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On the origin of fluorescence in bacteriophytochrome infrared fluorescent proteins

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Abstract

Tsien (*Science*, **2009**, 324, 804-807) has recently reported the creation of the first infrared fluorescent protein (IFP). It was engineered from bacterial phytochrome by removing the PHY and histidine kinase-related domains, by optimizing the protein to prevent dimerization and by limiting the biliverdin conformational freedom, especially around its D ring. We have used database analyses and molecular dynamics simulations with freely rotating chromophoric dihedrals in order to model the dihedral freedom available to the biliverdin D ring in the excited state; to show that the tetrapyrrole ligands in phytochromes are flexible and can adopt many conformations, however their conformational space is limited/defined by the chemospatial characteristics of the protein cavity. Our simulations confirm that the reduced accessibility to conformations geared to an excited state proton transfer may be responsible for the fluorescence in IFP, just as has been suggested by Kennis (*PNAS*, **2010**, 107, 9170-9175) for fluorescent bacteriophytochrome from *Rhodospseudomonas palustris*.

Introduction

Many bioluminescent¹ and fluorescent proteins^{2,3} are used as molecular imaging tools. One of the major shortcomings of these imaging agents is that they do not emit in the far red or infrared region of the spectrum and therefore, their use as *in vivo* tools is limited by the poor penetration of the excitation light.⁴ Since infrared wavelengths are minimally absorbed by hemoglobin, water and lipids,^{5,6} imaging agents that emit in the 650-900nm range would be ideally suited for *in vivo* imaging of deep tissue in live animals, so a search for red bioluminescent⁷⁻⁹ or fluorescent proteins¹⁰⁻¹² has been continuing unabated for the last 15 years. Tsien et al have recently reported the first infrared fluorescent protein. It was engineered from bacterial phytochrome,¹³ and has excitation and emission maxima of 684 and 708nm respectively (vs. 595 and 663nm for AQ14 the GFP-like protein with the highest peak emission wavelength¹⁴). Unfortunately, the quantum yield of 0.07 is low.

The infrared-fluorescent protein (IFP) is based on a bacteriophytochrome from *Deinococcus radiodurans*, which, with a single D207H mutation is red fluorescent.¹⁵ The bacteriophytochromes are part of the phytochrome superfamily that are found in plants, fungi and bacteria, where they are responsible for light-regulating responses such as seed germination, flowering and phototaxis.¹⁶⁻¹⁸ The phytochromes form homodimeric complexes with a linear tetrapyrrole, which is autocatalytically bound to a positionally conserved cysteine through the C3 vinyl side chain of pyrrole ring A (see Figure 1).

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Supporting Information Available Table 1S The pdb codes of the 47 protein crystal structures used Relibase+ search and clustered in Figure 4. Table 2S The Cavbase similarity scores relative to 2o9c. This information is available free of charge via the Internet at <http://pubs.acs.org>.

Phytochromes act as photointerconvertible light switches by alternating between the spectrally distinct red absorbing physiologically inactive Pr conformer and the active far red absorbing Pfr conformer. In the Pr state, the linear tetrapyrrole is cationic with all four pyrrole nitrogens protonated.^{19,20} Although the conversion from the Pr to the Pfr state is not well understood, it is thought to involve a Z to E isomerization of the C15-C16 bond between the C and D pyrrole rings;²¹⁻²³ deprotonation/reprotonation and three intermediate states (lumi-R; meta-R_a and meta R_c). Pfr can slowly, non-photochemically revert back to Pr or can rapidly be photoconverted back to Pr with far red light. Since fluorescence and signalling compete in phytochromes they have evolved to minimize their fluorescence quantum yield and to maximize their entry into the photocycle.²⁴

The bacteriophytochromes are excellent candidates for *in vivo* imaging agents, since they bind biliverdin IX_a, which is ubiquitous in all aerobic organisms as the initial intermediate in heme catabolism, is non-toxic and has negligible fluorescence on its own. *In vivo* the bacteriophytochromes use light to control gene expression.

The fluorescent truncated bacteriophytochromes used in IFPs were created by removing the PHY and histidine kinase-related domains, which convert the light energy to chemical energy used in signaling, by optimizing the protein to prevent dimerization, and by limiting the biliverdins conformational freedom, especially around its D ring (see Figures 1 and 2).¹³ The IFP's have been shown to be suitable for whole body mammalian imaging.¹³ In this work, we have analyzed the brightest of the IFP's, IFP1.4.

The following three paragraphs present different, but related explanations for IFP 1.4's fluorescence.

