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The fluorescent monomeric protein Kusabira Orange. Pressure effect on its structure and stability

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ABSTRACT

The structure and stability of the fluorescent protein monomeric Kusabira Orange (mKO), a GFP-like protein, was studied under different pressure levels and in different chemical environments. At different pH values (between pH 7.4 and pH 4.0) and under a pressure up to 600 MPa (at 25 °C), mKO did not show significant fluorescence spectral changes, indicating a structural stability of the protein. In more extreme chemical conditions (at pH 4.0 in the presence of 0.8 M guanidine hydrochloride), a marked reduction of mKO fluorescence intensity emission was observed at pressures above 300 MPa. This fluorescence emission quenching may be due to the loss of the intermolecular bonds and, consequently, to the de-structuration of the mKO chromophore structure. Since the electrostatic and hydrophobic interactions as well as the salt bridges present in proteins are usually perturbed under high pressure, the reduction of mKO fluorescence intensity emission is associated to the perturbation of the protein salt bridges network.

1. Introduction

Many cnidarians utilize fluorescent proteins as energy-transfer acceptors in bioluminescence. These highly fluorescent proteins are unique due to the chemical nature of their chromophore, which is composed of modified amino acid residues within the polypeptide. For example, by changing the amino acid residue 66 and/or the amino acid residues close to the chromophore, the color and fluorescence intensity of GFP (Green Fluorescent Protein) can be modified [1–3].

Many different biological and ecological functions have been proposed for bioluminescence [4,5], but the most parsimonious explanation is that bioluminescence is used as an anti-predator defensive stratagem. Sudden flashes in dark surroundings have been shown to startle, deter, and stun the prey. Bioluminescence induced by multifarious stimuli has long been observed and remains under investigation because of its great complexity. Many cnidarians emit light when they are mechanically disturbed. Proteins involved in the light emission process should resist to external perturbations, because the three-dimensional structure of protein molecules can exert a strong influence over the protein active site leading to inactivation of the light emission process [6].

