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Aggregation behavior of the red-absorbing oxazine derivative MR 121: A new method for determination of pure dimer spectra

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Abstract

In this Letter, we investigated the dimerization behavior of the red-absorbing oxazine derivative MR121. Thereby a new method to determine absorption spectra of pure non-fluorescent H-type dye dimers is presented. Therefore, the amino acid lysine is doubly labeled with the fluorescent dye in order to obtain an absorption spectrum mainly caused by dimers. By subtracting the fraction arising from monomeric dye, the dimer spectrum is generated. The extinction coefficient at absorption maximum (608 nm) was estimated to be 86 000 ± 2000 M^{-1} cm^{-1} per dye. Furthermore, the equilibrium constant K_D for the dimerization reaction is determined to be 3300 ± 300 M^{-1}.

1. Introduction

The phenomenon of aggregation and dimerization in aqueous solutions has been shown for a wide variety of fluorescent dyes, e.g., the xanthene [1,2], phenothiazine [3], squarine [4], BODIPY [5,6] and cyanine [7,8] dyes. Usually, the absorption band of dye dimers is significantly shifted and the fluorescence intensity is decreased, as can be explained by the excitation theory [9,10]. First, these phenomena attracted attention because they lead to complications in laser technologies [11,12], and also in medical applications, for example in photodynamic therapy of cancer [13]. The formation of dye dimers complicates the use of Beer's law, because the fraction of dimerized dyes, the extinction coefficient and the shape of the dimer absorption band are usually unknown. That often leads to difficulties in the interpretation of experiments.

In the recent decade, a number of applications have been developed, taking advantage of the fluorescence quenching caused by dye aggregation. To assay proteolytic enzymes, multi-labeled self-quenching substrates are used [14,15], whereby quenching is achieved partly by dimerization of the fluorescent labels and partly by dye tryptophan interactions [16]. A significant increase in fluorescence results from digestion of the protein by the enzyme. For detecting specific proteolytic enzymes, short peptide substrates labeled with two tetramethylrhodamines have been developed [17–19]. The dye-to-dye contact in the intact substrate diminishes the fluorescence of the participating dyes by more than 90%. Due to enzymatic cleavage of the enzyme-specific sequence of the substrate, the dyes dissociate and the fluorescence is no longer quenched. As a result, the activity of specific enzymes can be measured by detecting the increase of the fluorescence intensity. Also studies aimed at exploring distances between specific regions in proteins [20] and protein–peptide interactions [21] use dye dimerization.

Most applications use tetramethylrhodamine or BODIPY dyes absorbing light below 600 nm as aggregating chromophores. There should be several advantages of red-absorbing dyes compared to those absorbing

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at shorter blue and green wavelengths. One major advantage is the reduction of the background signal that limits the sensitivity. The major sources of background are fluorescence of impurities and elastic (Rayleigh) and inelastic (Raman) scattering. Both Raman and Rayleigh scattering are dramatically reduced by shifting to longer wavelength excitation because they are proportional to $1/\lambda$ [4]. Also the number of fluorescent impurities is significantly reduced with longer excitation and detection wavelength [22,23]. Using red-absorbing dyes minimizes the autofluorescence of biological samples so that individual antigen and antibody molecules have been detected in human serum samples [24,25]. A further important advantage is the availability of low-cost, energy-efficient, rugged diode lasers that can be used instead of the more expensive and shorter lived gas lasers. These advantages lead to the development of new fluorescent dyes absorbing and emitting above 620 nm but still having a sufficient fluorescence quantum yield [26]. Especially oxazine derivatives like MR121 or JA242 have been used in many applications, e.g., ultra sensitive detection of DNA [27], enzymes [28] and antibodies. Furthermore, they are used for the investigation of peptide dynamics and FRET experiments. In this article the dimerization behavior of the oxazine derivative MR121 is investigated. The absorption spectrum of the pure dye dimer is determined by subtracting the monomer fraction from a mixed spectrum obtained from a doubly labeled amino acid. Furthermore, the dimerization constant and the extinction coefficients are calculated.

2. Experimental

The fluorescence dye MR121 was kindly provided by K.-H. Drexhage (Universität-Gesamthochschule, Siegen, Germany). Its molecular structure and spectral properties are shown in Fig. 1. To investigate the dye dimer, the amino acid lysine was labeled with two chromophores. Therefore, the carboxyl group of the chromophore was converted into a *N*-hydroxysuccinimidyl ester using an equimolar amount of *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) in acetonitrile. For coupling the dyes to both amino groups of lysine, 20 μl of a 0.02 mM aqueous solution of the amino acid was mixed in 50 μl of carbonate buffer (0.1 M, pH 8.5) with a 10-fold excess of the activated dye (20 μl; 0.2 mM in acetonitrile). The reaction solution was incubated for 12 h at room temperature, and the product was purified by HPLC (Hewlett-Packard, Böblingen, Germany) using a reversed-phase column (Knauer, Berlin, Germany) with octadecylsilanehypersil C18. Separation was performed in 0.1 M triethylammonium acetate, using a linear gradient from 0% to 75% acetonitrile in 20 min.

