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Fluorescence lifetime distributions report on protein destabilisation in quenching experiments

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ABSTRACT

Tryptophan is the most often investigated intrinsic fluorophore due to its abundance in proteins and its sensitivity to different environmental conditions. Fluorescence quenching is a powerful method to study proteins and acrylamide is a frequently applied quencher in these investigations. Quenching experiments are sometimes distorted by the undesired protein–quencher interactions that can result in a misinterpretation of the results. Here we focused on the identification of the possible side-effects of acrylamide applying fluorescence lifetime measurements. To provide reference data for protein denaturation the fluorescence parameters were also recorded in the presence of different concentrations of guanidine hydrochloride. In circular dichroism experiments we characterized directly the acrylamide effect on the tertiary structure of the proteins. According to the obtained data in experiments with seven tryptophan-containing proteins the full width at half maximum (*FWHM*) of the fluorescence lifetime distribution is an appropriate parameter to monitor the undesired effects of acrylamide on the proteins.

1. Introduction

The method of fluorescence quenching was proved to be a powerful tool to provide information about protein dynamics and conformational changes [1–11]. The quenching of the intrinsic fluorescence of tryptophan by the neutral quencher acrylamide is commonly applied to characterize the microenvironment of fluorophores. Although both the theoretical background and the experimental protocols are well developed to perform and analyze such experiments, much less attention has been paid to the possible role of the direct interaction between the protein matrix and the acrylamide molecule. This interaction can result in the denaturation of the protein. In other cases the quencher induced changes of the environmental parameters of the solution, such as for example viscosity, can introduce artefacts to the experiments, and thus can lead to wrong conclusions.

Acrylamide and the protein matrix can interact in many ways: it was proposed that acrylamide could perform its effect by penetrating to the protein matrix [12,13]. Equilibrium dialysis measurements also provided evidences that proteins are able to bind acrylamide [14–17]. The binding of a considerable amount of acrylamide to human serum albumin, ovalbumin and cod parvalbumin III is in good agreement with the suggestions of the penetrating model [18]. It was also demonstrated that the number of binding sites is usually different for different proteins and sensitive to the pH and the presence of co-solvents such as glycerol [15]. Accordingly, it seems to be important to identify and consider the effect of acrylamide binding to the proteins. In these cases the interpretation of the results has to include the complexity of the quenching processes, i.e. the appearance of mechanisms different from simple collisional quenching.

Proteins in solution can adapt a number of conformational sub-states [19–21]. We used fluorescence lifetime distributions to characterize the conformational heterogeneity of the investigated proteins as described previously [22,23]. The theoretical basis of the correlation of the parameters resolved in the analysis assuming continuous lifetime distribution to the properties of proteins is provided by the landscape model, which was first proposed by Frauenfelder and co-workers [24]. The full width at half maximum (*FWHM*) of the fluorescence lifetime distributions was shown to be related to the distribution of the protein sub-states and to the dynamics of inter-conversion between them [25–29]. The width

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of these distributions measures the heterogeneity of the fluorophore population, i.e. the presence of a number of sub-states, which can be characterized by different fluorescence lifetimes. However, even a single tryptophan residue possess lifetime distributions indicating that the interpretation of these fluorescence parameters should involve the fundamental characteristic of the fluorophore itself.

The effect of guanidine hydrochloride (GuHCl) is well characterized by means of various experimental methods [30,31]. The conclusion of these experiments is that GuHCl can denature even relatively stable proteins. Nearby emission fluorescence and near-UV CD spectroscopy are simple and commonly used techniques to study the conformational changes in proteins. Both methods are sensitive to the local environment of the aromatic amino acids, e.g. Trp groups [32,33]. If the protein had more Trp residues, the measured fluorescence and CD signals are a superposition of the individual Trp-signals.

In this work our aim was to identify a parameter, which is informative concerning the undesired effects of acrylamide binding to proteins. This parameter was also aimed to be convenient to use in a sense that it can always be obtained in fluorescence lifetime quenching experiments without performing extra experiments. For this reason the set of fluorescence lifetime quenching and circular dichroism experiments were carried out. The influence of a denaturing agent (guanidine hydrochloride) was also studied to provide a reference system for the interpretation of the acrylamide effect. We found that the full width at half maximum (*FWHM*) of the fluorescence lifetime distributions is an appropriate parameter to monitor the effect of acrylamide on the proteins.

2. Materials and methods

2.1. Chemicals

HSA, BSA, lysozyme (from chicken egg white), hexokinase (Type III., Baker Yeast), phosphorylase b (from rabbit muscle), glycogen (Type III. From Rabbit Liver), tris-(hidroxy-methyl)amino-methane (Tris), acrylamide, L-trypthophan were obtained from SIGMA Chem. Co. (St. Louis, MO) and RNase T1 from Thermo Scientific. Adenosine-5′-triphosphate (ATP) and ß-mercaptoethanol (MEA) were obtained from MERCK (Darmstadt, Germany), and sodium azide (NaN_3) from FLUKA (Switzerland).

