STUDY OF THE ENHANCED GREEN FLUORESCENT PROTEIN FLUORESCENCE CHANGES DURING ITS DE- AND RENATURATION, AND UPON THE ADDITION OF NANOPARTICLES

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Abstract. The ability to track some structural changes in enhanced green fluorescent protein (EGFP) by observing its fluorescence makes EGFP a convenient object for studying the protein denaturation process and the influence of some factors on denaturation, in particular, the presence of nanoparticles. In this work, we studied the EGFP fluorescence during its de- and renaturation processes, as well as the influence of the addition of iron oxide nanoparticles on EGFP fluorescence and these processes. Kinetic measurements of denaturation revealed some details of this process. During renaturation, we managed to achieve a 60% recovery of EGFP fluorescence compared to the native protein. We also demonstrated significant effects of the presence of iron oxide nanoparticles. Iron nanoparticles approximately doubled the denaturation rate and suppressed protein renaturation.

Keywords: fluorescent proteins, protein denaturation and renaturation, nanoparticles.

List of Abbreviations

GFP – green fluorescent protein
EGFP – enhanced green fluorescent protein
GdHCl – guanidine HCl
TSC-IONP – iron oxide particles coated with sodium citrate
DLS – dynamic light scattering
TEM – transmission electron microscopy
RET – resonance energy transfer

Introduction

Green fluorescent protein (GFP), so called because of its emission at 508 nm, was first isolated from jellyfish *Aequorea Victoria* (Shimomura *et al.*, 1962). Since then, this protein has been modified in various directions, and today it and its derivatives are extremely widely applied in cell and molecular biology using the fluorescence microscopy method and fluorescence resonance energy transfer (Pollok & Heim, 1999; Tsien, 1998). The GFP structure is a cylinder formed by β-layers which is pierced by an α-helix running along the axis of the cylinder. Chromophore formed by residues 65–67, which are Ser-Tyr-Gly in the native protein, attached to the α-helix and placed in the center of the cylinder (Tsien, 1998). The so-called enhanced GFP (EGFP) is a mutant version of GFP (Phe64Leu, Ser65Thr), with improved spectral characteristics, as well as higher expression in mammalian cells (Cormack *et al.*, 1996; Zhang *et al.*, 1996). In this regard, this mutant version of GFP is extremely popular among researchers.

Due to the wide use of EGFP it is important to study the influence of various environmental factors on it, in particular, to study its fluorescent properties and the ability to restore the fluorescent signal during denaturation-renaturation. The process of denaturation-renaturation of protein molecules is important not only for fundamental science, but also for medicine, pharmaceutical and food industries (Kondakova *et al.*, 2023; Sarimov *et al.*, 2021; Sarimov, *et al.*, 2022a). Previously (Ward & Bokman, 1982), researchers were able to achieve 90% renaturation of the GFP protein. Similar studies have not been conducted for EGFP. At the same time, it is quite likely that despite being very similar in amino acid sequences, EGFP and GFP have structural and conformational differences (Saeed & Ashraf, 2009) and, accordingly, different dynamics of de- and renaturation.

Also, the ability to monitor some structural changes in EGFP by observing its fluorescence, in particular, under the influence of a denaturant, makes it a convenient object for studying the protein denaturation process as such, including its time course and the influence of various factors on denaturation, for example, the presence of nanoparticles.

In this work, we studied the fluorescence of EGFP during the processes of its de- and renaturation, as well as the effect of the addition of iron oxide nanoparticles on EGFP fluorescence and these processes.

Materials and Methods

EGFP obtaining

The recombinant EGFP-expressing E. Coli (DH5R) was formed by cloning the EGFP gene into an ampicillin-resistant pUC-derived plasmid vector.

The concentration of the resulting EGFP solution was determined by measuring its absorbance at 488 nm, taking into account that an extinction coefficient of EGFP at this wavelength nm is ~53000 $M^{-1}cm^{-1}$.

