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ORIGINAL PAPER

Fluorescence Quenching Determination of Uranium (VI) Binding Properties by Two Functional Proteins: Acetylcholinesterase (AChE) and Vitellogenin (Vtg)

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Abstract The interactions between uranium and two functional proteins (AChE and Vtg) were investigated using fluorescence quenching measurements. The combined use of a microplate spectrofluorometer and logarithmic additions of uranium into protein solutions allowed us to define the fluorescence quenching over a wide range of [U]/[Pi] ratios (from 1 to 3235) at physiologically relevant conditions of pH. Results showed that fluorescence from the two functional proteins was quenched by UO_2^{2+}. Stoichiometry reactions, fluorescence quenching mechanisms and complexing properties of proteins, i.e. binding constants and binding sites densities, were determined using classic fluorescence quenching methods and curve-fitting software (PROSECE). It was demonstrated that in our test conditions, the protein complexation by uranium could be simulated by two specific sites (L_1 and L_2). The obtained complexation constant values are log K_1= 5.7 (±1.0), log K_2=4.9 (±1.1); L_1=83 (±2), L_2=2220 (±150) for U(VI) – Vtg and log K_1=8.1 (±0.9), log K_2=6.6 (±0.5), L_1=115 (±16), L_2=530 (±23) for U(VI)-AChE (L_i is expressed in mol/mol of protein).

Keywords Acetylcholinesterase · Vitellogenin · Uranium · Fluorescence quenching · Complexing properties

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Introduction

Uranium, an element from the actinides series, is one of the heaviest naturally occurring elements on earth. Its distribution is ubiquitous but its concentration may be increased in some ecosystems due to anthropogenic activities [1]. In aerobic aqueous medium, within the physiological pH range (~5 to ~7.4), U(VI) hydrolyses and is mostly found in the form of hexavalent uranyl ions (UO_2^{2+}) [2, 3]. Thus, in biological media, UO_2^{2+} has the ability to form stable complexes with many ligands. In blood, UO_2^{2+} can bind strongly to plasma proteins and so can be transfered to the different organs [4–6]. In a previous study, we have studied the uranium binding with four metalloproteins known to be implied in the cation transport processes: human serum apo-transferrin (Apo-HTf), human serum albumin (HSA), rabbit liver metallothionein (MT) and equine-spleen apo-ferritin (Apo-EqSF) [6]. This work has been carried out using the fluorescence quenching principle which is the reduction of the fluorescence intensity of a given substance in solution, by the addition of another substance, called the quencher. Quenching may result from a variety of processes [7–9]; it can then be either dynamic, resulting from collision between the fluorophore and quencher, or static, resulting from the formation of a ground-state complex between the fluorophore and quencher, or both dynamic and static. It can reveal the accessibility of quenchers to fluorophore groups of proteins, can help to understand protein binding mechanisms and can provide important information on the nature of the binding phenomenon (number of available sites and binding constant) [6–9].

In the present work, we have chosen to use the fluorescence quenching method and the analytical procedure developed in our previous work [6] to the study of the complexation of uranyl ions with two functional proteins, acetylcholinesterase (AChE) and vitellogenin (Vtg). If none study has precisely

determined the complexation of these two proteins with uranium, some studies have demonstrated that AChE and Vtg could be affected by uranium exposure [10–12]. These two proteins, which are widely distributed throughout organisms, are successfully used at biomarkers of neurotoxic and xenobiotic contaminants [12, 13]. AChE is an important membrane-bound enzyme involved in the nerve impulse transmission across cholinergic synapses [14, 15] which is responsible for the hydrolyzation, in vivo, of acetylcholine into choline and acetic acid [14–17]. Concerning its metal chelation presence of Zn^{2+} binding sites has been recently demonstrated [15]. Vtg is the principal precursor of the egg-yolk proteins, vitellins, which provide energy reserves for the developing embryo in oviparous organisms [13, 18, 19]. Vtg is synthetized in the liver and is carried in the bloodstream to the oocytes of maturing females [18].

