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Spectrofluorometric Properties of N-Terminal Domain of Lumazine Protein from *Photobacterium leiognathi*

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Lumazine protein is a member of the riboflavin synthase superfamily and the intense fluorescence is caused by non-covalently bound to 6,7-dimethyl 8-ribityllumazine. To figure out the binding modes and the structure of the N-terminal domain of lumazine protein, the wild type of protein extending to amino acid 118 (N-LumP 118 Wt) and mutants of N-LumP 118 V41W, S48W, T50W, D64W, and A66W from *Photobacterium leiognathi* were purified. The biochemical properties of the wild type and mutants of N-LumP 118 proteins were analyzed by absorbance and fluorescence spectroscope. The peak of absorbance and fluorescence of lumazine ligand were shifted to longer wavelength on binding to N-LumPs. The observed absorbance value at 410 nm of lumazine bound to N-LumP 118 proteins indicate that one mole of N-LumP 118 proteins bind to one mole of ligand of lumazine. Fluorescence analysis show that the maximum peak of fluorescence of N-LumP S48W was shifted to the longest wavelength by binding with 6,7-dimethyl 8-ribityllumazine and was shown to the greatest quench effect by acrylamide among all tryptophan mutants.

Key Words : Bioluminescence, Lumazine, *Photobacterium*, Riboflavin

Introduction

Bioluminescence is a kind of photochemical reaction that involves radiation of the light energy during the organic chemical called luciferin is oxidized by the action of enzyme (Luciferase) inside living organism.[1,2] The luciferin which is a chemical engaged in the light emitting reaction of the light emitting living organism differs from each living body. The structure and function of the enzyme, luciferase are different from each other but there is a common point that the oxygen molecule(O_2) is engaged in every light emitting reaction.[3-5] The light emitting reaction in the bacteria involves the oxidation of the reduced riboflavin phosphate ($FMNH_2$) and long chain fatty aldehyde as shown in the following reaction scheme.[6-9]

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + light$$

The study on the light emitting reaction mechanism arisen by bacterial luciferase and intermediate compound was performed intensively. It originated from very slow turnover of this enzyme. First of all, $FMNH_2$ is combined to the enzyme and reacted with oxygen molecule (O_2) and it forms 4a-peroxy-flavin.[5-9] The intermediate then binds a long chain aldehyde to form an enzyme-flavin-oxygene aldehyde intermediate. The luciferase catalyzed reaction involves the oxidation of long chain aldehyde and $FMNH_2$ and results in the emission of blue-green light centered at 490 nm with 0.05-0.15 quantum efficiency.[5,7]

Riboflavin (vitamin B_2) biosynthesis genes were found at the downstream of *lux* operon, where genes involved in light emission are gathered in *Photobacterium leiognathi* species.[10,11] The riboflavin biosynthesis in bioluminescent bacteria is very important because it is the substrate of the light emitting reaction.[4-8] Lumazine protein is a fluorescent-colored protein and it was found in *Photobacterium phosphoreum* at the late of 1970.[12-14] The strong fluorescent protein forms non-covalent bond with 6,7-dimethyl-8-ribityllumazine (It was hereafter referred to as lumazine) which is known to be a direct precursor for biosynthesis of vitamin B_2.[13-15]

Lumazine protein is believed to act as an optical transponder in the emission of the light from light-emitting bacteria.[15-17] More clearly, it is considered to intensify the light by creating changes in light emitting reaction through providing energy to the bacterial luciferase.[15] The interference of lumazine protein, which is considered to play the role of optical transponder between the energy creation and light emitting stage, was suggested based on the phenomena such as the shorter wavelength shift in the maximum emission and the increase of quantum yield.[17]

Lumazine protein binds to fluorescent lumazine ligand with high affinity.[16,17] This protein is thought to be created through gene duplication,[18] as it shows about 30% amino acid identity with riboflavin synthase and is under the superfamily of riboflavin synthase.[18] The lumazine protein has a monomeric structure, which is different from a homotrimer protein of riboflavin synthase.[19,20] It was revealed that the monomeric protein folds into two closely similar domains that are structurally related by pseudo-C2 symmetry, whereby the entire domain topology resembles that of riboflavin synthase.[19] Riboflavin synthase binds to two molecules per protein, whereas lumazine protein bind to one molecule of 6,7-dimethyl-8-ribityllumazine (lumazine).[19,20] The lumazine protein has internal amino acid sequence homology between the amino terminal domain (LumP-N) and the carboxy terminal domain (LumP-C) (Fig. 1(a) and 1(b)), and it is expected that the ligand binds to either amino or carboxy

terminal half.

