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Interaction of a protein, BSA, and a fluorescent probe, Mag-Indo-1, influence of EDTA and calcium on the equilibrium

A-C. Ribou*, J. Vigo, P. Viallet, J-M. Salmon

Group of Quantitative Microfluorometry, Physical Chemistry Laboratory, University of Perpignan, 52 Avenue de Villeneuve, F-66860, Perpignan, France

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Abstract

Recent findings indicate that ion-chelator probes with tetracarboxylate structure bind proteins. It was suggested that these fluorescent probes are valuable tools to gain information on protein structure through the energy transfer from tryptophans to the bound probe. Here, the binding of the fluorescent probe Mag-Indo-1 to bovine serum albumin (BSA) was investigated. Mag-Indo-1 was reported previously to serve as a probe for magnesium cations ($K_d = 2.8 \times 10^{-4}$ M for zero ionic strength) which can also interact with calcium cations ($K_d = 7.5 \times 10^{-7}$ M). Probe complexation with protein results in a shift of the emission fluorescence spectrum of the probe from 480 to 457 nm. We used emission fluorescence techniques to monitor this interaction. Computational resolution of the complex fluorescence spectra and a new software to test the theoretical model were developed in our laboratory. This enabled us to calculate the number of interacting sites and the dissociation constants. The fluorescent probe Mag-Indo-1 binds at a singular site with high affinity ($K_d = 1.8 \times 10^{-7}$ M) to bovine serum albumin (BSA). Since proteins are known to bind several compounds unspecifically, we have studied the influence of EDTA as a competitor of the probe. Our findings suggest that the BSA binding site is identical for both Mag-Indo-1 and EDTA. We found that EDTA binds the protein with $K_d = 0.4 \times 10^{-3}$ M. We studied the influence of calcium and found that Mag-Indo-1 does not bind the calcium free Apo-protein anymore.

Keywords: Mag-Indo-1; BSA; Fluorescence; Probe; Calcium; Data analysis

* Corresponding author. Tel.: +33-4-6866-2113; fax: +33-4-6866-2144.
E-mail address: ribou@univ-perp.fr (A. Ribou)

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1. Introduction

Serum albumins are abundant proteins. The three-dimensional structure of human serum albumin (HSA) has been resolved [1]. However, for bovine serum albumin (BSA) no crystals suitable for X-ray diffraction studies have been obtained yet. Serum albumins are known to bind a variety of biological molecules. The binding involves hydrophobic, hydrophilic, cationic substances. The knowledge about proteins has profited from the use of these different ligands. The molecular interactions are often monitored using optical techniques. These methods are sensitive and relatively easy to use. Fluorescent spectroscopy is a valuable technique to study the binding of ligands to proteins. With BSA, different probes have been successfully used such as ANS [2–4], DAUDA [5], dansyl-Cl [6,7], Quin-2 [8], ADIFAB [9], Tb(III) [10] or probes covalently bound to BSA [11]. Direct intrinsic fluorescence of BSA was also used [5,12–15] and the energy transfer between an intrinsic chromophore and an external probe was studied [10,16]. This energy transfer was reported to give information about the protein structure. In some of those cases fluorescent measurements showed changes in the steady state fluorescence properties exhibited by free and liganded probe. Protein–probe interactions have also been shown to occur with calcium chelating dyes (e.g. Fura-2, Indo-1, Quin-2) [8,17–21]. Mag-Indo-1 is a well-known fluorescent probe with a cation cage part. Although designed as a specific magnesium chelator ($K_d = 0.28$ mM), it can bind other cations (calcium: $K_d = 0.75$ μM). Furthermore, Mag-Indo-1 can also bind proteins through a specific interaction with histidine residues [22]. Mag-Indo-1 bound to BSA has an emission maximum at a shorter wavelength (shift of 23 nm) than the free probe. At physiological pH, the Mag-Indo-1 (pK_a of 5,3) is in its deprotonated form (i.e. fluorescent form). This makes it an excellent probe for the investigation of protein structure or protein denaturation at physiological pH compared to the Indo-1 which has a pK_a close to 7.

In this paper, the binding of Mag-Indo-1 to BSA was investigated. While the absence of interfering compounds would be ideal, it is often not feasible in solution because usually ions are present. Here, we studied the interaction of the probe with native BSA in the presence of residual calcium in aqueous solution. Fluorescent spectral techniques were used to monitor the interaction. The resolution of the complex fluorescence spectra [23] together with a new software to test theoretical models, makes it possible to determine for the first time the apparent number of interaction sites and the dissociation constant. The next goal of the study was to assess the effects a complex environment has on the equilibrium and the spectroscopic characteristic. In the complex environment of living cells the cation concentrations can only be determined accurately with Mag-Indo-1 when a full spectra is recorded. Since proteins are always present, it is essential to know the characteristic spectrum of the protein bound probe. Calcium influences the structure of BSA [24] and subsequently the number of binding sites of BSA to Mag-Indo-1. Therefore, the probe-protein equilibrium was studied (i) with the calcium deficient Apo-protein; (ii) with EDTA as a calcium chelator; and (iii) under the addition of calcium. Since several authors used EDTA in millimolar concentrations to eliminate disturbances due to calcium, our findings will be useful to interpret these earlier results. Several possible interactions between Mag-Indo-1, BSA, EDTA and Ca will be described.

