Published in final edited form as:

J Phys Chem B. 2012 October 18; 116(41): 12426–12440. doi:10.1021/jp3060944.

Exploring Structural and Optical Properties of Fluorescent Proteins by Squeezing: Modeling High-Pressure Effects on the mStrawberry and mCherry Red Fluorescent Proteins

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Abstract

Molecular dynamics calculations of pressure effects on mStrawberry and mCherry fluorescent proteins are reported. The simulations reveal that mStrawberry has much floppier structure at atmospheric pressure, as evidenced by larger backbone fluctuations and coexistence of two conformers which differ by Ser146 orientation. Consequently, pressure increase has a larger effect on mStrawberry making its structure more rigid and reducing the population of one of the conformers. The most significant effect of pressure increase is in hydrogen-bonding network between the chromophore and the nearby residues. The quantum-mechanics/molecular mechanics calculations of excitation energies in mStrawberry explain the observed blue shift and identify Lys70 as the residue that has the most pronounced effect on the spectra. The results suggest that pressure increase causes initial increase of fluorescence yield only for relatively floppy fluorescent proteins, whereas the fluorescent proteins that have more rigid structures have quantum yield close to their maximum. The results suggest that low quantum yield in fluorescent proteins is dynamic in nature and depends on the range of thermal motions of the chromophore and fluctuations in the H-bonding network rather than on their average structure.

Introduction

Novel bioimaging techniques exploiting the unique properties of green fluorescent protein (GFP) have revolutionized many areas in life sciences.^{1, 2, 3} Driven by exciting applications, the photophysics of GFP and other wild-type and mutated photoactive proteins have been a subject of many experimental and theoretical studies.^{4, 5, 6} Yet, intrinsic electronic properties of the chromophores and their interactions with the protein matrix are not fully understood and the design of new FPs often proceeds in the dark. Moreover, even for the FPs with established properties detailed relationships between the structure and photophysical properties have not been entirely mapped out.

Among FPs used as genetically encoded fluorescent tags, the red-emitting FPs (RFPs)⁷ are of particular importance as suitable markers for mammalian deep tissue imaging.⁸ Most of the RFPs share a DsRed-like chromophore⁹ in which the π -system of the GFP chromophore

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is extended by additional N-acylimine moiety. However, the mechanism of the chromophore maturation and the factors determining color hues, one- and two-photon brightness, quantum yields, photostability, and other properties are not yet fully understood. For example, we do not know what is responsible for relatively low quantum yields and poor photostability of the RFPs from mFruit series.¹⁰ It was suggested¹⁰ that the apparent non-planarity of the extended chromophore may be responsible for lower quantum yields (e.g., 0.22 in mCherry versus 0.79 in wt-GFP). The twist and tilt angles characterizing the relative positions of the imidazolinone and phenolate rings in mCherry are 11 and 14 degrees, respectively, as compared to 0 and 4 degrees in wt-GFP. However, in the absence of an experimental or theoretical evidence it is not clear whether making the chromophore more planar would increase the quantum yield.

A possible explanation of low photostability is an apparent weakness of the β -barrel of mFruit RFPs between β_7 and β_{10} , likely due to the tetrameric nature of the parental DsRed. A recent computational study¹¹ suggested that this weakness leads to increased oxygen permeability.

Experimental interrogation of structure-function relationship in FPs is challenging because it is difficult to perform gradual and well-defined structural modifications in such complex systems. For example, single point mutations designed to test a specific structural hypothesis often lead to undesired consequences such as misfolded and/or immature FPs. High-throughput molecular evolution techniques^{12, 13} based on flow-cytometry allow one to tune-up properties of mutants; however, the structures of the resulting optimized FPs provide only an indirect evidence of the effect of specific structural changes. In this respect, theoretical modeling provides a powerful tool for testing different hypotheses of the structure-function relationship and for mapping out mechanistic details of the FPs photocycle.

Experimentally, gradual (and abrupt) structural modifications can be induced by applying hydrostatic pressure,¹⁴ which has been exploited in studies of interfaces, conformational dynamics, H-bonding and non-covalent interactions in various solids including crystals of amino-acids and proteins. Among other findings, these studies revealed¹⁴ that the length of H-bonds depends almost linearly on pressure, e.g., in L-serine, the N-O distance in this H-bond decreases by about $0.01 \text{ \AA} \cdot \text{GPa}^{-1}$ in the pressure range from ambient up to 10 GPa despite several phase transitions that are accompanied by a jump-wise increase in cell parameters.¹⁵ Shorter H-bonds (such as $\text{NH} \cdots \text{O}$) are less compressible than longer ones, and molecules in general become flatter as the pressure increases.¹⁴ Studies of proteins under pressure provide insight into compressibilities of different structural fragments of peptides and proteins (helices, sheets, turns, non-structured fragments, cavities).¹⁶ Globular proteins, like GFP, show less pressure-induced change as compared to fibrillar and amyloid structures.¹⁴ Pressure also affects other properties, such as solubility (which generally decreases) and acidity (which increases at higher pressure).^{14, 17}

Several studies of FPs under hydrostatic pressure have been reported.^{18, 19, 20, 21, 22, 23, 24, 25, 26, 27} If pressure-induced structural changes are well understood (which can be achieved by theoretical modeling), one can also learn about the effect of structure on photophysics from observing pressure dependence of optical properties, such as fluorescence wavelength and quantum yield. Such experiments have been carried out for the green,^{18, 19, 20, 21, 22} blue,^{21, 23, 24, 25} yellow,^{26, 27} as well as dsRed²¹ FPs. A companion paper²⁸ (Paper I) presents a recent experimental study that investigated the effect of pressure (up to 5 GPa) on the fluorescence yield and spectra of the RFPs from the mFruit family. Despite their similarity, the FPs investigated in this study (TagRFP-S, TagRFP-T, mCherry, mOrange2, mStrawberry, and mKO) exhibit somewhat different

trends.²⁸ TagRFP-S, TagRFP-T, mOrange2, and mStrawberry show an initial increase in fluorescence intensity upon the pressure increase up to 250–530 MPa, whereas fluorescence intensity of mCherry and mKO decreases. At higher pressures, the fluorescence intensity decreases dramatically for all the proteins. At moderate pressures, all FPs exhibit a small blue shift (≈ 0.005 – 0.01 eV). Finally, mOrange2 and mStrawberry were found to exhibit strong and abrupt changes in their fluorescence spectra at high pressures. The directions of spectral shifts are different, i.e., mStrawberry exhibits a blue shift of ≈ 0.1 eV, whereas fluorescence of mOrange2 is red-shifted by ≈ 0.08 eV.

The goal of this study is to characterize the effect of pressure on structure and, consequently, electronic properties of selected RFPs, to provide atomic-level interpretation of the observed trends.²⁸ Towards this goal, we focus on the two representative FPs, mStrawberry and mCherry. While they share an identical chromophore, mStrawberry features an initial increase of fluorescence intensity reaching maximum (1.8 relative to ambient pressure) at 350 MPa, whereas fluorescence of mCherry only decreases.²⁸ The pressure at which fluorescence reaches half its maximum value is 670 MPa and 300 MPa for mStrawberry and mCherry, respectively.²⁸ By understanding the differences between the two, we intend to identify structural elements that are essential for improved quantum yield. Modeling pressure-induced spectral shifts will help us to quantify the effect of neighboring residues, the structure of the chromophore and its binding pocket on the spectral properties of RFPs.

Our computational strategy is as follows. We perform molecular dynamics (MD) simulations to investigate pressure-induced structural changes. The MD results are validated by more reliable *ab initio* MD (AIMD) simulations in which the chromophore and neighboring residues are described by density functional theory (DFT). The structures from AIMD trajectories are used to compute the electronic spectra with the help of hybrid QM/MM (quantum mechanics/molecular mechanics) methods. Such spectra can be directly compared to the experiment, which provides validation of our computational protocols. Furthermore, we analyze the nature of the overall pressure-induced spectral shift in terms of contributions from individual interactions and specific structural changes, which provides insight into structure-function relationship in FPs.

Computational details

As described above, our computational study involves several steps: (i) MD simulations using a modified force-field; (ii) AIMD simulations using DFT; (iii) QM/MM excited-state calculations.

Starting with the X-ray structures¹⁰ of mStrawberry (pdb code: 2H5P) and mCherry (pdb code: 2H5Q), we carefully examined the cavities and protonation states of the protein groups. The *Dowser* program²⁹ was used to analyze the cavities and to fill them with water. Protonation states were determined manually by inspecting hydrogen-bonding network. In difficult cases *Propka* software³⁰ was used. At physiologic pH the chromophore is anionic and Glu215 is protonated in both mStrawberry and in mCherry.¹⁰ The CHARMM27 force field³¹ has been used to describe the protein. The parameters for the mStrawberry and mCherry chromophore were taken from: (i) anionic GFP chromophore's parameters for CHARMM22;³² (ii) acylimine parameters of the DsRed chromophore for OPLS;^{33, 34} and (iii) the retinal parameters (van der Waals parameters of acylimine nitrogen) for CHARMM27. The parameters are given in supporting information (SI).