In this study, the wild type and the tryptophan containing mutants of N-terminal domain of lumazine proteins from *Photobacterium leiognathi* were purified and analyzed the biochemical characteristics by the spectroscopic experiments.

Experimentals

Strain, Plasmid, Enzymes, and Chemicals. *Escherichia coli* XL-I blue purchased from Stratagene was used as a cloning strain. The DNA gene that codes for lumazine protein was obtained from Dr. Illarinov at University of Hamburg, who cloned it from *P. leiognathi* and inserted in pPhl36 plasmid.[21] pQE30 vector, from Qiagen Inc. was used as the vector due to its advantage of binding to Ni column in the 6X-His tagging system and the possibility of step by step elution with imidazole in separation and purification of proteins. The authentic ligand 6,7-dimethyl-8-ribityllumazine was synthesized by Prof. Fischer group at University of Hamburg in Germany.

Generation of Mutants of Amino-Terminal Domain of Lumazine Protein. V41W, S48W, T50W, D64W, and A66W mutants of N-terminal lumazine protein (N-LumP 118) were generated from the 118 genetic code of the wild type lumazine protein by point mutagenesis in a PCR reaction. The recombinant plasmid, containing pQE30 vector and the gene which codes for N-LumP 118 of wild type and mutant types,

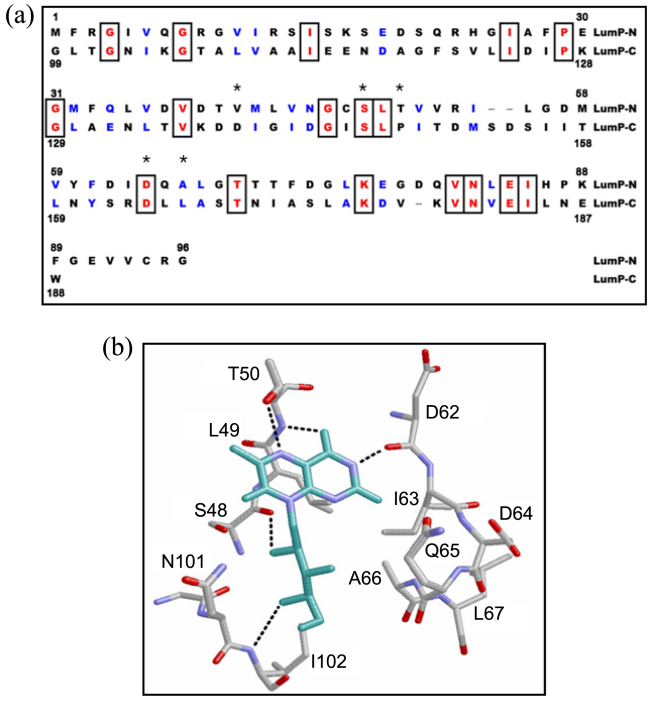

Figure 1. (a) Internal amino acid sequences between N-terminal half and C-terminal half of lumazine protein from *P. leiognathi.* Identical amino acids are marked in box and similar amino acids shown in grey letters. The asterisks indicate the amino acids changed to tryptophan by site directed mutagenesis. (b) Model of binding site topology of lumazine protein with 6,7-dimethyl-8-ribityllumazine.[20]

and other conditions were described published earlier.[23] The purification method by 6X-His tagging system was performed as described earlier.[23]

Removal of Ligand. Solutions containing 100 mM phosphate, pH 7.0, and the N-LumP protein were dialyzed against 100 mM phosphate pH 7.0, containing 4mM dithiothreitol and 6 M urea. For refolding, the apoprotein was dialyzed overnight against 100 mM phosphate, pH 7.0 containing 0.3 mM dithiothreitol and concentrated by ultrafiltration.

