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Förster energy-transfer studies between Trp residues of α_1-acid glycoprotein (orosomucoid) and the glycosylation site of the protein

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Abstract

Energy-transfer studies between Trp residues of α_1-acid glycoprotein and the fluorescent probe Calcofluor White were performed. Calcofluor White interacts with carbohydrate residues of the protein, while the three Trp residues are located at the surface (Trp-160) and in hydrophobic domains of the protein (Trp-25 and Trp-122). Binding of Calcofluor to the protein induces a decrease in the fluorescence intensity of the Trp residues accompanied by an increase of that of Calcofluor White. Efficiency (E) of Trp fluorescence quenching was determined to be equal to 45%, and the Förster distance R_o, at which the efficiency of energy transfer is 50%, was calculated to be 18.13 Å. This low distance and the value of the efficiency clearly indicate that energy transfer between Trp residues and Calcofluor White is weak.

Keywords: α_1-Acid glycoprotein; Trp residues; Calcofluor White; Fluorescence energy transfer

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 183 amino acids,[1] 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-glycosidic bond to the asparaginyl residues of the protein.[3]

α_1-Acid glycoprotein contains three Trp residues. One residue, Trp-160, is at the surface of the protein, and two are located in the protein matrix.[4] The three Trp residues contribute to the fluorescence of the protein.[4,5] The N-terminal fragment of the protein adopts a spatial conformation so that a pocket in contact with the buffer is induced, and to which Trp-25 residue belongs.[6] The five carbohydrate units are linked to the N-terminal fragment[7] and thus to the pocket.[6] Calcofluor White is a fluorescent probe that interacts preferentially with the glycan residues of α_1-acid glycoprotein.

In the present work, we carried out studies on Förster type energy transfer between Trp residues of α_1-acid glycoprotein and Calcofluor White. Our results show that energy transfer between Trp residues and Calcofluor White is weak. This is probably the result of binding of Calcofluor White to the carbohydrate residues and not to the protein matrix.

2. Materials and methods

α_1-Acid glycoprotein was purified as already described.[8] 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.[9] Calcofluor White was from Sigma Chemical Co. (St. Louis, MO, USA). Its concentration was determined spectrophotometrically using an extinction coefficient of 4388 M^{-1} cm^{-1} at 352.7 nm.[10] Absorbance data were obtained with a Varian DMS-100 S spectrophotometer using 1-cm pathlength cuvettes. Fluorescence spectra were recorded with a Perkin–Elmer LS-5B spectro-

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fluorometer. Titration experiments were performed at λ_{ex} of 295 nm. Binding of Calcofluor White to α_1-acid glycoprotein was obtained by adding aliquots of the fluorophore (3.3 μM) to a solution of 17 μM of α_1-acid glycoprotein.. The bandwidths used for the excitation and the emission were 5 nm. The quartz cuvettes had optical pathlengths equal to 1 and 0.4 cm for the emission and excitation wavelengths, respectively. Fluorescence spectra were corrected for the background intensities of the buffer solution. Corrections for the inner filter effect were not necessary since the absorption of Calcofluor were not significant at the excitation and emission wavelengths. All experiments were performed at 20 °C in 10 mM phosphate–0.143 M NaCl buffer, pH 7.

3. Results

In PBS buffer, free Calcofluor White displays a fluorescence that increases in presence of α_1-acid glycoprotein ($\lambda_{ex} = 295$ nm) (data not shown).

Fig. 1 shows emission spectra of a fixed amount of α_1-acid glycoprotein before and after addition of variable amounts of Calcofluor White, the excitation wavelength being 295 nm. As the Calcofluor concentration increases, its emission around 450 nm increases, while that of the Trp residues (at 333 nm) decreases. An isoemissive point is observed at 395 nm. The analysis of the decrease of the fluorescence intensity of the Trp residues yields a stoichiometry for the protein–Calcofluor complex equal to 1 (not shown). This value is equal to that we have already found.[10]

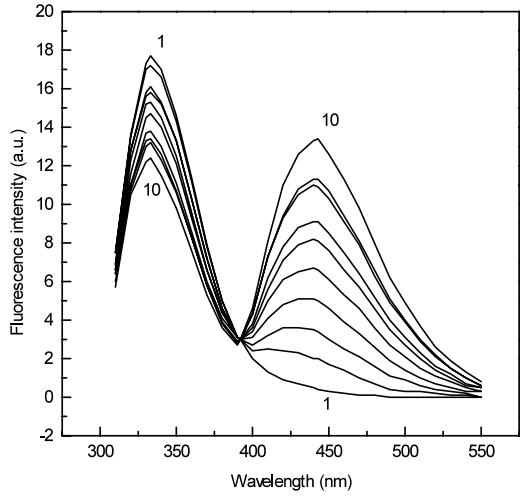

Fig. 1. Fluorescence spectra of α_1-acid glycoprotein. Before (spectrum 1) and after addition of Calcofluor White. λ_{ex}, 295 nm. Aliquots of 2.1 μM of Calcofluor White were added to a solution of 15 μM of α_1-acid glycoprotein. At spectrum 10, [Calcofluor White] = 19 μM.

