Fluorescence Emission Properties of S-Layer Enhanced Green Fluorescent Fusion Protein as a Function of Temperature, pH Conditions, and Guanidine Hydrochloride Concentration

José L. Toca-Herrera,*[,†,‡] Seta Küpcü,[§] Valerie Diederichs,[§] Gerald Moncayo,[||] Dietmar Pum,[§] and Uwe B. Sleytr[§]

Department of Chemical Engineering, Rovira i Virgili University, Av. Paisos Catalans, 43003 Tarragona, Spain, Biosurfaces Unit, CICBiomagune, Paseo Miramon 182, 20009 San Sebastian, Spain, Center for NanoBiotechnology, University of Natural Resources and Applied Life Sciences, 1180 Vienna, Austria, and Henry Wellcome Building of Molecular Physiology, Nuffield Department of Clinical Medicine, University of Oxford, Roosevelt Drive, Oxford OX3 7BN, United Kingdom

Received July 9, 2006; Revised Manuscript Received October 13, 2006

The fluorescent properties of the S-layer enhanced green fluorescent fusion protein (rSbpA$_{31-1068}$/EGFP) were investigated as a function of temperature, pH conditions, and guanidine hydrochloride concentration. These results were compared to the fluorescent properties of the recombinant enhanced green fluorescent protein (EGFP) and an equimolar mixture of the S-layer protein rSbpA and EGFP. The intensity of the fluorescence emission of the EGFP at 510 nm, after excitation at 490 nm, is not affected by the presence of rSbpA, either as a fusion partner or as a free protein in solution. In each of the three protein systems, the emission intensity at 510 nm reaches its maximum value between pH 7 and 9 at 20 °C and at 0 M guanidine hydrochloride. No fluorescence could be measured at pH 4 and 6 M guanidine hydrochloride. These results show that the S-layer fusion protein (rSbpA$_{31-1068}$/EGFP) is a suitable candidate for future applications in nanobiotechonology at a wide range of pH, temperature, and guanidine hydrochloride concentrations.

Introduction

Green Fluorescent Protein (GFP) is a naturally fluorescent 27 kDa protein with 238 amino acid residues from the jellyfish *Aequorea victoria*.[1] The discovery that GFP can form a functional fluorophore without other genes or cofactors[2] has led to its extensive use as a reporter of expression, with fluorescent fusion proteins being produced to monitor the expression and localization of many proteins.[3−5] Chemical studies of GFP and expression of cDNA for GFP in prokaryotes and eukaryotes have shown that the chromophore is a product of a post-translational modification of the primary structure, involving an auto-cyclodehydration of the tripeptide segment, -Ser^{65}-Tyr^{66}-Gly^{67}-, and an autoxidation of the resulting dihydro-ring system.[6,7] During the last few years some GFP mutants have been made to increase the fluorescence at the protein major peaks. The enhanced green fluorescent protein (EGFP) is a variation of the GFP.[8] EGFP presents several advantages against to wild-type GFP: (a) It fluoresces more intensely, and (b) expressed GFP has been found to be insoluble and nonfluorescent in bacterial systems.[9]

Among many different applications in biology, EGFP has been used to detect cell surface markers,[10] retroviral transfections,[11] and cytosolic and Golgi pH indicator.[12] However, in many cases it is necessary to find a method to attach EGFP to surfaces without changing its fluorescent properties. This goal can be achieved in two steps: (i) by engineering a fluorescent fusion protein using S-layer technology[5] and (ii) by finding the experimental conditions that lead to the recrystallization of such fusion proteins in two-dimensional (2-D) crystalline arrays.[13] The latter exploits the properties of the bacterial surface layers (S-layers), which are composed of identical (glyco)protein subunits and represent the outermost cell envelope component in many bacteria and archaea, presenting oblique, tetragonal, and occasionally hexagonal symmetry. It has been shown that isolated S-layer subunits are able to recrystallize on liposomes and polyelectrolytes, building supramolecular structures that adopt their original bacterial crystalline structures.[13,14] Thus S-layer proteins represent unique building blocks in molecular nanobiotechnology and biomimetics. The successful cloning and expression of the functional chimeric S-layer fusion protein (rSbpA$_{31-1068}$/EGFP) was reported in a previous paper.[5] This fusion protein was generated from a 750 bp nucleotide sequence encoding the EGFP and the 3′ end of the sequence encoding rSbpA$_{31-1068}$. Fusion of the N-terminus of EGFP to the C-terminus of rSbpA produced a fusion protein rSbpA$_{31-1068}$/EGFP that underwent correct folding and retained the ability to form regular arrays.