2. Material and methods

2.1. About equilibrium

When fluorescent spectra of the free probe L and the bound probe LP are different the dissociation constant can be calculated:

$$L + P \Leftrightarrow LP \qquad K_{dLP} = \frac{[\mathrm{L}] \times [\mathrm{P}]}{[\mathrm{LP}]} \tag{1}$$

where [L] is the concentration of free probe Mag-Indo-1 and [P] is the concentration of un-

bound BSA. This is done studying the evolution of the fluorescent spectra of L in presence of P.

Knowing K_d the relation can be used to calculate the concentration of P. However, (i) all new fluorescent bands caused by the binding of the probe with other compounds present in the solution must be known. The presence of fluorescent compounds other than L and LP forbid the use of the usual two-wavelength method for the calculation of the concentration of P [25]. (ii) The binding of molecules other than L to P at the same interaction site has to be considered and change the concentration of LP.

2.2. Chemicals

Bovine Serum Albumin (BSA) was purchased from Sigma Chemical Co, Mag-Indo-1 was purchased from Molecular Probe, Inc., water was de-ionized on an ion-exchanged column.

2.3. Fluorescence measurement

Emission fluorescence spectra were digitally recorded with a jobin-Yvon JY3D spectrofluorometer interfaced with a Tandon AT 286 microcomputer for data recording and monitoring the scanning monochromators. Band-pass of 4 and 10 nm were used for excitation and emission. Glass spectrocell of optical width 2 mm were used to avoid probe reabsorption. The solution was buffered with 1% HEPES at pH 7.2. Protonated and non-protonated Mag-Indo-1 ($pK_a = 5.3$) show two different fluorescent forms [21]. At 7.2, only the non-protonated form exists. Residual calcium was found in the water used for the experiments. Even with great care, calcium ions copurify with the water and come from the glassware surface. The glassware was washed with a concentrated solution of acid and rinsed with de-ionized water. Usually we found calcium concentration below 0.5 μM. The concentration of probe L was chosen constant and we varied the concentration $[P]_{total}$ of BSA. Two solutions were prepared: solution A containing Mag-Indo-1 (2 μM), buffer (1%) and residual calcium, solution B identical to solution A but containing different concentrations of BSA. Definite concentrations of protein were made by mixing the two solutions A and B. The fluorescent spectra were recorded for 12 BSA concentrations between 2×10^{-7} and 2×10^{-5} M (Fig. 1).

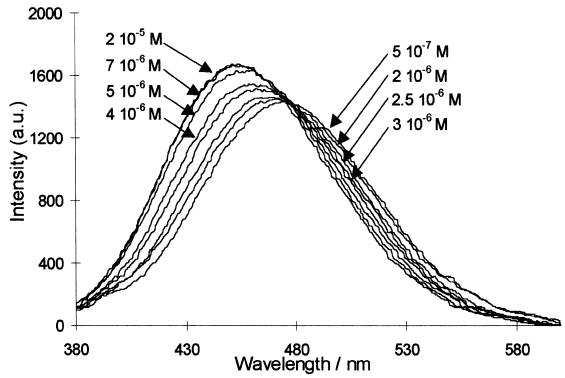


Fig. 1. Experimental spectra obtained for different concentrations of BSA added to a solution of 2 μM of Mag-Indo-1 in water. The x-axis represents the emission wavelength in nanometer. The y-axis represents the fluorescence intensity in arbitrary unit. Seven independent spectra were recorded separately and overlaid. The arrows pointing to the respective curves indicate the corresponding BSA concentration.

2.4. Data analysis

The complex fluorescent spectra were analyzed with a method described previously [23]. This method uses the spectra of all fluorescent components: free L, L link to BSA and L link to calcium obtained beforehand. The use of computer software allows quantifying all the fluorescent components of the fluorescent spectrum (Fig. 2). The reconstructed theoretical spectrum is compared to the experimental one through graphic (weighted residue curve) and numeric (chi-square) estimators. The data obtained as experimental intensities are represented by means of molar fraction X in Eq. (4).

Eq. (2) gives the molar fraction X of the free probe L:

$$X_L = \frac{[L]}{[L]_{total}} \ ,$$

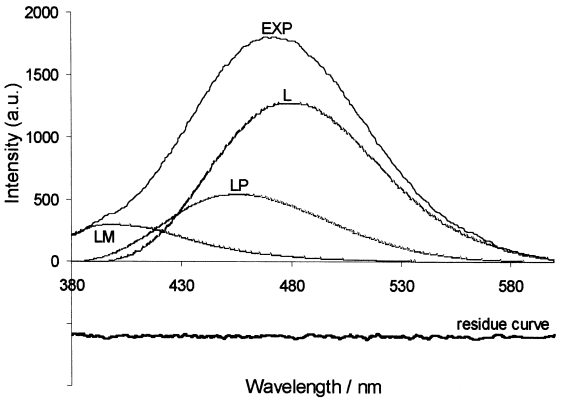


Fig. 2. Computational resolution of an experimental fluorescent spectra into the three components. On top of the *x*-axis, the emission spectra are displayed. Below the *x*-axis the difference between the experimental and calculated curves is shown. The *y*-axis is labeled as in Fig. 1. The experimental spectra is indicated by EXP. The computational resolution results in different intensity for the characteristic spectra LM (Mag-Indo-1 bound to the probe); LP (Mag-Indo-1 bound to the BSA); L (free probe).

$$\text{with } [\mathrm{L}]_{\text{total}} = [\mathrm{L}] + [\mathrm{LP}] + [\mathrm{LM}] \tag{2}$$

(with, [L] the free probe concentration, [LP] the bound to BSA probe concentration, and [LM] the bound to calcium probe concentration).

