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Interaction between calcofluor white and carbohydrates of α_1-acid glycoprotein

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Abstract

Interactions between the fluorescent probe, calcofluor white, and human serum albumin (HSA) and α_1-acid glycoprotein (orosomucoid) are compared. The two proteins have comparable isoelectric points, but α_1-acid glycoprotein is highly glycosylated (40% of glycans by weight), while the serum albumin is not. Binding of calcofluor to the proteins induces an increase in both the fluorescence anisotropy and the fluorescence intensity of the fluorophore. Also, we found that the calcofluor exhibits a fluorescence emission with a maximum located at 432, 415 or 445 nm, respectively, in the absence of proteins, in the presence of HSA, and in the presence of α_1-acid glycoprotein. The stoichiometries of the calcofluor–serum albumin and calcofluor–α_1-acid glycoprotein complexes are 2:1 and 1:1, respectively. The association constants are 0.04 and 0.15 μM^{-1}, respectively. The calcofluor does not interact with *Lens culinaris* agglutinin (LCA), although the protein has a hydrophobic site. Nevertheless, one cannot exclude that the binding of the fluorophore to the HSA is nonspecific. Our results, when compared with those obtained with calcofluor dissolved in the hydrophobic solvent isobutanol, and with the fluorescent probe, potassium 6-(*p*-toluidino)-2-naphthalenesulfonate (TNS), bound to α_1-acid glycoprotein, indicate that the emission of calcofluor bound to HSA occurs from a hydrophobic state, while that of calcofluor bound to α_1-acid glycoprotein occurs from a hydrophilic state. The fluorescence intensity of calcofluor decreases in the presence of carbohydrates isolated from α_1-acid glycoprotein, while it increases in the presence of α_1-cellulose. Thus, calcofluor interacts mainly with the glycan moiety of α_1-acid glycoprotein, and its fluorescence is sensitive to the secondary structure of the glycans.

Keywords: Calcofluor white; α_1-Acid glycoprotein; Carbohydrates, spatial organization; Fluorescence intensity quenching

1. Introduction

The human α_1-acid glycoprotein (orosomucoid) is a small acute-phase glycoprotein ($M_r = 41{,}000$) that is negatively charged at a 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].

Although the biological function of α_1-acid glycoprotein is still obscure, a number of activities of possible physiological significance have been described, such as the ability to bind the basic drug warfarin [3] and certain steroid hormones such as progesterone [4].

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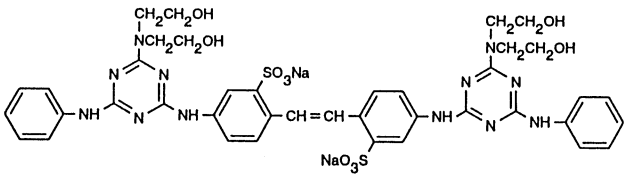
Calcofluor white is a fluorescent probe capable of making hydrogen bonds with β-(1→4) and β-(1→3) polysaccharides [5].



Structure of calcofluor white

The fluorophore shows a high affinity for chitin, cellulose and succinoglycan, forming hydrogen bonds with free hydroxyl groups [6].

In the presence of succinoglycan, a polymer of an octasaccharide repeating unit, consisting of galactose, glucose, acetate, succinate and pyruvate in a ratio of ≃1:7:1:1:1 [7], calcofluor fluoresces brightly as the result of its binding to the oligasaccharide [8].

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 [9–12]. However, in vitro, no experiments have been reported on the interaction between calcofluor and glycoproteins thus far.

Fluorescence is widely used to study interactions between a ligand and a protein or between two proteins. Fluorescence parameters such as anisotropy and intensity and position of the emission maximum are sensitive to the modifications occurring in the microenvironment of the fluorophore [13–16].

