

# **FLUORESCENT PROPERTIES OF THE KINDLING FLUORESCENT PROTEIN (KFP) AT ACIDIC PH VALUES**

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# Fluorescent properties of the kindling fluorescent protein (KFP) at acidic pH values

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## Abstract

Kindling fluorescent protein (KFP) is the photoswitchable protein which can be used in high-resolution microscopy and as a quencher in FRET-sensors. Fluorescent properties of KFP depend on pH value. In this paper we investigate the influence of pH on the spectral properties and kindling/quenching ability of KFP in the acidic pH region.

Shift to the acidic region leads to the increase of fluorescence intensity of KFP over time. The excitation spectrum has a new peak near 455nm, giving two peaks - 530 and 590nm – in emission spectrum. We can assume that this maximum corresponds to the appearance of protonated form of the KFP chromophore.

Analysis of fluorescence decay curves of KFP in H<sub>2</sub>O and D<sub>2</sub>O showed the presence of the kinetic isotope effect, which can be caused by the proton transfer from solvent molecules to the KFP chromophore, confirming the hypothesis that in the acidic pH region protonated form of KFP chromophore appears.

At acidic pH irradiation of KFP with green light doesn't lead to fluorescence increase, while blue light doesn't quench the fluorescence. It means that KFP is also in the bright form, and there is no conformational states of protein which can be quenched by blue light.

## Introduction

Fluorescent proteins are widely used as genetically encoded fluorescent markers for gene expression and localization of proteins in cells, as well as biosensors. Discovery of the photoswitchable proteins that change their properties upon irradiation with light of a certain wavelength, allowed to develop innovative methods of high-resolution microscopy. At low light intensities photoswitchable proteins with a low quantum yield before the photoactivation can be used as quenchers in FRET-sensors. Photoswitchable protein asCP595 was isolated from the anemone *Anemonia*

*Sulcata*. This protein is weakly fluorescent ( $\phi < 0,001$ ) chromoprotein with a maximum fluorescence at 595 nm. However, upon intense irradiation with green light this protein switches to the fluorescent form, significantly increasing the fluorescence quantum yield. Photoactivation of natural asCP595 is reversible, since switching off the green light irradiation leads to a gradual, and blue light irradiation – a rapid transition to nonfluorescent form of the asCP595. Inclusion of Ala148Gly mutation in asCP595 leads to protein named KFP, which has better contrast and increased relaxation time.

One of the factors that influence the spectral characteristics of fluorescent proteins is the pH value. Changing the pH shifts the equilibrium between the anionic and neutral forms of the chromophore. This phenomenon can be explained as the change of the protonation state of aminoacids near the chromophore affects the fluorescent properties of the protein[1].

Rusanov et al. [2] shown that the shift of pH to the alkaline region leads to a substantial change in the spectral characteristics of KFP. Thus, at neutral pH KFP practically does not fluoresce, whereas at pH above 9 a significant increase in fluorescence intensity can be observed, and this phenomenon is completely reversible. The absorption maximum at higher pH is shifted to shorter wavelengths, while the absorption decreases. These phenomena are interpreted by the authors as a shift of equilibrium between the different fluorescent and nonfluorescent conformations of the protein.

In this paper we investigate the influence of pH on the spectral properties of KFP in the acidic pH region. As in the case of alkaline pH, there is the appearance of new conformations of the protein, causing a change of its fluorescent properties.

## Materials and Methods

The purified protein KFP was dialysed against the 0.1 M CH<sub>3</sub>COONa, 0.2 M CH<sub>3</sub>COONH<sub>4</sub>, pH 5,7 buffer. Then the protein solution was titrated to the desired value of pH (from 5.3 to 3.3) and

measurements of the absorption spectra (Cary 300, “Varian”, USA), fluorescence excitation at  $\lambda_{em} = 640$  nm and emission spectra at  $\lambda_{exc} = 455$  and 530 nm (Cary Eclipse, “Varian”, USA).

Photoswitching and quenching of KFP was carried out by irradiating a sample of the protein in the 20 mM Tris-HCl, 150 mM NaCl, 500 mM Imidazole, pH 4,02 buffer with green (532 nm, power 5 mW) and blue (405 nm, power 80 mW) lasers. Fluorescence was measured by using the “Spektr-Cluster» setup (www.cluster.orc.ru). The source of the excitation light in this setup is a laser with a wavelength of 532 nm, coupled to optical fiber diameter of 110  $\mu$ m, and detection is performed by fiber-optic catheter, composed of six light-harvesting fibers with 110  $\mu$ m diameter, located around the source of the excitation light.

