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ENGINEERED GREEN FLUORESCENT PROTEIN AS A POTENTIAL METAL SENSOR

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ABSTRACT. The designed biosensor is based on a mutant form of green fluorescent protein (GFP). It is known that metal ions in close proximity to a cromophore group reduce the protein' s fluorescence intensity by fluorescence quenching. Using site directed mutagenesis, we formed a metal binding site near the protein's chromophore group. Thus, the mutant protein presents a new functional property, which allows its utilization in detection of metal ions.

Keywords: green fluorescent protein, metal binding, site-specific mutation, chromophore group, biosensor

INTRODUCTION

Green fluorescent protein (GFP) is a bioluminescent protein, that was isolated from the jellyfish *Aequorea victoria*. 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-acid residues (Ser65,Tyr66, Gly67), that are part of the protein's polypeptide chain [2]. The chromophore is generated only under conditions permissive of protein folding [3].

Site-directed mutagenesis investigations have revealed that fluorescence is very dependent on the three-dimensional structure of amino acid residues that enclose the chromophore group [4]. Denaturation of the protein results in the loss of fluorescence and mutations in residues immediately adjacent to the chromophore can significantly alter the fluorescent properties of the protein [5]. Moreover, the amino acid substitutions in regions of the polypeptide far removed from the chromophore can also affect the spectral characteristics of the protein [4].

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EGFP (Enhanced Green Fluorescent Protein) has two mutations (F64L, S65T) in the chromophore region and have a single excitation peak at 488 nm and fluoresces with greater intensity than wild type protein [6]. GFP are used as reporters of gene expression, tracers of cell lineage, and as fusion tags to monitor protein localization within living cells [7].

The structure of GFP was redesigned by using the genetic engineering tools, in such way that was created a metal binding site in the proximity of chromophore group [8, 9, 10]. As the presence of metal ions in close proximity to a chromophore can result in fluorescence quenching, this newly introduced functional property allows the protein to be used as a metal sensor in environmental systems [8]. These mutants represent a new class of protein-based metal sensors, thats can report metal ion concentrations without the use of any exogenous modification reagents.

It is a common practice in protein engineering to supplement recombinant 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 [11].

In earlier studies we examined the sensitivity of His-tagged EGFP for some metal ions. As a result of these studies we obtained that copper and ferrous ions in millimolar ranges reduced the fluorescent intensity of this protein. However for biosensor application of this protein further improvement of metal ion sensitivity and selectivity is. needed.

Starting from idea that the presence of metal ions in close proximity to the chromophore can result in fluorescence quenching, it is possible to create a prototype suitable for biosensor applications by engineering of a specific metal binding site in GFP structure that couples metal binding to modified fluorescent signals.

We investigated the structure of this protein for searching the target amino acid positions for substitution with histidines. These positions have to comply for two requirements: do not affect the formation of native conformation of this protein, and have to be in close proximity to chromophore group.

Therefore we designed and constructed a metal binding mutant of EGFP. We investigated the stability of mutant protein, and the sensitivity of protein fluorescence for quenching by some bivalent metal ions.

RESULTS AND DISCUSSION

Design of metal binding site on the surface of EGFP

The structure of GFP is known, so we can design directed modifications in the structure of GFP that confer a metal binding capacity to the GFP. The target site of modification is mostly on 7–10 β -sheets of the GFP β- barrel.

In this region of GFP can observe an irregularly large space between β -strands [10]. Therefore, this region is a potential target site for rational design of a metal binding site. As binding of analytes to this engineered binding site may alter the fluorescent properties of the GFP chromophore [11]. Taken these facts into consideration we choose two positions as targets for substitution with histidines, the serine in position 202 and the glutamine in position 204. These amino acids are close proximity to chromophore group.

For the designed mutant protein was constructed a structure model using the PyMOL molecular modelling package. Figure 1 show the structure model of the native EGFP and the mutant EGFP.