In this paper we compare the fluorescent properties of the fusion protein rSbpA$_{31-1068}$/EGFP with EGFP and an equimolar mixture composed of rSbpA and EGFP, as a function of temperature, pH value, and denaturant guanidine hydrochloride concentration. We also investigated the protein−protein interaction, that is, the influence of rSbpA on the fluorescent properties of EGFP.

Experimental Section

Equipment. Fluorescence measurements were carried out with a LS 55 Luminescence Spectrometer (Perkin-Elmer instruments). A quartz

* Author to whom correspondence should be addressed. E-mail: joseluis.toca@urv.net; jltocaherrera@cicbiomagune.es.
† Rovira i Virgili University.
‡ CICBiomagune.
§ University of Natural Resources and Applied Life Sciences.
|| University of Oxford.

10.1021/bm060661+ CCC: $33.50
Published on Web 11/15/2006

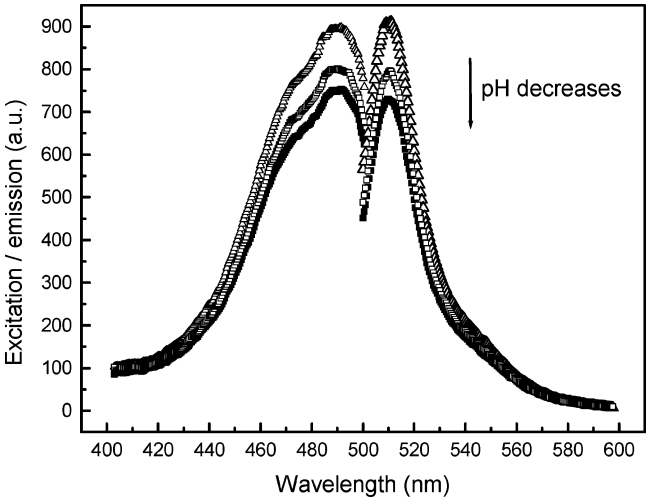

Figure 1. Typical fluorescence excitation (490 nm) and emission spectra (510 nm) of the fusion protein rSbpA/EGFP as a function of pH (9, 7, and 5.5). Note that the fluorescence intensity decreases at low pH. EGFP as well as the rSbpA + EGFP mixture presented the same behavior.

cuvette with a 1 cm path length was used with a minimum test volume of 2 mL. Special software (FL WinLab) recorded the spectra. Temperature-dependent measurements were performed with a Thermostat DC 30 (Thermo Haake).

Materials. Expression, isolation, and purification of EGFP ($M_n \approx$ 27 000) and rSbpA$_{31-1068}$/EGFP ($M_n \approx$ 136 000) have been described previously.[5] The stoichiometry of rSbpA$_{31-1068}$/EGFP in the fusion protein is 1:1.

Recombinant S-layer protein rSbpA ($M_n \approx$ 127 000) of *Bacillus sphaericus* CCM 2177 was produced according to the procedure described elsewhere.[15] All buffer solutions were prepared in Milli-Q water (18.2 MΩ cm^{-1}, pH = 5.5). Different buffer solutions were used: citric acid monohydrate (Merck) for pH 4, Tris-(hydroxymethyl) amino methane (Tris X−HCl) (Gerbu) for pH 7and 9, and Tris for pH 11. Guanidine hydrochloride (GHCl) was dissolved in Tris buffer (pH 7.2).

