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ORIGINAL PAPER

Energy Transfer Studies between Trp Residues of Three Lipocalin Proteins Family, α_1-Acid Glycoprotein, (Orosomucoid), β-Lactoglobulin and Porcine Odorant Binding Protein and the Fluorescent Probe, 1-Aminoanthracene (1-AMA)

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Abstract Energy transfer studies between Trp residues of α_1-acid glycoprotein, β-lactoglobulin and porcine odorant binding protein (OBP) and the fluorescent probe 1-aminoanthracene (1-AMA) were performed. 1-AMA binds to the hydrophobic binding sites of the three proteins inducing a decrease in the fluorescence intensity of the Trp residues accompanied by an increase of that of 1-AMA. Our results indicate that 1-AMA is in close contact with hydrophobic tryptophan residue of β-lactoglobulin (Trp 19) to the difference of its binding to OBP, where Trp residues are far from the pocket and to α_1-acid glycoprotein where three Trp residues are present at different areas of the protein.

Keywords α_1-acid glycoprotein · β-Lactoglobulin · Porcine odorant binding protein (OBP) · Tryptophan · 1- Aminoanthracene (1-AMA) · Förster energy transfer

Introduction

Lipocalins are small (160 to 180 amino acid residues) extracellular proteins sharing several molecular recognition properties such as binding of small hydrophobic molecules and formation of complexes with other soluble macromolecules. Lipocalins share sufficient similarity, in the form of short and characteristic conserved sequence motifs. These identical properties form the basis of a useful definition of family membership [1–4].

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Lipocalin proteins exhibit highly symmetrical all-β structure, dominated by a single eight-stranded antiparallel β-sheet, forming a continuously hydrogen bonded β-barrel, which encloses a ligand binding site composed of both an internal cavity and an external loop scaffold. Diversity observed in the cavity and scaffold gives rise to a variety of different binding modes, each capable of accommodating ligands of different size, shape, and chemical characters [1, 3, 5]. Also, despite a similar tertiary structure, differences do exist between nature of hydrophobic molecules bound to lipocalin proteins. For example, progesterone binds to α_1-acid glycoprotein but not to β-lactoglobulin. Difference in the ligand selection seems to be the result of amino acid residues surrounding the hydrophobic binding pocket [5]. Some lipocalin proteins display a narrow binding pocket, which correlates with the highly specific binding of a limited range of ligands, while other lipocalins contain an open and wide binding crevice and recognize a broad variety of different ligands [1].

Fluorophore 1-aminoanthracene (1-AMA) is widely used to study interaction between lipocalin family proteins and their ligands [6–12]. Aminoanthracene is known to be a cytotoxic compound [13] inducing DNA damaging and thus altering proteins structures.

Recently, we studied effect of 1-AMA binding on the structure of three lipocalin family proteins, dimeric β lactoglobulin, dimeric porcine odorant binding protein and monomeric α_1 – acid glycoprotein. Our results indicated that 1-AMA binding induces conformational modifications within β-lactoglobulin and porcine odorant binding protein, even at low fluorophore concentrations in regard to that of the protein. Experiments performed on α_1–acid glycoprotein in the same conditions as those applied on β-lactoglobulin and odorant binding protein show that 1-AMA does not modify α_1 – acid

glycoprotein structure / or conformation within the stoichiometric range [14].

β-Lactoglobulin is a small protein of 162 amino acid residues (Mr=18,400) [15]. It contains two Trp residues (Trp 19 and 61 residues) which are, respectively, buried in a hydrophobic region of the protein and solvent exposed. They both have equal hydrophobicity, but electronic environment differs.

OBP purified from pig nasal (pOBP) mucus is secreted in the nasal mucus of vertebrates, which convey odorants to their neuronal receptors [12]. It is a monomer of 157 amino acids [16, 17] containing one disulfide bridge between cysteines at positions 63 and 155. Burova et al. [18] showed that pOBP, such as bovine β-lactoglobulin, is dimeric at physiological pH (7.2) and monomeric at acidic pH. The protein contains one Trp residue at position 16 [19].

