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Monitoring methanol-induced protein unfolding by fluorescence anisotropy measurements of covalently labelled rhodamine probe*,**

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Abstract. We describe the use of an extrinsic fluorophore (rhodamine B isothiocyanate) as a versatile probe to measure rotational motions of proteins. To illustrate the usefulness of this probe, we describe the fluorescence anisotropy values of this fluorophore covalently linked to myoglobin protein measured in aqueous solutions of increased methanol content. Methanol-induced unfolding is revealed by the transition from constrained to free rotation of the covalently attached rhodamine B fluorophore.

1 Introduction

Protein conformational dynamics regulate protein function. Therefore, tools able to probe internal dynamics of proteins, in the picosecond to microsecond timescale are highly desirable. Fluorescence spectroscopy has become an established tool to investigate the dynamics and interactions of biomolecules [1]. In particular, fluorescence spectroscopy is an important tool for the characterization of protein folding [2]. As a possible route, a protein is labeled with appropriate fluorescent donor and acceptor probes and folding-induced changes in Förster Resonance Energy Transfer (FRET) are monitored [3]. However, the incorporation of two probes into a single protein for FRET studies can be difficult.

Fluorescence anisotropy is an alternative route for the characterization of protein folding [4]. Measurements are based on the principle of the excitation of fluorophores by polarized light resulting in polarized emission. The polarized emission is influenced by a number of processes, including motions that occur within the lifetime of the excited fluorophore. Both intrinsic [5] and extrinsic [6] fluorophores can be used for fluorescence anisotropy measurements. Due to the comparable timescale of rotational diffusion of biopolymers and the fluorescence lifetime of many fluorophores, fluorescence anisotropy has been reported for numerous biological applications. Information on the shape and size of proteins can be obtained and then have been used to measure protein–protein interactions [7], fluidity of membranes [8], binding and conformational dynamics [9].

In this paper, we aim at probing methanol induced unfolding of a protein by fluorescence anisotropy measurements using an extrinsic fluorophore. A simple and reliable synthesis for labeling myoglobin protein with visible rhodamine B chromophore is reported. Methanol-induced unfolding is revealed by the transition from constrained to free rotation of a covalently attached rhodamine B fluorophore.

2 Materials and methods

2.1 Labeling protocol

Myoglobin from equine skeletal muscle (Sigma-Aldrich) was labeled with rhodamine B isothiocyanate (Sigma-Aldrich) according to a protocol slightly modified from Hungerford et al. [10] as follow: reagents were dissolved so that their concentration ratio is 1. Typically, 34 mg of myoglobin (Mb) and 1.1 mg of rhodamine B isothiocyanate (RhB) were dissolved in 10 mL of 0.1 M sodium bicarbonate buffer (pH 9) while stirring continuously for 2 h

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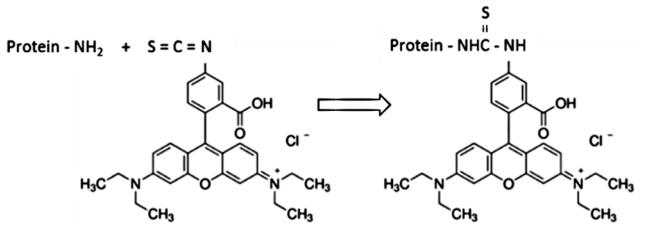
at room temperature. The reaction associated to the labeling is given by [11]:



In its native form, Mb is a globular protein having several available amine reactive groups [12]. The sequence chain of Mb and its 3D view are shown in Figure 1. Keeping the dye to protein ratio (D/P) around 1 is important to avoid any overlabeling of the protein; at most, only one dye is attached to the protein. Indeed, if the D/P ratio is greater than 1, it can lead to strong interactions between probe dye molecules grafted onto the same protein [10].

In order to remove any uncoupled RhB the solution was then dialyzed using washed tubular cellulose membranes with a nominal molecular weight cut-off at 6000–8000 Da (ZelluTrans/Roth) against 2 L of 10 mM Phosphate Buffered Saline (PBS) at pH 7 for one day. Several dialysis were performed until the absorption of RhB at 555 nm of the final supernatant was below the detection limit. Mixture of labeled protein and unlabeled protein was then recovered as is in PBS buffer as stock solution. Stock solution concentration is evaluated by absorption to $c_{stock} \sim 200\ \mu$M. Fluorescence measurements are performed after a 1:10 dilution.

