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Hyperactive Arg39Lys mutated mnemiopsin: implication of positively charged residue in chromophore binding cavity†

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Mnemiopsin, a Ca^{2+}-regulated photoprotein isolated from *Mnemiopsis leidyi*, belongs to the family of ctenophore photoproteins. These proteins emit blue light from a chromophore, which is tightly but non-covalently bound in their central hydrophobic core that contains 21 conserved residues. In an effort to investigate the role of Arg39 (the sole charged residue in coelenterazine binding cavity of ctenophore photoproteins) in bioluminescence properties of these photoproteins, three mutated forms of mnemiopsin 1 (R39E, R39K and R39M) were constructed and characterized. The results indicate that while the luminescence activity of R39K mutated mnemiopsin has increased about nine fold compared to the wild type, R39M and R39E mutated mnemiopsins have entirely lost their activities. The most distinguished properties of R39K mutated photoprotein are its high activity, slow rate of luminescence decay and broad pH profile compared to the wild type. The complete loss of bioluminescence activity in mutated photoproteins with negatively charged and aliphatic residues (R39E and R39M, respectively) shows that the presence of a positively charged residue at this position is necessary. The results of spectroscopic studies, including CD, intrinsic and extrinsic fluorescence measurements and acrylamide quenching studies show that, while the substitutions lead to structural rigidity in R39E and R39M mutated mnemiopsins, structural flexibility is obvious in R39K mutated mnemiopsin. The presence of a more localized positive charge on ε-amino group of Lys compared to guanidinium group of Arg residue in close proximity to the choromophre might affect its fixation in the binding cavity and result in increased bioluminescence activity in this mutated photoprotein. It appears that the polarity and flexibility of positively charged residue at this position finely tunes the luminescence properties of ctenophore photoproteins.

Introduction

Photoprotein mnemiopsin is a single subunit protein consisting of 206 amino acid residues.[1,2] This photoprotein, together with aequorin, obelin and other photoproteins that originate from cnidarians and ctenophores, are a subfamily of calcium-regulated proteins. These proteins, particularly cnidarian photoproteins aequorin and obelin, have been developed and exploited as markers or reporters for other biochemical processes in biological and biomedical research. In these proteins, the bioluminescence reaction is a single turnover event triggered by calcium ions and originates from an oxidative decarboxylation of a protein bound substrate, coelenterazine, resulting in the release of CO_2 and a flash emission of blue light.[3,4]

In addition to Ca^{2+}-regulated photoproteins, there is a distinct class of proteins in the EF-hand superfamily of Ca^{2+}-binding proteins, which are known coelenterazine-binding proteins (CBP). This Ca^{2+}-regulated protein contains coelenterazine bound within its inner cavity and is a part of the bioluminescent system in *Renilla*, which does not catalyze the oxidation of coelenterazine, but as a substrate binding protein upon the binding of calcium ions undergoes conformational changes, resulting in the release of coelenterazine for oxidative decarboxylation in the active center of luciferase. The results obtained from the crystal structure acquisition of holoCBP indicates that despite the low sequence identity, the structure of CBP closely resembles structures of Ca^{2+}-regulated photoproteins obelin, aequorin and clytin,[5,6] however, coelenterazine in CBP is found to be rotated over 90° compared to the peroxy coelenterazine orientation in obelin.[7,8]

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All the known Ca^{2+}-binding photoproteins are globular proteins, relatively small in size (21.4 to 27.5 kDa) with three "EF-hand" domains to bind Ca^{2+}, and accommodate a peroxidized coelenterazine in the central cavity of the protein. According to available crystal structures of Ca^{2+}-binding photoproteins (aequorin and obelin from cnidarians and berovin from ctenophores), the coelenterazine binding site is a highly hydrophobic cavity buried in the center of photoprotein that contains 21 conserved residues.[7–9] Hydrophobic interactions are considered as major factors in stabilizing the substrate in the binding pocket. In chromophore binding cavities of aequorin and obelin, in addition to hydrophobic residues, several hydrophilic side chains, such as His (His 16 and 169 of aequorin and 22 and 175 of obelin) and Tyr (Tyr 132 of aequorin and 138 of obelin) are also directed internally. These side chains form a network of hydrogen bonds that apparently stabilize the highly labile hydroperoxy coelenterazine. In ctenophore photoproteins (*e.g.* mnemiopsin and berovin) these residues are substituted by hydrophobic residues Leu, Phe and Met.[10–12]

The crystal structures of aequorin and obelin helped to clarify their supramolecular structures and also yielded important information on the characteristics of their active sites. Despite complete researches on cnidarian photoproteins, little efforts have been made on ctenophore photoproteins. Although these photoproteins functionally are identical with the cnidarian photoproteins, they are different in many of their physical properties.[13] In the attempt to understand bioluminescence in ctenophore photoproteins following cloning and characterization of mnemiopsins 1 and 2 from *Mnemiopsis Leidyi*,[1,2] we recently selected and modified a set of critical residues in mnemiopsin 1 using site directed mutagenesis.[14] As mentioned, in Ca^{2+}-regulated photoproteins the coelenterazine binding cavity is very hydrophobic and these proteins have high content of hydrophobic residues such as Leu, Ile and Trp in the chromophore binding cavity.

