FLUORESCENCE DYNAMICS OF N-TERMINAL TRYPTOFAN-X RESIDUES IN POLYPEPTIDE: pH RESPONSE

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The influence of the second amino acid on the pH response of the genetically encoded pH indicators is studied. The second Trp has been substituted by Ala and Glu to reduce self-quenching. Compared with the previously reported Trp-Trp-Ala-Ser (WWAS), the two new tetrapeptides, Trp-Ala-Ala-Ser (WAAS) and Trp-Glu-Ala-Ser (WEAS), have higher fluorescence quantum yields, longer fluorescence lifetimes, and more sensitive pH responses. As novel genetically encoded pH indicators, the N-terminal Trp–Ala and Trp–Glu could be fused to proteins for monitoring the environmental pH values during the studies of functional proteins.

Keywords: *fluorescent sensor, genetically encoded pH indicator, Trp-Ala-Ala-Ser, Trp-Glu-Ala-Ser, Trp-Trp-Ala-Ser.*

ДИНАМИКА ФЛУОРЕСЦЕНЦИИ N-ТЕРМИНАЛЬНЫХ ОСТАТКОВ ТРИПТОФАНА-X В ПОЛИПЕПТИДЕ: ВЛИЯНИЕ pH

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Исследовано влияние второй аминокислоты на зависимость от pH генетически закодированных pH индикаторов. Чтобы избежать самотушения, второй Trp заменен на Ala и Glu. По сравнению с ранее известным Trp-Trp-Ala-Ser (WWAS) два новых тетрапептида Trp-Ala-Ala-Ser (WAAS) и Trp-Glu-Ala-Ser (WEAS) имеют более высокие квантовые выходы флуоресценции, более длительные времена жизни флуоресценции и более чувствительную реакцию на pH. В качестве новых генетически кодированных индикаторов pH N-концевые Trp-Ala и Trp-Glu могут быть соединены с белками для мониторинга значений pH окружающей среды при исследовании функциональных белков.

Ключевые слова: *флуоресцентный датчик, генетически закодированный индикатор pH, Trp-Ala-Ala-Ser, Trp-Glu-Ala-Ser, Trp-Trp-Ala-Ser.*

Introduction. In cells, pH values in various organelles are different; for instance, the interior of lysosomes and endosomes are slightly acidic, whereas activated mitochondria and the cytosol are slightly basic [1–5]. Maintaining intracellular pH in physiological ranges is crucial to a lot of normal cellular events such as cell growth, apoptosis, ion transport, enzyme activity, endocytosis, etc. [6–12]. Abnormal pH values have been reported in pathological cells; thus, monitoring the real-time pH value is important in cytodiagnosis. Fluorescent probes are versatile tools for visualizing and detecting intracellular pH because of their intrinsic simplicity and selectivity. So far, various pH sensors, including small fluorescent organic molecules [13, 14],

nanoclusters [15, 16], nanodots [17], and fluorescent proteins [18], have been reported. However, small size pH sensors with high quantum yield, low toxicity, and high location selectivity are still highly needed. Genetically encoded pH indicators have drawn the researcher's interests because they can be easily targeted to specific proteins by genetics [18].

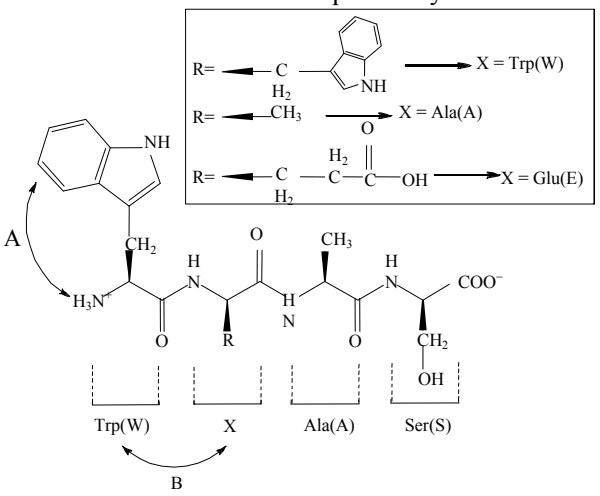
Tryptophan (Trp), as a natural amino acid and an intrinsic fluorescent probe, only responds at extreme pH conditions (pH < 4 and pH > 9), which are beyond the physiological pH range [19, 20]. In our previous report, N-terminal Trp-Trp dipeptides have been demonstrated to be effective genetically encoded pH indicators in the physiological pH range [21]. By comparing the fluorescence dynamics of Trp-Trp and its three derivatives, we proposed that the interactions between the exposed amino and the first Trp residual were pivotal for the pH response [21]. However, the performance of the N-terminal Trp may be influenced by its adjacent amino acid residue [22–25]. Recent studies by ultrafast fluorescence spectroscopy indicated that noncovalent interactions existed between adjacent Trp and Trp residues, which leads to lower fluorescence quantum yields and faster fluorescence decay [22]. Therefore, substituting the second Trp residue adjacent to the N-terminal Trp by other amino acids could potentially improve the performance of the genetically encoded pH indicator in the physiological pH range. In this paper, we designed two tetrapeptides, Trp-Ala-Ala-Ser (WAAS) and Trp-Glu-Ala-Ser (WEAS), by substituting the second Trp by Ala and Glu, and studied the pH response of these two tetrapeptides through steady-state fluorescence and time-resolved fluorescence spectroscopies. Both tetrapeptides showed higher fluorescence quantum yields and longer fluorescence lifetimes compared with previous reported N-terminal Trp-Trp dipetides. Thus, the pH response in the physiological pH range is more sensitive. The results suggest that the N-terminal Trp–Ala and Trp–Glu residues are better choices than the N-Trp-Trp residue in the design of genetically encoded pH probes.

