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Application of laser fluorimetry for determining the influence of a single amino-acid substitution on the individual photophysical parameters of a fluorescent form of a fluorescent protein mRFP1

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Abstract. **Individual photophysical parameters of the chromophore of a fluorescent protein mRFP1 and its two mutants (amino-acid substitution at position 66 – mRFP1/Q66C and mRFP1/Q66S proteins) are determined. For this purpose, apart from conventional methods of fluorimetry and spectrophotometry, nonlinear laser fluorimetry is used. It is shown that the individual extinction coefficients of the chromophore of proteins correlate (correlation coefficient above 0.9) with the volume of the substituted amino-acid residue at position 66 (similar to the positions of the absorption, fluorescence excitation and emission maxima).**

Keywords: *nonlinear laser fluorimetry, individual photophysical parameters, mRFP1, chromophore.*

1. Introduction

Fluorescence spectroscopy (fluorimetry) is widely used to study complex organic compounds [1]. However, conventional (linear) fluorimetric methods cannot provide complete information on fluorescent objects under study because of insufficient selectivity (fluorescence bands of most complex organic compounds are broad and structureless at room temperature [1]). Restraints on conventional methods become particularly obvious in investigations of protein fluorescence because this kind of fluorescence can simultaneously depend on several factors, and the protein specimen (ensemble of molecules) is a mixture of some spectrally nonidentical subensembles of protein molecules [2, 3]. For this reason, the data obtained by conventional fluorimetric methods does not permit unambiguous interpretation and allows only qualitative conclusions about the internal state of a protein molecule.

Methods of laser fluorimetry, in particular nonlinear laser fluorimetry [3 – 10], make it possible to expand considerably the possibilities of the fluorescence analysis of complex organic compounds. The method of nonlinear laser fluorimetry allows one to find individual photophysical parameters (lifetime, absorption cross section, singlet – triplet conversion rate, etc.) of fluorophores of complex organic compounds in the case when *a priori* information, which is necessary for conventional methods, is insufficient [3]. The above-mentioned parameters can be used for diagnostic purposes.

Fluorescent proteins (FPs) are an interesting and important class of proteins [11] and the application of nonlinear laser fluorimetry for their analysis allows one to arrive at new results which cannot be obtained by using conventional fluorimetric methods. This is a special class of proteins whose distinguishing property is the ability to form a chromophore (a heterogroup responsible for light absorption and fluorescence in the visible wavelength range) without involvement of any cofactors or ferments (except molecular oxygen [12]). It is this unique property that permits successful use of FPs as intracellular fluorescent markers for visualisation of intracellular processes [13].

In this paper, we study the influence of a single amino-acid substitution in mRFP1 on individual photophysical parameters of its chromophore. The 66th amino-acid residue (glutamine 66) was chosen as a position to be replaced. This residue participates in formation of the chromophore, and, hence, its substitution by another amino acid can change spectral and photophysical properties of the resultant mutant of the mRFP1 protein. The substitutes are serine and cysteine residues (mRFP1/Q66S and mRFP1/Q66C proteins, respectively). The individual extinction coefficient (the absorption cross section) of the fluorescent chromopore of proteins is shown to correlate with the volume of the substituted amino-acid residue at position 66, i.e. a higher extinction coefficient corresponds to a larger volume of the residue. We also determine the concentration of fluorescent molecules in the resultant solution of each FP. In addition to conventional fluorimetric methods, nonlinear laser fluorimetry is used in this paper.

2. Object

The method for fabrication and purification of proteins is described in [14]. All the experiments with the proteins were performed in a phosphate buffer (the concentration is 0.06 M, pH = 7.4, the temperature is $25 \pm 1\,^{\circ}$C). The Bradford method [15] was used to determine the initial concentration of the protein. When we made stationary spectral measurements and determined fluorescence lifetime, the protein concentration was 10^{-6} M; when we found the quantum yield of fluorescence, it was 4.8×10^{-10} M (mRFP1), 5.2×10^{-10} M (mRFP1/Q66S), $3.5 \times$

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10^{-10} M (mRFP1/Q66C); when we used nonlinear laser fluorimetry, the concentration was 10^{-8} M.