Sequence alignment of cnidarian and ctenophore photoproteins shows that the coelenterazine binding site in both cases is highly hydrophobic and that the distribution of residues is different between the two main types[1,2] (Fig. 1). Interestingly, while there are no charged amino acids in the coelenterazine binding cavity of cnidarian photoproteins, ctenophore photoproteins, including mnemiopsin, berovin, bolinopsin and BfosPP, have a positively charged residue (Arg) in their chromophore binding pocket.[9,15] The corresponding position in cnidarian photoproteins is occupied by a Met residue[11] (Fig. 1a).

In the present study, we introduce three mutations at this position in mnemiopsin (Arg39), in order to investigate the relevance of this positively charged residue for the bioluminescence characteristics of ctenophore photoproteins. In the first mutated protein, we replaced Arg39 with Met (corresponding residue in aequorin and obelin); in the second substitution we introduced a similarly charged residue (R39K), while the third one bears a residue with a negative charge (R39E). Three mutated proteins were thus obtained and comparative biochemical and bioinformatics studies with respect to WT (wild type) mnemiopsin were carried out.

Results and discussion

In the present study, site directed mutagenesis was used to make mutated forms of mnemiopsin at position 39 displaying different functional and structural properties. It is very important to note that in Ca^{2+}-regulated photoproteins, the coelenterazine binding cavity is highly hydrophobic.[1–3,7] While there is no charged residue in chromophore binding cavity of cnidarian photoproteins, the presence of a positively charged residue (Arg) in the coelenterazine binding site of ctenophore photoproteins is very interesting and noticeable.

In the attempt to compare the bioluminescence in two groups, in the first step, Arg39 was replaced with the corresponding residue in cnidarians (Met), therefore, R39M mutated mnemiopsin was constructed. Then, in order to investigate the effect of residue charge on mnemiopsin bioluminescence properties, a mutated protein containing a residue with negative charge was prepared (R39E mutated mnemiopsin). Finally, a third mutated photoprotein was designed by the substitution of Arg for the same charge (Lys) in order to determine the role of the residue side chain on bioluminescence properties.

Following the construction of structural models, their reliability was confirmed by model stereochemical quality checks. The results of local environment evaluation, calculated by Verify 3D and PSQS, accompanied by local geometry checks, calculated by ERRAT and ProCheck (ESI Table 1†), indicates a high quality for the models, which are thus suitable as tools for analyzing the 3D structures. Binding of the ligand in terms of interactions between coelenterazine and cavity residues in WT and mutated mnemiopsins were studied using structural analysis of the models.

Following the amplification of the constructs and transformation, mutations at specific residue were confirmed by sequencing. Over expression of apophotoproteins containing a His_6-tag was carried out in *E. coli* BL21 (DE3) and purification was efficiently performed by affinity chromatography using a Ni-NTA agarose column. The purified apo photoproteins migrate to around 27 kDa on SDS-PAGE, as reported for WT mnemiopsin 1.[1] Dialyzed apomnemiopsins were regenerated to mnemiopsins by incubation with coelenterazine and EDTA in the dark and finally, bioluminescence activities were determined.

Bioluminescence activity and emission spectra

The relative bioluminescence activities of semi-synthetic WT and mutated mnemiopsins have been summarized in Table 1. As shown in the Table 1, while activity of R39K mutated mnemiopsin increases more than nine times compared to semi-synthetic WT mnemiopsin, two other substitutions (R39M and R39E mutated mnemiopsins) lead to inactivation of the photoprotein, in other words these mutations disrupt the light emission process. According to sequence alignment results, the

