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A New-Generation Fluorescent-Based Metal Sensor – iLOV Protein

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The iLOV protein belongs to a family of blue-light photoreceptor proteins containing a light-oxygen-voltage sensing domain with a noncovalently bound flavin mononucleotide (FMN) as its chromophore. Owing to advantages such as its small size, oxygen-independent nature, and pH stability, iLOV is an ideal candidate over other reporter fluorescent proteins such as GFP and DsRed. Here, for the first time, we describe the feasibility of applying LOV domain-based fluorescent iLOV as a metal sensor by measuring the fluorescence quenching of a protein with respect to the concentration of metal ions. In the present study, we demonstrated the inherent copper sensing property of the iLOV protein and identified the possible amino acids responsible for metal binding. The fluorescence quenching upon exposure to Cu^{2+} was highly sensitive and exhibited reversibility upon the addition of the metal chelator EDTA. The copper binding constant was found to be 4.72 ± 0.84 μM. In addition, Cu^{2+}-bound iLOV showed high fluorescence quenching at near physiological pH. Further computational analysis yielded a better insight into understanding the possible amino acids responsible for Cu^{2+} binding with the iLOV protein.

Keywords: iLOV protein, copper, fluorescence quenching, metal sensor

Introduction

Metals, as environmental pollutants, have a great impact on biological organisms. Among them, heavy metals ubiquitous in nature generally serve as a major pollutant to the environment. Their toxicity and adverse effects on biological systems create the urge to develop various detection methods for such metals. In general, methods for determining the concentration of heavy metals in the environment and in living systems include atomic absorption spectroscopy and ICP-MS [22]. In both of these methods, depletion of the sample during the process has led researchers to search for suitable alternatives that would be able to easily detect even a trace amount of metal ions in a sensitive and accurate manner [20]. Recently, attention has been given to the exploitation of genetically encoded fluorescent proteins as fluorescent probes for the development of biosensors against heavy metals.

Genetically encoded fluorescent proteins are used as fluorescent probes in a broad range of applications such as *in vivo* cell imaging, and detection of protein-protein interactions and protein-polysaccharide conjugation, eventually leading to synthetic biology as well [1, 10, 21]. Inspired by these characteristics, protein engineers have devoted a lot of interest to the creation of new variants through the directed evolution and rational designing approach for expansion of pH stability, spectral properties, and so on, targeting for use in various functional applications [7]. Recently, a new red excitable monomeric fluorescent protein was used for detailed anatomical studies on myoblast differentiation in living mice [6]. Owing to its vast applications as a research tool, the exploitation of such fluorescent proteins for metal sensing purpose would be an easy and attractive approach for the development of metal biosensors. In earlier studies, genetically encoded sensors based on fluorescence resonance energy transfer (FRET) were used for the detection of Zn^{2+} in living cells [23]. In particular, DsRed possesses an inherent copper sensing property that

works through a fluorescence turn-off mechanism [19]. In addition, the engineering of EGFP with the metal-binding amino acid histidine has led to the development of a highly selective Cu^{2+} sensor [3]. Lately, the incorporation of metal-chelating unnatural amino acids into GFP has led to the development of a biosensor that can detect copper and zinc in living cells [2].

Recently, a class of blue light receptor proteins has overcome the fluorescent proteins of the last decade. These reported new-generation proteins contain a light, oxygen or voltage sensing domain (LOV) with a flavin mononucleotide (FMN) as the chromophore [4]. Upon irradiation with blue light at 450 nm, the FMN undergoes conformational changes that emit fluorescence at 495 nm [8]. Such FMN-dependent chromophore formation is highly advantageous owing to its natural occurrence in living systems and oxygen-independent nature. Recently, a variant derived through engineering of the LOV domain was designated as iLOV [4]. This iLOV variant has improved photo physical properties, rapid maturation of fluorescence, and increased thermal stability, which expand its successful application as a fluorescent reporter [15]. In the current decade, the molecular structure of the iLOV protein was also completely characterized and demonstrated with crystal structure as like other fluorescent proteins [5].