Experiment. Trp-Ala-Ala-Ser (WAAS), Trp-Glu-Ala-Ser (WEAS), and Trp-Trp-Ala-Ser (WWAS) with purity higher than 98% were purchased from GL Biochem and used without further purification. Na_2HPO_4 and NaH_2PO_4 were purchased from Sinopharm Chemical Reagent and used to prepare 0.1 M PBS buffers (pH 6.0–8.0). CH_3COOH and CH_3COONa were purchased from Snopharm Chemical Reagent and used to prepare the 0.1 M CH_3COO^- buffer (pH 5.5). Tris-HCl and Tris-Base were purchased from Sigma-Aldrich, and used to prepare the 0.1 M Tris buffer (pH 8.5, 9.0). $NaHCO_3$ and Na_2CO_3 were purchased from Sinopharm Chemical Reagent and used to prepare the 0.1 M $NaHCO_3$-Na_2CO_3 buffer (pH 9.5, 10.0). $CaCl_2$, $ZnSO_4$, $CuSO_4$, $FeSO_4$, $FeCl_3$, and $Al(NO_3)_3$ were purchased from Sinopharm Chemical Reagent. $MgSO_4$ was purchased from Shanghai Senhao Fine Chemical Co., Ltd. All chemicals were analytical grade. Ultrapure water with a high resistivity (>18.2 MΩ/cm) was used in all the experiments. LUDOX(R) TMA colloidal silica (34 wt. % suspension in H_2O) was purchased from Sigma-Aldrich and diluted to 0.34% to measure the instrument response function (IRF).

The steady-state absorption spectra were obtained with a UV-Vis spectrometer TU1901 (Beijing Purkinje General Instrument Co. Ltd.). The steady-state fluorescence spectra were characterized with a commercial fluorometer FluoroMax-4 (Horiba). The picosecond resolved fluorescence decay profiles were measured by a homemade TCSPC system, consisting of a 295 nm picosecond pulsed diode laser PDL 800-B (PicoQuant) with a repetition rate of 10 MHz, a single photon counting PMT detector PMA-165 (PicoQuant, Germany), and a standalone TCSPC electronics PicoHarp 300 (PicoQuant). The full width at half maximum (FWHM) of the IRF curve obtained from the scattering of SiO_2 particles was 190 ps for the TCSPC system. The details of this system have been described elsewhere [21, 22]. (20 μM) WAAS, WEAS, and WWAS were selected to perform the optical measurements to avoid self-quenching or re-absorption.

Results and discussion. *Steady-state fluorescence of tetrapeptides.* We first studied the steady-state fluorescence spectra of WAAS and WEAS in the physiological pH range as shown in Fig. 1. The fluorescence intensities of WAAS and WEAS increased by ca. 3.5-fold when the pH values were changed from 5.0 to 9.5 (Fig. 1, insets). The increased fluorescence intensity of both tetrapeptides as pH increased indicates that it has a good pH response in the physiological pH range. The quantum yields of WAAS and WEAS were measured under various pH values and compared with the previously studied WWAS [21]. The quantum yield values shown in Fig. 2 were measured relatively to that of tryptophan, which was generally regarded as 0.13 at pH 7.0 [26]. It is clear in Fig. 2 that WAAS and WEAS have higher luminous efficiency than WWAS in the physiological pH range. Under acid pH conditions, the quantum yields of WAAS and WEAS were only slightly higher than that of WWAS. However, under basic pH conditions, the quantum yields of WAAS and WEAS were much higher than that of WWAS. Because of the sharp increase of the fluorescence intensity and higher quantum yields, pH sensitivities of WAAS and WEAS are higher than for WWAS in the physiological pH range, which suggests that WAAS and WEAS are better pH indicators than