The objectives of this work were (i) to estimate, by fluorescence titration, the quenching mechanisms involved in the U/AChE and U/Vtg complexes (ii) to use PROSECE software to evaluate the complexing capacities and stability constants (L_i, K_i) of the different complexing sites of AChE and Vtg by fitting titration experimental data.

Experimental

Reagents

All solutions were prepared with high-purity de-ionised water (resistivity ≥ 18.2 MΩ cm) obtained from a doubly Milli-Q water purification system (Millipore Synergy 185 and Millipore Helix systems). All reagents were of the highest grade available from Sigma-Aldrich (St. Quentin Fallavier, France) and were used without any further purification. Vtg was obtained from whole-body homogenates of exposed zebrafish Vtg (zf-Vtg) and was purified according to the study of Brion et al. (2002) [18]. The purified Vtg has an apparent molecular weight of 375 KDa. Fraction of 1 mL were stored in 0.05 M carbonate-bicarbonate buffer, pH 9.6, at concentration of 537 $\mu g.mL^{-1}$ and T=−80 °C.

AChE from *Electrophurus electricus*, of which the molecular weight is 280 kDa, was obtained as a lyophilized powder (V-S type, Sigma C2888). AChE was a tetramer composed of four equal subunits of 70 kDa each .

Preparation of Solutions

HEPES buffer solution (50 mM) was prepared by dissolving powder in de-ionised water. The pH was then adjusted to 7.4 with NaOH (1 M). AChE and Vtg solutions were obtained by dissolving, or diluting, an appropriate amount of each protein in the HEPES buffer solution (0.05 M, pH = 7.4). Two uranyl working solutions (1.0 and 0.1 mM) were prepared daily from the uranyl stock solution (10 mM) diluted with de-ionised water. All solutions were kept in the dark at 4 °C.

Instrumentation

Fluorescence quenching experiments were realised using a microplate spectrofluorometer (SPECTRAmax GEMINI-XS, Molecular Device) equipped with a Xenon flash lamp (1 J/flash). Data obtained from the quenching of fluorescence experiments were collected with SoftMax Pro software (Molecular Devices). Microplates used for experiments were black walled and clear bottomed quartz 96-wells with a working well volume of 300 μL (cat n°730,009B-QG, Hellma, Paris, France).

General Procedure of Fluorescence Quenching Experiments

The same procedure than those used in [6] was carried out in this work. Briefly, for the two uranium-protein system studies, different amounts (1–20 μL) of the uranium solution were added to 280 μL of the specific protein solution in the microplate wells; the final volume being adjusted to 300 μL with HEPES buffer. Concentrations of uranium added followed a logarithmic law, (Δ(log[U]) = constant) and did not exceed 0.657 mM to keep the concentration below the limit of uranyl ions solubility in hepes and prevent from precipitation in wells. All experiments were carried out in duplicate. Final protein concentrations varied depending on the protein studied and its intrinsic fluorescence: AChE concentrations in the well ranged from 0.12 to 0.89 μM and Vtg from 0.05 to 0.30 μM. The [U]/[Pi] molar ratios tested therefore ranged from 1 to 1767 for the U - AChE system and from 16 to 3235 for the U - Vtg system.

Fluorescence measurements were performed at room temperature (22±1 °C) regulated by the apparatus directly within the microplate spectrofluorometer. One hour kinetic measurements showed that an incubation time of 20 min permits to obtain stable fluorescence signal intensity for each uranium-protein system studied as already observed in [6] (results not shown). Microplates were automatically shaken for 5 s before each reading. 2D emission spectra were realised to (1) determine the optimum $\lambda_{ex}/\lambda_{em}$ couple for each protein and (2) check that no shift of this couple was observed with the protein concentration and the uranium addition.

To check the stability of pH (i.e. 7.4) during the titration, the pH of the solution in the quartz wells was measured after quenching experiments with a microelectrode (MI-414, Microelectrodes Inc., Bedford, N.H., USA) connected to a conventional pH meter (Titralab TIM 900, Radiometer Analytical, Lyon, France). The quartz microplates were soaked daily in 0.5 M HNO_3 for 1 h and then rinsed five times with distilled water before use.