Results and Discussion

Influence of pH on the Emission Spectra of EGFP, rSbpA$_{31-1068}$/EGFP, and the Equimolar Mixture rSbpA + EGFP. Figure 1 shows typical excitation and emission spectra of rSbpA$_{31-1068}$/EGFP. Excitation takes place at 490 nm, while fluorescence is emitted at 510 nm. EGFP and the equimolar mixture rSbpA + EGFP were also excited at 490 nm and presented fluorescence emission at 520 nm (spectra not shown). The fluorescence intensity decreased at lower pH values. Figure 2 shows the values of fluorescence emission at 510 nm (excitation at 490 nm) as a function of pH value for EGFP, rSbpA$_{31-1068}$/EGFP, and the equimolar mixture rSbpA + GFP. The concentration of EGFP, 0.24 μM, was constant in each case. The measurements show that the intensity of EGFP, rSbpA$_{31-1068}$/EGFP, and rSbpA + EGFP rises with increasing pH value from 4 to 7, reaching a constant value between pH 9 and 11. At pH 4 neither excitation nor emission could be measured. These results are in agreement with those reported by Llopis et al.[12] for EGFP and Sawano and Miyawaki[16] for another GFP variant. The effect of reducing the pH is to quench the fluorescence rather than to shift the fluorescence peak due to a protonated chromophore.[8] In Figure 2 an exponential function has been used to fit and determine the differences in area below the

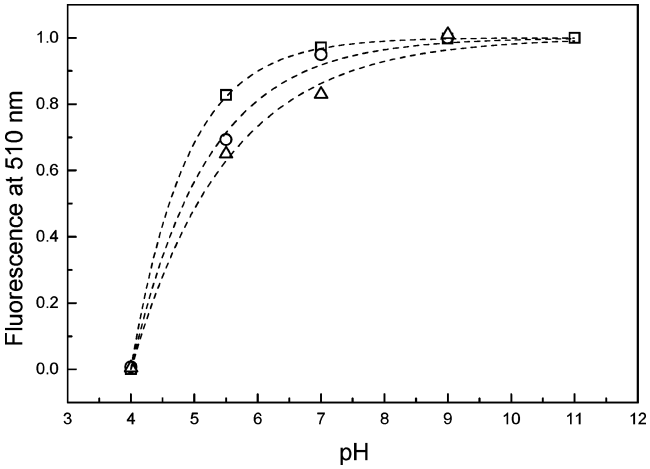

Figure 2. Fluorescence intensity as a function of pH value at room temperature. An exponential function $y = 1 - \exp[A(x_0 - x)]$, $x_0 = 4$, was chosen to integrate the area below the experimental points to quantify the loss in fluorescence, which is about 10% (see text and Table 1). The relative error of the experimental points was 5%: squares, EGFP; circles, rSbpA$_{31-1068}$/EGFP; triangles, rSbpA + EGFP.

Table 1. Loss in Fluorescence as a Function of pH Value[a]

protein sample	area	normalized value
EGFP	6.12	1
rSbpA$_{31-1068}$/EGFP	5.80	0.95
rSbpA + EGFP	5.50	0.90

[a] The area below the experimental points has been quantified by integration. Comparison between normalized values gives the percentage loss in fluorescence (taken EGFP as a reference), that is, 5% for the fusion protein and 10% for the equimolar mixture.

experimental points to quantify the percentage loss of fluorescence intensity. The obtained values are shown in Table 1; it can be seen that the equimolar mixture rSbpA + EGFP shows an approximately 10% loss of fluorescence with respect to that of EGFP. The fusion protein rSbpA$_{31-1068}$/EGFP, engineered for this study, has a 5% loss of fluorescence intensity. The results also show that the presence of rSbpA does not affect, in any case, significantly the fluorescent properties of EGFP. This fact is in agreement with studies carried out by Chalfie et al.,[2] where they showed that EGFP does not interfere with other macromolecules during cell growth (and function), retaining its fluorescent properties.

