Analysis of the Log-Normal Components of the Fluorescence Spectra of Protein-bound Prodan and Acrylodan

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Abstract—The steady-state fluorescence spectra of the probe prodan and the label acrylodan covalently bound to the cysteine residue in Lys-Cys-Phe tripeptide were recorded in a wide range of solvents with various polarities and then analyzed. It was shown that the shape of the spectral bands is described with a high accuracy by a log-normal function. For each of these fluorophores, as for other organic fluorophores, three shape parameters (the positions of the spectral maximum and of two points with the half-maximal amplitude) of the log-normal function were revealed to linearly related with each other. This enabled us to represent analytically the shape of the fluorescence band of each of the fluorophores in any environment as a unit-amplitude one-parameter log-normal function. The use of such an empirical one-parameter log-normal function in the component analysis of the composite fluorescence spectra was demonstrated by the examples of prodan complexes with bovine serum albumin, and covalent conjugates of acrylodan with actin and myosin subfragment 1.

Key words: fluorescence, component analysis, log-normal function, prodan, acrylodan, bovine serum albumin, myosin subfragment 1, actin

INTRODUCTION

Simultaneously with the development of the methods based on the intrinsic fluorescence of proteins, the techniques of studying proteins and other biological structures with the use of fluorescent dyes are being elaborated. With respect to the manner of their interaction with the structures, these dyes are divided into noncovalently bindable probes and covalently bondable labels [1]. Since the environment of the dyes in the structures and the structural rearrangements are often assessed by analyzing the changes in the emission spectra, it was of interest to check whether or not one can apply, in these cases, the methods and approaches developed for studying the composite fluorescence spectra of tryptophan residues in proteins, in particular, the techniques involving analytical description of the shapes of spectra by a log-normal function [2–6].

We have earlier [5, 6] succeeded in showing that the shape of the smooth asymmetrical fluorescence spectra of various fluorophores, much as that of the bands of absorption spectra [7], is described well by a four-parameter log-normal function

\[ f(v) = \begin{cases} f_m \exp\left\{-(\ln 2/\ln \rho)\ln^2 \left(\frac{a-v}{a-v_m}\right)\right\} & \text{at } v < a \\ 0 & \text{at } v \geq a, \end{cases} \]

where \( f_m = f(v_m) \) is the maximal intensity of the spectrum plotted versus the wave number, \( v_m \) is the...
position (wave number) of the maximum, \( v_m \) and \( v_\pm \) are the positions of the points where the intensity is the half of its maximal value, \( H = v_\pm - v_m \) is the spectral half-width, \( p = (v_m - v_+)/(v_+ - v_-) \) is the empirical asymmetry parameter, and \( a = v_m + H p/(p^2 - 1) \). The area under curve (1) is

\[
S = f_m \int [H \sqrt{2 \pi c} \exp(c^2/2) \rho/(\rho^2 - 1)] = f_m S_0(v_m, v_+, v_-),
\]

where \( c = \ln(\rho)/2\ln2 \), and \( S_0 \) is the area under this curve at \( f_m = 1 \).

Characterization of the spectra by function (1) enables one to find the parameters (wave numbers) \( v_m, v_+ \), and \( v_- \) with a high accuracy under different conditions of recording the emission spectra, which, in its turn, has allowed us to disclose some regularities related to the nature of the factors that influence the fluorescence [5, 6].

In this work, we investigated the shapes and the positions of the fluorescence spectra of two derivatives of dimethylaminonaphthalene—the probe prodan, which has been proposed and synthesized by Weber [8], and the covalent label acrylodan, which has been scrutinized by Prendergast [9]—in a number of solvents with substantially dissimilar polarities. Over the last years, prodan [8, 10–18] and acrylodan [9, 19–21] have enjoyed increasing popularity as tools for exploring protein and membrane systems. Because of the large dipole moments of these fluorophores in the excited state (e.g., according to various estimates [8, 22–24], the dipole moment of prodan is 10–20 D), the positions of their fluorescence spectra are strongly dependent on the dielectric constant of the medium and the rate of its dielectric relaxation.