The mKO is the monomeric version of Kusabira Orange, a GFP-like protein, isolated from the stony coral *Fungia concinna* and emitting bright orange fluorescence. The mKO structure is a typical beta barrel architecture with a fully mature chromophore containing a 3-thiazoline ring as a third ring that accounts for the fluorescence properties of the protein [7]. Fig. 1a shows a schematic representation of the secondary structure topology, consisting of 15 secondary structure elements, mainly beta strands. Fig. 1b shows the 3D structure in two orientations, with secondary structures elements and the chromophore in evidence. The structural stability of mKO appears enhanced, in comparison to beta barrels structures of other proteins analyzed as counterparts, by the presence of a higher number of salt bridges and H-bonds. Table 1 shows the number of H-bonds and salt bridges observed into the structure of mKO and of bovine and porcine Odorant Binding Protein (bOBP and pOBP, respectively). In fact, OBPs belong to the lipocalin family. This class of proteins displays a beta barrel tertiary structure organization, 70–80% of the total number of amino acid residues [8], even if OBPs do not share a significant primary structure similarity. From a general point of view, this observation suggests that mKO may be more stable than bOBP and pOBP despite that these proteins are very stable to the denaturing action of GdnHCl [9] and reveal a particularly high thermostability having a denaturation temperature of 90 °C [10]. A more detailed analysis of the position of the salt bridges may offer additional information. The interactions that constitute the network of salt
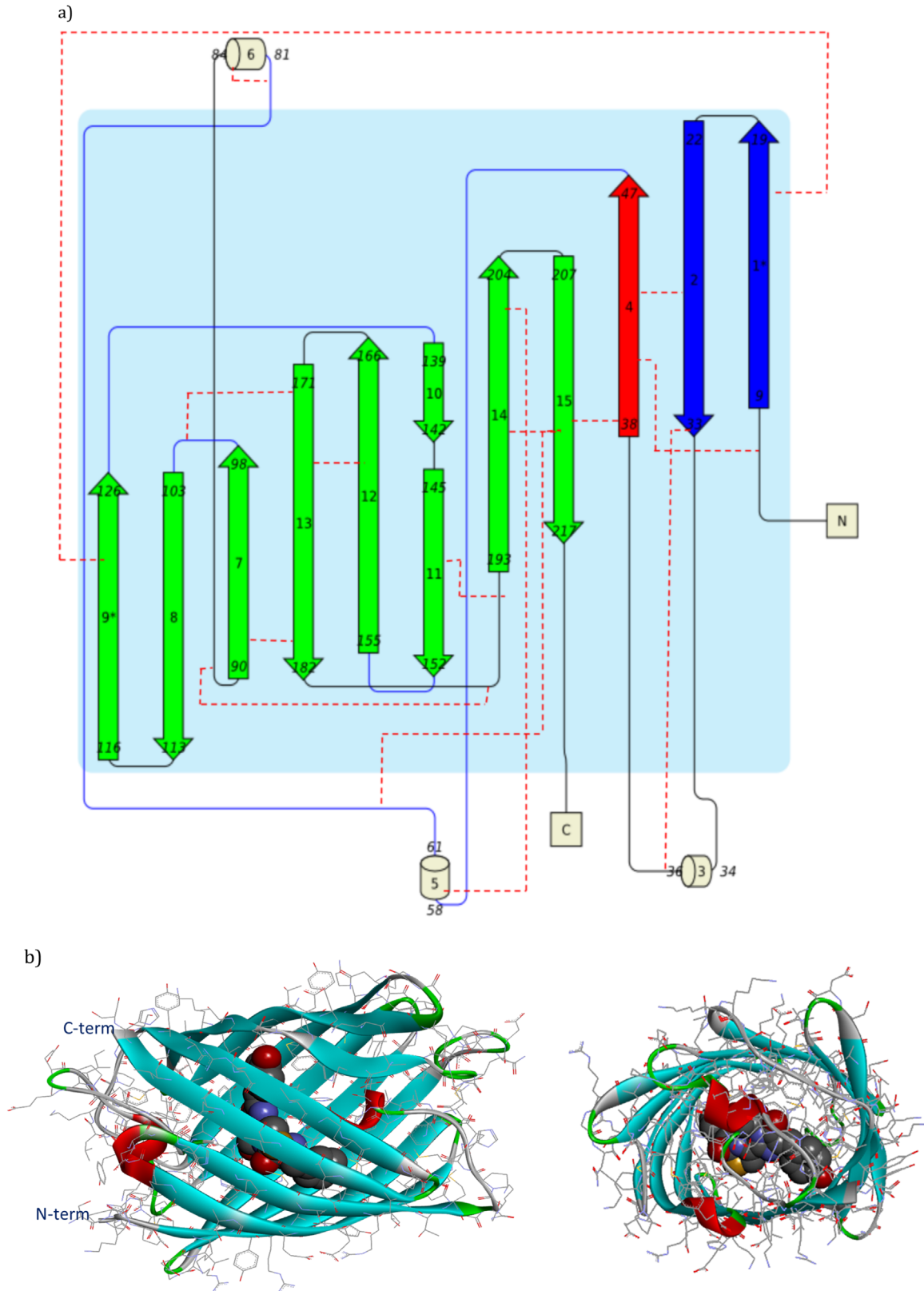
Fig. 1. (a) Secondary structure schematization of mKO. Each secondary structure element has a progressive number in the middle, and the first and last residue numbers at extremities. Arrows indicate beta strands, cylinders indicate helices. Dashed lines indicated the network of predicted salt bridges. (b) 3D structure of mKO, with two different orientations. Secondary structure is represented with beta strands (arrows colored in cyan) and helices (cylinders colored in red), while the chromophore in the middle of the structure is represented as spacefill atoms with standard colors (C=gray, N=blue, O=red, S=yellow). 3D structure of mKO protein from *VerrilloFungia concinna* extracted from the Protein Data Bank archive (PDB code: 3MGF). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Salt bridges and H-bond interactions in mKO, compared to bovine and porcine Odorant Binding Protein (bOBP and pOBP, respectively).