Fluorescence spectra recording

The fluorescence spectrum was recorded using a JASCO spectrofluorimeter FP-8300 (JASCO Applied Sciences, Canada) with a resolution of 1 nm in the cell with optical path of 10 mm. The total volume of all measured samples was 1.5 mL. The sensitivity of the detector varied depending on the concentration of the protein; if it was necessary to compare the signal intensities of different samples, the sensitivity of the detector was chosen to be equal in their measurements (Matveeva *et al.*, 2022).

Kinetic measurements were performed with interval time of 0.1 min (scan time – 0.05 min, waiting time – 0.05 min). The excitation wavelength was fixed at 490 nm, the emission wavelength was recorded in the range of 500-600 nm.

Nanoparticles preparation

Iron oxide particles (Fe_3O_4) coated with sodium citrate (TSC-IONP) were obtained by chemical precipitation of oxide with ammonium hydrate from an aqueous solution of a mixture of iron chloride salts. Nanoparticles form self-organizing stable clusters ~10 nm and 50–80 nm in size, consisting of NPs 3 nm in size. Stability was monitored using the dynamic light scattering method. Detailed data on the process of TSC-IONP synthesis, as well as their characterization by dynamic light scattering (DLS) and transmission electron microscopy (TEM), are given in (Sarimov *et al.*, 2022b).

EGFP de- and renaturation

In all cases, denaturation of the EGFP protein was carried out at room temperature during 30 min in a 6 M solution of guanidine HCl (GdHCl). Renaturation of protein was carried out at room temperature during 60 min in water.

Results

The study of EGFP fluorescence during its denaturation

At first, fluorescence spectra of native 0.02 g/L EGFP water solution were recorded (Fig. 1A).

The main fluorescence peak was observed at 510 nm (emission) at 488 nm excitation (Ex488nm/Em510nm). In addition to the main fluorescence peak, a peak Ex277nm/Em511nm (minor peak) was observed. There was also a peak commonly associated with aromatic residues with a characteristic excitation wavelength in the region of 280 nm (Fig. 1B).

Next, fluorescence spectra of denatured 0.02 g/L EGFP were recorded (Fig. 1C). Since we are not able to directly observe conformational changes of a protein, but only observe its fluorescence, by denaturation here and below we mean a partial or complete loss of the protein's ability to fluoresce. In consequence of denaturation, complete quenching of the main and minor fluorescence peaks was observed. At the same time, the aromatic residues peak became more (approximately 2 times) intense (Fig. 1D), changed shape and its maximum shifts towards longer wavelengths (from Ex276nm/Em306nm to Ex281nm/Em312nm).

To observe the process of denaturation over time, we measured the fluorescence kinetics (carried out interval scan measurements) of this process. We were able to observe the gradual quenching of fluorescence with time upon denaturation of EGFP and found that the quenching time significantly depended on the protein concentration (Fig. 2).

At a EGFP concentration of 0.05 g/L the intensity of the main maximum (Ex490nm/ Em510nm) decreased by 2 times in less than half a minute; at a concentration of 0.08 g/L a 2-fold decrease in intensity occurred in 5 minutes; at a concentration of 0.1 g/L – in 12 minutes and at a concentration of 0.15 g/L

