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Monitoring Labeling Reactions Using Fluorescence Polarization

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Abstract: Fluorescence techniques are widely applied in protein research owing to their specificity and sensitivity, but require prior fluorescent labeling. Here we show a novel approach to optimize labeling protocols by monitoring labeling reactions using fluorescence polarization: the larger molecular mass of the fluorescent protein conjugate compared to the dye alone results in an increase in fluorescence anisotropy during the reaction. Thereby, labeling of lysozyme with fluorescein isothiocyanate or carboxyfluorescein succinimidyl ester could be monitored and the influence of parameters such as the pH could be quantitatively assessed. Moreover, the impact and kinetics of side reactions such as hydrolysis were determined. This new method is rapid, easy to implement, and generically applicable.

Keywords: Fluorescence anisotropy · Fluorescence polarization · Fluorescent labeling · Lysozyme

1. Introduction

Owing to their sensitivity and specificity, fluorescence methods enjoy a broad interest in bioanalytics for the detection, quantification and analysis of proteins.^[1] Most proteins, however, are not intrinsically fluorescent and require prior labeling with fluorophores. A wide array of methods exists to covalently bind fluorescent labels to proteins^[2,3] like the reaction between fluorescein isothiocyanate (FITC) or carboxyfluorescein succinimidyl ester (NHS-fluo) and the ε-amino group of lysine residues. Despite the important impact of labeling on the performance of fluorescent studies, protein labeling is usually carried out with no particular optimization by letting the reagents react for several hours or overnight using empirical protocols.^[3,4] A few reports have studied the kinetics of labeling, but used complex techniques such as MALDI-TOF^[5,6] and capillary electrophoresis.^[7,8] There is therefore a need for a simple method that allows the generic monitoring of labeling reactions.

Fluorescence polarization (FP) is a technique that enables the detection of the increase of the molecular mass of a fluorescent molecule.^[9] FP monitors the degree of polarization of the fluorescence emitted by a fluorophore excited using polarized excitation light. This is usually quantified with an anisotropy value, r , which varies between 0 and 0.4 and critically depends on the speed of rotation of the molecule and therefore on its size. Generally, small molecules exhibit large anisotropy values whereas large ones have small anisotropy values.

Here we investigated the possibility to use FP to monitor labeling reactions, to optimize reaction parameters such as pH, and to assess the impact of side reactions such as hydrolysis. As

the fluorescent reagent is a small molecule and the fluorophore–protein conjugate is much larger, the attachment of the fluorophore to the protein resulted in an increase of the anisotropy, which could be continuously monitored. The prototypical protein lysozyme was labeled using FITC or NHS-fluo under varying conditions and the kinetics of labeling was assessed using FP. Our results demonstrate the applicability of FP as a generic method for the monitoring of labeling reactions.

2. Experimental

2.1 Reagents

Fluorescein isothiocyanate (F7250) and egg-white lysozyme (L6876) were purchased from Sigma-Aldrich, carboxyfluorescein succinimidyl ester (46410) from Thermo Scientific.

2.2 Monitoring of Labeling Reactions

Stock solutions of the fluorescent reagents were prepared in DMSO at a concentration of typically 250 μM and freshly diluted in buffered water directly prior to the experiment. Solutions of lysozyme were prepared directly in the reaction buffer. The pH was fixed at 7.4 using a PBS buffer or at 9.1 or 11.3 using a borate buffer. Measurements were initiated by mixing 100 μl of the solution of fluorescent conjugate with 100 μl of the solution of lysozyme within the well of a 96-well microplate. The reaction concentrations varied between 10 to 100 μM for the fluorescent reagent and 20 to 200 μM (0.3–3 g/l) for lysozyme. Fluorescence anisotropy was measured every 30 s using a SpectraMax Paradigm (Molecular Devices).

For the analysis of the hydrolysis reaction, 100 μl of a 179 μM solution of NHS-fluo at pH 8.3 were dispensed in 6 wells of a 96 well-microplate. Every ten minutes, 100 μl of a 372 μM solution of lysozyme at pH 8.3 were added to a new well.