The positions of the maxima of the fluorescence spectra of the dyes in different solvents that were found in this work virtually coincided with the literature data for both acrylodan [9] and prodan [8, 10]. Like the emission spectra of other fluorophores in organic solvents [5, 6], the prodan and acrylodan spectra plotted versus the wave number are characterized by a linear relationship between three shape parameters (the position \( v_m \) of the spectral maximum, and the positions \( v_+ \) and \( v_- \) of two points with the half-maximal amplitude), despite the complex character of the emission of fluorophores of this class [25, 26]. This relationship suggests that the shape of the elementary fluorescence band of each of these dyes in various environments is determined only by the position of its maximum.

The problem of resolving a composite spectrum into components representable analytically as some mathematical functions is an incorrect one [27]. Uniquely solving such problems becomes much more feasible if the values of some band parameters are obtained in independent measurements [28] or theoretical investigations [29]. Unique relationships between band parameters reduce the number of parameters in (1) that should be determined down to at least as two (the position of the maximum and the amplitude thereat), and the composite fluorescence spectrum becomes describable as a sum of elementary log-normal components.

For correctly assessing the components of the composite fluorescence spectra of tryptophan residues in proteins, different resolution algorithms have been employed. In the simplest of these algorithms, the contributions and the positions of the maxima of the analytical one-parametric log-normal components are determined by the least squares method [2], and the minimizing functional contains the term that characterizes the linearity of the Stern–Volmer equation for fluorescence quenching by external quenchers. In this work, the same methods were used for resolving the composite spectra of prodan and acrylodan.

The goodness of fit of the resolution of the emission spectra of the fluorescent dyes in protein systems was checked by recording the fluorescence spectra of prodan complexes with bovine serum albumin of various compositions, and conjugates of acrylodan with myosin subfragment 1, and also with G-actin at low ionic strength and after its polymerization into F-actin.

**EXPERIMENTAL**

**Reagents.** In this work, we used prodan and acrylodan produced by Molecular Probes Inc., Eugene, OR, USA; and also the following spectrophotically pure solvents: cyclohexane, benzene, dioxane, chlorobenzene, chloroform, acetone, acetonitrile, dimethyl formamide, propylene glycol, ethanol, and methanol (all from Aldrich Chemical Co., Milwaukee, WI, USA).
Fig. 1. Fluorescence (left) and absorption (right) electrophoregrams in a tricine-polyacrylamide gel for (1) the actin-acrylodan conjugate stained with Coomassie blue, (2) the products of its splitting out by trypsin, and (3) the purified conjugate of acrylodan with the tripeptide Lys-Cys-Phe.

Proteins. Trypsin, chymotrypsin, and bovine serum albumin (BSA) (all of Sigma, St. Louis, MO, USA) were employed. Before experiments, BSA was defatted [30], and purified of dimers and aggregates by the previously described procedure [31]. Rabbit skeletal muscle myosin was extracted by the method of Tonomura et al. [32]. Myosin subfragment 1 (S1) was produced by chymotrypsinolysis of myosin, and two isomers of S1 were separated chromatographically on DEAE cellulose [33]. The experiments were carried out with the A1 isomer. Actin was isolated by a published protocol [34].