KFP quenching kinetics were obtained by simultaneously focusing the green and blue lasers on a KFP sample in the quartz cell with 0,3 mm optical path. The spectra were measured every 700 ms, and the exposure time was 50 ms. Beams crossing were adjusted by obtaining the minimum fluorescence intensity of the KFP sample during irradiation with both lasers. Laser beam power was measured using a power meter 2936-C (Newport).

To determine the kinetic isotope effect sample of KFP in the buffer 20 mM Tris-HCl, 150 mM NaCl, 500 mM Imidazole, pH 7,6 was titrated to pH 3,94, and then 10  $\mu$ l of the sample was added to 90  $\mu$ l of H<sub>2</sub>O and D<sub>2</sub>O, respectively. Lifetimes were measured by TCSPC method on FluoTime 200 (PicoQuant GmbH, Germany) using a picosecond laser (wavelength 405 nm) as a source of excitation light. Data analysis was performed using FluoFit software (PicoQuant GmbH, Germany). Kinetics of decay were captured at 520 nm wavelength.

## Results and discussion

### *pH-dependence of fluorescent properties of KFP*

The absorption spectra of the KFP show a decrease in the intensity of the absorption peak at 570 nm over time, whereas intensity of 455 nm peak gradually increased (Fig.1).

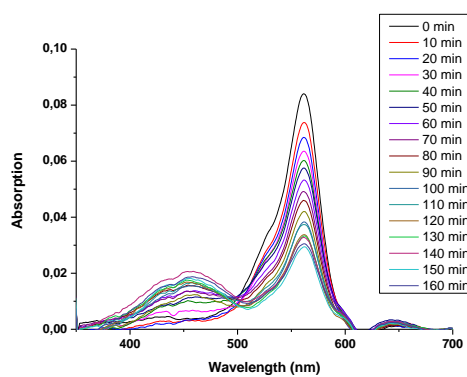


Figure 1. Time dependence of KFP’s absorption spectrum at pH 4,1.

Excitation spectra of KFP also demonstrate an increase of the fluorescence intensity over time, and in addition to the main excitation peak at 560 nm, there is another, previously undescribed peak – at 455 nm (Fig. 2).

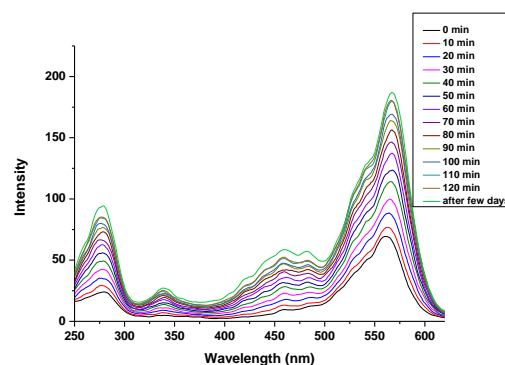


Figure 2. Time dependence of KFP’s excitation spectrum at pH 4,5.

As for the fluorescence spectra, the excitation at 530 nm gives one maximum at 600 nm, which is typical for KFP (Fig. 3).

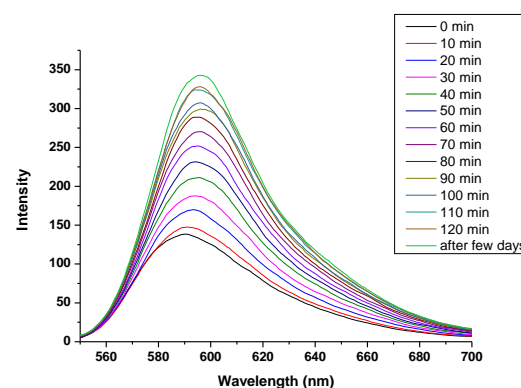


Figure 3. Time dependence of KFP’s fluorescence spectrum, excitation at 530 nm, pH 4,5.

But fluorescence spectra at 455 nm excitation have two peaks - in addition to the main peak (600 nm) there is new peak - at 530 nm, which is not described previously (Fig. 4).