The MD simulations were performed using NAMD³⁵ in isobaric-isothermal ensemble with Langevin dynamics and Nose-Hoover barostat. Five different MD simulations with periodic boundary conditions were performed starting from the X-ray structures (protonated and hydrated) at pressures varying from 0.1 MPa to 1500 MPa (0.1 MPa, 300 MPa, 600 MPa,

900 MPa, 1200 MPa, and 1500 MPa). The periodic cell contained approximately 30,000 water molecules and was approximately $70 \times 70 \times 70$ Å in size (at normal pressure). In each simulation, the trajectory was first equilibrated for 10 ns. Then the protein structure was reexamined and production runs of 50 ns were performed. A time step of 1 fs was used in all MD simulations and the intervals between the collected data (energies and coordinates) was 50 ps.

To test whether high-pressure trajectories were fully equilibrated in the above protocol, we also performed MD simulations (mStrawberry) at 1200 MPa starting from the equilibrated snapshots from slightly lower pressure (900 MPa) rather than the X-ray structure. No differences were observed between the two protocols confirming that our high-pressure results were fully equilibrated.

Each MD simulation was followed by Born-Oppenheimer *ab initio* MD (AIMD)³⁶ simulations in the NVT ensemble³⁷ performed with the *CP2K* package.³⁸ The starting structure for AIMD was a snapshot from an MD calculation at a given pressure. The simulation box size was exactly as in the MD simulations to mimic the pressure. The AIMD trajectories were equilibrated for 1–3 ps at constant $T=300$ K maintained by Nosé-Hoover thermostat chain (significant heating of the QM subsystem was observed). Following equilibration, the production runs (of 8–10 ps) were performed to collect snapshots. The time step of 1 fs was used (as in the MD simulations).³⁹ The QM level of theory in AIMD was BLYP and BLYP with empirical dispersion correction (BLYP-D, Ref. 40), as implemented in the *CP2K* package. The Goedecker-Teter-Hutter pseudopotentials⁴¹ and the QZV2P (aug-QZV2P on heteroatoms) basis were used. The multigrid approach was used.⁴² The cutoff of the finest grid level was 350 Ry.

From these AIMD trajectories, the snapshots were selected with intervals of 0.25 ps for excitation energy calculations. In order to achieve faster convergence with respect to the number of snapshots, the structures taken from the snapshots were optimized (using the same level of theory, BLYP-D/QZV2P//CHARMM27); these locally optimized structures were used in the excited-state calculations.

Two different QM parts were tested in the AIMD simulations, as illustrated in Figure 1. The first one, called SmallQM-AIMD, includes only the chromophore, whereas the second one, called BigQM-AIMD, contains the chromophore and several nearby residues (Phe65, Glu215, Arg95), side chains of Lys70, Ser146 and Ile197 residues and nearby water molecules. The position of the link atoms are specified in SI. We found that AIMD using SmallQM leads to too long H-bonds (see discussion below). At ambient pressure, we performed two sets of the AIMD calculations following local optimization of the snapshots: one using SmallQM and one using BigQM. At other pressures, we performed AIMD followed by the local optimization of the snapshots using BigQM.

Interestingly, the MD simulations of mStrawberry at atmospheric pressure have revealed two coexisting conformations involving a flip of Ser146, as shown in Figure 2. In the first conformation (Conf I), which is the same as in the X-ray structure, there is an H-bond between the hydroxyl of Ser146 and the phenolate's oxygen of the chromophore. In Conf II, this bond is broken, and the chromophore forms the H-bond with a nearby water.

The AIMD trajectories that were initiated from the MD snapshots corresponding to Conf I do not capture the interconversion between the two conformations because they are too short (≈ 10 ps). Thus, in order to evaluate optical properties corresponding to Conf II, we performed additional AIMD calculations starting from the MD snapshots corresponding to the Conf II structures and following the same protocol as above (i.e., AIMD annealing,

equilibrium simulation, and local QM/MM optimization of the selected MD snapshots). The resulting structures were used to compute the spectroscopic properties of Conf II.

An accurate calculation of the relative populations of the two conformations, which is desirable for computing the average absorption maximum, is a difficult task. We performed a quick estimate based on a different H-bond pattern in the two conformations. By visual inspection of the MD snapshots, we noted that Ser146 tend not to form an H-bond with the chromophore in Conf II. Thus, we split the frames of the MD trajectories into two sets using Ser146-PhO distance as the criterion (if the distance is below 3.5 Å, the snapshot is classified as Conf I). This allowed us to analyze the H-bond patterns for each conformation separately and to estimate relative populations of Conf I and Conf II at different pressures. We note that at very high pressures our estimate may not be reliable as the interconversion between the two conformations might be too slow to be captured by the MD simulations and because the protein structure becomes too perturbed for such a simple criterion to work.

At ambient pressure, the estimated population for Conf I is 59 %; at higher pressures it becomes more dominant. In the second conformation (Conf II, 41 % at 0.1 MPa), Ser146 is twisted by 100 degrees with respect to Conf I breaking the H-bond with Ser146; in this configuration, phenolic oxygen forms a second H-bond with a nearby water. The pressure has strong effect on the relative populations of Conf I and Conf II, e.g., the population of Conf I becomes 99 and 92 % at 300 and 600 MPa, respectively. At 900 MPa, the population of Conf I drops down to 56 % (as estimated by simple snapshot counting). The reliability of this estimate may be affected by significant structural changes at high pressure. We note that average population of the Ser146-PhO H-bond is 78 % at 900 MPa suggesting a larger population of Conf I. Since H-bonding network and distances between the chromophore and nearby charged residues are different in these two conformations, changes in their relative populations may affect optical properties.

Electronic spectra were computed, first, for the isolated chromophore and, second, for the entire mStrawberry protein extracted from AIMD. The geometry of the isolated model chromophore was optimized with the ω B97X-D range-separated hybrid functional⁴³ and with the 6-31+G(d,p) basis set. The optimization in gas phase yields the structure with the planar GFP-like part of the chromophore, however, the acylimine moiety is non-planar (the structure is given in SI). Using gas-phase calculations, we also estimate inaccuracies in structural parameters by comparing the BLYP-D optimized structure with a more reliable ω B97X-D one (see Table S10 in SI).

All vertical excitation energies were computed using the SOS-CIS(D) method⁴⁴ (same-opposite-spin configuration interaction singles with a perturbative account of double excitations) with the cc-pVDZ+ basis derived from the standard cc-pVDZ basis⁴⁵ by augmenting it by one set of diffuse functions taken from the 6-31+G(d,p) basis set.⁴⁶ The basis set dependence of excitation energies is discussed in SI (we also used aug-cc-pVDZ); the variations due to basis sets were about 0.03 eV.

The locally optimized snapshots from AIMD/BLYP-D at selected pressures were used to compute the QM and QM/MM excitation energies to simulate absorption maximum. For benchmark purposes, we also considered AIMD/BLYP snapshots and compared the differences between BigQM and SmallQM. The effect of dispersion and choice of the QM part on structures and excitation energy is discussed in SI.

In excited-state calculations, various choices of the QM part have been tested, as discussed in SI. We found that SmallQM (exactly the same as in the SmallQM-AIMD calculations) yields the same results as the calculations using a larger QM part. The QM part was capped with hydrogens using the Hydrogen Link Atom scheme, as described in the SI.

The MM part in AIMD and excited-state calculations was described by the CHARMM27 atomic charges.³¹ The charges of the frontier atoms (the MM atoms which are linked to the QM part) were set to zero. The original CHARMM27 charges were redistributed among the neighboring MM atoms/charges which are linked by at least two bonds to the frontier atom.

The calculations of the isolated chromophore and all QM and QM/MM excitation energy calculations were performed with the *Q-Chem* package.⁴⁷

Results and discussion

Validation of structures

Table 1 lists the selected structural parameters of the mStrawberry chromophore from the X-ray structure, and from the MD and AIMD calculations at 0.1 MPa, as well as the ω B97X-D structure of the bare (gas-phase) chromophore. SI also gives a BLYP-D structure. The differences between the ω B97X-D and BLYP-D structures are relatively minor, e.g., the discrepancies in bondlengths do not exceed 0.02 Å. The largest difference in torsional angles (about 2 degrees) is observed for the acylimine angle, whereas P and I angles are within 0.3 degrees from each other.

From the present set of calculations, we consider BigQM-AIMD/BLYP-D calculations to be of the highest quality. The difference between MD and AIMD allows us to evaluate the quality of the MD parameters. The differences between the X-ray structure and AIMD is likely due to limitations of the former (see, for example, a recent work from Thiel and co-workers on DsRed which demonstrated that X-ray structures can be considerably refined by using a QM/MM rather than an MD-based guiding algorithm⁴⁸).

