Construction of a Chimeric Histidine6-Green Fluorescent Protein: Role of Metal on Fluorescent Characteristic

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Abstract

A chimeric green fluorescent gene encoding six histidine residues at the 5/-end was constructed by genetic engineering. Synthetic oligonucleotides (5/-AGCTTACACCATCACCATCACCATGCGAG CTCTGCGGTAC- 3/ and 5/- CGCAGAGCTCGCATGGTGATGGTGATGGTGTA- 3/) were fused in-frame to the 5/-terminal end of the green fluorescent gene (GFPuv). The bacterially expressed chimeric fluorescent protein (His6GFP) was purified by immobilized metal (Zn^{2+}) affinity chromatography. The purified protein was tested for metal binding and the group of divalent metal ions (Zn^{2+}, Cu^{2+}, Cd^{2+}, and Ni^{2+}) was shown to reduce fluorescence emission of green fluorescent protein. Interestingly, the fluorescence of His6GFP was more sensitive to metal ions than the native GFP particularly with copper and zinc. The fluorescence emission of His6GFP declined to half in the presence of 150 μM $CuSO_4$ or 3.1 mM $ZnCl_2$ while native GFP retained 50% of its fluorescence at 450 μM $CuSO_4$ or 5 mM $ZnCl_2$. This infered that binding of metal to the histidine region of the chimeric protein affected the fluorescence emission. In this study, a linear correlation between the fluorescent intensity of His6GFP and low metal concentrations was also demonstrated.

1. Introduction

In recent years, the green fluorescent protein (GFP) from the Pacific Northwest jelly fish, *Aequorea victoria*, has been applied in many facets e.g. as reporter molecule for monitoring gene expression [1, 2, 3], as fusion tag to monitor protein localization [2, 4, 5], and as measurement tool for protein-protein interactions [6]. The GFP is a relatively small monomeric protein composed of 238 amino acids with a molecular mass of 27-30 KDa [7, 8, 9]. The wild type GFP absorbs ultraviolet (UV) or blue light maximally at 395 nm and emits green light at 509 nm [7]. Therefore, it can be easily detected by irradiation using standard long-wave UV light sources. Furthermore, substrates or cofactors are not required for the fluorescence formation. The fluorescence is caused by an internal chromophore via the posttranslational oxidation of residues Ser^{65}, Tyr^{66}, and Gly^{67} within a hexapeptide at positions 64-69 [10]. The chromophore is resistant to a variety of hazardous conditions including high temperature, extreme pH, and proteases [11]. Even in drastic acidic/basic conditions or highly potent denaturants (guanidine hydrochloride), GFP regains its natural fluorescence after removal of the drastic condition [11, 12].

Recently, a cDNA encoding GFP from cnidarian was cloned and functionally expressed in bacteria [1, 13, 14]. This has, therefore, opened a new approach of investigations in to prokaryotes and development in molecular biology. Many characteristics of the protein have been rapidly explored, e.g. the structural basis of chromophore formation [15, 16, 17, 18, 19, 20], mutagenesis for fluorescence improvement [21, 22, 23, 24, 25], and deletion mapping [26, 27]. Effects of redox reaction on

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the fluorescence molecule have also been reported [28, 29]. However, the effects of metal ions on the fluorescent properties are still not fully revealed.

Therefore, in this study we aim to construct a chimeric green fluorescent protein that has an avidity for metal ions and to study the effects of metal ions on the fluorescent properties of the purified recombinant protein.

2. Materials and methods

2.1 Bacterial strains and plasmid

Escherichia coli (*E. coli*) strains TGI (lac-pro), SupE, thi1, hsd D5/F/ tra D36, proA$^+$B$^+$, lacI, lacZ, M15, (ung$^+$, dut$^+$) was used as host. The plasmid pGFPuv (Clontech Laboratories, U.S.A) was used to construct the chimeric gene.

2.2 Enzymes and chemicals

Restriction endonucleases, T4 DNA ligase and molecular weight marker (λ/ *Eco*RI + *Hin*dIII) were obtained from Promega and Biolabs. Chelating Sepharose Fast Flow, Sephacryl S-200 gel filtration column and DEAE Sepharose fast flow gel from Pharmacia Biotech, Sweden. All other chemicals were of analytical grade and commercially available.