The field of application and properties of FPs are considered in detail in [11–13]. Note that today over 200 types of FPs are known; however, the most popular for practical applications are FPs whose fluorescence is shifted to the red (red FPs) and whose molecules are monomers [12]. The mRFP1 protein possesses these properties [16], which made it an object of the research.

The formation of a fluorescent molecule of red FPs is a complicated process (usually called maturation) consisting of several stages [16, 17]. At some stages, intermediate protein forms* (the so-called blue and green [16] forms, denoted below as B- and G-forms) are produced, which remain in the resultant specimen (solution) of protein. In other words, the solution of red FPs is an ensemble of protein molecules consisting of several chemically nonequivalent subensembles (a mixture of different inseparable spectral forms of FPs), namely, mature (R-form) and immature (B- and/or G-form) forms.

Note that the simultaneous presence of different protein forms in the solution makes it difficult to determine individual photophysical parameters of the chromophores of these forms with the help of conventional spectroscopic methods (traditional fluorimetry, spectrophotometry, etc.). These methods allow one to determine only integral optical characteristics of a chromophore reflecting the total concentration of protein molecules rather than partial concentrations of each form [12]. As shown below, the use of nonlinear laser fluorimetry can help overcome these limitations.

3. Experimental

3.1 Nonlinear laser fluorimetry

A laser fluorimeter described in [6, 8] was used in the experiment. A pulsed frequency-doubled 532-nm Nd : YAG laser with a pulse repetition rate of 10 Hz was used as the radiation source (the second harmonic of the fundamental laser radiation). The laser radiation parameters were as follows: the laser pulse duration was 12 ns, the pulse energy was 2 mJ and the beam diameter was 800 μm. A MUM monochromator (Russia) coupled to a FEU-100 photomultiplier (Russia) was used to detect fluorescence. Fluorescence was detected at 610 nm. The fluorescence intensity was measured when the photon flux density F of laser radiation changed from 7×10^{23} to 8×10^{25} s^{-1} cm^{-2} (see [10] to find the definition of F). With F varying within this range, the noise intensity did not exceed 15 %. A mixing system (a magnetic stirrer) was used to prevent the accumulation of photoproducts (the volume of protein solution was 3 mL). A method for measuring the saturation curve is given in [9]. Special tests were performed to establish that the specimens were not damaged in the experiments.

3.2 Conventional (linear) spectroscopy

A picosecond fluorometer based on a Nd : YAG laser (laser wavelength 532 nm, pulse duration 20 ps, beam diameter 5 mm, pulse energy 160 μJ) was used to measure fluorescence decay times. An Agat SF 3M streak camera (VNIIOFI, Russia) was employed as a detector. The error in determining the fluorescence lifetimes with this kind of fluorometer and time intervals of several nanoseconds did not exceed 5 %.

Apart from laser facilities, we used in experiments a Cary 100 spectrophotometer (Varian Inc., USA) for measuring absorption spectra, and a Cary Eclipse spectrofluorimeter (Varian Inc., USA) with the spectral slit width of 5 nm for measuring excitation and emission spectra of fluorescence.

4. The method of nonlinear laser fluorimetry

The method of nonlinear laser fluorimetry was proposed in paper [4], its modern version is described in detail in papers [5–10]. Here, we only outline it. The method uses the effect of fluorescence saturation which is manifested when complex organic molecules are excited by high-power laser radiation.

The theory of nonlinear laser fluorimetry is based on equations describing the kinetics of concentration variation of molecules of complex organic compounds which are at the corresponding energy states. Under assumption that the diluted solution of complex organic compounds is excited by laser radiation with the parameters presented in section 3.1, the system of kinetic equations for populations n_1, n_2 and n_3 of the ground (S_0 – level 1), first excited singlet (S_1 – level 3) and first excited triplet (T_1 – level 2) energy states has the form:

$$\begin{aligned}
\frac{\partial n_1(t,\boldsymbol{r})}{\partial t} &= -F(t,\boldsymbol{r})\sigma[n_0(t,\boldsymbol{r}) - n_3(t,\boldsymbol{r}) - n_2(t,\boldsymbol{r})] + \frac{n_3(t,\boldsymbol{r})}{\tau_{31}},\\
\frac{\partial n_3(t,\boldsymbol{r})}{\partial t} &= F(t,\boldsymbol{r})\sigma[n_0(t,\boldsymbol{r}) - n_3(t,\boldsymbol{r}) - n_2(t,\boldsymbol{r})] - \frac{n_3(t,\boldsymbol{r})}{\tau_{31}}\\
&\quad - K_{32}n_3(t,\boldsymbol{r}),\\
\frac{\partial n_2(t,\boldsymbol{r})}{\partial t} &= K_{32}n_3(t,\boldsymbol{r}),\\
n_0 &= n_1 + n_2 + n_3\,,
\end{aligned} \tag{1}$$

where $F(t,\boldsymbol{r})$ is the photon flux density of exciting radiation at the coordinate point $\boldsymbol{r}$ at instant of time t; σ is the absorption cross section of the fluorophore; n_0 is the total concentration of organic molecules; $\tau_{31} \equiv 1/(K_{31} + K'_{31})$; K_{31} and K'_{31} are the rates of radiative and nonradiative transitions from state S_1 to S_0; K_{32} is the rate of singlet–triplet conversion. For this model, the full lifetime of a molecule in S_1 state (the fluorescence decay time) is determined as $\tau_3^{-1} \equiv K_3 = \tau_{31}^{-1} + K_{32}$.

By solving system (1) analytically or numerically, we can find the concentration $n_3(t,\boldsymbol{r})$ of molecules and determine the number of fluorescence photons emitted from the volume V after the action of the laser pulse:

* The following terminology is used in the paper: the blue protein form (B-form) is a protein molecule whose chromophore has the absorption maximum at 360–420 nm and does not fluoresce (in some red FPs this form demonstrates very weak fluorescence at 450 nm [17]); the green protein form (G-form) is a protein molecule whose chromophore has the absorption maximum at 450–520 nm and does not fluoresce [16] or fluoresces in the green region of the spectrum (the maximum is at 500 nm [12]); the red protein form (R-form) is a protein molecule whose chromophore has the absorption maximum at 550–600 nm and fluoresces in the red region of the spectrum.

$$N_{\mathrm{Fl}} = K_{31} \int_0^{\infty} \mathrm{d}t \int_V n_3(t, \boldsymbol{r}) \mathrm{d}\boldsymbol{r}. \quad (2)$$

At small values of the photon flux density F (intensity) of exciting radiation, the dependence N_{Fl} is a linear function of F (i.e. $N_{\mathrm{Fl}} \sim F$). However, when F increases, this dependence stops being linear, which means that fluorescence reaches saturation.

There are a few mechanisms explaining the effect of fluorescence saturation. In this paper we consider model (1) in which saturation is caused by a finite lifetime of fluorescent molecules in the excited state and by singlet–triplet conversion. In making this choice, we took the following reasons into account: (i) the proteins in the sample solution are monomers (one molecule contains one chromophore [16]), hence, the saturation mechanism is absent, which can be caused by the energy exchange between chromophores inside a single molecule; (ii) at the given parameters of the laser radiation (see section 3.1) the probability of two-photon excitation for red FPs [18] is small compared to the mechanisms under study and contributes little to fluorescence saturation. Therefore, these two mechanisms in (1) were not taken into account. For most complex organic compounds described by model (1), saturation appears at $F > 10^{22}$ cm^{-2} s^{-1}.

The essence of nonlinear laser fluorimetry consists in determining (by solving the inverse problem) the photophysical parameters of complex organic compounds [parameters σ, K_{32} and τ_3 in model (1)] from the dependence $N_{\mathrm{Fl}}(F)$ (specifically, a nonlinear part of the dependence), which is called the fluorescence saturation curve. In experiments, it is convenient to normalise the number of detected fluorescence photons N_{Fl} to the reference signal N_{Ref}, which can represent a part of exciting radiation directed to the reference channel of the detection system by a beamsplitter (as in our experiments) or a Raman scattering signal from water molecules. In this case, one has to deal with the dependence $[\Phi(F)]^{-1} = N_{\mathrm{Ref}}/N_{\mathrm{Fl}}$ [which is also called a saturation curve, $\Phi(F)$ is the fluorescence parameter] rather than $N_{\mathrm{Fl}}(F)$. In the absence of saturation, Φ stops being dependent on F and tends to a constant which is denoted by Φ_0 [$\Phi_0 \equiv \lim_{F\to 0} \Phi(F)$] [3, 9].

