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Determination of structural elements on the folding reaction of mnemiopsin by spectroscopic techniques

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ABSTRACT

Mnemiopsin 1 is a member of photoprotein family, made up of 206 amino acid residues. These Ca^{2+}-regulated photoproteins are responsible for light emission in a variety of marine cnidarians and ctenophores. They composed of an apoprotein, a single polypeptide chain of 25 kDa, molecular oxygen and the non-covalently bound chromophore. In this study, we examined whether three mutations, namely R39K, S128G and V183T affect the thermodynamic stability as well as refolding and unfolding kinetics of mnemiopsin 1. Conformational stability measurements using fluorescence and far-UV CD spectroscopies revealed that all variants unfold in multi-step manner in which the secondary and tertiary structures are lost in different steps. However kinetic studies showed that point mutation S128G destabilizes both kinetic intermediate and native conformation; while, these structural elements are stabilized in V183T. We also found that the stability of folded and intermediate states increases in R39K. We concluded that the initial packing of helical segments within the protein structure is more facilitated when Lys with smaller side chain is present in the protein chain.

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1. Introduction

Ca^{2+}-regulated photoproteins are responsible for light emission in a variety of marine cnidarians and ctenophores. These photoproteins consist of an apoprotein, a single polypeptide chain of 25 kDa, molecular oxygen and the non-covalently bound chromophore called peroxy-coelenterazine which is located in the highly hydrophobic cavity. All Ca^{2+}-binding photoproteins have the same compact globular structure containing three "EF-hand" calcium-binding sites [1–3]. Upon binding of Ca^{2+} ions with the Ca^{2+}-binding EF-hand motif, the tertiary structure of protein changes which results in the oxidation of the non-covalently bound coelenterazine to coelenteramide, yielding carbon dioxide (CO_2) and blue light.

Aequorin and obelin are two well-known and highly-studied photoproteins isolated from cnidarians *Aequorea* and *Obelia*, respectively. However, photoproteins from ctenophores remain largely unexplored [4].

The applications of photoproteins span a wide spectrum including tracking the location and concentration of Ca^{2+}, nucleic acid hybridization assays, investigating the signal transduction pathways, protein–protein interaction, imaging of living cells, and discovery of novel drugs [5–7].

Photoproteins have attracted much research interest in both basic and diagnostic areas because of their unique biophysical properties such as low background noise, superior detection sensitivity and harmless applications including lack of cellular toxicity, hazard-free handling, and non-invasive nature [8,9].

Mnemiopsin was first isolated and characterized from the luminous ctenophore *Mnemiopsis* sp. in 1970s [10–12]. Additionally, molecular cloning and expression of cDNA coding for two isoforms of mnemiopsins from *Mnemiopsis leidyi* and further characterization of related photoproteins have recently been performed [13]. In an attempt to understand the structural properties related to bioluminescence in ctenophore photoproteins, we previously investigated functional properties of a variety of critical residues in mnemiopsin 1 using site directed mutagenesis [14]. It has 206 amino acids and bears high homology (sequence identity 90%) to berovin, as the only structurally determined ctenophore photoprotein (PDB ID: 4MN0) [4,12].

The crystal structure of native aequorin (PDB ID: 1EJ3) also reveals a highly hydrophobic core containing 21 residues that stabilize the chromophore. Three sets of tyrosine, histidine and tryptophan residues form three triads that interact with the chromophore through hydrogen bonds and π–π interactions and are responsible for the stabilizing of coelenterazine but in mnemiopsin tryptophan, methionine and phenylalanine form this triad. Due to the fact that bioluminescence activity is highly dependent on the stability of chromophore within the pocket, bioluminescent properties of photoproteins may be substantially altered by changing these critical residues [15–18].

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Nowadays equilibrium unfolding experiments on wild-type (WT) and mutants are widely used for determining the conformational stability of proteins. This approach evaluates how a mutation changes the free energy of unfolding of a protein ($\Delta\Delta G_{equilibrium}$) [19]. Protein engineering analysis with stopped-flow fluorescence measurements and chevron plot analysis may also be used to characterize transition states and intermediate structures of refolding reaction. These methods can compare the effect of mutations on the conformational stability of native and intermediate states as well as folding rate interpreted in terms of the changes in the free-energy barrier ($\Delta\Delta G_{TS}$) [20–22].

In this study, we performed thermodynamic stability and kinetics of refolding and unfolding measurements on the WT and the three mutants of mnemiopsin 1. Sequence alignment of mnemiopsin, obelin as well as aequorin indicates that the corresponding residues in the positions 128 and 183 of mnemiopsin in aforementioned proteins are Gly and Thr; respectively. So, in our previous studies, Ser128 and Val183 in mnemiopsin were replaced by Gly and Thr, respectively. In the other mutant, Arg39 was replaced by Lys as another charged residue with smaller side chain. Hence; the selection of mutants is based on the evolutionary conservation of selected residues according to the resulting data of multiple sequence alignment [18]. In comparison with the WT protein, these modifications led to an increase in the activity of R39K and a decrease in the activities of S128G and V183T. The aim of this study is to determine the importance of the above-mentioned residues on the folding pathway and conformational stability of mnemiopsin. According to thermodynamic studies using fluorescence and far-UV CD spectroscopy measurements, we found that all protein variants are unfolded in different steps in urea denaturation experiments so that the tertiary structure disarrays prior to the secondary contacts.