The average bond lengths of the chromophore agree reasonably well between different methods and the X-ray structure. The largest difference between BigQM-AIMD and X-ray is for the C-N bond of acylimine (0.144 Å, the X-ray structure has a shorter bond), the variations in other bonds are less than 0.1 Å (0.001–0.07 Å). The differences between MD and AIMD are slightly larger, C-N of acylimine giving the largest difference. It is instructive to compare bond alternation patterns, as they reflect the degree of delocalization in the π -system of the chromophore due to resonance interaction and conjugation. In the canonical representation of the chromophore (see Figure 1), the (C-O)_{Ph} and (C-C)_{Ph} bonds are formally single bonds, whereas (C=O)_{Im} and (C=C)_{Im} are double bonds. The resonance interaction^{49, 6} reduces the difference between formally single and formally double bonds. For example, in the anionic GFP chromophore the C-C-C bridge has almost perfect allylic character,⁶ the CC and CO bond alternations are 0.016 Å and 0.016 Å, respectively.^{49, 50} As one can see from the optimized structure of the isolated mStrawberry chromophore (last column in Table 1), the two bridge CC bonds are almost the same, and the (C=C)_{Im} is even slightly longer than (C-C)_{Ph}. The CC and CO bond alternation in BigQM-AIMD are 0.004 and 0.026 Å, respectively. For the CC bonds, both X-ray and MD give larger values, 0.019 and 0.023 Å, respectively. The CO bond alternation in the X-ray structure is 0.094 Å, and in MD — 0.006 Å. In sum, the AIMD calculations yield the most delocalized structure of the chromophore featuring significant bond order scrambling. In order to reproduce these effects in MD, the respective force field parameters need to be properly adjusted. The X-ray structure is likely to reflect the deficiencies of the underlying guiding MD parameters.⁴⁸

The torsional angles show some variation. The largest differences are observed for the acylimine angle, which is 19 degrees smaller in AIMD than in X-ray, and is 50 degrees larger in MD than in AIMD. The difference in P and I torsional angles between AIMD and X-ray are 10 and 3 degrees, respectively. We note that these differences are much larger than variations in structural parameters computed by BLYP-D and ω B97X-D (see SI). We

also notice that the sign of the P-torsional angle is different (between the calculations and X-ray). In order to test whether AIMD was trapped in a local minimum, we performed AIMD calculations starting from structures with torsional angles similar to those in the X-ray structure, and these calculations also converged to structures with the same torsional angles we obtained from the AIMD calculations initiated from the MD snapshots. Thus, we conclude that our torsional angles are likely to be more accurate than those derived from X-ray.

As far as the structural parameters of the chromophore are concerned, there is no perceivable differences between BigQM and SmallQM AIMD (and between BLYP and BLYP-D). However, the H-bonding network around the chromophore is significantly affected by the QM size, as illustrated by the average lengths of selected H-bonds collected in Table 2. As one can see from the results for atmospheric pressure, SmallQM-AIMD overestimates the lengths of H-bonds by about 0.46 Å relative to BigQM-AIMD. The biggest difference is for the H-bond between Ser146 and PhO for which the smallQM value is 0.98 Å larger than the BigQM one. The BigQM-AIMD and the X-ray values agree well. The difference between the MD and BigQM-AIMD values is much smaller, i.e., 0.17 Å on average (MD tends to overestimate). Thus, we employ the MD results to calculate the average number of H-bonds at different pressures (Table 3 and Table 4).

Pressure-induced structural changes

We begin by analyzing the Root Mean Square Deviation (RMSD) from the reference X-ray structure computed by averaging over snapshots from the 50 ns long MD trajectories run at 0.1 MPa, 300 MPa, 600 MPa, 900 MPa, 1200 MPa, and 1500 MPa. The RMSD quantifies: (i) differences between the average structure from the dynamical calculations and the reference; (ii) the thermal fluctuations in dynamical calculations. For example, if the average structure is the same as the reference one, then RMSD will still be non-zero due to thermal fluctuations. The width of RMSD or the standard deviation of all RMSD values roughly indicates the range of thermal motions, although it may also reflect the presence of several conformers.

The comparison of RMSD average values at various pressure and their standard deviation is shown in Figure 3 for mCherry and mStrawberry (the respective RMSD distributions are given in SI). To analyze structural changes, we break down the overall RMSD into backbone's RMSD (bb-RMSD, red traces) and all heavy atoms RMSD (ha-RMSD, blue traces). As one can see, the standard deviation (bars) of the backbone RMSD decreases as the pressure increases for mStrawberry (squares). This suggests that the range of thermal motions is reduced at elevated pressure. mCherry (triangles) shows smaller average values and more narrow distributions. The average RMSD shows a more complex trend (green traces). Up to 300 MPa the average RMSD decreases from 1.1 Å to 0.92 Å in mStrawberry; and up to 600 MPa from 0.93 Å to 0.81 Å in mCherry, likely, due to a restricted range of motion. Above 600 MPa, it increases up to 1.1 Å in mStrawberry or 1.0 Å in mCherry suggesting that the protein undergoes overall structural changes.

As one can see, the values for mStrawberry and mCherry show different trends. To better analyze them, consider a more detailed analysis of bb-RMSD and ha-RMSD for mStrawberry, which is presented in Figure 4. The top panel of Figure 4 shows the distribution of the bb-RMSD of the β -barrel ('Surface') and of the α -helix ('Core').⁵¹ Each plot includes the average values and bars indicating the range including 90% of all values. The surface and the core parts of the protein behave differently. The bb-RMSD is larger for the surface than for the core illustrating that the surface is more flexible. It appears that the core is well protected by the barrel and is, therefore, less sensitive to the pressure effects. At atmospheric pressure, the surface and the core bb-RMSDs for mStrawberry including 90%

of the values are spread between 0.8–1.2 Å, and 0.4–1.0 Å, respectively. With the increase of pressure the RMSD distributions narrow down (less thermal motion). For the surface bb-RMSD, we observe a notable drift to larger values (up to 1.15 Å for the average values). The average value of the core bb-RMSD shows a smaller drift — it stays around 0.6 Å.

The protein is not really denatured even at 1500 MPa in this time-scale — the RMSD of 1 Å indicates relatively small structural changes. Furthermore, once pressure is released, the structures relax back to the original structure for both MD and AIMD simulations (this is observed after several tens of picoseconds). Denaturation typically takes place on a slower time scale (microseconds and longer), and the reversibility of the structural changes in our simulations suggests that these fast changes are not directly on the pathway to denaturing. Moreover, GFP as a globular protein seems to be resistant to pressure, e.g., the secondary structure of the wild-type GFP does not denature up to 1.3 GPa.¹⁸

Let us now consider the behavior of the side chains of mStrawberry (ha-RMSD) outside and inside the β -barrel (shown in the bottom of Figure 4). The core ha-RMSD also includes the chromophore. The side chains show similar trends as the backbone (although, the values of ha-RMSD are larger than bb-RMSD) — the motions and structure of the surface groups are more sensitive to pressure than those of the core. The surface ha-RMSD differs from the surface bb-RMSD and the core ha-RMSD between 900 and 1500 MPa. Figure 5 compares mStrawberry's bb-RMSD and ha-RMSD. As one can see, the variation between the surface and the core is about twice as large for ha-RMSD than for bb-RMSD. However, the surface ha-RMSD shows a different behavior than other RMSD analysis above 900 MPa. The core bb-RMSD and ha-RMSD are similar showing that both the backbone and the side chains inside the β -barrel do not move much and are not very sensitive to pressure. Thus, the chromophore, which is deeply buried inside the barrel, is not expected to exhibit significant pressure-induced structural changes, which is confirmed by the analysis (below) of the I and P torsional angles.

As far as the bb-RMSD is concerned, mCherry exhibits similar trends as mStrawberry, as illustrated in Figure 3 (green traces). mCherry's bb-RMSD and ha-RMSD differ slightly from those of mStrawberry. The bb-RMSD decreases up to 600 MPa (up to 300 MPa for mStrawberry) and at higher pressure the trend of increasing RMSD is similar for both proteins. We note that for mCherry the backbone's and side chains' average RMSD values as well as the spread of RMSD values are smaller than in mStrawberry. For example, at 0.1 MPa, the average bb-RMSD values in mStrawberry and mCherry are 1.15 and 0.65 Å, respectively. The X-ray B factor, which indicates the range of thermal motion, is larger for mStrawberry (19.42 Å²) than for mCherry (15.65 Å²). In sum, the MD simulations show that the range of thermal motions is smaller in mCherry, in agreement with the X-ray data. Consequently, the pressure-induced structural changes are less prominent in mCherry.

