FLUORESCENCE QUENCHING ANALYSIS OF HISTIDINE-TAGGED ENHANCED GREEN FLUORESCENT PROTEIN

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Proteina cu fluorescenţă verde (EGFP) nu necesită adăugarea unor substrate exogene pentru emisia luminii fluorescente. La capătul N- terminal al genei codificatoare a proteinei EGFP s-a fuzionat o regiune specifică de şase histidine, astfel proteina fluorescentă recombinată (His6-EGFP), obţinută prin expresie heterologă în sistem bacterial, s-a purificat prin cromatografie de afinitate cu ioni metalici imobilizaţi (Ni^{2+}). S-a testat efectul unor ioni metalici bivalenţi asupra fluorescenţei His6-EGFP. S-a observat că ionii de cupru, chiar în concentraţii mici, reduc intensitatea fluorescenţei emise a His6-EGFP. S-a evaluat şi stabilitatea structurală a acestei proteine în condiţii reducătoare în prezenţă de clorhidrat de guanidină.

Enhanced green fluorescent protein (EGFP) can emit fluorescent light without the addition of any exogenous substrate. A six histidine coding region was fused to the N -terminal end of the EGFP coding gene, thus the recombined fluorescent protein (His6-EGFP), obtained by heterologous expression in a bacterial system was purified by immobilized metal (Ni^{2+}) affinity chromatography. The effect of some bivalent metal ions was tested on the fluorescence of His6-EGFP. Copper ions, even in low concentration, are shown to reduce the fluorescence emission of His – tagged EGFP. The stability of this protein was also assessed in the presence of the reducing agent guanidine hydrochloride.

Keywords: fluorescence, metal ion, chromophore, denaturation, quenching

1. Introduction

Green fluorescent protein (GFP) is a bioluminescent protein, that was isolated from the jellyfish *Aequorea victoria*, and it is an exceptionally versatile and useful tool in cell biology and biotechnology [1].

The color originates from a fluorescent entity, a chromophore group, that is generated in the protein's interior by interaction of three consecutive amino-

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acid residues (Ser65,Tyr66, Gly67), that are part of the protein's polypeptide chain [3]. The chromophore is generated only under permissive conditions of protein folding. The polypeptide must be able to obtain its native three-dimensional structure to become visibly fluorescent. Chromophore formation in GFP-like proteins is a highly unusual posttranslational modification that spontaneously follows protein folding and requires molecular oxygen. What is unique to the fluorescent protein is that the location of this peptide triplet resides in the center of a remarkably stable barrel structure consisting in 11 beta-sheets folded into a tube [2].

Fluorescence occurs after a molecule known as a chromophore absorbs photons. The molecule is raised to an excited state, as a result of electron transfer from a ground state to a higher energy level. As the electrons drop back to the ground state, they emit energy in the form of light. The intensity of fluorescence can be decreased by a wide variety of processes. Such decreases in intensity are called quenching [1].

GFPs are used as reporters of gene expression, tracers of cell lineage, and as fusion tags to monitor protein localization within living cells. The native GFP has some deficiencies such as low brightness, a significant delay between protein synthesis and fluorescence development. This protein was re-engineered, several mutations have been made to the wild type protein (wtGFP) to improve one or more characteristics of the protein [4]. The new variants have shifted excitation and emission wavelength, creating different colors for different applications [2].

Enhanced green fluorescent protein (EGFP), also known as GFPmut 1:23, has two mutations in the chromophore region and have a single excitation peak at 488 nm and fluoresces with greater intensity than the wild type protein [3]. The excitation maximum of the green fluorescent protein is shifted in the cyan region by introducing a single point mutation, by serine substitution at position 65 with a threonine residue (S65T). The GFP-S65T mutant also has an added advantage of acquiring fluorescence approximately four times faster than the wild-type GFP [2]. The only drawbacks for using EGFP are a slight sensitivity to pH and a weak tendency to dimerize [3]. 2 -5 GFP contains three additional mutations (V163A, I167T and S175G), which increase the thermal stability and facilitate the *in vivo* maturation [11].

The major advantage of using GFP-like reporters is that GFP is inherently fluorescent and its fluorescence is not species-specific, while other bioluminescent reporters require additional proteins, substrates, or cofactors to emit light. Another advantage of GFP is its ability to alter its stability and spectral properties through structural alterations, and thus produce mutants with improved fluorescence intensity, thermostability, and chromophore folding [2].

