ORIGINAL PAPER

# Effect of 1-Aminoanthracene (1-AMA) Binding on the Structure of Three Lipocalin Proteins, the Dimeric β Lactoglobulin, the Dimeric Odorant Binding Protein and the Monomeric $\alpha_1$-Acid Glycoprotein. Fluorescence Spectra and Lifetimes Studies

**Daniel Kmiecik · Jihad René Albani**

**Abstract** We studied effect of 1-aminoanthracene (1-AMA) binding on the structures of dimeric β lactoglobulin, dimeric odorant binding protein (OBP) and monomeric $\alpha_1$-acid glycoprotein (lipocalin family proteins) by monitoring fluorescence excitation spectra and measuring fluorescence lifetimes of the tryptophan residues of the proteins. Results show that binding of 1-AMA to β lactoglobulin and OBP modifies their conformation even at low probe concentration compared to that of the proteins. Structural modification induces a red shift of the fluorescence excitation spectra maximum of tryptophan residues accompanied with an increase of the third fluorescence lifetime and a decrease of its pre-exponential factor. These effects were not observed for $\alpha_1$-acid glycoprotein, probably as the result of carbohydrate presence. These data raise doubts concerning use of 1-AMA as a probe to study biological properties of β lactoglobulin and OBP.

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## Introduction

Fluorescent probes are widely used to study proteins properties (structure and dynamics) [1, 2], protein-protein interaction [3] and binding of ligands to proteins [4]. Many fluorophores are used such as tryptophan residues, 6,p-toluidinylnaphthalene 2-sulfonate (TNS), fluorescein, etc. [5–7]. Tryptophan is an intrinsic fluorophore which fluorescence properties are modified upon ligand binding. Extrinsic fluorophores such as TNS and fluorescein are added at low concentrations to the proteins and it is known that their binding to proteins does not modify or alter the protein structure or dynamics. One of the widely used fluorophore to study interaction between lipocalin family proteins and their ligands is 1-aminoanthracene (1-AMA) [8–15]. Aminoanthracene is known to be a cytotoxic compound [16] inducing DNA damaging and thus altering proteins structures. In all performed studies with lipocalin protein family using 1-AMA as a fluorophore, no structural studies have been undertaken in order to see whether the fluorophore disrupts proteins structures or not.

In the present work, we performed binding studies with fluorescence spectra and lifetimes between 1-AMA and three lipocalin family proteins, dimeric β-lactoglobulin, dimeric porcine odorant binding protein and monomeric $\alpha_1$-acid glycoprotein. β-Lactoglobulin is a small protein of 162 amino acid residues (Mr=18,400) which tertiary structure possesses a pocket (calyx) where hydrophobic ligands can easily bind [17]. β-lactoglobulin contains two tryptophan residues, one (Trp-19) is surrounded by a hydrophobic environment and the second (Trp-61) in a hydrophilic one [18]. Both tryptophan residues contribute to the protein fluorescence.

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 [19, 20] containing one disulfide bridge between cysteines at positions 63 and 155. Burova *et al.* [21] 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 [22].

$\alpha_1$-Acid glycoprotein (orosomucoid), a small acute phase glycoprotein (Mr=41,000), consists of a chain of 183 amino acids [23] contains 40% carbohydrate by weight and has up to 16 sialic acid residues (10–14% by weight) [24]. Five heteropolysaccharide groups are linked via an N-glycosidic bond to the asparaginyl residues of the protein [25].

$\alpha_1$-Acid glycoprotein contains three Trp residues which all contribute to the protein fluorescence [26, 27]. In the absence of crystallographic data, tertiary structure of $\alpha_1$-acid glycoprotein was investigated by fluorescence spectroscopy revealing the 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 [28]. Thus, $\alpha_1$-acid glycoprotein pocket contains a hydrophobic domain where ligands such as progesterone and TNS can bind and a hydrophilic one formed mainly by the glycosylation site of the protein [29, 30]. Thus, chemical and physical properties of the pocket in $\alpha_1$-acid glycoprotein are different from those of other lipocalin family as the result of the carbohydrate residues presence. In the present work, fluorescence data obtained on β-lactoglobulin and odorant binding protein indicate that 1-AMA binding induces conformational modifications within the protein, even at low fluorophore concentrations in regard to the protein concentration. Experiments performed on $\alpha_1$-acid glycoprotein in the same conditions as those applied on β-lactoglobulin and odorant binding protein show that 1-AMA does not modify $\alpha_1$-acid glycoprotein structure / or conformation within the stoichiometric range.

## 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 $\alpha_1$-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 (Fig. 1). Proteins concentrations were determined at 278 nm with the following extinction coefficients: $17.6\times10^3$M$^{-1}$cm$^{-1}$ for bovine β-lactoglobulin, [31], 29.7 mM$^{-1}$cm$^{-1}$ for human $\alpha_1$-acid glycoprotein [32] and 12200 M$^{-1}$cm$^{-1}$ for OBP [33]. In the whole manuscript, β-lactoglobulin and odorant binding protein concentrations are expressed in dimer.

Absorbance data were obtained with a Varian DMS-100 S (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 [34, 35].

Fluorescence lifetime measurements were obtained with a Horiba Jobin Yvon FluoroMax-4-P, (Horiba-Jobin Yvon, Longjumeau, France) using the time correlated single photon counting method. A Ludox solution was used as scatter. Excitation was performed at 296 nm with a nanoLED. Each fluorescence decay was analyzed with one, two, three and four lifetimes and then values of χ2 were compared in order to determine the best fit. A minimal value of $\chi^2$ indicates the best fit. A $\chi^2$ value that approaches 1 indicates a good fit. For example, let us consider the value of $\chi^2$ equal to 1.054, 1.06 and 1.1 when analysis is done with 1, 2 and 3 lifetimes, respectively. One lifetime should be considered as the best description of the decay
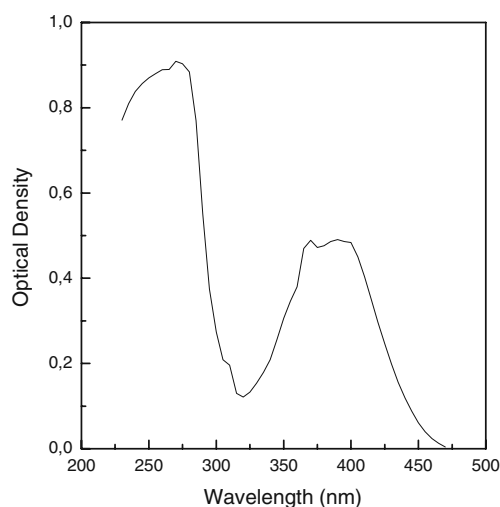