Oxygen-dependent fluorescent proteins generally exhibit less fluorescence or are nearly non-fluorescent in oxygen-deprived environments, and hence have constrained utility in particular cases, such as for understanding the role of Fe^{2+} and Zn^{2+} in cerebral ischemia along with the roles of various other transition metal ions in hypoxic conditions. Therefore, the applicability of earlier fluorescent proteins remains a challenging task for elucidating detailed insights that could explain the functional importance of metal ions in the field of anaerobic biology. From this perspective, the new class of oxygen-independent FMN-based chromophores forming the LOV protein variants could be utilized efficiently to detect and dissect the major cellular events under oxygen-deprived conditions [8]. Herein, the utility of the FMN chromophore-based iLOV protein for metal sensing application was demonstrated for the first time since its discovery. The results obtained provide insight into understanding the mechanism of fluorescence quenching, and indicate the probable amino acids involved in Cu^{2+} binding (Fig. 1).

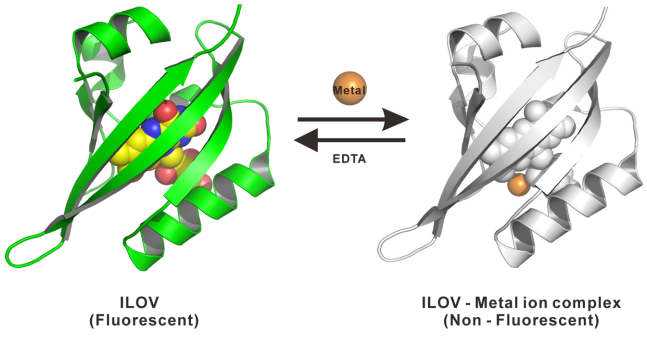

Fig. 1. Proposed schematic representation of the metal-sensing iLOV protein.

Binding of Cu^{2+} to the FMN chromophore leads to non-fluorescence of the iLOV protein.

Materials and Methods

The host bacterium *Escherichia coli* (*E. coli*) strain XL1-blue (Stratagene, CA, USA) was used for plasmid DNA preparation. *E. coli* cells with plasmids were grown aerobically in Luria-Bertani (LB) broth (Difco Labororatories, Detroit, MI, USA) or on LB agar plates, supplemented with appropriate antibiotics for the selection of transformants. The pQE80L plasmid and the nickel-nitrilotriacetic acid (Ni-NTA) affinity column were purchased from Qiagen (Valencia, CA, USA). Isopropyl-β-D-thioagalactopyranoside (IPTG) (Carbosynth) and all the other chemicals used were purchased from Sigma-Aldrich, Korea.

Construction of Plasmid for iLOV Gene

The protein sequence of the engineered LOV domain-containing protein was retrieved from an earlier report that introduced iLOV as the subject of the manuscript [4]. The gene encoding for iLOV was chemically synthesized after codon optimization to comply with the codon preferences of *E. coli* (Bioneer Inc., Daejon, South Korea). The synthetic gene was supplied by Bioneer Inc. after cloning into the pGEM-T easy vector (Promega, Madison, WI, USA). The synthetic iLOV gene was cut with the restriction enzymes *Bam*HI and *Hin*dIII and then inserted into the IPTG-inducible expression vector pQE80L. The plasmid was further transformed into *E. coli* (BL21) cells.

Protein Expression and Purification

The BL21 transformants were grown at 37°C in 1 L of LB broth containing 100 μg/ml of ampicillin. When the OD_{600} reached 0.6, the protein expression was induced by adding IPTG to a final concentration of 1 mM. The culture was then incubated at 37°C. After 7 h of induction, the cells were harvested and washed twice with distilled water. Following centrifugation, the cell pellet was re-solubilized in 5 ml of Lysis buffer (300 mM NaCl and 5 mM imidazole) at pH 7.0. The cells were then subjected to ultrasonic disruption for 20 min, followed by centrifugation at 16,000 rpm for 30 min at 4°C. The soluble fraction containing the iLOV protein was further purified at 4°C on a Ni-NTA agarose resin obtained from Qiagen (Hilden, Germany), as directed in the manufacturer's manual.