WWAS. As illustrated by the processes *A* and *B* in scheme 1, the interactions between the fluorescent indole group and its neighboring exposed amino group ($-NH_3^+$) and the second amino acid residues (–X–) are pivotal to the pH response of these pH probes. The group $-NH_3^+$ rich in acid environments is an efficient quencher to indole, while the group $-NH_2$ rich in basic environments is not. For individual Trp molecules, the pKa of $-NH_3^+$ is ca. 9.5 (Fig. S1, supporting information). However, in these dipeptides the second amino acid residues make the pKa of $-NH_3^+$ move to the physiological pH range. The fluorescence of Trp can also be influenced by its neighboring amino acids. For example, energy transfer exists between adjacent Trp and Trp residues, which leads to lower fluorescence quantum yields and faster fluorescence decay [22].



Scheme 1. Chemical structures and acronyms of the tetrapeptides WWAS, WAAS, and WEAS.

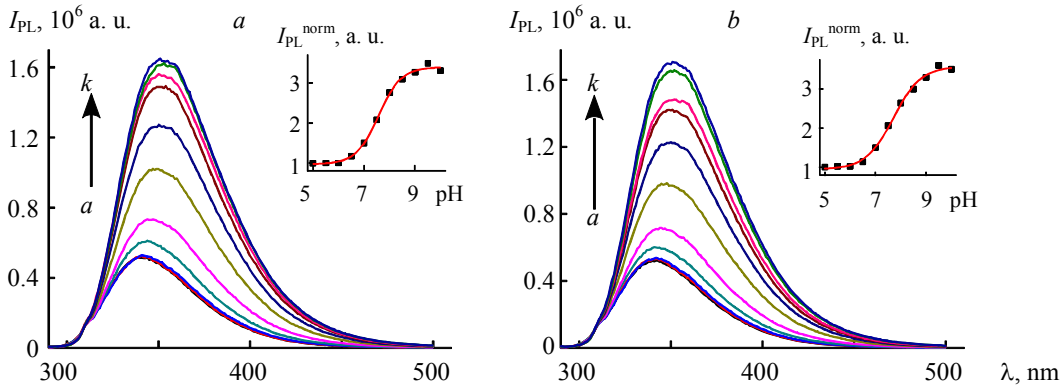


Fig. 1. Steady-state fluorescence (λ_{exc} = 280 nm) spectra of WAAS (a) and WEAS (b) in buffers with pH 5.0 (1), 5.5 (2), 6.0 (3), 6.5 (4), 7.0 (5), 7.5 (6), 8.0 (7), 8.5 (8), 9.0 (9), and 10 (10). The insets in (a) and (b) show the dependence of the normalized fluorescence intensity on the pH values. The squares are experimental data, and the curves show the fitting by a sigmoidal function $y = a + b/(1 + e^{-k(pH - c)})$. The parameters (*a*, *b*, *c*, and *k*) are 0.97, 2.42, 7.57, 2.26 for panel (a) and 0.94, 2.63, 7.71, 1.83 for panel (b), respectively.

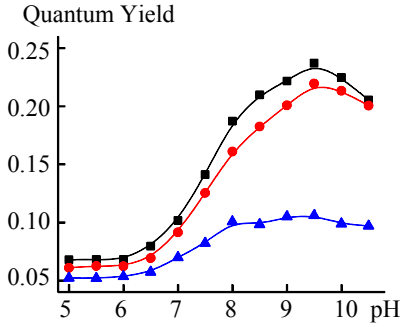


Fig. 2. The quantum yields of WAAS (■), WEAS (●), and WWAS (▲) under various pH buffer solutions. The quantum yield values were determined relatively to that of tryptophan (0.13 at pH 7.0) [26].

When the second Trp residue was substituted by some other residues, such as Ala or Glu, the process of energy transfer from the first Trp residue was hindered to a certain degree, which promotes radiative transition and leads to higher fluorescence quantum yields.