A torsional angle distribution analysis of mStrawberry shows that unlike the gas-phase chromophore, the average structure of the chromophore inside the protein is slightly non-planar — the average values from MD of the P and I angles are 4.8 and 6.6 degrees, respectively (see Figure S2 in SI). The definition of P and I angles are given in Table 1. We observe that up to 900 MPa both I and P distributions are similar. Above 900 MPa the P torsion angle shifts slightly toward zero degree (more planar structure). The I torsion angle undergoes similar change, but only at 1500 MPa. Flattening of molecules under high pressure has been noted before.¹⁴ Overall, the pressure-induced variations in the values of these angles are remarkably small. Thus, we conclude that these changes are unlikely to have much effect on the optical properties.

In sum, pressure-induced structural changes are larger for the surface of the β -barrel, whereas the effect on the chromophore is small. However, as demonstrated below, the increased pressure considerably changes the H-bonding network and the amount of water inside the barrel as well as relative populations of different conformers (mStrawberry), which ultimately affects optical properties of the protein.

To quantify the changes in the H-bonding network, we compute average populations of H-bonds between the chromophores and the nearby residues and water molecules. Population of 100% means that there is one hydrogen bond formed, on average. Table 3 and Table 4 show the average number of H-bonds formed by the chromophore derived from the MD trajectories. We define an H-bond when D-H...A distance is less than 2.8 Å and the D-H...A angle is less than 30 degrees. Obviously, the number of H-bonds depends on the cutoff, as illustrated in Table S6 in SI, however, the overall trend remains the same. As discussed in the previous section, the average lengths of the H-bonds are similar in MD and BigQM-AIMD. In the MD simulations, the bond lengths have been averaged over 50 ns trajectory for both Conf I and Conf II.

For mStrawberry the average total number of H-bonds varies quite notably with pressure. There is a slight increase in the total number (from 2.4 to 2.7) up to 600 MPa, probably because there is less thermal motion, so H-bonding network is less perturbed by large-amplitude motions, and then it decreases at 900 MPa.

To better understand the pressure-induced changes in the H-bonding network, we consider average populations of specific H-bonds in mStrawberry and in mCherry (Table 3 and Table 4, Figure 6). In the MD simulations of mStrawberry at 0.1 MPa, the phenolate oxygen (PhO) has, on average, slightly more than one H-bond (1.15) with either water (0.74) or Ser146 (0.41). mCherry's PhO has a similar number of H-bonds (1.11) with the major contribution from Ser146 (0.72) against 0.56 from the nearby water molecule. A larger difference between mStrawberry and mCherry is observed for the imidazolinone's oxygen (ImO). It forms 0.57 bonds with either Arg95 (0.52) or water (0.04) for mStrawberry and forms more than one bond (1.10) in the mCherry protein with either Arg95 (0.59) or water (0.52). ImN forms a transient H-bond with Glu215 (0.02 and 0.06) and the acylimine's oxygen is H-bonded to water (0.48 and 0.37 bonds) for mStrawberry and mCherry, respectively. The number of these H-bonds formed by PhO and ImO increase up to 600 MPa for mStrawberry, whereas it is mainly constant for mCherry (see Figure 6). For example, at 300 MPa, the number of H-bonds formed by PhO and ImO are 1.32 and 0.47. The respective number of H-bonds in mCherry is larger, i.e., 1.44 and 0.73. At 1200 MPa we observe a clear decrease in the number of bonds for mStrawberry: the populations of PhO and ImO H-bonds drops down to 1.22 and 0.27. At 1200 MPa, one new H-bond appears between ImO and Lys70 (0.04). Such effects are not observed for mCherry. In mStrawberry, pressure increases the number of H-bonds formed by ImN and AcO slightly, i.e., by 0.07 and 0.03, respectively. In contrast, at higher pressure the number of H-bonds formed by ImN and AcO in mCherry, shows a different trend, at higher pressure, the former increases by 0.25 and the latter — decreases by 0.32.

As discussed in Section, the H-bond pattern in mStrawberry reflects the presence of two conformations. In Conf I, which is the dominant one, the hydroxyl from Ser146 is oriented towards the phenolate of the chromophore forming an H-bond, whereas Conf II does not have such a bond; instead, the phenolic oxygen is forming two H-bonds with water molecules, as illustrated in Figure 2. Table 3 shows the H-bond population for both conformations (see Section for details). We observe that at all pressures, the number of H-bonds formed between PhO and water is, on average, higher by 0.16 in Conf II than in Conf I, as shown in Figure 2. The number of the H-bonds formed by ImO is rather constant (0.43)

in Conf I up to 1200 MPa, whereas in Conf II it increases from 0.52 at ambient pressure to 0.68 at 600 MPa. This is directly related to a smaller number of H-bonds between ImO and water in Conf II. The number of bonds formed by ImN is similar for both conformations at ambient pressure, but increases up to 0.22 for Conf II at higher pressures.

In sum, the main trend for mStrawberry is that the number of H-bonds formed by PhO and ImO increases at intermediate pressures and decreases at high pressures, with the respective values being 1.2/5.8 at 0.1 MPa, 1.6/0.8 at 600 MPa, and 1.0/0.2 at 1500 MPa. The relative population of Conf II also decreases with pressure. Overall, it appears that the pressure increase up to 900 MPa increases structural rigidity of mStrawberry (less backbone fluctuations, decreased population of Conf II, higher populations of H-bonds), whereas at 900 MPa its structure is collapsed such that the local environment of the chromophore is considerably perturbed, which is reflected by the significant changes in the H-bonding pattern.

A completely different behavior is observed for mCherry in which the number of H-bonds formed by PhO and ImO is larger than one for almost all pressures and is relatively constant. Moreover, there is no evidence of the multiple conformations in mCherry. The existence of two conformations in mStrawberry and changes in their relative populations with pressure may play a crucial role in understanding its optical properties and explaining the differences between mCherry and mStrawberry.

The effects of H-bonding on spectral properties of the GFP chromophore has been discussed in the literature.^{52, 53, 4, 54} A recent computational study of microhydrated model GFP chromophores has shown that H-bonding with water molecules interacting with phenolate and/or imidazolinone oxygens can lead to 0.1–0.3 eV spectral shifts.⁵⁴ As will be discussed below, in addition to pressure-induced changes in the H-bonding pattern, the distances between the chromophore and other nearby charged residues (such as Lys70) are strongly affected by pressure, which has an important effect on the optical properties.

The number of water molecules in the vicinity of the chromophore shows a very interesting trend (Figure 7) for mStrawberry. As pressure increases up to 900 MPa, the average number (as well as maximum number) of water molecules around the mStrawberry chromophore decreases (average from 12.5 to 10, and maximum — from 21 to 15). This is because the surface groups fluctuate less, thereby decreasing the permeability of the β -barrel. However, above 900 MPa the number of water molecules in the chromophore pocket increases — the pressure becomes sufficiently high to squeeze water inside (this is a known effect of pressure^{55, 56, 57}). The increased number of water molecules around the protein can affect H-bonding network and induce solvatochromic shifts and can also affect quantum yield. For example, the GFP chromophore exhibits significant solvatochromic shifts, i.e., the absorption of the anionic form of GFP is 2.60 eV, whereas the absorption of the anionic HBDI in solutions at high pH is blue-shifted by 0.2–0.3 eV.^{58, 59} Moreover, fluorescence of solvated FP chromophores is suppressed by fast radiationless relaxation.⁴

The number of water molecules in the vicinity of the mCherry chromophore is, on average, smaller than that for mStrawberry (Figure 7), and, as expected, the distribution is more narrow. For example, at ambient pressure the average number of water molecules around the chromophore is 12.5 for mStrawberry and 11 for mCherry; the maximum number of water molecules within 5 Å of the chromophore are 19 and 10, respectively. Consequently, changes in the number of water molecules upon the increase of pressure are less pronounced for mCherry; the number of waters remains relatively constant (around 11 ± 1). The initial decrease in the number of water molecules around the mCherry chromophore is very small (from 11 to 10), and it increases back to 11 at 500 MPa. This might be the key to the

observed trend in quantum yield — initial decrease in water molecules around the mStrawberry chromophore results in an increased quantum yield, whereas in mCherry, there is no considerable change at intermediate pressures and, therefore, there is no increase in the quantum yield. In addition, large changes in quantum yield for mStrawberry might be due to changes in relative populations of the two conformations, e.g., if Conf II has smaller quantum yield, then reducing its weight with pressure will result in the overall increase of the fluorescence yield.

Spectroscopy (mStrawberry)

The isolated mStrawberry model chromophore (see Figure 1) has a planar structure. The computed (SOS-CIS(D)/cc-pVDZ+) excitation energy equals 2.14 eV, and the oscillator strength, f_L , is 1.49. The MOs describing this $\pi \rightarrow \pi^*$ transition are shown in SI (Figure S5).