In the present work we report on our studies of the nature of the interaction between calcofluor and α_1-acid glycoprotein by comparing the fluorescence spectra of free calcofluor with those of the following complexes: calcofluor–α_1-acid glycoprotein, calcofluor–human serum albumin (HSA) and calcofluor–*Lens culinaris* agglutinin (LCA) (a lentil lectin). The HSA and α_1-acid glycoprotein have similar isoelectric points; however, serum albumin does not contain any glycan, while α_1-acid glycoprotein is highly glycosylated. The lentil lectin is a tetramer of molecular weight of 52,570 Da, which is not glycosylated [17].

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 described already [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 [19].

The pronasic glycopeptides (one or two amino acids) from α_1-acid glycoprotein have been prepared following the procedure of Muramatsu et al. [20]. Briefly, the sample (10 mg) was dissolved in 10 mL of Tris–HCl (0.14 M), 14 mM $CaCl_2$, pH 8, with 5 mg pronase (Pronase F, E. Merck, Darmstadt) and was kept at 37 °C for 72 h. Pronase (2.5 mg) was then added after 24 and 48 h. The glycopeptide was desalted onto a BioGel P2 column equilibrated with twice-distilled water. In order to yield a large amount of glycopeptide, this procedure was repeated three times.

LCA, HSA and calcofluor were from Sigma Chemical Co.. The concentration of LCA was determined spectrophotometrically at 280 nm ($E^{1\%}_{1\,cm} = 12.5$ [21]). The concentration of HSA was determined spectrophotometrically using

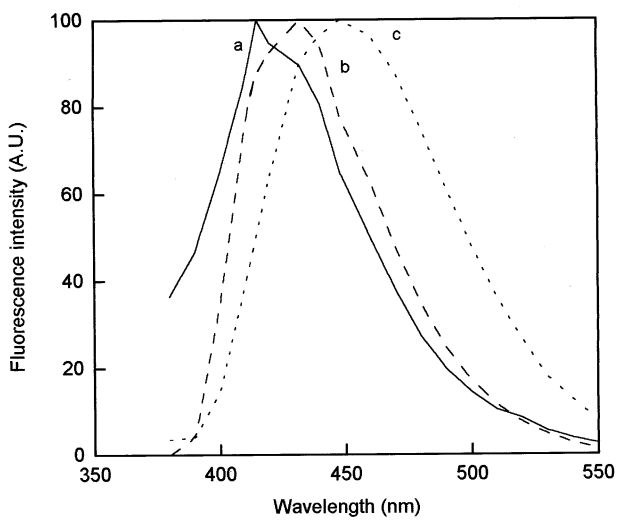


Fig. 1. Normalized emission spectra of 0.9 μM of calcofluor free in 10 mM phosphate, 0.143 M NaCl buffer, pH 7 (b) (λ_{max} 432 nm), in the presence of 50 μM of HSA (λ_{max} 415 nm) (a) and of 250 μM of calcofluor in the presence of 8.5 μM of α_1-acid glycoprotein (λ_{max} 447 nm) (c). λ_{ex} 300 nm and temperature = 20 °C.

an extinction coefficient of 4.2×10^4 M^{-1} cm^{-1} at 280 nm [22], and the concentration of calcofluor was determined spectrophotometrically using an extinction coefficient of 4388 M^{-1} cm^{-1} at 352.7 nm. 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. 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 [23,24]. Finally, fluorescence spectra were corrected for the background intensities of the buffer solution.

Steady-state anisotropy was measured with the same Perkin–Elmer fluorometer used for the emission spectra acquisition. Bandwidths used for excitation and emission were both 5 nm.