3. Results and Discussion

Labeling reactions were usually performed with a ten-fold excess of lysozyme to ensure mono-labeling of the protein and a nearly constant concentration of unlabeled lysozyme. Monitoring of labeling reactions was however not restricted to these conditions and could be generally performed using any ratio of lysozyme/fluorescent reagent and under various concentrations (data not shown). Examples of labeling kinetics using either FITC or NHS-fluo are shown in Fig. 1. Assuming a pseudo-first-order kinetics, the curves were fitted using

$$r = r_{\max} - \Delta r \cdot \exp(-k \cdot t) \quad (1)$$

where r is the anisotropy, r_{\max} the maximal value of the anisotropy, Δr the change in anisotropy, t the time and k the pseudo-first-order rate constant, which under these conditions was three times larger for the reaction with NHS-fluo ($k_{\text{NHS-fluo}} = 0.33 \pm 0.02 \text{ min}^{-1}$) than for FITC ($k_{\text{FITC}} = 0.131 \pm 0.008 \text{ min}^{-1}$). Labeling with NHS-fluo and FITC was typically completed within 10 min, respectively 30 min. However, the rate constants varied with the

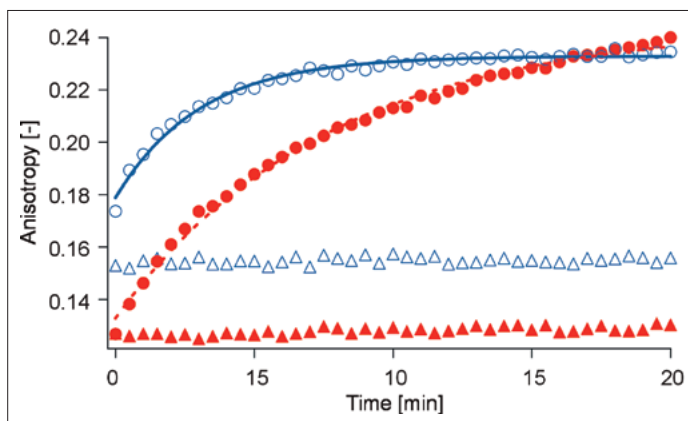


Fig. 1. Monitoring of labeling reactions: Fluorescence anisotropy was monitored after mixing lysozyme at a final concentration of $17 \mu\text{M}$ with either FITC (red filled circles) (pH 9.1) or NHS-fluo (blue empty circles) (pH 8.3) at a final concentration of $1.7 \mu\text{M}$. The time required for the initiation of the instrument resulted in a small delay of less than a minute that hindered monitoring of the reaction just after initiation. In absence of lysozyme, no change in anisotropy was observed for FITC (red filled triangles) or for NHS-fluo (blue empty triangles). The experimental curves for FITC (red dashed line) and NHS-fluo (blue solid line) were fitted using Eqn. (1) showing that the rate constant for the labeling reaction with NHS-fluo was three time larger than for FITC.

concentration and ratio of lysozyme as the underlying kinetics is second order.

The change in anisotropy was a consequence of the lower rotational mobility of fluorescein after conjugation. The final maximal anisotropy r_{max} is the weighted average of the anisotropy of the fluorescein-protein conjugate $r_{\text{conjugate}}$ and of the fluorescent reagent $r_{\text{fluorophore}}$, i.e. $r_{\text{max}} = x_{\text{conjugate}} \cdot r_{\text{conjugate}} + (1 - x_{\text{conjugate}}) \cdot r_{\text{fluorophore}}$, where $x_{\text{conjugate}}$ is the fraction of the fluorescent reagent attached to the protein. For a particular protein at a fixed concentration, r_{max} gave direct information on the reaction yield. The reaction with FITC resulted in a slightly higher yield ($r_{\text{max, FITC}} = 0.245$) than the reaction with NHS-fluo ($r_{\text{max, NHS-fluo}} = 0.233$).

Labeling of the ϵ -amino group of lysine is known to depend on the pH.^[3] Lower pH values result in protonation of the amino group and reduction of its nucleophilicity. This is apparent in Fig. 2, where the rate of the reaction at pH 7.4 was lower ($k_{\text{FITC, pH 7.4}} =$