Labeled Lys-Cys-Phe tripeptide. This tripeptide containing acrylodan bound covalently to the thiol group of the cysteine residue was obtained by splitting off the C-terminal tripeptide of G-actin by trypsin [35]. For this purpose, G-actin labeled specifically with acrylodan was incubated with trypsin at the trypsin-to-actin weight ratio 1:20 for 30 min at room temperature. The reaction was terminated by introducing soybean trypsin inhibitor. Then actin was polymerized by adding 50 mM KCl, 2 mM MgCl₂, and phalloidin for 2 h, whereupon it was sedimented in a Beckman TL100.1 ultracentrifuge. The tripeptide-containing supernatant was lyophilized under vacuum. Before measurements, the dry labeled peptide was dissolved in a small volume of dimethyl formamide and was purified by passing through a Sephadex G-10 column equilibrated with the solvent. Figure 1 presents the fluorescence and absorption traces in a Tricine-polyacrylamide gel for the actin-acrylodan conjugate stained with Coomassie Brilliant Blue, the products of its cleavage with trypsin, and the purified conjugate of acrylodan with the tripeptide. The electrophoresis was performed by the method of Schagger and von Jagow [36], which permits one to separate peptides and fluorescent dyes with es on the order of 1 kDa. Lane 1 shows that the labeled actin sample contains no free acrylodan. Lane 2 shows that trypsin splits off a fluorescent peptide with a molecular mass about 1 kDa. Lane 3 demonstrates that, after purification, only the fluorescent tripeptide (~1 kDa) remains.

Conjugate of acrylodan with S1. This conjugate was synthesized by incubation of 10 μM S1 with a 50% molar excess of acrylodan for 12 h in ice. As shown earlier, under these conditions, thiol-specific fluorescent labels are bound with a high degree of specificity to Cys707 of the heavy chain of myosin [37]. In incubation of S1 with a fivefold molar excess of acrylodan, the degree of labeling was 2 mol of acrylodan per 1 mol of S1. Measurements of the intensity of the fluorescent traces in the gel revealed that 0.5 mol of acrylodan was bound to the basic light chain of myosin, which contains only a single cysteine (Cys178), and that 1.5 mol of acrylodan was bound to the heavy chain of S1 (apparently, the labeling of Cys707 was complete, and Cys697 was labeled by ~50%).

Actin-acrylodan conjugate bound covalently through Cys374. This conjugate was obtained by the published procedure [19]. The labeled proteins were purified of the free dye by dialysis and gel filtration.

Concentrations of proteins and prodan. The concentrations were measured spectrophotometrically, with the molar extinction coefficients taken as follows: for prodan in water, A₅56 = 14,500 M⁻¹ cm⁻¹ [8]; for S1, A₅430 = 7.5 cm⁻¹ [37]; for G-actin, A₅430 = 6.3 cm⁻¹ [38]; and for BSA, A₅430 = 6.67 cm⁻¹ [31]. The concentrations of the conjugates of the proteins with acrylodan were determined by the Bradford method [39], using the standard samples (Bio-Rad).
ANALYSIS OF LOG-NORMAL COMPONENTS

Spectral measurements. The absorption spectra were recorded with Shimadzu UV1600U (Japan) and Specord UV-VIS (Carl Zeiss, Jena, Germany) spectrometers. The fluorescence spectra were recorded with SLM Aminco SPF-500C and Perkin-Elmer SPF-44B spectrofluorimeters, with fluorescence of the dye solutions being excited at 360 nm. The spectral width of the slits for excitation and emission did not exceed 3 nm. The fluorescence spectra were corrected for the spectral sensitivity of the devices. The fluorescence spectrum of quinine sulfate in 1 N H₂SO₄ [40] was employed as a standard for calculating the correction factors. The intensities of the corrected spectra were proportional to the number of photons emitted in unit wavelength range per unit time.

Processing and analysis of fluorescence spectra. The spectra were transformed into plots on the wave number scale in the conventional manner by the relation \( I(\nu) = I(\lambda)\lambda^2 \). The experimental spectra were approximated by the log-normal function (1) using the optimization program based on the Marquardt method [41]. The goodness of fit of the resolution was estimated from the root-mean-square deviation and the uniformity of the distribution of residuals over the spectrum.

The algorithms of resolving composite spectra [2, 42, 43] are grounded on the following notions. Composite spectra are sums of one-parametric log-normal functions (up to three ones) of type (1) with regard for dependences (5) and (5a) (see below):

\[
I(\nu) = a_1 f(\nu_{m1}) + a_2 f(\nu_{m2}) + a_3 f(\nu_{m3}),
\]

where the parameters to be found are the maximal amplitudes \( a_i \) and the positions \( \nu_{m_i} \) of the maxima of the components. The spectra were approximated sequentially by one, two, or three components, and the result with the minimal root-mean-square error of decomposition was taken as the best approximation. The contribution \( A_i \) of the \( i \)th component to the total emission spectrum was assessed as the ratio of the area \( S_i \) under this component that is computed by equation (2), to the sum of the areas under all the components.