Methods of Data Analysis

Fluorescence Quenching Mechanisms

The dynamic or static nature of the fluorescence quenching was investigated for the U - AChE and U - Vtg systems. Graphs were plotted according to the Stern-Volmer equation [8]:

$$F_0/F = 1 + k_q\tau_0[Q] = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the relative fluorescence intensities of AChE and Vtg in the absence and in the presence of uranium respectively; [Q] the quencher concentration (M), here [Q] = [U]; k_q the biomolecular quenching rate constant (M^{-1} s^{-1}); τ_0 the lifetime of the fluorophore in the absence of uranium (s) (10^{-8} s for protein [8]); and K_{SV} the Stern-Volmer constant (M^{-1}).

If the evolution of F_0/F plots, according to the concentration of quencher, is linear for the whole range of quencher concentrations, fluorescence quenching can be attributed either to being purely dynamic, or purely static. In contrast, if the ratio F_0/F is not linear and shows an upward curve at higher quencher concentrations, the fluorescence quenching mechanism can be attributed to the presence of simultaneous dynamic and static quenching. In the latter situation, from the bimolecular quenching rate constant value k_q, determined in the linear range of the F_0/F ratio, the initial fluorescence quenching mechanism can be determined. Typically, if k_q is much higher than 10^{10} M^{-1} s^{-1}, i.e., the upper value possible for diffusion-limited quenching in most solutions at room temperature [8, 9], the fluorescence quenching mechanism is initially a static one (and so K_{SV} is called K_a) whereas with the lower k_q values it is initially a dynamic quenching.

Fitting Model: PROSECE Software

The experimental fluorescence data obtained were also analysed with the PROSECE software, which allows the determination of complexing properties using a discrete model of binding sites distribution [20–22]. This software has previously been successfully applied to the determination of binding parameters between uranyl ions and metalloproteins [6]. Thus, U - AChE and U - Vtg complexes stability constant (K_i) and site density (L_i) values were obtained using the same algorithm [6]. PROSECE fitting establishes the optimal number of binding sites and the quantum yield (Q_{Li}) of the non-complexed ligand form (L_i) was optimized assuming that the complexed form (U - L_i) is non-fluorescent ($Q_{U\text{-}Li}$ equals to 0) as the fluorescence intensity measured throughout the titration tends toward 0.

Results and Discussion

Spectral Characteristics of AChE and Vtg Proteins

As mentioned in the ***General procedure*** section, the first experiments were realised to determine the optimal $\lambda_{ex.}/\lambda_{em.}$ combination for AChE and Vtg. Values obtained for the optimum $\lambda_{ex.}/\lambda_{em.}$ combinations were of 278/334 nm for AChE and 279/328 nm for Vtg (Fig. 1).

According to the literature data [8, 9], excitation and emission wavelengths obtained for the two proteins ($\lambda_{ex.}/\lambda_{em} \approx 280/330$ nm) were characteristic to the presence of tryptophan (Trp, an aromatic amino acid) in their structure. Concerning AChE, this result is in agreement with the structure of enzymes belonged to the cholinesterase family which is known to contain some Trp in its amino acid structure [23]. For Vtg, the presence of Trp was not revealed in most amino-acid chain of vertebrate species [24]. According to this last study, Trp was detected only in plasma of Japanese eel (*Anguilla japonica*) and represents only 0.39 % of the total quantity of the amino-acid structure.

The fluorescence intensity varied linearly with the protein concentration (mean correlation coefficients (r^2) higher than 0.99) in the range of 0.1–1 μM for AChE and in the range of 0.05–0.30 μM for Vtg (data not shown). The proteins were therefore neither affected nor denatured in the concentration range studied. This was of paramount importance for fitting the data obtained during the quenching experiments and interpretation.

