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Short sequence-paper

Effect of high pressure and reversed micelles on the fluorescent proteins

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Abstract

Two physico-chemical perturbations were applied to ECFP, EGFP, EYFP and DsRed fluorescent proteins: high hydrostatic pressure and encapsulation in reversed micelles. The observed fluorescence changes were described by two-state model and quantified by thermodynamic formalism. ECFP, EYFP and DsRed exhibited similar reaction volumes under pressure. The changes of the chemical potentials of the chromophore in *bis*(2-ethylhexyl)sulfosuccinate (AOT) micelles caused apparent chromophore protonation changes resulting in a fluorescence decrease of ECFP and EYFP. In contrast to the remarkable stability of DsRed, the highest sensitivity of EYFP fluorescence under pressure and in micelles is attributed to its chromophore structure.

Keywords: Chemical potential; Reaction volume; Chromophore protonation; Thermodynamics

The green fluorescent protein (GFP) from the jellyfish *Aequorea v.* is a widely used reporter in studies of gene expression, protein dynamics and localization [1]. Crystallographic structures of the wild-type GFP and its enhanced mutants (ECFP, EGFP and EYFP) have revealed that the GFP tertiary structure resembles a β-can [2,3]. This compact structure makes GFP fluorescence rather stable under a variety of conditions including the treatment with detergents and pH [4], proteases [5] and temperature [6]. Recently, a red GFP-like protein DsRed from the corallimorph *Discosoma* sp. has been cloned [7]. Although DsRed monomers fold, similarly to GFP [9], into β-cans they further form an obligate tetramer [10].

Here we have employed two perturbations—high pressure and micelles—to study the behavior of the DsRed and GFP mutants in order to observe changes (if any) in the fluorescence properties. The fluorescence changes were analyzed phenomenologically into thermodynamic quantities by assuming a two-state model of protein conformations:

$$\mathrm{N} \underset{k_r}{\overset{k_f}{\rightleftarrows}} \mathrm{A} \tag{1}$$

where N and A designate native and affected state of the FP, respectively; k_f and k_r are forward and reverse rate constants, respectively, so the equilibrium constant $K = k_r/k_f$. Both pressure and micellar experiments were interpreted using an equilibrium approximation. The dependence of the equilibrium constant K_p on pressure p was determined from the principle of Le Chatelier [12]:

$$\left.\frac{\partial \ln K_p(p, T)}{\partial p}\right|_p = -\frac{\Delta v}{RT} \tag{2}$$

where T is temperature, R is the universal gas constant, and Δv is a change in the reaction volume (per mole). To calculate K_p from the fluorescence intensities for various pressures we have employed two approximations depending on the form of the intensity–pressure curve. If the pressure dependence of the fluorescence intensity shows a linear increase at higher

Abbreviations: GFP, green fluorescent protein; ECFP, EGFP and EYFP, enhanced cyan, green and yellow fluorescent proteins, respectively; DsRed, red fluorescent protein; FP, fluorescent protein; AOT, sodium *bis*(2-ethylhexyl)sulfosuccinate; *w*, water-to-AOT molar ratio

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pressure, we propose only form A to be fluorescent, and at atmospheric pressure forms N and A are in equilibrium. When the intensity ceases to change at high pressures, the protein is almost completely transformed into form A with its fluorescence I_{as}:

$$K_p = \frac{I_p}{I_{as} - I_p} \quad (3)$$

where I_p is intensity at pressure p, I_{as}—intensity that does not change anymore with increase of pressure. On the other hand, if the curve has a sigmoidal behavior and the intensity drops upon increase of the pressure, we propose that both forms N and A are fluorescent, and at atmospheric pressure almost only the form N is present. A pressure increase produces the form A, which has lower fluorescence I_{as}:

$$K_p = \frac{I_p - I_{atm}}{I_{as} - I_p} \quad (4)$$

where I_p and I_{as} are as above and I_{atm} is the intensity at atmospheric pressure (0.1 MPa). The equilibrium constant K_{p0} at $p = 0.1$ MPa and $T = 298$ K was used to determine a change of Gibbs energy under standard conditions:

$$-RT\ln K_{p0} = \Delta G^0 \quad (5)$$

We consider AOT micelles to affect the equilibrium constant of chromophore protonation K_a. The protonation leads to the loss of fluorescence of the GFP variants [13]. We propose a standard chemical potential μ^0 of the particles involved in the protonation reaction (H^+, protonated (FP) and deprotonated (FP^-) proteins) to depend on the hydration extent w. For small changes of w the dependence is linear: $\mu^0_w = \mu^0_{(w=0)} + bw$, where b is a particle-specific parameter defining the energy change. Thus, the dependence of the apparent K_a from w [12]:

$$-RT\ln K_a = \Delta G^0 \mid_{w=0} + wB \quad (6)$$

where B is a sum of parameters b for H^+ and FP^- subtracted by the b for FP. To calculate the corresponding K_a the following equation was used:

$$K_a = \frac{[H^+] I_w K_f}{K_f + [H^+] - I_w K_f} \quad (7)$$

where I_w is relative fluorescence intensity at certain w and K_f—known chromophore protonation constant of a fluorescent protein in the absence of AOT.

The experiments were performed with the plasmids encoding FPs followed by polyhistidine tags constructed as described elsewhere [8]. The plasmids were transfected into *E. coli* BL21(DE3) (Invitrogen), and recombinant proteins were purified by Ni–NTA agarose (Qiagen). Protein concentrations were adjusted to 0.05 mg/ml in 50 mM Tris–HCl buffer, pH 8.0. To reach a desired water-to-surfactant molar ratio, w, in reversed micelles, 2 μl of FP stock solution and previously determined volume of the same buffer containing 120 mM KCl were added to 1 ml of 100 mM sodium *bis*(2-ethylhexyl)sulfosuccinate (AOT, Sigma) in isooctane, and the mixture was shaken as described [11]. ECFP, EGFP, EYFP and DsRed were excited at 440, 480, 510 and 555 nm, and emission was recorded at 475, 510, 529 and 585 nm, respectively. The studies at high pressure were performed using the Spex-Fluorolog 3.2.2 spectrofluorimeter (Jobin-Yvon Horiba), equipped with a high-pressure cell (ISS, Inc.). The FP samples were pressurized for at least 10 min prior measurements. All measurements were performed at room temperature (298 K).

DsRed fluorescence was found to be the most tolerant to the high hydrostatic pressure. The increase from 0.1 to 300 MPa resulted maximally in 8% increase of its fluorescence intensity (Fig. 1A). In contrast, ECFP raised its emission intensity up to 24% upon changing the pressure from 0.1 to 200 MPa, and then the intensity became almost constant. EYFP showed a continuous decrease of its emission intensity upon pressure elevation,

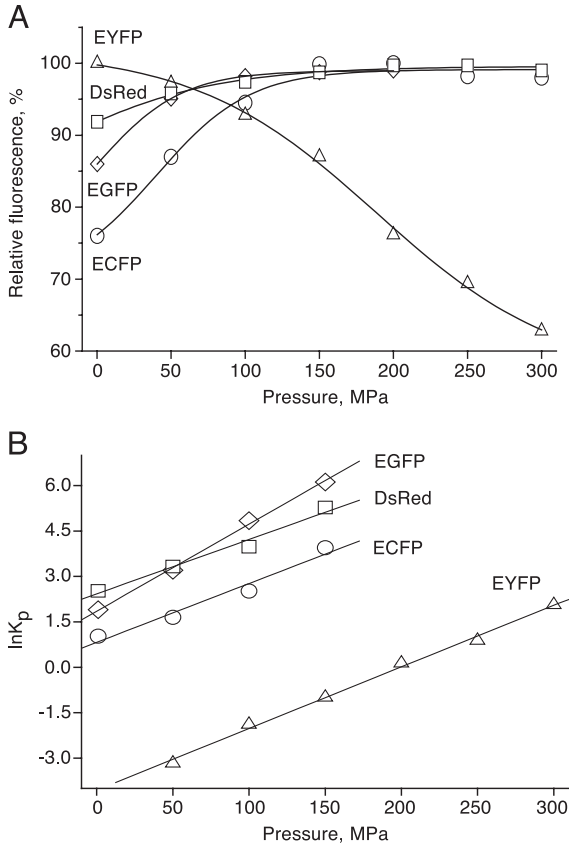

Fig. 1. Effect of pressure on the fluorescence intensity of ECFP (circles), EGFP (diamonds), EYFP (triangles), and DsRed (squares) in the range 0.1–300 MPa. Data for 0.1 MPa correspond to atmospheric pressure. Changes in the fluorescence intensity (A) and dependence of the equilibrium constants K_p (B) upon pressure are presented.