Time-resolved fluorescence of tetrapeptides. The time-resolved fluorescence spectra of WAAS and WEAS were also measured under various pH conditions. Figure 3 show the time-resolved fluorescence decay curves of WAAS and WEAS measured in buffers with various pH values. A tri-exponential decay model ($I(\lambda,t) = \sum_{i=1}^{3}\alpha_i(\lambda)e^{-t/\tau_i}$) was used to fit the decay curves, where α_i is the fractional amplitude, and τ_i is the respective lifetime. The average fluorescence lifetime was obtained through $\hat{\tau} = \sum_{i=1}^{3}\alpha_i\tau_i^2 \Big/ \sum_{i=1}^{3}\alpha_i\tau_i$. The insets in Fig. 3 show that the average fluorescence lifetimes of WAAS and WEAS increased with pH values as well. A sigmoidal function $y = a + b/(1 + e^{-k(\mathrm{pH}-c)})$ could be used to describe the dependence of fluorescence lifetimes on pH values. By comparing the fluorescence lifetimes of WAAS and WEAS with that of WWAS, an obvious difference was noticed. At pH 9.5, the average fluorescence lifetimes were 5.0 ns for WAAS and WEAS, but 2.8 ns for WWAS. At pH 5.0, the average fluorescence lifetimes of WAAS, WEAS, and WWAS were 1.4, 1.3, and 1.4 ns, respectively. The fluorescence lifetimes of WAAS and WEAS also gave higher pH sensitivity than WWAS. When the pH values were increased from 5.0 to 9.5, the fluorescence lifetime of WAAS and WEAS increased by 3.8 fold but by 2 fold for WWAS. One advantage of the time-resolved fluorescence measurement is that the fluorescence lifetime is stable over the variation of many experimental factors, such as the fluctuation of excitation power, concentration of fluorophore, and the detection efficiency of the optical–electronic system. Thus, the pH values could be quantitatively determined from the lifetimes of the WAAS/WEAS and the sigmoidal function.

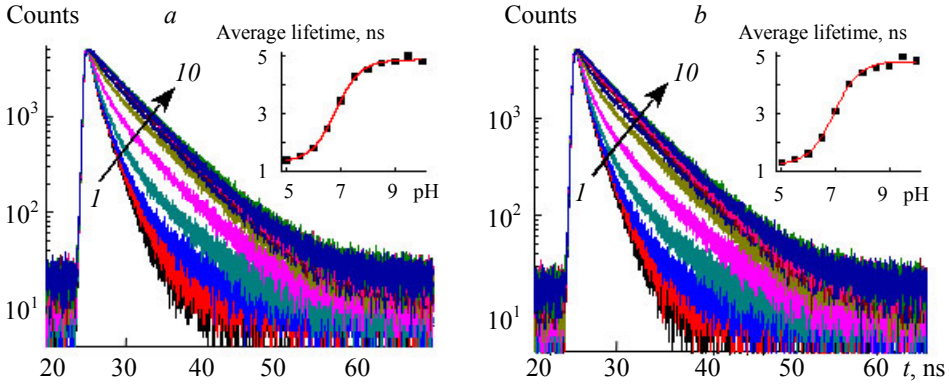


Fig. 3. Time-resolved fluorescence decay curves of WAAS (a) and WEAS (b) in buffers with pH 5.0 (1), 5.5 (2), 6.0 (3), 6.5 (4), 7.0 (5), 7.5 (6), 8.0 (7), 8.5 (8), 9.0 (9), and 10 (10). The insets in (a) and (b) show the dependence of the average fluorescence lifetimes on the pH values. The squares are experimental fluorescence lifetimes. They are 1.4, 1.5, 1.8, 2.5, 3.4, 4.3, 4.5, 4.7, 4.8, 5.0, 4.8 ns for WAAS (panel a) and 1.3, 1.4, 1.6, 2.2, 3.1, 4.0, 4.4, 4.6, 4.7, 5.0, 4.8 ns for WEAS (panel b) for the pH values increased from 5.0 to 10.0 with a step of 0.5. The experimental fluorescence lifetime values may be fitted by a sigmoidal function $y = a + b/(1 + e^{-k(\mathrm{pH}-c)})$. The parameters (a, b, c, and k) are 1.24, 3.57, 6.97, 2.18 for panel (a) and 1.32, 3.54, 6.82, 2.19 for panel (b), respectively. λ_{exc} = 298 nm and λ_{det} = 350 nm.