Spectroscopic Analysis

Spectroscopic analysis of the fluorescence from the purified iLOV protein was carried out. First, 100 µl of 3 µM protein was prepared in 10 mM MOPS buffer at pH 7.4. The fluorescence readings were then recorded on a Perkin Elmer LS-55 spectrofluorometer. Using an emission wavelength at 520 nm, the excitation spectra of the ilOV protein was recorded in the range from 400 to 500 nm. Similarly, emission spectra were also recorded in the range from 470 to 600 nm after excitation at 450 nm.

Metal Sensing Assay

As an initial study, the screening of different metal ions with iLOV was performed in order to identify any specific affinity for the selective binding of metal ions. To test this, 100 µl of 6 µM protein (in 10 mM MOPS buffer at pH 7.4) was prepared and treated with the same volume containing 200 µM of metal ions. After 30 min of incubation at 25ºC, the samples were excited at 450 nm and the emitted fluorescence was measured at 495 and 520 nm.

Determination of Dissociation Constant for Copper

To perform the Cu^{2+} binding study, 100 µl of copper solution at varying concentrations (0–200 µM) was added to 100 µl of 6 µM iLOV protein (in 10 mM MOPS buffer at pH 7.4). Fluorescence measurements were recorded and the baseline was corrected. Dissociation constants were then calculated by plotting the $[F/F_0]$ against the different Cu^{2+} concentrations. The fluorescence was analyzed by exciting the sample at 450 nm, and the emitted fluorescence was measured at 495 nm.

EDTA-Mediated Fluorescence Recovery

In order to study the effects of the metal-chelating agent EDTA on the fluorescence spectra and the reversibility of metal binding, simultaneous addition of different concentrations of EDTA to 3 µM protein (in 10 mM MOPS buffer pH 7.4) was carried out, with and without the addition of Cu^{2+} to the final concentration of 100 µM. The samples were excited at 450 nm, and the emitted fluorescence was recorded at 495 and 520 nm.

Results

iLOV Expression and Purification

The plasmid encoding the gene for iLOV was cloned into the pQE80L vector, which works under the control of the T5 promoter. BL21 cells bearing the iLOV plasmid yielded good expression under the IPTG induction, which produced a greenish color. The expressed iLOV protein was purified using the Ni-NTA affinity column. Wash buffer containing 5 mM imidazole was used to remove the nonspecifically bound proteins. The purified protein fractions were then collected and dialyzed against 1× PBS buffer overnight in order to remove the imidazole completely. The imidazole-free protein was then concentrated using an Amicon PM-10 ultrafiltration unit and then stored at 4ºC until used in further analysis. SDS-PAGE analysis was carried out in order to visualize the expressed protein (Fig. 2). The purified iLOV protein gave a single clear band on the gel.

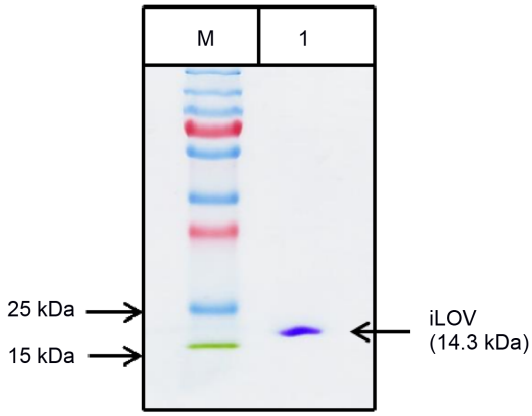

Fig. 2. SDS-PAGE analysis of purified iLOV protein.
Lane M represents the molecular weight marker. Lane 1 contains the purified iLOV protein after Ni-NTA affinity chromatography.