Fluorescence Quenching of AChE and Vtg by Uranium

Quenching Spectra of AChE and Vtg

Fluorescence titrations on AChE and Vtg were achieved by addition of a solution of UO_2^{2+} in 0.05 M HEPES at pH 7.4. An example of spectra obtained for one concentration of each protein is presented in Fig. 1.

As shown in Fig. 1, the fluorescence intensities of the two proteins decrease concomitantly with increasing UO_2^{2+} concentration. Furthermore, the spectra show that there are no significant λ_{em} shifts with the addition of uranyl ions for any of the two proteins. This data indicates that UO_2^{2+} could interact with AChE and Vtg and quench their fluorescence without changing the microenvironment properties of the fluorophore site.

Stoichiometry of the U/Pi Complexes

The evolution of the fluorescence intensity versus the molar ratio [U]/[Pi] was studied in order to determine the stoichiometry of the binding reactions between AChE/Vtg and uranium. As an example, Fig. 2 shows typical titration curves of two

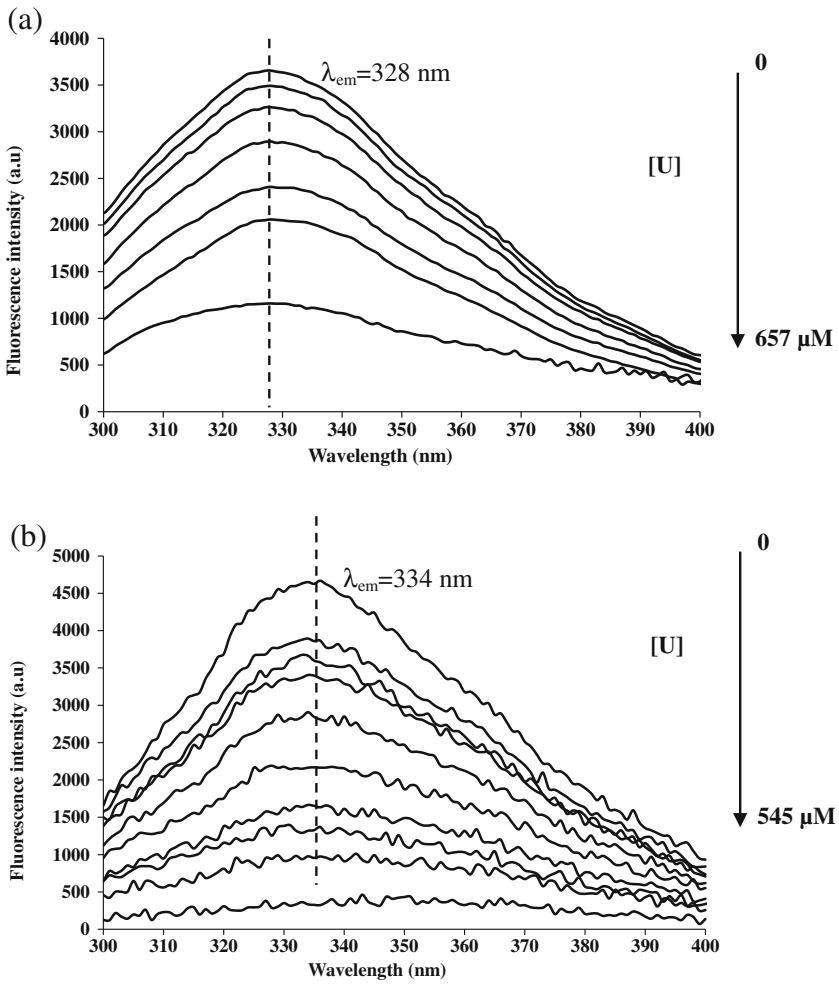

Fig. 1 Fluorescence spectra of 0.30 µM Vtg (**a**) and 0.89 µM AChE (**b**) in 0.05 M HEPES (pH = 7.4) in the presence of increasing uranium at ambient conditions ($\lambda_{ex.}$=278 nm for AChE and 279 nm for Vtg, excitation and emission slits are 2 mm)