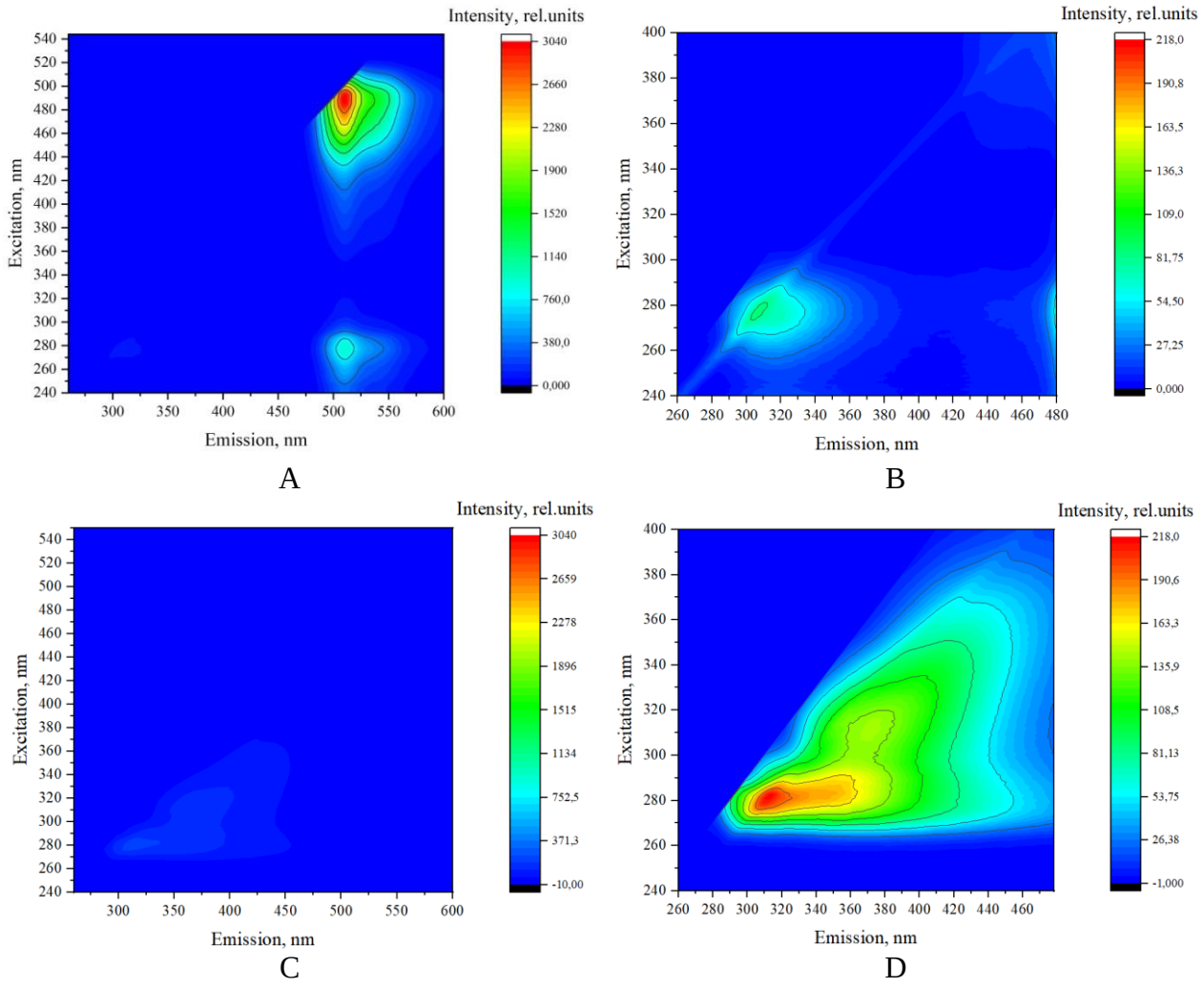

Fig. 1. Typical fluorescence spectra of 0.02 g/L EGFP solutions. A: wide range spectrum of native EGFP in water; B: Ex240-400nm/Em260-480nm range spectrum of native EGFP in water; C: wide range spectrum of denaturated EGFP in GdHCl; D: Ex240-400nm/Em260-480nm range spectrum of denaturated EGFP in GdHCl. Ex240-400nm/Em260-480nm range spectra separately demonstrate the aromatic amino acids fluorescence region, since its relative intensity is low and indistinguishable over the wide range spectra

the time of falling intensity by 2 times was more than 1 hour, and complete quenching of fluorescence did not occur at all.

The study of EGFP renaturation

Next, we investigated the possibility of restoring the fluorescence (renaturation) of a denatured protein by diluting its solution in GdHCl with water by 10 and 50 times. In both cases, the incubation time after dilution was one hour. In the first case, the fluorescence signal was not restored, while at a 50-fold dilution (in this case, the final concentration of EGFP was 0.002 g/L) we found a recovery of the fluorescence intensity (Fig. 3) in the amount of 60% of the initial signal.

