogeneity may affect the interaction between extrinsic probes such as TNS or Calcofluor and the protein. For example, the multiple fluorescence intensity decay observed for the calcofluor would be the result of the microheterogeneity of the carbohydrate residues. However, the multiexponential decay was also observed for calcofluor free in water. Therefore, the four lifetimes observed are intrinsic to the calcofluor and probably due to different possible conformations of Calcofluor. Its binding to α_1-acid glycoprotein increases the average lifetime considerably. Looking closer at the data, it is revealed that this increase is largely due to an increase in the fractional intensities of the two longest lifetimes. This increase is the result of the type of interaction between the fluorophore and the carbohydrates of the glycoprotein. The results obtained with Calcofluor are in good agreement with those found with TNS: the protein microheterogeneity does not play any role in the interaction of the protein with TNS and is not responsible of the heterogeneous fluorescence of the fluorophore [33].

Dynamics of carbohydrate residues, whether free in solution or bound to a protein, are usually studied by ^{1}H NMR [34]. The present work allows one for the first time to follow the dynamics of a carbohydrate moiety using a fluorescence approach. In fact, our results show that the red-edge excitation spectra and anisotropy measurements are sensitive to the changes that occur in the microenvironment of the Calcofluor, i.e., within the carbohydrate residues.

In the red-edge excitation spectral studies, it is the Calcofluor environment consisting mainly of glycan residues and solvent dipoles that is relaxing around the excited Calcofluor. In the fluorescence anisotropy experiments, on the other hand, the displacement of the emission dipole moment of the Calcofluor is monitored. In the first approach, it is the environment that is either fluid or rigid. In the second approach, the restricted reorientational motion of the fluorophore is followed.

Absence of red-edge excitation shift for Calcofluor when bound to sialylated α_1-acid glycoprotein meets with the fact that emission occurs from a relaxed state, i.e., motions around Calcofluor do exist. This result is in good agreement with that found from the anisotropy experiment, i.e., the Calcofluor exhibits free motion. The apparent rotational

correlation time ($\Phi_A = 7.5$ ns) obtained from the Perrin plot is the result of the global rotation of the protein and the local motion of the Calcofluor.

In the presence of asialylated α_1-acid glycoprotein, a red-edge excitation shift of 8 nm is observed. This indicates that the microenvironment of the fluorophore exhibits restricted motion. Therefore, the carbohydrates in the vicinity of Calcofluor in the asialylated α_1-acid glycoprotein are rigid and do not show any segmental motion.

Anisotropy data as a function of temperature (Fig. 2(b)) yield a rotational correlation time (19 ns) close to that (14 ns) theoretically calculated for asialylated α_1-acid glycoprotein. Thus, in the asialylated protein, the Calcofluor is tightly bound to the carbohydrate residues and follows the global motion of the protein.

When Calcofluor is dissolved in a solvent such as water, a red-edge excitation shift in the emission does not occur (data not shown). Thus, the spectral properties of calcofluor either bound to α_1-acid glycoprotein or dissolved in a liquid isotropic solvent are not equivalent. Calcofluor fluorescence is sensitive to the polarity of the surrounding environment and to the dynamics of this microenvironment.

Calcofluor free in solution fluoresces with an emission maximum of 432 nm. When bound to sialylated or asialylated α_1-acid glycoprotein, it emits with a maximum equal to 437 or 440 nm. Also, the average fluorescence lifetime of calcofluor, whether bound to sialylated or to asialylated protein, is almost the same. Thus, the presence of the sialic acid residues on α_1-acid glycoprotein does not affect the fluorescence parameters of the bound Calcofluor, although an interaction between the fluorophore and the terminal glycan residues occurs. In fact, our results show that this interaction increases the local dynamics of Calcofluor.

The rotational correlation time of the carbohydrate side chains α_1-acid glycoprotein (largely 2-acetamido-2-deoxy-D-glucose and *N*-acetylneuraminic acid), calculated from ^{1}H NMR experiments [35,36], is near 0.1 ns [16]. The rotational correlation time of α_1-acid glycoprotein is equal to 16 ns. Thus, the carbohydrate side-chain residues are highly mobile compared to the protein.

The present work clearly shows that the sialic acid residues are highly mobile, inducing the mobility of Calcofluor, while the other glycan residues exhibit restricted motions. This result is in good agreement with the finding that in most glycoprotein structures known to date, only the 1–4 sugar residues most proximal to the glycosylation site are immobilized [37].

We recently showed that the fluorescence of calcofluor can be used to study the structure and the spatial organization of the glycans [12], while the present work deals with the possibility of using fluorescence properties of calcofluor to study the dynamics of carbohydrate residues. Until now, structure and dynamics of glycans were studied mainly with NMR and X-ray diffraction studies. Our work shows that fluorescence spectroscopy can be a complementary approach toward studying the molecular dynamics of the glycan moiety.