At atmospheric pressure, the experimental absorption maximum for mStrawberry is 2.16 eV (at pH<9.5). Table 6 presents the computed QM/MM excitations energies for the two conformations averaged over several snapshots, as described in Section (all calculations were performed using snapshots from the BigQM-AIMD trajectories). The overall absorption maximum was computed by taking a weighted average of the average excitation energies for the two conformations with the weights estimated as described in Section. The convergence of excitation energies as function of the number of snapshots at 0.1 and 900 MPa is presented in SI (Figure S6). Figure 8 shows the distribution of excited energies at 0.1 and 900 MPa depending of the conformation.

At low pressure, the two conformers have noticeably different excitation energies, i.e., the absorption of Conf II is red-shifted by ~0.1 eV relative to Conf I, whereas at high pressure the spectral difference is less pronounced and Conf II has higher excitation energy.

At atmospheric pressure, the computed excitation energy for Conf I, which corresponds to the X-ray structure, is 2.14 eV; E_{ex} for Conf II is considerably lower (2.02 eV). Thus, averaging over the two conformations, whose estimated populations at 0.1 MPa are 59 % and 41 %, we obtain 2.09 eV. This value is within 0.07 eV from the experimental absorption maximum, which is well within anticipated error bars of the calculations. Note that reducing the population of Conf II (which is red-shifted relative to Conf I) will lead to the apparent blue shift in absorption maximum.

Interestingly, the pressure effect on the excitation energies of the two conformers is different. Conf I yields consistent red-shift (−0.02 eV), whereas excitation energy of Conf II is blue-shifted by 0.13 eV. As described in the companion paper,²⁸ a small blue shift (0.005–0.01 eV) is observed with moderate pressure increase for all FPs from the mFruit family. At higher pressures, the spectra become broader, and some FPs exhibit larger shifts, i.e., mStrawberry exhibits large blue shift (+0.10 eV), whereas mOrange — a red one (−0.08 eV).

The mStrawberry calculations suggest that the overall pressure effect on the absorption/emission wavelength is determined by the two trends: (i) changes in excitation energies for each conformation (Conf I exhibits red-shift, whereas Conf II exhibits a blue shift); and (ii) changes in the relative population of Conf I/Conf II (Conf I becomes more dominant).

Our calculations suggest that small blue shifts at moderate pressures originate from tightening the H-bonding network and reducing the distance between Lys70 and the chromophore, as observed for Conf II-like structures. As explained below, the distance between the chromophore and Lys70 appears to be very important for the magnitude of the

shift. The blue shift can further increase if the population of Conf II decreases. The differences between FPs at higher pressures may be due to different ratio of two or more conformations. Therefore, an accurate estimate of the shifts requires estimate of relative populations of the two conformations. This is a very challenging task as it requires extensive sampling and free-energy calculations. Our crude estimates based on the H-bond populations suggest that the population of Conf II reduces from 41 % to 4 % leading to a blue shift of +0.04/+0.03 eV, in a reasonable agreement with the experimental value (+0.10 eV).

We observe a moderate effect of pressure on oscillator strengths, i.e., about 4% increase at 300 MPa and a 6% decrease at 900 MPa for Conf I (we do not take into account possible changes in the Franck-Condon factors). Thus, pressure-induced change in absorption cross section is moderate, and the observed increase of fluorescence quantum yield must be dominated by increased quantum yield due to the restricted range of motion and/or changes in relative populations of different conformations. The comparison between mCherry and mStrawberry suggests that Conf II may have much lower quantum yield; thus, reducing its population with pressure may result in an increased fluorescence yield.

To further analyze the computed shift and the effect of H-bonding on excitation energies, we analyze the changes in NBO atomic charges of the chromophore upon excitation (see Table 7). The table also includes the difference between the charges in the first singlet excited state and the ground state (Δ). When Δ is positive, the ground state has more negative charge than the excited state, and, consequently, the ground state is more stabilized by the H-bonds resulting in a blue shift in the absorption spectrum.

As one can see, the $S_0 \rightarrow S_1$ excitation leads to large changes in the charge on the bridge. The changes on phenolate and imidazolinone oxygens are smaller — both loose about 0.03e upon excitation. Thus, we expect that H-bonds formed with these two oxygens will result in a blue shift (larger stabilization of S_0). Likewise, imidazolinone's nitrogen gains 0.12e upon excitation. Therefore, we expect that H-bonding with this group will result in a red shift. Note that changing the distance between the bridge and the nearby charged residues is expected to produce significant effects on the excitation energy.

To verify this reasoning and to better understand pressure-induced spectral shifts, we performed the QM/MM excitation energy computations using one representative snapshot extracted from BigQM-AIMD at low (0.1 MPa) and high (900 MPa) pressures. In these calculations, the MM part included only the CHARMM27 charges of the selected residues (Ser146, Arg95, Glu215, and Lys70) around the chromophore. These residues were chosen because they form H-bonds with the chromophore (Ser146, Arg95, and Glu215) or because they are expected to affect the excitation energies due to their charges (Lys70). The results for both conformations are summarized in Table 8. At atmospheric pressure the shifts induced by specific interactions are similar for Conf I and Conf II, e.g., relative to isolated chromophore (chr), they are chr+Glu215 (−0.04 eV and −0.02 eV), chr+Arg95 (+0.32 eV and +0.31 eV), and chr+Ser146 (0.00 eV and 0.01 eV). These shifts are consistent with the analysis of partial charges described above — Glu215 is H-bonded to ImN and Arg95 (Ser146) forms H-bond with ImO (PhO, Conf I). At 900 MPa the same trend is observed concerning the influence of these residues on E_{ex} , we see no major differences between the two conformations. However, the effect of Lys70 is very different depending on the conformation and on the pressure: at atmospheric pressure the shift in chr+Lys70 is +0.31 eV and +0.14 eV for Conf I and II, respectively, whereas at higher pressure (900 MPa), it is +0.23 eV for Conf I and +0.30 eV for Conf II. This is due to electrostatic interactions between the NH_3^+ group of Lys70 and the carbonyl group of the imidazolinone ($\text{C}-\text{O}_{\text{Im}}^-$) that

hosts significant negative charge due to the resonance stabilization of the anionic chromophore.

As Lys70 is responsible for the difference in pressure-induced spectral changes of the two conformers, the weighted excitation energies of the chr+Lys70 for both conformations should also lead to the experimentally observed blue shift. In fact, the chr+Lys70 weighted values at atmospheric pressure and at 900 MPa are 2.28 eV and 2.30 eV, respectively. This blue shift is similar to that obtained for the calculations of the entire protein, e.g. +0.03 eV (Table 6).

We note that Lys70 is a highly conserved residue in RFPs, and its crucial role for the red chromophore maturation has been recently predicted by theory and confirmed by directed mutagenesis.⁶⁰

To better understand the role that Lys70 and other residues around the chromophore have on the spectroscopic properties, the electrostatic potential (ESP) has been computed at each nuclei of the mStrawberry chromophore (only heavy atoms participating in the π -system are considered, for the numbering scheme see Figure 1). Figure 9 compares the ESP of the entire system (protein and solvent/counterions environment). The pressure increase induces significant changes of ESP at each chromophore nuclei for both conformations, which is expected as the system is compressed and also becomes less flexible. The ESP changes due to conformations are larger at atmospheric pressure. We observe a similar effect on excitation energies (Figure 8). The larger differences between Conf I and Conf II are at atmospheric pressure for the oxygen of the imidazolinone (atom number 9), and at high pressure — for oxygen of the phenolate (atom number 18). We also note that for all heavy atoms of acylimine (atom numbers 1–4) the trends for Conf I and Conf II are opposite at both pressures.

A more detailed analysis can be performed by computing the ESP of each individual residue around the chromophore, i.e., Glu215, Arg95, Lys70, Ser146 and water molecule within 2.8 Å of the chromophore (Figure 10). As for the residue-by-residue spectroscopic analysis, only one representative snapshot for each conformation is chosen at 0.1 MPa and at 900 MPa. This analysis can be related to Figure 9 to understand the origin of the previous finding and to Table 3 in which the H-bond population formed by the chromophore and the nearby residues are given. Comparing the two figures, the major difference is the ESP due to Lys70 for both conformations and to the water molecules (only for Conf II), while the ESP behavior of other residues does not depend on the conformation.

At atmospheric pressure we observe that the large change is mainly due to Lys70 which is responsible for the large difference at the oxygen of the imidazolinone (atom number 9) in Figure 9. This positively charged residue is located, on average, 3.72 Å away of atom number 9 in Conf I, whereas in Conf II the distance is 1.48 Å longer (Table 9). Therefore, ESP of Lys70 is larger for Conf I than for Conf II at atmospheric pressure. At 900 MPa, the opposite behavior is observed: the ESP due to Lys70 is higher for Conf II than for Conf I, this is directly related to the average distances between the chromophore and Lys70 in Conf I and Conf II, 4.57 Å and 3.21 Å, respectively. A similar trend is observed for the central bridge carbon (atom number 11), as illustrated by the data in Table 9.