Based on a linear dependence of the protein concentration on the fluorescence intensity (in the analyzed range of concentrations, Fig. 4), this number can be considered as the percentage of renaturation. The intensity error was calculated by repeated measurements of the fluorescence intensity of identical samples and was ~10%.

The position of the fluorescence maxima after renaturation does not change (within error) (Table 1). Note that the intensity of the minor peak falls by the same factor as the intensity of the main peak falls (~2.5 times).

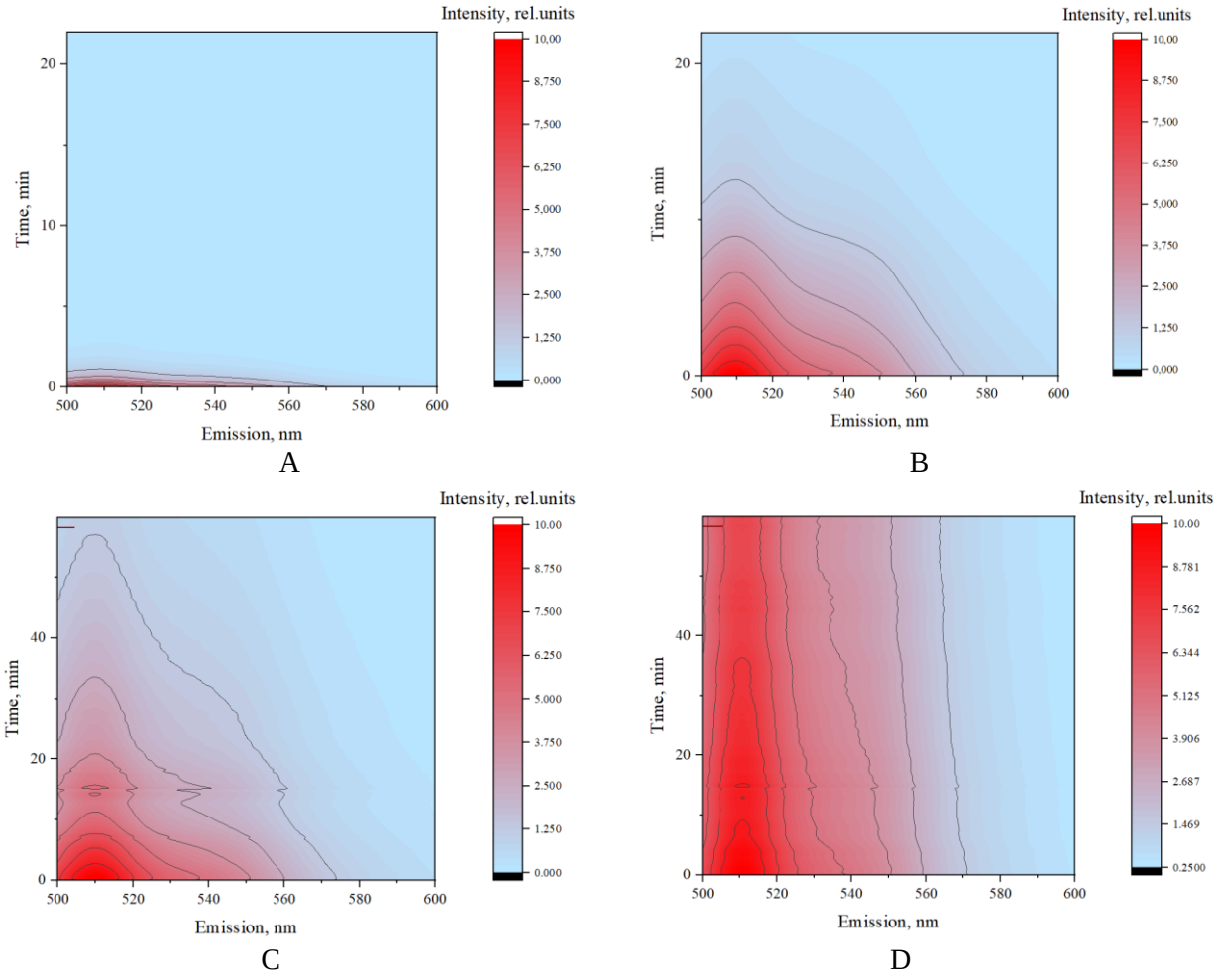

Fig. 2. Interval scan measurements of EGFP denaturation. A: 0.05 g/L EGFP solution; B: 0.08 g/L EGFP solution; C: 0.1 g/L EGFP solution; D: 0.15 g/L EGFP solution