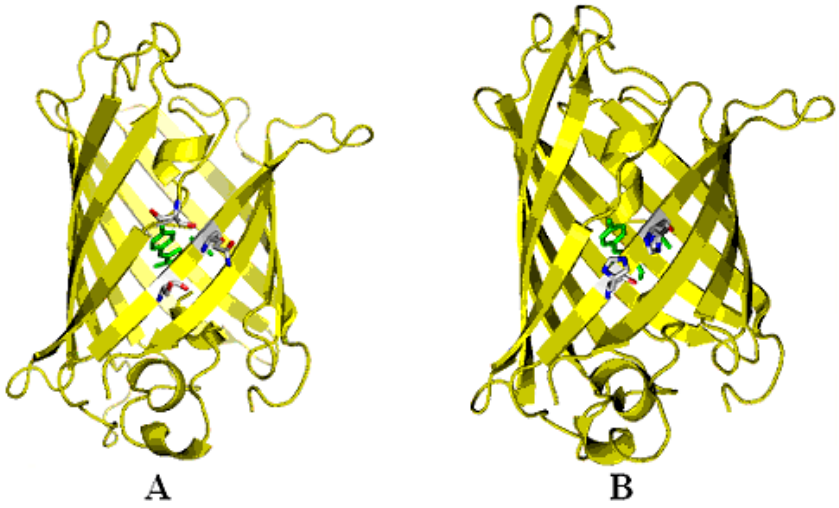

Figure 1. The 3 D structure of EGFP**.** A- native form**,** B- mutant form. The chromophore group of the protein are colored green, A - in gray are represented the target site for the mutation (202 serine and the 204 glutamine), B- in gray are represented the introduced hisitidines in positions 202 and 204

Expression and purification of the mutant protein

The mutant EGFP contains a His tag at the N terminal end of the polypeptid chain, that help in the purification. The mutant protein was purified by adsorption on a Ni-charged polymer matrix. The molecular weight of the mutant EGFP is 28 kDa. Figure 2 shows the result of the protein expression and purification, verified by SDS polyacrylamide gelelectroforesis.

The mutant protein was produced in relativly large quantities and by affinity chromatography purification we obtained the mutant protein in high purity.

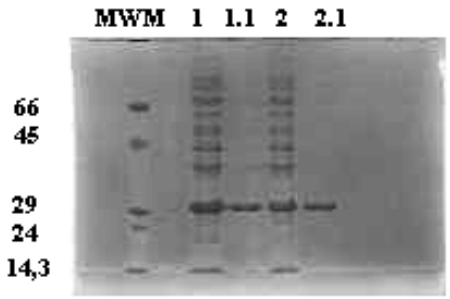

Figure 2. The verification of the mutant protein expression and purification. MWM- molecular weight marker, 1, 2- proteins in the cell lysate, 1.1, 1.2. – purified EGFP mutant

Analysis of mutated EGFP denaturation in guanidine solution

GFP gives strong fluorescence only when the chromophore is kept inside the protein barrel. Since the fluorescence of GFP is linked to its properly folded structure, it is therefore possible to use the fluorescence of GFP as an indication of its properly folded structure *[12]*.

In this work, we have used the fluorescence spectroscopy to study the denaturation of mutated EGFP by guanidine hydrochloride. Guanidine hydrochloride (GuHCl) is chaotropic agent, which disrupts the arrangement of water molecules around the hydrophillic regions of proteins to cause the proteins to denature. It apparently disrupts hydrogen bonds, which hold the protein in its unique structure. However, there also is evidence suggesting that GuHCl may disrupt hydrophobic interactions by promoting the solubility of hydrophobic residues in aqueous solutions.

Temporal change of the relative fluorescence intensity at different GuHCl concentrations is shown in Figure 3.

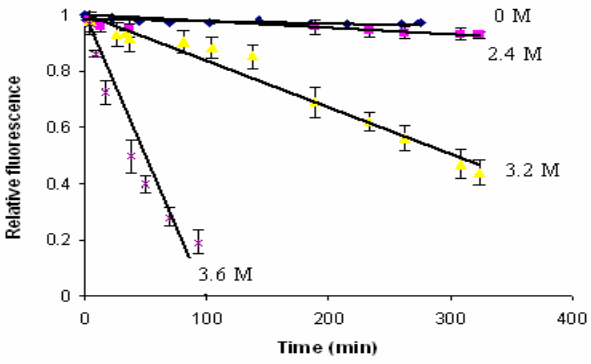

Figure 3. Temporal change of the fluorescence intensity at different guanidine hydrochloride concentrations

It was observed that the fluorescence intensity decreases during denaturation. As the GFP molecule unfolds, the chromophore converts into a non-fluorescent state. Water molecules penetrate into the protein's interior and protonate the chromophore. The protonated chromophore does not give fluorescence.

Protein denaturation was also observed in different concentration of guanidine hydrochloride, after incubation at room temperature for 20 hours. The results of denaturation are shown in Figure 4.