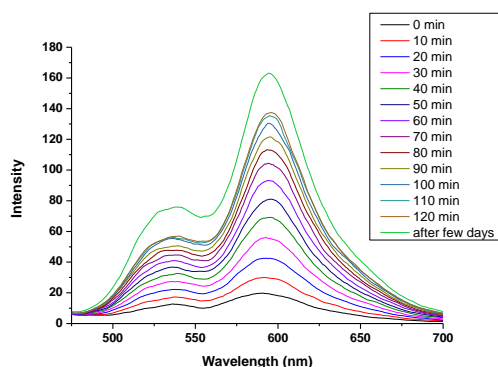


Figure 4. Time dependence of KFP's fluorescence spectrum, excitation at 455 nm, pH 4,5.

Also at pH lower than 4,5 the fluorescence intensity of KFP increases more than 2 times at 530 nm excitation, while excitation at 455 nm results in more than an order of magnitude increase of intensity.

At low pH, the fluorescence of the protein decreases over time, indicating that KFP was denatured. While shifting the pH from acidic to neutral region the rate of increase of fluorescence KFP also increases, reaching a maximum at pH 4,3, and at pH 7,3 the intensity is almost zero, which corresponds to the transition to nonfluorescent form of KFP.

Thus, in acidic pH region there is a spontaneous kindling of KFP. According to the changes in absorption spectrum of KFP, where 570 nm peak decreases over time, and 455 nm peak appears, it can be suggested that at acidic pH small fraction of the protein changes its protonation state from the anionic to neutral form of chromophore. Results of the quantum-chemical calculations of the spectral properties of KFP with neutral chromophore prove this hypothesis [3]. The excitation spectrum of KFP has a new peak near 455nm, giving two peaks - 530 and 590nm - in emission spectrum. The highest intensity of KFP's fluorescence increase KFP is observed at pH 3,9 and 4.3 for the 530 and 590 nm peaks, respectively. In the fluorescence spectra of KFP, obtained by excitation at 455nm, in addition to the characteristic maximum around 600nm, there is another, not previously described - about 530 nm. According to the quantum-chemical calculations, the neutral form of KFP's chromophore is non-fluorescent [3], so we can assume that this maximum corresponds to the appearance of protonated form of the KFP's chromophore which is formed as a result of the proton transfer in the excited state. So, this

hypothesis can be proved by kinetic isotope effect measurements.

### **Fluorescence decay kinetics of KFP in H<sub>2</sub>O and D<sub>2</sub>O**

Analysis of fluorescence decay curves of KFP in H<sub>2</sub>O and D<sub>2</sub>O showed that the initial curves for these samples are different, namely, the fluorescence decay kinetics of the sample in D<sub>2</sub>O is somewhat delayed compared with the kinetics of the sample in H<sub>2</sub>O (Fig. 5).

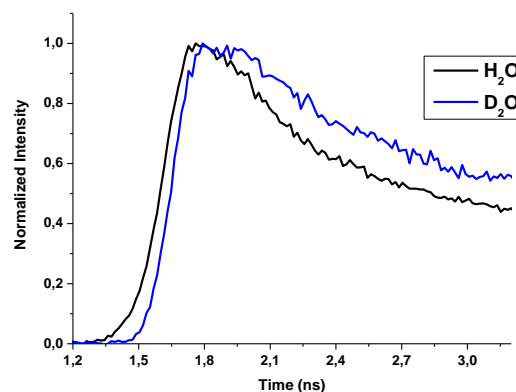


Figure 5. Fluorescence decay curves of KFP in H<sub>2</sub>O and D<sub>2</sub>O, pH 3,94.

These results indicate the presence of the kinetic isotope effect, which confirms the hypothesis that in the acidic pH region protonated form of KFP chromophore appears by means of the proton transfer in the excited state. That proton transfer from solvent molecules to the KFP chromophore causes the difference in the fluorescence decay kinetics of KFP in H<sub>2</sub>O and D<sub>2</sub>O.

### **Kindling and quenching of KFP in acidic pH region**

According to Rusanov et al. [2], at pH 8,8 KFP practically does not fluoresce, whereas under intense green light intensity of its fluorescence increases several times. While shifting to the alkaline region the appearance of bright fluorescent components, which are not kindled by the intense green light, can be observed. At acidic pH values there is a similar pattern - KFP initially has a high fluorescence quantum yield, and the irradiation by green light increases fluorescence by no more than 10% (Fig. 6).