Fig. 1 Absorption spectrum of 24.94 μM 1-AMA dissolved in ethanol. $\varepsilon_{280\ nm}$=35.45 mM$^{-1}$cm$^{-1}$ and $\varepsilon_{380nm}$=19.08 mM$^{-1}$cm$^{-1}$

curve since there was no real improvement in $\chi^2$ value when the experimental decay was fitted with one, two, three or four lifetimes [34, 36–38].

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

## Results

Absorption spectrum of 1-aminoanthracene (1-AMA)

Figure 1 displays absorption spectrum of 1-AMA dissolved in 100% pure ethanol solution. Molar extinction coefficient was determined at 280 and 380 nm by dissolving a known weight of 1-AMA in ethanol and recording the absorption spectrum of the solution. The values of $\varepsilon$ obtained at 280 and 380 nm were found equal to 35.45 mM$^{-1}$cm$^{-1}$ and 19.08 mM$^{-1}$cm$^{-1}$, respectively.

Interaction between 1-aminoanthracene and β-lactoglobulin

### Fluorescence emission spectrum of β-lactoglobulin with 1-aminoanthracene

Figure 2 displays corrected emission spectra of 30 μM β-lactoglobulin dimer tryptophan residues ($\lambda_{ex}$=295 nm) in absence and presence of increasing concentrations of 1-AMA. We notice that emission intensity of the tryptophan residues decrease is accompanied by an increase of the emission intensity of 1-AMA ($\lambda_{max}$=520 nm). Position of the tryptophan maximum in absence of 1-AMA (spectrum 1)

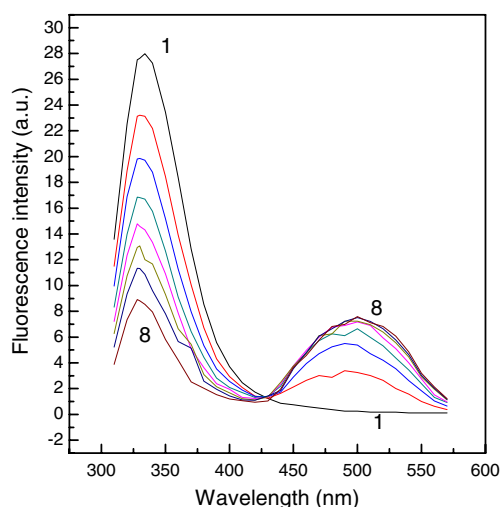is equal to 335 nm, while in presence of 20 μM AMA, it shifts to 328 nm (spectrum 8). This blue shift is the result of the increasing hydrophobicity around tryptophan residues. We did not study in this work the percentage of quenching by 1-AMA of each of the two tryptophan residues. Intensity increase of bound 1-AMA is the result of the extrinsic fluorophore binding to the protein and to energy transfer from tryptophan residues to the extrinsic fluorophore. The plot of intensity increase of 1-AMA as a function of 1-AMA concentration is displayed in Fig. 3 (plot a). The dissociation constant of AMA-β-lactoglobulin complex is obtained according to Eq. 1

$$\frac{1}{I_F} = \frac{K_d}{[AMA]I_{F\max}} + \frac{1}{I_{F\max}} \qquad (1)$$

where [AMA] is equal to the added 1-AMA concentration and $I_F$ is the fluorescence intensity at certain 1-AMA concentration, and $I_{F\max}$ is fluorescence intensity at infinite 1-AMA concentration. The $K_d$ obtained is equal to 3.345±0.5 μM.

### Fluorescence excitation spectrum of β-lactoglobulin with 1-aminoanthracene

Structural modification, if any, within a protein, can be monitored by recording the fluorescence excitation spectrum of the Trp residues in the absence and in the presence of different 1-AMA concentrations. In fact, the fluorescence excitation spectrum characterizes the electron distribution of the molecule in the ground state. Since 1-AMA does not emit at 340 nm, thus, at this wavelength, only the excitation spectrum of the Trp residues will be recorded. Therefore,
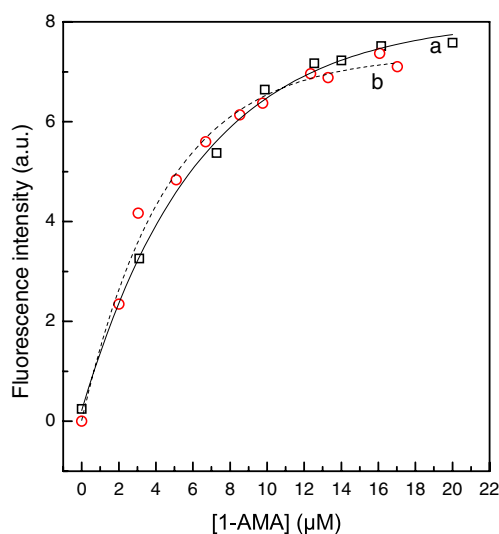


**Fig. 2** Titration of 30 μM β-lactoglobulin dimer with 1-aminoanthracene. $\lambda_{ex}$=295 nm. Position of the tryptophan maximum in absence of 1-AMA (spectrum 1) is equal to 335 nm, while in presence of 20 μM AMA, it shifts to 328 nm (spectrum 8). Corresponding [1-AMA] concentrations from spectra 1 to 8 are: 0, 3.1, 7.27, 9.87, 12.55, 14, 16.16 and 20 μM, respectively



**Fig. 3** Fluorescence intensity increase of 1-AMA bound to 30 μM β-lactoglobulin dimer observed at 500 nm (squares) (**a**) and to 9.5 μM α₁-acid glycoprotein observed at 520 nm (circles) (**b**). $\lambda_{ex}$=295 nm

any modification of the fluorescence excitation spectrum in the presence of 1-AMA would be the result of a structural modification of the protein in the ground state.