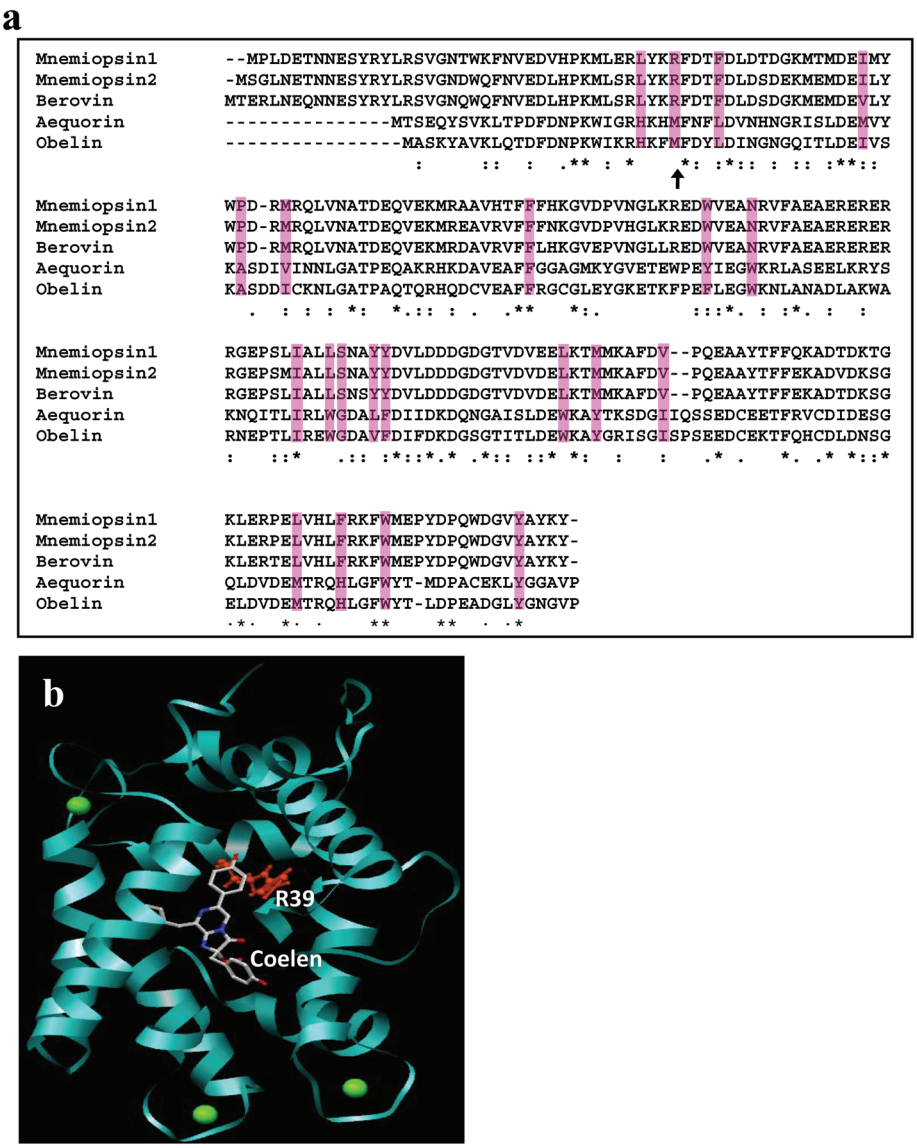

Fig. 1 (a) Multiple sequence alignment of ctenophore (mnemiopsin 1: GQ231544; mnemiopsin 2: GQ884175 and berovin: CS050690) with cnidarian photoproteins (aequorin: P07164 and obelin: AF394688). 21 conserved residues of cavity (based on cnidarian photoproteins) have been shown inside the boxes. The position of the mutations has been identified with the arrow. (b) Predicted 3D structure of mnemiopsin 1 showing the structural position of Arg39 relative to the chromophore. Aequorin structural information (PDB ID: 1EJ3) was used in order to insert coelentrazine molecule within the constructed model. Ca^{2+} ions are shown as the green spheres.

Table 1 Characteristics of mnemiopsin and its mutated forms

Photoprotein	Relative activity[a] (%)	λ_{max} (nm)	λ_{max} shoulder (nm)	Opt pH	pH range[b]	C_{50} [b]
WT	100	480	500	9.3	8.8–9.8	$10^{-4.8}$
R39E & R39M	<1	—	—	—	—	—
R39K	900	480	500	9.5	8.2–10.2	$10^{-4.1}$

[a] Specific activity of WT semi-synthetic mnemiopsin was 3.190×10^9 RLU per s per mg protein and its initial count in the absence of calcium was also 382 167 RLU s^{-1}. [b] Respectively the ranges of pH and Ca^{2+} concentration that 50% of activity is seen.

residue at this position is highly conserved in the coelenterazine binding cavity in each type of photoprotein (Fig. 1a). Because the substitutions of Arg with negatively charged (R39E) and aliphatic residue (R39M) result in inactive mutated photoproteins, the importance of the presence of positive charge at this position is demonstrated and it appears that Lys is more suitable candidate to locate in this position than the Arg residue.

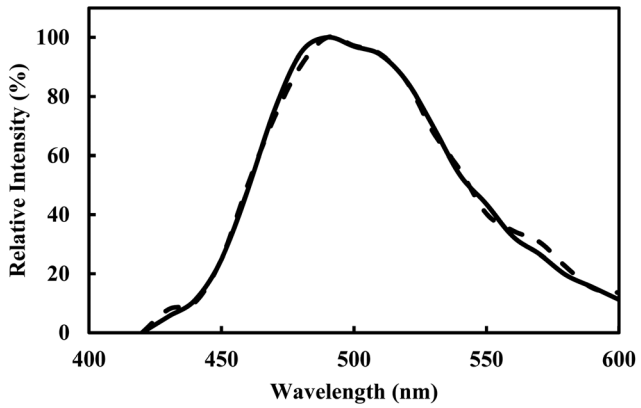

Fig. 2 The bioluminescence emission spectra of the WT (—) and R39K mutated mnemiopsin (---) at pH 9.0. Emission spectra were obtained as described in the experimental procedures.

Substitution of the corresponding residue in aequorin (Met 19) with polar (Cys and Ser) and hydrophobic residues (Ala and Ile) resulted in inactive mutated mnemiopsins. While relative luminescence activity of M19C mutated aequorin was 6% and its decay rate was faster than WT aequorin, mutation did not affect the bioluminescence λ_{max}. The other mentioned mutated photoproteins completely lost their bioluminescence activities.[11] To date, the role of this residue on bioluminescence of cnidarians has not been determined and no report has been published on the substitution of the Met with charged residues in aequorin or obelin.

