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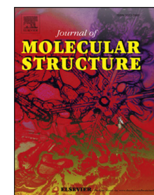

Adjustment of local conformational flexibility and accessible surface area alterations of Serine[128] and Valine[183] in mnemiopsin

Forough Hakiminia [a], Maryam Molakarimi [b], Khosrow Khalifeh [c], Zohreh Jahani [d], Reza H. Sajedi [b], Bijan Ranjbar [a, *]

[a] Department of Biophysics, Faculty of Biological Sciences, Tarbiat Modares University, P.O. Box 14115-175, Tehran, Iran
[b] Department of Biochemistry, Faculty of Biological Sciences, Tarbiat Modares University, P.O. Box 14115-175, Tehran, Iran
[c] Department of Biology, Faculty of Sciences, University of Zanjan, P.O. Box 45195-313, Zanjan, Iran
[d] Department of Biology, Faculty of Sciences, University of Guilan, Rasht, Iran

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ABSTRACT

We used a combination of experimental and bioinformatic studies to elucidate the importance of Serine^{128} and Valine^{183} on the activity and thermal stability of mnemiopsin 1 by substitution of S^{128} and V^{183} with glycine and threonine, respectively (S128G and V183T mutants). Luminescence emissions of S128G and V183T were reduced to 71.6% and 46.6% with respect to the original activity of the wild type protein. According to circular dichroism (CD) measurements, compactness of mutants decreased in comparison with wild type (WT) protein. Differential scanning calorimetry (DSC) indicated that T_m values of thermal unfolding are not changed significantly upon mutation. Herein, we suggest that the protein variants unfold through molecular association and intermediate states. Bioinformatic studies revealed that local fluctuation of residues in S128G increased with respect to WT protein. However, S128G mutation leads to increment of the accessible surface area of lysine188. Therefore, this change is thermodynamically favorable. Finally, both experimental and theoretical studies showed a delicate balance between all structural alterations, determining total conformational stability of the protein.
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1. Introduction

Various marine cnidarians and ctenophores utilize Ca^{2+}-regulated photoproteins as a source of bioluminescence. These photoproteins are made of a single chain polypeptide as apoprotein, which forms a hydrophobic binding pocket in their tertiary structures for peroxy-coelenterazine. This pocket is highly hydrophobic and is made by residues originating from different helices [1,2]. Such photoproteins emit blue light upon binding of calcium ions to motifs with helix-loop-helix structure and highly conserved 12 residues calcium binding loop (EF-hand motifs). Calcium binding induces conformational changes followed by oxidation of the bound coelenterazine to coelenteramide and release of CO_2 [3].

Researchers have focused their attention on photoproteins due to unique properties in light emission. Nowadays, techniques based on photoproteins are used frequently because of their detection sensitivity and low background noise level, making them useful label in tracking the location of calcium ions and nucleic acid hybridization assays [4–6].

Mnemiopsin is a Ca^{2+}- binding photoprotein from *Mnemiopsis leidyi* that belongs to the Ctenophores group. Mnemiopsin 1, having 206 amino acids, shows high homology (sequence identity 90%) with berovin as another ctenophore photoprotein, which structurally has been characterized (PDB ID: 4MN0) [7].

This group of ctenophores functionally is identical to many properties of Coelenterate photoproteins. In contrast, the ctenophore photoproteins are extremely sensitive to UV and visible light over the range of their absorption spectrum. Comparison of the two most famous groups of calcium-regulated photoproteins has shown unexpectedly high degree of structural similarity, regardless of their low sequence identity [8]. Up to now, crystal structures of several photoproteins such as aequorin, obelin and clytin have been solved. Based on the knowledge on the crystal structures and hydrogen-bond network in the binding cavity, a mechanism have been proposed to explain how Ca^{2+}binding might trigger the bioluminescence reaction and different excited states [9,10].

The crystal structure of native aequorin (PDB ID: 1EJ3) revealed

* Corresponding author.
E-mail address: ranjbarb@modares.ac.ir (B. Ranjbar).

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that there is a hydrophobic core containing 21 residues with three sets of tyrosine, histidine and tryptophan for stabilizing coelenterazine via hydrogen bonds and π-π interactions. It is notable that bioluminescence activity is related to the stability of substrate in this pocket [11]. According to previous studies on coelenterate group, it has been demonstrated that replacing the residues such as His^{16}, Met^{19}, Try^{82}, Trp^{86}, Trp^{108}, Phe^{113} and Tyr^{132} with another residues in aequorin would result in a different pattern of hydrogen bonds and π-π interactions, followed by a considerable change in the activity, wavelength and half-life of the emitted light [11].

Beside amino acid residues exist in the cavity, the substrate itself plays a major role in characterizing the bioluminescence features in photoproteins. This is mostly because of its tendency for binding to calcium ions. In aequorin, the phenolic OH group of the 2-substituent of coelenterazine is stabilized through hydrogen bond network between W1 molecule, the Oγ atom of T^{166}, and the carbonyl oxygen of I^{105}. This network breaks upon binding of calcium ion [12].