Figure 4 displays the fluorescence excitation spectrum of Trp residues of β-lactoglobulin in the absence and presence of increasing concentrations of 1-AMA. We observe a significant decrease in the intensity of the excitation spectrum accompanied by a shift of the recorded spectrum, from 280 nm in absence of 1-AMA to 290 nm in presence of 20 μM of 1-AMA. This red shift clearly indicates that binding of 1-AMA to β-lactoglobulin induces conformational modifications within the tryptophan environment and mainly within the binding site of the protein. Therefore, β-lactoglobulin conformation is not the same in absence and in presence of 1-AMA. In this case, the dissociation constant measured from the emission spectra (Fig. 2) has no significant meaning (see also discussion).

*Fluorescence lifetimes measurements*

In absence and presence of 1-AMA, fluorescence intensity, I(λ,t), of Trp residues in β-lactoglobulin can be adequately represented by a sum of three exponentials. In absence of 1-AMA,

$$I(\lambda, t) = 0.412e^{-t/0.747} + 0.425e^{-t/2.1} + 0.164e^{-t/5.678}$$

where 0.412, 0.425 and 0.164 are the pre-exponential factors, $0.747\pm0.05$, $2.1\pm0.07$ and $5.678\pm0.27$ ns are the decay times and $\lambda$ is the emission wavelength (340 nm) ($\chi2=0.922$, $\lambda_{ex}=296$ nm). The two shortest lifetimes

correspond to what we can usually observe for Trp residues in Protein or for free tryptophan in solution [39, 40], while the longest lifetime is the result of the interaction of the Trp residue within its environment. The mean fluorescence lifetime calculated from four experiments was found equal to 3.466 ns.

The mean fluorescence lifetime is the second order mean [34]:

$$\tau_o = \Sigma f_i \tau_i \qquad (2)$$

and

$$f_i = \alpha_i \tau_i / \Sigma \alpha_i \tau_i \qquad (3)$$

where $\alpha_i$ are the preexponential terms, $\tau_i$ are the fluorescence lifetimes and $f_i$ the fractional intensities. $\lambda_{em}=340$ nm.

Analyzing the decay curve with two lifetimes yields a $\chi2$ equal to 2.1. And fitting the data with four exponentials did not yield better $\chi2$ values than the fitting with three lifetimes.

Increasing 1-AMA concentrations in the β-lactoglobulin solution induces a dramatic increase in the longest fluorescence lifetime of the tryptophan residues. The two shortest fluorescence lifetimes are not affected by 1-AMA presence. The mean fluorescence lifetime increases as the result of the important increase of the longest fluorescence lifetime (Fig. 5). When the stoichiometry 2:1 of the 1-AMA-β-lactoglobulin complex is reached, i.e., two moles of 1-AMA for one dimer of β-lactoglobulin, we notice that the longest lifetime increase stops. Modification of the longest fluorescence lifetime in presence of 1-AMA is in good agreement with the results obtained when the fluorescence excitation spectrum of β-lactoglobulin was
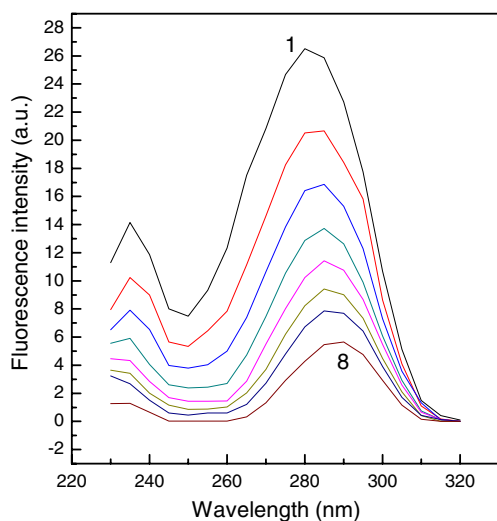


**Fig. 4** Fluorescence excitation spectra of 30 μM β-lactoglobulin dimer with 1-AMA. $\lambda_{em}=340$ nm. Spectrum 1: peak=280 nm. Spectrum 8: peak=290 nm. Corresponding [1-AMA] concentrations from spectra 1 to 8 are: 0, 3.1, 7.27, 9.87, 12.55, 14, 16.16 and 20 μM, respectively. The position of the peak shifts to 285 nm in presence of 0.3 μM of 1-AMA
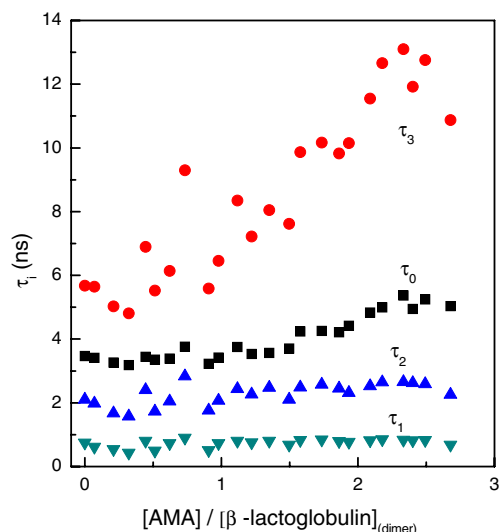


**Fig. 5** Fluorescence lifetimes variation of β-lactoglobulin tryptophan residues as a function of [1-AMA] / [β-lactoglobulin] ratio. $\lambda_{ex}=$ 296 nm and $\lambda_{em}=340$ nm

recorded in presence of increasing concentrations of 1-AMA. In both experiments, we are following structural modifications within β-lactoglobulin as the result of 1-AMA binding to β-lactoglobulin (see also discussion).