Analysis and Quantification of N-LumP 118 Protein. The concentrations of purified proteins were determined by using Qubit™ from Invitrogen. Using the approximate molecular weight determined by SDS-PAGE analysis as a reference. Each quantified protein was diluted to 100 μM and 500 μL of it was mixed with 500 μL of lumazine 500 μM. The resultant mixture was incubated all day at 4 °C. Dialysis was then carried out on the incubated mixture for 8 h using 500 mL of 50 mM NaH_2PO_4 buffer at pH 7.0.

Ligand Binding. The protein and its ligand lumazine were mixed together at a concentration ratio of 1:5, after which the resultant mixture was evenly shaken and dialyzed in a dialysis bag at 4 for 8 h. After dialysis, the protein was concentrated to a volume of 1 mL.

Absorbance and Fluorescence Spectroscopy. The absorbance spectra of the proteins were analyzed at a wavelength of range spanning 250 nm to 650 nm using Agilent 8453 Spectrophotometer. FP-750 series Fluorescence Spectrometer (JASCO) was used in measuring (scanning) the emission spectra of the mutant proteins containing just one tryptophan amino acid residue (N-LumP 118 V41W, S48W, T50W, D64W, A66W) at 280 nm of fixed excitation. In addition, the emission spectra for each protein followed by the addition of 0.05, 0.1, 0.2, 0.3, 0.5, 0.7 M acrylamide were measured, respectively.

Modeling of N-LumP. A three-dimensional model of N-LumP was built using Chemfinder, Chemdraw, and Bioclipse program from Chembiooffice (Cambridge Soft, USA) based on the sequence homology to the riboflavin synthase. Bound of 6,7-dimethyl 8-ribityllumazine was modeled into N-LumP on the basis of riboflavin synthase from PDB (protein data bank acc. Code 1KZL).

Results

Recent studies[19,20] have shown that, amino acids of Asn 101 and Ile 102, which are above the N-terminal region of lumazine protein (Fig. 1(a)), have been discovered to be involved in binding to the lumazine ligand with Ser 48, Thr 50, Asp 64, and Ala 66 (Fig. 1(b)). Therefore, in order to synthesize the gene which codes for N-terminal lumazine protein (N-LumP 118) containing the region of these amino acids of asparagine 101 and isoleucine 102 was constructed and the protein purified. Furthermore, in order to investigate the effective spectroscopic analysis of mutant lumazine proteins, site directed mutagenesis of amino acids such as valine 41, serine 48, threonine 50, aspartic acid 64, and alanine 66 (Fig. 1(b)), which are located at the binding site of lumazine

protein, were performed. This method included the replacement of the above named amino acids with tryptophan and resulted in the synthesis of tryptophan-mutant forms of N-LumP. Since N-LumP wild type does not possess tryptophan amino acid, the fluorescence properties of new generating endogenous tryptophan residues at different positions can serve as local intrinsic probes for investigating the dynamic nature of the protein.

As the first experiment of analysis of spectroscopic properties of wild type and mutant of N-LumP, the possibility of binding of N-terminal lumazine protein (N-LumP 118) to lumazine ligand was investigated by measuring the absorbance from the UV-visible domain of the protein. As shown in Figure 2, the following solutions 27 μM N-LumP 118 Wt and N-LumP 118 A66W separated and purified protein bound to lumazine fluorophore after incubation, N-LumP 118 A66W protein 27 μM without incubation of ligand, and free lumazine 30 μM were prepared. They were then scanned at wavelengths using Agilent 8453 spectrophotometer. N-LumP 118 A66W protein, which did not add to lumazine, recorded a tiny peak (characteristic of the chromophore) at 410 nm. It is presumed that the reason why the separated and purified protein without incubation of lumazine (dotted line showing slight chromophore peak at 410 nm) is that the lumazine ligand had not been dissociated entirely.