3. Results

Fluorescence parameters of calcofluor free in solution and bound either to α_1-acid glycoprotein or to human serum albumin.—Fig. 1 exhibits the normalized fluorescence emission spectra of calcofluor free in the phosphate–NaCl buffer (b), in the presence of a saturated concentration of α_1-acid glycoprotein (c) and of HSA at a concentration close to saturation (a). The emission maximum of the fluorophore shifts from 432 nm in buffer to 447 or to 415 nm, respectively, in the presence of α_1-acid glycoprotein or HSA. The red shift (15 nm) of the maximum observed in presence of α_1-acid glycoprotein compared with the maximum obtained in water indicates that the microenvironment of the excited state of calcofluor on α_1-acid glycoprotein is hydrophilic. The blue shift (17 nm) observed in presence of the serum albumin corresponds to an emission from a hydrophobic environment. Thus, the interaction of calcofluor with serum albumin arises from molecular energy transitions different from those present in the interaction between the fluorophore and α_1-acid glycoprotein.

An increase in the fluorescence intensity of calcofluor was observed upon its interaction with both proteins (not shown). The emission maximum of calcofluor on HSA (415 nm) is close to that (413 nm) observed when the fluorophore is dissolved in isobutanol.

At 0.9 μM of calcofluor and in the presence of a saturated concentration of α_1-acid glycoprotein (45 μM) we observed an increase of the fluorescence intensity. However, this enhancement was not accompanied by a shift in the emission maximum (data not shown).

Upon increasing the concentration of calcofluor to 5 μM, and when calcofluor is in excess compared to α_1-acid glycoprotein (for example, 200 μM of calcofluor in presence of 5 μM of protein), the shift to 447 nm is obtained (Fig. 1). Thus, it seems that a minimum concentration of calcofluor is necessary to observe the red shift.

Anisotropy (A) at 20°C of calcofluor free in solution increases from 0.1830 ± 0.0007 to 0.1982 ± 0.0007 and 0.2094 ± 0.0014 in the presence of α_1-acid glycoprotein and HSA, respectively. The increase in anisotropy indicates that the calcofluor interacts with both proteins [25].

Binding parameters of the calcofluor–α_1-acid glycoprotein and calcofluor–HSA complexes.—The interaction between calcofluor and α_1-acid glycoprotein or HSA induces a decrease in the fluorescence intensity of the Trp residues of the proteins (λ_{ex} 295 nm). In order to determine the binding pararameters (stoichiometry and the association constant) of the two complexes, a titration of a constant amount of each protein with calcofluor was performed, following the intensity decrease of the Trp residues (Fig. 2).

The dissociation constants of the calcofluor–protein complexes were determined by fitting the data to Eq. (1), obtained from the balance of total fluorescence:

$$\Delta F/F_0 = (\Delta F_{max}/F_0)(L_b/P) \quad (1)$$

where ΔF, ΔF_{max}, F_0, L_b and P are the fluorescence change for a concentration L of calcofluor, the maximum fluorescence change at saturation of the protein with calcofluor, the

