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Carbohydrate Research 338 (2003) 1097–1101
CARBOHYDRATE RESEARCH
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Relation between the secondary structure of carbohydrate residues of α_1-acid glycoprotein (orosomucoid) and the fluorescence of the protein

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Received 9 December 2002; accepted 12 February 2003

Abstract

We studied in this work the relation that exists between the secondary structure of the glycans of α_1-acid glycoprotein and the fluorescence of the Trp residues of the protein. We calculated for that the efficiency of quenching and the radiative and non-radiative constants. Our results indicate that the glycans display a spatial structure that is modified upon asialylation. The asialylated conformation is closer to the protein matrix than the sialylated form, inducing by that a decrease in the fluorescence parameters of the Trp residues. In fact, the mean quantum yield of Trp residues in sialylated and asialylated α_1-acid glycoprotein are 0.0645 and 0.0385, respectively. Analysis of the fluorescence emission of α_1-acid glycoprotein as the result of two contributions (surface and hydrophobic domains) indicates that quantum yields of both classes of Trp residues are lower when the protein is in the asialylated form. Also, the mean fluorescence lifetime of Trp residues decreases from 2.285 ns in the sialylated protein to 1.948 ns in the asialylated one. The radiative rate constant k_r of the Trp residues in the sialylated α_1-acid glycoprotein is higher than that in the asialylated protein. Thus, the carbohydrate residues are closer to the Trp residues in the absence of sialic acid. The modification of the spatial conformation of the glycans upon asialylation is confirmed by the decrease of the fluorescence lifetimes of Calcofluor, a fluorophore that binds to the carbohydrate residues. Finally, thermal intensity quenching of Calcofluor bound to α_1-acid glycoprotein shows that the carbohydrate residues have slower residual motions in the absence of sialic acid residues.

Keywords: α_1-Acid glycoprotein; Trp residues; Calcofluor white; Sialic acid; Fluorescence lifetimes; Fluorescence quantum yield; Microenvironment polarity

1. Introduction

The human 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).[1] Five heteropolysaccharide groups are linked via an N-glycosylic bond to the asparaginyl residues of the protein.[2] The protein contains tetra-antennary as well as di- and triantennary glycans.

Calcofluor white is a fluorescent probe that binds preferentially to the glycan residues of α_1-acid glycoprotein.[3] The interaction is dependent on the spatial conformation of the glycans.

α_1-Acid glycoprotein contains three Trp residues, one at the surface and two in the protein matrix.[1,2] The three Trp residues contribute to the fluorescence of the protein.[4,5]

In the present work, we studied the relation that exists between the secondary structure of carbohydrate residues of α_1-acid glycoprotein and the fluorescence of the protein. Therefore, we determined and compared the fluorescence parameters such as lifetimes, quantum yields and radiative constants of Trp residues in the sialylated and asialylated forms. Our results show that asialylated α_1-acid glycoprotein possesses quantum yields and lifetimes lower than those in the sialylated

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0008-6215/03/$ - see front matter
doi:10.1016/S0008-6215(03)00075-2

protein. Also, the radiative rate constant k_r of the Trp residues are lower in the asialylated form than in the sialylated one, i.e., the polarity of the microenvironment of the Trp residues increases in the asialylated protein. The non-radiative rate constant k_{nr} is lower in the sialylated form; thus, the molecular interaction within the protein is more important in the asialylated form. These results suggest that the carbohydrate residues are closer to the Trp residues in the absence of sialic acids, i.e., the carbohydrate residues have a secondary structure that differs whether the sialic acid residues are present or absent. A conclusion confirmed by the values of the fluorescence lifetimes of Calcofluor bound to sialylated and asialylated proteins and by the thermal intensity quenching of Calcofluor. In fact, thermal intensity quenching of Calcofluor (this work) along with anisotropy studies[6] indicate that local motions and flexibility of the carbohydrate residues vary with their secondary structure.

2. Materials and methods

α_1-Acid glycoprotein was purified from serum by a successive combination of ion-displacement chromatography, gel-filtration and ion-exchange chromatography as already described.[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.[8]

Calcofluor white was from Sigma Chemical Co. Its concentration was determined spectrophotometrically using an extinction coefficient of 4388 M^{-1} cm^{-1} at 352.7 nm.[3]

Absorbance data were obtained with a Perkin–Elmer-555 spectrophotometer using 1-cm pathlength cuvettes.