The decrease of the fluorescence intensity of the Trp residues in the presence of Calcofluor White is the result of a Förster type energy transfer from the Trp residues to the extrinsic probe. The efficiency of this energy transfer depends on three parameters: (1) the distance R between the donor (Trp residues) and the acceptor (Calcofluor White); (2) the spectral overlap between the fluorescence of the donor, and the absorption of the acceptor and (3) the orientation factor κ^2, which gives an indication on how the dipoles of acceptor in the fundamental state and donor in the excited state are aligned.

Fig. 2 displays the normalized fluorescence emission spectrum of Trp residues in α_1-acid glycoprotein (a) and absorption spectrum of Calcofluor (b). From the overlap of the two spectra we have calculated the overlap integral J[11–13]

$$J(\lambda) = \frac{\int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int_0^\infty F_D(\lambda)d\lambda} \quad (1)$$

J was found equal to 0.447×10^{-14} M^{-1} cm^3.

The Forster distance R_o (in Å) at which the efficiency of energy transfer is 50% was calculated with Eq. (2):

$$R_0 = 9.78 \times 10^3[\kappa^2 n^{-4} Q_D J(\lambda)]^{1/6} \quad (2)$$

where κ^2 is the orientation factor ($= 2/3$), n the refractive index ($= 1.33$) and Q_d the average quantum yield ($= 0.064$).[14] R_o is equal to 18.13 Å. This small distance suggests the presence of a weak energy transfer between Trp residues and Calcofluor White.

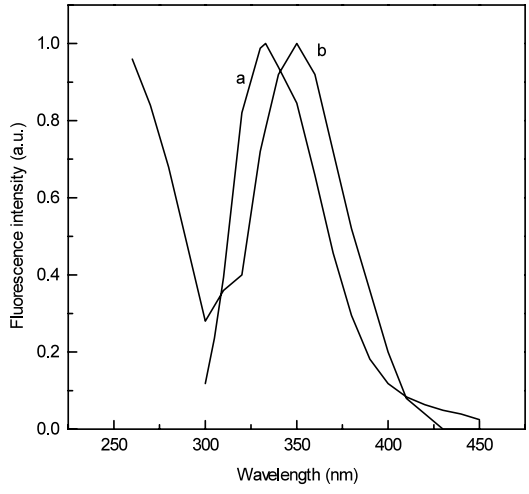

Fig. 2. Fluorescence emission spectrum of Trp residues of 15 μM of α_1-acid glycoprotein (a) and absorption spectrum of 5 μM of Calcofluor (b). Buffer, PBS, pH 7.

The efficiency of quenching (E) is equal to

$$E = 1 - \frac{I}{I_o} = 1 - \frac{\tau}{\tau_o} \quad (3)$$

where τ and I are the mean fluorescence lifetime and intensity in the absence (τ_o, I_o) and in the presence of Calcofluor White (τ and I).

The value of E calculated at infinite concentrations of Calcofluor White was obtained by plotting $1/E$ as a function of 1/[Calcofluor White] (Fig. 3). E was found equal to 0.45.

The distance that separates the donor from the acceptor was calculated using Eq. (4):

$$R = R_0 \left(\frac{1 - E}{E} \right)^{1/6} \quad (4)$$

R is equal to 18.72 Å.

The value of 0.45 found for E suggests that the energy-transfer mechanism from Trp residues to Calcofluor occurs within a time close to the fluorescence lifetime. The constant rate of the energy transfer (k_t) can be calculated from Eqs. (5) and/or (6):

$$k_t = (1/\tau_o)(R_o/R)^6 \quad (5)$$

and

$$k_t = 1/\tau_o - 1/\tau_r \quad (6)$$

where τ_r is the radiative lifetime. $1/\tau_r$ and $1/\tau_o$ are equal to 0.028×10^9 and 0.4376×10^9 s^{-1}, respectively.[14] Eqs. (5) and (6) yield very close values for k_t, 0.435×10^9 s^{-1} and 0.409×10^9 s^{-1}, respectively. We notice that k_t is $47 \pm 1\%$ of the sum of the three rate constants of Eq. (6) confirming the value (45%) found for E (Fig. 3).