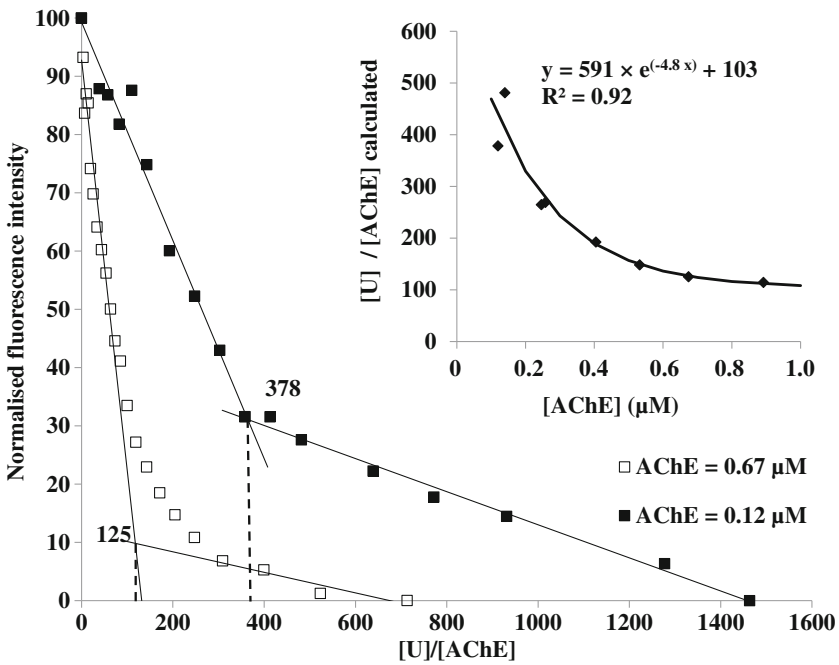

Fig. 2 Plots of AChE titrated with UO_2^{2+}. Conditions: [AChE] = 0.12 and 0.67 µM pH = 7.4 - [HEPES] = 50 mM - T = 22±1 °C - Incubation time: 20 min. Inset figure: calculated [U]/[AChE] with the "graphic method" function of [AChE]

different concentrations of AChE (0.12 and 0.67 μM) quenched by UO_2^{2+}. Stoichiometry of the reaction is determined by a commonly used method in the literature called 'graphic method' [6, 8, 25, 26].

Analysis of the curves obtained in Fig. 2 indicates that for the different AChE concentrations the sharp break occurs at two different molar ratio [U]/[AChE] of 378 (±46) and 125 (±12) for [AChE] 0.12 and 0.67 μM, respectively. The uncertainties are calculated by taking into account the standard error on the slope and on intercept of the two lines. As shown in the inset Fig. 2 an exponential relationship was obtained between the calculated [U]/[AChE] ratio at the nick point vs the different [AChE] tested. For Vtg experiments the graphic method gives a [U]/[Vtg] of 714 (±70) (varying from 653 to 754 for the four tested concentrations of Vtg) (Online Resource 1).

The modification of calculated [U]/[AChE] vs [AChE] could be explained by the fact that with 'graphic method' only one protein complexation site could be taken into account. If the protein has several complexation sites only partial information could be obtained on the stoichiometry reaction as already discussed in [6]. The results obtained suggested that AChE has at least two binding sites. Only the experiments conducted with weak AChE concentrations give information on the maximum binding capacity of AChE, while for the highest AChE concentrations only a portion of the binding sites are activated. In this way, by using the empirical exponential relationship given in the inset Fig. 2 we can consider that for a very weak [AChE] (≈0) the maximum calculated [U]/[AChE] is equal to 694. For Vtg (Online Resource 1) the results suggested that only one biding site could be present or the range of concentrations investigated did not permit to observe the activation of different binding sites. The obtained results are summarised in Table 1.

Even though the 'graphic method' could give information about the complexation between uranium and proteins, anything can be deduced about the nature of either the quenching mechanism which occurred or the complexes formed.