However, N-LumP 118 Wt and N-LumP 118 A66W which was incubated with lumazine recorded a high peak of 410 nm (Fig. 2). This pattern is the same as the full length lumazine protein that the absorbance peak of the lumazine protein slightly increase and red shift by binding the lumazine ligand.[20] These results indicate that the wild type as well as the mutant N-terminal lumazine proteins maintain the capability of binding to the lumazine ligand during incubation. In order to determine the accurate ratio of binding; the value of maximum absorption and literature[21,24] value of molar extinction coefficient: (ε_{410} = 10,800 $M^{-1}cm^{-1}$) were substituted into Lambert-Beer's law equation to calculate the amount of lumazine that bound to the ligand. The value of 30 μM was obtained from the calculation and this corresponds to the concentration of the protein deduced; hence the molar ratio of binding of N-LumP protein to lumazine was found to be 1:1. These results are correlated with the fact that the full lumazine protein bound to equvalent concentration of the lumazine ligand calculated by the absorbance change.[20] In addition, this data also support that the binding site of lumazine ligand is located at the N-terminal half.

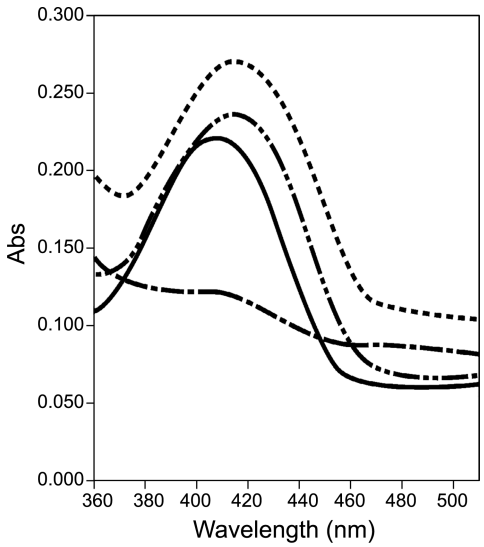

Figure 2. Absorbance spectra of 6,7-dimethyl-8-ribityllumazine, free or in complex with N-terminal domain of lumazine protein (N-LumP 118). Solid line, free 6,7-dimethyl-8-ribityllumazine; Dash-two dotted line; N-LumP Wt after incubation with lumazine ligand; Dotted line, N-LumP 118 A66W after incubation with lumazine ligand; Dash-dotted line, N-LumP 118 A66W without incubation of lumazine ligand. The proteins and the ligand concentration is 27 μM, respectively.

Under these conditions, the binding properties of several mutant N-terminal lumazine proteins (N-LumP 118) were analyzed, after incubation with lumazine ligand, by comparing the absorption spectra obtained from the spectrophotometer (data not shown). It was observed that the dissociated lumazine ligand bound to the protein, and the λ_{max} (maximum absorption) of lumazine generally shifted over a longer wavelength. Maximum absorption peak of free lumazine and lumazine by binding with various N-LumP 118 proteins are as follows. Lumazine, 407 nm; lumazine with N-LumP 118 wt, 412 nm; N-LumP 118 V41W, 412nm; N-LumP 118 S48W, 415 nm; N-LumP 118 T50W, 409 nm; N-LumP 118 D64W, 409 nm; N-LumP 118 A66W, 415 nm.

Out of the recombinant proteins, N-LumP S48W mutant protein recorded the longest distance of wavelength and N-LumP T50W mutant protein recorded the shortest wavelength of maximum absorbance peak. It can be inferred that the presence of another chromophore (amino acid tryptophan) in the mutant protein, resulted in a change in the light absorption of the three dimensional light environment of lumazine ligand chromophore. Moreover, it was observed from Figure 3 that, at a fixed wavelength of 410 nm, the emission spectra of fluorophore (lumazine ligand) from wild type LumP 118 protein recorded a slightly increased brightness in fluorescence as compared to that of the dissociated fluorescent lumazine ligand.