Free tetrapyrroles do not fluoresce. However, in the IFPs the tetrapyrrole is bound to the protein, its conformational freedom is restricted by the protein matrix, and the excited chromophore cannot undergo fluorescence quenching non adiabatic crossing because the bound tetrapyrrole is unable to adopt the conformation found at the conical intersection. This behavior is common in fluorogenic monomethine bridged dyes^{25,26} and chromophores.²⁷⁻³⁰

Recently, Kennis et al.²⁴ reported on their femtosecond time-resolved absorption spectroscopy studies of a bacteriophytochrome from *Rhodospseudomonas palustris* (RpBphP3), which is fluorescent. The fluorescence is associated with an excited-state lifetime of RpBphP3 that is significantly longer than that of other phytochromes. The extended lifetime is due to constraints placed on the rotation of the pyrrole D ring by its hydrogen bonding interactions with the surrounding protein matrix. The authors suggest that the longer excited-state lifetime of RpBphP3 is due to the restricted D ring rotation that is in turn responsible for a decreased excited-state proton transfer (ESPT). It is proposed that in all phytochromes twisting around the methine bridge between the C and D rings facilitates deprotonation of the A, B or C pyrroles, which leads to rapid internal conversion to the ground state. The fluorescence of RpBphP3 is explained by the fact that the dihedral freedom of the C-D methine bridge is limited, the C15-C16 bond is not twisted, therefore the light induced deprotonation of the A, B or C pyrroles is reduced, and fluorescence quenching intersystem crossing from the deprotonated excited state to ground state does not occur.²⁴

Many years ago we noticed that the chromophore cavity in GFP-like proteins is not complementary to a planar chromophore and that the protein matrix exerts a twisting force on the chromophore.³¹ Recently, Mathies et al.³² used femtosecond stimulated Raman spectroscopy to examine the structural evolution of the excited state in GFP. They found that the dominant motion is a phenolic wag of the chromophore that is directed to a transition

state perfectly aligned for ESPT by aforementioned twisting force. In GFP the protein matrix exerts a twisting force on its chromophore, upon excitation the chromophore responds to the twisting force adopting a new conformation that is a prerequisite for ESPT from the phenolic (state A) to the green fluorescent phenolate (state B) form of the chromophore. A similar prerequisite proton alignment might be required for fluorescence quenching ESPT in RpBphP3.²⁴

The first two mechanisms predict that IFP1.4 will have reduced dihedral conformational space in the excited state relative to the *Deinococcus* bacteriophytochrome wild type, however, according to the third mechanism an altered dihedral space would be more important. We have used molecular dynamics simulations with freely rotating chromophoric dihedrals to model the dihedral freedom around the C-D methine bridge of biliverdin in the excited state.

Experimental

The coordinates of the *Deinococcus* bacteriophytochrome crystal structure (2O9C¹⁵) were obtained from the Protein Data Bank (PDB)³³. The protein preparation workflow and Epik v2.0109³⁴ were used with hydrogen bond optimization to add hydrogen atoms to protein and solvent atoms as required. The starting structure for the IFP, for which no crystal structure has been determined, was calculated by graphically mutating the *Deinococcus* bacteriophytochrome structure (2O9C¹⁵) so that it has the same primary sequence as the reported IFP1.4¹³ and undertaking a conformational search. The OPLS_2005 force field of MacroModel v9.0016³⁵ was used. The force field was validated using the optimized ab initio (DFT/B3LYP/6-31g(d)) structures of the PΦB chromophore without propionate chains and the small model fragments reported by Kaminski et al.³⁶ The following mutations were performed D207H, A288V, I208T, V186M, M54V, L311K, T135I, L195M, H196Q, G119A, S307E, L314G, V318R. Conformational searches were conducted using the combined Monte Carlo torsional variation and low mode method.^{37,38} The flexible dihedral angles of all the side-chains of the mutated residues that are within 8.00 Å of the chromophore were randomly rotated by between 0 and 180° (residues 207, 288, 208, 186, 311) and all solvent molecules in that sphere were randomly rotated and translated by between 0 and 1.00 Å in each Monte Carlo (MC) step.³⁹ 15,000 MC steps were taken in each search. Structures within 50 kJ/mol of the lowest energy minimum were kept, and a usage directed method³⁸ was used to select structures for subsequent MC steps. Structures found in the conformational search were considered unique if the least squared superimposition of equivalent non-hydrogen atoms found one or more pairs separated by 0.25 Å or more. The lowest energy structure obtained in the search was further subjected to a 5,000 step large scale low mode conformational search.^{40,41}

The final structures obtained from the MC search (Monte Carlo torsional and molecular position variation and large scale low mode) or fully minimized pdb structures were used to initiate molecular dynamics (MD) simulations with freely rotating N_C-C₁₄-C₁₅-C₁₆ and C₁₃-C₁₄-C₁₅-C₁₆ dihedrals (for τ_{CD}), and C₁₄-C₁₅-C₁₆-N_D and C₁₄-C₁₅-C₁₆-C₁₇ dihedrals (for the ϕ_{CD})(V1 = V2 = V3 = 0.000).

MD simulations were carried out in the NPT ensemble at 300K and 1 bar with 1.5fs steps using Desmond⁴². All molecular dynamics calculations used the OPLS_2005 force field with freely rotating τ_{CD} and ϕ_{CD} dihedrals and SHAKE constrained hydrogens. Two thousand and eighty three structures were sampled in each 10ns MD simulation. Each structure was in an orthorhombic simulation box of 0.15M NaCl and SPC waters, with a 10 Å solvent buffer between the protein surface and the boundary. Minimizations and pre-equilibrium simulations were done using the default Desmond/multisim relaxation protocol.