The final D/P ratio was determined using the molar extinction coefficients of Mb at 408 nm (188 000 M^{-1} cm^{-1}) [13] versus RhB one at 555 nm (106 000 M^{-1} cm^{-1})[14].

2.2 Fluorescence measurements

An Oxxius Slim 532-300 laser (Oxxius Inc., Santa Clara, CA, U.S.A.) was used for excitation. Fluorescence intensities were determined by the steady-state fluorescence spectra maximum recorded by an Ultracompact spectrophotometer Econic (B&WTek Inc., Newark, DE, U.S.A.) with a resolution of 1.5 nm via an optical fiber [15,16].

A polarizing beamsplitter cube and a half-wave plate were used to tune the linear polarity of the light on the excitation line. A linear broadband polarizer in the range 500–720 nm was used for the collection. The alignment of the setup has been performed using the pure diffusion of the polarized light by a solution containing latex nanoparticles in suspension. For each fluorescence anisotropy measurements, the correction factor G (defined below) was reevaluated. Vertically or horizontally excitations were tuned by rotating the half-wave plate by 45° according to the wanted configuration and vertically or horizontally emissions were collected by rotating the linear polarizer by 90° (see Figure 2).

Fluorescence lifetimes of samples were measured by a HORIBA Scientific's hybrid spectrofluorometer Fluoromax, using a pulsed nanoled excitation at 372 nm and collecting the emitted light at 580 nm with a bandwidth of 5 nm [17,18].

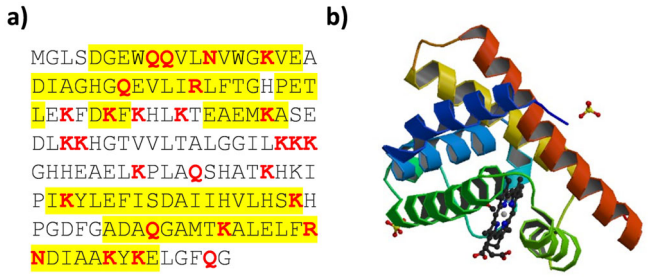


Fig. 1. (a) Sequence chain of myoglobin. Amine groups are noted in red and α-helix regions are highlighted in yellow (b) 3D view of myoglobin, reprinted from Protein Data Bank (ref: 5CMV).

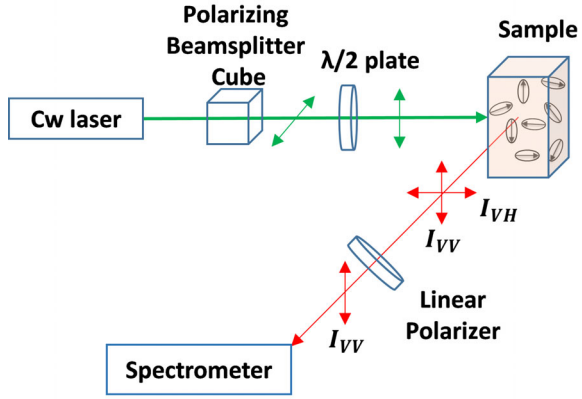


Fig. 2. Schematic view of the experimental setup to measure fluorescence anisotropy r. The excitation and detection polarization are here vertical. The $\lambda/2$ plate and the linear polarizer are mounted on rotation plates.

2.3 Anisotropy fluorescence – principle

When a fluorophore interacts with an electromagnetic wave, it can absorb a photon through the coupling of the electromagnetic field to its transition dipole moment. If the orientation of the electric filed vector of the excited light is parallel to the transition dipole moment of the fluorophore, the fluorophore can be excited efficiently. Hence, by exciting an ensemble of randomly oriented molecules with a linearly polarized light, only molecules that are aligned with the direction of the polarized excitation light are excited, so called photoselection [19].