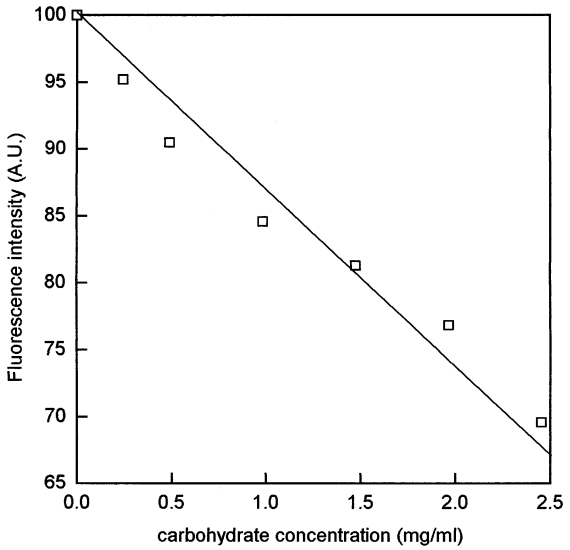


Fig. 2. Titration curves of α_1-acid glycoprotein (17 μM) (a) and of HSA (10.5 μM) (b) with calcofluor. The number n of binding sites was calculated by plotting $\Delta F/\Delta F_{max}$ vs. the calcofluor concentration. The equation used is $\Delta F/\Delta F_{max} = nL/(P + K_d)$, where ΔF is the fluorescence change for a concentration L of calcofluor, ΔF_{max} is the maximum fluorescence change at saturation, P the protein concentration and K_d is the corresponding dissociation constant of the complex. The value of n was found equal to 1.1 ± 0.1 and 2.1 ± 0.1 for the calcofluor–α_1-acid glycoprotein and calcofluor–HSA complexes, respectively. In both experiments, the excitation wavelength was 295 nm, and the emission wavelengths were 333 and 340 nm, when the titration was performed in the presence of α_1-acid glycoprotein and HSA, respectively. Temperature = 20 °C.

fluorescence intensity of the Trp residues in absence of calcofluor, the concentration of bound calcofluor and the total concentration of the protein, respectively. The concentration of bound calcofluor can be calculated from the root of the quadratic Eq. (2) arising from the definition of the binding constant:

$$L_b = 0.5[(P + L + K_d) + \{(L + P + K_d)^2 - 4LP\}^{1/2}] \quad (2)$$

Replacing Eq. (2) in Eq. (1) gives Eq. (3):

$$\Delta F/F_0 = (\Delta F_{max}/F_0)[(P + L + K_d) + \{(L + P + K_d)^2 - 4LP\}^{1/2}]/2P \quad (3)$$

ΔF_{max} was obtained by plotting $1/\Delta F$ as a function of 1/[calcofluor] (not shown). We found ΔF_{max} equal to 0.3348 and 0.78 for the calcofluor–α_1-acid glycoprotein and calcofluor–HSA complexes, respectively.

The dissociation constants were found equal to 6.6 ± 0.5 μM, i.e., an association constant of 0.15 μM^{-1} and to 25 ± 1 μM, i.e., an association constant of 0.04 μM^{-1} for the calcofluor–α_1-acid glycoprotein and calcofluor–HSA complexes, respectively. Thus, the affinity of calcofluor to α_1-acid glycoprotein is higher than its affinity to HSA.

The number of interacting sites was obtained from the slope of $\Delta F/\Delta F_{max}$ versus the calcofluor concentration. The equation used is that derived in Ref. [26]:

$$x = nL/(P + K_d) \quad (4)$$

where $x = \Delta F/\Delta F_{max}$ and n is the number of sites. The value of n was found equal to 0.8 ± 0.1 and 2.1 ± 0.1 for the calcofluor–α_1-acid glycoprotein and calcofluor–HSA complexes, respectively.

Nature of the interaction of calcofluor with α_1-acid glycoprotein and HSA.—Addition of LCA to calcofluor does not modify the fluorescence observables (intensity, position of the maximum and anisotropy) of calcofluor. Thus, the fluorophore does not bind to the LCA, indicating that it is not just binding to any hydrophobic site. Nevertheless, one cannot exclude that the binding of the fluorophore to the HSA is nonspecific, since serum albumin has the characteristic of binding many types of ligands.

Addition of carbohydrate residues extracted from α_1-acid glycoprotein to a solution of calcofluor induces a decrease in the fluorescence intensity of the fluorophore (Fig. 3). This decrease clearly indicates that interaction between the glycans and calcofluor is occurring. The same result was obtained when the carbohydrates originate from other sources than α_1-acid glycoprotein (not shown). However, in the presence of α_1-acid glycoprotein, the fluorescence intensity of calcofluor increases, while in the presence of free glycans, it decreases. In order to clarify this problem, we compared the fluorescence emission spectra of calcofluor in the presence of free carbohydrates and of α_1-cellulose (Fig. 4). We notice that with α_1-cellulose, the fluorescence intensity of calcofluor increases, a phenomenon similar to that observed when the fluorophore is bound to α_1-acid glycoprotein.