References

[1] T. Kute, U. Westphal, *Biochim. Biophys. Acta*, 420 (1976) 195–213.
[2] K. Schmid, H. Kaufmann, S. Isemura, F. Bauer, J. Emura, T. Motoyama, M. Ishiguro, S. Nanno, *Biochemistry*, 12 (1973) 2711–2724.
[3] M.L. Friedman, K.T. Schlueter, T.L. Kirley, H.B. Halsall, *Biochem. J.*, 232 (1985) 863–867.
[4] I. Nicollet, J.P. Lebreton, M. Fontaine, M. Hiron, *Biochim. Biophys. Acta*, 668 (1981) 235–245.
[5] J. Albani, *Biophys. Chem.*, 44 (1992) 129–137.
[6] J.R. Albani, *J. Biochem.*, 116 (1994) 625–630.
[7] J.R. Albani, *Spectrochim. Acta Part 1*, 55 (1999) 2353–2360.
[8] I.D. Rattee, M.M. Greur, *The Physical Chemistry of Dye Absorption*, Academic Press, New York, 1974, pp. 181–182.
[9] H. Macda, N. Ishida, *J. Biochem.*, 62 (1967) 276–278.
[10] J.E. Gonzalez, G.M. York, G.C. Walker, *Gene*, 179 (1996) 141–146.
[11] T.F. Hogan, R.S. Riley, J.G. Thomas, *J. Clin. Lab. Anal.*, 11 (1997) 202–207.
[12] J.R. Albani, Y.D. Plancke, *Carbobydr. Res.*, 318 (1999) 193–200.
[13] R.F. Chen, *Anal. Biochem.*, 19 (1967) 374–387.
[14] J.R. Lakowicz, H. Cherek, *Biochem. Biophys. Res. Commun.*, 99 (1981) 1173–1178.
[15] J.R. Albani, *Spectrochim. Acta., Part A*, 54 (1998) 175–182.
[16] J.R. Albani, *J. Fluoresc.*, 8 (1998) 213–224.
[17] J.R. Albani, *Biochim. Biophys. Acta*, 1291 (1996) 215–220.
[18] Y. Plancke, M. Dautrevaux, G. Biserte, *Biochimie*, 60 (1978) 171–175.
[19] T.F. Busby, K.C. Ingham, *Biochim. Biophys. Acta*, 871 (1986) 61–71.

[20] J.R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Plenum, New York, 1983.
[21] J. Albani, *Arch. Biochem. Biophys.*, 243 (1985) 292–297.
[22] R.D. Spencer, Fluorescence Lifetimes: Theory, Instrumentation and Application of Nanosecond Fluorometry. Ph.D Thesis, 1970, University of Illinois at Urbana-Champaign (University Microfilms Intl. editions, Ann Arbor, MI, accession no. AAG7114956).
[23] R. Vos, R. Strobbe, Y. Engelborghs, *J. Fluoresc.*, 7 (1996) 33S–35S.
[24] A. Sillen, R. Vos, Y. Engelborghs, *Photochem. Photobiol.*, 64 (1996) 785–791.
[25] G. Desie, N. Boens, F.C. De Schryver, *Biochemistry*, 25 (1986) 8301–8308.
[26] A. Sillen, Y. Engelborghs, *Photochem. Photobiol.*, 67 (1998) 475–486.
[27] 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.
[28] G. Weber, *Biochem. J.*, 51 (1952) 145–155.
[29] E.A. Burstein, N.S. Vedenkina, M.N. Ivkova, *Photochem. Photobiol.*, 18 (1973) 263–275.
[30] W.O. McClure, G.M. Edelman, *Biochemistry*, 5 (1966) 1908–1919.
[31] J.R. Albani, A. Sillen, Y. Engelborghs, M. Gervais, *Photochem. Photobiol.*, 69 (1999) 22–26.
[32] U. Westpha, *Steroid–Protein Interaction*, Ch. 13, Springer-Verlag, New York, 1971, pp. 375–433.
[33] J. Albani, R. Vos, K. Willaert, Y. Engelborghs, *Photochem. Photobiol.*, 62 (1995) 30–34.
[34] D.F. Wyss, J.S. Choi, J. Li, M.H. Knoppers, K.J. Willis, A.R.N. Arulanandam, A. Smolyar, E.L. Reinherz, G. Wagner, *Science*, 269 (1995) 1273–1278.
[35] J.D. Bell, J.C. Brown, J.K. Nicholson, P.J. Sadler, *FEBS Lett.*, 215 (1987) 311–315.
[36] K. Akiyama, E.R. Simons, P. Bernasconi, K. Schmidt, H. Van Halbeck, J.F.G. Vliegenthart, H. Haupt, G.H. Schwick, *J. Biol. Chem.*, 259 (1984) 7151–7154.
[37] D.F. Wyss, G. Wagner, *Curr. Opin. Biotechnol.*, 7 (1996) 409–416.