2.2. Protein preparation

The proteins purchased from SIGMA Chem. Co. and Thermo Scientific were used without further purification. Prior to the fluorescence experiments the lyophilized HSA, BSA, lysozyme, RNase T1, phosphorylase b and hexokinase were dissolved into 4 mM TRIS buffer, pH 7.4. Actin was purified according to Spudich and Watt [34], and stored in 4 mM Tris–HCl, pH 8.0, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM MEA and 0.005% NaN_3 (buffer A). Glycogen as a reference protein was dissolved in distilled water just before usage to exploit its Trp-free nature in Trp fluorescent lifetime measurements. The protein concentration was 25–30 μM in the samples.

2.3. Fluorescence lifetime measurements

Fluorescence lifetimes were measured as a function of acrylamide or guanidine hydrochloride concentration at 20 °C. Fluorescence lifetime measurements were carried out on a Horiba Jobin–Yvon Nanolog spectrofluorometer operating in time correlated single photon counting (TCSPC) mode. To excite the protein sample a 295 nm peak wavelength pulsed LED (NanoLED 295, Horiba Jobin Yvon) was used that generated ~1 ns pulses. After exciting tryptophan the fluorescence emission was collected at 340 nm. The instrument response function (IRF) had a *FWHM* of ~1 ns measured by using freshly prepared glycogen solution. Measurements were completed when 10,000 counts were collected in the peak channel (Fig. 1). The temperature of the cuvette holder was controlled by Thermo Scientific AC200 – A25 bath circulatory system. We used 10 mm × 3 mm quartz cuvettes for the measurements.

2.4. Data analysis

The analysis of the raw data was performed using FluoFit (PicoQuant) analysis software, that implements nonlinear least square minimization based on the Levenberg–Marquardt algorithm. The data of fluorescence lifetime measurements were fit by continuous (Gaussian) lifetime distributions. Gaussian distribution (ρ_G) of lifetime of the tryptophans was resolved from the intensity (I) decay curves according to the following equation:

$$I(t) = \int_{-\infty}^{t} IRF(t') \int_{-\infty}^{\infty} \rho_G(\tau) e^{-\frac{t-t'}{\tau}} d\tau dt'$$

$$\rho_G(\tau) = \sum_{i=1}^{n} \frac{A_i}{\sigma_i (2\pi)^{0.5}} e^{-\frac{1}{2}\left(\frac{\tau-\tau_i}{\sigma_i}\right)^2}; \ \sigma_i = \frac{FWHM_i}{(8ln2)^{0.5}}$$

where τ_i and A_i are the centre lifetime and amplitude of the *i*th distributed component, respectively with a distribution width of $FWHM_i$. σ_i represents the standard deviation of the Gaussian of the *i*th component. The fit was obtained by assuming one lifetime component in all cases.

The goodness of fitting was determined from the value of the reduced χ^2 [35]. The full width at half maximum (*FWHM*) is 2.345σ, which value can reflect the distribution of the protein conformations (sub-states) [25–27].

2.5. Fluorescence quenching experiments

In quenching experiments, the fluorescence lifetime of tryptophans of seven different proteins was measured by titrating acrylamide (to the protein samples) by increasing the concentration from 0 M to 2 M. Time resolved fluorescence experiments are sensitive only for the dynamic quenching. To describe the dynamic quenching of fluorescence, the classical Stern–Volmer equation can be used [36]:

$$\tau_o/\tau = 1 + K_{SV}[Q] \quad (1)$$

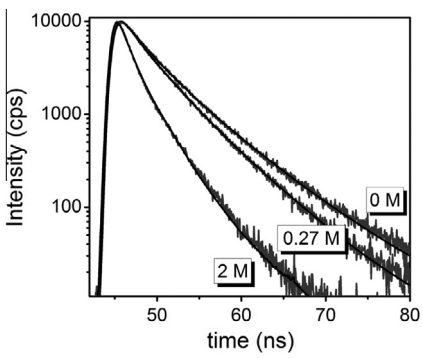

Fig. 1a. Fluorescence decay of tryptophan emission in hexokinase in the presence of 0, 0.27 and 2.0 M acrylamide at 20 °C. Gaussian fits to the experimental data are also shown and gave fluorescence lifetimes of 3.31 ns, 2.95 ns and 1.31 ns with *FWHM* values of 6.76 ns, 3.03 ns and 3.86 ns, respectively.