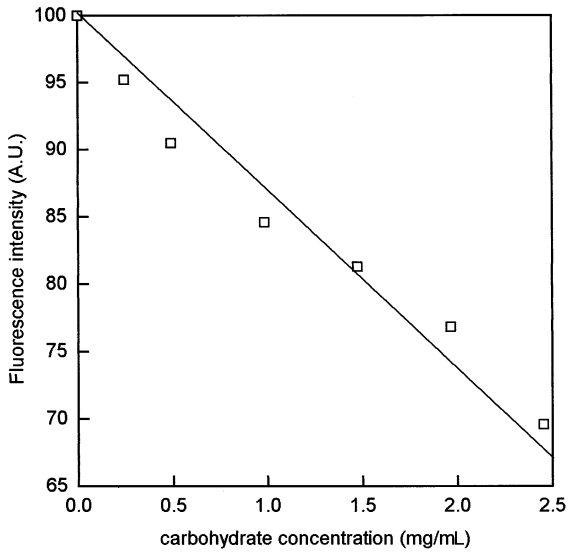


Fig. 3. Titration of 10 μM of calcofluor with a glycopeptide (one to two amino acids) from α_1-acid glycoprotein. λ_{ex} 300 nm and λ_{em} 435 nm.

4. Discussion

The fluorescence emission maxima of calcofluor dissolved in water and in isobutanol are located at 435 and 415 nm, respectively. Also, the fluorescence intensity is three times higher when the fluorophore is in isobutanol

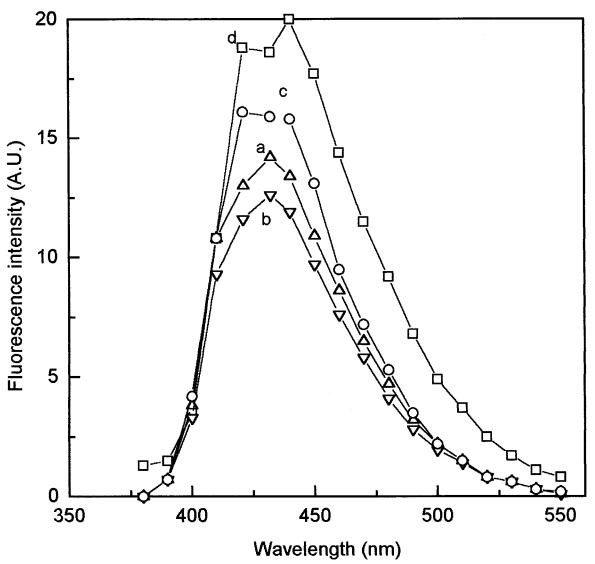


Fig. 4. Fluorescence emission spectra of 10 μM of calcofluor in water (a), in the presence of a glycan (b), in the presence of α_1-cellulose (c) and in the presence of α_1-acid glycoprotein (d). λ_{ex} 300 nm. α_1-cellulose was added to the cuvette containing calcofluor with the glycan, and α_1-acid glycoprotein was then added to the cuvette containing the fluorophore, the glycans and α_1-cellulose.

than in water. Thus, calcofluor emission is sensitive to the polarity of the solvent. This characteristic is common to many fluorophores such as TNS [27], Trp residues [28] and flavin [29]. However, the fluorescence emission maxima of the above fluorophores are also viscosity dependent. Thus, the solvent polarity scale is insufficient to describe the spectral properties of a fluorophore in a protein (case of Trp residues) or bound to a protein (case of TNS). In fact, when the fluorophore is surrounded by a rigid or viscous environment, or when it is bound tightly to a protein, its fluorescence emission will be located at short wavelengths. In this case, the emission occurs from a non-relaxed state, and the spectrum obtained will be identical to that obtained when the emission occurs from a hydrophobic environment such as isobutanol. Therefore, emission of calcofluor on HSA may be the result of an emission from a hydrophobic binding site and/or a highly rigid binding site. We did not attempt in this work to elucidate this question. When the binding site or when the microenvironment of the fluorophore displays free motions, the emission will occur from a relaxed state, yielding a fluorescence spectrum with a maximum located at high wavelengths compared with that observed in a rigid or a hydrophobic environment. Therefore, the emission of calcofluor bound to α_1-acid glycoprotein could be the result of an emission from a hydrophilic binding site and/or a highly dynamic one. The shift observed for calcofluor from 432 nm in water to 447 nm when bound to α_1-acid glycoprotein can in no way be the result of increasing dynamics of the fluorophore. Otherwise, calcofluor would have a higher degree of freedom when bound to α_1-acid glycoprotein than when it is free in solution. This is not logical. Therefore, the only explanation of this red shift is that the microenvironment of calcofluor on α_1-acid glycoprotein is highly polar. This polarity is the result of the spatial organization of the carbohydrates of the protein, providing a complex and a highly polar microenvironment. Therefore, calcofluor interacts preferentially with the glycans, although an interaction with the protein moiety cannot be excluded.