If the fluorophore moves during the life span of its excited state (rotational Brownian motion) the emitted light polarization will be modified. To quantify this loss of polarization, we define the fluorescence anisotropy r as

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I_{VV} corresponds to the fluorescence intensity of vertically polarized excitation and vertically polarized

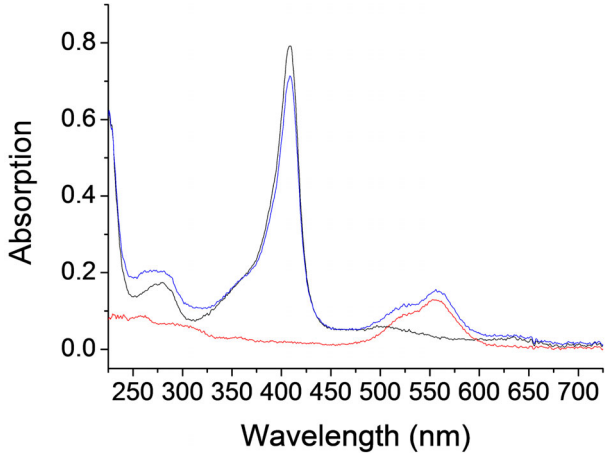


Fig. 3. Absorption spectra of (black) Mb, (red) RhB and (blue) RhB-labeled Mb in 10 mM PBS buffer.

emission, I_{VH} the vertically polarized excitation and horizontally polarized emission and G is a correction factor defined by

$$G = \frac{I_{HV}}{I_{HH}}. \tag{2}$$

Assuming that rotational diffusion is the significant process that results a change of polarization, Perrin equation gives an expression of the fluorescence anisotropy

$$r = \frac{r_0}{1 + \tau/\theta} \tag{3}$$

where r_0 is the limiting anisotropy that would be measured in the absence of rotational diffusion – it refers to the existence of an angle between the absorption and the emission transition dipole moments – τ is the lifetime of the fluorophore and θ the correlation time for the rotational process for a spherical object that can by expressed from the Stokes–Einstein equation [20]:

$$\theta = \frac{\eta V}{kT} \tag{4}$$

where η is the viscosity of the solution, k the Boltzmann constant, T the temperature and V the volume of the rotating object.

3 Results and discussion

3.1 Characterization of the sample

Absorption spectra of Mb, RhB and of the solution of the complex (RhB-labeled Mb) right after the final dialysis in 10 mM Phosphate Buffer Solution (PBS) are shown in Figure 3.

The characteristic Soret band at 408 nm denotes the presence of the heme group on the Mb. The existence of the RhB absorption pick at 555 nm for the complex solution right after the final dialysis clearly indicates that the labeling occurs efficiently. We can estimate by ratiometry of the respective absorbance at 555 and 408 nm that the D/P ratio is approximately 1/3.

Table 1. Fluorescence lifetime of RhB alone and RhB-labeled Mb in water/MeOH mixture in 10 mM PBS buffer.

Vol. % of MeOH	Fluorescence lifetime (ns)	
	Rhodamine B isothiocynate	Rhodamine B isothiocynate labeled on myoglobin
0	1.75	1.73
10	1.87	1.88
20	1.95	1.98
30	2.02	2.04

Mass spectrometry (MS) measurements of RhB-labeled Mb in water (PBS) are shown in Figure S1a. Characteristic peaks of holo-Mb in its native form with a low charge state distribution are clearly predominant (9^+, 8^+ and 7^+), along with one-dye labeled proteins also visible in their native form with the same charge state distribution with a typical ratio consistent with the D/P ratio estimated by absorbance ratiometry.

Circular dichroism (CD) measurements of RhB-labeled Mb in water (PBS) are displayed in Figure S2. Both mass spectra and CD spectra confirm that the presence of a covalently attached dye onto the protein does not affect the structure of the protein, regarding either its charge state distribution (cf. mass spectrometry) or its secondary structure (cf. CD spectra).

Fluorescence lifetimes of the different samples are reported in Table 1.