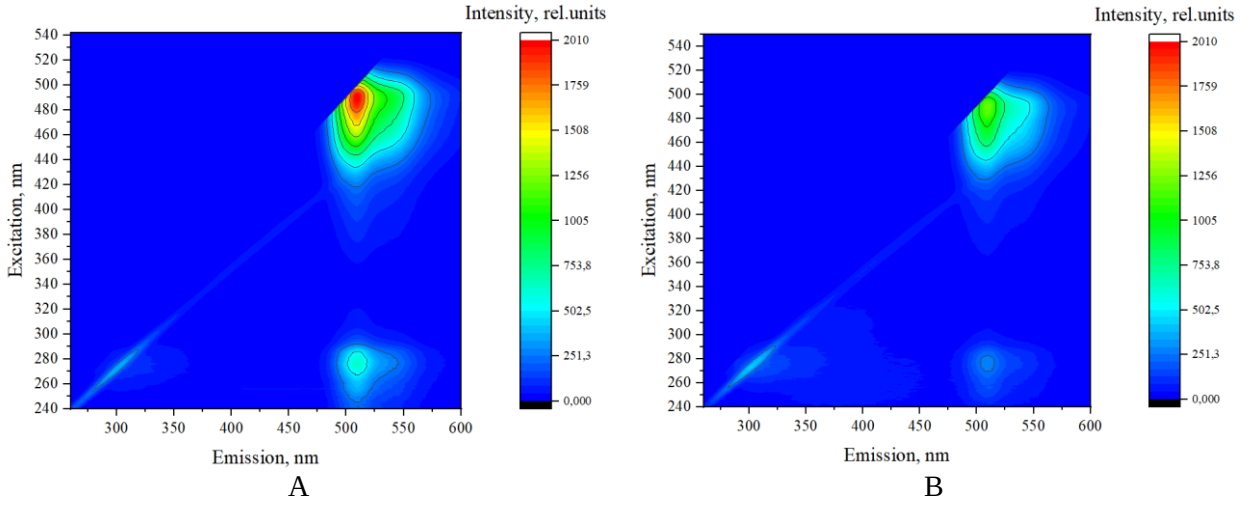

Fig. 3. Typical fluorescence spectra of 0.002 g/L EGFP solutions. A: spectrum of native EGFP in water; B: spectrum of EGFP renatured by 50 times dilution with water