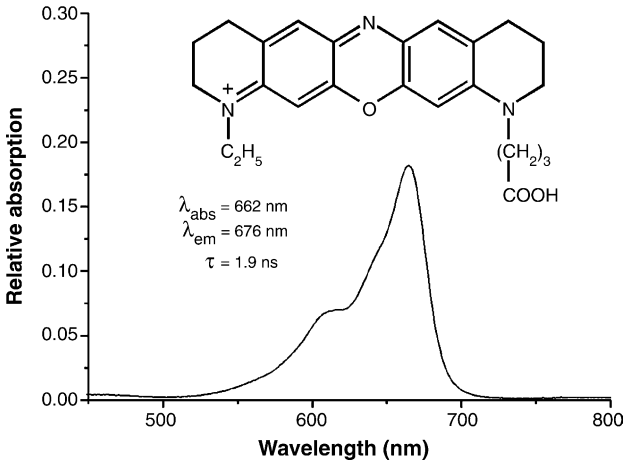

Fig. 1. Structure and absorption spectrum of the oxazine derivative MR 121. Absorption maximum is 662 nm, emission maximum 690 nm and the extinction coefficient in ethanol 125 000 M^{-1} cm^{-1}.

Absorption spectra were taken on a Cary 500 UV–Vis–NIR spectrometer (Varian, Darmstadt, Germany). All measurements were carried out in PBS buffer (140 mM NaCl, pH 7.0) using glass cuvettes with an optical pathway of 10 and 0.05 mm, respectively. Fluorescence and excitation spectra were performed using a Cary Eclipse fluorescence spectrometer (Varian, Darmstadt, Germany).

3. Results and discussion

The aim of this Letter was to investigate the aggregation of the oxazine derivative MR121 in aqueous solution. To imitate physiological conditions we choose phosphate-buffered saline (PBS, 140 mM NaCl, pH 7.0), but we obtained very similar results under other conditions, e.g., in pure water. Fig. 2 shows the absorption spectra of MR121 recorded at different concentrations from 3.0×10^{-5} to 1.0×10^{-3} M. At lower dye concentrations ($<10^{-6}$ M), the shape of the spectrum does not change and can be regarded as the monomer spectrum with an absorption maximum at 662 nm. Additionally there is a shoulder at 608 nm with a two-thirds lower extinction coefficient. Due to formation of dye aggregates at higher concentrations, the relative magnitude of the 608 nm band increases significantly and finally becomes more intense than the 662 nm band. Excitation theory predicts that equal excited-state energy levels of two monomer dyes split into two different levels upon dimerization. The two resulting energy levels lie below and above the former energy level of the monomer excited state. The transition to the lower state is forbidden for parallel sandwich dimers (H-type), whereas transition to the higher state is forbidden for head-to-tail dimers (J-type). Therefore, blue-shifted absorption bands are characteristic for H-type dimers

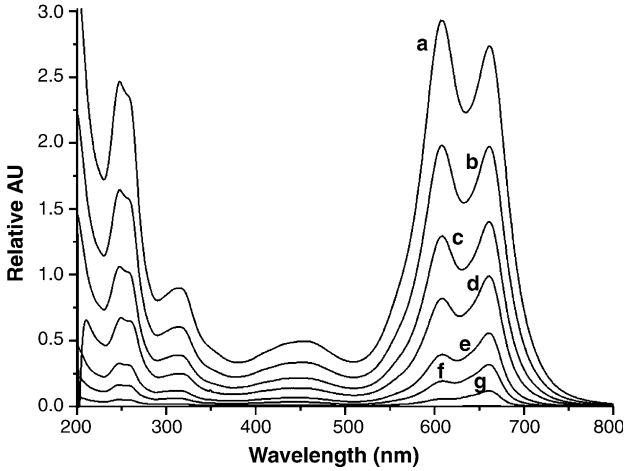

Fig. 2. Absorption spectra of MR121 in PBS (140 mM NaCl, pH 7.0) at 25 °C. The dye concentrations were (a) 1.0×10^{-3} M, (b) 7.3×10^{-4} M, (c) 4.6×10^{-4} M, (d) 2.8×10^{-4} M, (e) 1.4×10^{-4} M, (f) 7.0×10^{-5} M and (g) 3.0×10^{-5} M. Measurements were carried out in glass cuvettes with an optical pathway of 0.05 cm.