Influence of Temperature on the Emission Spectra of EGFP, rSbpA$_{31-1068}$/EGFP, and the Equimolar Mixture rSbpA + EGFP. Figure 3 shows the behavior of the emission spectra as a function of temperature for (a) EGFP, (b) rSbpA$_{31-1068}$/EGFP, and (c) rSbpA + EGFP. The emission intensity of EGFP in every case decreases linearly with increasing temperature as the sample is heated from 10 to 70 °C, with an average decrease of 50% of the fluorescence intensity observed over this temperature range. Ward and co-workers found a similar behavior for GFP variants.[17] The values of the different slopes, ranging from −0.0101 to −0.0108, obtained for the three samples, are shown in Figure 3. These values indicate that the decay in emission of EGFP is similar for each sample. (The relative error of the experimental points was 5%.) The heat-induced decrease in fluorescence intensity is a reversible process, although the measured kinetics could not be the same due to the fact that cooling the sample took approximately 10 min longer than heating.

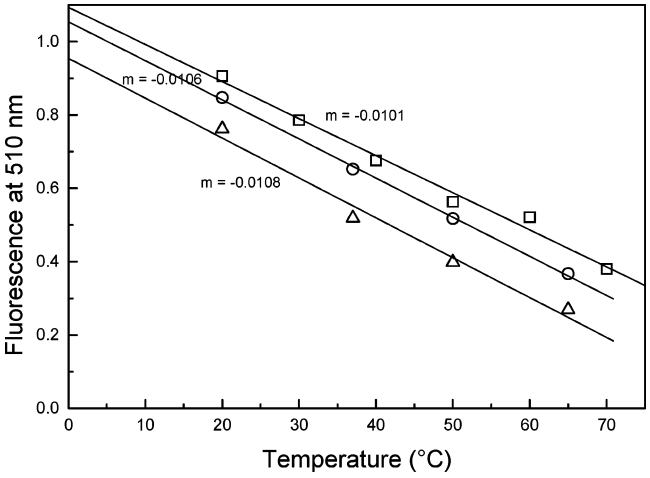

Figure 3. Fluorescence intensity at 510 nm. The slopes of the straight lines, denoted by *m* with a variation lower than 7%, show that the fluorescent properties of EGFP are independent of the fusion partner. The relative error of the measurements is 5%. Note that at the biological temperature of 37 °C the fluorescence efficiency is about 75% of the value at 20 °C: squares, EGFP; circles, $rSbpA_{31-1068}$/EGFP; triangles, rSbpA + EGFP.

The increase of the temperature seems to affect the fluorescence intensity in two ways. On one hand, the rise of the temperature increases the probability of unfolding EGFP as well as rSbpA from their native states by decreasing the barrier of the transition estate along the coordinate reaction. On the other hand, collisions between molecules increase fluorescence quenching. These results show the thermostability of the engineered fusion protein (**r**$SbpA_{31-1068}$/EGFP) and its ability to refold. In former experiments,[18] it has been found that wild-type SbpA, the main building block of the fusion protein presented in this study, preserves its 2-D crystalline structure at 60 °C. This fact together with the thermal dependence $rSbpA_{31-1068}$/EGFP makes this fusion protein a valuable cell membrane marker for thermal studies.