Binding Properties of the U/Pi Complexes

In order to determine the quenching mechanisms occurring between the two functional proteins and uranium, the fluorescence titration data were investigated using the Stern-Volmer plots. Figure 3 shows the results obtained for the F_0/F ratio evolution against U concentration for AChE (0.14 and 0.67 μM) and Vtg (0.11 and 0.20 μM).

The Stern-Volmer F_0/F plots of AChE and Vtg deviated from linearity towards the y-axis at higher uranium concentrations which indicating a combination of dynamic and static quenching. As shown in the inset of Fig. 3, for low uranium concentrations, Stern-Volmer graphics are linear for AChE and Vtg. Based on these experimental data, biomolecular quenching rate constants k_q and Stern-Volmer constants K_{SV} are deduced from Eq. (1) in this linear range of F_0/F ratio. k_q data was found to vary from 3 10^{11} to 2 10^{12} M^{-1} s^{-1} for Vtg and from 2 to 5 10^{12} M^{-1} s^{-1} for AChE for the different concentrations tested. These values were 10^1–10^2 fold higher than the upper value possible for diffusion-limited quenching (~10^{10} M^{-1} s^{-1}) suggesting that the fluorescence quenching is initiated by static quenching, starting with the formation of a complex between uranyl ion and proteins.

Due the nature of quenching occurring, the Stern-Volmer constant K_{SV} could be assimilated to a binding constant [6]. Thus, log K_{SV} values were calculated to be 4.4 (±0.2) and 3.9 (±0.4) for AChE and Vtg respectively. The comparison of these values with values obtained in our previous study [6] seemed to show that uranium binding by these protein is in the same order of magnitude than other metalloproteins (Apo-HTf > **AChE** > HSA > MT - **Vtg** > Apo-EqSF). However, using this Stern-Volmer approach only the linear range of F_0/F ratio can be used thus excluding the high uranium concentration range values. In addition the number of binding sites and their density could not be obtained by this approach.

For the two U(VI)-protein complexes studied, two discrete models of binding site distribution were tested by PROSECE

Table 1 Fitting results obtained for the complexation of uranium(VI) with the two proteins (AChE and Vtg) using 'Graphic method' or PROSECE software

		AChE		Vtg	
Graphic method	Stoichiometry	Min. 694/1		714/1	
	Complexation reaction	694U+AChE <=>U_{694}AChE		714U+Vtg <=>U_{714}Vtg	
PROSECE		L_1 site	L_2 site	L_1 site	L_2 site
	Binding constant (log K_i)	8.1 (±0.9)	6.6 (±0.5)	5.7 (±1.0)	4.9 (±1.1)
	Li (mol Li/per mol of Pi)	115 (±16)	530 (±23)	83 (±2)	2220 (±150)

Numbers of specific complexation sites taken into account for PROSECE fitting: 2. (Error corresponds to the standard deviation σ_d)