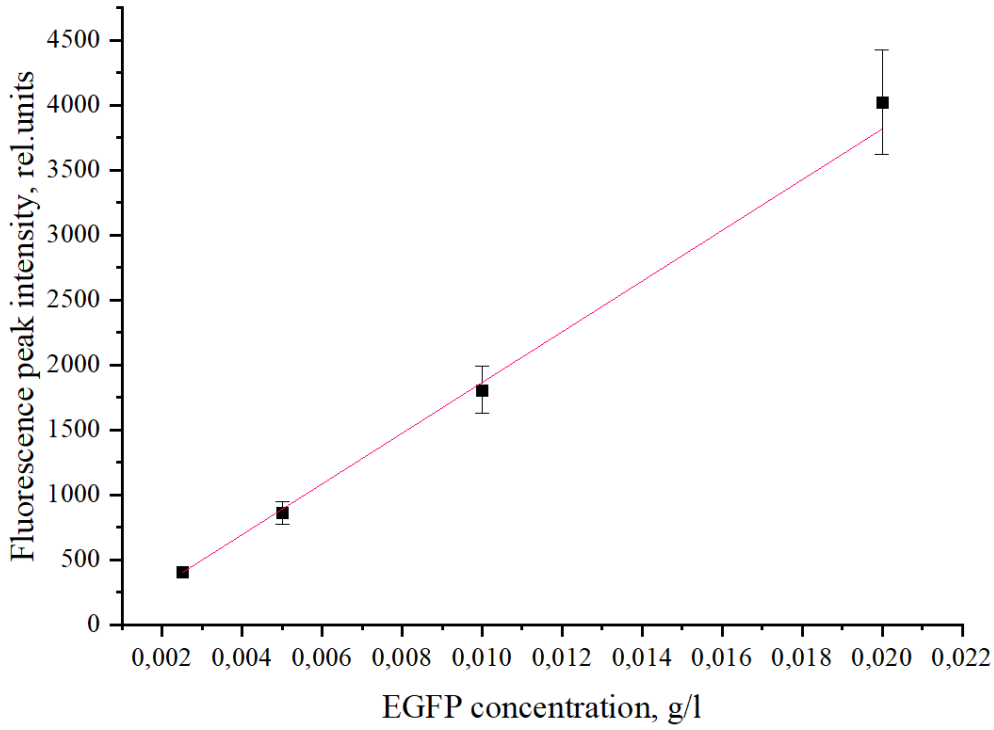

Fig. 4. Demonstration of a linear dependency of the EGFP concentration on intensity of its fluorescence. Intensity measurement error (approximately 10%) was calculated based on several independent measurements

Table 1

Excitation and emission maxima for the main and minor peaks and their intensity (intensity measurement error was calculated based on several independent measurements)

	1 (main) peak			2 (minor) peak		
Renatured EGFP	Ex_{1R}	Em_{1R}	I_{1R}	Ex_{2R}	Em_{2R}	I_{2R}
	488 ± 1	509 ± 1	1234 ± 34	275 ± 1	509 ± 1	286 ± 6
Native EGFP	Ex_{1N}	Em_{1N}	I_{1N}	Ex_{2N}	Em_{2N}	I_{2N}
	489 ± 1	509 ± 1	3105 ± 21	276 ± 1	510 ± 1	722 ± 68
Ratio of fluorescence peak intensities of native and renatured proteins			I_{1N}/ I_{1R}			I_{2N}/ I_{2R}
			2.5			2.5

Impact of adding TSC-IONP

Effect of TSC-IONP on the native EGFP fluorescence spectrum

The effect of adding different amounts of TSC-IONP on the EGFP fluorescence spectra was studied. It was preliminarily found that nanoparticles form ~10 nm clusters in solution. The equal amount of protein (final concentration – 0.02 g/L) was added to the solutions of nanoparticles in concentrations of 10 nm clusters 10^{13}, 10^{14} and 2×10^{14} mL^{-1}. For comparison, the number of EGFP molecules (at the concentration used) is ~10^{14} mL^{-1} and the sizes of clusters and protein molecules are of the same order (several nm). It was found that when TSC-IONP were added, the position of the EGFP main fluorescence maximum changed: it drifts towards longer wavelengths (Table 2).

Table 2

The EGFP main fluorescence maximum position at different concentrations of TSC-IONP

TSC-IONP concentration, clusters/mL	Excitation wavelength, nm	Emission wavelength, nm
0 (EGFP water solution)	489 ± 1	510 ± 1
10^{13}	490 ± 1	510 ± 1
10^{14}	492 ± 1	512 ± 1
2×10^{14}	494 ± 1	512 ± 1