If in measurements of Φ_0 we use the Raman scattering band of water molecules as a reference signal, then we can find the fluorescence quantum yield η of a complex organic compound (the method is described in [19] for the first time). Thus, the determination of the fluorescence quantum yield requires measurement of the entire spectrum of secondary emission which holds both the fluorescence band of the sample under study and the Raman scattering band of water (for this purpose a low-concentration solution that provides comparable intensities of the two bands is used in experiments).

It is necessary to point out two distinctive features of nonlinear laser fluorimetry: (i) as the method implies detection of fluorescence photons [see (2)], the photophysical parameters derived from the saturation curve relate only to a fluorescent molecule of the complex organic compound; (ii) information on the concentration of fluorescent molecules is not used in deriving the photophysical parameters from the saturation curve [3, 5].

Thus, nonlinear laser fluorimetry allows one to determine individual photophysical parameters of a molecule of the fluorescent subensemble in the case when the following complex situation takes place: (i) the sample under study is a multicomponent ensemble of molecules from the complex organic compounds, the absorption bands of its subensembles overlapping (i.e. when the sample is excited, all the subensembles absorb light); (ii) the concentrations of molecules from the subensembles in the mixture are *a priori* unknown; (iii) among the subensembles, only molecules of one of them fluoresce.

Note that the third condition can be less severe: the molecules of all the subensembles can fluoresce, but it is necessary for the fluorescence spectra of these subensembles not to overlap. In this case, it is possible to determine the individual photophysical parameters of molecules from subensembles of complex organic compounds by detecting the fluorescence at a wavelength lying away from the overlap of the spectra.

What is said above is an advantage of this method that distinguishes it from conventional spectroscopic methods, in which it is necessary to know *a priori* the concentration of absorbing and/or fluorescent molecules of complex organic compounds (e.g., methods of spectrophotometry, correlation spectroscopy [20]) for determining individual photophysical parameters.

5. Results

5.1 Analysis of the absorption and excitation spectra

One can see from the absorption spectra of proteins under study (Fig. 1) that in the wavelength range from 370 to 650 nm, there exist three absorption bands, which are explained by the presence of three spectral forms in the solution of each protein [16, 17], i.e. R-, G- and B-forms (the corresponding absorption maxima lie in the wavelength ranges 561–584 nm, 503–507 nm and 377–384 nm depending on the protein form). One can also see that the absorption bands of G- and R-forms overlap for all three samples. Excitation of fluorescence in the absorption band of each form indicates that G- and B-forms do not fluoresce in all of the three protein samples, the R-form fluoresces with maxima at 607 (mRFP1), 585 (mRFP1/Q66C) and 561 nm (mRFP1/Q66S).

Note that the presence of the three protein forms can be qualitatively seen in the absorption spectrum (Fig. 1). However, the quantitative analysis of the concentration

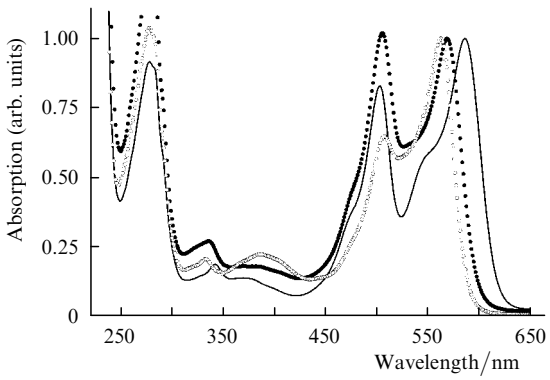

Figure 1. Absorption spectra of mRFP1 (solid curve), mRFP1/Q66C (●) and mRFP1/Q66S (○) proteins. The spectra are normalised to the optical density at the absorption maximum of the R-form of proteins [584 nm (mRFP1), 568 nm (mRFP1/Q66C) and 562 nm (mRFP1/Q66S)].