2.3 Gene construction

Cloning procedures were performed as described by Maniatis et al. [30]. Purification of plasmid DNA was done by using a Wizard^{TM} Plus Minipreps DNA Purification system Kit (Promega). All oligonucleotides were synthesized by the Biomolecular Unit, Lund University, Sweden.

To generate His6GFP, oligonucleotides (5/-AGCTTACACCATCACCATCACCATGCGA GCTCTGCGGTAC- 3/ and 5/- CGCAGAGCT CGCATGGTGATGGTGATGGTGTA-3/) encoding six histidine residues and containing a *Sac*I site were annealed to derive a piece of double stranded DNA which was inserted between the *Hin*dIII and *Kpn*I sites of pGFPuv yielding plasmid pHis6-GFPuv.

2.4 Crude protein preparation

Both native and chimeric fluorescent proteins were isolated from cultures of *E. coli* TGI carrying plasmids pGFPuv or pHis6-GFPuv. The cultures were initiated in LB broth supplemented with 100 mg/L ampicillin. GFP gene expression was induced with isopropyl-β-D-thiogalac-topyranoside (IPTG) (1mM final concentration) at OD 550 = 0.1. Late log phase cultures were spun at 10,000 g for 5 min and the cell pellets were resuspended in 10 mM sodium phosphate buffer, pH 7.4. The cell suspensions were sonicated and debris was removed by centrifugation (10,000 g, 5 min).

2.5 Purification of native GFP by DEAE anion exchange chromatography and gel filtration

The native GFP was purified by two steps column chromatography. Crude homogenate was loaded on a DEAE anion exchange column (DEAE Sepharose fast flow gel) and eluted by a zero to 1 M NaCl gradient in 10 mM phosphate buffer, pH 7.4. Fractions were collected and loaded on a gel filtration column (Sephacryl S-200) equilibrated with phosphate buffer. Fractions containing green fluorescence activity were pooled and stored at –20^{o}C until use.

2.6 Purification of His6GFP by immobilized metal affinity chromatography, IMAC [31]

Crude extract was loaded on a Metal Chelating Sepharose 6B column (0.7 X 8 cm) charged with $ZnCl_2$ after washing with at least 10 column volumes of distilled H_2O and equilibration with 50 mM phosphate buffer, pH 7.4. Bound protein was eluted by applying a linear pH gradient of 20 mM phosphate buffer, pH 7.4-3.5. Green fluorescence fractions were then collected.

2.7 Fluorescence assay, protein determination and polyacrylamide gel electrophoresis

Fluorescence was assayed by excitation at near UV or blue light at 395 nm and emission at 509 nm was measured by spectrofluorometry (Shimadzu uv2000).

Purity of green fluorescent protein was determined by calculating the absorbance ratio of *A*395/*A*280. A value approaching 1 or greater indicated high degree of purity [32].

SDS-PAGE was performed on a 12% polyacrylamide gel in a Tris-glycine, pH 8.3 discontinuous buffer system as described by

Laemmli [33]. Protein concentration was quantitated by using dye binding assay (Biorad) and bovine serum albumin was used as standard.

2.8 Effect of metal ions on fluorescent intensity

To study the effect of metal ions on fluorescent intensity, aliquots of purified native and chimeric GFP were adjusted to a fluorescence intensity of 3500 A.U. Samples were then incubated with various concentrations of metals ($CdCl_2$, $ZnCl_2$, $CuSO_4$, and $NiCl_2$) in phosphate buffer on ice for 60 min. The fluorescent emission was then assayed.

3. Results

3.1 Chimeric gene construction

The chimeric gene His6-GFPuv consisting of a DNA linker encoding six histidine residues fused to the 5′- terminal end of the GFPuv gene was constructed as illustrated in Figure 1.The plasmid pGFPuv was digested with *Hin*dIII and *Kpn*I and the large plasmid fragment was purified by agarose gel electrophoresis (0.7%). Synthetic oligonucleotides were then ligated to the fragment, generating the plasmid pHis6-GFPuv. The inserted linker also contained an unique *Sac*I cleaving site to check the integrity of the construct.