The final concentration of 3 µM of purified iLOV protein was taken for spectral studies, including analysis of both the fluorescence excitation and emission patterns. A fluorescence excitation scan was performed from 400 to 500 nm. By measuring emission at 470 nm, two excitation peaks were observed: a major excitation maximum at 450 nm and a minor shoulder peak at 480 nm. By further

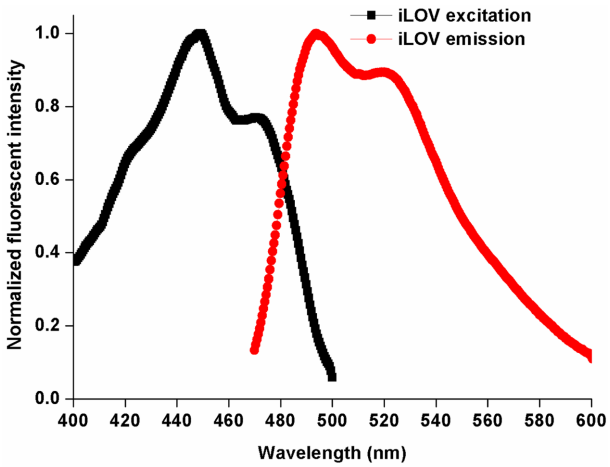

Fig. 3. Excitation and emission spectra of iLOV fluorescence.
The fluorescence excitation spectrum (black color) was obtained by measuring emission at 495 nm, while the fluorescence emission spectrum (red color) was measured under excitation at 450 nm. iLOV (3 µM) was dissolved in 10 mM MOPS buffer (pH 7.4).

exciting the protein at 450 nm with blue light irradiation, two emission peaks were observed in the range of 470 to 600 nm. The major emission maxima occurred at 495 nm, while a small shoulder peak was observed at 520 nm (Fig. 3). These results matched well with those of previous reports [4].

Metal Selectivity Assay

As an initial study, a metal selectivity assay was carried out on the iLOV protein using different monovalent and divalent metal ions. The results demonstrated that the fluorescence of iLOV was not affected by the addition of 0.1 mM of Ba^{2+}, Ca^{2+}, K^{+}, Li^{+}, Mg^{2+}, Mn^{2+}, and Na^{+}. Other heavy metal ions such as Cd^{2+}, Co^{2+}, Pb^{2+}, and Zn^{2+} showed only insubstantial fluorescence response. Surprisingly, the addition of 100 μM of Cu^{2+} exerted a maximum fluorescence quenching of around 80%, which proves the inherent copper binding ability of iLOV (Fig. 4). Among various metal ions, copper is generally considered as one of the most important transition metal ions, playing a crucial role in cellular homeostasis and the regulation of various metalloenzymes within the cell [12]. Moreover, the governing of copper metabolism includes a lot of transporters. One such important transporter is the P-type ATPase copper-transporting ATPase 1 (ATP7A), involved in copper homeostasis. Genetic defects associated with this gene lead to Menkes disease and occipital horn syndrome [12]. With such a high significance of copper and the inherent ability of the iLOV protein investigated herein to be quenched in the presence of copper, further exploration into the mechanism of fluorescence quenching in the presence of Cu^{2+} was carried out.

Fluorescence Quenching-Based Analysis of Copper-Protein Interactions

A copper binding study was performed by adding different concentrations of Cu^{2+} to the iLOV protein. A calibration curve was generated by plotting the fluorescence quenching against varying copper concentrations. At the concentration of 100 μM copper, approximately 80% of the fluorescence was quenched (Fig. 5). The quenching was highly rapid, and a plateau was obtained in around 20 min. It was therefore concluded that 20 min was sufficient for the iLOV protein to completely lose its fluorescence in the presence of 100 μM Cu^{2+} (Fig. 5). The following experiments on samples containing iLOV and Cu^{2+} were measured after 30 min. The copper concentrations detected in contaminated sites are usually in the millimolar and micromolar range in biological samples. The good relationship observed between the fluorescence quenching and Cu^{2+} concentration provides the firm belief that iLOV has the potential to be used for the detection of Cu^{2+} present in both environmental and biological samples. In order to determine the binding affinity of Cu^{2+},