ratio of these protein forms and the determination of individual photophysical parameters of their chromophore with the help of only conventional methods are problematic. For example, determining the absorption cross section σ (or, more often, extinction coefficient ε (M^{-1} cm^{-1}) = $2.6\times 10^{20}\sigma$ (cm^{-2}) of the chromophore of the protein R-form from the absorption spectrum requires the concentration of this form in the solution to be known, while conventional biochemical methods (e.g., the Bradford method or Lowry method [15]) allow one to find only the total concentration of protein molecules in a solution rather than its particular form. In this case, determining the absorption cross section from the absorption spectra with the help of expression $2.3D = \sigma C_0 l$ (D is the optical density, l is the solution thickness), where C_0 (in cm^{-3}) is the total concentration of all protein molecules (all three forms), as a result, helps to determine the integral absorption cross section of FPs. This is explained by the fact that the preparative separation of the forms (and, consequently, the determination of the partial concentrations) is rather difficult. The physical-chemical properties (the molecular weight, diffusion coefficient, etc.) of the molecules of these forms are almost identical; the differences consist only in the chemical structure of the chromophore of these forms. In this situation, a method is needed which does not require the concentrations of protein molecules to be *a priori* known for determining the individual photophysical parameters of a specific chromophore. As was mentioned above, this method is nonlinear laser fluorimetry.

5.2 Use of nonlinear laser fluorimetry for determining the photophysical parameters of the chromophore of the protein R-form

Generally speaking, the method of nonlinear laser fluorimetry allows one to determine all three molecular photophysical parameters, σ, t_3 and K_{32}, mentioned in section 4. However, the results of numerical simulation [21] showed that this is possible only when the laser radiation pulse exciting fluorescence has specific parameters which ensure sufficiently high practical stability of the solution of the inverse problem. In this research, we reduced the number of parameters to two: for each of the three protein samples we determined σ and K_{32}, while τ_3 was measured independently with the picosecond laser fluorometer. It turned out that all three samples exhibit single-exponential fluorescence decay kinetics. The recovered times τ_3 are presented in Table 1.

The experimental setup described in section 3.1 was used to measure the fluorescence saturation curves. The obtained dependences of Φ^{-1} on F are given in Fig. 2. Solving the inverse problem of nonlinear fluorimetry for each saturation curve at given τ_3, we defined σ and K_{32} for each protein sample. The techniques of artificial neural networks and the variational method [7, 21] were employed to solve the inverse problems. The results are presented in Table 1 (in terms of the quantum yield to the triplet state $\eta_T = K_{32}/K_3$).

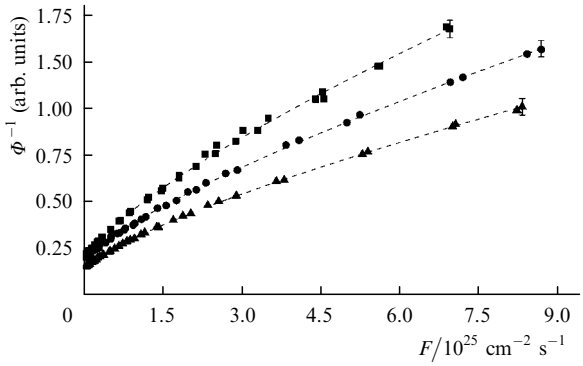

Figure 2. Fluorescence saturation curves for mRFP1 (■), mRFP1/Q66S (●) and mRFP1/Q66C (▲) proteins. The dashed curves are the saturation curves obtained by solving the direct problem [3 – 10] for the parameters τ_3, $\sigma_R^{(532)}$ and η_T from Table 1.