In the past decade, scientists have become increasingly aware of the importance of cation–π interactions on protein structure and stability, molecular recognition, binding of ligands to protein, stabilizing reaction substrates or intermediates and enzyme catalysis.[16,17] In WT and mutated constructed models, the positive charge of Arg and Lys residues is located in the proximity of coelenterazine, which may bring about the formation of cation–π interactions (Fig. 1b). In comparison with Arg, which distributes positive charge on the guanidinium group, the charge of the Lys ε-amino group is less dispersed but is a more localized positive charge that may favour cation–π interactions between chromophore and Lys in R39K mutated mnemiopsin. This change might affect the fixing of the chromophore and enhance its stabilization in the binding cavity and it may result in increased bioluminescence activity in this mutated form.

The *in vitro* bioluminescence spectra of semi-synthetic WT and R39K mutated mnemiopsins are depicted in Fig. 2. These measurements were carried out at pH 9.0 and room temperature. As shown in the figure, no change in the maximum wavelength for the bioluminescence spectra of the mutated mnemiopsins was observed (Table 1), suggesting that, while this residue plays very crucial role in bioluminescence activity of mnemiopsin; it is not involved in determining the color emission properties.

Decay time

Measurements of decay time for the semi-synthetic photoproteins showed that the decay rate of light emission for R39K mutated mnemiopsin was slower than WT mnemiopsin's (Fig. 3a). Longer decay time of the semi-synthetic mutated photoprotein is probably due to the factors such as interactions of chromophore with chromophore binding cavity residues. On the other hand, increased decay time of the semi-synthetic mutated photoprotein can be likely due to a decrease in its calcium sensitivity compared to semi-synthetic WT (see below). The relationship between decay time and calcium sensitivity has been elucidated previously.[14,18]

pH profile

The profile of activity *vs.* pH for semi-synthetic WT and R39K mutated mnemiopsin is presented in Fig. 3b. Optimum pHs were determined by injecting 10 μl of regeneration mixture into a 40 mM $CaCl_2$ solution with pHs ranging from 7.0 to 11.0 (0.5 and 0.2 unit intervals from 7.0 to 8.5 and 8.5 to 10.0, respectively), followed by a bioluminescence activity assay. The optimum pH for semi-synthetic WT mnemiopsin was obtained at pH 9.3 that was in agreement with previous studies.[1,14] The optimum pH of R39K mutated mnemiopsin showed a 0.2 shift toward alkaline pH (Table 1) and its pH profile curve was

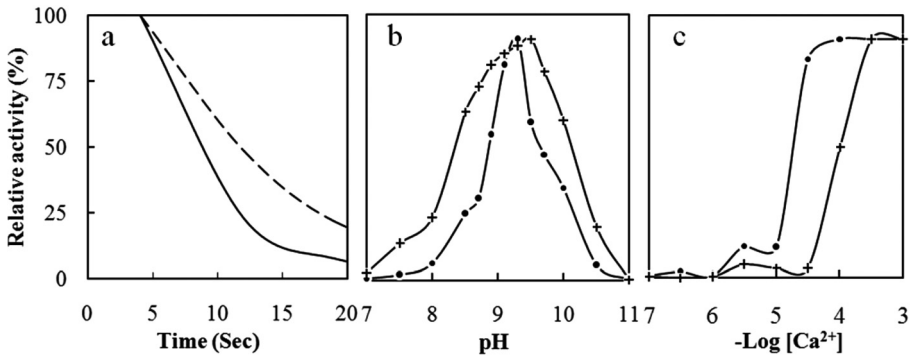

Fig. 3 (a) Comparison of decay rates of WT (—) and R39K mutated mnemiopsins (---). The residual activity was reported as a percentage of the original activity. (b) pH optima for light intensity assays of WT (•—•) and R39K mutated mnemiopsins (×—×). (c) Relationship between Ca^{2+} concentration and the initial light intensity of WT (•—•) and R39K mutated mnemiopsins (×—×).

higher and more broader than semi-synthetic WT's, *i.e.* in contrast with WT mnemiopsin, that regeneration takes place only at a narrow pH range around 9.0, the mutated photoprotein showed lower sensitivity to pH particularly between pHs 8.5 to 10.0 as the calculated relative bioluminescence activities in this range were more than 65%.

It is known that unlike the other photoproteins, the bioluminescence activity of mnemiopsin is more sensitive to the pH changes and mnemiopsin regeneration takes place only at a narrow pH range around 9.0.[14] Therefore, the construction of such variant of mnemiopsin with high activity in a broad pH range is very significant and could expand the applications of the photopretein. To logically discuss the difference in the optimum pH values, the pK_a values for titrable residues presented in the cavity of WT and mutated mnemiopsins were calculated by the MacroDox program. According to sequence alignment studies, it has been revealed that mnemiopsin 1 has four ionizable residues in its cavity, including Arg39, Tyr131, Tyr132 and Tyr202. According to the results, it appears that a decrease in pK_a of three titrable residues (Lys39, Tyr132 and Tyr202) in the coelenterazine binding cavity of R39K mutated mnemiopsin is likely involved in the pH-dependent behavior of the mutated photoprotein compared to WT.