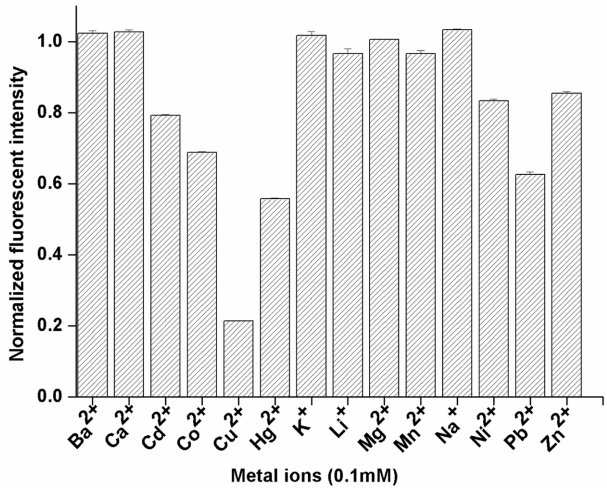

Fig. 4. Metal selectivity assay of iLOV protein with different metal ions.

Fluorescent emission intensities were recorded at 495 nm after excitation of the sample at 450 nm. iLOV (3 μM) was incubated in 10 mM MOPS buffer (pH 7.4) containing 0.1 mM metal ions for 30 min.

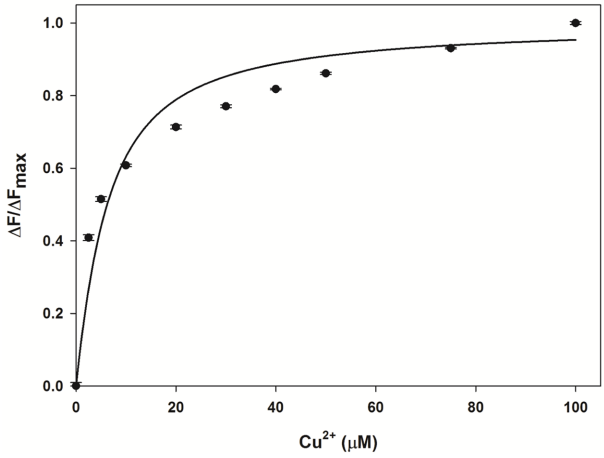

Fig. 5. Plot of $\Delta F/\Delta F_{max}$ against different copper concentrations, fitted using the equation $\Delta F/\Delta F_{max} = (K_d + [P] + [Cu] \pm \sqrt{[(K_d+[P]+[Cu^{2+}])2–(4[P][Cu^{2+}]/2[P]}$.

Here, ΔF is the change in the measured fluorescence and ΔF_{max} is the maximum fluorescence emission change at 495 nm upon excitation at 450 nm. iLOV (3 μM) was incubated in 10 mM MOPS buffer (pH 7.4) with various concentrations of Cu^{2+} for 30 min.

Table 1. The K_d values of copper-binding fluorescent proteins.

Protein	K_d	Reference
iLOV	4.72 μM	This work
HcRed	3.6 μM	[18]
DsRed Express	5.4 μM	[17]
drFP583	14.80 μM	[9]
Rmu13	10.90 μM	[9]
Dopa containing GFP	5.6 μM	[2]
Engineered EGFP	16 nM	[3]

the copper dissociation constant of the protein was calculated using the equation below:

$$\Delta F/\Delta F_{max} = (K_d + [P] + [Cu] \pm \sqrt{[(K_d+[P]+[Cu^{2+}])2-(4[P][Cu^{2+}]/2[P]}$$

where ΔF is the change in measured fluorescence, ΔF_{max} is the maximum fluorescence change, [P] is the total protein concentration, K_d is the dissociation constant of the iLOV protein, and $[Cu^{2+}]$ is the total copper concentration. The curve of $\Delta F/\Delta F_{max}$ against copper concentration was fitted using the above equation. The K_d value of iLOV towards Cu^{2+} was found to be 4.72 ± 0.84 μM. The reported K_d values of other copper-binding fluorescent proteins are listed in Table 1. Based on the K_d values presented in Table 1, the K_d value obtained from the wild-type inherently copper-sensing iLOV protein is highly promising, because it can be employed for the detection of copper in low oxygen conditions, whereas those listed are not suitable owing to the strict requirement of molecular oxygen for chromophore formation.