Note the following: (i) as was said in section 5.1, the solution of each of the three proteins contains fluorescent and non-fluorescent forms; because in measurements of τ_3, σ and K_{32} fluorescence photons were detected, the resultant values of these parameters correspond to the R-form for all three protein samples; (ii) in this scheme of nonlinear laser fluorimetry the 532-nm laser pulses were used for exciting fluorescence (see section 3), and, hence, σ is the absorption cross section of the protein R-form at 532 nm, i.e., $\sigma \equiv \sigma_R^{(532)}$.

5.3 Determining the concentrations of fluorescent and non-fluorescent forms of proteins

Using both the results of section 5.2 and spectrophotometric measurements, we can determine the concentrations of fluorescent and non-fluorescent molecules of proteins under study.

It is known [22] that the equilibrium between the B- and G-forms of FPs can be broken under the action of external factors (e.g., irradiation by light whose wavelength lies in the absorption band of the G-form). Using this property of red FPs, it is theoretically possible to find the ratio of concentrations of all three forms (as was done in [22] for the mRFP1 protein). However, it was mentioned above that only the R-form fluoresce in all three protein samples and it is its fluorescence that finds a practical application for the mRFP1 protein [12, 16]. The B- and G-forms are the by-products of maturation and theoretically supposed to be absent. Therefore, it is important first of all to know characteristics of the R-form to predict spectral properties

Table 1. Individual photophysical parameters of the R-form and the fraction of fluorescent molecules in the protein samples.

Protein	$\sigma_R^{(532)*}/10^{-16}$ cm^2	$\sigma_R^{(570)**}/10^{-16}$ cm^2	ε_R^{max}/mM^{-1} cm^{-1}	τ_3/ns	η	η_T^*	n_R/n_0^{**} (%)
mRFP1	3.4 ± 0.6	6.5 ± 1.1	215 ± 40	3 ± 0.15	0.24 ± 0.03	$0 - 0.02$	26 ± 6
mRFP1/Q66C	3 ± 0.5	5.1 ± 0.8	135 ± 20	2.8 ± 0.14	0.19 ± 0.04	$0 - 0.02$	17 ± 6
mRFP1/Q66S	2.2 ± 0.5	3 ± 0.5	85 ± 13	2.9 ± 0.14	0.20 ± 0.04	0.05 ± 0.02	34 ± 6

Notes: $\sigma_R^{(532)}$ and $\sigma_R^{(570)}$ are the absorption cross section of the protein R-form at 532 nm and 570 nm; ε_R^{max} is the extinction coefficient of the chromophore of the protein R-form at the absorption maximum (see Fig.1); τ_3, η and η_T are the fluorescence decay time, quantum yield of fluorescence and quantum yield to the triplet state of a particular protein sample, respectively; n_R/n_0 is the fraction of fluorescent protein molecules (R-form) in the solution.

* Determined from the fluorescence saturation curve; ** determined by solving system of equations (3) (see below).

of the mRFP1 protein. The fact that the information on non-fluorescent forms is less important allows us to simplify the measurement procedure, in particular, to avoid additional measurements of the absorption spectra after the action of external factors. In this case, it becomes possible to determine the concentration of the R-form and the total concentration of the B- and G-forms.

Indeed, given above-mentioned assumptions, we can write the following system of equations:

$$\begin{aligned} &\frac{\Phi_0^{(570)}}{\Phi_0^{(532)}}\frac{\sigma_{RS}^{(570)}}{\sigma_{RS}^{(532)}} = \frac{\sigma_R^{(570)}}{\sigma_R^{(532)}}, \\ &C_R\sigma_R^{(570)} = 2.3D^{(570)}l^{-1}, \\ &C_{GB}\sigma_{GB}^{(532)} + C_R\sigma_R^{(532)} = 2.3D^{(532)}l^{-1}, \\ &C_R + C_{GB} = C_0, \end{aligned} \tag{3}$$

where C_R, $C_{GB} \equiv (C_G + C_B)$ are the concentrations of the R-form and the total concentration of the B- and G-forms in the solution (in cm^{-3}); $\sigma_{GB}^{(570)}$ and $\sigma_{GB}^{(532)}$ are the integral absorption cross section of the chromophore of the non-fluorescent forms at 570 and 532 nm; $D^{(570)}$, $D^{(532)}$ and $\sigma_{RS}^{(570)}$, $\sigma_{RS}^{(532)}$ are the optical density of the protein solution and Raman scattering cross section of water [3, 19] at 570 and 532 nm, respectively.