Calcium titration

In Ca^{2+} binding photoproteins, although calcium regulates bioluminescence emission but it is not essential for emission, and photoproteins alone gives a very low level of light emission called the "calcium-independent luminescence". The light intensity is increased up to 1 million fold or more with the addition of calcium.[19] There are three EF-hand Ca^{2+}-binding consensus sequences in these proteins.[1,2,7,8] High Ca^{2+} sensitivity has been led to photoprotein applications as Ca^{2+} indicators for a variety of purposes, and especially in tracking the location and concentration of calcium ions in real time in biological systems.[20] We examined the Ca^{2+} sensitivity of semi-synthetic WT and mutated mnemiopsins by the injection of photoprotein into the Ca^{2+} solutions ($10^{-8.5}$ to $10^{-3.5}$ M). As seen in Fig. 3c, calcium sensitivity curve for R39K mutated mnemiopsin shifted toward higher concentrations of calcium, which indicates lower Ca^{2+} sensitivity of the mutated photoprotein compared to semi-synthetic WT (Table 1), thus it can be used for the detection of higher Ca^{2+} concentrations.

Structural characterization of WT and mutated apomnemiopsins

CD spectra. CD spectra of WT and mutated apomnemiopsins were obtained in Tris buffer (50 mM, pH 9.0) and used to evaluate the secondary structural changes induced by substitutions. As indicated in Fig. 4a, the far-UV CD spectra show changes in the secondary structures of the three mutated apoforms compared to WT. While the secondary structure contents have noticeably increased in R39E compared to WT apomnemiopsin, CD spectrum of R39M mutated apomnemiopsin shows only slight difference as a little increase in the secondary structures compared to WT. However, the far-UV spectrum of the R39K mutated apomnemiopsin shows major alterations compared to WT photoprotein. There is a loss in the characteristic alpha helical minima at 208 nm and 222 nm, which is evident in the WT and two other mutated apomnemiopsins. In the view of the difficulties caused by excessive noise below 195 nm, it is not possible to obtain reliable estimates of the beta sheet contents of the photoprotein samples.

Fluorescence measurements. Because of the intrinsically high fluorescence sensitivity of tryptophan residues to the polarity of microenvironments, it was used for monitoring the conformational changes in 3D structures of WT and mutated apomnemiopsins. As shown in Fig. 4b, increase in intrinsic fluorescence intensity was observed for R39E and R39M mutated mnemiopsins, which indicates an increase in tertiary structure of these mutated forms, whereas it decreased in R39K mutated photoprotein compared to WT apomnemiopsin. It appears that substitutions may bring tryptophan residues to more hydrophobic (R39E and R39M mutated mnemiopsins) or hydrophilic environments (R39K mutated mnemiopsin). In other words, the results show mutation-induced conformational changes in each of three mutated photoproteins compared to WT mnemiopsin and presumably indicate structural compactness in R39E and R39M mutated mnemiopsins, and conversely the structural flexibility of R39K mutated mnemiopsin. These results are in good agreement with CD results.

ANS anion is conventionally considered to bind to preexisting hydrophobic (nonpolar) surfaces of proteins, primarily through its nonpolar anilinonaphthalene group. Such binding is followed by an increase in ANS fluorescence intensity, similar to that occurring when ANS is dissolved in organic solvents. For this reason ANS is used to study the surface hydrophobicity of proteins. In other words, the enhancement of ANS fluorescence reflects more exposed hydrophobic patches on protein surface.[21,22] Fig. 4c shows ANS fluorescence spectra of WT and mutated apomnemiopsins. As shown in the figure, ANS florescence intensity has clearly decreased in these mutated proteins compared to WT apomnemiopsin. The results, obtained from the calculation of total ASA values for hydrophobic residues of WT and mutated constructed models of mnemiopsin, are also in good agreement with the results of ANS fluorescence. From R39E and R39M mutated apomnemiopsins, we deduce that the structural conformation of the mutated apomnemiopsins is compact and hydrophobic patches are buried, which in turn results in reduced ANS fluorescence intensity. It can represent an increase of tertiary structure of these mutated photoproteins in comparison with WT, and moreover it is consistent with CD and intrinsic fluorescence results for these two mutated proteins. On the other hand, although structural changes are obvious in R39K mutated mnemiopsin and the CD and fluorescence results indicate an open conformation for this mutated form compared to WT mnemiopsin, its ANS florescence spectrum has significantly decreased unexpectedly. To explain this observation, it can be said that the mutation-induced conformational changes likely result in lower environmental accessibility of protein hydrophobic patches.