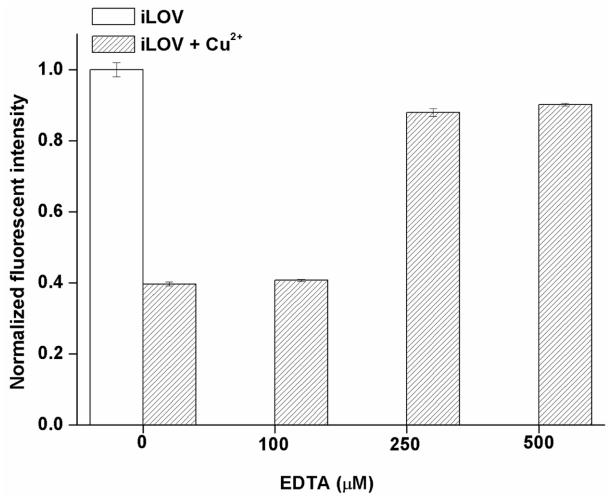

Fig. 6. Influence of EDTA on iLOV-Cu^{2+}-bound non-fluorescent complex.
Different concentrations of EDTA were added to iLOV (final concentration, 3 μM) in solution, which was quenched by the addition of 100 μM Cu^{2+}.

EDTA-Mediated Fluorescence Recovery and pH Study

The reversibility of metal binding to iLOV was examined in the presence of the metal-chelating agent EDTA, as shown in Fig. 6. The quenched fluorescence intensity in the presence of 0.1 mM Cu^{2+} returned to 90% of its original fluorescence within 2 min upon the addition of EDTA, when allowed to equilibrate. This is because EDTA, which has higher binding affinity, removes the Cu^{2+} from the non-fluorescent complex to form a more stable EDTA–Cu^{2+} complex, releasing the iLOV protein to regain its original fluorescence.

The effects of pH on iLOV and Cu^{2+} binding were analyzed by monitoring the fluorescence change in the presence and absence of Cu^{2+}. In the absence of Cu^{2+}, iLOV emitted fluorescence over a broad pH range (pH 4.0–11.0), retaining more than 80% of the fluorescence emission upon 30 min of incubation at both pH 4.0 and 10.0. The pH tolerance of iLOV fluorescence is highly exceptional, and may be suitable to allow its use as a fluorescent probe for exploring acidophilic microorganisms. In the presence of Cu^{2+}, a gradual decrease in fluorescence was observed from pH 7.0 to 10.0. The fluorescence quenching from pH 7.0 to 9.0 remained more or less the same with respect to 100 μM Cu^{2+}. This interesting result indicates the possible utility of iLOV for sensing Cu^{2+} in this broad pH range.

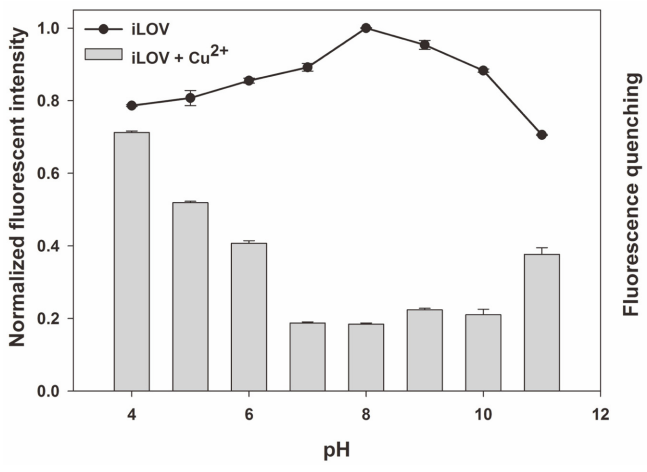

Fig. 7. Effect of pH on the fluorescence intensity of iLOV and the fluorescence quenching of iLOV by Cu^{2+}.
The line representation shows fluorescent intensity of iLOV in the absence of Cu^{2+}. The bar chart represents the fluorescent quenching of iLOV in the presence of 100 μM Cu^{2+}.

