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Correlation between fluorescence and structure in the orange-emitting GFP-like protein, monomeric Kusabira Orange

A. Ausili [a,*], M. Staiano [a], A. Marabotti [b], G. D'Auria [a,b], J.C. Gómez-Fernández [c], A. Torrecillas [c], A. Ortiz [c], S. D'Auria [a,*]

[a] Laboratory for Molecular Sensing, IBP-CNR, Naples, Italy
[b] Department of Chemistry and Biology, University of Salerno, Fisciano (SA), Italy
[c] Departamento de Bioquimica y Biologia Molecular-A, University of Murcia, Spain

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ABSTRACT

The mKO is the monomeric version of Kusabira Orange, a GFP-like protein emitting bright orange fluorescence at 559 nm. This protein shows the characteristic β-barrel motif typical of the fluorescent protein family which it belongs to, similar spectral properties to the tetrameric form and an exceptional photo-stability to pH changes. Here, we demonstrate that mKO in solution at physiological pH exhibits a secondary structure analogue to that of the crystal. Moreover, we describe the thermal unfolding, revealing an outstanding structural stability with a denaturation temperature close to 90 °C and identifying the existence of a thermodynamic intermediate. The denaturation process of mKO results to be absolutely irreversible because of the complete lost of the native structure and the consequent aggregation, while the presence of the intermediate state is most likely due to coexistence of two different species of mKO, with protonated and deprotonated chromophore respectively, that affects the fluorescence properties and the structural stability of the protein.

1. Introduction

The use of fluorescent proteins (FPs) had and still has a fundamental importance in cell and molecular biology. Application of FPs in living cell imaging, protein co-localization, gene expression imaging, cellular processes monitoring or bio/sensing allowed to make visible several different biological phenomena and at present it is an essential tool in various disciplines of biological research [1–5]. Recently, a novel and promising application of FPs has been proposed by Kiyonaka et al., who have developed a genetically encoded GFP-base thermosensor to visualize the thermoregulation in living cells [6]. More than half of a century has passed from the discovery of the first FP, the green fluorescent protein (GFP), isolated from the jellyfish *Aequorea victoria* [7], and nowadays a wide number of FPs of different colors, brightness and light absorbing ability as well as their engineered variants, are available [8–10]. All FPs share an amazing and characteristic structure that makes these proteins fluorescent and provides them a remarkable stability: A perfect β-barrel structure, also named β-can, formed by 11 β-strands surrounds the chromophore composed by three amino acids and situated in a coaxial α-helix. Other four helical segments protect the upper and lower extremities of the barrel which, as the most soluble proteins, consists of a hydrophobic core while the polar residues are oriented toward the outside [8,11–14]. This structure provides the indispensable rigidity and protection to the chromophore preventing its non-radiative deactivation [15]. As previously said, the chromophore is formed by three amino acids located at the central α-helix at positions 65–67. Post-translational modifications of these residues lead to the chromophore maturation which occurs in different times depending on the protein nature and involves a number of steps. After the correct folding of the FP apo-protein that facilitates the chromophore formation, an autocatalytic cyclization and a dehydration of the tri-peptide occur and at a later stage it undergoes an oxidation step. Finally, the process leads to the creation of an imidazolinone ring forming a highly conjugated system that corresponds to the complete maturation of the chromophore where the hydrogen bonding network, electron-stacking and hydrophobic interactions determine color, intensity and photo-stability of the different FPs [15–17]. Despite the advances in FPs technology and the creation of hundreds of new variants, most of these proteins belong to the green, cyan and yellow spectral classes. However, on occasion these spectral regions are not ideal for some specific needs [9]; for this reason,

* Corresponding authors. Address: Institute of Protein Biochemistry, CNR, Via Pietro Castellino, 111, 80131 Napoli, Italy. Tel.: +39 0816132312; fax: +39 0816132273 (A. Ausili). Tel.: +39 0816132250; fax: +39 0816132273 (S. D'Auria).
E-mail addresses: a.ausili@ibp.cnr.it (A. Ausili), s.dauria@ibp.cnr.it (S. D'Auria).

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at present, more attention is focused on orange- and red- emitting FPs. Among orange FPs, Kusabira Orange (KO) is one of the first cloned, most used and commercially available. Originally, KO was isolated from the stony coral *Fungia concinna* (known in Japanese as Kusabira-Ishi) and derived through the introduction of ten residues to the N-terminus in order to provide the protein with a bright orange fluorescence [18]. KO presents a complex homotetrameric structure and a fast chromophore maturation with excitation and emission maxima at 548 and 561 nm, respectively [18]. KO was further engineered by introduction of additional 22 mutations to create a monomeric version. The resulting protein (named mKO) maintained the original brilliance and fluorescence characteristics with a slight shift in the emission maximum (559 nm) and showed a molecular weight of 28.1 kDa [18]. The crystal structure of mKO was also resolved at high resolution and it showed the typical FP motif but with a novel three-rings chromophore that arise from the Cys65-Tyr66-Glu67 tri-peptide [19]. In this context, we wanted to analyze the mKO secondary structure and investigate the effect of the temperature on the protein structure as well as the relationship between the chromophore stability and fluorescence by using different biophysical methods.