This analysis explains the origin of the blue shift in mStrawberry. The excitation energies of both conformations are driven by Lys70. The short distance between Lys70 and ImO (as well as the bridge) in Conf I at atmospheric pressure and in Conf II at 900 MPa strongly stabilizes the ground state of the chromophore inducing a blue shift, as compared to Conf I at 900 MPa and Conf II at 0.1 MPa, respectively.

As far as other neighboring residues are concerned, Glu215 increases the ESP for the atom number 6 (ImN), which is expected. The ESP of Glu215 and Ser146 does not change significantly with the conformation and with pressure. The ESP of Arg95 exhibits the same trend for both conformations at all pressures. This last observation is consistent with the residue-by-residue analysis of the shifts in excitation energies described above.

The average value of the bridge-Lys70 distance in mCherry decreases strongly (from 6.5 to 4.5 Å) with pressure increase from 0.1 MPa to 600 MPa suggesting an overall spectral blue shift.

Conclusions

Our results for mStrawberry and mCherry show that pressure has a significant effect on the number of water molecules and H-bonding network around the chromophore, as well as the distance between the chromophore and Lys70, whereas the structure of the chromophore itself is not strongly affected. The key difference between the two FPs is their structural rigidity, with mStrawberry being more floppy. In addition to larger structural fluctuations, mStrawberry also features two different conformers with almost equal populations at ambient pressure. The conformers, which differ by the flip of Ser146 residue, have different optical properties (Conf II is red-shifted relative to Conf I) and exhibit different spectral shifts with pressure. At higher pressures, Conf I, which is similar to the X-ray structure, becomes more dominant. Thus, the overall pressure effect on the absorption/emission wavelength in mStrawberry is determined by the two trends: (i) changes in excitation energies for each conformation (Conf I exhibits a small red-shift, whereas Conf II exhibits a blue shift); and (ii) changes in the relative population of Conf I/Conf II (Conf I becomes more dominant). The computed spectral shift in absorption (+0.04 eV) agrees well with the experimentally observed shift in fluorescence (+0.09 eV).

The origin of the shift is explained by analyzing partial charges of the chromophore in the ground and excited states and by computing ESP of the protein environment. These calculations reveal the crucial role of a highly conserved Lys70. Increased pressure leads to significant changes in its position, the effect being different for different conformations. This residue strongly stabilizes the anionic form of the chromophore that has significant negative charge on the imidazolinone's oxygen due to resonance stabilization. The pressure changes the distance between the chromophore and Lys70, which leads to the blue shift in both conformations. We note that Lys70 is a highly conserved residue in RFPs and is important for the red chromophore maturation.⁶⁰

The mCherry is less flexible and exhibits less thermal fluctuations at atmospheric pressure. Moreover, it does not have multiple conformers. Consequently, the effect of pressure on its structure is smaller. We note that in mCherry, the distance between Lys70 and the chromophore decreases with pressure, consistently with the experimentally observed blue shift.

Our results suggest that pressure increase causes initial increase of fluorescence yield only for relatively floppy FPs, whereas the FPs that have more rigid structures have quantum yield close to their maximum. The strong increase in quantum yield in some FPs may be indicative of multiple conformers present at ambient pressure. The calculations also suggest that the origin of the low quantum yield in some FPs is dynamic in nature and depends on the range of thermal motions of the chromophore and fluctuations in the H-bonding network rather than on its average structure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is conducted under the auspices of the *iOpenShell* Center for Computational Studies of Electronic Structure and Spectroscopy of Open-Shell and Electronically Excited Species. AIK is supported by the National Science Foundation through the CHE-0951634 grant. A.I.K. is deeply indebted to the Dornsife College of Letters, Arts, and Sciences and the WISE program (USC) for bridge funding support. AIK also acknowledges support from the JILA visiting fellowship and the Bessel Research Award from the Humboldt foundation. PC is supported by the GM096903 grant from the National Institute of General Medical Sciences of National Institute of Health. VAM and AVN thank the Research Computational Center of the M.V. Lomonosov Moscow State University and acknowledge a partial support from the Russian Foundation for Basic Research (project 10-03-00085). We are thankful to Prof. Mathias Weber for stimulating discussions and sharing his results prior to publication.

References

1. Tsien RY. Annu Rev Biochem. 1998; 67:509. [PubMed: 9759496]
2. Zimmer M. Chem Rev. 2002; 102:759. [PubMed: 11890756]
3. Wang Y, Shyy JY-J, Chien S. Ann Rev Biomed Eng. 2008; 10:1. [PubMed: 18647110]
4. Meech SR. Chem Soc Rev. 2009; 38:2922. [PubMed: 19771336]
5. Sample V, Newman RH, Zhang J. Chem Soc Rev. 2009; 38:2852. [PubMed: 19771332]
6. Bravaya K, Grigorenko BL, Nemukhin AV, Krylov AI. Acc Chem Res. 2012; 45:265. [PubMed: 21882809]
7. Subach FV, Verkhusha VV. Chem Rev. 2012 in press. 10.1021/cr2001965
8. Lin MZ, McKeown MR, Ng H-L, Aguilera TA, Shaner NC, Campbell RE, Adams SR, Gross LA, Ma W, Alder T, Tsien RY. Chem Biol. 2009; 16:1169. [PubMed: 19942140]
9. Gross LA, Baird GS, Hoffman RC, Baldrige KK, Tsien RY. Proc Nat Acad Sci. 2000; 97:11990. [PubMed: 11050230]
10. Shu X, Shaner NC, Yarbrough CA, Tsien RY, Remington SJ. Biochem. 2006; 45:9639. [PubMed: 16893165]
11. Chapagain PP, Regmi C, Castillo W. J Chem Phys. 2011; 135:235101. [PubMed: 22191901]
12. Haidinger W, Szostak MP, Beisker W, Lubitz W. Cytometry. 2001; 44:106. [PubMed: 11378860]
13. Maksimow M, Hakkila K, Karp M, Virta M. Cytometry. 2002; 47:243. [PubMed: 11933014]
14. Boldyreva EV. Acta Cryst Sec A. 2007; 64:218.
15. Boldyreva EV, Sowa H, Seryotkin YuV, Drebuschak TN, Ahsbahs H, Cherny-shev VV, Dmitriev VP. Chem Phys Lett. 2006; 429:474.
16. Boldyreva, EV. Berlin: Springer; 2007. p. 169
17. Hamann SD. Annu Rev Phys Chem. 1964; 15:349.
18. Scheyhing CH, Meersman F, Ehrmann MA, Heremans K, Vogel RF. Biopolymers. 2002; 65:244. [PubMed: 12382285]
19. Ehrmann MA, Scheyhing CH, Vogel RF. Lett Appl Microbiol. 2001; 32:230. [PubMed: 11298931]
20. Leiderman P, Huppert D, Remington SJ, Tolbert LM, Solntsev KM. Chem Phys Lett. 2008; 455:303.
21. Verkhusha VV, Pozhitkov AE, Smirnov SA, Borst JW, van Hoek A, Klyachko NL, Levashov AV, Visser A. Biochim Biophys Acta - Gen Subj. 2003; 1622:192.
22. Malavasi NV, Foguel D, Bonafe CFS, Braga C, Chura-Chambi RM, Vieira JM, Morganti L. Process Biochem. 2011; 46:512.
23. Muring K, Krasnenko V, Miller S. J Luminesc. 2007; 122:291.
24. Muring K, Deich J, Rosell FI, McAnaney TB, Moerner WE, Boxer SG. J Phys Chem B. 2005; 109:12976. [PubMed: 16852610]
25. Herberhold H, Marchal S, Lange R, Scheyhing CH, Vogel RF, Winter R. J Mol Biol. 2003; 330:1153. [PubMed: 12860135]