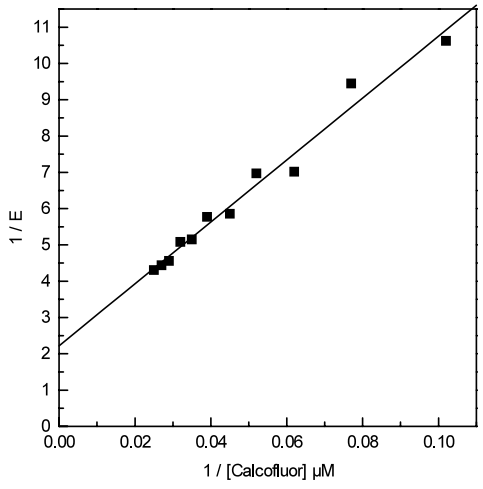

Fig. 3. Determination of the efficiency (E) of energy transfer from Trp residues of α_1-acid glycoprotein to Calcofluor White. The value of the efficiency determined at the extrapolation is equal to 0.45.

4. Discussion

Different mechanisms could account for the quenching of Trp residues fluorescence of α_1-acid glycoprotein upon binding of Calcofluor White. One such mechanism is energy transfer between the tryptophans to Calcofluor White. Another possibility is a conformational change induced by Calcofluor White binding, altering by that the environment around the tryptophans. Also, both mechanisms could occur simultaneously. At the concentrations of Calcofluor White and α_1-acid glycoprotein used in the present work, important conformational changes around Trp residues can be ruled out.[5] In fact, the position of the maximum (333 nm) of the Trp residues does not change in presence of Calcofluor White (Fig. 1). The presence of an isoemissive wavelength means that the stoichiometry of the α_1-acid glycoprotein–Calcofluor White complex is 1:1. This result is in good agreement with our previous results.[10]

Calcofluor White interacts preferentially with the carbohydrate residues of α_1-acid glycoprotein and thus binds to the glycosylation site of the protein and does not bind directly to the protein matrix.[15] This binding property of the probe and the presence of the carbohydrate residues around the extrinsic fluorophore[15] decreases the quenching efficiency of the fluorescence of Trp residues. Although energy transfer does exist between the Trp residues and Calcofluor, it is weak as it is revealed by the values of E (0.45) and R_o (18.13 Å). The calculated distance R (18.72 Å) between donor and acceptor has no physical meaning because of the presence of three potential donor tryptophan residues located at different areas of the protein.

In a recent work, we studied the binding of progesterone to α_1-acid glycoprotein by following the fluorescence intensity quenching of Calcofluor bound to the carbohydrate residues. Our results have indicated that the pocket of α_1-acid glycoprotein contains two zones, one hydrophobic and the second hydrophilic.[15] The hydrophobic domain is formed by amino acids where ligands such as progesterone and fluorescent probe 6-(*p*-toluidino)-2-naphthalenesulfonate (TNS) can bind. The hydrophilic domain concerns the glycosylation site of the protein where Calcofluor binds.

Energy transfer studies between Trp residues of α_1-acid glycoprotein and TNS allowed one to calculate a value for R_o equal to 28 Å. This value is very high and reveals the presence of an efficient energy transfer between the Trp residues and TNS that occurs specifically inside the formed α_1-acid glycoprotein–TNS complex.[16] Thus, TNS binding to α_1-acid glycoprotein differs from that of Calcofluor. TNS binds to the protein matrix in the hydrophobic region, while Calcofluor White interacts mainly with the carbohydrate residues at the glycosylation site. Binding to the protein matrix could favor the energy transfer mechanism. Also,

TNS is a small molecule compared to Calcofluor White. This could allow a better orientation and space interaction of the dipoles intervening in the energy transfer mechanism.

Therefore, the value of R_o will depend on the position of the acceptor molecule (TNS or Calcofluor) in the pocket and on the relative acceptor dipole–donor dipole orientation. The presence of three Trp residues renders the analysis of the data very complex. In fact, we are observing an energy-transfer mechanism that is occurring from the three Trp residues to the acceptor molecules. In this case, the distance measured does not allow one to establish a structural map. However, it can be used to follow important structural modifications within the protein.

Homology modeling of α_1-acid glycoprotein revealed the presence of a pocket within the protein where progesterone can bind.[17] This result confirms the model we have already described concerning the presence of a pocket where different ligands can bind.[6] The authors[17] revealed also the presence of Trp-122 residue within the pocket and in close proximity to the progesterone. In the absence of any crystallographic data, and due to the difficulty of preparing different types of mutants, the different spectroscopic studies would be complementary and useful to describe in details the structural properties of α_1-acid glycoprotein.

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