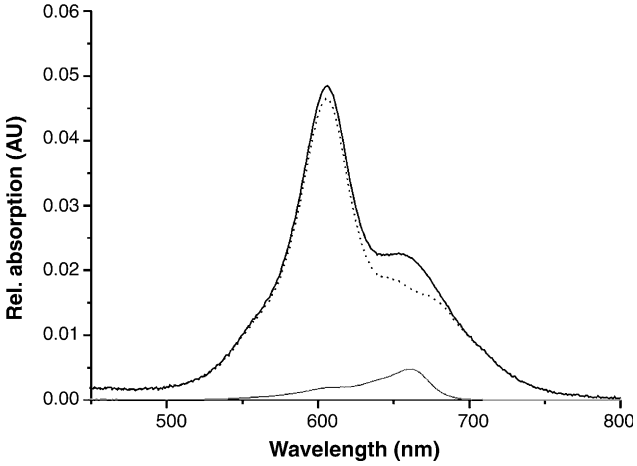

Fig. 3. Absorption spectra of doubly MR121-labeled lysine (black solid line) and of pure MR121 (gray solid line) at 25 °C. Both probes were dissolved in PBS and concentrations were chosen so as to obtain the same fluorescence intensities. The difference spectrum (dashed line) is the absorption spectrum of pure dye dimers.

and red-shifted bands for J-type dimers, respectively. In solution only the H-type dimer is usually observed for most fluorescent dyes.

We used the concentration-dependent spectra to determine the K_D value, which describes the dimerization equilibrium

$$2M \rightleftarrows D \quad K_D = [D]/[M]^2,$$

where D and M are the equilibrium concentration of the dye dimers and monomers, respectively.

Each spectrum is composed of individual monomer and dimer components by linear combination of the respective spectra [29]. Therefore, it is necessary to know the shapes of the monomer as well as the dimer spectrum. The monomer spectrum shown in Fig. 1 is well known. To identify the dimer spectrum, two chromophores were coupled to the amino acid lysine. Due to the close proximity of both dyes most of them form dimers. To determine the concentration of the monomeric chromophores, we assumed that the fluorescence of dimers is neglectable, which is the case for H-type aggregates only. Therefore, we can prepare a dye solution with a fluorescence intensity corresponding to the doubly labeled lysine solution and record the respective absorption spectrum. By separating both spectra, we obtain a pure dimer spectrum (Fig. 3). The spectrum shows an absorption maximum at 608 nm, a significant red-shifted shoulder at approx. 660 nm and a very small blue-shifted shoulder at approx. 560 nm. The assumption that the two chromophores coupled to the lysine form only non-fluorescent H-type dimers that are also supposed for the free dye in solution is justifiable because the fluorescence spectra (data not shown) as well as the excitation spectra (Fig. 4) of the doubly-labeled lysine and the lower concentrated dye solution are identical. Even if dye dimers have a significant fluorescence emission, they do not necessarily change the fluorescence spectrum. However, they should affect the excitation spectrum due to different absorption properties of both H-type and J-type dimers. Furthermore, both excitation spectra are similar to the absorption spectrum of free MR121, indicating fluorescence emission from non-aggregated chromophores. With the knowledge of the spectral shapes of the pure dye monomer and pure dimer, the mixed absorption spectra of the highly concentrated dye solution from Fig. 2 can be divided into a monomer and a dimer part. Therefore, a monomer

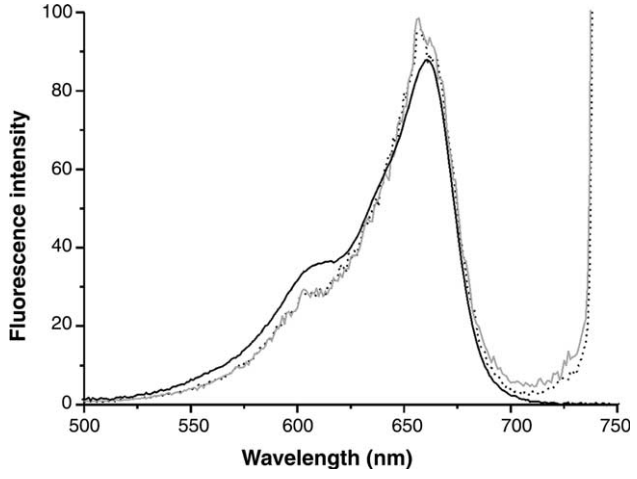

Fig. 4. Excitation spectra of a doubly MR121 labeled lysine (gray solid line) and pure MR121 (dashed line). Both samples are dissolved in PBS and distinctive concentrations were chosen to obtain the same fluorescence intensities. Measurements were taken at 25 °C and the emission wavelength was fixed at 750 nm. For comparison, an absorption spectrum (black solid line) of a 10^{-7} M MR121 solution is shown.