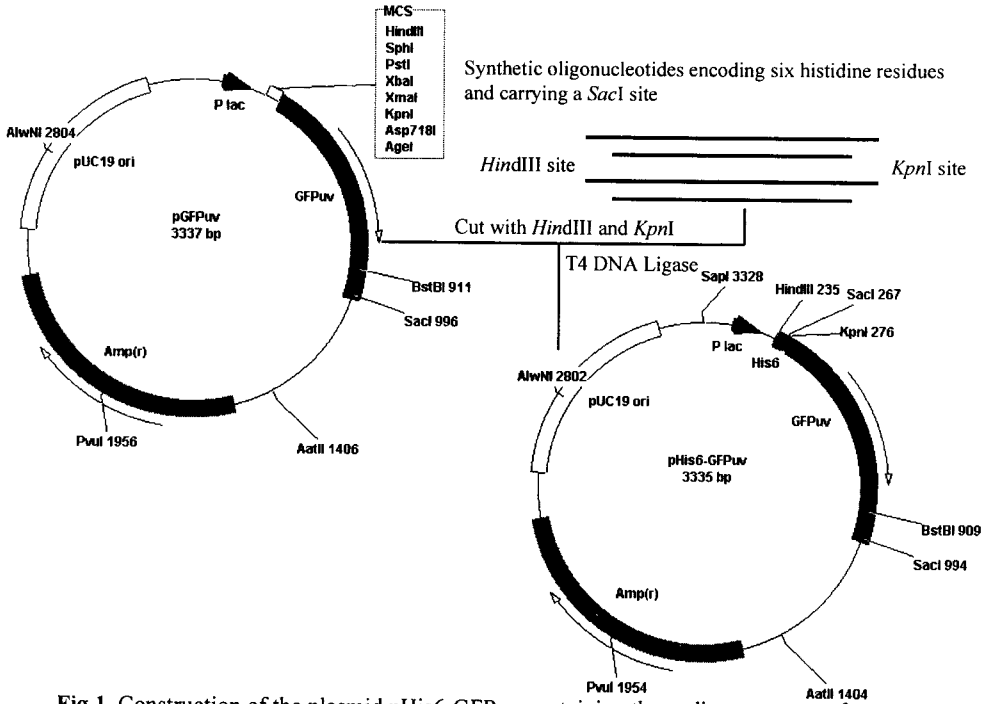

Fig.1 Construction of the plasmid pHis6-GFPuv containing the coding sequences for green fluorescent protein and six histidine residues inserted in-frame at the 5′-end of the GFP gene.

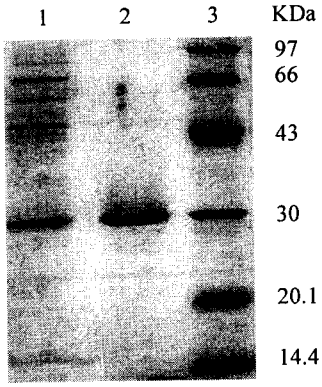

Fig.2 SDS-PAGE of purified native GFP and His6GFP. Lane 1 native GFP purified by both DEAE anion exchanger and gel filtration columns, lane 2 His6GFP purified by immobilized metal (Zn^{2+}) affinity chromatography, lane 3 molecular mass marker proteins.

3.2 Gene expression and protein purification

The chimeric gene was expressed in *E. coli* TGI cells. His6GFP was simply detected by its fluorescence emission in the culture when stimulated with UV light. The fluorescence excitation (λ max at 395 nm) and emission spectra (λ max at 509 nm) was identical to those of the native GFP (data not shown). The His6GFP possessed dual characteristics of both metal binding and fluorescent properties. Therefore, it was purified by binding to a IMAC-Zn column and eluted at pH 4.0. The native GFP without metal binding properties was not bound to the column. Purity and molecular weight of both the native and the engineered GFP are illustrated in Figure 2. Their estimated molecular weight is 30 KDa which corresponds to the calculated sizes (29.484 and 29.358 KDa for the native and the engineered GFP, respectively). The ratios of absorbance at 395 and 280 nm of the purified proteins were 0.96 chimeric for the GFP and 0.71 for the native GEP and indicate the higher purity of the climeric GFP.