The increase in the longest fluorescence lifetime in presence of 1-AMA is accompanied by a decrease in its pre-exponential value. This decrease is important and reaches a plateau at a stoichiometry of 2 moles of 1-AMA for 1 β-lactoglobulin dimer (Fig. 6). The pre-exponential values of the two shortest lifetimes are affected by 1-AMA binding to β-lactoglobulin but their variations do not correspond to that observed for the pre-exponential of the longest lifetime (Fig. 7).

### Interaction between 1-AMA and $\alpha_1$-acid glycoprotein

#### *Fluorescence emission spectrum of $\alpha_1$-acid glycoprotein with 1-aminoanthracene*

Increasing concentrations of 1-AMA to a solution of 7 µM $\alpha_1$-acid glycoprotein induces a decrease in the fluorescence emission of the protein tryptophan residues accompanied with an increase of the emission intensity corresponding to 1-AMA (data not shown).

Figure 3 (plot b) displays the emission intensity increase of 1-AMA in presence of $\alpha_1$-glycoprotein at different extrinsic fluorophore concentrations. Determination of the dissociation constant of 1-AMA-$\alpha_1$-glycoprotein complex was calculated from the plot of the inverse data (not shown) as described by Eq. 1. The value of the dissociation constant $K_d$ of 1-AMA-$\alpha_1$-glycoprotein complex was found equal to 6.45±0.4 µM.

**Fig. 7** Variation of the two shortest fluorescence lifetime pre-exponentials of β-lactoglobulin tryptophan residues as a function of [1-AMA] / [β-lactoglobulin] ratio. $\lambda_{ex}$=296 nm and $\lambda_{em}$=340 nm

#### *Fluorescence excitation spectrum of $\alpha_1$-glycoprotein with 1-aminoanthracene*

Figure 8 displays the fluorescence excitation spectrum of Trp residues of $\alpha_1$-glycoprotein in the absence and presence of increasing concentrations of 1-AMA. We observe a significant decrease in the intensity of the excitation spectrum and absence of a shift up to [AMA] / [$\alpha_1$-glycoprotein] ratio equal to 1.30. Above this ratio, a
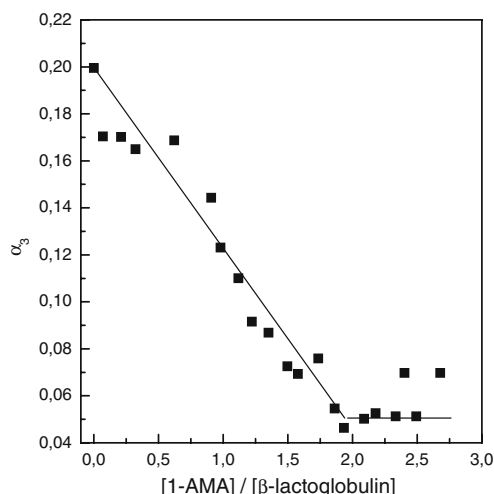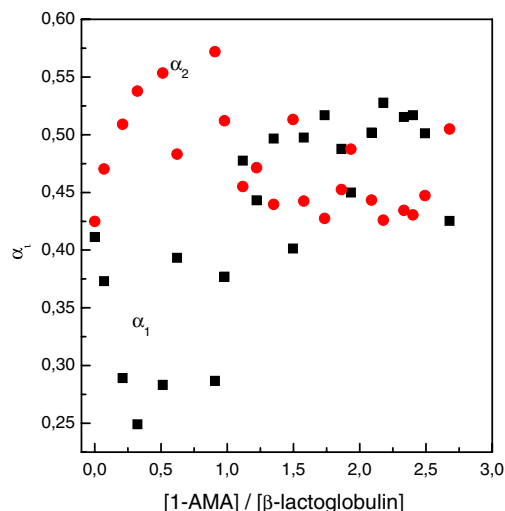
**Fig. 6** Variation of longest fluorescence lifetime pre-exponential of β-lactoglobulin tryptophan residues as a function of [1-AMA] / [β-lactoglobulin] ratio. $\lambda_{ex}$=296 nm and $\lambda_{em}$=340 nm
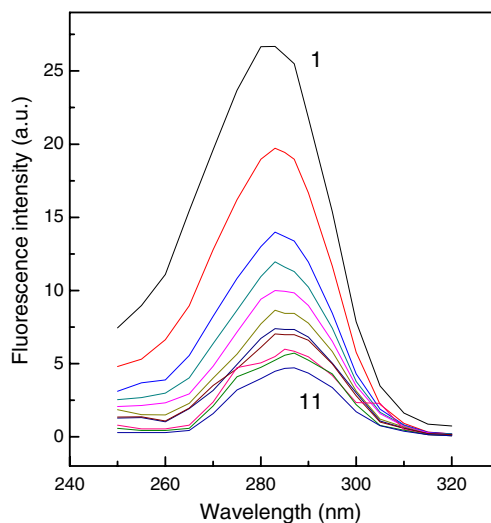
**Fig. 8** Titration of 9.5 µM $\alpha_1$-acid glycoprotein with 1-AMA following excitation spectra of the tryptophan residues. $\lambda_{em}$= 340 nm. Spectrum 1: $\lambda_{max}$=283 nm. Spectrum 11: $\lambda_{max}$=287 nm. Corresponding [1-AMA] concentrations from spectra 1 to 11 are: 0, 2, 3.05, 5.1, 6.68, 8.52, 9.76, 12.35, 13.29, 16.07 and 17 µM, respectively. The stoichiometry of the complex is reached at plot 6. The slight red shift is observed when the ratio [1-AMA] / [$\alpha_1$-acid glycoprotein] is equal to or higher than 1.3 (plots 8 to 11)

shift occurs to 285 then to 287 nm. Thus, to the difference of β-lactoglobulin, binding of 1-AMA to $\alpha_1$-glycoprotein does not induce, within the stoichiometric range, any structural modification within the glycoprotein. Presence of 40% carbohydrate residues on $\alpha_1$-glycoprotein would be the principal reason for that (see also discussion).