From the experimental data we obtain the value of [L]/[LP] and [L]/[LM] with Eq. (3):

$$\frac{[\mathrm{L}]}{[\mathrm{LP}]} = \left(\frac{I_L}{I_{LP}}\right) \times \frac{1}{R_{L/LP}} \tag{3}$$

(I_L and I_{LP} are the intensity of the L and LP signal obtained after analysis, $R_{L/LP}$ is the quantum yield ratio obtained for the starting spectra and $R_{L/LP} = \varepsilon_L \Phi_L / \varepsilon_{LP} \phi_{LP} = 0.87$).

Eq. (4) gives the molar fraction X as a function of the experimental data:

$$X_L = \frac{1}{1 + \left(R_{L/LP} \times \dfrac{I_{LP}}{I_L}\right) + \left(R_{L/LM} \times \dfrac{I_{LM}}{I_L}\right)} \tag{4}$$

We calculated X_{LP} and X_{LM} in a similar way.

After analysis of the fluorescence spectra we obtained an experimental curve of molar fraction as a function of the total protein concentration (Figs. 3–6).

2.5. Theoretical model

A theoretical model is constructed to simulate the experimental environment. A system of equilibrium equations defines the binding model, and hypotheses are made about individual species involved in these interactions. We developed two ways to test the assumed theoretical model:

2.5.1. Direct mathematical resolution

The direct resolution of the system of equations and unknowns [Eqs. (1) and (2)] is only possible with a simple model. We give an example of the resolution of the system of L, P and M (L = Mag-Indo-1, P = BSA and M = residual calcium in water solution) when the probe L binds to P and M. Two equations for LM and LP [Eq. (1)] have to be solved. We make the assumption that there are n equivalent interaction sites in BSA (same $K_{\mathrm{d\,LP}}$). The second assumption is that the calcium does not interact with BSA. It is fulfilled when the concentration of calcium is low. After mathematical resolution we find Eq. (5):

$$[\mathrm{P}]_{\text{total}} = [[\mathrm{L}]_{\text{total}} \times X_L + Kd_{LP}] \times \left[\frac{1}{X_L} - 1 - \frac{[\mathrm{M}]_{\text{total}}}{[\mathrm{L}]_{\text{total}} \times X_L + Kd_{LM}}\right] \times \frac{1}{n} \tag{5}$$

The molar fraction X_L is connected, in Eq. (5), to the BSA concentration $[\mathrm{P}]_{\text{total}}$ with concentration $[\mathrm{M}]_{\text{total}}$, apparent number of interaction sites n and equilibrium constants $K_{\mathrm{d}LM}$, $K_{\mathrm{d}LP}$ as variables. To compare the experimental and the theoretical curves, the variables $K_{\mathrm{d}LP}$,n and $[\mathrm{M}]_{\text{total}}$ were computed with the solver of MS Excel [26].

2.5.2. EQUIL program

A second method was constructed to test complicated models that cannot be solved by direct mathematical resolution. We will call the pro-

gram EQUIL. We assume that all reactions can be separated in elementary reactions with stoechiometric coefficient of 1. The elementary reaction [as in Eq. (1)] can be described on the kinetic point of view by a differential equation:

$$\frac{\mathrm{d}LP}{\mathrm{d}t} = k^{-}[\mathrm{L}] \times [\mathrm{P}] - k^{+}[\mathrm{LP}] = \frac{\mathrm{d}\xi}{\mathrm{d}t} \qquad (6)$$

We obtained as many differential equations as reactions. The system of differential equations is then resolved with respect to time with the known numerical method RKCK (Runge Kutta embedded by CASH and KARP [27]). The progress of the reaction is measured by comparison of the dissociation constant obtained from the concentration given by the RKCK resolution and the theoretical dissociation constant. The estimation of the equilibrium state is done by $(K_{\mathrm{dtheo}} - K_{\mathrm{d\,exp}})/(K_{\mathrm{dtheo}} + K_{\mathrm{dexp}})$. Theoretical and experimental data are compared and the input values for EQUIL are adapted until the calculated curve fits with the experimental. This method allows studying more complicated models. It was, however, generally necessary to use the simplest model to find a rough estimation of the dissociation constant. With EQUIL, the three experimental curves of X_L, X_{LP} and X_{LM} are available for the fit and give more complete results. In case a direct mathematical resolution was possible, it was used before curve fitting with EQUIL. All models presented here are tested with EQUIL.

3. Results

3.1. Native protein and Mag-Indo-1

Experimental measurements were done with native BSA. Addition of BSA results in a decrease of the fluorescence signal of *LM*, the metal chelate, and of *L*, the free probe. This decrease is associated with an increase of the fluorescence signal of the BSA/Mag-Indo-1 complex. These results are represented in Fig. 1. The chosen model is a three components system: Mag-Indo-1 binds calcium as well as proteins with *n* possible binding sites in the protein. Resolution of the model by the simplest method demonstrates a 1/1 binding between the fluorescent probe and the protein. The dissociation constant is $K_{\mathrm{d}LP} = 0.25$ μM. $K_{\mathrm{d}LM}$ was determined elsewhere to be 0.7 μM [28]. The standard deviation of the molar fraction X_{LP} increases with high protein concentrations (upper part of the curve, Fig. 3). In this case, the intensities of *L* and *LM* are very small. This induces a loss in the accuracy of the resolution. We calculated the dissociation constant (0.25 ± 0.1 μM) and the calcium concentration (approx. 10^{-7} M). The use of EQUIL permits the testing of the model on three curves. To obtain the correct X_{LM} curve, the quantity of calcium was increased to $2.5\ 10^{-7}$ M. As a result, the dissociation constant is $K_{\mathrm{d}LP} = 0.18$ μM.