The first equality in (3) reflects the fact that the quantum yields (expressed in terms of the fluorescence parameter Φ_0 [13, 19]) upon excitation of the protein solution at 532 and 570 nm are the same. The second and third equalities are the optical density (determined from the absorption spectrum of the proteins) written in terms of the concentration of protein molecules absorbing light at 570 and 532 nm and in terms of their absorption cross section. The fourth is the total concentration of protein molecules in the solution determined by conventional methods [15].

In system of equations (3) the wavelength of 570 nm was chosen so that the optical density D measured with the spectrophotometer be determined only by the absorption of only the fluorescent R-form of the protein. The value 532 nm was chosen in this paper because in our experimental realisation of nonlinear laser fluorimetry radiation at 532 nm was used to excite fluorescence. For this reason the absorption cross section that we found from the saturation curve (section 5.2) corresponds to 532 nm.

In system (3) the sought-for quantities are C_R, C_{GB}, $\sigma_R^{(570)}$, $\sigma_{GB}^{(532)}$, while experimentally measured values are $\Phi_0^{(570)}$, $\Phi_0^{(532)}$ $D^{(570)}$, $D^{(532)}$, l, C_0, $\sigma_R^{(532)}$ (the latter is the absorption cross section found by means of nonlinear laser fluorimetry). Solving system (3) for the unknowns, it is possible to determine the concentrations and absorption cross sections of the protein R-form. The computation results for the three samples are given in Table 1.

Note that apart from concentrations of the fluorescent and non-fluorescent molecules, another important result drawn from (3) is the cross section $\sigma_R^{(570)}$, which allows one to find the maximum value of the individual absorption cross section σ_R^{max} (or the extinction coefficient ε_R^{max}, which is more convenient for comparison with data from literature) of the chomophore of the protein R-form. This value can be calculated using the absorption spectrum and relation $D^{max}/D^{(570)} = \varepsilon_R^{max}/\varepsilon_R^{(570)}$, where D^{max} is the optical density at the maximum of the absorption band of the R-form. The result is presented in Table 1.

6. Discussion

One can see from Table 1, at the absorption maximum of the R-form of the mRFP1 protein (at 584 nm) $\varepsilon_R^{max} = (215 \pm 40)\ mM^{-1}\ cm^{-1}$, which is four times the value given in paper [16]. Most likely, this difference is due to the fact that the authors of [16] calculated the extinction coefficient using the total protein concentration (and, therefore, found the integral extinction coefficient) rather than the partial concentration (which is the case in our research). As a result, the determination of the partial concentration of fluorescent molecules allowed us to find the individual extinction coefficient of the chromophore of the R-form. In the general case, the determination of photophysical parameters of FPs with the help of integral characteristics of the sample is incorrect, which can be proved by several examples. In particular, the published values of the extinction coefficient of red FP chromophores differ: 75 $mM^{-1}\ cm^{-1}$ per a polypeptide chain for the DsRed protein [12, 16], 120 $mM^{-1}\ cm^{-1}$ per a polypeptide chain for tdimer2(12) [16], 50 $mM^{-1}\ cm^{-1}$ for mRFP1 [16] and 90 $mM^{-1}\ cm^{-1}$ for mStrawberry [23], despite the fact that the chromophores of these proteins are considered chemically identical.

However, it should be noted that we did not take into account photochemical processes (photoionisation, photobleaching, etc. [8, 22]) in our model of fluorescence response generation (1). The efficiency of these processes in the three protein samples under study may be different. When efficient enough, the photochemical processes may contribute noticeably to fluorescence saturation. In this case their omission can result in the saturation curve giving an overstated value of the absorption cross section and, therefore, overstated quantity of ε_R^{max}. The contribution of photochemical processes to the determined photophysical parameters will be taken into account in future researches. The first step in this direction is made in our paper [22] where we took into account photobleaching (treated as a two-stage process) in the mRFP1 protein. It follows from [22] that for mRFP1 this process has a small efficiency [compared to other processes allowed for in (1)] and does not distort noticeably the previous results.