Measurements show that attached dye fluorescence lifetimes are similar to those of dye alone in the same solution. Thus the protein backbone does not seem to affect the lifetime of the rhodamine dye. This might indicate a dye labeling at the surface of the protein which does not significantly affect the excitation lifetime. Furthermore, measured fluorescence lifetime decreases when increasing the water proportion in the solution revealing that the RhB fluorescence is quenched by water.

RhB alone in a PBS displays a fluorescence anisotropy of $r = 0.046 \pm 0.005$ whereas for the RhB-labeled protein $r = 0.199 \pm 0.005$. If we assume that Mb displays a globular shape in its native form in PBS, we can estimate that the system radius of gyration is equal to $r_g = 1.6 \pm 0.1$ nm considering a limiting anisotropy $r_0 = 0.3$, and a viscosity $\eta = 8.95 \times 10^{-4}$ Pa s. This value is consistent with the values reported in references [12,21].

3.2 Parameters' influence on fluorescence anisotropy

According to expressions (3) and (4), fluorescence anisotropy is affected by the following main physical parameters:

(i) Temperature is a key parameter regarding Brownian motion. For high temperature, objects motion is faster resulting in a loss of anisotropy.

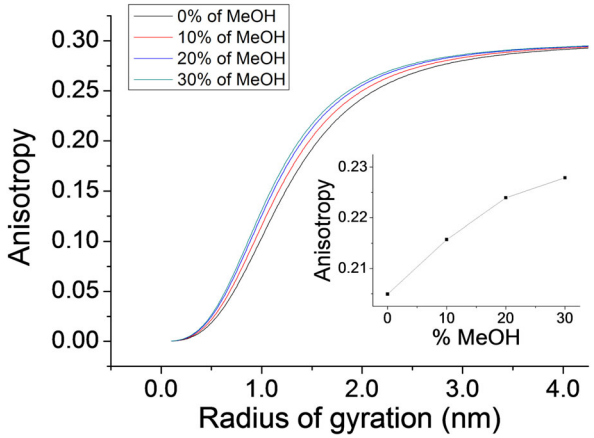


Fig. 4. Calculated fluorescence anisotropy regarding the radius of gyration of a system. Inset: Anistropy for a given radius of gyration (r_g = 1.6 nm) regarding the methanol proportion. Values of τ and η have been reevaluated as follow: for 0% of MeOH, $\tau = 1.73$ ns, $\eta = 8.95 \times 10^{-4}$ Pa s; for 10% of MeOH, $\tau = 1.88$ ns, $\eta = 11.55 \times 10^{-4}$ Pa s; for 20% of MeOH, $\tau = 1.97$ ns, $\eta = 13.92 \times 10^{-4}$ Pa s; for 30% of MeOH, $\tau = 2.03$ ns, $\eta = 15.40 \times 10^{-4}$ Pa s.

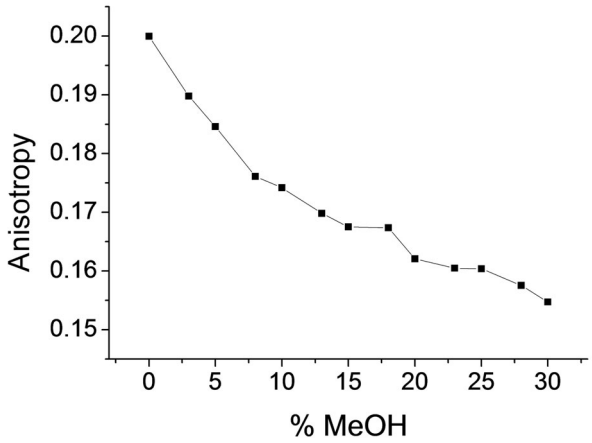


Fig. 5. Fluorescence anisotropy of RhB-labeled Mb in water/MeOH mixture.

(ii) The size of the moving objects also greatly influences the anisotropy. The bigger an object is, the slower its rotation will be, resulting in an increase in anisotropy.
(iii) High viscosity tends to slow down the rotation motion inducing an anisotropy increase.
(iv) It is also clear that the fluorescence lifetime plays a role in the depolarization process. For short lifetime, the molecule reemits light rapidly so that it does not rotate enough to induce a great depolarization.