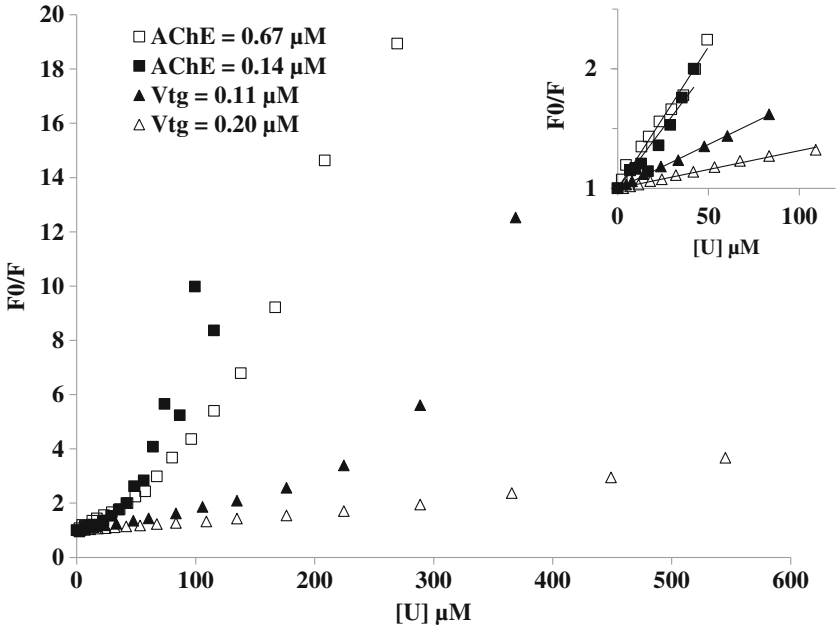

Fig. 3 Plots of AChE and Vtg titrated with UO_2^{2+}. Conditions: [AChE] = 0.14 and 0.67 µM - [Vtg] = 0.11 and 0.20 µM- pH = 7.4 - [HEPES] = 50 mM - T=22± 1 °C - Incubation time: 20 min

to fit the quenching of fluorescence at different titration points, i.e., a first fitting with one specific complexation site and a second with two specific complexation sites. In both cases, the U-L_i considered stoichiometry was a 1:1 one.

Results obtained showed that distribution with two binding sites (L_1 and L_2) was optimal to simulate all the titrations obtained between the two proteins and uranium. Each protein complexing site was defined by a binding site density (mole of U(VI) complexed per mole of Pi) and a stability constant (log K) towards uranyl ions, i.e., four unknown parameters for each protein. The values of these four parameters were optimized using PROSECE, by fitting experimental fluorescence data obtained for each protein concentrations. The parameters values optimized for each protein were then averaged and summarized in Table 1. Results of fitting by the proposed model of the experiments carried out on AChE are shown as example in Fig. 4 (for Vtg figure is available in Online Resource 1).

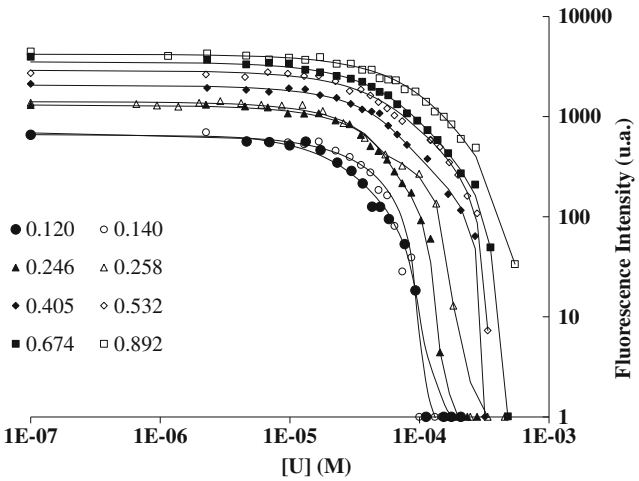

Fig. 4 Experimental fluorescence intensities acquired by the uranium logarithmic addition to the eight different AChE concentrations (µM) at pH 7.4 *(open and closed geometric forms)* and PROSECE corresponding fitting *(solid lines)* with models using two specific complexation sites (log/log scale for axis)

According to the binding constant values obtained and summarized in Table 1, for each protein studied we established a 'strong' (L_1) and a 'weak' (L_2) binding site. Results in Table 1 also show that L_1, the 'strong' binding sites, are sites with the lower number of U(VI) atoms complexed per mole of protein, and L_2, the 'weak' binding sites, are sites with the higher number of U(VI) atoms complexed per mole of protein. PROSECE results showed that these two proteins have a very high number of binding sites compared with the protein previously studied (except Apo-EqSF) [6] with the highest cumulated binding site density for Vtg (2307± 155 mol of U(VI) per mole of Vtg by addition of site 1 and site 2 densities) compared to AChE (645±39 mol of U(VI) per mole of AChE by addition of site 1 and site 2 densities). However, this last one has the highest L_1 and L_2 biding constants (log K_1=8.1±0.9 and log K_2=6.6±0.5), whereas Vtg has values of log K_1=5.7±1.0 and log K_2=4.9±1.1. Thus, combinations of binding constants and binding site densities (i.e. L_i x K_i) reported in Table 1 suggest that uranium would be bound preferentially with AChE. More comparing with the data obtained for metalloproteins [6] AChE has the highest capacity complexation (**AChE > Vtg** > Apo-HTf > Apo-EqSF > HSA > MT).