In experiments on fluorescence quenching by iodide, the Stern–Volmer constants \( K_{svi} \) were estimated from the dependences of the areas \( S_y \) under the components on the quencher concentration \( c_i \):

\[
S_y = \frac{S_{yi}}{1 + K_{svi}c_i},
\]

where \( S_{yi} \) is the area under the \( i \)th component in the absence of quencher.

RESULTS

Effect of Solvent on the Shape and the Position of the Fluorescence Spectra: Determination of the Relationship between the Shape Parameters of the Log-Normal Function

Each of the fluorescence spectra of prodan and acrylodan in the conjugate with the tripeptide in solvents of various polarities is a single wide structureless asymmetrical band, which is described well by the four-parameter log-normal function (1). The small amplitude and the uniform distribution of the deviations between the experimental intensities and the calculated values of the log-normal function indicate a good fit of the approximation even for the far tails of the spectrum. Tables 1 and 2 list the shape parameters (the positions of the maxima and of the half-maximal amplitude points) of the prodan and acrylodan spectra...
Table 1. Parameters of log-normal function (1) that approximates the prodan fluorescence spectra in various solvents (excitation at 360 nm)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>(v_m), cm(^{-1})</th>
<th>(v_+), cm(^{-1})</th>
<th>(v_-), cm(^{-1})</th>
<th>(H), cm(^{-1})</th>
<th>RMSE*</th>
</tr>
</thead>
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<tr>
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</tr>
<tr>
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<td>24596</td>
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</tr>
<tr>
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<td>Dioxane + ethanol (1:2 volume ratio)</td>
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<td>Ethanol</td>
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<td>Ethanol + water (1:1 volume ratio)</td>
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<td>Dioxane + water (1:2 volume ratio)</td>
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<td>Ethylene glycol</td>
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<td>20800</td>
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<td>Ethanol + water (1:2 volume ratio)</td>
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<td>17781</td>
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</tr>
<tr>
<td>Water</td>
<td>18847</td>
<td>20143</td>
<td>17337</td>
<td>2806</td>
<td>0.0076</td>
</tr>
</tbody>
</table>

* Root-mean-square error of approximation of the spectrum by a unit-amplitude log-normal function.

plotted versus the wave number. The positions of the spectral maxima that were obtained in this work virtually coincide with the literature data for both prodan [8, 10, 11], and the acrylodan conjugate with the tripeptide (the literature data are referred to the acrylodan conjugate with 2-mercaptoethanol [9, 20]).

Figure 3a displays the relationships between the parameters \(v_m\), \(v_+\), and \(v_-\) of the emission spectra of prodan and acrylodan in different solvents. In the same solvent, the maxima of the spectra of the acrylodan label are always shifted toward longer wavelengths in comparison with those of prodan, but the dependences of \(v_+\) and \(v_-\) on \(v_m\) for these fluorophores are very close, albeit not completely identical.

Detailed analysis of the positions of the half-maximal amplitude points and also their differences (spectral widths) showed (Fig. 3b) that, depending on the position (wave number) of the maximum, one can separate two linear portions, which approximately correspond to the spectra recorded in aprotic \((v_m \gtrsim 20,500 \text{ cm}^{-1})\) and protic \((v_m \lesssim 20,500 \text{ cm}^{-1})\) solvents. This correlates with the data of Rottenberg.

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Table 2. Parameters of log-normal function (1) that approximates the fluorescence spectra of the acrylodan conjugate with the cysteine residue in the Lys-Cys-Phe tripeptide in various solvents (excitation at 360 nm)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>( \nu_m ), cm(^{-1} )</th>
<th>( \nu_* ), cm(^{-1} )</th>
<th>( \nu_\gamma ), cm(^{-1} )</th>
<th>( H ), cm(^{-1} )</th>
<th>RMSE*</th>
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<td>Dioxane + methanol (1:1 volume ratio)</td>
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</table>

* Root-mean-square error of approximation of the spectrum by a unit-amplitude log-normal function.