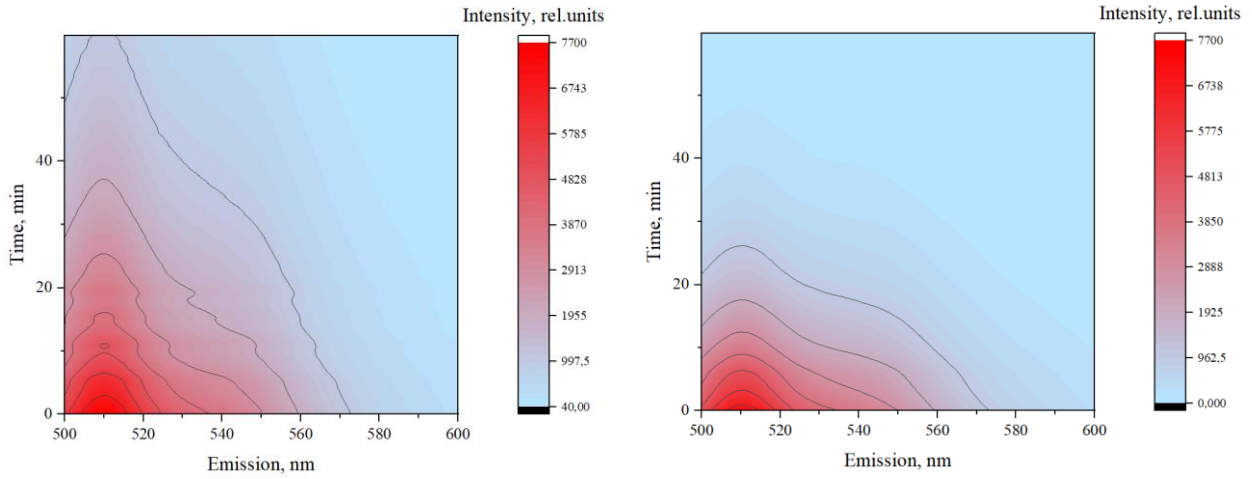

Fig. 5. Interval scan measurements of 0.1 g/L EGFP solution denaturation. A: without TSC-IONP; B: with TSC-IONP

Effect of TSC-IONP on EGFP de- and renaturation

We also investigated the effect of nanoparticles on protein denaturation time. 0.1 g/L EGFP solution was denatured without and with TSC-IONP (Fig. 5). The concentration of nanoparticles in the latter case was 10^{13} clusters/mL. It can be seen from the data presented that denaturation proceeds faster in the presence of nanoparticles (about 2 times). Note that it is difficult to make accurate estimates of the denaturation time due to the difficulty of providing sufficiently close initial times of kinetic measurements for different samples.

Interesting results were obtained when comparing EGFP renaturation without and with TSC-IONP (Fig. 5). EGFP renaturation with TSC-IONP was carried out by diluting 50 times a solution of a protein denatured in the presence of nanoparticles with a solution of TSC-IONP (at a concentration of ~10^{13} clusters/mL). The final protein concentration (after dilution) was 0.002 g/L. The spectrum of the thus «renatured» protein shows complete quenching of its fluorescence.

Discussion

The main fluorescence peak detected corresponds to the available data on EGFP fluorescence (Patterson *et al.*, 1997) within a measurement error of ±1 nm. The presence of minor peak was explained earlier by the existence of two different conformations of this protein observed in its crystalline structure. This is analogous to what is seen for GFP wild type, which displays two fluorescence peaks – a major around Ex400nm for the protonated form and a minor at Ex475nm for the unprotonated state (Dos Santos *et al.*, 2019). We can attribute fluorescence peak in the region of 280 nm (Fig. 1B) to tyrosine residues (Ghisaidoobe & Chung, 2014), because the excitation and emis-

sion wavelengths of the peak maximum are close to 276 nm and 306 nm respectively. It is known that resonance energy transfer (RET) from the tryptophan residue to the chromophore making tryptophan fluorescence faintly visible in the native GFP (Visser *et al.*, 2005). The proximity of the tryptophan residue to the chromophore (distance ~10 Å) in the EGFP structure is shown in the Fig. 6.