2. Materials and methods

2.1. Materials

Deuterium oxide (99.9% ^2H_2O) was purchased from Aldrich (Madrid, Spain). All other reagents and solvents were commercial samples of the highest purity. Monomeric Kusabira Orange was expressed and purified according to previously reported procedures [20].

2.2. Fourier transform infrared spectroscopy

An amount of 1.5 mg of mKO dissolved in 10 mM Tris–HCl buffer pH 7.4 was concentrated into an approximate volume of 40 μl by centrifugation in an Amicon Ultra-0.5 Centrifugal Filter with Ultracel-10 membrane (Millipore) at 7000 g and at 4 °C. Then, a further 400 μl of the same buffer but prepared in ^2H_2O was added, the sample was centrifuged again, and the solution was re-concentrated. This procedure was repeated 5 times to completely replace the original buffer. Before the last concentration the protein was incubated overnight at 4 °C in order to maximize the H–^{2}H exchange and then the sample volume was brought to 40 μl used for the Fourier transform infrared spectroscopy (FTIR) experiment. The p^{2}H value was measured with a standard pH electrode, and the value was corrected according to p^{2}H = pH + 0.4 [21].

The concentrated protein sample was placed in a thermostated GS20500 cell equipped with CaF_2 windows and 25 μm Teflon spacers used with a water heating jacket cell holder GS20710 (Graseby-Specac Ltd, Orpington, Kent, UK). FTIR spectra were recorded by means of a Bruker Vector 22 Fourier transform infrared spectrometer using a liquid nitrogen-cooled MCT detector and a normal triangular apodization function. A total of 128 scans were performed for each spectrum with a nominal resolution of 2 cm^{-1}. A sample shuttle accessory was used to obtain the average background and sample spectra. At least 24 h before and during data acquisition, the sample chamber of the spectrometer was continuously purged with dry air. Sample and buffer were scanned between 24.2 and 97.4 °C at 4.9 °C intervals with a 5 min delay between each scan for the stabilization of the temperature inside the cell using an external bath circulator interfaced to the spectrometer's computer. A further measurement was acquired cooling down the sample at 24.2 °C in order to check the reversibility of the thermal denaturation. The temperature of the cell was controlled by a thermocouple placed directly onto the CaF_2 windows. Spectra were collected using the "Opus" software from Bruker and processed using Grams/32 software (Galactic Industries Corp., Salem, NH). Subtraction of ^2H_2O buffer from the sample spectrum was performed interactively removing the ^2H_2O bending absorption close to 1220 cm^{-1} and maintaining a flat baseline between 2000 cm^{-1} and 1300 cm^{-1} [22,23], second derivative spectra were calculated over a 9 data-point range (9 cm^{-1}) and the parameters of the deconvoluted spectra were set with a γ value of 7 and a smoothing length of 70.

The quantitative analysis of the protein secondary structure was performed by curve fitting of the amide I' band [24,25] using the peak fitting module of the Origin software (OriginLab Corporation, Northampton, MA 01060, USA). The band shape was set to a Gaussian curve, and the fitting was obtained by iteration in two steps, the first iteration was performed fixing the peak positions as obtained by second derivative and deconvolution, while in the second step the bands were free [26].

2.3. Differential scanning calorimetry

For the experiments of differential scanning calorimetry (DSC) a quantity of around 0.5 mg of protein dissolved in a total volume of 600 μl of 10 mM Tris–HCl buffer pH 7.4, was degassed for 10 min and then loaded into the calorimeter at a final protein concentration of 17.8 μM. Thermograms were acquired by means of a Microcal VP Differential Scanning Calorimeter (VP-DSC) (Microcal, Northampton, MA, USA). The same buffer employed to dissolve the protein was used in the reference cell. The samples were scanned over a temperature range from 20 to 100 °C and with a scan heating rate of 30 °C/h. The thermogram data were recorded and analyzed with the Microcal Origin 5.0 software (Microcal Software Inc., Northamptom, MA). The reversibility of the thermal transitions was checked by performing cooling and re-heating runs in the same temperature range. Since the denaturation process was irreversible the baseline was determined by using the second heating run and then subtracted from the first heating run (raw thermograms are reported in Fig. 5 inset) [27].

2.4. Fluorescence measurements

Steady state fluorescence experiments were carried out on a Jasco FP-8600 fluorescence spectrofluorometer equipped with a constant-temperature cell holder and with the temperature controlled by using a circulating bath Thermo Haake P5. mKO was selectively excited at 548 nm and the emission was recorded from 555 to 650 nm with 2.5 and 5 nm slit widths for excitation and emission, respectively. Spectra of mKO in 10 mM Tris–HCl pH 7.4 were acquired at increasing temperatures, first from 25.5 to 64.2 °C and, after cooling back the sample, from 25.5 to 90 °C at 4.3 °C intervals, incubating five minutes at each temperature before measurements. The spectra reported are the average of three scans.

3. Results

In Fig. 1 is presented the structure of mKO resolved at high resolution [19]. The presence of a π−π stacking interaction between the imidazole ring of the residue His197 and the phenol ring of chromophore, and of a cation-π interaction with the charged moiety of Arg69 are considered important for stabilization of the anionic form of the chromophore and tin turn responsible for the fluorescence properties of mKO.