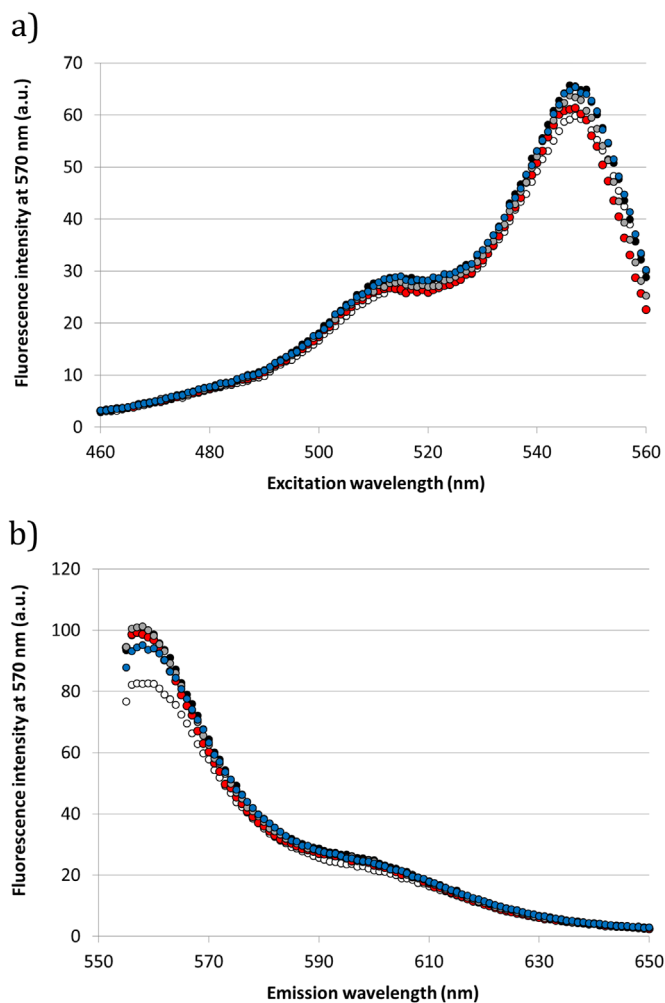|	mKO	bOBP	pOBP
Salt bridges	14	7	7
H-bonds (intra-protein total)	187	129	117
H-bonds (main-main)	134	87	82
H-bonds (side-side)	29	14	10
H-bonds (main-side)	24	28	25
Interactions per 100 amino acids			
Salt bridges	6.5	4.4	4.7
H-bonds (intra-protein total)	86.6	81.6	78.5
H-bonds (main-main)	62.0	55.1	55.0
H-bonds (side-side)	13.4	8.9	6.7
H-bonds (main-side)	11.1	17.7	16.8

a)

b)

Fig. 2. Influence of high pressure on the fluorescence intensity of mKO (pH 7.4, 25 °C). (a) excitation spectra of mKO with an emission wavelength fixed at 570 nm; (b) emission spectra of mKO with an excitation wavelength fixed at 548 nm. Pressure levels: (○) 10 MPa; (●) 100 MPa; (•) 200 MPa (●) 400 MPa and (●) 600 MPa. Spectra are the mean of three measurements.