It is clear from the data that the shift observed for β-lactoglobulin excitation spectrum (Fig. 4) occurs at a ratio of 0.3 (1-AMA to β-lactoglobulin), much before the stoichiometry of the complex (2 AMA for 1 β-lactoglobulin dimer) is reached. Concerning $\alpha_1$-glycoprotein, the position of the excitation peak remains unchanged up to 1.3 [1-AMA] / [$\alpha_1$-glycoprotein] ratio (Fig. 8). This clearly indicates that addition of 1-AMA to $\alpha_1$-acid glycoprotein up to the stoichiometric range (1:1) does not affect the protein conformation as it is the case for β-lactoglobulin.

*Fluorescence lifetimes measurements*

In absence and presence of 1-AMA, fluorescence intensity, I(λ,t), of Trp residues in $\alpha_1$-acid glycoprotein can be adequately represented by a sum of three exponentials. In absence of 1-AMA,

$$I(\lambda, t) = 0.230e^{-t/0.795} + 0.625e^{-t/2.445} + 0.145e^{-t/6.18}$$

where 0.230, 0.625 and 0.145 are the pre-exponential factors, $0.795\pm0.03$, $2.445\pm0.06$ and $6.18\pm0.77$ ns are the decay times and λ is the emission wavelength (335 nm) ($\chi2=1.03$, $\lambda_{ex}=296$ nm). The mean fluorescence lifetime calculated from three experiments was found equal to 3.61 ns.

Analyzing the decay curve with two lifetimes yields a $\chi2$ equal to 2.5. Also, it was not possible to fit the data with four exponentials.

Increasing 1-AMA concentrations in $\alpha_1$-acid glycoprotein solution up to 0.6 [1-AMA] / [$\alpha_1$-glycoprotein] ratio, does not affect none of the three fluorescence lifetimes. However, a slight increase of the third lifetime (from 6 to 8 ns) occurs up to 1.2 [1-AMA] / [$\alpha_1$-glycoprotein] ratio. This increase is simply a variation correlated to statistical measurements since the data shown are from three different experiments. Beyond the 1.2 ratio, the value of the longest lifetime increases faster (Fig. 9). The absence of a significant increase in the longest fluorescence lifetime of $\alpha_1$-acid glycoprotein in presence of 1-AMA up to the stoichiometry of the complex (1:1) is in good agreement with the results obtained when the fluorescence excitation spectrum of $\alpha_1$-acid glycoprotein was recorded in presence of different 1-AMA concentrations. In both experiments, structural modifications are not observed within $\alpha_1$-acid glycoprotein as the result of 1-AMA binding to the glycoprotein within the stoichiometric range (see also discussion).

The slight increase in the longest fluorescence lifetime of $\alpha_1$-acid glycoprotein tryptophan residues in presence of
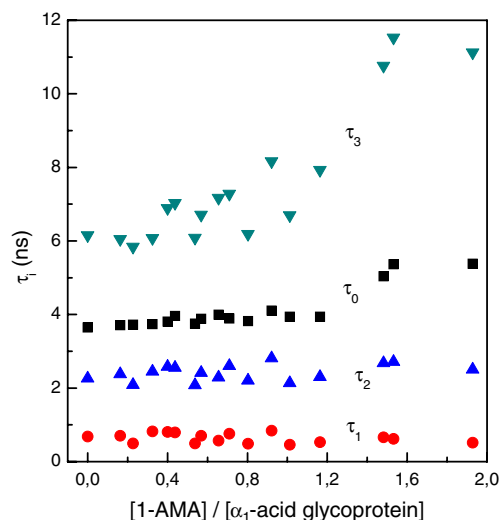


Fig. 9 Fluorescence lifetimes variation of $\alpha_1$-glycoprotein tryptophan residues as a function of [1-AMA] / $\alpha_1$-glycoprotein] ratio. $\lambda_{ex}=$ 296 nm and $\lambda_{em}=335$ nm. The data are from 3 experiments

1-AMA is accompanied by a decrease in its pre-exponential value ($\alpha_3$). However, to the difference of the results obtained with β-lactoglobulin, this decrease does not reach a plateau at the stoichiometry (1:1) of the complex (Fig. 10). Variation of $\alpha_3$ affects the values of $\alpha_1$ and $\alpha_2$ (Fig. 11) differently from that was observed for β-lactoglobulin (Fig. 7). The global similar variation (a decrease) of $\alpha_3$ in both $\alpha_1$-acid glycoprotein and β-lactoglobulin in presence of 1-AMA, is a clear indication to the fact that the longest lifetime along with its pre-exponential value characterize the interaction between tryptophan residues with the protein matrix confirming our previous results [39, 40]. Any modification of the
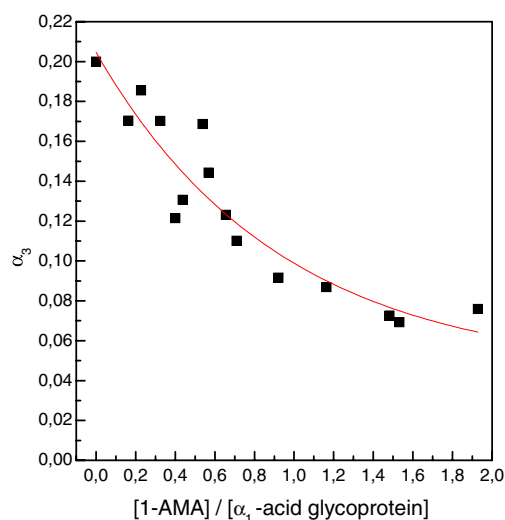


Fig. 10 Variation of longest fluorescence lifetime pre-exponential of $\alpha_1$-glycoprotein tryptophan residues as a function of [1-AMA] / [$\alpha_1$-acid glycoprotein] ratio. $\lambda_{ex}=296$ nm and $\lambda_{em}=335$ nm
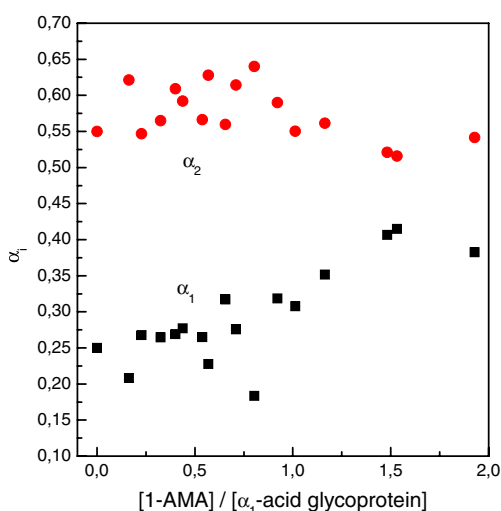
Fig. 11 Pre-exponential variations of the two shortest fluorescence lifetimes of $\alpha_1$-acid glycoprotein tryptophan residues as a function of AMA / glycoprotein ratio



Fig. 12 Titration of OBPa (2.7 µM dimer) with 1-aminoanthracene following the fluorescence increase at 490 nm of the probe. $\lambda_{ex}$= 280 nm and $\lambda_{em}$=490 nm

conformation within the protein will affect both long lifetime and its pre-exponential factor.