![Fig. 3](image-url)  
Fig. 3. (a) Dependences of the positions \( \nu_* \) and \( \nu_\gamma \) of the half-maximal amplitude points and (b) the spectral half-width \( H = \nu_* - \nu_\gamma \) on the positions \( \nu_m \) of the maxima of the fluorescence spectra of circles prodan and (triangles) the acrylodan conjugate with the Lys-Cys-Phe tripeptide in various solvents (by the data of Tables 1 and 2).
for protic solvents,

\[ v^- = 0.8858v_m + 687.31 \text{ [cm}^{-1}\text{]} \]  
\[ v^* = 1.1151v_m - 832.92 \text{ [cm}^{-1}\text{]} \]  
(5)

for aprotic solvents,

\[ v^- = 0.965v_m - 930.19 \text{ [cm}^{-1}\text{]} \]  
\[ v^* = 0.936v_m + 2832.70 \text{ [cm}^{-1}\text{]} \]  
(5a)

The linear correlation coefficient in all the cases exceeds 0.99.

The width of the spectra of the dyes in protic solvents decreases with increasing their polarity (Fig. 3b). This trend has been noted in the literature for both prodan [8] and the acrylodan conjugate with 2-mercaptoethanol [9, Table 1] in various alcohols and water. However, for the prodan spectra, this trend is observed only in protic solvents; and in aprotic ones, the spectral width remains constant within the accuracy of the experiment.

The fluorescence of aqueous solutions of prodan and the acrylodan label is quenched by iodide without changing the position and the shape of the spectrum, with the Stern–Volmer constants 2.0 and 1.95 M$^{-1}$, respectively (see Fig. 6a).

Thus, the empirical parameters of the spectral shape—the half-width and the asymmetry—and, hence, the shape of the steady-state fluorescence spectra of two different 6-acyl derivatives of dimethylaminonaphthalene are uniquely determined by the position of the spectral maximum. This allows one to regard equation (1) with allowance made for dependences (5) and (5a) as a sufficiently accurate analytical expression for the shape of the prodan and acrylodan fluorescence spectra, i.e., the spectra of uniform fluorescence of these dyes in various media. In its turn, this enabled us to use the programs of resolution into log-normal components that are based on these equations, for analyzing the composite fluorescence spectra of the prodan probe and the acrylodan label in proteins (see Experimental). For this purpose, we employed the programs written previously for resolving the spectra of tryptophan residues in proteins into log-normal components [2, 5, 6, 43], with the only difference that the dependences of \( v^- \) and \( v^* \) on \( v_m \) therein...
ANALYSIS OF LOG-NORMAL COMPONENTS

Fig. 5. Approximation of (points) the fluorescence spectra of the (a) 1:1 and (b) 2:1 acrylodan-S1 conjugates by (solid lines) the sums of two one-parameter log-normal components at various concentrations of the quencher KI. (a) [KI] = (1) 0, (2) 0.05, (3) 0.15, (4) 0.20, (5) 0.30. (b) [KI] = (1) 0, (2) 0.05, (3) 0.10, (4) 0.15, (5) 0.20, and (6) 0.30. Dotted lines are the log-normal components of the spectra. Along the ordinate are the fluorescence intensities $F$ (rel. units).

were replaced by (5) and (5a). Let us further consider examples of application of these programs.