3.2. Apo-protein and Mag-Indo-1

To decrease the concentration of residual calcium we used a drastic method consisting of a dialysis against EDTA solution. The EDTA solution (5 mM) was changed two times every 24 h. Subsequently, pure water was used (two times every 12 h) to remove residual EDTA [29]. The final protein concentration was calculated from the absorption at 277 nm (BSA: $\varepsilon_{277} = 38\,400$,

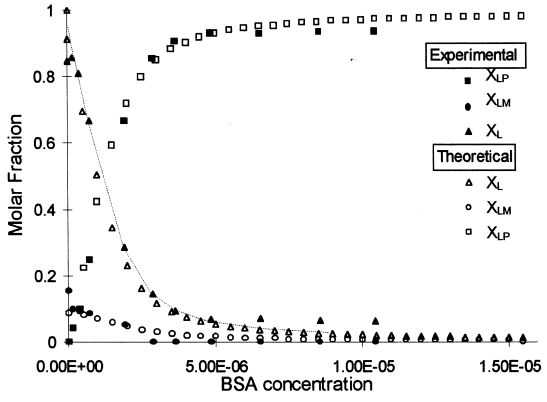


Fig. 3. Amount of Mag-Indo-1 bound to BSA in the presence of residual calcium measured by fluorescence. The *x*-axis represents the concentration of BSA in the solution in M. The *y*-axis represents the three different molar fractions. Three different data sets are shown: experimental data indicated by full symbol, theoretical data obtained with EQUIL program indicated by hollow symbol and direct mathematical resolution for X_L as dotted line.

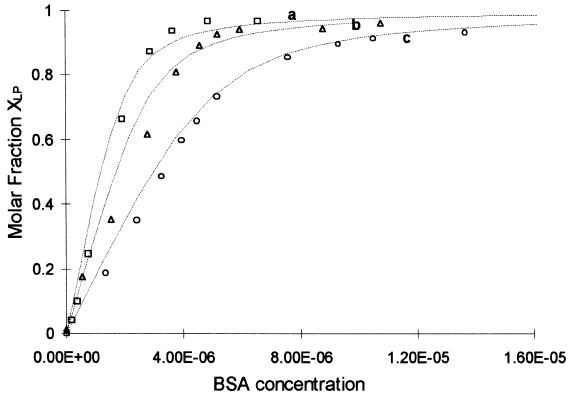
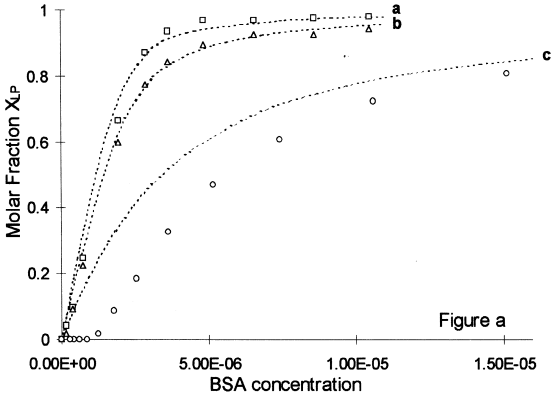


Fig. 4. Influence on the dialyses on the binding of the probe to the protein. The *x*-axis represents the concentration of BSA in the solution in M. The *y*-axis represents the molar fraction ($X_{LP} = [LP]/[L]_{total}$). Three pairs of theoretical and experimental curves are shown: hollow symbols indicated the experimental curves, dotted line the results of the EQUIL calculation. X_{LP} obtained with the commercial probe (hollow square, line a), X_{LP} obtained after the BSA dialyzed 1 day (hollow triangle, line b), X_{LP} obtain after the BSA dialyzed 2 days (hollow circle, line c). Theoretical points obtained with EQUIL program based on the hypothesis of BSA denaturation.

$M = 60\,000$) [30]. The experimental curves were obtained as outlined in Section 2. The simulation with a model of three components (L, P, M) allows to give an apparent number of interaction sites and a dissociation constant. The dissociation constant was found in each case equal to the dissociation constant of the native protein, 0.18 μM. But the apparent number of interaction sites n decreases depending on the dialyze time. We obtained $n = 1$ for the native BSA and $n = 0.4$ for the BSA dialyzed for 2 days (results shown in Fig. 4). Investigation of the influence of dialysis on the interaction of BSA to Mag-Indo-1 showed that the apparent number of interaction sites n decreases with the dialysis time. $n = 0.7$ after 1 day, 0.4 after 2 days and 0.18 after 3 days (Fig. 4).

3.3. Systematic study: BSA, Mag-Indo-1 and EDTA

EDTA a commonly used chelator have been suggested to bind protein [31]. EDTA is not fluorescent and EDTA/protein interaction cannot be measure directly by fluorescent methods. However, since EDTA interferes with the binding of Mag-Indo-1 to protein our method can be used to monitor its influence indirectly. Fluorimetric determinations with different concentrations of