Similarly, it is possible to convert this fluorescent protein into a biosensing element that is capable to monitor metal ions. Metals in close

proximity to chromophore are known to quench fluorescence in a distance-dependent manner [5]. The structure of GFP was redesigned by using the genetic engineering tools in such a way a metal binding site is created in the proximity of chromophore group [5 – 8]. As the presence of metal ions in close proximity to a chromophore can result in fluorescence quenching, this newly introduced functional property allow the protein to be used as a metal sensor in biological and environmental systems [6].

It is a common practice in protein engineering to supplement recombining proteins with hexa-histidine tags to facilitate their purification. It was shown that the presence of such a tag by itself increases the sensitivity of GFP fluorescence for quenching by copper ions [9, 10]. However, conflicting data were reported on the efficacy of Cu ion as quencher.

Isarankura-Na-Ayudhya *et al.* [10] found that in the presence of 1000 μM Cu^{2+} the fluorescence intensity decreased by about 60%, while Jung *et al*. [9] reported about 8% decrease under similar conditions. Most probably the reason of the discrepancy is the different mutant form of GFP; Isarankura-Na-Ayudhya *et al.* used EGFP, while Jung et al investigated 2-5 GFP. This form contains three so called surface-located folding mutations, which improve the *in vivo* maturation of the fluorescent form.

In order to clarify this conflict, we investigated again the effect of bivalent metal ions on the fluorescent properties of EGFP, and the stability of EGFP structure in solutions with high guanidine concentration studied as well.

2. Experimental

2.1. Cloning and expression of His-tagged EGFP

The gene encoding EGFP was cloned into a bacterial plasmid, pET 15b, in such a way that at the N terminal end of GFP a hexahistidine coding region was fused. The resulting plasmid pET-EGFP was a generous gift of L. Radnai (Dept. of Biochemistry, Eötvös Loránd University, Budapest, Hungary). BL21 Star (DE3) was used for expression *E. coli* strain. The results of the expression were analyzed by SDS-PAGE.

The obtained recombining protein was purified by immobilized metal (Ni^{2+}) affinity chromatography. The protein, containing an N-terminal 6xHistidine tag, was purified by affinity chromatography, by adsorption on a Ni-charged polymer matrix (Porfinity IMAC Ni-charged Resin, BioRad). Purified protein was dialyzed against 50 mM sodium phosphate buffer.

2.2. Analysis of His –tagged EGFP denaturation in guanidine solution

Different concentrations of guanidine hydrochloride solution (0.5-5M) have been used in these experiments. The protein concentration in the denaturing solutions was in low micromolar range. After 16 hours incubation at room

temperature, the fluorescence intensity of denatured EGFP was measured by a FluoStar OPTIMA fluorimeter. Excitation wavelength was 485 nm and emission wavelength was 520 nm.

2.3. Fluorescence quenching by metal ions

Fluorescence measurements were carried out using a Fluo Star OPTIMA fluorimeter at 298 K. Excitation and emission wavelengths were assayed at 485 and 520 nm, respectively. Samples containing the protein (His6EGFP) in low μM range in 50 mM Na_2HPO_4, pH 7, were mixed with $CuSO_4$, $ZnSO_4$, and $FeSO_4$, or dissolved in ultra pure water to yield the final concentrations in the range of 0.1–1000 μM.

3. Results and discussion

3.1. Analysis of His-tagged EGFP denaturation in guanidine solution

Guan idine hydrochloride (GuHCl) is a chaotropic agent, which disrupts the arrangement of water molecules around the hydrophillic regions of proteins determining the proteins to denature (unfold). It apparently disrupts hydrogen bonds, which hold the protein in its unique structure, so disrupt the molecular structure of the protein. Protein unfolds because guanidine hydrochloride forms hydrogen bonds with all the groups on the protein which can form hydrogen bonds.

GFP gives strong fluorescence only when the chromophore is kept inside the protein barrel. In this work, the fluorescence spectroscopy has been used to study GFP denaturation by guanidine, as well as the effects of different fluorescence quenchers. Temporal change of the relative fluorescence intensity at different guanidine concentrations is shown in Figure 1.