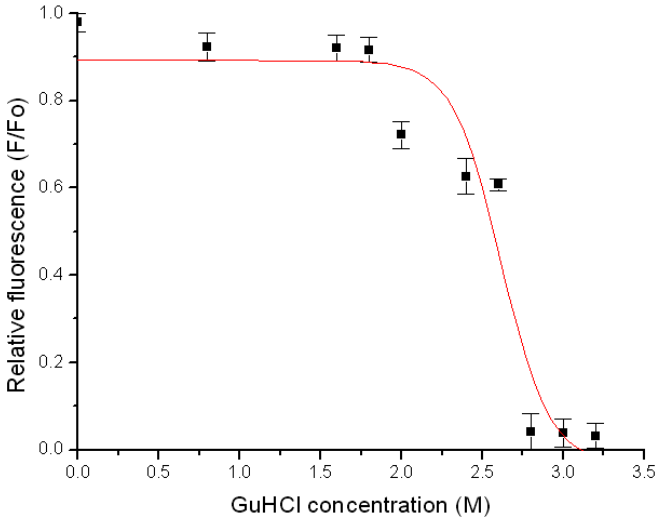

Figure 4. Fluorescence of EGFP after 20 hours incubation in guanidine hydrochloride solutions

The equilibrium fluorescence data for the GuHCl induced unfolding of EGFP can be approached with a curve describing two-state equilibrium. The midpoint of transition is at 2,41 M GuHCl.

Effect of bivalent metal ions on fluorescence of EGFP

The intensity of fluorescence can be decreased by a wide variety of processes. Such decreases in intensity are called quenching. Quenching can occur by different mechanisms. Collisional quenching (dynamic) occurs when the excited-state fluorophore is deactivated upon contact with some other molecule in solution, which is called the quencher. In this case the fluorophore is returned to the ground state during a diffusive encounter with the quencher [13].

We investigated the effect of various concentrations (0.5 μM to 500 μM) of divalent cations (Fe^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}) on the fluorescent emission of the mutated EGFP.

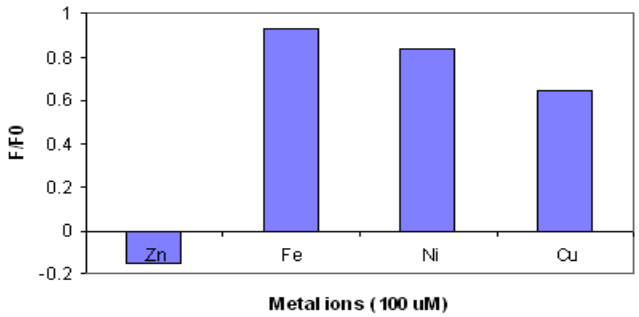

Figure 5. Relative fluorescence of mutated EGFP in the presence of different metal ions

As represented in Figure 5, copper ions exerted the strongest suppressing effect on the fluorescent intensity of mutant EGFP compared to another metal ions. The remaining fluorescence of approximately 60% was found in the 0,1 mM $CuSO_4$ solutions. Meanwhile, 93% of fluorescent intensity remained in 0,1 mM $FeSO_4$ solutions and in 0,1 mM $NiSO_4$ solution the remaining fluorescence was 84%. Surprisingly in the $ZnSO_4$ solution the fluorescence intensity of mutant protein increased by 15%.

We investigated the effect of different concentration of copper ions on fluorescence emission of mutant and native EGFP. It was observed that the fluorescence intensity of mutated EGFP decreased proportionally with the increasing of copper ion quantity in solutions, as show Figure 6. As this figure show the mutant protein's fluorescence intensity decreased more significantly than the fluorescence of native protein.

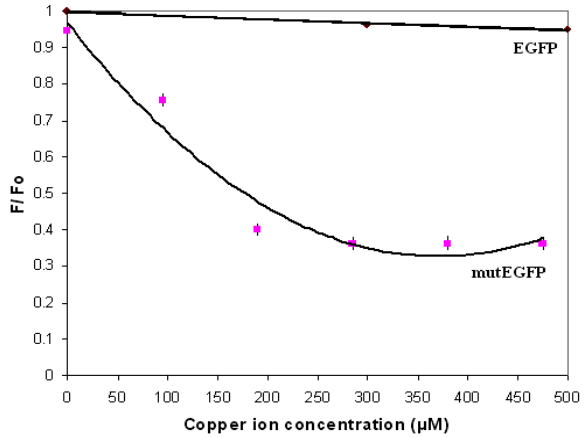

Figure 6. Relative fluorescence of native and mutant EGFP in different copper ion solution

In the case of mutant EGFP the fluorescence quenching occurred by copper ions is proportionate to copper ion concentration, in the range of 50 μM - 400 μM copper ion concentration.