bridges are evidenced in Fig. 1a. Six out of fourteen interactions involve at least one amino acid located in a segment without secondary structure. This means that regions commonly considered flexible, due to the lack of secondary structures, are stabilized in this protein by salt bridges. Moreover, some of these interactions occur between remote amino acids, in terms of sequence position, which means that they contribute to stabilize the whole structure by preventing unfolding of the backbone
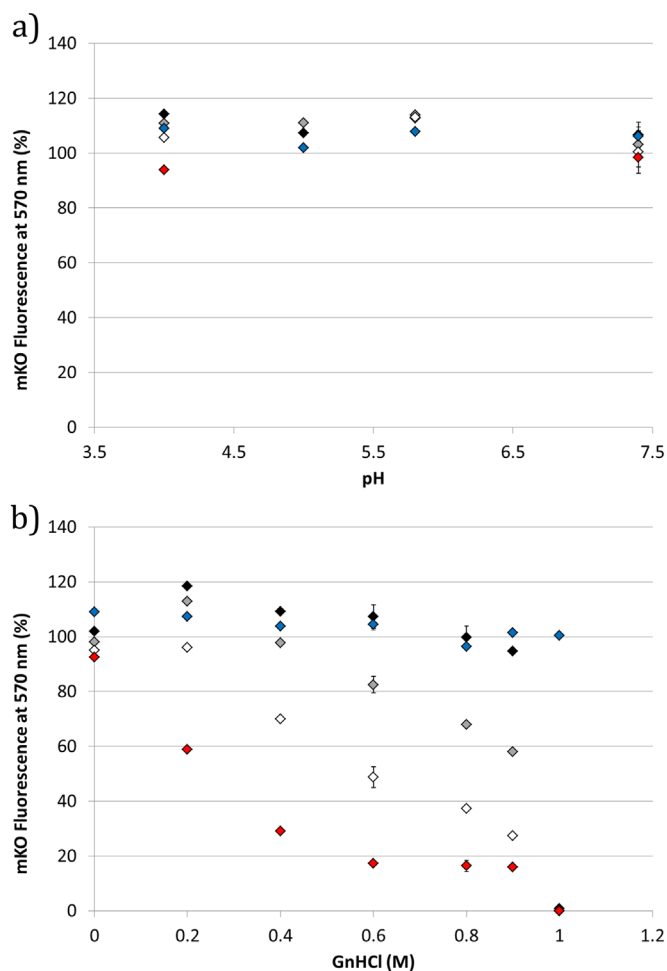
a)

b)

Fig. 3. Influence of pH (a) or GdnHCl concentration (b) on the fluorescence intensity of mKO under pressure. Excitation at 548 nm, emission at 570 nm. (a) mKO suspended in Tris–HCl (10 mM, pH 7.4) or acetate buffer (200 mM, pH 5.8, 5.0 or 4.0); (b) mKO suspended in acetate buffer (200 mM, pH 4.0). Pressure levels: (◆) 100 MPa; (•) 200 MPa; (◆) 400 MPa; (◇) 500 MPa and (◆) 600 MPa. Data are the mean of at least three measurements.
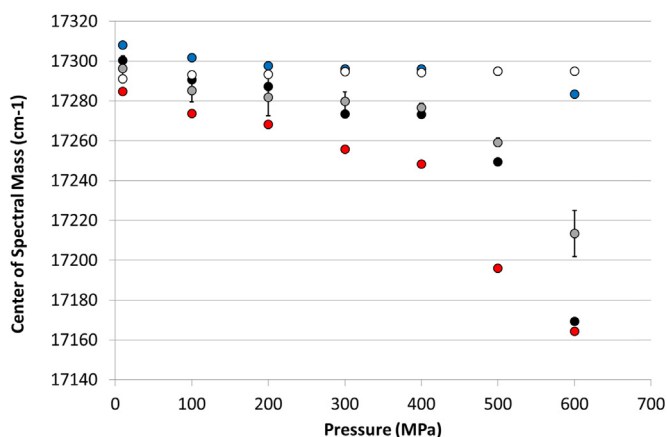

Fig. 4. Influence of GdnHCl concentration on the center of spectral mass (CSM) of mKO (pH 4.0) as a function of pressure. mKO suspended in acetate buffer (200 mM, pH 4.0) Excitation at 548 nm, emission between 550 and 650 nm. GdnHCl concentrations: (○) 0.2 M; (●) 0.4 M; (●) 0.6 M; (•) 0.8 M and (●) 0.9 M. Data are the mean of at least three measurements.