In addition, the mutant N-LumPs showed a slightly decreased fluorescence to 70-80% of the fluorescence brightness of the lumazine ligand were observed by binding N-LumP. This result is contrary to the fluorescence brightness of N-terminal domain of riboflavin synthase (N-RS), which showed a rapid decrease to be quenched under 10% on binding of lumazine to the wild type as well as mutant proteins of N-RS, respectively.[24] These results of different fluorescent characteristic between N-LumP and N-RS reflect that their own tertiary structure of N-LumP and N-RS protein.

As described above, each mutant N-terminal lumazine protein (N-LumP 118) contains one tryptophan amino acid. Hence, in order to analyze the three-dimensional region of tryptophan on each protein, the fluorescence emission spectra of tryptophan on each protein were measured (Fig. 4).

At 280 nm a fixed excitation was carried out for lumazine

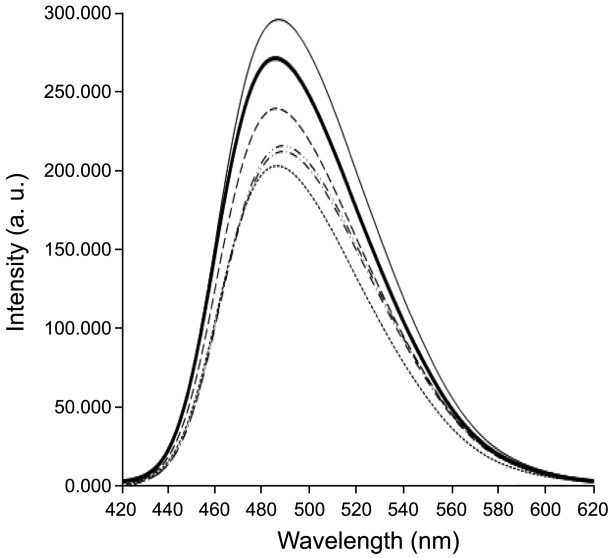

Figure 3. Fluorescence emission spectra of N-LumP 118 mutant proteins bound to lumazine. Excitation at 408 nm. Thick solid line, free lumazine (3 μM); Thin solid line, N-LumP 118 wt after incubation with lumazine; Dotted line, N-LumP 118 V41W; Dashed line, N-LumP 118 S48W; Dash-dotted line, N-LumP 118 T50W; Dash-two dotted line, N-LumP 118 D64W.

ligand, that showed some amount of fluorescence, together with mutants of N-LumP 118 proteins and their respective fluorescence emission spectra were scanned. From these results, it was observed that the highest peak of the relative fluorescence intensity of each mutant protein showed slight differences in their respective emission wavelength .

Especially, the highest emission peak of N-Lump 118 S48W was observed to have longer wavelength than that of N-LumP 118 V41W, T50W. Furthermore, in order to obtain information about the three-dimensional spatial arrangement of tryptophan amino acid, the quenching of tryptophan's fluorescence by acrylamide was investigated. A final concentration of 10 μM of mutants of N-LumP 118 proteins bound to lumazine ligand were prepared and 0.05, 0.1, 0.2, 0.3, 0.5, and 0.7 M of acrylamide were added to enhance the change of tryptophan's fluorescence. Consequently, the quenching constant of all quenched mutants of N-LumP 118 protein could be determined (Fig. 5(a)).

In order to accurately compare the extent of quenching, Stern-Volmer plot was plotted. The graph shows the concentration of acrylamide added (on the X-axis), fluorescence intensity (before the addition of acrylamide of different concentrations), divided by the fluorescence intensity (after the addition of different concentrations of acrylamide) on the Y-axis (Fig. 5(a)). From this plot, it was observed that N-LumP 118 S48W recorded the highest degree of quenching of its fluorescence intensity. This implies that acrylamide had a huge effect on N-LumP 118 S48W, because its structure has an exposed surface. It was also observed that trypto-