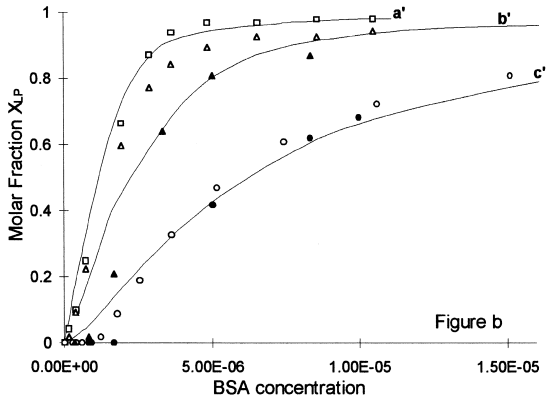


Fig. 5. Influence of EDTA on the binding of Mag-indo-1 to BSA. Axes are labeled as in Fig. 4. Fig. 5a: Spectrum recorded immediately after the mixture of the components. Three pairs of theoretical and experimental curves are shown: hollow symbols indicate the experimental curves, dotted line the results of EQUIL for the model of four equilibria (*LP*, *LM*, *EM*, *EP*). No EDTA [hollow square, line (a)], EDTA 5×10^{-4} M [hollow triangle, line (b)], EDTA 5×10^{-3} M [hollow circle, line (c)]. Fig. 5b: Spectrum recorded after incubation of 1 h. For comparison three experimental data sets of Figure a are included in Figure b (hollow square for no EDTA, hollow triangle for 5×10^{-4} M EDTA, hollow circle for 5×10^{-3} M EDTA). The full triangle (EDTA 5×10^{-4} M) and full circle (EDTA 5×10^{-3} M) represent data after the incubation. Dotted line represents the result of EQUIL program after introduction of the equilibrium Apo + Ca ⇔ *P*.

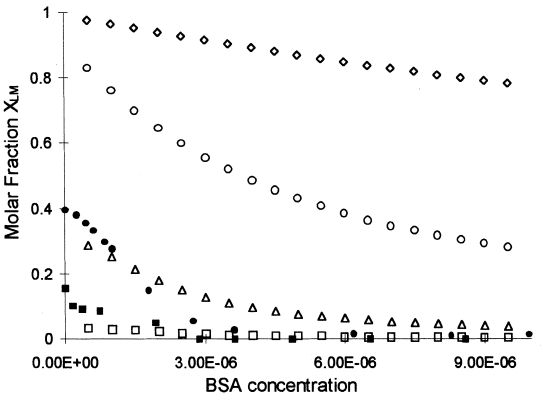


Fig. 6. EQUIL calculation predicting the influence of calcium on the equilibrium $L + P \Leftrightarrow LP$ under the assumption no interaction between BSA and calcium exist. The *x*-axes represent the concentration in BSA in the solution in M/l. The *y*-axes represent the molar fraction ($X_{LM} = [LM]/[L]_{total}$). Six independent curves are shown. Hollow symbols indicate theoretical EQUIL data. The molar fraction X_{LM} increases when calcium is added: square ($Ca^{2+} = 10^{-7}$ M), triangle ($Ca^{2+} = 10^{-6}$ M), circle ($Ca^{2+} = 10^{-5}$ M), diamond shape ($Ca^{2+} = 10^{-4}$ M). Full symbols show experimental results for calcium concentration of 2.5×10^{-7} (square) and 2×10^{-5} M (circle).

EDTA (0 M, 5×10^{-4} M and 5×10^{-3} M) were done. Increase of the concentration of EDTA results in a decrease of protein molar fraction, X_{LP} (Fig. 5). This decrease shows that EDTA changes the equilibrium and the previous model cannot be employed anymore. Accordingly, we propose an interaction between EDTA and BSA. We first developed a model consisting of two equilibria:

$$E + P \Leftrightarrow EP \quad K_{dEP} \tag{7}$$

$$L + P \Leftrightarrow LP \quad K_{dLP} \tag{8}$$

where L is the probe, P the BSA and E the EDTA. In this model we supposed that calcium ions do not interfere with any of the compounds. This assumption is correct since the resolution of the fluorescent spectra gave 0 for the concentration of LM. A mathematically resolution of the model was possible because it is a system of two equations [Eqs. (7) and (8)]. The total concentration in protein is a function of the molar fraction X_{LP}:

$$[P]_{total} = P + \frac{P \times [L]_{total}}{K_{dLP} + P} + \frac{P \times [E]_{total}}{K_{dEP} + P} \ ,$$

$$\text{with } P = \frac{K_{dLP}}{1/X_{LP} - 1} \tag{9}$$

We tested the complete model (calcium, EDTA, BSA, Mag-Indo-1) with EQUIL. The model involved four equilibria since calcium adds two new equilibria (LM and EM). In that case we used $K_{d\ EDTA\text{-}Ca} = 0.01$ μM [32]. We obtained exactly the same result as for the model with two equilibria. The protein/probe interaction is described by a 1/1 binding and the dissociation constant K_{LP} is equal to 0.18μM found without EDTA. We determined the dissociation constant of BSA and EDTA to be $K_{d\ EDTA\text{-}P} = 0.4$ mM (Fig. 5a). Finally, two series of experiments (with EDTA = 5×10^{-4} and 5×10^{-3} M) were carried out at 25°C: 30 min incubation, 1 and 2 h prior to registration of the spectra (Fig. 5b).

3.4. EQUIL model limitation

Hirshfield et al. [8] had focused on the study of the binding of a 'Quin2' probe to HSA by spectroscopic methods. They suggested two binding sites between the probe and the protein with dissociation constants similar to ours. They noted the decrease of the number of Quin2 bound to HSA when calcium was added. We decided to use the EQUIL program to test the model of three components (L, LM, LP) with increasing concentrations of calcium. The theoretical results are represented in Fig. 6. Increasing concentrations of calcium result in a decrease of the curve of molar fraction X_{LP}. This decrease explained by the competitive binding of the probe with both calcium and protein is in accordance with Hirsfields' results. We obtained experimental data (for $Ca = 5 \times 10^{-6}$ M) to compare them with the theoretical data. The quantity of calcium that binds the probe is lower than expected. This can be the expression of a strong interaction of calcium with the protein (low dissociation constant). A better knowledge of the calcium and BSA interaction is needed to make an assumption on the model and

to reduce the number of unknowns to enter in EQUIL.