We can calculate the predicted evolution of RhB emission anisotropy regarding the size of the rotating system (i.e. the radius of gyration) for a given temperature in a water/MeOH mixture in the range 0–30% of methanol [22]. Results are given in Figure 4 taking into account viscosity and lifetime modifications due to different solvent conditions. It is shown that for a given size, fluorescence anisotropy of a system tends to increase with the methanol proportion. Indeed, the anisotropy increase induced by the change of viscosity [22] counterbalances the anisotropy loss due to the augmentation of the fluorophore lifetime.

3.3 Probing unfolding of myoglobin by fluorescence anisotropy measurements

Alcohols are well-known solvents that highly weaken hydrophobic interaction leading to strong denaturation of proteins [23–25]. In order to probe protein dynamic events due to methanol (MeOH) addition, fluorescence anisotropy of RhB-labeled Mb has been measured as a function of methanol proportion at room temperature. Results shown in Figure 5 clearly indicate a depolarization of fluorescence emission when increasing the methanol proportion.

According to the previous discussion, a loss of anisotropy may be explained by a reduction of the size of the object. However, this is inconsistent with ion mobility spectrometry measurements performed on Mb. Indeed, for each charge state, several conformers are observed, and a global augmentation of the cross-section occurs when the solvent is changed from water to water/methanol [26]. MS measurements on Mb in different solvent conditions (Figure S1b) show that the average charge state distribution of Mb tends to increase with methanol which is consistent with a partial unfolding of the protein. Moreover, the signature of apo-Mb induced by the liberation of the non-covalent heme group is observed in MS spectra and is a signature of a partial opening of the protein. Furthermore, CD analysis reveals a slight denaturation from α helix to unordered structure when increasing the methanol proportion (see Table S1). All these considerations are favorable to a partial unfolding of the protein leading to an augmentation of its radius of gyration.

Thus, the drop in anisotropy can only be explained as an effect of the protein unfolding. The decrease is attributable to a global unfolding which allows the fluorophore to rotate independently and freely from the protein backbone. Several models have been developed in the literature to discuss processes where independent motions of the transition dipole moment (protein chain or covalently attached chromophore) are considered [27–29]. This phenomenon has already been observed on depolarization tryptophan UV emission. Indeed, the decrease was attributable to a great extent of unfolding which releases the tryptophan residue from the hydrophobic core of the protein allowing the indole ring to rotate independently of the protein [4]. However, the anisotropy value of the system stays significantly bigger than the fluorophore's one alone in solution because the fluorophore is still covalently attached to the protein (see Figure S3 for more details). The circular dichroism spectra reported for Mb have signatures that are typical for helical structure [26]. One may argue that structuration of Mb in α-helix upon addition of methanol avoids a strong interaction of the rhodamine B dye with the protein peptidic skeleton.

4 Conclusion

A simple and reliable synthesis for labeling a protein with a visible chromophore is reported. Optical characterization of the system is performed through absorption spectrometry in the visible range, mass spectrometry, circular dichroism, fluorescence lifetime and fluorescence anisotropy measurements. It is shown that at most, one rhodamine B isothiocyanate molecule is grafted to myoglobin via an amine group to avoid any possible interactions within the molecules onto the same protein and no effect of the grafting can be detected regarding the main structure of the protein. Moreover, unfolding events of the protein induced by organic solvent like methanol can be probed by fluorescence anisotropy measurements. Such events are revealed by the transition from "constrained" to "free" rotation of the attached fluorophore.

The present study suggests that it should be possible, with fluorescent techniques using extrinsic fluorophores to obtain information in kinetic studies of protein folding and conformational changes.

Supporting information

Mass spectrum of RhB-labeled Mb in water (PBS) and Mb in different solvent conditions. Circular dichroism spectra of Mb and RhB-labeled Mb in water (PBS). Average values of the secondary structure for RhB-labeled Mb in different solvent conditions. Protocol and absorption spectra of RhB-labeled Mb in water (PBS) with and without the presence of methanol.

Author contribution statement

A.S. performed the experiments. A.S. and R.A. interpreted the results and wrote the draft manuscript. F.B., M.G., P.D., R.A. supervised the experiment, data interpretation and preparation and editing of the manuscript.

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