α_1-Acid glycoprotein (orosomucoid), a small acute phase glycoprotein (Mr=41,000), consists of a chain of 183 amino acids [20] contains 40 % carbohydrate by weight and has up to 16 sialic acid residues (10–14 % by weight) [21]. Five heteropolysaccharide groups are linked via an N-glycosidic bond to the asparaginyl residues of the protein [22]. α_1-Acid glycoprotein contains three Trp residues which all contribute to the protein fluorescence [23, 24]. In the absence of crystallographic data, tertiary structure of α_1-acid glycoprotein was investigated by fluorescence spectroscopy revealing presence of a pocket where different ligands can bind. Also, the five carbohydrate units are linked to the pocket giving it both hydrophobic and hydrophilic properties [25]. Thus, α_1-acid glycoprotein pocket contains a hydrophobic domain where ligands such as progesterone and 6,p-toluidinylnaphtalene-2-sulfonate TNS can bind and a hydrophilic one formed mainly by the glycosylation site of the protein [26, 27]. Chemical and physical properties of the pocket in α_1-acid glycoprotein are different from those of other lipocalin family proteins as the result of carbohydrate residues presence.

The aim of the present work is to find out the relative position of 1-AMA toward the tryptophan residues of the three lipocalin proteins. For this matter, energy transfer experiments were performed between Trp residues of the proteins and 1-AMA. In the three proteins, the extrinsic fluorophore binds within the hydrophobic pocket [14]. Stoichiometry of 1-AMA-α_1-acid glycoprotein is 1:1, while those of 1-AMA-β-lactoglobulin and 1-AMA-OBP are 2 mol of AMA for 1 dimer [14].

Data obtained in the present work show that in β-lactoglobulin, Förster distance determination between 1-AMA and Trp residues was not possible; therefore, the ligand is in close contact or in proximity with hydrophobic Trp residue (Trp 19) located within the pocket. In OBP, energy transfer experiments allowed to calculate the distance that separates the sole Trp residue of the protein (Trp 16) from 1-AMA. This means that this tryptophan residue is not within the pocket such as Trp −19 residue of β-lactoglobulin. The Förster distance calculated for α_1–acid glycoprotein is a mean one between 1-AMA and the three tryptophan residues.

Materials and Methods

1-AMA (purity>90 %) was from Fluka Sigma –Aldrich (Saint Quentin Fallavier, France). Bovine β-lactoglobulin (purity >90 %) was from Sigma –Aldrich (Saint Quentin Fallavier, France). Odorant binding protein was a generous gift from Dr. Patricia Nagnan Le Meillour (Laboratoire de Glycobiologie, University of Lille 1). Human α_1-acid glycoprotein (purity> 99 %) was prepared by Professor H. Debray (actually retired) of University of Lille 1 or bought from Sigma-Aldrich (Saint Quentin Fallavier, France) (purity=99 %).

1-AMA concentration was determined spectrophotometrically using an extinction coefficient equal to 35.45 mM^{-1} cm^{-1} at 280 nm [14]. Proteins concentrations were determined at 278 nm with the following extinction coefficients: 17.6×10^3 M^{-1} cm^{-1} for bovine β-lactoglobulin, [28], 29.7 mM^{-1} cm^{-1} for human α_1-acid glycoprotein [29] and 12,200 M^{-1} cm^{-1} for OBP [18]. In the whole manuscript, β-lactoglobulin and odorant binding protein concentrations are expressed in dimer.

Absorbance data were obtained with a Varian DMS-100S (Les Ulis, France) spectrophotometer using 1-cm pathlength cuvettes. Fluorescence spectra were recorded with a Perkin–Elmer LS-5B spectrofluorometer (Perkin-Elmer, Courtaboeuf, France). 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. Observed fluorescence intensities were first corrected for the dilution, and then corrections were made for the inner filter effect as described [30, 31].

All spectral experiments were performed at 20 °C in 10 mM Tris pH 7.5, or in 10 mM phosphate buffer, pH 7.

Results and Discusssion

Titration of a fixed protein concentration with variable amounts of 1-AMA induces a decrease in the fluorescence intensity emission of the tryptophan residues (335 nm) accompanied with an increase of the emission intensity of 1-AMA at 520 nm for α_1-acid glycoprotein, 500 nm for β-lactoglobulin and 490 nm for OBP [14]. These data allowed us to determine the dissociation constants of the extrinsic fluorophore and the proteins (K_d=3.345, 6.45 and 5 μM for β-lactoglobulin-AMA, α_1-acid glycoprotein-AMA and OBP-AMA complexes, respectively) [14] indicating a possible energy transfer between Trp residues of the proteins and 1-AMA.

Energy transfer can be put into evidence by recording fluorescence excitation spectrum of 1-AMA in presence of the proteins at an emission wavelength equal to 480 nm. At this wavelength, only emission of 1-AMA is observed. Therefore, if energy transfer occurs from Trp residues to the extrinsic fluorophore, recording excitation spectra will yield a peak at 275–278 nm characterizing aromatic amino acids of the proteins.