Interference experiments. The interference of metal ions on the WAAS and WEAS pH indicators was also studied by the steady-state and time-resolved fluorescence techniques under the physiological conditions (PBS buffer, pH 7.4, 10 mM). In the presence of some metal ions, such as Mg^{2+}, Al^{3+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, and Zn^{2+}, fluctuation of fluorescence intensity can be observed for WAAS (Fig. 4a) and WEAS (Fig. 4c). Especially, in the presence of Fe^{2+} and Cu^{2+}, an obvious fluorescence quenching was observed. However, even in the presence of various metal ions, the time resolved fluorescence decay curves overlapped well with the control curves (Fig. 4b,d), and no remarkable changes in the fluorescence lifetime were observed, which implies that the fluorescence lifetimes of WAAS and WEAS could be used to quantitively measure the pH values without interference of metal ions. These phenomena indicate that the fluorescence of WAAS and WEAS may be quenched by metal ions through static quenching. Especially Fe^{2+} and Cu^{2+} may combine with the WAAS and WEAS tetrapeptides and form some complexes with non-radiative processes [27].

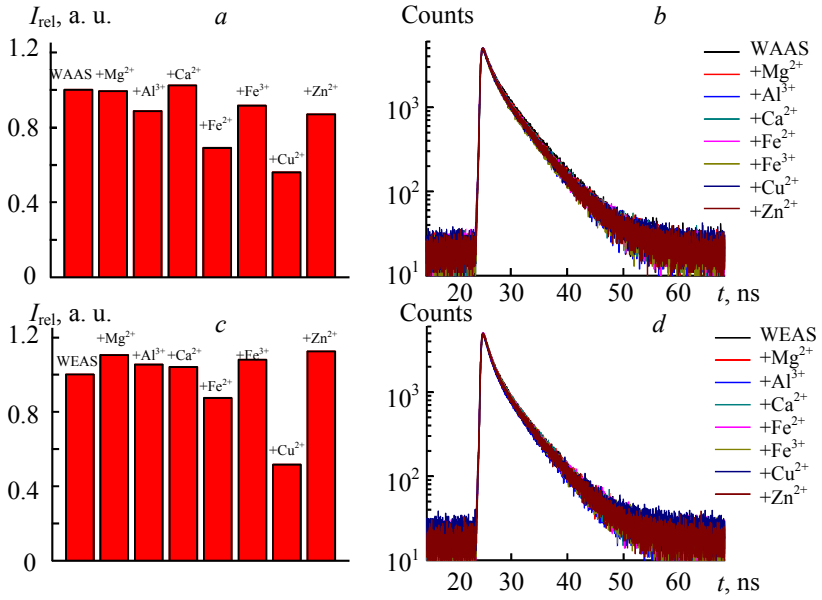


Fig. 4. Relative variations of steady-state fluorescence (λ_{exc} = 280 nm) intensity of WAAS (a) and WEAS (c) in the presence of various metal ions, and time-resolved fluorescence decay curves of WAAS (b) and WEAS (d) in the presence of various metal ions, λ_{exc} = 298 nm and λ_{dec} = 350 nm. The ratio of tetrapeptides to metal ions was 1:1. All these experiments were performed in PBS buffers (pH 7.4, 10 mM).

Conclusion. N-terminal Trp-Trp has been demonstrated as a genetically encoded pH indicator in the physiological pH range, and the N-terminal Trp was found pivotal for the pH response. In this paper, we substituted the second Trp with Ala and Glu, and studied the influence of the second amino acid. Compared with the WWAS, the two new tetrapeptides, Trp-Ala-Ala-Ser (WAAS) and Trp-Glu-Ala-Ser (WEAS), have higher fluorescence quantum yields, longer fluorescence lifetimes, and more sensitive pH responses. At pH 9.5, the fluorescence quantum yields were found to be 0.24 for WAAS and 0.22 for WAES, but only 0.1 for WWAS. The fluorescence lifetimes were 5.0 ns for WAAS and WAES but 3.0 ns for WWAS at pH 9.5. When pH values were changed from 5.0 to 9.5, the fluorescence intensities were increased by ca. 3.5-fold for WAAS and WEAS but by 2-fold for WWAS. The fluorescence lifetime of WAAS and WEAS was increased by 3.8-fold but by 2 fold for WWAS. These results indicate that the second amino acid can influence the optical properties of the genetically encoded N-terminal Trp-X pH indicators. Although the absorption in the UV range limits its applications in cells, this research affords two novel genetically encoded pH indicators, the N-terminal Trp–Ala and Trp–Glu. They could be fused to proteins for monitoring the environmental pH values during the studies of functional proteins.

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The authors declare that they have no conflict of interest.

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