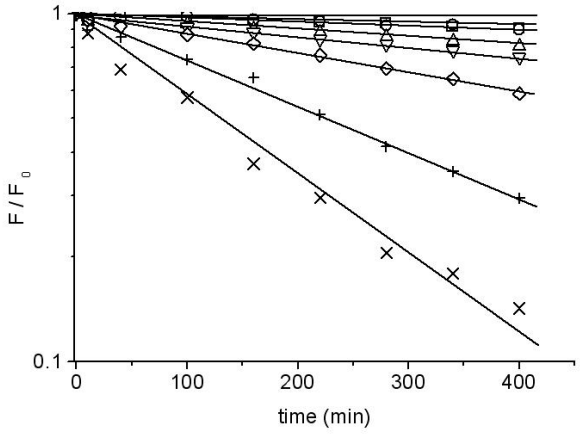

Fig.1.Temporal change of the fluorescence intensity at different GuHCl concentrations (from top to bottom): 0 M (no symbols; 1 M (□); 1.2 M (○); 1.6M (Δ); 1.8 M (∇); 2.0 M (◊); 2.2 M (+); 2.4 M (x)

The fluorescence intensity decreases during denaturation, as one can observe. As the GFP molecule unfolds, the chromophore converts into a non-fluorescent state. Water molecules penetrate into the protein molecule and protonate the chromophore. The protonated chromophore does not give fluorescence.

The decrease of fluorescence follows a first order kinetics. The rate constant for this process depends on the GuHCl concentration. The rate constant is about $8x10^{-3}$ min^{-1} in the presence of 2.4 M GuHCl. Jung et al. [9] measured the rate constant of the fluorescence decrease during denaturation of the protein and they found a value of $8x10^{-3}$ min^{-1} for 2-5 GFP in 4 M GuHCl. We got similar value but at significantly lower (2.4M) GuHCl concentration.

Protein denaturation was observed in different concentration of guanidine hydrochloride, after incubation at room temperature for 16 hours. The results of denaturation are shown in Fig. 2.

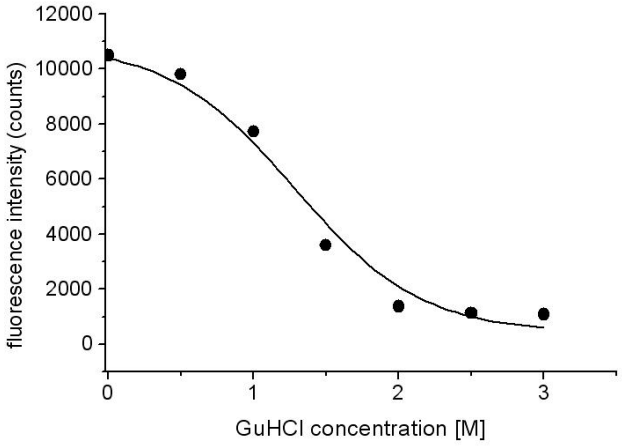

Fig. 2. Fluorescence of EGFP after 16 hours incubation in guanidine hydrochloride solutions

The equilibrium fluorescence data for the GuHCl induced EGFP unfolding can be approached with a curve describing the two-state equilibrium. The EGFP proved to be more sensitive to GuHCl induced denaturation, the midpoint of transition in our experiment was at 1.32 M GuHCl, while Jung et al. [9] reported 3.2M for 2-5 GFP. In the light of this stability difference, further studies are required to solve the mechanism of the GuHCl induced fluorescence decrease.

3.2. Fluorescence quenching by metal ions

Quenching can occur by different mechanisms. Collisional (dynamic) quenching occurs when the excited-state fluorophore is deactivated upon contact

with some other molecules in solution, which are called quenchers. In this case the fluorophore is returned to the ground state during a diffusive encounter with the quencher [1]. Cu^{2+} ion is a dynamic quencher interacting with excited states of chromophore. Copper ions penetrate inside the barrel and quench fluorescence through direct interactions with the chromophore [10].

We investigated the effect of different concentrations of copper ions on EGFP fluorescence emission. It was observed that the fluorescence intensity of EGFP decreased significantly in 1mM copper ion solutions, as shown in Fig.3. The fluorescence quenching by copper ions is proportional to copper ion concentration in the range of 50 μM - 1000 μM.