which finally resulted in about 63% of the initial value. The fluorescence behavior of EGFP was rather analogous to that of DsRed and ECFP, the emission increased for about 13% from 0.1 to 200 MPa followed by its stabilization. While ECFP, EGFP and DsRed did not exhibit detectable changes in their fluorescence spectra, EYFP displayed a 4–5-nm red shift during the increase of pressure from 0.1 to 300 MPa. The fluorescence changes for all FPs were reversible—release of high pressure completely recovered the fluorescence intensity and spectral shape. The respective equilibrium constants K_p were calculated from the above formulas (3) and (4). In the case of EYFP the I_{as} in the corresponding formula (4) was supposed to be about 63% of the fluorescence at atmospheric pressure—in our experiments we could not reach the state when the fluorescence did not change anymore upon the pressure increase, although one can clearly see the curve approaching such a state. The dependencies of $\ln K_p$ were linear upon pressure (Fig. 1B), the fitted lines had nearly similar slopes for ECFP, EYFP and DsRed that resulted in virtually the same reaction volumes $\Delta v = 49.6$ ml/mol calculated from Eq. (2) (Table 1). The Δv value for EGFP differed 1.5 times that possibly was caused by the smaller size of its chromophore [1]. On the other hand, the ΔG^0 values exhibited both positive and negative changes for DsRed, EGFP, ECFP and EYFP, respectively (Table 1).

Reverse micelles are micrometer-sized droplets of water surrounded by a monolayer of surfactant in a nonpolar solvent. DsRed fluorescence was almost insensitive to the differences of the micellar size upon a change of w from 10 to 30. EGFP intensity exhibited very small changes, less than 7% within all w range. In contrast, EYFP displayed convincing fluorescence decrease with decreasing w that resulted in about 45% lower intensity at hydration extent 10 (Fig. 2A). ECFP behaved quite similar to EYFP but with somewhat smaller effect—about 25% at $w = 10$. The shape of ECFP and EYFP emission spectra and their maxima remained the same. To calculate the apparent K_a according to Eq. (7) the pK_f values in the absence of AOT were taken equal to 4.7 and 6.5 for ECFP and EYFP, respectively [14]. At small values of w the $\ln K_a$ depended linearly upon w (Fig. 2B) indicating the validity of our approach. The calculated apparent pK_a values in AOT were higher, its maximal shifts were 2.7 and 1.3 at $w = 10$ for ECFP and EYFP, respectively. $\Delta G^0|_{w=0}$ values were almost equal for ECFP and EYFP (Table 1) as expected from rather similar protein matrix of the β-cans surrounding the chromophores. On the other hand, the parameter B, characterizing changes of the chromophore chemical potentials, was twice larger for EYFP reflecting its higher sensitivity to the micellar environment.

Table 1
Parameters characterizing fluorescence changes under high hydrostatic pressure and in reversed micelles

Protein	High pressure			Reversed micelles	
	K_{p0}	Δv, ml/mol	ΔG^0, kJ/mol	B, kJ/mol	$\Delta G^0\|_{w=0}$, kJ/mol
EGFP	6.69	− 74.3	− 4.7	n.d.	n.d.
ECFP	2.79	− 49.5	− 2.54	− 0.89	51.3
EYFP	1.73×10^{-2}	− 49.6	10.0	− 0.42	48.9
DsRed	12.5	− 49.6	− 6.24	n.d.	n.d.