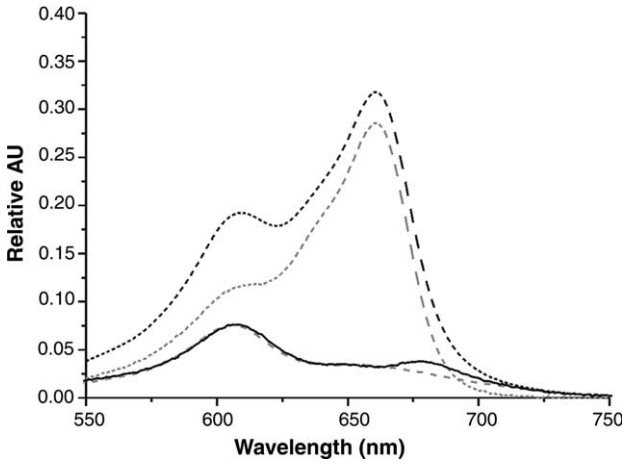

Fig. 5. Absorption spectrum of a 7.0×10^{-5} M MR121 at 25 °C in PBS (black dashed line) using a cuvette with an optical pathway of 0.05 cm. The monomer spectrum of MR multiplied (gray dashed line) and the dimer spectrum (gray solid line) obtained from Fig. 3 are multiplied with appropriate factors. Both factors were adjusted until the spectrum (black solid line obtained by subtracting the monomer (gray dashed line) from the mixed spectrum (black dashed line) matches the dimer spectrum (gray solid line) best.

spectrum recorded from a 10^{-7} M dye solution and multiplied by an appropriate factor is subtracted from the mixed spectrum. The multiplication factor is adjusted until the resulting spectrum fits the determined dimer spectrum best. This procedure is shown in Fig. 5 for the mixed spectrum of a 7.0×10^{-5} M MR121 solution (Fig. 2f). The resulting spectrum (black solid line; Fig. 5) matches with the pure dimer spectrum (gray solid line; Fig. 5) except for a small band at approx. 680 nm which is possibly due to a small amount of J-type dye dimers usually showing a red-shifted absorption band. To determine the concentration of monomer dye molecules, the extinction coefficient in water is required. In ethanol an extinction coefficient of 125 000 M^{-1} cm^{-1} was measured. Due to the lower solubility of MR121 in water it is not possible to dissolve an accurate amount of solid dye powder in a defined volume of water. Therefore, we diluted 2 μl of MR121 dissolved in DMF in 1 ml of ethanol and water, respectively. By measuring the ratio of the absorption maxima we estimated the extinction coefficient in water to be 105 000 M^{-1} cm^{-1}. For each mixed spectrum (Fig. 2), the appropriate monomer spectrum is determined and the concentration is calculated. The dimer concentration is estimated by subtracting the monomer concentration from the overall concentration. The concentrations and resulting K_D values are summarized in Table 1. The equilibrium constant is estimated to be 3300 ± 300 M^{-1} corresponding to most green-absorbing rhodamine derivatives having dimerization constants from 1.300 M^{-1} (Rhodamine B) [30] to 18 000 M^{-1} (6′ isomer of iodoacetamido-tetramethylrhodamine) [31]. From the calculated dimer concentration and the obtained dimer spectra, the extinction coefficient from the dimer at 608 nm can be estimated to be 86 000 ± 2000 M^{-1} cm^{-1} per dye.

Table 1
For each spectrum of Fig. 2 overall concentration, absorption caused by monomer dyes, concentrations of monomers and dimers and the resulting K_D values are summarized

c(MR121) [M]	Abs. (Monomer)	c(Monomer) [M]	c(Dimer) [M]	K_D [M^{-1}]
1.0×10^{-3}	1.78	3.39×10^{-4}	3.30×10^{-4}	2875
7.0×10^{-4}	1.35	2.57×10^{-4}	2.21×10^{-4}	3348
4.6×10^{-4}	1.01	1.92×10^{-4}	1.34×10^{-4}	3645
2.8×10^{-4}	0.77	1.46×10^{-4}	0.66×10^{-4}	3099
1.4×10^{-4}	0.46	8.76×10^{-5}	2.62×10^{-5}	3411
7.0×10^{-5}	0.28	5.33×10^{-5}	0.83×10^{-5}	2930

The absorption of the monomers is estimated by a method described in Fig. 5. For the calculation of the monomer concentration, an extinction coefficient of 105 000 M^{-1} cm^{-1} was assumed (optical pathway was 0.05 cm). To calculate the dimer concentration the difference between monomer concentration and overall concentration has to be halved. The dimerization constant K_D can be calculated from the mass-action expression [$K_D = c(\text{dimer})/c^2(\text{monomer})$].

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