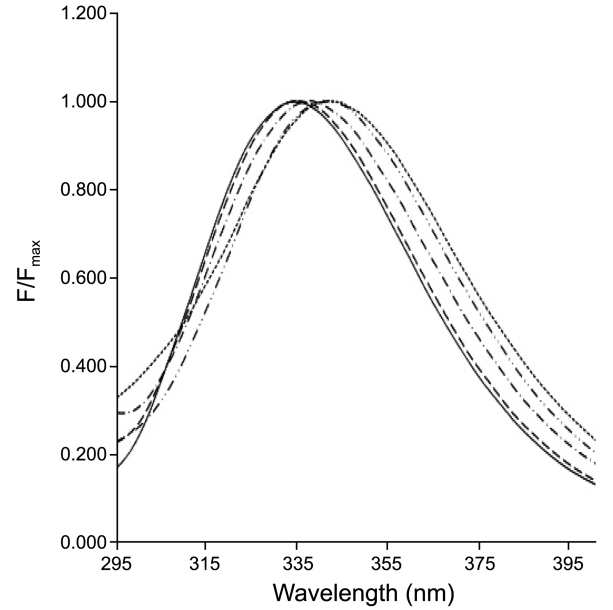

Figure 4. Normalized fluorescence spectra of mutant N-LumP 118 proteins. Excitation at 280 nm, protein 3 μM. Solid line, N-LumP 118 V41W (maximum emission peak at 334 nm); Dotted line, N-LumP 118 S48W (max. at 341 nm); Dashed line, N-LumP 118 T50W (max. peak at 335 nm); Dash-dotted line, N-LumP 118 D64W (max. peak at 337 nm); Dash-two dotted line, N-LumP 118 A66W (maxi. peak at 342 nm).

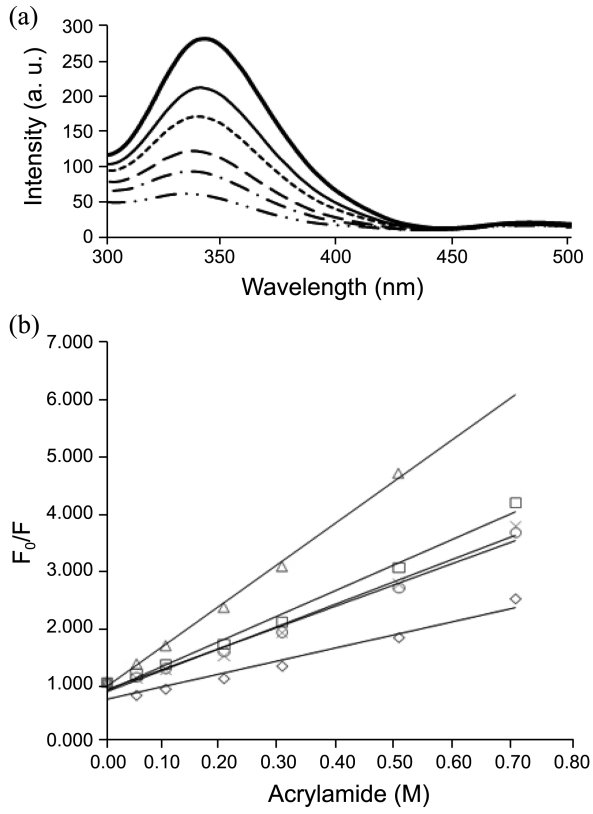

Figure 5. (a) Fluorescence quenching of mutant N-LumP 118 S48W Protein by adding acrylamide. Excitation at 280 nm and 10 μM protein (a) Thick solid line, N-LumP 118 S48W without acrylamide; Thin solid line, 0.5 M acrylamide; Dotted line, 1 M acrylamide; Dashed line, 2 M acrylamide; Dash-dotted line, 3 M acrylamide; Dash-two dotted line, 5 M acrylamide. (b) Stern-Volmer plot of fluorescence quenching studies with various mutants of N-LumP by acrylamide. N-LumP 118 V41W (◇), N-LumP 118 S48W (△), N-LumP 118 T50W (○), N-LumP 118 D64W (□), N-LumP 118 A66W (×).

phan fluorescence of N-LumP 118 V41W protein showed less effects of quenching of by acrylamide (Fig. 5(b)). By using Stern-Volmer equation, the K_{sv} Stern-Volmer constant of mutant N-LumP 118 protein was deduced.

$$F_0/F = 1 + K_{sv}[Q]$$

F_0 : fluorescence intensity without quencher
F: fluorescence intensity at the concentration quencher
[Q] : concentration of quencher
K_{sv} : Stern-Volmer quenching constant.