Our kinetic data indicate that the stability of intermediate state increases in R39K and V183T mutants, while in S128G variant it is the same as WT protein. The conversion of intermediate state to native structure as the rate limiting step in the refolding reaction is also speeding up in V183T and R39K relative to WT protein.

2. Materials and methods

2.1. Expression, purification and activity determination of His-tagged apo-mnemiopsin

Expression and purification of His-tagged apo-mnemiopsins and its luminescence activity were carried out as described previously [14]. pET28a expression vector containing apo-mnemiopsin 1 gene (GenBank accession No. GQ231544) from *M. leidyi* was obtained from the Caspian Sea, northern Iran [12] and its mutants were used for overexpression of the apo-photoproteins in *Escherichia coli* BL21 (DE3).

2.2. Stability measurements

Fluorescence and far-UV CD spectroscopies were used for monitoring urea-induced unfolding of mnemiopsin. The buffer used contains 50 mM NaH_2Po_4, 300 mM NaCl, pH 8.0 and 25 °C.

At first, urea stock solutions (0–9 M) were prepared in buffer. Then, 100 μg/ml concentration of protein was incubated at different concentrations of urea (0–8 M). Fluorescence measurement was performed by fluorescence spectroscopy with excitation at 280 nm and emission spectra were recorded between 300 and 450 nm (both slits of excitation and emission were set to 5 nm). For CD measurements, the concentration of protein was 200 μg/ml [23,24].

It is noticeable that agents such as urea at high concentrations absorb too strongly CD data below 210 nm even using cells of short pathlength. This, of course, is not a problem if changes in the CD signals at 222 or 225 nm are used to assess the unfolding of a protein. Hence, using data of 222 nm wavelengths in this study is not problematic for CD investigations [25].

All experiments were also measured by circular dichroism technique at far-UV region (far-UV CD). In these wavelengths, we can monitor the changes of the secondary structure of WT and mutants of mnemiopsin [26]. The analysis of CD equilibrium denaturation curves was the same as fluorescence-based curves. Standard deviations are calculated based on four or five replicates of experiments.

Mnemiopsin is a monomer and only one protein concentration (100 μg/ml for fluorescence and 200 μg/ml for CD experiments) was used for the final analysis. However, several concentrations in the range of experimental conditions were tested to verify that the protein does not aggregate at higher protein concentrations. Results show that the equilibrium denaturation curves of different concentrations were superimposable, demonstrating that the stability of protein is independent of protein concentration (from 100 to 200 μg/ml concentration of protein) [27].

The observed sigmoid-like equilibrium curves were fitted by KaleidaGraph analysis software into Eq. (1); considering a two state model [28]:

$$F_{350} = \frac{\{(\alpha_N + \beta_N[\mathrm{Urea}]) + (\alpha_D + \beta_D[\mathrm{Urea}])\} \times \exp((m_{D-N}([\mathrm{Urea}] - [\mathrm{Urea}]_{50\%}))/RT)}{\{1 + \exp((m_{D-N}([\mathrm{Urea}] - [\mathrm{Urea}]_{50\%}))/RT)\}} \quad (1)$$

where F_{350} is the fluorescence intensity at 350 nm as a function of [Urea], α_N and α_D are the intercepts, and β_N and β_D are the slopes of the baselines. $[Urea]_{50\%}$ is the concentration of urea at which half of the protein is denatured, R is the gas constant and T is the temperature in Kelvin. m_{D-N} is defined as a constant that is proportional to difference in the solvent accessible surface area between the native and denatured states. The relationship between the free energy of unfolding in the urea and buffer as well as the concentration of denaturants is given by Eq. (2) [29]:

$$\Delta G_{D-N}^{[Urea]} = \Delta G_{D-N}^{H_2O} - m_{D-N}[\mathrm{Urea}]. \quad (2)$$

Moreover, the free energy of unfolding at the absence of urea, $\Delta G_{D-N}^{H_2O}$, can be calculated by Eq. (3):

$$\Delta G_{D-N}^{H_2O} = m_{D-N}[\mathrm{Urea}]_{50\%}. \quad (3)$$

2.3. Kinetic experiments

A Biologic μ-SFM-20 fluorescence detected stopped-flow, equipped with a 0.8 cm cuvette (FC-08) (excitation 280 nm, emission 350 nm) was used for kinetic measurements.

For refolding studies, unfolded protein in 50 mM NaH_2Po_4, 300 mM NaCl, pH 8.0 containing high concentration of urea was diluted with refolding buffer containing different concentrations of urea ranging from 0 to 5 M. For unfolding experiments, protein was unfolded by mixing one volume of protein in 50 mM NaH_2Po_4, 300 mM NaCl, pH 8.0 with 6 volumes of buffer containing different concentrations of urea.

Kinetic traces were analyzed by fitting to exponential function of Eq. (4) using Biokine software (Ver. V4.49-1):

$$F(t) = at + b + \sum_{i}^{N} C_i \exp(\pm k_i t) \quad (4)$$

where F(t) is the fluorescence signal at time t, C_i is the amplitude, k_i is the rate constant, a is the slope of the drift and b is the offset of kinetic curve corresponding to the baseline.

According to the biological meaning and the accuracy of fitting, kinetic traces may be fitted to a single or double exponential function by using simplex method and setting $N = 1$ or $N = 2$ into Eq. (4), respectively [19].

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