As was discovered earlier [14], for the R-form proteins under investigation, the position of the maximum of the absorption band, fluorescence excitation and emission depends on the substituted amino-acid residue at position 66 and correlates well with the volume of this residue (Fig. 3). The position of the maximum moves to the red with increasing the volume of the residue. A similar correlation exists for the specific extinction coefficient of the R-form chromophore (Fig. 3): a higher extinction coefficient corresponds to a larger volume of the residue (the correlation coefficient is over 0.9). There is no such dependence for the integral extinction coefficient (at the maximum of the absorption band of the R-form).

The obtained result does not contradict the statement that in the case of a polar amino acid at position 66 (which are the serine and cysteine used in the research), its side radical can form hydrogen bonds with the side radicals of glutamine-213 and glutamate-215 [14]. These radicals in turn belong to the protein shell (the β-barrel) and are rather

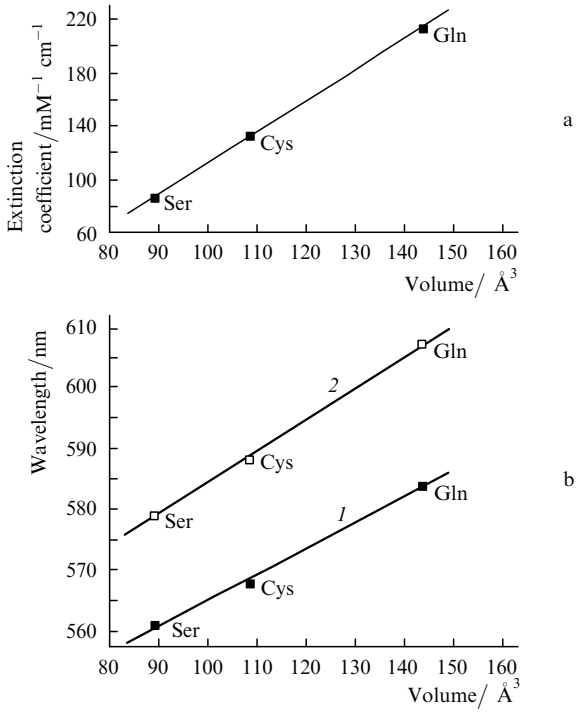

Figure 3. The dependence of the extinction coefficient (a), the position of absorption/excitation maximum (*1*), the fluorescence emission maximum (*2*) (b) of the protein R-form on the volumes of the amino-acid residue at position 66; Ser is serine, Cys is cysteine, Gln is glutamine.

rigidly bonded to it [14]. A change in the geometry of a side radical at position 66 will cause a change in the chromophore geometry, namely, the imidazolidine ring, and therefore, a change in specific optical (position of the spectra of absorption, fluorescence excitation and emission) and photophysical parameters of mutants. In the case of nonpolar amino acids there may be no such effect, which is to be proved later.

At present active researches are being carried out to obtain new types of red monomeric FPs with improved properties [14, 23]: with the fluorescence spectrum in a longer-wavelength range, a higher quantum yield of fluorescence, greater brightness, better photo- and pH-stability. At the same time, there is no full understanding of how the structure of red FPs affects their optical or photophysical parameters, which is required for the development of this field. There is no method that could help us to predict the properties of a particular FP using the information about its structure. Therefore, the results of the research on the properties of mutant proteins containing a single amino-acid substituent can be used to tackle the general problem of the development of an algorithm of construction of red FPs with predefined properties.

7. Conclusions

We have employed the laser fluorimetry method to determine individual photophysical parameters of the chromophore of the red fluorescent form of the fluorescent protein mRFP1 and its mutants (mRFP1/Q66C and mRFP1/Q66S). The concentration of the fluorescent component in the solutions of these three proteins has been found. We have discovered the growing dependence of the individual extinction coefficient of the chromophore of the protein R-form (at the maximum of the absorption band) on the volume of the amino acid at position 66, which may be helpful in predicting the properties of new mutants of red fluorescent proteins and synthesising proteins with predefined properties.

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