The Stern-Volmer constants for acrylamide quenched N-LumP 118 mutants protein are as follows. N-LumP 118 V41W, 1.2 M^{-1} ; N-LumP 118 S48W, 6.8 M^{-1}; N-LumP 118 T50W, 2.9 M^{-1} ; N-LumP 118 D64W, 3.6 M^{-1} ; N-LumP 118 A66W, 2.8 M^{-1}. By comparing the values obtained, N-Lump 118 S48W recorded about 5.5 times acrylamide quenching than N-LumP 118 V41W. The variation of the slope *Ksv* of the plots reflects the different overall accessibility of the intrinsic tryptophan residue to solvent.

Discussion

With some molecules, the absorption of photon is followed by the emission of light at a longer wavelength. This emission is called fluorescence, with protein's fluorescence measurement can give information about conformation, binding sites, solvent interactions, degree of flexibility, and intermolecular distance.[2,25] The exact location and properties of the mutual reaction between specific molecules inside the living body is very important in new medicine development. This excites the fluorescent protein and produces an image at a specific wavelength. Cells and bacteria containing fluorescent protein (for example, Green Fluorescent Protein: GFP) expressed gene adopts such method in *in vivo* imaging.[25,26]

The skills of FRET (Fluorescence Resonance Energy Transfer) and FP (Fluorescence Polarization) are well developed technologies used to measure the mutual reaction of the proteins in biopolymers.[27] Green Fluorescent Protein (GFP) is utilized in the study of gene expression and protein mutual reaction in the eukaryotic cell. But in the in-depth pursuit of the study on the lumazine protein, which is a fluorescent protein coded by *lux* gene from marine bioluminescence bacteria, it is necessary to find its expression in the prokaryotic cell only.

Although we have succeed in over-expression and purification of N-terminal domain of lumazine protein reaching to 96 amino acid,[22] to more precise study on binding and structure, we intended to generate the gene coding for N-lumP extending 118 amino acids with creating tryptophan amino acid residue. It was observed that the N-terminal lumazine protein wild type (N-LumP 118 Wt) bound to its lumazine ligand according to the 1:1 molar ratio. V41W, S48W, T50W, D64W, and A66W mutants of N-LumP 118