Interaction of Prodan with Bovine Serum Albumin

Figure 4a presents the fluorescence spectra of free prodan in water and prodan complex with BSA at different BSA-to-prodan molar ratios. Serum albumins contain a set of sites of binding of aromatic compounds with substantially dissimilar affinities [44]. With raise in the protein concentration (at constant prodan content), the portion of the dye bound to the protein rises; and at high enough BSA-to-prodan molar ratios, most of molecules of the probe are bound at the main, most potent site of binding of aromatic ligands. This is accompanied by emergence and relative enhancement of the fluorescence band of bound prodan in the region of 450 nm. Each of the spectra recorded at the tested BSA-to-prodan molar ratios (0.22, 0.62, 2.25, and 4.5) contains an isoemission point at ~495 nm, similarly to what has been observed by Weber and Farris [8]. The presence of the isoemission point suggests that prodan in the system under investigation exists only in two states: in the free state (or in the state of weak binding to the protein surface) and in the state when it is bound to the weakly polar binding site, whose polarity, as one can judge from the spectral maximum, is close to that of acetonitrile or acetone (see Table 1). Unlike the spectrum of the free probe in water, these emission spectra are resolved into two log-normal components (Fig. 4a, dotted lines). The maxima of these components are at 522–526 and 440–456 nm, and their contributions are dependent on the BSA-to-prodan molar ratio (Fig. 4b). Analysis of the changes in the contributions of the components in terms of the single-site binding model disclosed that the equilibrium constant of complex formation is about $10^{-5}$ M$^{-1}$, which agrees with the estimate made by Weber and Farris [8], with the fluorescence yield of prodan in the complex with BSA being 3.5 times higher than that of the free dye.

Fluorescence of Acrylodan in Conjugate with S1

At the acrylodan-to-S1 molar ratio 1:1, the dye is bound to the most reactive thiol group of Cys 707 [45]. Figure 5a displays the emission spectra of this conjugate. The maximum of the spectrum is at 505 nm (19,658 cm$^{-1}$), which considerably (by ~25 nm) differs from the position of the maximum of the spectrum of the acrylodan conjugate with the tripeptide in water. The spectral half-width is 3,350 cm$^{-1}$, which is 250 cm$^{-1}$
larger than the expected half-width of the acrylodan spectrum at this position of the maximum according to dependences (5) and (5a). In comparison with the spectra of the acrylodan conjugate with the tripeptide in simple solvents, the spectrum of the acrylodan conjugate with S1 has a flatter short-wavelength tail, as in the spectrum of the acrylodan conjugate with parvalbumin [9]. This could mean that the spectrum of the conjugate is not uniform. Indeed, this spectrum is reliably resolved into two log-normal components with the maxima at 451.5 ± 3 and 506 ± 1 nm and the respective contributions of 11 ± 3 and 89 ± 3% (Table 3).

Figure 5a shows the fluorescence spectra recorded at various KI concentrations and a constant ionic strength of 0.3 M. With raising the concentration, the fluorescence intensity in the long-wavelength spectral tail declines, and that in the short-wavelength region rises. The maximum of the composite spectrum is shifted by 2 nm toward shorter wavelengths, with an isoemission point arising at 470 nm. In the absence of quencher, the spectrum is described by the sum of two log-normal components with the maxima at 451.5 ± 3 and 506 ± 1 nm, and the contributions 11 ± 3 and 89 ± 3%, respectively. With changing the KI concentration to 0.3 M, the maximum of the short-wavelength component is shifted toward longer wavelengths, from 451.5 to 454 nm (-3 nm), and the position of the maximum of the long-wavelength component remains virtually invariable (505 ± 2 nm) (Fig. 6b). These components are plotted as the dotted lines in Fig. 5a. With increasing the iodide concentration, the contribution of the long-wavelength component decreases pursuant to the Stern–Volmer law with $K_{SV} = 1.02 ± 0.07$ M$^{-1}$ (Figs. 6a, 6b), and the contribution of the short-wavelength component rises linearly. The accessibility of the fluorophore emitting in the long-wavelength range for iodide is half that in the model conjugate (in water, $K_{SV} = 1.95 ± 0.09$ M$^{-1}$), which correlates with the poor accessibility of Cys707 in S1 [46]. The short-wavelength component is similar to the spectrum of the model conjugate of acrylodan in chloroform, and the long-wavelength component is close to the spectrum of the model conjugate of acrylodan in ethanol.