4. Discussion

Protein–probe interactions have been shown to occur with calcium chelating dyes. The binding results in emission spectra changes. In the case of Mag-Indo-1, it was shown that the carboxyl group interact with the histidine residues of the protein [22]. Bovine serum albumin (BSA) has 16 histidine residues. Our experiments with native protein and Mag-Indo-1 allowed us to determine that the commercially available BSA shows only one histidine residue strongly linked to Mag-Indo-1. It is possible that other histidine residues bind to the probe with a higher dissociation constant (moderate interaction) but we could not detect them. The idea that other interaction sites exist is in agreement with the value of dissociation constants found in proteins other than BSA [16,22].The Mag-Indo-1 binds the BSA probably at the A site [16]. Other binding sites with higher dissociation constants are expected on the outer surface of the protein. The presence of a high affinity site does not allow quantification of the low association constants of the other sites.

Using Mag-Indo-1 and the Apo-protein instead of native BSA we found apparent numbers of interaction sites < 1. Binding sites are either present ($n = 1$) or not present ($n = 0$). Thus, we interpret the value $n = 0.4$ as 60% of the protein molecules do not bind anymore the probe. Dialysis against water (without EDTA) for the same time has no effect. The number of sites is one in that case. This proves that the decrease in the number of interaction sites results from the loss of calcium. EDTA in solution cannot be the cause for the reduction of bound protein since (i) it was removed by dialysis; and (ii) it showed a completely different effect when the experiments were done in the presence of EDTA as described in Section 3.3. We propose the hypothesis that irreversible changes in the protein structure take place during or after the loss of calcium. In its new structure the BSA is not able to bind the probe. To confirm this assumption, we increased the dialysis time. Longer dialyzes times decreased the apparent number of interaction sites representing 70, 40 and 18% of the remaining native protein. However, it was impossible to recover the initial protein ($n = 1$) either by simple addition of calcium or by incubation with calcium (37 then 56°C, up to 2 days). When longer dialyzes were applied the number of sites continued to decrease. This behavior cannot be explained by an equilibrium. The fact that we were not able to show the reversibility of the phenomenon (folding) suggests a denatured state (U). No change in the absorption spectra has been observed indicating constant protein concentration. The Apo-protein obtained by dialysis does not bind anymore to the probe in the A site, this is due to changes in its structure. The binding of Mag-Indo-1 with the histidine residue (A site) is certainly stabilized by the surrounding amino acids since we found a dissociation constant in micromolar range. When the environment changes, the probe/histidine binding is less stabilized and the dissociation constant is higher. The difference in probe binding between the native and denaturated BSA allows monitoring the denaturation of BSA at physiological pH with fluorescent measurements using the Mag-Indo-1 probe. Therefore, Mag-Indo-1 can be a useful tool to monitor the denaturation kinetics [24] of globular protein [33] by fluorescence spectroscopy.

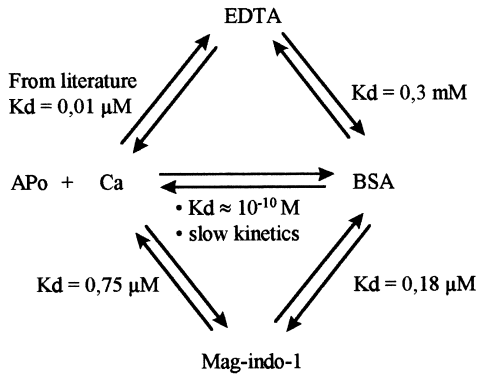
The systematic study with BSA, Mag-Indo-1 and EDTA (Section 3.3) was carried out for several reasons: (i) initially, the experiments were done in the presence of EDTA to eliminate the interaction of calcium and BSA. This is commonly recommended in the literature but the results were unsatisfactory; (ii) the hypothesis proposed in Section 3.2, that a new protein structure is generated in the presence of EDTA could be tested — and finally ruled out; and (iii) this was an opportunity to test our model on a more complex system.

Our experimental results showed that BSA/Mag-Indo-1 and BSA/EDTA interactions occurred as a 1/1 binding in the same A site. The dissociation constant $K_{LP} = 0.18$ μM with and without EDTA. For the first time we determined the dissociation constant of BSA and EDTA, K_d

$_{EDTA\text{-}P}$ = 0.4 mM. The fluorescent study shows the binding of EDTA to BSA by the displacement of Mag-Indo-1 from its binding site. This gives direct evidence for the binding of EDTA to proteins and confirms the interaction mentioned by other authors [31]. The BSA binding sites for Mag-Indo-1 and EDTA are identical under the conditions used in the experiments. The relatively low binding affinity of EDTA requires high concentrations to displace other ligands. However, millimolar concentrations of EDTA are commonly applied in biochemical experiments. Our findings indicate that under these conditions the fluorescent probe is displaced from the protein. We emphasize the fact that EDTA has to be used with care because the presence of EDTA disrupts the Mag-Indo-1/BSA interactions. High concentration of EDTA result in the formation of a probe/EDTA complex. Our method (data analysis and program to test model) allows the detection of the interaction of the probe with the protein even in a complex system of other interacting components like EDTA. However, the four equilibrium theoretical model does not fit with the experimental results below 1×10^{-5} M of BSA and EDTA concentration of 5 mM. Accordingly, we adapted the theoretical model. While in previous experiment no degradation of protein occurred the higher quantity of EDTA (5×10^{-3} M) might lead to a degradation. Two new hypotheses were made and a new model was developed. In the first hypothesis we considered a new equilibrium between the native BSA and an intermediate BSA, I, which has a new structure (no more interaction with Mag-Indo-1):