26. Barstow B, Ando N, Kim CU, Gruner SM. *Proc Nat Acad Sci*. 2008; 105:13362. [PubMed: 18768811]
27. Barstow B, Ando N, Kim CU, Gruner SM. *Biophys J*. 2008; 97:1719. [PubMed: 19751677]
28. Pozzi EA, Schwall LR, Jimenez R, Weber JM. *J Phys Chem B*. 2012 submitted.
29. Zhang L, Hermans J. *Proteins*. 1996; 24:433. [PubMed: 9162944]
30. PROPKA. <http://propka.ki.ku.dk>
31. Foloppe N, MacKerell AD Jr. *J Comput Chem*. 2000; 21
32. Reuter N, Lin H, Thiel W. *J Phys Chem B*. 2002; 106:6310.
33. Dmitrienko DV, Vrzheschch EP, Drutsa VL, Vrzheschch PV. *Biochemistry (Moscow)*. 2006; 71:1133. [PubMed: 17125463]
34. Olsen S, Smith SC. *Chem Phys Lett*. 2006; 426:159.
35. Phillips JC, Braun R, Wang W, Gumbart J, Tajkhorshid E, Villa E, Chipot C, Skeel RD, Kale L, Schulten K. *J Comput Chem*. 2005; 26:1781. [PubMed: 16222654]
36. Marx, D.; Hutter, J. Cambridge University Press; New-York, USA: 2009.
37. Because the AIMD QM/MM code does not have constant pressure option, the pressure in these calculations was maintained by keeping V and T constant.
38. Cp2k. <http://cp2k.berlios.de>
39. A smaller time step (0.5 fs) has been also used. Both 0.5 fs and 1 fs time steps trajectories were very similar to each other.
40. Grimme S. *J Comput Chem*. 2004; 25:1463. [PubMed: 15224390]
41. Goedecker S, Teter M, Hutter J. *Phys Rev B*. 1996; 54:1703.
42. Laino T, Mohamed F, Laio A, Parrinello M. *J Chem Theory Comput*. 2005; 1:1176.
43. Chai J-D, Head-Gordon M. *Phys Chem Chem Phys*. 2008; 10:6615. [PubMed: 18989472]
44. Rhee YM, Head-Gordon M. *J Phys Chem A*. 2007; 111:5314. [PubMed: 17521172]
45. Dunning TH. *J Chem Phys*. 1989; 90:1007.
46. Hehre WJ, Ditchfield R, Pople JA. *J Chem Phys*. 1972; 56:2257.
47. Shao Y, Fusti-Molnar L, Jung Y, Kussmann J, Ochsenfeld C, Brown S, Gilbert ATB, Slipchenko LV, Levchenko SV, O'Neill DP, Distasio RA Jr, Lochan RC, Wang T, Beran GJO, Besley NA, Herbert JM, Lin CY, Van Voorhis T, Chien SH, Sodt A, Steele RP, Rassolov VA, Maslen P, Korambath PP, Adamson RD, Austin B, Baker J, Bird EFC, Daschel H, Doerksen RJ, Dreuw A, Dunietz BD, Dutoi AD, Furlani TR, Gwaltney SR, Heyden A, Hirata S, Hsu C-P, Kedziora GS, Khalliulin RZ, Klunziger P, Lee AM, Liang WZ, Lotan I, Nair N, Peters B, Proynov EI, Pieniazek PA, Rhee YM, Ritchie J, Rosta E, Sherrill CD, Simmonett AC, Subotnik JE, Woodcock HL III, Zhang W, Bell AT, Chakraborty AK, Chipman DM, Keil FJ, Warshel A, Hehre WJ, Schaefer HF III, Kong J, Krylov AI, Gill PMW, Head-Gordon M. *Phys Chem Chem Phys*. 2006; 8:3172. [PubMed: 16902710]
48. Hsiao YW, Sanchez-Garcia E, Doerr M, Thiel W. *J Phys Chem B*. 2010; 114:15413. [PubMed: 20977248]
49. Epifanovsky E, Polyakov I, Grigorenko BL, Nemukhin AV, Krylov AI. *J Chem Theory Comput*. 2009; 5:1895.
50. Polyakov I, Epifanovsky E, Grigorenko BL, Krylov AI, Nemukhin AV. *J Chem Theory Comput*. 2009; 5:1907.
51. 'Core' and 'Surface' bb-RMSD include the backbone atoms of the α -helix and the ones of the β -barrel, respectively. 'Core' ('Surface') ha-RMAD are sidechain atoms from the residues located inside (outside) the β -barrel and within 7 Å of their backbone atoms.
52. Sniegowski J, Phail M, Wachter R. *Biochem Biophys Res Commun*. 2005; 332:657. [PubMed: 15894286]
53. Jung G, Wiehler J, Zumbusch A. *Biophys J*. 2005; 88:1932. [PubMed: 15613627]
54. Zuev D, Bravaya K, Makarova M, Krylov AI. *J Chem Phys*. 2011; 135:194304. [PubMed: 22112079]
55. Day R, García AE. *Proteins*. 2008; 70:1175. [PubMed: 17847086]

56. Collins MD, Hummer G, Quillin ML, Matthews BW, Gruner SM. Proc Nat Acad Sci. 2005; 102:16668. [PubMed: 16269539]
57. Meinhold L, Smith JC, Kitao A, Zewail AH. Proc Nat Acad Sci. 2007; 104:17261. [PubMed: 17956984]
58. Voityuk AA, Kummer AD, Michel-Beyerle ME, Rösch N. Chem Phys. 2001; 269:83.
59. Dong J, Solntsev KM, Tolbert LM. J Am Chem Soc. 2006; 128:12038. [PubMed: 16967932]
60. Bravaya KB, Subach O, Korovina N, Verkhusha VV, Krylov AI. J Am Chem Soc. 2012; 134:2807. [PubMed: 22239269]

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Figure 1. Structure of the mStrawberry chromophore. The panel shows the hydrogen-capped SmallQM (left) and BigQM (right) parts used in the AIMD calculations. The atom numbering scheme used in ESP analysis is given for SmallQM.

Figure 2.
The two conformations that differ by the flip of Ser146 observed in the MD simulations of mStrawberry: Conf I (left) and Conf II (right).

Figure 3. Comparison between backbone (green) and heavy-atom (blue) RMSD (Å) relative to the X-ray structure at different pressures for mStrawberry (squares) and mCherry (triangles). The bars on the plot represent the standard deviation values.

Figure 4.

Top: Backbone RMSD ($\text{\AA}$) relative to the X-ray structure at different pressures for the surface (left) and core (right) parts of mStrawberry. Bottom: Heavy-atom RMSD ($\text{\AA}$) relative to the X-ray structure at different pressures for the surface (left) and core (right) parts of mStrawberry. Small squares with bars show average values; boxes enclose 25%–75% range; and bars indicate 5–95% range.

Figure 5. Comparison between backbone (black) and heavy-atom (red) RMSD ($\text{\AA}$) relative to the X-ray structure at different pressures for mStrawberry. Core atoms are denoted by squares, surface atoms — by circles.

Figure 6.

Average populations of the H-bonds formed by PhO (black), ImO (red), ImN (green), and AcO (blue) for mStrawberry (top) and mCherry (bottom). The top and bottom left panels show results averaged over the equilibrium MD trajectories for mStrawberry (top) and mCherry (bottom). Top right panel shows average values computed for Conf I (squares) and Conf II (triangles) of mStrawberry.

Figure 7. Distribution of number of water molecules within 5 Å from the chromophore at different pressures. Left: mStrawberry; right: mCherry.

Figure 8. Histograms show the distribution of the excitation energies, one bin is equal to 0.02 eV. Left panel: $P=0.1$ MPa; right panel: $P=900$ MPa.

Figure 9.

The electrostatic potential (ESP) of the protein and solvent environment at the nuclei of the mStrawberry chromophore at 0.1 MPa and 900 MPa. The atom numbering scheme is given in Figure 1. The solid and dashed lines denote ESP for Conf I and Conf II, respectively.

Figure 10.

The contributions of the selected residues into the total ESP of the protein/solvent environment at the nuclei of the mStrawberry chromophore at 0.1 MPa (left) and 900 MPa (right). The atom numbering scheme is given in Figure 1. The solid and dashed lines denote ESP for Conf I and Conf II, respectively.

Table 1

Structural parameters for the mStrawberry chromophore at atmospheric pressure from the X-ray structure, MD (average values), AIMD (average values), AIMD (average values) and QM calculations. Bonds in angstrom, angles in degrees.

	Xray	MD	BigQM BLYP-D	AIMD SmallQM BLYP-D	BigQM BLYP	QM
Bonds						
(C ₁₇ -O ₁₈) _{Ph}	1.292	1.251	1.285	1.273	1.285	1.244
(C ₈ =O ₉) _{Im}	1.198	1.244	1.259	1.258	1.257	1.235
(C ₁₂ -C ₁₁) _{Ph}	1.392	1.421	1.406	1.408	1.407	1.393
(C ₁₁ =C ₁₀) _{Im}	1.373	1.398	1.402	1.404	1.406	1.398
(C ₄ =N ₃) _{acyl}	1.334	1.301	1.294	1.292	1.297	1.282
(C ₁ -N ₃) _{acyl}	1.243	1.153	1.387	1.399	1.389	1.384
(C ₁ =O ₂) _{acyl}	1.244	1.230	1.236	1.240	1.236	1.217
Angles						
α (C ₁₂ -C ₁ =C ₁₀) _{bridge}	131.49	135.9	133.1	133.8	133.8	131.6
Torsion						
P(C ₁₃ -C ₁₂ -C ₁₁ =C ₁₀)	-6.2	4.8	3.8	3.8	3.1	-0.46
I(C ₁₂ -C ₁₁ =C ₁₀ -N ₆)	9.3	6.6	6.7	3.6	6.7	-0.31
Acylimine(C ₄ =N ₃ -C ₁ =O ₂)	107.4	138.1	88.1	78.7	86.7	81.2

See Figure 1 for the definition of atomic labels.