Cu^{2+}-Binding Site of iLOV

In order to find the possible metal-binding site in the structure of iLOV, the crystal structure of the protein from the PDB database (PDB ID 4EES) was investigated. Molecular modeling studies of the iLOV protein revealed the key residues interacting with the FMN chromophore. The chromophore was found to be crowded with polar uncharged amino acids, especially Asn. The amine and carbonyl groups of these two such amino acids, residing at 401 and 425, may be the probable Cu^{2+} coordination sites that interact with the FMN and thereby cause the resulting fluorescence quenching (Figs. 8A and 8B)

Discussion

Recently, tremendous efforts have been carried out to develop fluorescent protein-based metal sensors. One of the critical factors for advancement in the field of biosensors is the discovery of novel fluorescent proteins that are able to sense metal ions both sensitively and selectively. In general, highly sensitive and selective fluorescent protein-based Cu^{2+} sensors are being developed continually through two main methods. The most common approach is by engineering fluorescent proteins such as GFP with the addition of metal-binding amino acids such as His, Glu, Asp, and Cys near the chromophore [11, 19]. The other approach is exploiting unnatural amino acids, which have been used earlier as a tool for genetic code expansion, and were also proven to be useful as a copper biosensor [2]. Although the aforementioned methods have provided useful snapshots as metal sensors, they are strictly restricted to use in aerobic conditions owing to their dependency on molecular oxygen for chromophore formation. As a result, it is necessary to devise alternative methods or use novel fluorescent proteins for the detection of metal ions in living biological systems to provide an understanding of the dynamic changes influenced by metals in low oxygen conditions. Therefore, discovery of novel fluorescent proteins that can selectively detect metal ions with high sensitivity in anoxic conditions has significant applications. In this regard, the oxygen-independent chromophore forming the iLOV protein was investigated herein for metal sensing applications, and its inherent copper sensing mechanism was demonstrated, which can efficiently detect copper in the micromolar range.

The pH factor commonly plays a crucial role in living cells, being almost neutral in the cytosol but mostly acidic in the lysosome. Genetically encoded GFP-based pH sensors have already been successfully employed for the detection