### Interaction between 1-AMA and odorant binding protein

*Fluorescence emission spectrum of odorant binding protein with 1-aminoanthracene*

Addition of increasing concentrations of 1-AMA to 2.7 µM odorant binding protein dimer induces a decrease in the fluorescence intensity at 340 nm (Trp residue) accompanied with an increase of the fluorescence intensity at 490 nm (1-AMA) (not shown). Binding of 1-AMA to OBP does not modify the emission peak position of the tryptophan residue indicating that binding site of 1-AMA is not necessarily in proximity of OBP Trp residue. In fact, analysis of OBP structure has shown that the Trp residue does not belong to the internal cavity forming the binding site for ligands [21, 23]. Intensity increase of bound 1-AMA is the result of the extrinsic fluorophore binding to the protein and to energy transfer from tryptophan residue to the extrinsic fluorophore. The plot of intensity increase of 1-AMA as a function of 1-AMA concentration is displayed in Fig. 12. The dissociation constant of 1-AMA-OBP complex obtained according to Eq. 1 is equal to 5±0.25 µM.

*Fluorescence lifetimes measurements*

In absence of 1-AMA, fluorescence intensity, I($\lambda$,t), of Trp residue in OBP can be adequately represented by a sum of three exponentials:

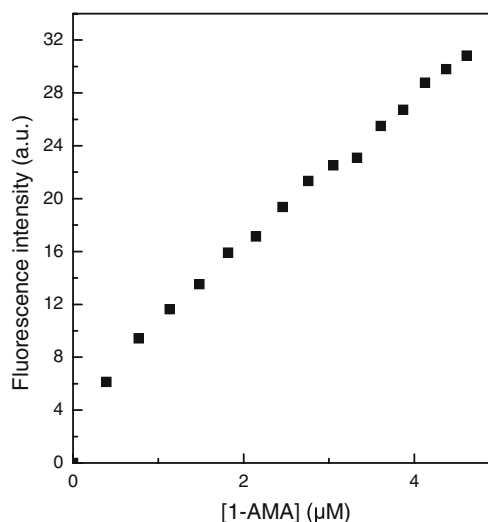$$I(\lambda, t) = 0.357e^{-t/0.858} + 0.534e^{-t/2.761} + 0.109e^{-t/9.201}$$

where 0.357, 0.534 and 0.109 are the pre-exponential factors, 0.858±0.05, 2.761±0.07 and 9.201±0.27 ns are the decay times and $\lambda$ is the emission wavelength (340 nm) ($\chi2$=1.167, $\lambda_{ex}$=296 nm).

Analyzing the decay curve with two lifetimes yields a $\chi2$ equal to 2.5. However, in presence of 1-AMA and to the difference of β-lactoglobulin and $\alpha_1$-glycoprotein, fitting the data with four exponentials yields better $\chi2$ values than the fitting with three lifetimes indicating that 1-AMA modifies the global conformation around the Trp residue of OBP. For example, when the ratio [1-AMA] / [OBP] reaches 0.33, fluorescence intensity decay of OBP can be described as

$$I(\lambda, t) = 0.153e^{-t/0.617} + 0.6e^{-t/1.862} + 0.188e^{-t/3.98}$$
$$+ 0.059e^{-t/16}$$

($\chi2$=1.152). Analyzing the data with 3 lifetimes yields a $\chi2$ value equal to 1.214. At high [1-AMA] / [OBP] ratios, for example at 1.7, $\chi2$ values obtained were 1.180 and 1.466 with four and three lifetimes, respectively.

Also, increasing 1-AMA concentrations in OBP solution induces an important increase in both longest and mean fluorescence lifetimes of the tryptophan residue. When the stoichiometry 2:1 of 1-AMA-OBP complex is reached, i.e., two moles of 1-AMA for one dimer of OBP, we notice that the longest and mean lifetimes increase stops (Fig. 13 and 14). The increase in the longest fluorescence lifetime in presence of 1-AMA is accompanied by an increase in its pre-exponential value (not shown). The pre-exponential values of the two shortest lifetimes are not significantly affected by 1-AMA binding to OBP.
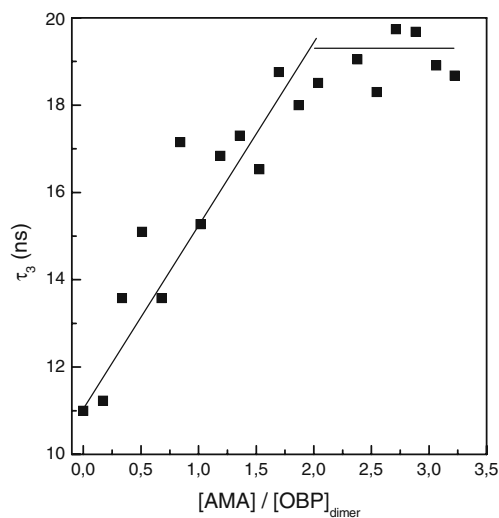
Fig. 13 Variation of the third fluorescence lifetime of OBP tryptophan residues with the [1-AMA] / [OBP] ratio. $n=2$ monomer of AMA for 1 dimer of OBP