$$P \Leftrightarrow I + Ca \quad K_{dPI} \tag{10}$$

The model is resolved with EQUIL. The curve of the molar fraction as a function of the total protein concentration with EDTA = 5 mM fits better with this new model. We obtained a dissociation constant $K_{dPI} = 10^{-10}$ M (Fig. 5b). However, under the same assumption that gave a correct curve for 5 mM, the experimental curve for EDTA = 0.5 mM is different from the theoretical curve (Fig. 5). This led to the second hypothesis that this equilibrium [Eq. (10)] is not yet reached when the fluorescent spectrum is recorded. To confirm this hypothesis, time depending experiments were done. The mixture was incubated up to 2 h prior to registration of the spectra. As expected, the experimental data gradually approached the simulated curve (5×10^{-4} M EDTA). No changes were noted for EDTA = 5 mM. This allowed to give an estimation of the dissociation constant: $K_{dPI} \sim 10^{-10}$ M for the reversible $P \Leftrightarrow I$. In contrast to the reversible $P \Leftrightarrow I$, irreversible denaturation appears during dialyzes, Section 3.2. This second reaction is label $I \rightarrow U$. A three state model $N \Leftrightarrow I \Leftrightarrow U$ (N, native protein; I, intermediate; U, denatured state) has been proposed earlier [24,34–37]. Here we present an estimation of the dissociation constant for the reversible step of BSA denaturation, $K_{dPI} \sim 10^{-10}$ M.



Scheme 1. Observed interaction between Mag-Indo-1, BSA and calcium.

Energy transfer from the intrinsic chromophore of BSA (tryptophan) to the probe was shown previously in our laboratory [16]. The occurrence of energy transfer can be different in each protein. Tryptophan and the interaction site (i.e. the probe) have to be in close physical proximity. However, qualitative data cannot be obtained without prior information about the dissociation constant and the interfering ligands in solution. This paper describes a method to get this information and to use the Mag-Indo-1 probe for the investigation of protein structure in solution. In addition, the results presented here allow the simplification of the possible interaction

between BSA, Mag-Indo-1, EDTA and calcium as shown in Scheme 1.

References

[1] H.X. Min, D.C. Carter, Atomic structure and chemistry of human serum albumin, Nature 358 (1992) 209–215.

[2] G.W. Peng, W.L. Chiou, Study on the binding of trichloromonofluoromethane by bovine serum albumin using a fluorescent probe technique, Pharmacology 14 (1976) 58–66.

[3] B. Nerli, G. Pico, Influence of the medium conditions on the 1-anilino-8-naphthalene sulfonate-bovine serum albumin binding, Arch. Int. Physiol. Biochim. Biophys. 102 (1994) 5–8.

[4] N.A. Avdulov, S.V. Chochina, V.A. Daragan, F. Schroeder, K.H. Mayo, W.G. Wood, Direct binding of ethanol to bovine serum albumin: a fluorescent and ^{13}C NMR multiplet relaxation study, Biochemistry 35 (1996) 340–347.

[5] A.E. Thumser, J.E. Voysey, D.C. Wilton, The binding of lysophospholipids to rat liver fatty acid-binding protein and albumin, Biochem. J. 301 (1994) 801–806.

[6] K. Takeda, I. Yoshida, K. Yamamoto, Changes of fluorescence lifetime and rotational correlation time of bovine serum albumin labeled with 1-dimethylaminonaphthalene-5-sulfonyl chloride in guanidine and thermal denaturations, J. Protein Chem. 10 (1991) 17–23.

[7] D.C. Wilton, The fatty acid analogue 11-(dansylamino)undecanoic acid is a fluorescent probe for the bilirubin-binding sites of albumin and not for the high-affinity fatty acid-binding sites, Biochem. J. 270 (1990) 163–166.

[8] K.M. Hirshfield, D. Toptygin, G. Grandhige, H. Kim, B.Z. Packard, L. Brand, Steady-state and time-resolved fluorescence measurements for studying molecular interactions: interaction of a calcium-binding probe with proteins, Biophys. Chem. 62 (1996) 25–38.

[9] G.V. Richieri, A. Anel, A.M. Kleinfeld, Interactions of long-chain fatty acids and albumin: determination of free fatty acid levels using the fluorescent probe ADIFAB, Biochemistry 32 (1993) 7574–7580.

[10] Y.J. Jin, W.L. Li, Q.R. Wang, Tb(III) as a fluorescent probe for the structure of bovine serum albumin, Biochem. Biophys. Res. Commun. 177 (1991) 474–479.

[11] J. Yuan, K. Matsumoto, Synthesis of a new tetradentate beta-diketonate-europium chelate and its application for time-resolved fluorimetry of albumin, J. Pharm. Biomed. Anal. 15 (1997) 1397–1403.

[12] Y. Moriyama, D. Ohta, K. Hachiya, Y. Mitsui, K. Takeda, Fluorescence behavior of tryptophan residues of bovine and human serum albumins in ionic surfactant solutions: a comparative study of the two and one tryptophan(s) of bovine and human albumins, J. Protein Chem. 15 (1996) 265–272.

[13] V.S. Danilov, S.N. Orlov, V.G. Rebrov, V.A. Fedin, A.S. Shevchenko, Study of the structural lability of biomembranes and their components by the fluorescent analysis method. IV. Kinetic characteristics of the binding of unsaturated fatty acids with bovine serum albumin, Biofizika 21 (1976) 1013–1018.