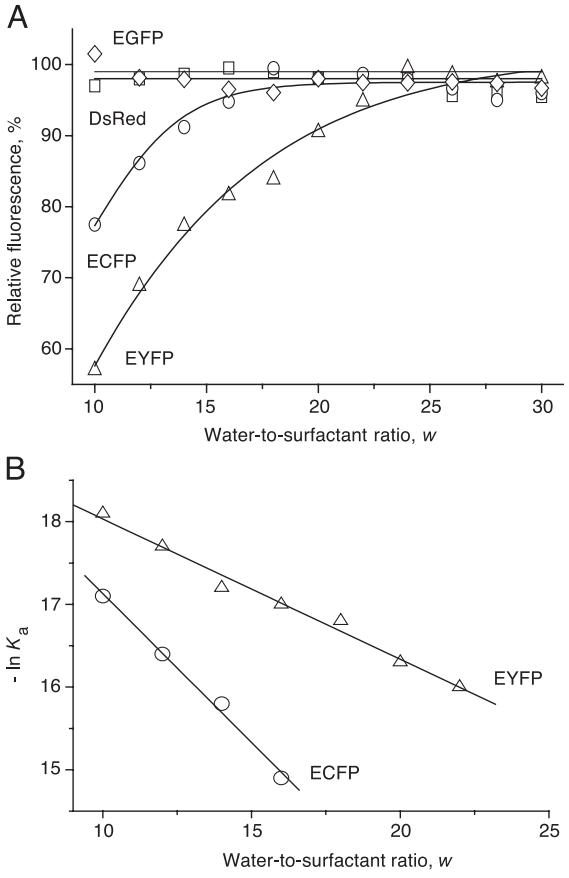

Fig. 2. Effect of micellar restrictions on fluorescence of ECFP (circles), EGFP (diamonds), EYFP (triangles), and DsRed (squares). Dependence of fluorescence intensities on the water content, w, of AOT reversed micelles in isooctane (A) and dependence of the equilibrium constants K_a (B) are presented. K_a values for EGFP and DsRed were not calculated because of the small intensity changes.

In conclusion, the outstanding stability of DsRed as compared to all GFPs would allow to use it in vivo as population marker for organisms living in the extreme conditions, and as advanced genetic reporter in high-pressure and micellar biotechnological applications, whereas EYFP can be used as a fluorescent sensor.

Acknowledgements

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References

[1] R.Y. Tsien, The green fluorescent protein, Ann. Rev. Biochem. 67 (1998) 509–544.

[2] M. Ormö, A.B. Cubitt, K. Kallio, L.A. Gross, R.Y. Tsien, S.J. Remington, Crystal structure of the *Aequorea victoria* green fluorescent protein, Science 273 (1996) 1392–1395.

[3] R.M. Wachter, M.A. Elsliger, K. Kallio, G.T. Hanson, S.J. Remington, Structural basis of spectral shifts in the yellow-emission variants of green fluorescent protein, Structure 6 (1998) 1267–1277.

[4] S.H. Bokman, W.W. Ward, Renaturation of *Aequorea* green fluorescent protein, Biochem. Biophys. Res. Commun. 101 (1981) 1372–1380.

[5] M. Chalfie, Y. Tu, G. Euskirchen, W.W. Ward, D.C. Prasher, Green fluorescent protein as a marker for gene expression, Science 263 (1994) 802–805.

[6] W.W. Ward, H.J. Prentice, A.F. Roth, C.W. Cody, S.C. Reeves, Spectral pertrubations of the *Aequorea* green fluorescent protein, Photochem. Photobiol. 35 (1982) 803–809.

[7] M.V. Matz, A.F. Fradkov, Y.A. Labas, A.P. Savitsky, A.G. Zaraisky, M.L. Markelov, S.A. Lukyanov, Fluorescent proteins from nonbioluminescent *Anthozoa* species, Nat. Biotechnol. 17 (1999) 969–973.

[8] V.V. Verkhusha, H. Otsuna, T. Awasaki, H. Oda, S. Tsukita, K. Ito, An enhanced mutant of red fluorescent protein DsRed for double labeling and developmental timer of neural fiber bundle formation, J. Biol. Chem. 276 (2001) 29621–29624.

[9] M.A. Wall, M. Socolich, R. Ranganathan, The structural basis for red fluorescence in the tetrameric GFP homolog DsRed, Nat. Struct. Biol. 7 (2000) 1133–1138.

[10] D. Yarbrough, R.M. Wachter, K. Kallio, M.V. Matz, S.J. Remington, Refined crystal structure of DsRed, a red fluorescent protein from coral, at 2.0-A resolution, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 462–467.

[11] M.A. Uskova, J.W. Borst, M.A. Hink, A. van Hoek, A. Schots, N.L. Klyachko, A.J.W.G. Visser, Fluorescence dynamics of green fluorescent protein in AOT reversed micelles, Biophys. Chemist. 87 (2000) 73–84.

[12] W. Greiner, L. Neise, H. Stoecker, Thermodynamics and Statistical Mechanics, Springer-Verlag, New York, 1995.

[13] J. Llopis, J.M. McCaffery, A. Miyawaki, M.G. Farquhar, R.Y. Tsien, Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 6803–6808.

[14] G. Patterson, R.N. Day, D. Piston, Fluorescent protein spectra, J. Cell. Sci. 114 (2001) 837–838.