Fluorescence spectra were recorded with a Perkin–Elmer LS-5B spectrofluorometer. The bandwidths used for the excitation and the emission were 2.5 nm. The quartz cuvettes had optical pathlengths equal to 1 and 0.4 cm for the emission and excitation wavelengths, respectively. Fluorescence spectra were corrected for the background intensities of the buffer solution. Corrections for the inner filter effect were not necessary as a consequence of the low values of the optical densities at the excitation and emission wavelengths.

Fluorescence lifetimes data of Calcofluor and Trp residues of α_1-acid glycoprotein have been already published.[6,9]

Quantum yields were determined relative to tryptophan in water according to the method of Parker and Rees.[10]

$$Q_{prot} = \frac{A_{Trp}\, I_{prot}}{A_{prot}\, I_{Trp}}\, Q_{Trp} \quad (1)$$

where I is the integrated intensity over the wavelength region of 300–400 nm, A is the absorbance at 295 nm, and the quantum yield Q_{Trp} for tryptophan in water is taken as 0.14.[10]

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

3. Results and discussion

Quantum yields of the sialylated and asialylated α_1-acid glycoprotein are 0.0645 and 0.0385, respectively. Since, emission of α_1-acid glycoprotein can be interpreted in terms of two contributions (surface and hydrophobic domains),[4,5] it should be possible to estimate the quantum yield of each class of Trp residues.

α_1-Acid glycoprotein contains three Trp residues that contribute to the fluorescence of the protein.[4,5] The two hydrophobic Trp residues participate equally to the 'blue' emission,[4] thus one can write:

$$Q = \frac{2Q_{blue} + Q_{red}}{3} \quad (2)$$

Table 1 shows the values of Q, Q_{blue} and Q_{red} for the sialylated and asialylated α_1-acid glycoproteins.

We notice that the quantum yields of asialylated α_1-acid glycoprotein are lower than those obtained for the sialylated protein. Therefore, asialylation of the protein induces a quenching of the fluorescence quantum yield of α_1-acid glycoprotein of both classes of Trp residues.

Table 2 shows the values of the fluorescence lifetimes of the Trp residues of the sialylated and asialylated α_1-acid glycoproteins. The longest lifetime is attributed to the surface residue (Trp-166 residue) while the 1.4 ns lifetime is attributed to the blue Trp residue (Trp-25 residue).[11] We notice that the asialylation of α_1-acid glycoprotein induces a quenching of the fluorescence lifetimes.

The efficiency of quenching is equal to

Table 1
Fluorescence quantum yield of the global emission (Q), the red Trp residues (Q_{red}) and the hydrophobic Trp residues (Q_{blue}) for the sialylated and asialylated α_1-acid glycoproteins

	Sialylated α_1-acid glycoproteins	Asialylated α_1-acid glycoproteins
Q	0.0645 ± 0.005	0.0385 ± 0.0036
Q_{red}	0.0780 ± 0.007	0.0462 ± 0.0085
Q_{blue}	0.0585 ± 0.006	0.0346 ± 0.0022

The values are from six experiments.

Table 2
Fluorescence lifetimes (in ns) of the Trp residues of the sialylated and asialylated α_1-acid glycoproteins

	τ_1	f_1	τ_2	f_2	τ_3	f_3	$\langle\tau\rangle$
Sialylated α_1-acid glycoprotein	0.354 ± 0.034	0.101 ± 0.05	1.664 ± 0.072	0.66 ± 0.03	4.638 ± 0.342	0.238 ± 0.01	2.285
Asialylated α_1-acid glycoprotein	0.197 ± 0.037	0.07 ± 0.01	1.42 ± 0.05	0.65 ± 0.02	3.61 ± 0.15	0.28 ± 0.03	1.948

$$E = 1 - \frac{\tau_t}{\tau_o} = 1 - \frac{Q_t}{Q_o} \quad (3)$$

where τ and Q are the mean fluorescence lifetime and quantum yield in the absence (τ_t and Q_t) and in the presence of sialic acid (τ_o and Q_o).

Table 3 shows the values of E calculated from the mean lifetime and quantum yield and from the lifetime and quantum yield of the surface and hydrophobic Trp residues. The value of E calculated from the quantum yield is higher than that measured from the lifetime. Therefore, fluorescence lifetimes of Trp residues of α_1-acid glycoprotein are the result of an energy transfer Förster type or by electron transfer to neighboring amino acids and of the molecular collisions of the Trp residues with their environments.