organization. Interestingly, these interactions create links between the first three beta strands elements and the other part of the protein. As reported for GFP [11], different folding pathways have
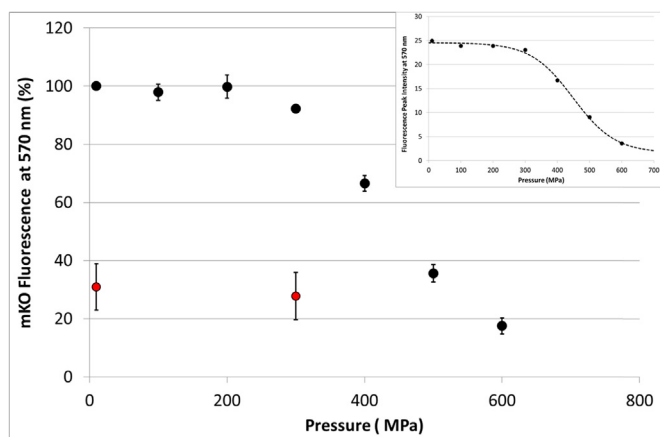
Fig. 5. Influence of high pressure on the fluorescence intensity of mKO in drastic conditions (pH 4.0, 0.8 M GndHCl, 25 °C). Excitation wavelength fixed at 548 nm and emission wavelength fixed at 570 nm. (●) fluorescence intensity for pressure increase (●) fluorescence intensity for pressure decrease. Data are the mean of 9 measurements from 3 independent experiments. The dotted line in the inset is a fit of experimental data.

been proposed. The unfolding of GFP may proceed with a primary unfolding, starting from the C-terminal beta strand, and a secondary unfolding starting from the N-terminus beta strand. Moreover, another possible pathway considers that the unfold starts with the disruption of the first three beta strands. In both cases, the salt bridge network in mKO blocks the C-terminus strand as well as the three N-terminus ones, with a consequent effect increasing the stability of the protein by opposing the proposed unfolding mechanisms.

Pressure effects on biological systems are governed by Le Chatelier's principle, which states that at equilibrium, a system tends to minimize the effect of any external factor by which it is perturbed. Pressure favors processes that are accompanied by negative volume changes [12]. While the covalent bonds are pressure insensitive (up to 1000–1500 MPa), the electrostatic and hydrophobic interactions are destabilized under pressure. Furthermore, pressure induces a volume reduction of proteins associated to a collapse of voids and an increase in protein hydration [13,14]. Atomic structure of proteins pressurized at pressures up to a few hundred MPa [15,16] indicate that atoms in protein molecules are typically displaced by ≈ 0.1–1 Å from their ambient pressure positions. Pressure in the same range can also significantly modify protein function [17]. For example, the flash decay rate of firefly luciferase is reduced [18], the R to T transition in human hemoglobin is biased [19] and the morphinone reductase activity is substantially increased [20]. These observations suggest that the exact positioning of atoms, especially in the active sites of catalytic proteins, is an important feature of protein operation and that this positioning is subject to environmental perturbation. The chemical diversity of chromophores makes these proteins an interesting target for investigating their reactions to physical stimuli.

The main goal of the present study was to investigate the fluorescent mKO protein, as a model system, under high-pressure perturbation. To study the effect of pressure on the mKO protein structure and function, the *in situ* fluorescence spectroscopy in the pressure range between 0.1 MPa and 600 MPa was used.

2. Material and methods

2.1. Materials

Guanidine hydrochloride (GdnHCl) was purchased from Sigma-Aldrich (MO, USA). All other reagents and solvents were A grade commercial samples. All solutions were made with MilliQ water. Monomeric Kusabira Orange was expressed and purified as previously described [21].