3.3 Effect of metal ions on fluorescent intensity

The effect of the group of divalent metal ions (Zn^{2+}, Cu^{2+}, Cd^{2+}, and Ni^{2+}) on the fluorescent emission of both native and chimeric GFP is shown in Figure 3. The fluorescence intensities of both proteins were markedly reduced by all four metals tested. The His6GFP was more sensitive than the native GFP to low concentration of zinc or copper. The fluorescence emission of His6GFP was reduced to half in the presence of 150 μM $CuSO_4$ or 3.1 mM $ZnCl_2$ while native GFP retained 50% of its fluorescence at 450 μM $CuSO_4$ or 5 mM $ZnCl_2$. Cadmium and nickle provided comparable inhibitory effects to the chimeric and the native GFP. The inhibitory effects of metal ions to the fluorescence of His6GFP are concentration dependent (Figure 4). Linear correlations for zinc, copper, cadmium, and nickle ranged from 0-3 mM, 0-120 μM, 0-1000 μM, and 0-150 μM, respectively.

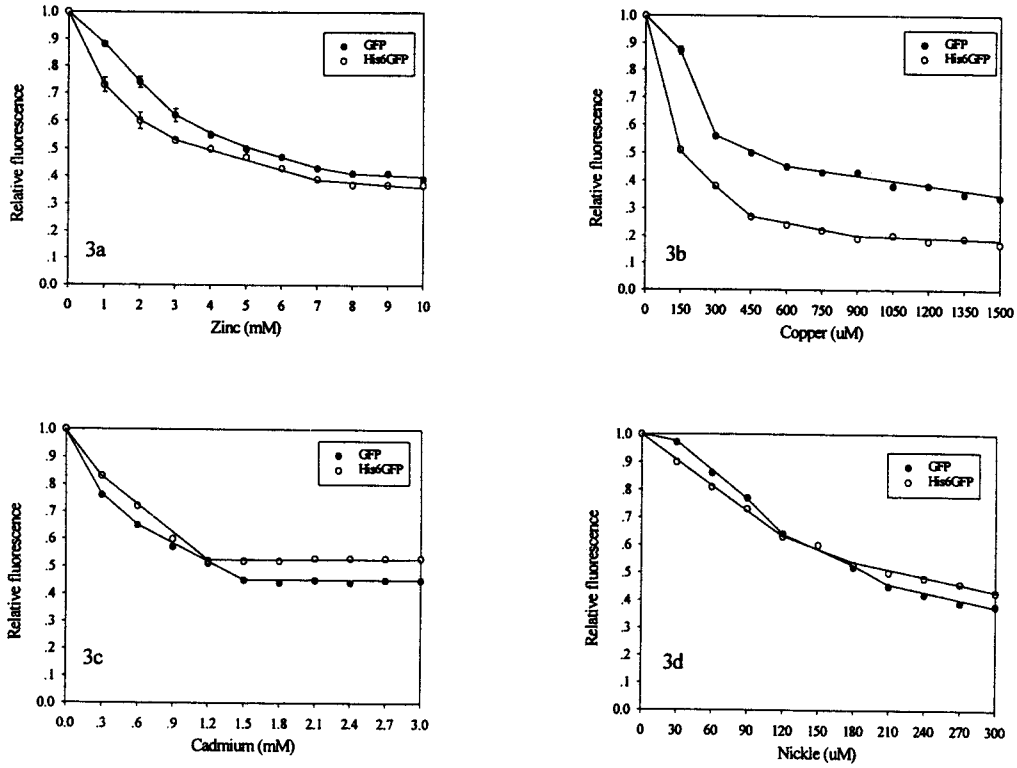

Fig.3 Effect of zinc (3a), copper (3b), cadmium (3c), and nickle (3d) on the fluorescent emission of native GFP and chimeric His6GFP.

4. Discussion

A chimeric gene encoding six histidine residues and green fluorescent protein (His6-GFPuv) was successfully constructed and expressed in *E. coli*. The GFPuv was soluble in the cytoplasm and inclusion bodies were not observed as found in the case of wild type GFP [25]. The excitation and emission wavelengths of native and chimeric GFP were proved to be the same. This indicates that the hexapeptide of the internal chromophore is not affected by the metal-binding region inserted at the N-terminal end [14, 18, 34]. It also supports the notion that extension at the N-terminus does not disrupt the barrel formation of the folded structure of GFP [19, 27].