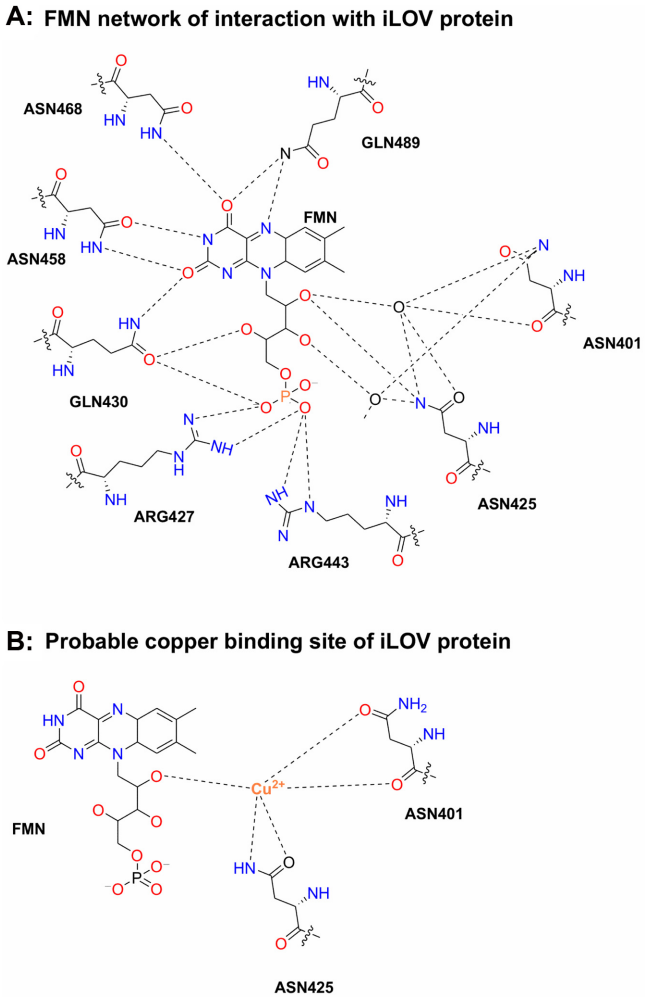

Fig. 8. (**A**) The FMN chromophore interaction of iLOV protein with its surrounding residues and (**B**) 2D-structural analysis of the probable Cu^{2+}-binding site of the iLOV protein.

of pH changes in the mitochondria, Golgi, and cytoplasm [14]. However, since GFP is highly pH sensitive, it exhibits weak fluorescence in acidic environments for the detection of cellular processes such as autophagy [13]. In comparison with GFP, the advantage of the iLOV protein was shown herein, as it maintained good fluorescent tolerance towards a broad pH range. More than 75% of the fluorescence intensity was retained in both acidic (pH 4 and 5) and alkaline conditions (pH 10 and 11), as depicted in Fig. 7. Therefore, the iLOV protein, which shows good fluorescence tolerance over broad operational pH ranges, can effectively be used for the detection of metal ions in cells, which generally experience both acidic and physiological conditions.

In addition to selectivity, the sensitivity of iLOV towards copper was also remarkably high. The obtained K_d value of

4.72 ± 0.84 μM shows higher affinity for copper binding. With this great affinity and the inherent fluorescence quenching towards copper shown herein, iLOV could be efficiently utilized for the monitoring of copper ions in low oxygen environment conditions. As presented in Table 1, more sensitive variants with much lower K_d values towards Cu^{2+} could be developed by mutating the amino acids near the chromophore with metal-binding amino acids through the site-directed mutagenesis approach. For the successful utilization of iLOV for metal sensing in practice, protein reusability is considered to be one of the crucial parameters. With high affinity towards Cu^{2+}, treatment with the metal-chelating agent EDTA demonstrated that successful recovery of the bound metal from the iLOV protein is practical under equilibrated conditions, as shown in Fig. 6. Moreover, the addition of EDTA to the protein itself did not lead to any fluorescence quenching, which demonstrates the EDTA did not denature the protein. In addition, results obtained from the computational analysis provided basic information on the interaction of Cu^{2+} with the FMN chromophore. Commonly, amines and thiol group side chains containing amino acids such as His, Cys, Asp, and Glu are found to have high affinity towards copper ions. To a further extent, Asn and Gln can also bind specifically with copper. In addition it is reported that structural motifs for the copper-binding activity also contains Asn [19]. Furthermore, structural scanning of iLOV shows that the FMN chromophore is filled with amino acids Asn, Gln, and Arg. Among the amino acids residing closer to the FMN chromophore, it was found that the amine and carbonyl groups from the Asn residues at positions 401 and 425 might yield the coordination site for Cu^{2+} that interacts with the FMN chromophore, as shown in Fig. 8. From the above-discussed computational analysis, it was hypothesized that Cu^{2+} forms a complex with the specific amino acids on the iLOV protein, leading to the fluorescence quenching.

In conclusion, we have demonstrated the application of the FMN chromophore-based fluorescent protein iLOV- as a selective metal sensor for the detection of copper ions. To the best of our knowledge, this is the first report describing the potentiality of iLOV, which can selectively and intrinsically bind copper in an efficient and reversible manner. With high sensitivity towards the detection of copper in micromolar range concentrations and being non-toxic in nature, iLOV may serve as a diagnostic tool for the measurement of intracellular copper ions in cells devoid of oxygen. With the results obtained from this groundwork, further engineering of FMN-binding amino acids through the study of canonical and noncanonical amino acids may lead to an iLOV variant with better spectral characteristics. Moreover, such engineering might open a new gateway for the development of various biosensors with efficiency for the detection of toxic heavy metals in contaminated sites, as well as for other real-time applications under anoxic conditions.

Acknowledgments

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