Interaction between calcofluor and α_1-acid glycoprotein differs from that observed between TNS and α_1-acid glycoprotein. TNS binds to the protein on a rigid binding site [30]. Also, the fluorescence maximum of TNS has been shown to shift to shorter wavelengths upon binding to α_1-acid glycoprotein [9]. Comparing this result with those obtained in this work, one may conclude that the microenvironment of the binding site of calcofluor on α_1-acid glycoprotein differs from that of the binding site of TNS.

α_1-Cellulose possesses a well-defined secondary structure, while smaller free carbohydrates in solution do not. However, when bound to the protein moiety of α_1-acid glycoprotein, the glycans have a defined structure in space. This secondary structure will interact with calcofluor differently than in its absence, i.e., when the glycans are free in solution.

The interaction between calcofluor and the highly hydrophilic α_1-acid glycoprotein glycans will induce the important red shift (15 nm) in the emission of the fluorophore (Fig. 1).

Calcofluor is a symmetric molecule with two triazol rings and two primary alcohol functions on both sides of an ethylene bridge. At low concentration of fluorophore the binding to the glycan moiety of α_1-acid glycoprotein occurs by one end of the fluorophore, the shift observed being dependent on the ratio of calcofluor to α_1-acid glycoprotein. Whereas, for calcofluor in excess compared with α_1-acid glycoprotein, the two equivalent parts of calcofluor are involved in the binding, thus affording bridges between distant alcohol functions of the glycan residues. In this case, we observe a red shift of 15 nm. All N-linked sugars such as those of α_1-acid glycoprotein, contain a hexasaccharide $Man_3GlcNAc_2$ core. The heterogeneity of N-linked glycans originates primarily from the presence, absence, type or length of the sugar side chains attached.

Identical binding parameters and the same fluorescence variation were obtained for the calcofluor when bound to α_1-acid glycoprotein molecules prepared from different human sources and by different methods. Therefore, the heterogeneity due either to the protein backbone or to the glycan antennae does not play any fondamental role in the interaction between the calcofluor and α_1-acid glycoprotein. We found also that this 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 [31].

Calcofluor interacts with different glycan structures, mainly with structures containing galactose, glucose and mannose. Since α_1-acid glycoprotein is rich in these residues, the interaction with calcofluor should be expected. Moreover, our work suggests that the spatial organization of the glycans plays an important role in the interaction between calcofluor and α_1-acid glycoprotein.

Performing steady-state measurements of emission anisotropy between -40 and 20 °C, allows us to derive parameters characteristic of the environment of the rotating unit, such as the thermal coefficient of the frictional resistance to the rotation of the fluorophore and the characteristic amplitude at which the surrounding microenvironment (in our case, the carbohydrate residues), become the determinant of that frictional resistance [32–34].

Our next goal is to study the dynamics of calcofluor bound to α_1-acid glycoprotein at low and at high concentrations of calcofluor compared with α_1-acid glycoprotein. Red-edge excitation spectra experiments and Perrin plots as a function of temperature and as a function of sucrose concentration and anisotropy decay will yield information on the dynamics of calcofluor and its microenvironment.

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