The inserted histidine region acted as a binding site for metal ions which in our case was also effectively used as in previous reports for tag affinity purification [14,19,28,34,35,36]. Importantly, the creation of the metal-binding site was beneficial for studying the effects of metals on fluorescence properties. Many metals were shown to reduce the fluorescence intensity of native GFP in our finding. However, the divalent transition metals (Cu^{2+} and Zn^{2+}) exerted relatively more effects on His6GFP (Figure 3). This infers a potentiation effect of the metal and histidine region complex upon the reduction of fluorescence emission. It can be suggested that the transition metal complex is changing the electrostatic environment of the chromophore into a reduced state which is non fluorescent. In other studies, reductants such as dithionite and ferric ions reduced the fluorescence emission of GFP, probably by rehydrogenating the chromophore [15, 28, 29, 37].

Our finding of a linear correlation of metal binding and the fluorescence emission of His6GFP at low metal concentrations opens up

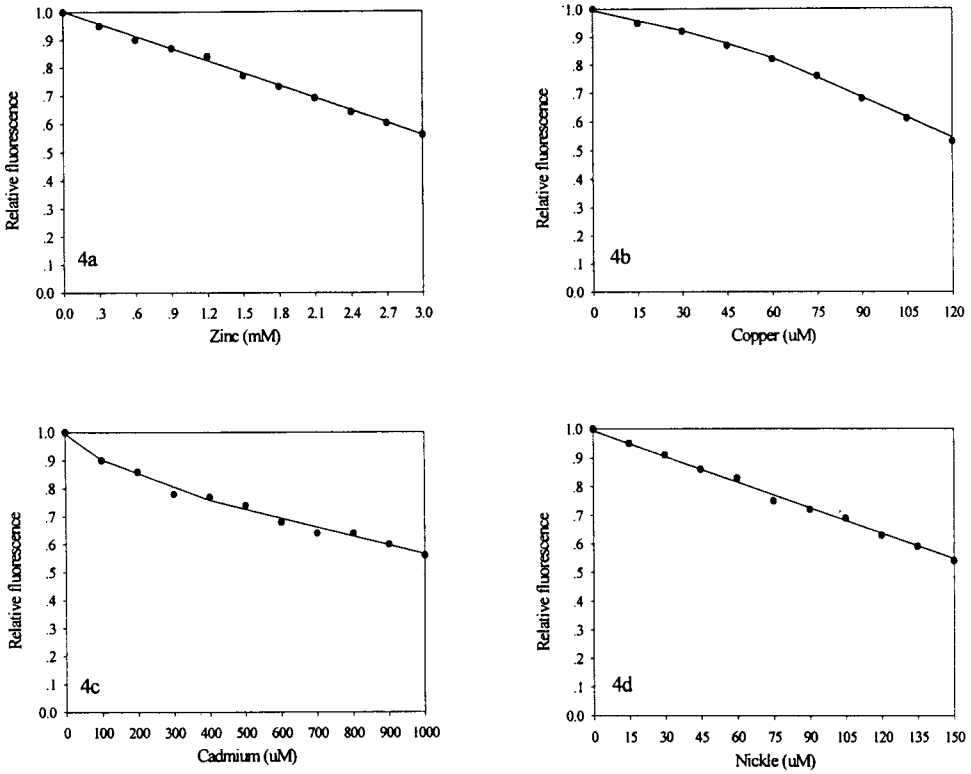

Fig.4 Linear correlation between relative fluorescence of His6GFP and low concentrations of zinc (4a), copper(4b), cadmium (4c), and nickle (4d).

a new approach on metal quantitation (Figure 4). Determination could simply be done by measuring the fluorescence emission or could be further developed into a biosensor [35, 38, 39]. Sensitivity and specificity of the metal binding could be further maximized by insertion of an engineered metal specific region instead of the non-specific polyhistidine. Other feasible applications might include the tagging of metals for nuclear imaging and usage as an intracellular calcium sensor [40, 41].

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