Table 2

Average lengths of selected H-bonds (Å) between the mStrawberry chromophore and nearby residues at different pressures.

Acceptor	Donor	Xray	SmallQM-AIMD	BigQM-AIMD	MD	
0.1 MPa						
PhO	Ser146	2.63	3.72	2.74	3.13	
ImO	Arg95-1	3.57	3.80	3.79	3.41	
ImO	Arg95-2	2.79	3.23	2.76	2.86	
ImN	Glu215	2.75	3.20	2.84	3.42	
Acceptor	Donor	MD				
		300 MPa	600 MPa	900 MPa	1200 MPa	1500 MPa
PhO	Ser146	2.72	2.88	3.74	3.63	3.93
ImO	Arg95-1	3.32	3.26	3.35	3.15	3.10
ImO	Arg95-2	2.86	2.89	2.89	2.96	2.96
ImN	Glu215	3.06	3.16	2.94	2.95	3.15

Table 3

Populations (%) of different H-bonds between the mStrawberry chromophore and the surrounding protein groups and water molecules at different pressures (MPa) computed from the MD trajectories. The following definition of an H-bond is used: D-H...A distance is less than 2.8 Å and the D-H...A angle is less than 30 degrees. The top part is the total H-bond population derived from the entire trajectory, the center and bottom parts give the H-bond populations computed for snapshots corresponding to Conf I and Conf II, respectively (see text).

Acceptor	Donor	0.1 MPa	300 MPa	600 MPa	900 MPa	1200 MPa	1500 MPa
PhO ^a	W	74.1	65.3	73.9	73.4	69.4	85.8
PhO ^a	Ser146	40.5	75.7	84.2	78.2	52.9	11.1
ImO ^{a,b,c}	Arg95-1	0.6	6.5	3.3	1.2	24.2	4.1
ImO	Arg95-2	51.7	37.5	41.9	37.0	1.0	11.4
ImO	W	4.2	2.7	36.9	28.1	1.6	0.6
ImN ^a	Glu215	2.3	5.9	7.1	8.8	8.3	9.3
AcO ^{a,d}	W	48.0	55.9	20.6	32.4	65.2	51.1
Acceptor	Donor	0.1 MPa	300 MPa	600 MPa	900 MPa	1200 MPa	1500 MPa
PhO	W	64.24	66.73	72.37	69.90	68.33	77.73
PhO	Ser146	68.74	78.26	84.44	81.43	58.75	40.11
ImO	Arg95-1	1.49	3.69	10.07	3.26	19.37	16.54
ImO	Arg95-2	42.33	38.66	34.87	39.95	15.60	35.99
ImO	W	16.11	19.04	22.75	29.81	10.37	0.22
ImN	Glu215	1.21	3.68	4.19	9.13	10.84	16.29
AcO	W	45.46	33.24	27.81	37.49	65.02	54.50
Acceptor	Donor	0.1 MPa	300 MPa	600 MPa	900 MPa	1200 MPa	1500 MPa
PhO	W	87.80	82.04	86.50	82.18	87.71	91.65
PhO	Ser146	-	-	-	-	-	-

Acceptor	Donor	Conformation II				
		0.1 MPa	300 MPa	600 MPa	900 MPa	1200 MPa
ImO	Arg95-1	0.31	1.76	0.05	6.18	5.46
ImO	Arg95-2	51.55	59.15	67.59	48.43	51.22
ImO	W	8.30	3.87	0.57	1.68	3.28
ImN	Glu215	1.69	5.99	12.20	22.04	20.10
AcO	W	49.81	41.20	15.71	51.47	61.09

^aPhO, ImO, and AcO denote phenolate's, imidazolinone's, and acylimine's oxygens, respectively. ImN denotes imidazolinone's nitrogen.

^bArg95 can form two different H-bonds using two of its NH groups.

^cImO and Lys70 form an H-bond at 1200 MPa (4.3%).

^dAcO and Ser69 form an H-bond at 900 MPa (0.3%)

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Table 4

Populations (%) of different H-bonds between the mCherry chromophore and the surrounding protein groups and water molecules at different pressures computed from the MD snapshots. The following definition of an H-bond is used: D-H...A distance is less than 2.8 Å and the D-H...A angle is less than 30 degrees.

Acceptor	Donor	0.1 MPa	300 MPa	600 MPa	900 MPa	1200 MPa	1500 MPa
PhO	W	55.90	64.26	69.93	70.85	65.65	75.59
PhO	Ser146	71.80	80.10	70.03	74.13	85.56	69.79
ImO	Arg95-1	1.30	1.12	0.41	2.40	0.84	0.59
ImO	Arg95-2	57.60	46.19	62.49	55.57	49.21	72.87
ImO	W	51.90	25.38	47.30	44.65	24.45	25.47
ImN	Glu215	5.90	15.03	17.33	23.14	24.45	26.90
AcO	W	36.70	21.83	32.52	42.79	37.72	3.55

Table 5

Average lengths of selected H-bonds (Å) between the mCherry chromophore and nearby residues at different pressures.

Acceptor	Donor	Xray		MD		MD		
		0.1 MPa		300 MPa		600 MPa		900 MPa
PhO	Ser146	2.65	2.98	2.75	2.94	2.90	2.67	2.92
ImO	Arg95-1	3.58	3.45	3.36	3.35	3.34	3.34	3.28
ImO	Arg95-2	2.81	2.80	2.85	2.77	2.80	2.81	2.74
ImN	Glu215	2.65	3.00	2.91	2.91	2.88	2.87	2.86

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Table 6

Average QM/MM SOS-CIS(D)/cc-pVDZ+ excitation energies ($\langle E_{\text{ex}} \rangle$, in eV), their standard deviation (Δ), relative intensity ($I^{\text{rel.}}$) against 0.1 MPa and the number of snapshots (N) for Conf I and II at different pressures (MPa). E_{ex} of the model chromophore is 2.14 eV.

Conf	Pressure	$\langle E_{\text{ex}} \rangle$	Δ	$I^{\text{rel.}}$	N
I	0.1	2.14	0.02	-	28
	300	2.13	0.04	+4%	21
	900	2.12	0.02	-2%	25
II	0.1	2.02	0.04	-	15
	300	2.11	0.03	+1%	22
	900	2.15	0.06	0%	15
Weighted	0.1	2.09			
	300	2.13			
	900	2.13			

The experimental absorption maximum is 2.16 eV.¹⁰

Table 7

The NBO charges computed for the ground and the first singlet excited state of the isolated mStrawberry chromophore.

	PhO	Bridge	Im	Thr	Acyl	PhO(O)	Im(O)	Im(N)	Acyl(O)	Acyl(N)
S_0	-0.56	0.23	-0.58	0.06	-0.16	-0.79	-0.82	-0.53	-0.73	-0.66
S_1	-0.39	-0.06	-0.36	0.06	-0.26	-0.76	-0.78	-0.64	-0.73	-0.66
$\Delta(S_1 - S_0)$	0.17	-0.29	0.22	0.00	-0.10	0.03	0.03	-0.12	0.00	0.00

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Table 8

Average SOS-CIS(D)/cc-pVDZ+ excitation energies (eV) computed from one representative BigQM-AIMD/BLYP-D snapshot at high and low pressures. The variation (Δ) between pressures for both conformations are also shown (in eV). Only the charges of the selected residues were included (see text).

Excitation energies							
	0.1 MPa		900 MPa		Δ		
	Conf I	Conf II	Conf I	Conf II	Conf I	Conf II	
Chr	2.04	2.04	2.07	2.05	0.03	0.01	
Chr+Glu215	2.00	2.02	2.03	2.04	0.03	0.02	
Chr+Arg95	2.36	2.35	2.40	2.42	0.04	0.07	
Chr+Lys70	2.35	2.18	2.30	2.35	-0.05	0.17	
Chr+Ser146	2.04	2.05	2.07	2.05	0.03	0.01	
Chr+Arg95+Glu215	2.33	2.34	2.38	2.42	0.05	0.08	
Chr+Arg95+Glu+Lys70	2.61	2.51	2.57	2.70	-0.04	0.19	
Chr+Arg95+Glu215+Lys70+Ser216	2.61	2.51	2.57	2.70	-0.04	0.19	
Protein	2.14	2.02	2.11	2.15	-0.03	0.19	

Table 9

Relevant average distance ($\text{\AA}$) between the mStrawberry chromophore and nearby residues computed from the BigQM-AIMD trajectories.

Acceptor	Donor	0.1 MPa		900 MPa	
		Conf I	Conf II	Conf I	Conf II
Bridge	Lys70	4.05	6.72	4.60	4.09
	Lys70	3.71	5.19	4.57	3.21
ImO	Arg95-1	3.84	3.90	3.77	3.60
ImO	Arg95-2	2.83	2.80	2.76	2.76