This local motion is indicated by the fact that the value of E calculated from the quantum yields of each class of Trp residues is equal to that calculated from the mean quantum yield and is higher than the value of E obtained from the lifetimes. This means that the two classes of Trp residues display local motions, a result that confirms the one obtained with the anisotropy measurements.[5]

The fact that the fluorescence parameters of α_1-acid glycoprotein decrease in the asialylated form, indicates that the glycans are closer to the protein matrix in the absence of the sialic acids than in their presence. Removal of the sialyl groups causes the carbohydrate residues to contract closer to the core of the protein. This is likely to be the case, since the sialyl group is charged. Thus, the microenvironment of the Trp residues of α_1-acid glycoprotein should be more polar in the asialylated form. In this case, the radiative rate constant k_r

$$k_r = 1/\tau_r = Q/\langle\tau\rangle \quad (4)$$

and the non-radiative rate constant k_{nr}

$$k_{nr} = (1/Q)/\langle\tau\rangle \quad (5)$$

of the sialylated and asialylated α_1-acid glycoprotein are not the same. The values of k_r and k_{nr} for the sialylated and asialylated α_1-acid glycoprotein calculated from the mean values of Q and τ and of each class of Trp residues are shown in Table 4.

The radiative rate constant of indole increases when the polarity of the environment decreases.[12] We note from Table 4 that the k_r of the sialylated α_1-acid glycoprotein is higher than that of the asialylated protein. Thus, presence of sialic acids decreases the polarity of the environment of the Trp residues. This suggests that the carbohydrate residues are closer to the Trp residues in absence of sialic acids.

This is also observed for the surface (Trp-166) and hydrophobic residues, since the k_r calculated for each class of Trp residue are higher for the sialylated α_1-acid glycoprotein than for the asialylated protein. This means that asialylation of α_1-acid glycoprotein affects the secondary structure of the whole carbohydrates of the protein, i.e., asialylation induces a motion of the glycans toward the protein matrix. Since the polarity of the microenvironment of Trp-25 is also affected, then this residue is close to the carbohydrate residues and is not buried in the protein core. These results are in good agreement with the model we have already described: the N-terminal side chain adopts a spatial conformation so that a pocket in contact with the buffer is induced and to which Trp-25 residue belongs. The five carbohydrate units are linked to the pocket, i.e., the glycosylation site belongs to the pocket.[13]

Comparing the k_{nr} values obtained for the sialylated and asialylated α_1-acid glycoprotein, we notice that the k_{nr} of the sialylated protein is lower than that of the asialylated one (see Table 4). Thus, the molecular interaction in α_1-acid glycoprotein is more important in the asialylated protein, i.e., in the absence of sialic acid residues the carbohydrate residues are closer to the protein matrix and thus to the Trp-25 and Trp-166 residues.

Table 3
Values of E calculated from the mean lifetime and quantum yield and from the lifetimes and quantum yields of the surface (longest lifetime) and hydrophobic Trp residue ($\tau = 1.8$ or 1.4 ns)

$E_{(\langle\tau\rangle)}$	$E_{(\tau)(\text{blue})}$	$E_{(\tau)(\text{red})}$	$E_{(Q)}$	$E_{(Q)(\text{blue})}$	$E_{(Q)(\text{red})}$
0.148	0.147	0.220	0.403	0.408	0.408

Table 4
Values of the radiative (k_r) and non-radiative (k_{nr}) rate constants calculated from the mean values of the quantum yield (Q) and lifetime ($\langle\tau\rangle$) and from the values of Q and τ of the red Trp residues and the hydrophobic Trp residues for the sialylated and asialylated α_1-acid glycoproteins

	$\langle k_\tau \rangle$	$\langle k_{nr} \rangle$	$k_{r(red)}$	$k_{r(blue)}$	$k_{nr(red)}$	$k_{nr(blue)}$
Sialylated α_1-acid glycoprotein	0.028	0.409	0.0168	0.035	0.198	0.566
Asialylated α_1-acid glycoprotein	0.0197	0.494	0.0128	0.0244	0.2642	0.679

All our data, lifetime, quantum yield, k_r and k_{nr} indicate that in the asialylated α_1-acid glycoprotein, compared to the sialylated protein, a fluorescence quenching of the Trp residues is occurring as the result of a strong interaction with the carbohydrate residues. In the asialylated form, the carbohydrate residues are closer to the protein surface and matrix than in the sialylated form.