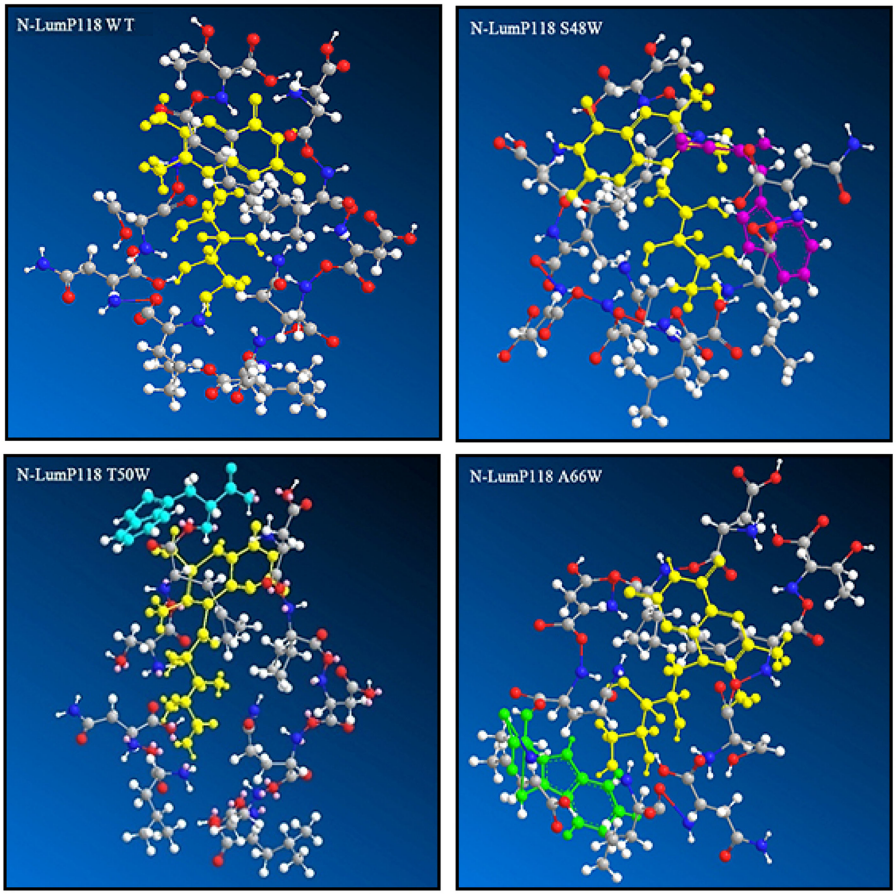

Figure 6. 3D binding model of wild type and mutants lumazine protein bound to lumazine modified 3-D model from previous paper.[24]

also recorded similar phenomenon as the above. The maximum absorption wavelength of the lumazine chromophore was shifted to a longer wavelength as the wild type and mutant N-LumP 118 proteins were also bound to the ligand.

Tryptophan generated proteins showed slightly different emission values for the maximum peak of fluorescence intensity. The extent of conformational change affecting the accessibility of individual tryptophan residues was tested with acrylamide through the quenching experiment. Acrylamide diminishes the fluorescence of tryptophan through the collision with tryptophan or complex formation. From the results obtained, amongst the tryptophan mutated proteins, N-LumP 118 S48W especially, was most sensitive to acrylamide (Fig. 5(b)). In the case of N-LumP 118 S48W protein, the maximum peak was shifted to the longest wavelength according to the degree of binding with the free lumazine (Fig. 3) and the emission fluorescence of its tryptophan appeared to have the longest wavelength (Fig. 4). For tryptophan residues, the intensity and spectra of their fluorescence emission are dependent on the polarity and microenvironment. To show a correlation of these experimental results with the orientation and distance of the chromophores, the 3-D modeling of the proteins are constructed (Fig. 6).

In biophysical chemistry, in-depth study of the ligand of lumazine and the amino acid tryptophan is useful for tracing of the distance and direction which are located at the different positions. Therefore, many tryptophan containing mutated N-LumPs 118 were separated and purified in this research study. Their individual binding characteristics were analyzed with ligands in order to obtain information about their structure and mode of binding.

The lumazine protein shows similar biochemical and structural properties with riboflavin synthase. Despite the relatively low sequence similarity, the tertiary structure of lumazine protein resembles the typical riboflavin synthase fold.[19] Therefore, such study can suggest the starting point of developing the inhibitor for the reasonable enzyme (riboflavin synthase) by revealing of the function of protein and this can be applied in the development of high throughput screening system, which is used for the development of many lumazine derivatives with possibility as antibiotics.

Conclusion

The spectroscopic properties of the wild type and mutants N-terminal lumazine protein (N-LumP 118 Wt, V41W, S48W, T50W, D64W, and A66W) were studied to cheek the binding mode and the fluorescent characteristics. N-LumP and lumazine ligand bind according to the 1:1 molar ratio. Wild type or mutant N-LumP 118 proteins almost maintained its fluorescence intensity of lumazine even when it bound to the luminous ligand. Amongst several mutant proteins purified, N-LumP 118 S48W when bound to the ligand, showed a shift to the longest wavelength and also the largest effect of acrylamide quenching.

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