Figure 1 displays fluorescence excitation spectra of 1-AMA free in 10 mM Tris buffer pH 7 and bound to α_1-acid glycoprotein, β-lactoglobulin and OBP. We observe, in presence of the three proteins, an important peak at 275–278 nm as the result of energy transfer that is occurring within each complex. Efficiency of this energy transfer depends on three parameters, the distance R between donor (Trp residues) and acceptor (1-AMA), spectral overlap between fluorescence emission spectrum of the donor and absorption spectrum of the acceptor and orientation factor κ^2 which gives an indication on the alignment of the acceptor dipoles in the fundamental state and donor dipoles in the excited state. Figure 2 displays normalized fluorescence emission spectrum of Trp residues in α_1-acid glycoprotein, (a) and absorption spectrum of 1-AMA bound to α_1-acid glycoprotein (b). From the overlap of the two spectra we have calculated the overlap integral J [32]:

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

For OBP and α_1-acid glycoprotein, J was found equal to 1.862×10^{-14} M^{-1} cm^3 and 1.398×10^{-14} M^{-1} cm^3, respectively. For β-lactoglobulin, we did not calculate any J, because energy transfer occurring between Trp residue(s) of the protein and 1-AMA was not Förster type, as shown in Fig. 3.

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

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

where κ^2 is the orientation factor (= 2/3), n the refractive index (= 1.33) and Q_d the average quantum yield (= 0.1 and 0.065 for OBP and α_1-acid glycoprotein, respectively.) R_o is equal to 26.5 and 23.5 Å for OBP and α_1-acid glycoprotein, respectively.

Quenching efficiency (E) is equal to

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

where I_o and I are intensities in absence and presence of 1-AMA, respectively.

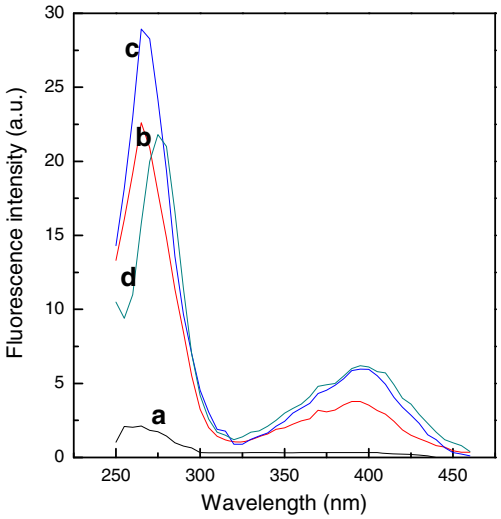

Fig. 1 Corrected fluorescence excitation spectra of 5.5 μM 1-AMA in 10 mM Tris pH 7 buffer (**a**), of 5.5 μM 1-AMA in presence of 17 μM α_1-acid glycopotein (**b**), of 2.75 μM 1-AMA in presence of 30 μM β-lactoglobulin dimer (**c**) and of 5.5 μM 1-AMA in presence of 5 μM OBP (**d**) λem=480 nm

Value of E calculated at infinite concentrations of 1-AMA was obtained by plotting 1/E as a function of 1/[1-AMA] (Fig. 3). E was found equal to 0.477, 0.495 and 1.38 for OBP-1-AMA, α_1-acid glycoprotein–1-AMA and β-lactoglobulin–1-AMA, respectively. Value of E found for energy transfer between β-lactoglobulin and 1-AMA is higher than 1. This means that Förster energy transfer is not occurring between Trp residues and 1-AMA and the extrinsic fluorophore is in close contact with intrinsic fluorophore. Since β-lactoglobulin contains two Trp residues, one within the hydrophobic pocket of the protein (Trp 19) and the second

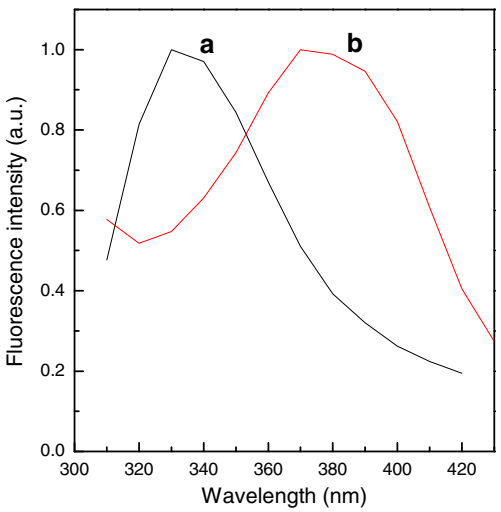

Fig. 2 Normalized emission spectrum (**a**) of tryptophan residues of α_1-acid glycoprotein (λex=295 nm) and of absorption spectrum of 1-AMA-α_1-acid glycoprotein (**b**). Experiments were performed in 10 mM Tris buffer, pH 7