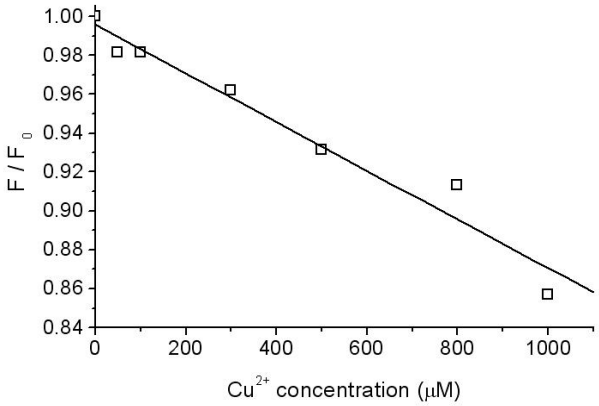

Fig. 3. EGFP's fluorescence quenching by copper ions

We also investigated the effect of various concentrations (0.5 μM to 500 μM) of divalent cations (Fe^{2+}, Zn^{2+}) on the fluorescent emission of the His6-GFP.

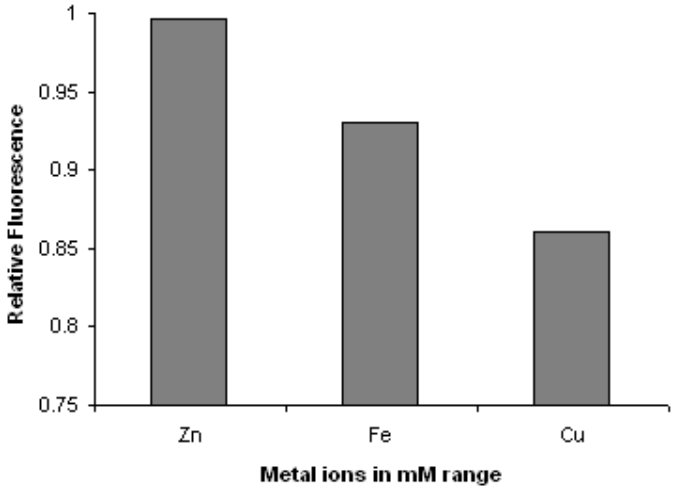

Fig. 4. EGFP's fluorescence quenching by different bivalent metal ions

As represented in Fig. 4, copper ions exerted the strongest suppressing effect on the fluorescent intensity of EGFP compared to the other metal ions. The remaining fluorescence of approximately 85% was found in the 1 mM $CuSO_4$ solutions. Up to 90% of fluorescent intensity remained in 1mM $FeSO_4$ solutions, while $ZnSO_4$ exerted practically no quenching.

4. Conclusions

Formation of nonfluorescent ground state complex constitutes the main cause for the static quenching. During the GFP denaturation by guanidine, the non-fluorescent state is formed by protonation. The chromophore group is protonated by water giving rise to the non-fluorescent form. Our data suggest a simple one step transition between the two states: native and unfolded protein.

The rate constant of the fluorescence decrease during denaturation of the protein was similar with that measured by Jung and it has been obtained at significantly lower GuHCl concentration (2.4M)

The His-tagged EGFP proved to be most sensitive to the bivalent copper ions, compared to the other investigated metal ions (Cu^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+}). In the case of EGFP, a linear correlation between fluorescence quenching and copper ion concentration in the range of 50 μM- 1000 μM has been obtained. The quenching was more than 15% at 1000 μM.

2-5 GFP Cu^{2+} is a significantly weaker quencher; the quenching was of about only 8% at 1000 μM. It is reasonable to suppose that the more efficient quenching by Cu^{2+} correlates with the higher sensitivity of EGFP to GuHCl denaturation. A more flexible structure allows easier penetration of the quencher.

At present it is not clear what could be the reason of the extremely efficient quenching reported by Isarankura-Na-Ayudhya et al. [10].

Sensitivity and specificity of EGFP for the metal binding could be increased by insertion of an engineered metal specific region instead of the non-specific polyhistidine-tag. The next stept step of this research is the improving of the sensitivity of EGFP for copper ion by creating a metal binding region near the chromophore group of EGFP. This metal binding site will be introduced by substituting some amino acids with histidine, using site directed mutagenesis. It is also intended to develop a fluorescent protein based biosensor for copper ion determination in environmental samples.

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