Influence of Guanidine Hydrochloride Concentration on the Fluorescence Properties of EGFP, $rSbpA_{31-1068}$/EGFP, and the Equimolar Mixture rSbpA + EGFP. Figure 4 shows the influence of the denaturant agent GHCl on the emission intensity at 510 nm of EGFP, $rSbpA_{31-1068}$/EGFP, and rSbpA + EGFP. These measurements were carried out after 20 min of equilibration. The fluorescence intensity is at its maximum when no guanidine hydrochloride is present in solution. From Figure 4, it can be seen that the decrease in fluorescence intensity is approximately 35−40% for a concentration of 2 M GHCl, approximately 70% for 3 M GHCl, and approximately 90% for 4 M GHCl. No fluorescence emission could be measured for 6 M GHCl, indicating that EGFP had lost its initial folded conformation and activity due to the disruption of the interactions that stabilize the anionic form of the chromophore. Fukuda et al.[19] reported that the fluorescence emission of GFP vanished at 6.5 M GHCl, while in a more recent study Campanini et al.[20] found that encapsulated green fluorescent protein (mut2) in silica in wet nanoporous silica gels unfolded at 6 M GHCl. Our results show that the unfolded state of both the EGFP (and rSbpA) proteins at 6 M GHCl led to a decrease in the fluorescence intensity. The results also show that the fusion partner rSbpA does not influence the fluorescent properties of EGFP.

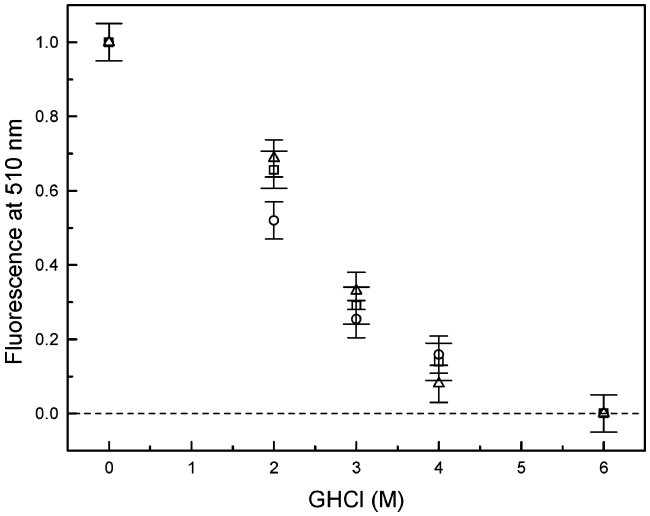

Figure 4. Fluorescence intensity at 510 nm as a function of GHCl concentration. The EGFP fluorescence intensity vanishes at 6 M for the three protein systems studied. At this concentration rSbpA is normally unfolded, but its folding state does not seem to influence the fluorescent properties of EGFP: squares, EGFP; circles, $rSbpA_{31-1068}$/EGFP; triangles, rSbpA + EGFP.

Conclusions

The fluorescence emission of the EGFP, the S-layer fusion protein $rSbpA_{31-1068}$/EGFP, and an equimolar mixture EGFP + rSbpA has been investigated as a function of the temperature, pH, and GHCl concentration. It has been found that rSbpA does not influence the fluorescence emission at 510 nm of the EGFP, either as a fusion partner or free in solution. For the three protein systems, the emission intensity of EGFP at 510 nm reaches its maximum between pH 7 and 9, at 20 °C, and when no GHCl is present in solution. No fluorescence could be detected at pH 4 and 6 M GHCl.

In this study we have shown, in particular, that the engineered S-layer fusion protein $rSbpA_{31-1068}$/EGFP is suitable for in vitro applications. We conclude that the fluorescent properties of the fusion protein $rSbpA_{31-1068}$/EGFP are equivalent to the fluorescent properties of EGFP. This fact together with the ability of the fusion protein to recrystallize on different supports building model membranes makes this fusion protein a useful tool for nanobiotechnology. Thus, $rSbpA_{31-1068}$/EGFP could be used as a label to visualize and measure specific interactions of S-layers with cell membrane components or other macromolecules.