CONCLUSIONS

During the GFP denaturation by guanidine hydrochloride, the protein unfolds and the chromophore group become into the surface of the protein, where is protonated by the water molecules. The protonated form is non-fluorescent. The guanidine hydrochlorid induced unfolding process enter into an equilibrium state after 20 hours incubation. It is evolved an equilibrium between the native and the denaturated form of protein.

Metal quenching process is highly concentration and distance dependent. To design a sensitive fluorescent protein-based biosensor for metal ions the structural orientation of metal binding site and its distance towards the GFP fluorophore have to be taken into consideration The location and distance of the imidazole ring need to be very close to the GFP fluorophore (>5 A°) in order to effectively quench the fluorescence [10].

Cu^{2+} ion is known as dynamic quencher interacting with excited states of chromophore. Copper ions penetrate inside the barrel and quench fluorescence through direct interactions with the chromophore [10]. Both the mutant EGFP and native EGFP show the highest sensitivity for copper ions. In the case of native EGFP we got a linear correlation between fluorescence quenching and copper ion concentration in the range of 50 μM - 1000 μM. At 1000 μM the quenching was more than 15%. The mutant protein is quenched at a much lower metal concentration than the native protein. This indicates that metal ions binds to the created metal binding site and this site is close enough to quench the fluorescence of EGFP.

The mutant EGFP is more sensitive to copper ions, than the native form. So we can conclude that due to the directed mutagenesis the sensitivity of EGFP for copper ions was increased significantly.

Further studies are needed to assay different metal ions, and to increase the affinity of the metal binding site.

EXPERIMENTAL SECTION

Site-directed mutagenesis

A histidine substituted EGFP mutant (S202H/S204H) was constructed using QuikChange® Site-Directed Mutagenesis Kit (Stratagene). Specific mutations were introduced into the pET- EGFP plasmid, by substitution of two triplets in EGFP coding sequence with triplets that encode histidines. This plasmid (pET-EGFP) was a generous gift of L. Radnai (Dept. of Biochemistry, Eötvös Loránd University, Budapest, Hungary).

The mutagenic primers coding for the desired mutations were as follows: S202H, Q204H-forward primer- CTACCTGCACACCCACTCCGC CCTGAGC, S202H, Q204H-reverse primer- GCTCAGGGCGGAGTG GGTGTGCAGGTAG. PCR mutagenesis was carried out according to the manufacturer's recommendations (Stratagene). Incorporation of the oligonucleotide primers generated a mutated plasmid containing staggered nicks. The PCR products were digested by DpnI to eliminate the template plasmid, which did not contain the desired mutations and was transformed into supercompetent *XL1 Blue* cells.

Expression of the mutant protein

The mutant plasmid was isolated from transformed *XL1 Blue* cells, harbouring the mutated plasmid. The resulting pET-EGFPmut plasmid was cloned into *E. coli* cells. Expression of the mutant proteins was carried out in *E. coli* strain BL21 Star (DE3). The results of the expression were analyzed by SDS-PAGE.

The obtained recombinant protein was purified by immobilized metal (Ni^{2+}) affinity chromatography. The protein, containing an N-terminal 6xHistidine tag, was purified by adsorption on a Ni-charged polymer matrix (Porfinity IMAC Ni-charged Resin, BioRad). Purified protein was dialyzed against 20 mM sodium phosphate buffer, with pH 7.

Structural stability analysis of mutated EGFP

In these experiments we used different concentration of guanidine hydrochloride solutions (0,5-5 M). The protein quantity in the denaturing solutions was 10 micromols. After 20 hour incubation at room temperature (298 K), the fluorescence intensity of denatured mutant EGFP was measured by a FluoStar OPTIMA fluorimeter, the excitation wavelength was 485 nm and emission wavelength was 520 nm. For the fluorescence measurments we used U-bottomed 96 well Greiner microtiter plates. The denaturing solution contained the corresponding guanidine hydrochloride dissolved in 20 mM sodium phosphate buffer (pH 7). The final volume of samples in the microplate wells was 100 microliters. All measurments were executed at 298 K.

Effect of bivalent metal ions on fluorescence of EGFP

Fluorescence measurements were carried out using a FluoStar OPTIMA fluorimeter at 298 K. Excitation and emission wavelengths were assayed at 485 and 520 nm, respectively. Samples containing the protein (mutant EGFP) in low μM range in 20 mM Na_2HPO_4, pH 7, were mixed with $CuSO_4$, $ZnSO_4$, $NiSO_4$ and $FeSO_4$ dissolved in ultrapure water to yield the final concentrations in the range of 10–500 μM. The final volume of samples in the microplate was 100 microliters.

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