### Table 3. Parameters of the log-normal components that describe the fluorescence spectra of the acrylodan conjugate with S1 (excitation at 360 nm)

<table>
<thead>
<tr>
<th>State</th>
<th>$\lambda_{max}, \text{nm}$ (±3)</th>
<th>$A_r$ **</th>
<th>$\lambda_{max}, \text{nm}$ (±1)</th>
<th>$K_{SVr}$ *, M$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylodan + S1 (1:1)</td>
<td>451.5</td>
<td>11 ± 3</td>
<td>506</td>
<td>1.07 ± 0.07</td>
</tr>
<tr>
<td>Acrylodan + S1 (2:1)</td>
<td>454</td>
<td>6 ± 1</td>
<td>512</td>
<td>2.8 ± 0.14</td>
</tr>
<tr>
<td>Acrylodan + S1 (1:1) + F-actin</td>
<td>453</td>
<td>16 ± 3</td>
<td>501</td>
<td>—</td>
</tr>
</tbody>
</table>

* $K_{SVr}$ is the Stern–Volmer constant for quenching of long-wavelength component 2 by iodide (at the wavelength $\lambda_{max}$).

** $K_{SV}$ is the contribution of the short-wavelength component.
Along with binding to Cys707, acrylodan in the 2:1 acrylodan–S1 conjugate is also bound partially to Cys697 of S1 and to Cys178 of the light chain of myosin (see Experimental). Therefore, the fluorescence spectra of acrylodan in the 2:1 acrylodan–S1 conjugate differ significantly from its spectra in the 1:1 acrylodan–S1 conjugate (Fig. 5b): the spectral maximum is shifted toward longer wavelengths to 511 nm, and its width decreases to 3,238 cm⁻¹. The spectrum is described by the sum of two log-normal components with the maxima at 454 ± 3 and 512 ± 1 nm, and the contributions 6 ± 1 and 94 ± 1%, respectively (Table 3, Fig. 6c). In the presence of iodide, as for the 1:1 conjugate, the long-wavelength component is quenched, and the short-wavelength component builds up, with the isoemission point being at 476 nm (Fig. 5b). Component analysis of the spectra of the 2:1 conjugate in the presence of iodide at the concentrations up to 0.3 M revealed that, with raising the iodide content, the position of the maximum of the long-wavelength component remains virtually invariant and is ~511 nm, and the position of the maximum of the short-wavelength component is shifted from 454 to 460 nm (Fig. 6c). The intensity of the long-wavelength component diminishes in accordance with the Stern–Volmer law with $K_{SV} = 2.8 \pm 0.14$ M⁻¹ (Fig. 6a), which is ~40% larger than $K_{SV}$ for the model conjugate in water. This can be due to the much easier accessibility of the emitting acrylodan fluorophore in the 2:1 conjugate. The crystal structure [46] suggests that, unlike Cys707 and Cys697 of S1, Cys178 is readily accessible for solvent; therefore, one can suppose that it is the fluorophore bound to this cysteine residue that makes the main contribution to the long-wavelength component of the fluorescence spectrum of the 2:1 conjugate.

The increase in the contribution of the short-wavelength component from 6 to 15% with raising the iodide concentration (Fig. 6b) is most likely to be due to the chaotropic action of the iodide ion on the hydrophobic interactions in the protein–acrylodan–solvent system [47], which causes structural changes in the environment of the fluorophores. Most probably, the different effect of iodide on the components of the spectra is not related to the fact that acrylodan emits from two excited states, as it has been assumed earlier [16, 48]. Rather, it is associated with the fact that the environment of acrylodan bounded covalently to the cysteine residues is labile enough and can occur in two equilibrium structural states, which differ in polarity of the environment of the fluorophore and in its accessibility to solvent.