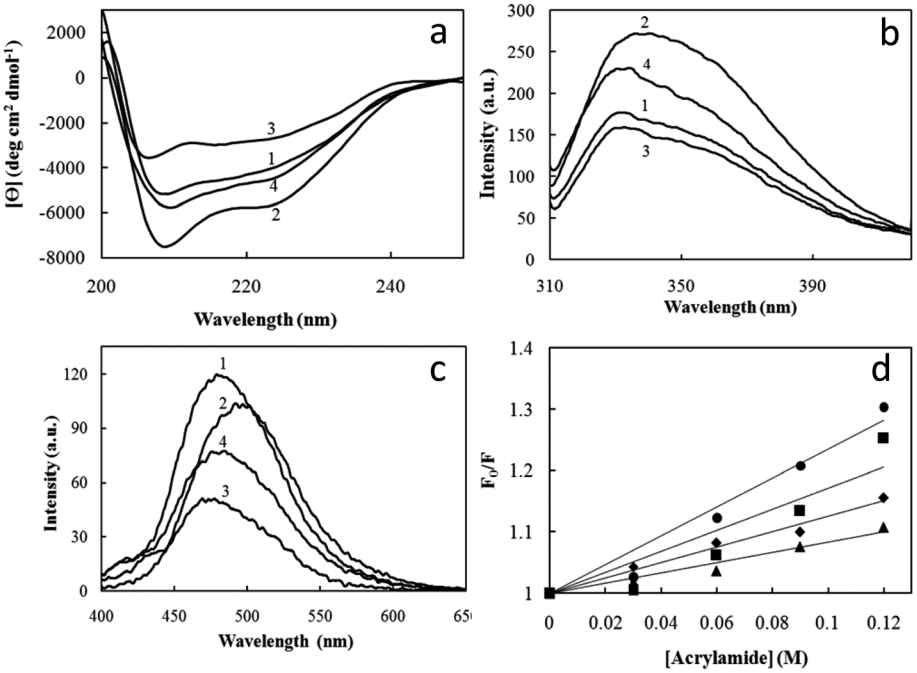

Fig. 4 (a) Far-UV CD spectra for the WT (1) and mutated forms of apomnemiopsin [R39E (2), R39K (3) and R39M (4)]. The concentration of protein used for the far-UV CD spectrum was 0.2 mg ml^{-1} and apomnemiopsins equilibrated in 0.05 M Tris buffer (pH 9.0) containing 0.005 M NaCl at 25 °C. (b) Intrinsic fluorescence spectra of tryptophan residues for the WT (1) and mutated apomnemiopsins [R39E (2), R39K (3) and R39M (4)]. The spectra were measured at room temperature and the same buffering condition as the CD measurement. The protein concentration was 15 μg ml^{-1}. (c) Extrinsic fluorescence spectra using ANS for the WT (1) and mutated apomnemiopsins [R39E (2), R39K (3) and R39M (4)]. Spectra were recorded at the same conditions as the intrinsic fluorescence measurements and 30 mM ANS. The excitation wavelength was 350 nm. (d) Stern–Volmer plots of WT (◆) and mutated apomnemiopsins [R39E (▲), R39K (●) and R39M (■)] apomnemiopsins were obtained by quenching with acrylamide. The excitation and emission wavelengths were 295 and 340 nm, respectively. The protein was dissolved in 0.05 M Tris-base buffer (pH 9.0) and the protein concentration was 30 mg ml^{-1} in all samples.

Fluorescence quenching by acrylamide. A very informative experiment in the protein field is quenching by the addition of a quencher molecule or ion to the solution. Acrylamide quenching has been extensively used to determine the degree of exposure of Trp residues in proteins. The results of CD and fluorescence studies show that mutations likely result in structural compactness in R39E and R39M mutated mnemiopsins and local structure opening in R39K mutated mnemiopsin. For complementary investigation and in order to reveal the difference in the surface accessibility of tryptophan residues as a result of mutation, we measured the quenching of tryptophan fluorescence of WT and mutated apomnemiopsins with acrylamide.[23] According to the Stern–Volmer plots shown in Fig. 4d, while R39M mutated apomnemiopsin is quenched almost similar to WT, R39K and R39E mutated apophotoprotein, respectively, show higher and lower quenching compared to WT. An increased slop of Stern–Volmer plot in R39K mutated protein indicates that the substitution changes the integrity of protein and more effectively exposes the fluorophore to the quencher than WT and other mutated mnemiopsins. In other words, mutation leads to an increase in the structural flexibility in this mutated form, whereas in R39E mutated apomnemiopsin had a decreased slope of the Stern–Volmer plot as a result of increased structural rigidity of the mutated photoprotein compared to WT mnemiopsin.

From the entire spectroscopic data, it was seen that the changes of amino acids in mnemiopsin have great impact on both its secondary and tertiary structure microenvironment. The stability and integrality of protein's tertiary structure is important for protein to put its function into practice.

Materials and methods

Chemicals

The restriction enzyme *Dpn*I was obtained from Fermentas (Fermentas, Vilnius, Lithuania). cp-coelenterazine was purchased from Sigma (St. Louis, MO, USA). Kanamycin and isopropyl-D-thiogalactopyranoside (IPTG) were obtained from Invitrogen (Carlsbad, CA, USA). The Ni-NTA agarose was provided by Qiagen (Qiagen, Hilden, Germany). Plasmid and PCR purification kits were purchased from Bioneer (Bioneer, Seoul, Korea). All the other chemicals were obtained from Merck (Darmstadt, Germany). Nucleotide sequencing was performed using an automatic sequencer (Eurofins MWG Operon, Germany) based on dideoxy chain termination/cycle sequencing on ABI 3730XL sequencing machines. Reproducibility of the data presented in this paper was confirmed by repeating the experiments at least three times. The data presented here are typical experimental data.