2.2. Fluorescence measurements under high pressure

Fluorescence measurements were carried out at 25 °C using an SLM Series 2 spectrofluorometer (Aminco Bowman, Foster City, CA), modified to accommodate a thermostated high pressure optical cell [22]. The HP-cell was equipped with 8-mm-thick sapphire windows. mKO dispersion was prepared at 0.29 mg/mL in different buffers: Tris–HCl buffer (10 mM, pH7.4) or acetate buffer (200 mM, pH 5.8, 5.0 or 4.0). For some experiments, GdnHCl was added to mKO dispersions at different final concentration (0.2–1 M). The sample was placed in a 5 mm diameter quartz cuvette, closed at the top with a flexible polyethylene film that was attached by a rubber O-ring. The cuvette was placed into the thermostated HP-cell filled with deionised water as pressure transmitting medium. Pressure was then gradually increased from 10 to 600 MPa via a manual piston pump. Following each pressure increment (steps of 100 MPa), the sample was equilibrated 3 min before spectral recording. All spectra were recorded as a mean of three repetitive scans. If not stated otherwise, mKO was excited at 548 nm and emission scans were collected between 555 and 650 nm. To record excitation spectra between 460 and 570 nm, the emission wavelength was fixed to 570 nm.

2.3. Thermodynamic parameters

The pressure-induced fluorescence spectral changes were quantified by determining the emission intensity, I, at a characteristic maximum wavelength, and calculating the center of spectral mass, CSM, using Eq. (1):

$$\mathrm{CSM} = 1 \bigg/ \left(\sum \left(\nu_i \times I_i \right) \bigg/ \sum \left(I_i \right) \right) \tag{1}$$

where I_i is the fluorescence intensity emitted at a wavenumber ν_i. The CSM parameter reflects the mean exposure of tryptophan residues to water [23].

The thermodynamic parameters were evaluated by fitting the intensity pressure profiles according to Eq. (2):

$$I = \left(I_f - I_u \right) \bigg/ \left[1 + e^{-\left(\left(\Delta G_u^0 + p.\Delta V_u \right)/RT \right)} \right] + I_u \tag{2}$$

where I_f and I_u are the fluorescence intensities of the folded and unfolded states, respectively, and I the observed fluorescence intensity at pressure p; ΔG_u^0 and ΔV_u are the free energy and volume changes of unfolding at 0.1 MPa, respectively [24]. Alternatively, the thermodynamic parameters were evaluated from the CSM pressure profiles in an analogous way, replacing I by CSM in Eq. (2).

Table 2
Thermodynamic parameters of mKO unfolding.

GdnHCl (M)	I_u	I_f	$\Delta G°$ (kJ/mol)	ΔV_u (mL/mol)	$P_{(1/2)}$ (MPa)	R^2
0.8	24.56 ± 0.42	1.62 ± 1.45	16.97 ± 2.21	-37.84 ± 5.51	448	0.9972

3. Results and discussion

3.1. Influence of high pressure on the fluorescence of mKO

The influence of high pressure, up to 600 MPa, on the excitation or on the emission spectra of mKO at pH 7.4 and 25 °C is presented on Fig. 2a or b, respectively. No significant fluorescence spectral changes were detected under pressure up to 600 MPa, neither in the excitation, nor in the emission mode, suggesting that mKO remains correctly folded in this range of pressure levels. It appears therefore that the applied pressure did not destabilize the numerous salt bridges responsible for maintaining the tertiary structure of mKO. These results differ from those previously published on the investigation of the influence of high pressure, up to 2 GPa, on the fluorescence intensity of mKO at pH 7.4 (50 mM tris, 50 mM NaCl) reporting a continuous decrease of the fluorescence intensity of mKO upon pressure increase [25]. At atmospheric pressure or under pressure, the excitation spectra of mKO (Fig. 2a) with an emission wavelength fixed at 570 nm, present 2 peaks: a small one around 513 nm and a higher one around 548 nm. The evolution of the mKO fluorescence intensity at 570 nm, as a function of pressure, was similar at these two emission wavelengths. In the rest of the paper (except where noted), an excitation wavelength of 548 nm was chosen since it induced higher emission intensities than an excitation at 513 nm.