The results obtained and the conclusions drawn from the data on the Trp residues are confirmed by the fluorescence lifetimes measurements obtained on Calcofluor. In fact, the mean fluorescence lifetime of Calcofluor bound to the carbohydrate residues of sialylated α_1-acid glycoprotein decreases from 4.8 to 3.9 ns in the asialylated one.[6] The efficiency of quenching is equal to 0.1875, a value close to that (0.148) found for the Trp residues. Therefore, in presence of sialic acids, the spatial conformation of the carbohydrate residues is different from that observed in their absence. Asialylated α_1-acid glycoprotein possesses the carbohydrate structure and backbone closer to that of the protein matrix than the sialylated form, inducing by that a decrease in the fluorescence lifetime of Calcofluor as the result of the molecular interaction between the carbohydrate residues and the protein matrix.

Increasing the temperature from 6 to 35 °C induces a decrease in the fluorescence intensity emitted by Calcofluor bound to the sialylated and asialylated forms of α_1-acid glycoprotein (Fig. 1). The intensity decrease is more important in the sialylated form of the protein. This decrease is not accompanied by a shift in the emission maximum wavelength (data not shown). The fluorescence of Calcofluor itself shows no thermal quenching (Fig. 1a). Therefore, thermal quenching is not related to environmental polarity alone. The presence of a structured conformation (carbohydrate residues) around the fluorophore induces the sensitivity of the fluorophore to temperature. The origin of this thermal quenching is the flexibility of the carbohydrate residues. Our results indicate that this flexibility is more important for the sialylated α_1-acid glycoprotein than for the asialylated one. This result is in good agreement with the fact that anisotropy studies and red-edge excitation spectra have shown that sialic acid displays important local motions, while the carbohydrate residues proximal to the glycosylation site have restricted motions.[6]

Since Calcofluor binds to the pocket of α_1-acid glycoprotein,[14] the thermal quenching observed could also be the result of a local flexibility that originates from both the carbohydrate residues surrounding the fluorophore and the amino acids of the glycosylation site. In fact, flexibility and local motions were observed for Trp residues in both sialylated and asialylated α_1-acid glycoprotein.[8,11] Therefore, the interaction between the carbohydrate residues and the protein matrix does not necessarily induce the compactness of the matrix. In fact, asialylated α_1-acid glycoprotein possesses the same physiological properties as those observed for the sialylated form such as binding of progesterone[15] and propranolol.[16]

In conclusion, the present work describes the relation that exists between the spatial conformation of the carbohydrate residues of α_1-acid glycoprotein and the fluorescence of the Trp residues of the protein. This

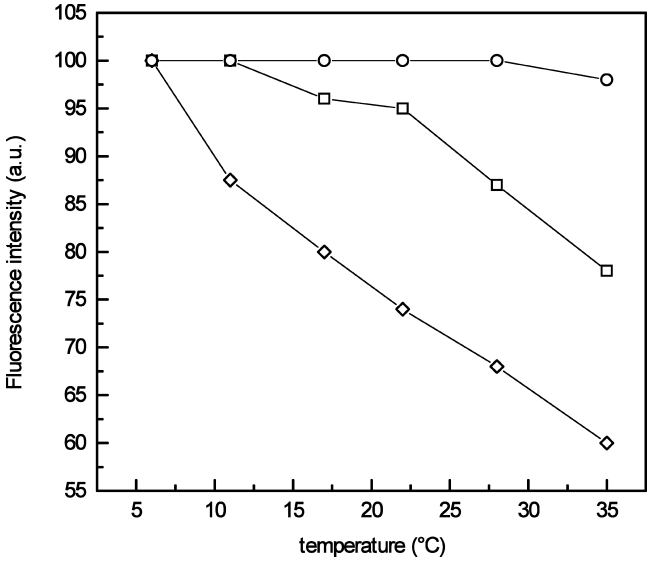

Fig. 1. Fluorescence thermal quenching of Calcofluor free in solution (a), bound to sialylated α_1-acid glycoprotein (b) and bound to asialylated α_1-acid glycoprotein (c). The concentration of the fluorophore (5 μM) is equal to that of the proteins. $\lambda_{ex} = 330$ nm and $\lambda_{em} = 435$ nm. Temperature = 20 °C.

relation is specific to α_1-acid glycoprotein and can in no way be generalized, since in each protein the Trp residues have their own microenvironments that differ from a protein to another.

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