Borejdo et al. have demonstrated that S1 is bound to F-actin with the high affinity (1.2×10⁷ M⁻¹) [49]. Figure 7 shows the fluorescence spectrum of the 1:1 acrylodan–S1 conjugate (1.5 μM) in the presence of 6 μM F-actin. As compared to the spectrum of the 1:1 acrylodan–S1 conjugate, the maximum of the spectra of the complex with F-actin is shifted toward shorter wavelengths (to 496 nm), and the spectral width rises to 3,420 cm⁻¹. The log-normal components that fit this spectrum best have maxima at 453 and 501 nm, and their contributions are 16 ± 3 and 84 ± 3%, respectively (Table 3). In distinction from the spectrum of the conjugate with S1, in the spectrum of the complex of the conjugate with F-actin, the contribution of the short-wavelength component increases without changing the position of the band, and the long-wavelength component is shifted by 6 nm toward shorter wavelengths. In binding of S1 to F-actin, the polarity and/or the relaxation rate of the environment of acrylodan bound to Cys707 is likely to decline noticeably.
Figure 8 displays the absorption and fluorescence spectra of acrylodan bounded covalently to Cys393 of G- and F-actins. The spectral maxima are at 491 nm (20,237 cm⁻¹) and 468 nm (21,236 cm⁻¹) for the G- and F-actins, respectively. These findings virtually coincide with Mariott's data (492 and 465 nm, respectively [9]). Both spectra are narrow (3,276 and 2,832 cm⁻¹, respectively) and essentially one-component. The positions of the maxima and the widths of the spectra of the conjugates with G- and F-actins are somewhat dependent on the excitation wavelength. With decreasing this wavelength from 380 to 350 nm, the spectra are shifted toward shorter wavelengths by 0.5 and 0.3 nm, respectively, and their widths increase to 3,318 and 2,896 cm⁻¹, respectively. The considerable hypsochromic shift of the acrylodan label during G-actin polymerization apparently reflects the insertion of the C-terminal portion of the monomer into the region of contact between subunits of F-actin.

**DISCUSSION**

Prodan and acrylodan are sensitive reporter groups in studying structural physical properties of their environments in proteins and lipids. The shape and the position of the spectra of these dyes, as of most of organic fluorophores, are strongly dependent on the properties of the dielectric environment: the dipole moments and the density of dipoles, the dielectric constant, the dipole relaxation rate, the capability for hydrogen bonding in the excited state [50–53]. The investigations into the nature of the great Stokes shift of the fluorescence spectra of these dyes have disclosed that the emission of derivatives of 6-acetyldiaminonaphthalenes has the complex photophysical character [22, 24, 54, 55].

We succeeded in showing that, for prodan and acrylodan, as for other organic molecules [5, 6], the positions of the spectral maxima and of the half-maximal amplitude points are uniquely related with each other; hence, the shape of the emission spectrum is determined by a single parameter (the position of the maximum) of the log-normal function, and the shape of the elementary fluorescence spectrum can be analytically represented as one-parameter function. Such a significant diminution in the number of the parameters to be found has enabled us to create the efficient programs for the component analysis of the composite fluorescence spectra of prodan and acrylodan on the basis of the previously developed algorithms of analyzing the fluorescence spectra of tryptophan residues in proteins.

However, the exploration of the effect of solvent on the spectral shape parameters has revealed the anomalous character of the dependence of the spectral half-width on the position of the maximum. A similar behavior has earlier been noted for the emission spectra of coumarin 135 [56]. The narrowing of the spectrum with raising the polarity of protic solvents has been explained by the response to the redistribution of the charge of the solvation shell of the fluorophore in the excited state, which is polarized with increasing the dipole moment of the excited state. For prodan, this shell comprises 2–4 solvent molecules [12]. Obviously, this has complicated the mathematical description of the shape of the emission spectra, and has forced us to supplement the component analysis algorithm with two sets of equations (5) and (5a), which linearly relate the position of the spectral maximum with the positions of the half-maximal amplitude points. Nonetheless, the above results have corroborated that the emission spectra of probes and labels can be analyzed by resolving them into log-normal components, and have demonstrated that the resolution of composite spectra into log-normal components gives additional information on the system under examination.
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