[14] R. Chambert, M.F. Petit-Glatron, Reversible thermal unfolding of *Bacillus subtilis* levansucrase is modulated by Fe^{3+} and Ca^{2+}, FEBS Lett. 275 (1990) 61–70.

[15] J.S. Johansson, R.G. Eckenhoff, P.L. Dutlon, Binding of halothane to serum albumin demonstrated using tryptophan fluorescence, Aneshesiology 83 (1995) 316–324.

[16] P.M. Viallet, T. Vo-Dinh, T. Bunde, A.-C. Ribou, J. Vigo, J.-M. Salmon, Fluorescent molecular reporter for the 3-D conformation of protein sub-domains: the Mag-Indo-1, J. Lumin. 9 (1999) 153–161.

[17] M. Konishi, A. Olson, S. Hollingworth, S.M. Baylor, Myoplasmic binding of fura-2 investigated by steady-state fluorescence and absorbance measurements, Biophys. J. 54 (1988) 1089–1104.

[18] C.S. Owen, R.L. Shuler, Spectra evidence for non-calcium interactions of intracellular indo-1, Biochem. Biophys. Res. Commun. 163 (1989) 328–333.

[19] N. Kurebayashi, A.B. Harkins, S.M. Baylor, Use of fura red as an intracellular calcium indicator in frog skeletal muscle fibers, Biophys. J. 64 (1993) 1934–1960.

[20] H. Ikenouchi, G.A. Peeters, W.H. Barry, Evidence that binding of Indo-1 to cardiac myocyte protein does not markedly change K_d for Ca^{2+}, Cell Calcium 12 (1991) 415–422.

[21] B. Morelle, J.M. Salmon, J. Vigo, P. Viallet, Proton, Mg^{2+} and proteins as competing ligands for the fluorescent probe Mag-Indo-1: A first step to the quantification of intracellular Mg^{2+} concentration, Photochem. Photobiol. 58 (1993) 795–802.

[22] J. Chahboun, J.-M. Salmon, P. Viallet, Are histidine rings the main potential sites of the interaction between proteins and the fluorescent Mg^{2+} indicator Mag-indo-1? J. Photochem. Photobiol. B 33 (1996) 125–130.

[23] J.-M. Salmon, J. Vigo, P.M. Viallet, Resolution of complex fluorescence spectra recorded on single unpigmented living cells using a computerized method, Cytometry 9 (1988) 25–32.

[24] P. Haezebrouck, K. Noyelle, H. VanDael, Equilibrium and kinetic folding of pigeon lysozyme, Biochemistry 37 (1998) 6772–6780.

[25] C.S. Owen, Comparison of spectrum-shifting intracellular pH Probes 5′ (and 6′)-carboxy-10-dimethylamino-3-hydroxyspiro[7H-benzo[*c*]xanthene-7,1′(3′H)-isobenzofuran]-3′-one and 2′,7′-biscarboxyethyl-5(and 6)-carboxyfluorescein, Anal. Biochem. 204 (1992) 65–71.

[26] Microsoft Windows 98, Microsoft Excel 97 SR-2

[27] P. Gorniac, Solving the daisyworld equations with adaptive stepsize control (Web Page), available at http://www.geog.ubc.ca/numeric/labs/lab5.html, accessed 1998, 1996

[28] P.M. Viallet, T. Vo-Dinh, T. Bunde, J. Pesco, J. Vigo, J.-M. Salmon, Intracellular compartmentalization of

Indo-1 and Mag-indo-1: Consequences on intracellular calcium and magnesium sensing and imaging for biomedical applications, SPIE 2976 (1997) 105–116.
[29] G. Benzonana, J.-P. Capony, J.-F. Pechere, The binding of calcium to muscular parvalbumins, Biochim. Biophys. Acta 278 (1972) 100–116.
[30] P. Haezebrouck, W. Noppe, H. VanDael, I. Hanssens, Hydrophobic interaction of lysozyme and alfa-lactalbumin from equine milk whey, Biochim. Biophys. Acta 1122 (1992) 305–310.
[31] P. Bryan, P. Alexander, S. Strausberg, F. Schwarz, W. Lan, G. Gilliland, D.T. Gallagher, Energetics of folding subtilisin BPN, Biochemistry 31 (1992) 4937–4945.
[32] A. Fabiato, F. Fabiato, Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells, J. Physiol. 75 (1979) 463–505.
[33] C. Giancola, C. De Sena, D. Fessas, G. Graziano, G. Barone, DSC studies on bovine serum albumin denaturation. Effects of ionic strength and SDS concentration, Int. J. Biol. Macromol. 20 (1997) 193–204.
[34] R. Jaenicke, Protein folding: Local structures, domains, subunits and assemblies, Biochemistry 30 (1991) 3147–3161.
[35] R. Rudolf, R. Siebendritt, G. Nesslauer, A.K. Sharma, R. Jaenicke, Folding of an all-beta-protein: Independant domain folding in II-crystallin from calf eye lens, Proc. Natl. Acad. Sci. USA 87 (1990) 4625–4629.
[36] A. Sali, E. Shakhonovich, M. Karpus, How does protein fold? Nature 369 (1994) 248–251.
[37] K. Kuwajima, M. Mitani, S. Sugai, Characterization of the critical state in protein folding. Effects of guanidine hydrochloride and specific Ca^{2+} binding on the folding kinetics of alpha-lactalbumin, J. Mol. Biol. 206 (1989) 547–561.