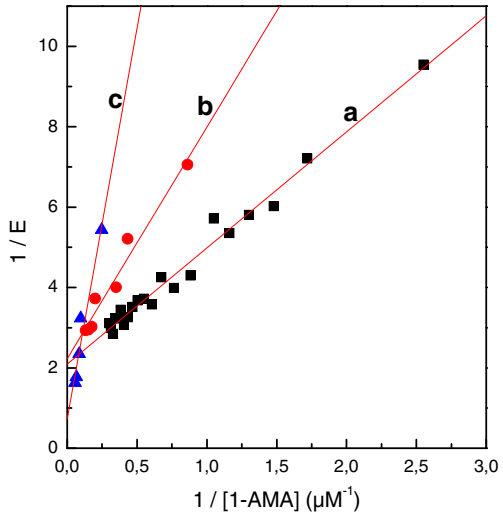

Fig. 3 Determination of energy transfer efficiency (E) between Trp residues of OBP (**a**) α_1-acid glycoprotein (**b**) and β-lactoglobulin (**c**) and 1-AMA. λex=295 nm and λem=330 nm. (a): 1 / E=2.095 thus E=0.477; b: 1 / E=2.02, thus E=0.495; c : 1 / E=0.723, thus E=1.38

at the aperture of the pocket within a hydrophilic region (Trp 61), 1-AMA cannot be in contact simultaneously with both tryptophan residues. 1-AMA is a hydrophobic ligand and binds necessarily to a hydrophobic site; therefore it is located within β-lactoglobulin pocket [14] and thus is in close contact with Trp 19 residue as indicated by the calculated energy transfer efficiency. This leads us to the fact that fluorescence of Trp 61 residue is totally quenched or / and far from 1-AMA. The same results were obtained when interaction between calcofluor white and β-lactoglobulin was studied and where the authors showed that protein emission occurs from Trp 19 residue [33]. Absence of emission of Trp 61 residue of β-lactoglobulin could be explained by the fact that this tryptophan is not excited as the result of its close interaction with disulfide bridge or any other amino acids [34].

At neutral pH, β-lactoglobulin and OBP are dimers. Therefore, one could wonder whether energy transfer calculation between Trp residue(s) (donor) and 1-AMA (acceptor) are applied to the monomers or to the dimers. First of all, it is important here to remind that each monomer binds 1-AMA molecule and thus in a first approach, one could consider that energy transfer calculations are obtained within a monomer. In order to check our assumption, energy transfer experiments were repeated on β-lactoglobulin −1-AMA complex at pH 2, 7 and 10 in 10 mM phosphate buffer. At pH 2, β-lactoglobulin is a monomer in a molten state. At pH 7, the protein is 100 % dimer and at pH 10, β-lactoglobulin is in both states, monomer and dimer. Experiment showed that at the three pHs, energy transfer is equal to or higher than 100 % (Fig. 4). Therefore, energy transfer calculations are obtained within a monomer.

For OBP and α_1-acid glycoprotein, the distance that separates donor from acceptor was calculated using Eq. 4:

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

R is equal to 26.9 and 23.6 Å for OBP and α_1-acid glycoprotein, respectively. Since OBP contains one Trp residue, calculated distance characterizes that existing between 1-AMA location within the pocket and Trp 16 residue. Thus, intrinsic fluorophore is far from the pocket interior. This result is in good agreement with analysis studies of OBP structure which showed that Trp 16 residue does not belong to the protein internal cavity forming the binding site for ligands [18].

Distance calculated for α_1-acid glycoprotein has no physical meaning because of the presence of three potential donor tryptophan residues located at different areas of the protein [23, 24]. The value of R (23.6 Å) obtained in the present work using 1-AMA is in the same range of those obtained when 2,p-toluidinyl-naphthalene-6-sulfonate (TNS) or calcofluor white were used as extrinsic probes (28 and 18 Å, respectively) [35, 36].