## Discussion

The results obtained in the present study show that 1-Aminoanthracene disrupts β-lactoglobulin and OBP tertiary structures even at very low fluorophore / protein concentrations ratios. Tryptophan 19 residue in β-lactoglobulin is facing into the base of the hydrophobic pocket while tryptophan 61 is within the extremely mobile loop at the mouth of the pocket [17]. Thus, the two tryptophan residues in β-lactoglobulin are in positions which render them very sensitive to structural modification within and near the pocket. This high sensitivity would affect tryptophan residues fluorescence observables such as the excitation
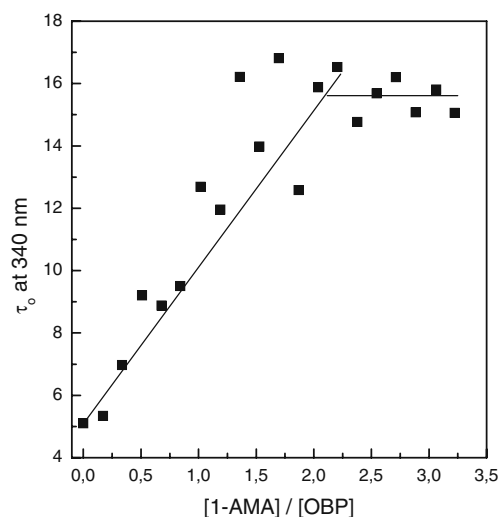


Fig. 14 Mean fluorescence lifetime of Trp residue of OBP at different [1-AMA] / [OBP] ratios. $\lambda_{ex}=296$ nm. $n=2$ monomer of AMA for 1 dimer of OBP

spectrum and fluorescence intensity decay parameters (lifetimes and pre-exponentials). Conformational modifications are observed from the fluorescence excitation spectrum of the protein recorded at different concentrations of 1-AMA and from the modification of the longest fluorescence lifetime of the tryptophan residues and of its pre-exponential component. In the absence of any conformational modification within the protein, the fluorescence excitation spectrum should not display any shift of its maximum. Thus, the important shift (10 nm) (Fig. 4) observed for β-lactoglobulin in presence of 1-AMA is a clear indication that binding of the extrinsic probe disrupts the protein conformation. Also, β-lactoglobulin structural modification was put into evidence by measuring fluorescence lifetimes of the protein tryptophan residues at different 1-AMA concentrations. Binding of the extrinsic fluorophore increases the longest fluorescence lifetime and decreases its pre-exponential component. Values of the two parameters reach a plateau up to the complex stoichiometry (2 moles of 1-AMA for 1 β-lactoglobulin dimer). The fact that only the longest fluorescence lifetime is modified upon the structural modification confirms our previous works on protein fluorescence lifetimes and where we showed that the two lifetimes measured for free tryptophan in water ($\approx 0.5$ and 2.5 ns) are found in almost all proteins [39]. Thus, these two values are in a certain way independent of the surrounding environment of tryptophan, revealing that they characterize intrinsic properties of the fluorophore, i.e. specific substructures formed upon excitation. The third fluorescence lifetime found in most of the proteins would then characterize the interaction existing between the fluorophore and surroundings amino acids [39–42].

One should pay attention here that stoichiometry of 1-AMA to β-lactoglobulin is 2:1 (Fig. 6) while that of 1-AMA to $\alpha_1$-acid glycoprotein is 1:1 (Fig. 10). Therefore increase of fluorescence lifetime in β-lactoglobulin occurs when [1-AMA] / [β-lactoglobulin] ratio is already equal to 0.3 (Fig. 5) while this increase is not observed for $\alpha_1$-acid glycoprotein until this ratio reaches 1.2 (Fig. 9). Thus, it is possible to use 1-AMA as a fluorophore to study binding of ligands to $\alpha_1$-acid glycoprotein. However, in presence of β-lactoglobulin, it should be used at low concentrations to avoid any modification in the conformation of the protein.

Emission spectra of β-lactoglobulin dimer in presence of 1-AMA (Fig. 2) do not show any shift to the longest wavelengths (350–355 nm). This means that protein denaturation in presence of 1-AMA is not occurring. However excitation spectra clearly indicate that addition of 1-AMA to β-lactoglobulin induces conformational changes in the protein structure. Excitation spectrum characterizes tertiary structure of a molecule in the ground state. Although structural modification does not mean here protein denaturation, still β-lactoglobulin structure in the

absence of 1-AMA is not the same in its presence. In this case, the dissociation constant measured characterizes the interaction between the extrinsic fluorophore and β-lactoglobulin within a structure that is different from the native structure in the absence of 1-AMA. At pH 7.5, β-lactoglobulin is a dimer, the pocket of each monomer makes a barrel (calyx) where 1-AMA binds. However, this calyx is narrow and thus 1-AMA, although attracted by the hydrophobic binding site of β-lactoglobulin, modifies the binding site and in consequence the protein structure. Therefore, 1-AMA would be able to induce a specific binding site that fits to its size. In other terms, hydrophobic binding site adopts a structure that is imposed by the bound ligand (in this case, 1-AMA).

In the case of $\alpha_1$-acid glycoprotein, carbohydrate residues protect protein structure of the binding effect of 1-AMA. It is already known that presence of the important amount of carbohydrate residues in $\alpha_1$-acid glycoprotein (40% of carbohydrate by weight) explains the resistance and the weak antigenicity of the protein, the carbohydrates acting as protective shield. In fact, it has been shown on five different glycoproteins, that carbohydrate residues stabilize the conformation of the protein matrix and prevent the unfolded or partially folded protein molecules from aggregation [43]. Also, it is important here to indicate that emission peak of 1-AMA bound to $\alpha_1$-acid glycoprotein is located at 520 nm compared to 500 and 490 nm for β-lactoglobulin and OBP, respectively. This means that binding site of 1-AMA on $\alpha_1$-acid glycoprotein is more hydrophilic than that of the other two proteins. 1-AMA binding site on OBP is the most hydrophobic which facilitates binding of the extrinsic fluorophore.