3.2. Influence of pH and GdnHCl concentration on the high pressure stability of mKO

Decrease the pH of the mKO dispersion from 7.5 to 5.0 had no significant effect on the protein HP-stability up to 600 MPa at 25 °C, since no change in fluorescence spectra at 570 nm was observed in this range of pressure (Fig. 3a). When the pH was fixed at 4.0, a slight decrease of the mKO fluorescence was only observed at 600 MPa, indicating that mKO remained folded even in these conditions (Fig. 3a). In order to induce the destabilization of the protein structure, GdnHCl was added, at different concentrations, to the mKO dispersion at pH 4.0 (Fig. 3b). In these conditions at low pressure level, 100 or 200 MPa, the mKO fluorescence was not affected by the presence of GdnHCl even at the highest concentrations of 0.9 or 1 M. At higher pressure levels, a progressive decrease of the mKO fluorescence intensity was observed when the concentration of GdnHCl increased from 0.2 to 1 M, indicating a likely destabilization of the protein structure. At 1 M of GdnHCl, the intrinsic fluorescence of mKO was completely and irreversibly lost at pressure above 200 MPa, probably due to protein unfolding. The influence of GdnHCl concentration on the center of spectral mass (CSM) of mKO (pH 4.0) as a function of pressure is presented on Fig. 4. CSM analysis indicates that the 0.8 or 0.9 M GdnHCl concentrations were the most suitable conditions to study mKO pressure-induced unfolding, these concentrations being previously reported to be sub-denaturing for this protein at atmospheric pressure [26]. From here on, all experiments were therefore conducted in the presence of 0.8 M GdnHCl.

3.3. Pressure–induced unfolding of mKO

The evolution of the mKO fluorescence intensity at 570 nm as a function of pressure, in drastic chemical conditions (pH4.0, 0.8 M GdnHCl), is presented in Fig. 5. mKO exhibited a strong and sigmoidal decrease in fluorescence intensity for a pressure level increase from 300 to 600 MPa, that can be attributed to a loss of intramolecular bond structure. At 600 MPa, the highest pressure, mKO has lost more than 80% of its maximum fluorescence intensity and after the pressure release to atmospheric pressure, the protein did not recover its initial fluorescence intensity. These

results suggest that the reduction in mKO fluorescence intensity under high pressure may be due to pressure-induced disruption of the mKO chromophore salt bridges and the hydrogen-bonding network. The protein can be expected to unfold or undergo at least strong chromophore pocket deformation at high pressures [27,28]. These data also suggest a single essentially non-reversible (or a very slowly reversible) unfolding step. High pressure changes the H-bonded networks and pressure-induced denaturation is thought to involve forcing water molecules into the hydrophobic regions of the molecule, disrupting its structure [27]. In our study, it was verified that the spectra as a function of pressure did not vary with time up to 3 h after high pressure treatment. This suggests that an equilibrium between the native and the pressure-unfolded protein was attained at each pressure. The finding that the intensity and CSM profiles as a function of pressure were not or only partly reversible might be explained by a very slow refolding reaction. However, we observed that 3 h after the pressure release, the spectrum of the native protein was still not recovered. This could indicate that the rate of structural changes is extremely slow at atmospheric pressure. The pressure $p_{1/2}$ at which fluorescence intensity decreases to half of its maximum value can serve as a stability measurement for the chromophore environment. As shown in Table 2 presenting the thermodynamic parameters of mKO unfolding, mKO seems to have a fragile pocket. Indeed, the $p_{1/2}$ is increased by 30 MPa when the GndHCl concentration is decreased from 0.9 to 0.8 M. This rather strong variation indicates that the pressure-induced protein unfolding is strongly dependent on the GndHCl concentration. According to the above observations, a volume change was observed. The value of ΔV_u is reasonable for pressure-induced protein unfolding (Table 2). In conclusion, it can be clearly observed that, the presence of a higher number of salt bridges and H-bonds in the chromophore pocket is of high importance for the pressure behavior of mKO. Indeed, only in drastic chemical conditions (at high-pressure levels and in the presence of 0.9 M GdnHCl) the protein unfolds since mKO chromophore pocket becomes distorted, leading to a decreased resistance toward high pressure.

Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.06.002.

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