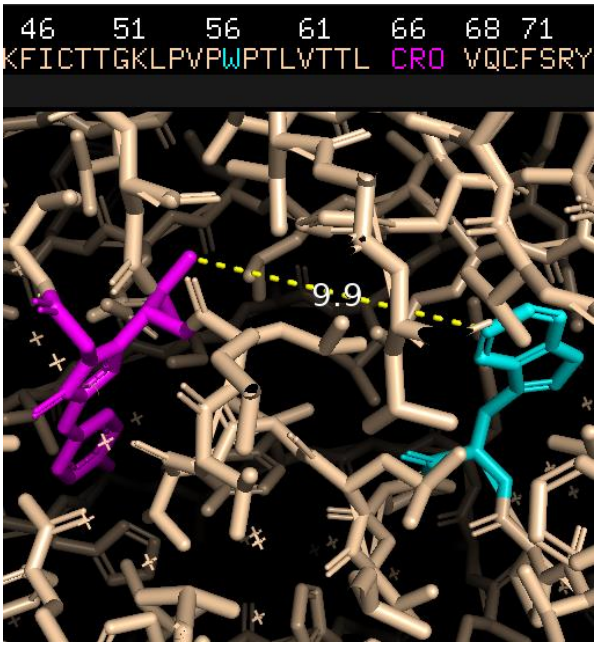

Fig. 6. The image of the chromophore (magenta) and tryptophan (cyan) in the structure of the protein, obtained in the PyMOL program. At dashed line shows the distance between them in Å

Due to denaturation, the aromatic residues peak became more intense (Fig. 1D) and shifts towards longer wavelengths. This may be due to the addition of a contribution from the tryptophan fluorescence, whose fluorescence peak maximum has a longer emission wavelengths than that of tyrosine (Khrustalev *et al.*, 2021). This may indicate the removal of the tryptophan residue at a greater distance from the chromophore during denaturation, which makes the energy transfer from tryptophan less efficient. In general, it can be argued that the environment of the chromophore changes significantly during protein denaturation and the contacts of the chromophore with the some protein regions are disrupted: it is known that the isolated GFP chromophore (as the naked molecule) is not fluorescent (Remington, 2011).

It was found that with an increase EGFP concentration its fluorescence quenching time in the presence of denaturant increases. In all experiments, protein molar concentration is many times less than that of the denaturant ((number of GdHCl molecules)/(number of EGFP molecules) ~ 10^6), and, even taking into account the fact that several denaturant molecules interact with one protein molecule, the change in the ratio (number of GdHCl molecules)/(number of EGFP molecules) with a change in protein concentration can be neglected. We believe that the observed dependence of quenching time on the protein concentration is caused by the formation of complexes (agglomerates) of protein molecules with an increase in its concentration in the studied range. In this case, EGFP molecules buried deep into such a complex are protected from the action of a denaturant, and the observed quenching of fluorescence occurs due to the interaction of GdHCl with protein molecules located on the surface of the complexes.

The possibility of fluorescence restoration suggests that the presence of the denaturant does not cause irreversible changes in EGFP, and most protein molecules probably assume its original conformation after removal of GdHCl. This is evidenced by the absence of shifts in the EGFP fluorescence maxima after renaturation.

When TSC-IONP were added, the position of the EGFP main fluorescence maximum changed. This may be due to a change in the environment with which the protein chromophore interacts. It is known (Tsien, 1998) that several polar groups and structured water molecules are buried adjacent to the chromophore. It is possible that the nanoparticles, through interaction with the outer surface of the protein, change this environment of the chromophore. Note that the presence of nanoparticles coated with sodium citrate increases the pH of the solution. Thus, in the work (Sarimov *et al.*, 2022b), the TSC-IONPs solution at a concentration of 10^{13} clusters/mL had a pH of 8.6. However, although changes in pH affect the fluorescence intensity of GFP and some of its mutants, the position

of the excitation and emission maxima (Ex488nm/Em510nm) does not change significantly (Kneen *et al.*, 1998).

Conclusions

Thus, we were able to demonstrate partial (~60%) EGFP renaturation and elucidate some details of its denaturation-renaturation process. We also established the effect of the addition of iron oxide nanoparticles on EGFP fluorescence as well as de- and renaturation processes. We observed a slight red shift of the main protein fluorescence maximum upon the addition to native protein of TSC-IONP. We also found, that denaturation is accelerating (about 2 times) in their presence (at TSC-IONP concentration of ~10^{13} clusters/mL and protein concentration of 0.1 g/L). It was also discovered that when carrying out the denaturation-renaturation process in the presence of TSC-IONP, it is not possible to achieve EGFP renaturation (at TSC-IONP concentration of ~10^{13} clusters/mL and protein concentration of 0.002 g/L).

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