Site directed mutagenesis can be used to investigate the effects of a set of residues on the structure and function of the photoprotein [13,14]. In this study, due to the importance of the hydrogen bond in the stability at C2 position of coelenterazine, and high homology between mnemiopsin and coelenterate photoproteins in sequence, S^{128} and V^{183} in mnemiopsin were replaced by corresponding amino acids in aequorin including G^{109} and T^{166} and the structural features as well as bioluminescence properties of the variants were compared with those of the WT protein [13]. The effect of mutation on the secondary structural content of mnemiopsin was determined by far-UV CD measurements. Changes in T_m values between the mutant enzymes and WT were determined by differential scanning calorimetry. Moreover, bioinformatic tools were used for comparing the structural features of wild type and mutants.

2. Material and methods

2.1. Construction, expression, and purification of the WT and mutant apomnemiopsins

To investigate the effect of conserved residues on the structure and bioluminescence properties of menmiopsin, mutants were constructed by the quick change method [15]. Plasmid of apomnemiopsin1 gene (GeneBank accession No.GQ231544) was used as template for the reaction. This reaction was done by sequential steps: denaturation at 95 °C for 5 min; 22 cycles of 95 °C for 1 min; 72 °C for 13 min and final extension at 72 °C for 1 min. The products were purified by a PCR purification kit for removing redundant primers. The fragments were digested with *Dpn1* to digest native parental plasmids and those fragments transformed to *Escherichia coli* BL21(BE3) (Novagen, Madison, WI.USA) by chemical methods for each mutation [16]. At the end, plasmids of mutations were verified by DNA sequencing. To characterize the properties of menmiopsin and its mutants, histidine-tagged apoproteins were expressed in the *E. coli* cells strain BL21(BE3), followed by efficient purification on a Ni-NTA agarose column. Under heat-denatured conditions, the purified apoproteins would migrate to around 27 kDa, on SDS-PAGE as reported for WT apomnemiopsin 1. Apomnemiopsin variants were converted to mnemiopsin by incubating with coelenterazine and EDTA in dark conditions. Finally, specific bioluminescence activities were determined.

2.2. Preparation of photoproteins

To produce the protein variants from their apomnemiopsins, purified proteins were diluted by 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0, containing 10 mM EDTA (buffer 1) and mixed with a given volume of coelenterazine analogue in a microtube. The mixtures were vortexed and placed at 4 °C in dark condition for 16 h [13].

2.3. Determination of bioluminescence activity

Bioluminescence activities of the WT and mutant photoproteins were determined by adding 10 µl of the regeneration mixture into a glass tube, containing 40 µl of 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0 (buffer 2). The tube was then placed in a luminometer (Sirius tube luminometer, berthold detection system, Germany). By injecting 50 µl of buffer 3 (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0 containing 40 mM $CaCl_2$, pH 8.0) into the sample solution, the luminescence intensity was measured [13,17].

2.4. Measurement of Ca^{2+} sensitivity

The required volume of purified recombinant photoprotein was dissolved in 50 mM NaH_2PO_4 (pH 8.0), containing 0.01 mM EDTA, 0.1% bovine serom albumin and 150 mM NaCl and incubated at 4 °C for 16 h. A volume of 10 µl of the mixture was added to 40 µl buffer 2 in a glass tube and 50 µl of various concentrations (from 10^{-3} to 10^{-7} M) in 50 mM NaH_2PO_4 (pH 8.0) was injected into the tube. The luminescence intensity was determined with a luminometer [13].

2.5. Experimental methods

DSC experiments were carried out in phosphate buffer, containing 50 mM NaH_2PO_4, 300 mM NaCl, and pH 8.0 at 25 °C and an apoprotein concentration of 0.4 mg/ml, using a Nano DSC III microcalorimeter. The heating rate was fixed at 1 °C/min with pressure of 3 atm. Final baseline was defined by recording baseline for the both reference and sample cells. DSC profile was analyzed by Nano Analyze software™. This software allows to extract native and denatured lines based on excess C_p (denoted $<C_p>$) [18–21].

Jasco J-715 spectropolarimeter (Japan) was used for recording CD spectra and the results were presented as molar elipticity, [θ] (degree cm^2 $dmol^{-1}$) using the equation $[\theta]_\lambda = (\theta \times 100\ MRW/Cl)$ by Jasco software. In this equation "MRW is a Mean amino acid Residue Weight, C is the protein concentration in mg/ml and l and θ represent the length of the light path in cm and the measured elipticity in degree, respectively". Data were smoothed and secondary structural contents were calculated according to YANG standards [22,23].

2.6. Theoretical methods

Due to the lack of access to structural information on mnemiopsin, mutations on mnemiopsin were selected according to sequence alignment of mnemiopsin (from ctenophore photoproteins) and coelenterate photoproteins including aequorin, obelin and semi synthetic aequorins.

The structure of berovin (PDB ID: 4MN0) was used as template for generating 3D structural models of mnemiopsin 1, using MODELLER program (Ver. 9.14) [24]. Furthermore, structural information of aequorin (PDB ID: 1EJ3) was used in order to insert a coelenterazine molecule within the constructed models. The quality of the models was evaluated by SaliLab Model Evaluation Server at http://modbase.compbio.ucsf.edu/evaluation/ [25–28]. The RosettaBackrub server (http://kortemmelab.ucsf.edu/backrub) was also used for modeling the structures of the mutants. It initiates from a user-provide single input protein structure in PDB format. Eventually, server creates structural models of mutants in PDB format [29].

Protein interactions calculator server (PIC server: http://pic.

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