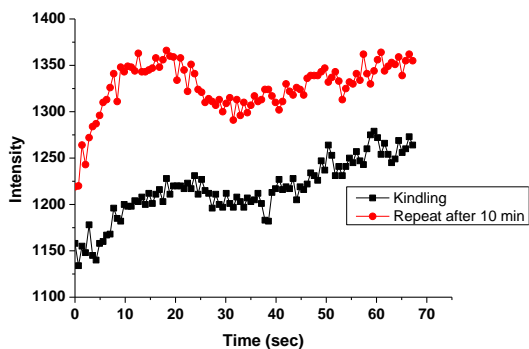


Figure 6. KFP kindling at 532 nm laser irradiation, pH 4,0.

Irradiation by green laser followed by switching on the blue laser (405 nm wavelength) does not lead to fluorescence quenching of KFP (Fig. 7).

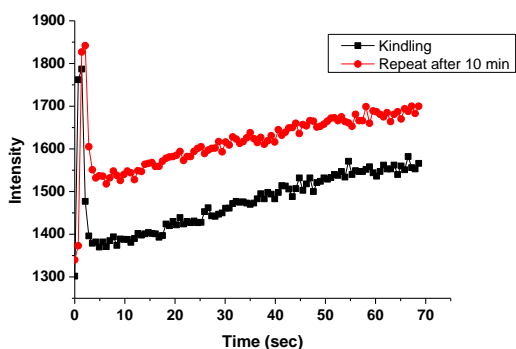


Figure 7. KFP kindling at green (532 nm) laser followed by switching on the blue (405 nm) laser.

The same result was obtained while irradiating the sample with green and blue lasers simultaneously. If we irradiate KFP only by blue laser, the fluorescence intensity increases, but to a lesser extent than in the case of a green laser. This phenomenon does not depend on the power of blue laser (Fig. 8).

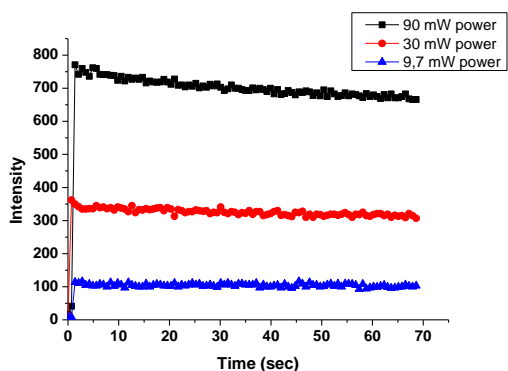


Figure 7. KFP kindling at blue (405 nm) laser irradiation with different laser power.

Thus, at acidic pH KFP is in the bright form, and its irradiation with blue laser doesn't lead to fluorescence quenching, in contrast to the alkaline pH region. So, at acidic pH there is no form of KFP which can be quenched by blue light.

## Conclusions

The pH shift in the acidic region leads to the increase of fluorescence intensity of KFP over time.

Two new spectral forms of KFP were detected at acidic pH values. First form is characterized by absorption at 450 nm and can be identified as the fraction with the neutral form of the chromophore, second form fluoresces at 530 nm, and its occurrence can be explained by the formation of the cationic form of the chromophore as a result of the proton transfer in excited state. Analysis of fluorescence decay curves of KFP in H<sub>2</sub>O and D<sub>2</sub>O showed the presence of the kinetic isotope effect, which can be caused by the proton transfer from solvent molecules to the KFP chromophore, confirming the hypothesis that in the acidic pH region protonated form of KFP chromophore appears.

Irradiation of KFP with green light doesn't lead to fluorescence increase, while blue light doesn't quench the fluorescence.

## References

- [1] R. Bizzarri, R. Nifosi, S. Abbruzzetti, W. Rocchia, S. Guidi, D. Arosio, G. Garau, B. Campanini, E. Grandi, F. Ricci, C. Viappiani, and F. Beltram (2007), Green Fluorescent Protein ground states: the influence of a second protonation site near the chromophore, *Biochemistry*, 46 (18), 5494-5504
- [2] A.L. Rusanov, V.A. Mironov, A.S. Goryashenko, B.L. Grigorenko, A.V. Nemukhin, and A.P. Savitsky (2011), Conformational Partitioning in pH-Induced Fluorescence of the Kindling Fluorescent Protein (KFP), *J. Phys. Chem. B*, 115(29), 9195-201
- [3] I. Topol, J. Collins, V. Mironov, A. Savitsky and A. Nemukhin (2012), Modeling Absorption of the Kindling Fluorescent Protein with the Neutral Form of the Chromophore, *Int. J. Quantum Chem.*, 112, 2947-2951