β-Lactoglobulin belongs to the lipocalin family and thus, the effect observed on β-lactoglobulin structure upon 1-AMA binding has been detected also on proteins such as odorant binding proteins. In fact, our results show that 1-AMA binding to OBP, modifies fluorescence lifetimes of the only Trp-residue of the protein. In presence of 1-AMA, fluorescence intensity decrease with time can be best described with four lifetimes instead of three in the absence of 1-AMA. This means clearly that binding of 1-AMA to OBP modifies its local and global conformation. In the same conditions, when 1-AMA was added to β-lactoglobulin, it did not induce any appearance of a fourth fluorescence lifetime. This may be explained by the fact that binding sites and interactions of 1-AMA with the two proteins are not the same. Nevertheless, in both cases, the two protein conformations are disrupted in presence of 1-AMA.

1-AMA is widely used as a probe to study interaction between odorant binding proteins ($OBP_s$) and hydrophobic ligands such as 2-iso-butyl-3-metoxypyrazine (IBMP), dihydromyrcenol (DHM) or benzophenone (BZP). In general, hydrophobic ligands are added to $OBP_s$-1-

AMA complex and binding of these ligands to the proteins is followed by analyzing the fluorescence intensity decrease of bound 1-AMA. Looking to the literature very carefully, one can notice that, in order to perform their experiments, the different authors measure the dissociation constant of the 1-AMA-OBP complex by titrating 1 μM of protein with concentrations of 1-AMA that go from 0.076 to 5 μM (see for example ref.11). Based on the data we obtained in the present work, OBP tertiary structure in presence of the concentrations used of 1-AMA is different from the native tertiary structure of the protein. However, some authors considered that their proteins structures are identical either in presence or in absence of 1-AMA. Their conclusion is based on the fact that emission peak does not shift to high wavelengths (350 or 355 nm) upon 1-AMA binding (see for example ref.15). One should remind here that emission spectrum is not so sensitive to structural modifications as it is the case for the excitation spectrum or fluorescence lifetimes. Therefore, upon slight or important structural modifications within the protein, recording the emission spectrum will not yield any information on these modifications.

Another problem occurs when 1-AMA is used as a probe. In fact, displacing 1-AMA from its binding site on OBP is usually performed in the following conditions: 0.5 μM or 1 μM OBP samples are incubated with a fixed amount of 1-AMA (3 μM) and increasing concentrations of odorants (0.39–50 μM) are then added. First of all, the [1-AMA] / [OBP] ratios used would in principle modify protein structure. Thus, the OBP studied in presence of 1-AMA should have different structure than the non-modified protein. Secondly, displacement of 3 μM of 1-AMA with 50 μM of ligand clearly means that the ligand, in order to displace the 1-AMA, needs to modify the structure of the OBP of the 1-AMA-OBP complex. If the interaction between 1-AMA and the other hydrophobic ligands are competitive, this means that small concentrations of the ligands are sufficient to displace 1-AMA from its binding site. Also, in the case of a real competitive interaction, affinity of the ligands to OBP should be around 100 times that of 1-AMA to OBP. See for example, displacement by hemin of the fluorophore 2,p-toluidinylnaphthalene-6-sulfonate (TNS) from its binding site on apocytochrome $b_2$ core [44] and on $\alpha_1$-acid glycoprotein [28]. In both cases, TNS fluorescence decreases to zero when the stoichiometry of the proteins-hemin complex reaches 1:1. In the studies on OBP, it is clear, from the concentrations of hydrophobic ligands used, that experiments performed do not fit with competitive interactions. The large concentrations of ligands modify OBP structure so that 1-AMA can thus be released from the binding site. This fact is in good agreement with our results on both β-lactoglobulin and OBP that show that binding of 1-AMA to the lipocalin

proteins induces important structural modifications within the proteins. Since important concentrations of ligands are needed to displace 1-AMA from its binding site, this means that hydrophobic binding site follows a conformation imposed by 1-AMA presence. Added ligand would exert a pressure on the protein so that new structural rearrangements within the protein and within the binding site occur in order to release 1-AMA to the solution. Our conclusion is in good agreement with the fact 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]. Also, the close dissociation constants values of 1-AMA-OBP complex and ligands-OBP complex are in good agreement with the fact that conformational modifications are occurring within the protein upon 1-AMA displaced with the "supposed" competitive ligands.

In conclusion, our present work shows that binding of 1-AMA to β-lactoglobulin and OBP, two lipocalin proteins family, induces conformational modifications within the proteins. The results indicate that before the stoichiometry of the complex is reached (2 moles of 1-AMA for 1 lipocalin protein dimer), structural modifications are occurring within the proteins. Experiments performed on $\alpha_1$-acid glycoprotein, a monomer that contains 40% of carbohydrate by weight, showed that structural modification of the protein upon 1-AMA binding is not occurring at least up to the stoichiometric range (1 mole of 1-AMA for 1 $\alpha_1$-acid glycoprotein). Thus, one should question the ability of using 1-AMA as a probe to study interaction of lipocalin proteins family with their ligands and the meaning of the measured $K_d$ of the 1-AMA-protein and of the ligands-protein complexes.

Fluorophores have been defined by Edelman and McClure [45] as "small molecules which undergo changes in one or more of their fluorescence properties as a result of noncovalent interaction with a protein or other molecules". Such fluorophores, when added to macromolecules such as proteins, should not modify their local or global conformation. Only in this case, a fluorophore is considered as a good probe to study, for examples, allosteric transitions, protein-protein interactions and structural alterations which accompany protein denaturation. Therefore, the present work indicates clearly that 1-AMA is not a suitable fluorophore to study interaction between β-lactoglobulin or OBP and their ligands. At the best and in order to avoid any conformational modification of the protein, low concentrations of 1-AMA compared to those of the proteins should be used. This is not the case for $\alpha_1$-acid glycoprotein since conformational modification within the protein does not occur within the stoichiometric range. Although $\alpha_1$-acid glycoprotein belongs to the lipocalin family, such

as β-lactoglobulin and OBP, presence of important amount of carbohydrate on $\alpha_1$-acid glycoprotein (40% by weight) modifies the protein pocket conformation rendering it larger and thus more suitable to bind different types of ligands such as 1-AMA, calcofluor white [27] or progesterone [30, 46].

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