For the U - AChE complex, PROSECE results seem to be in line with the results obtained by the graphic method. For the highest [U]/[Pi] ratio the two sites are activated with the saturation of the strong site and some complexation at the weak

site, whereas for the lowest [U]/[Pi] ratio only strong sites are activated (see Table 1 and Fig. 2). This result explains that depending on the [Pi], the [U]/[Pi] obtained by graphic method is different. For the U-Vtg complex, the site identified by the 'graphic method' was shown to be an 'artificial' site resulting of a combination of the density values of the two sites obtained by PROSECE. The binding constants identified by PROSECE (Table 1), were higher than binding constants K_{sv} obtained with Stern-Volmer regression curves. Such differences between the two methods could be explained by the fact that only one fluorescence quenching mechanism (dynamic or static) could be determined using the Stern-Volmer regression curves, themselves obtained only with the linear portion of the titration curve. Moreover the Stern-Volmer regression plot is based on the total concentration of the quencher while to obtain a real complexation constant it is necessary to plot F_0/F vs free concentration of quencher. To obtain K_{SV} value this information is not available and so a lot of study replaced directly the free concentration of quencher by the total concentration of quencher (e.g. [27]). In the case of a large excess of quencher this hypothesis is true, while in our study the free uranium concentration is certainly overestimate and so K_{SV} underestimated.

Due to uncertainties and limitation of the "graphic method" or the Stern-Volmer plot, binding parameters obtained by PROSECE seemed to be more confident than the classical graphic methods. A user-friendly version of PROSECE, called ProMCC, was recently developed and is available free of charge [28]. Fitting of fluorescence quenching data using ProMCC will be implemented soon (the actual ProMCC version is restricted to metal titration assessed using voltammetric analysis), allowing the users to easily treat such data as performed in the present study.

No results have been found in literature concerning the binding properties of these two proteins with uranium. Then, no comparison can be done with our data. However comparison with our previous study [6] shows that the binding capacity of these two proteins is quite high and is in the same order of the binding capacity than Apo-EqSF (i.e. 525). Vtg is a precursor from which are derived the smaller more readily characterized yolk proteins lipovitellin (LV) and phosvitin (PV) [29]. Both of these proteins have been previously shown to contain metals [30–32] and possibly to play a part in metal bioavailability [33] but neither site densities nor association constants have been calculated in these studies. The role of phosphorylation on uranium complexation has been emphasized through numerous studies of speciation [e.g. 34, 35]. Vtg is a highly phosphorylated protein, particularly with its phosvitin part which has been shown to complex U [36]. pH has also been demonstrated as a very important parameter for the result of U complexation [34]. The results of this study suggest that uranium could be transfer between the different organs by these proteins. This result for divalent cation has been already observed for Cd^{2+}transport by Vtg from liver to the ovary during the period of vitellogenesis [37]. In the biological point of view, metal exposure, especially with cadmium, resulted in an increase in vitellogenin concentration in hemolymph of the shore crab Carcinus maenas [38]. On the contrary, waterborne uranium exposure of fish Danio rerio had been shown to decrease the Vtg synthesis [11] with potential reproduction perturbations. About brain, AChE activity had been shown to increase after waterborne exposure to U but the mechanism is still unknown and the implication of U complexation has not been tested yet [10].

Conclusion

In this paper, the interactions of two functional proteins (AChE and Vtg) with uranium were studied by fluorescence quenching measurements using a microplate spectrofluorometer. This system, which has been used previously for the determinations of the complexing properties of four metalloproteins with uranium, enabled us to fully characterize the U - AChE and U - Vtg complexing properties over a wide range of [U]/[Pi] ratios and at physiological pH conditions (7.4). Using the PROSECE software this study proposes for the first time values of binding parameters for these two functional proteins with uranium. The results obtained show that uranium can be strongly associated with these two functional proteins.

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