Site-directed mutagenesis, protein expression and purification

Site-directed mutagenesis was performed using the Quick Change method.[24] The plasmid containing the gene of apomnemiopsin 1 (GenBank accession No. GQ231544) was used as a template for the reaction. The mutagenesis primers are listed in ESI Table 2.† The following parameters were employed: denaturation at 95 °C for 5 min; 22 cycles of 95 °C for 1 min; 60 °C for 1 min; 72 °C for 13 min and final extension at 72 °C for 10 min. Subsequently, the amplified products were purified using a PCR purification kit to remove redundant primers, and the fragments digested with *Dpn*I in order to digest native parental plasmids, then the products transformed to *E. coli* BL21 (DE3) (Novagen, Madison, WI, USA) by a chemical method[25] for each mutation. The plasmids harboring mutations were verified by DNA sequencing.

His_6-tagged WT and mutated apomnemiopsins were expressed from pET28a in *E. coli* BL21 (DE3) host cells and induction was performed with a final concentration of 1 mM IPTG. After the purification of recombinant photoproteins using a Ni–NTA resin, the eluted fractions were collected for SDS-PAGE analysis and showed a purity of 95%. Finally, the dialysis of the collected fractions and determination of protein concentrations were carried out as described previously.[14]

Bioluminescence activity and decay half-life time

The purified and dialyzed apomnemiopsins were incubated with a given volume of coelenterazine analogue (8 mM in final volume, 10-fold molar excess of coelenterazine to apomnemiopsins) in a microtube with 50 mM Tris buffer (pH 9.0) containing 10 mM EDTA at 4 °C in the dark for 16 h. The luminescence activities of the semi-synthetic WT and mutated photoproteins were determined by adding 10 μl of the regeneration mixture to a glass tube containing 40 μl of 50 mM Tris–base, pH 9.0 (buffer 2). The tube was then placed in a luminometer (Sirius tube luminometer, Berthold Detection System, Germany). By the injection of a volume of 50 μl of buffer 3 (50 mM Tris-base containing 40 mM $CaCl_2$, pH 9.0) into the sample solution, the luminescence intensity was measured. Furthermore, for the determination of the half decay time of semi-synthetic photoproteins, samples were prepared in the same manner as mentioned above and a microplate reader (Berthold Detection System/Orion II) was used. A volume of 50 μl of buffer 3 was injected into the sample solution and the bioluminescence signals were collected at 0.1 s and at 20 s intervals. The residual activity for each photoprotein is reported as a percentage of the original activity. All the reported data are the mean of three replications that are corrected for the blank.

Determination of bioluminescence emission spectra

Bioluminescence emission spectra of semi-synthetic WT and R39K mutated mnemiopsin were measured on a Synergy H4 fluorescence plate reader (injection rate 270 μl s^{-1}, sensitivity 150; set 4 mm, emission step: 10, BioTek, USA). The instrument was capable to obtain spectra from flash reactions of luminescent samples that emit in the 400 to 700 nm range. The bioluminescence emission spectra of the samples were obtained by placing 50 μl of the regenerated mixture in a 96-well microtiter plate, which was then placed into the instrument. A volume of 50 μl of buffer 50 mM Tris-base containing 40 mM $CaCl_2$, pH 9.0 was injected into the sample solution in order to trigger the light emission. The luminescence signal was collected over a 10 s period of time. Measurements were carried out at room temperature.

Photoprotein characterization

For determination of the Ca^{2+} sensitivity, the required volume of purified apophotoprotein was dissolved in 50 mM Tris–base (pH 9.0) containing 0.01 mM EDTA, 0.1% bovine serum albumin and 150 mM NaCl, and then incubated at 4 °C for 16 h.[14] 10 μl of the mixture was added to 40 μl of 50 mM Tris–base, pH 9.0 in a glass tube and 50 μl of various Ca^{2+} concentrations ($10^{-8.5}$ to $10^{-4.0}$ M) with 50 mM Tris–base (pH 9.0) was injected into the tube. The luminescence intensity was determined by a luminometer (Sirius tube luminometer, Berthold Detection System, Germany).

Moreover, the optimum pH of activity for photoprotein was measured by the incubation of apophotoprotein in a mixed buffer having pH range of 6.5 to 11.0. The mixed buffer was prepared using various amounts of 50 mM Tris, 100 mM glycine and 100 mM succinic acid. The remaining activities were recorded as a percentage of the original activity.

Structural analysis of the WT and mutated apomnemiopsins

Intrinsic and extrinsic fluorescence. All the structural studies were carried out on purified and dialyzed apomnemiopsins. Fluorescence studies were performed at room temperature using a Perkin Elmer luminescence spectrometer LS 55. Intrinsic fluorescence was recorded using 15 μg ml^{-1} apophotoprotein (protein buffer was 50 mM Tris-base, pH 9.0, containing 5 mM NaCl). Emission spectra were read against a control sample and scanned between 300 and 420 nm. The excitation wavelength was 295 nm. Extrinsic fluorescence studies were carried out with ANS as a fluorescent probe. The final concentration of the ANS in the protein solutions was 30 mM and samples were incubated with the hydrophobic probe for 5 min. The ANS emission was scanned between 380 and 700 nm with an excitation wavelength of 350 nm in a 1 cm path length quartz cell.