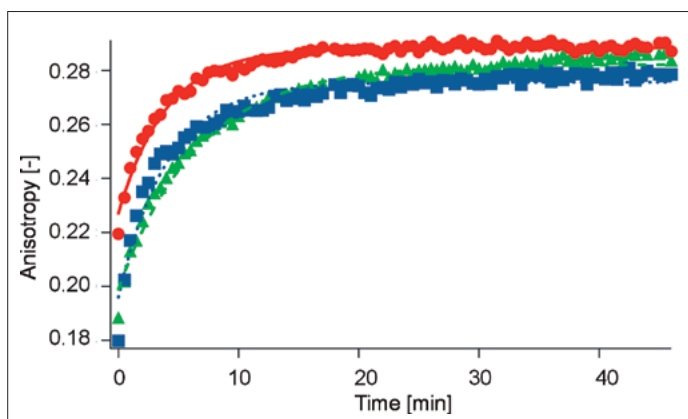


Fig. 2. Influence of the pH: Fluorescence anisotropy was monitored after mixing lysozyme at a final concentration of $112 \mu\text{M}$ with FITC at a final concentration of $14 \mu\text{M}$ at a pH of 7.4 (green triangles), 9.1 (red circles) or 11.3 (blue squares). The experimental curves were fitted using Eqn. (1) for pH 7.4 (green dashed line), 9.1 (red solid line) and 11.3 (blue dotted line) showing that lower pH resulted in slower reaction rates, while higher pH had lower yields. The optimal pH was 9.1.

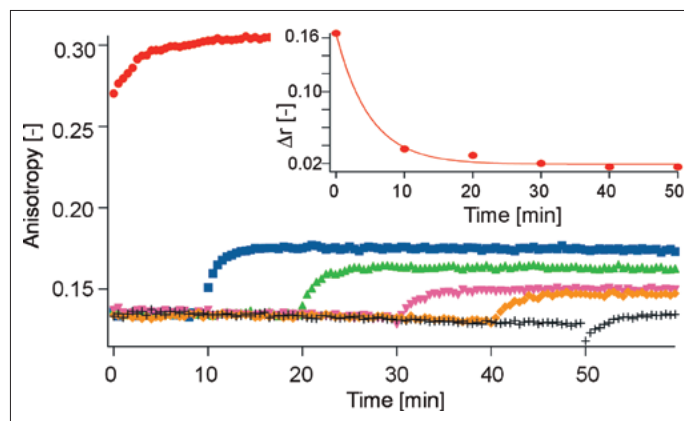


Fig. 3. Hydrolysis of NHS-fluo in presence of water: Labeling was performed after increasing incubation times of NHS-fluo in water. The resulting decrease in the maximal anisotropy r_{max} allowed the quantification of the hydrolyzed fraction of NHS-fluo. Insert: the decrease of the maximal change in anisotropy Δr with increasing incubation time (red circle) allowed quantification of the hydrolysis rate using an exponential fit (red solid line).

$0.151 \pm 0.008 \text{ min}^{-1}$) compared to the rate of the reaction at pH 9.1 ($k_{\text{FITC, pH 9.1}} = 0.25 \pm 0.02 \text{ min}^{-1}$) or at pH 11.3 ($k_{\text{FITC, pH 11.3}} = 0.24 \pm 0.02 \text{ min}^{-1}$). On the other hand, higher pH accelerated the rate of hydrolysis and decreased the overall yield of the reaction as seen by the lower r_{max} of 0.276 for the reaction at pH 11.3 (Fig. 2) compared to the r_{max} of 0.288 at pH 9.1.

Hydrolysis had a strong impact on the overall reaction yield. The rate of hydrolysis could be determined by incubating the fluorescent reagent with water before adding the protein (Fig. 3). With increasing incubation times, the maximal anisotropy r_{max} , which is proportional to the fraction of fluorescein attached to the lysozyme, gradually decreased following a first-order kinetics (insert of Fig. 3). A fit using an exponential curve yielded the rate constant for hydrolysis $k_{\text{hydrolysis}} = 0.20 \pm 0.03 \text{ min}^{-1}$. This value was comparable to the overall rate of reaction under these conditions of $k_{\text{reaction}} = 0.83 \pm 0.03 \text{ min}^{-1}$: labeling and hydrolysis were therefore competing. In general, the best yields were obtained at high concentrations and excess of lysozyme.

In conclusion, this new method using FP is characterized by its simplicity and allows the rapid optimization of labeling conditions. For instance, labeling reaction times are strongly exaggerated in experimental protocols. In addition, it enables the quantitative assessment of side reactions. We speculate that this approach might be used more generally to monitor any coupling reaction by attaching a fluorescent probe to one of the reactive group.

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