Acknowledgment. J.L.T.H. is a Ramon y Cajal senior research fellow and thanks the Ramon y Cajal programme of the Spanish Government. This work was partially supported by the Air Force Office of Scientific Research (Project No. F49620-03-1-0222). The authors thank K. Melzak, M. Delcea, and S. Moreno-Flores for reading the manuscript. This paper is dedicated to Margit Sara.

References and Notes

(1) Morin, J.; Hastings, J. *J. Cell. Physiol.* **1971**, *77*, 313−318.
(2) Chalfie, M.; Tu, Y.; Euskirchen, G.; Ward, W. W.; Prasher, D. C. *Science* **1994**, *263*, 802−805.
(3) Cha, H. J.; Dalai, N. G; Pham, M.-Q.; Bentley, W. E. *Biotechnol. Prog.* **1999**, *15*, 283−286.
(4) Brevis, N.; Phelan, A.; Webb, J.; Drew, J.; Elliot, G.; O'Hare, P. *J. Virol.* **2000**, *74*, 1051−1056.
(5) Ilk, N.; Küpcü, S.; Moncayo, G.; Klimt, S.; Ecker, R. C.; Hofer-Warbinek, R.; Egelseer, E. M.; Sleytr, U. B.; Sara, M. *Biochem. J.* **2004**, *379*, 441−448.
(6) Niwa, H.; Inoyue, S.; Hirano, T.; Matsuno, T.; Kojima, S.; Kubota, M.; Ohashi, M.; Tsuji, F. I. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 13617−13622.

(7) Nishiuchi, Y.; Inui, T.; Nishio, H.; Bódi, J.; Kimura, T.; Tsuji, F. I.; Sakakibara, S. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13549−13554.
(8) Tsien, R. T. *Annu. Rev. Biochem.* **1998**, *67*, 509−544.
(9) Cormack, B. P.; Valdivia, R. H., Falkow, S. *Gene* **1996**, *173*, 33−38.
(10) Kusser, K. L. and Randall T. D. *J. Histochem. Cytochem.* **2003**, *51*, 5−14.
(11) Tahara-Hanaoka, S.; Sudo, K.; Ema, H.; Miyoshi, H.; Nakauchi, H. *Exp. Hematol.* **2002**, *30*, 11−17.
(12) Llopis, J.; McCaffery, M.; Miyawaki, A.; Farquhar, M. G.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6803−6808.
(13) Toca-Herrera, J. L.; Krastev, R.; Bosio, V.; Küpcü, S.; Pum, D.; Fery, A.; Sara, M.; Sleytr, U. B. *Small* **2005**, *1*, 339−348.
(14) Sleytr, U. B.; Pum, D.; Györvary, E.; Schuster, B.; Küpcü, S.; Sára, M. *Prog. Colloid. Polym. Sci.* **2002**, *121*, 57−61.
(15) Ilk, N.; Völlenkle, C.; Egelseer, E. M.; Breitwieser, A.; Sleytr, U. B.; Sára, M. *Appl. Environ. Microbiol.* **2002**, *68*, 3251−3260.
(16) Sawano, A.; Miyawaki, A. *Nucleic Acids Res.* **2000**, *28*, e78.
(17) Ward W. W.; Prentice, H. J.; Roth A. F.; Cody C. W.; Reeves S. C. *Photochem. Photobiol.* **1982**, *35*, 803−808.
(18) Toca-Herrera, J. L.; Moreno-Flores, S.; Friedmann, J.; Pum, D.; Sleytr, U. B. *Microsc. Res. Tech.* **2004**, *65*, 226−234.
(19) Fukuda, H.; Arai, M.; Kuwajina, K. *Biochemistry* **2000**, *39*, 12025−12032.
(20) Campanini, B.; Bologna, S.; Cannone, F.; Chirico, G.; Mozarelli, A.; Bettati, S. *Protein Sci.* **2005**, *14*, 1125−1133.

BM060661+