Values of 0.477, 0.495 found for E for OBP and α_1-acid glycoprotein suggest that energy transfer mechanism from Trp residues to 1-AMA occurs within a time close to the fluorescence lifetime. The constant rate of the energy transfer (k_t) can be calculated from Eq. 5:

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

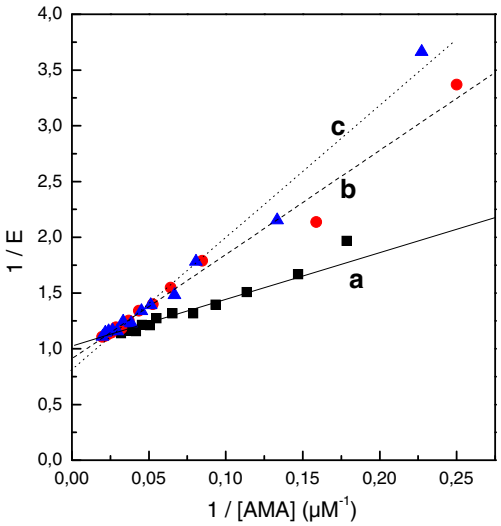

Fig. 4 Energy transfer efficiency (E) between Trp 19 residue of β-lactoglobulin and 1-AMA bound to the protein. Experiments were performed in 10 mM phosphate buffer at pH 2 (**a**), 7 (**b**) and 10 (**c**). λex=295 nm and λem=330 nm. pH 2 : 1 / E=1.02353. E=0.977. pH 7 : 1 / E=0.91123. E=1.097. pH 10 : 1 / E=0.81321. E=1.23

where τ_o is the mean fluorescence lifetime (= 4.778 ns for OBP [37] and 2.285 ns for α_1-acid glycoprotein [38]. Equation 5 yields a value for k_t equal to 0.4266×10^9 s^{-1} for OBP and 0.19×10^9 s^{-1} for α_1-acid glycoprotein.

Radiative constant $1/\tau_r$ can be calculated from Eq. 6:

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

$1/\tau_r$ is found equal to 0.0188×10^9 and 0.011×10^9 s^{-1} for OBP and α_1-acid glycoprotein, respectively. We notice that k_t is 45 % of the sum of the three rate constants of Eq. 6 for OBP and 48.7 % for α_1-acid glycoprotein, confirming the values (47.7 and 49.5 %) found for E for OBP and α_1-acid glycoprotein, respectively (Fig. 3). Thus, energy transfer between Tryptophan residues and 1-AMA, in both proteins, is occurring within a time almost equal to fluorescence lifetime. Finally, it is important to remind here that different mechanisms could account for the quenching of Trp residues fluorescence of a protein upon binding of a ligand. One such mechanism is energy transfer between tryptophans and extrinsic fluorophore. Another possibility is a conformational change induced by ligand binding, altering by that the environment around the tryptophans. Also, both mechanisms could occur simultaneously. We have shown that binding of 1-AMA to OBP and β-lactoglobulin alters the structure of the ligand binding site, the hydrophobic pocket of both proteins [14]. In this case, energy transfer parameters measured for OBP are not necessarily those expected in the absence of any local structural modification. This conclusion is in good agreement with previous ones showing that hydrophobic binding site of OBP is highly mobile and can adopt the structure of the bound ligand as it is shown by the electron density map of OBP pocket in presence of different ligands [11]. However, for α_1-acid glycoprotein, presence of large amount of carbohydrate (40 % by weight) modifies protein pocket conformation rendering it larger and thus more suitable to bind different types of ligands such as 1-AMA [14], progesterone [21, 27] and calcofluor white [24].

In conclusion, energy transfer experiments described in the present work allows us to conclude that although OBP, β-lactoglobulin and α_1-acid glycoprotein belong to one family, the lipocalins one, they do not have the same tertiary structures. Also, data obtained with β-lactoglobulin indicates clearly that Trp 19 residue is the main emitting fluorophore. It is also evident from values of the energy transfer efficiency that pocket structures of β-lactoglobulin and OBP are different. For α_1-acid glycoprotein, presence of 40 % carbohydrate by weight renders the pocket more flexible and larger than those of β-lactoglobulin and OBP. Finally, it is interesting to note that although fluorescence excitation spectrum reveals presence of energy transfer from Trp 19 residue of β-lactoglobulin to 1-AMA bound to β-lactoglobulin, our data show that this does not mean that a Förster energy transfer is necessarily occurring.

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