Circular dichroism. Circular dichroism (CD) spectra were measured with a JASCO J-715 spectropolarimeter (Tokyo, Japan) using solutions with apo photoprotein concentration 0.2 mg ml^{-1} (200 to 250 nm) in 50 mM Tris buffer (pH 9.0). Measurements were carried out at room temperature. The results were expressed as molar ellipticity $[\theta]$ (deg cm^2 $dmol^{-1}$), based on a mean amino acid residue weight (MRW) of apophotoproteins. The molar ellipticity was determined as $[\theta] = (\theta \times 100MRW)/(cl)$, with c being the protein concentration in mg ml^{-1}, l is the light path length in centimeters, and θ is the measured ellipticity in degrees at a wavelength λ. Noise in the data was smoothed using the JASCO J-715 software, includ-

ing the fast Fourier-transform noise-reduction routine, which allows the elimination of noisy spectra without distorting their peak shapes.[26,27]

Acrylamide quenching. Fluorescence quenching was carried out *via* the addition of final concentrations 30 to 120 mM acrylamide, with 30 mM intervals, to 0.02 mg ml^{-1} of protein solutions. The fluorescence emission was scanned between 300 and 440 nm with an excitation wavelength of 295 nm in Perkin Elmer luminescence spectrometer LS 55. Quenching data were analyzed in terms of the Stern–Volmer constant, KSV, which was calculated from the ratio of the unquenched and the quenched fluorescence intensities, F_o/F, using the relationships $F_o/F = 1 + KSV[Q]$. [Q] is the molar concentration of the quencher.[28]

Sequence analysis and bioinformatic studies. Similarity searches, the derivation of nucleotide and amino acid sequences and multiple sequence alignment studies were performed as described previously.[14] 3D structural models of holo WT and mutated mnemiopsins were constructed using the MODELLER program Ver.9v2, and berovin (PDB ID: 2HPK) was selected as template for its high homology with mnemiopsin (sequence identity 90%). Moreover, aequorin structural information (PDB ID: 1EJ3) was also used in order to insert a coelentrazine molecule within the constructed models. The models were constructed by optimizing the probability objective function and simulated annealing. Ten models were generated for each of the WT and mutated mnemiopsins. The structures displaying the lowest objective function value were selected as the final structural models. To validate the quality of the models, programs such as the Protein Structure Quality Score (PSQS) (http://www1.jcsg.org/psqs), ERRAT, Verify3D, and ProCheck (http://nihserver.mbi.ucla.edu/SAVS/) were used.

Calculations of pK_a. The MacroDox program (ver. 2.0.2)[29] was used to calculate pK_a of cavity residues of WT and R39K mutated mnemiopsins. All the pK_a values were calculated in ionic strength 10 mM and temperature 298 K at various pH values based on the Tanford-Kirkwood calculation.

Conclusion

In this study, for the first time, three types of mutations based on the charge properties of residues at position 39 of mnemiopsin 1 (Arg) were designed and constructed. Regarding Ca^{2+} binding photoproteins, the coelenterazine binding cavity is highly hydrophobic, the presence of charged residue in the cavity of ctenophore photoproteins is very interesting and even may be indicative of differences in light emission in ctenophore photoproteins compared to cnidarian photoproteins. It offers a new insight into the mechanism of light emission in photoproteins. Because the substitutions at this position (Arg 39) strongly affect bioluminescence activity in mnemiopsin, it appears that corresponding residue has a crucial role in the bioluminescence of ctenophore photoproteins. On the other hand, because of complete loss of bioluminescence activity in the mutations with negatively charged and aliphatic residues (R39E and R39M, respectively), the presence of a positively charged residue at this position is necessary, but it appears that side chain of Lys is more suitable than the side chain of Arg for association in required interactions in the cavity. The presence of Lys in this position not only causes a large increase in activity but also more effectively decreases the decay rate and increases pH range of activity compared to semi-synthetic WT mnemiopsin. On the other hand, our spectroscopic studies show that whilst the substitutions lead to structural rigidity in R39E and R39M mutated mnemiopsins, structural flexibility is obvious in the R39K mutated protein. Considering the importance of conformational changes for bioluminescence triggering in Ca^{2+} binding photoproteins, it is likely that mutation has induced a closed conformation in R39E and R39M mutated mnemiopsins. These changes may severely interfere with the Ca^{2+}-induced conformational changes and thus it has inhibited the light emission in related mutated photoproteins. In contrast, the presence of Lys positive charge, likely with a more appropriate conformation compared to Arg (in WT mnemiopsin), and also a more flexible structure in R39K compared to WT photoprotein, can be the desired factors for improved bioluminescence characteristics of this mutated photoprotein. Thus, it appears that this position is a critical point for protein function and finely tunes the ctenophore photoprotein's bioluminescence activity through the polarity and flexibility of a positively charged residue. Finally, it is again emphasized that the present study is the only report from a mutated mnemiopsin with very high activity and improved kinetic properties. More studies, especially the structure determination of WT mnemiopsin and R39K mutated mnemiopsin will undoubtedly help us to understand, in more detail, the mechanisms of bioluminescence and also the precise identification of the role of the desired charged residue in the light emission process of ctenophore photoproteins.

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