The area of the convex hull* of the ϕ and τ graphs was calculated. The smallest convex set containing 80% of all ϕ and τ points was also determined.

The Cambridge Structure Database (CSD) v5.30 as released in November 2008, was searched with Conquest.⁴³

Xcluster⁴⁴ was used for the cluster analysis. Proximity matrices were obtained by determining the pairwise distances between structures, using the rms displacement after optimal rigid-body superposition between pairs of atoms of the 25 non-hydrogen tetrapyrrole atoms shown in Figure 3.^{45,46}

Cavbase, a method for describing and comparing protein binding pockets in terms of exposed physico-chemical properties, was used.^{47,48} It is a modular extension of the protein-ligand database Relibase+ v3.0.^{49,50}

Results and Discussion

Theoretically open-chain tetrapyrroles can adopt 64 different isomers. According to the crystal structures of *Deinococcus* and *Rhodospseudomonas* bacteriophytochrome the biliverdin is in the $ZZZ_{\text{syn,syn,anti}}$ conformation in the Pr form.^{15,51}

In order to get an idea of the dihedral space available to the **ground state** of free biliverdin all biliverdin analogs in the Cambridge Structural Database (CSD)⁵² were examined. There are 12 crystal structures⁵³⁻⁵⁹ of linear conjugated tetrapyrroles in the CSD with the substructure shown in Figure 3. In 11 structures the tetrapyrroles adopt the ZZZ_{sss} isomer shown in Figure 3, which has all amine hydrogens pointed in towards the center of the molecule, one is in the ZZE_{sss} conformation (DIVBIT01)⁵⁴. None of the structures are planar, the average absolute dihedral angle around the three monomethine bridges in all 12 structures is 7.2°, and all but one of the tetrapyrroles are helical. These 12 structures represent some of the conformations free ground state biliverdin can adopt.

To examine the effect of the protein matrix on biliverdin we used the protein database (pdb)³³ and Relibase+^{49,60} to analyze the conformations of all the tetrapyrroles with the substructure shown in Figure 3.

The Relibase+ search of the pdb found 47 protein crystal structures (Table S1) that contained 385 acyclic tetrapyrroles moieties, such as phycocyanobilin, biliverdin IX α , phycoerythrobilin and phycocourobilin (many proteins contained more than one tetrapyrrole). Due to the significantly better resolution of small molecule crystal structures (CSD) vs protein structures (pdb) it was possible to assign the conformations/configurations of all the free tetrapyrroles in the CSD. However, it is not a trivial and necessarily constructive process to assign the single/double bond character of the monomethine bridges in all the protein structures. Therefore, the conformational freedom of the tetrapyrroles embedded in protein matrixes was assessed using cluster analysis.

The aim of cluster analysis is to place objects into groups, also called clusters, in such a way that all objects within a cluster are very similar and that all the objects in different clusters are very dissimilar to each other. In this study we used the Xcluster program⁴⁴, an agglomerative, hierarchical, single-link method. The first step, in such a cluster analysis, is to find a similarity criterion and arrange all the molecules in a generic ordering according to

*The convex hull or convex envelope for a set of points X in a real vector space V is the minimal convex set containing X . The convex hull of the ϕ and τ graphs can be visualized as the area encompassed by an imaginary elastic band that is stretched to surround all the datapoints.

their similarities. In clustering level 1 all the structures are generically ordered and placed in separate clusters. In each of the following clustering levels the two molecules that are most similar to each other are joined to form a cluster. The separation ratio,⁴⁴ distance maps and mosaics were used to find the ideal clustering criteria and optimal clustering levels. All 25 non-hydrogen atoms in the tetrapyrrole backbone were superimposed and the rms displacement between their positions in the different clusters was used as a clustering criteria. The cluster separations obtained in this way were superior to those found with dihedral comparisons or any other atomic superimpositions. The best clustering separation occurred at clustering level 377, at which the 385 structures were grouped into 9 clusters. The average structures from each of the 9 most significant clusters are shown in Figure 4. The members of clusters 7 and 8 are all bacteriophytochromes, while those in cluster 1 are mainly phycocyanin and phycorythrins.

In order to establish whether the protein cavity around the tetrapyrrole moiety was responsible for the conformations adopted in the crystal structure a Cavbase⁴⁷ comparison of the 385 ligands found in the pdb search was conducted. Cavbase is a method for describing and comparing protein binding pockets in terms of exposed physico-chemical properties. The Cavbase similarity scores (relative to 2o9c, see Table S2) followed the same clustering trends as the ligand superimposition clustering discussed above. While the protein cavity dimensions and properties dictate the tetrapyrrole conformation, no relationship between the sequence homology of the phycoprotein and the conformational families adopted by the ligands that are shown in Figure 4 was found. Interestingly a Cavbase search of the whole pdb revealed that the cavity dimensions of a number of barrel structures such as porins, antifreeze proteins and the red GFP-like fluorescent proteins have a higher Cavbase similarity score to *Deinococcus* bacteriophytochrome (2o9c) than the members of clusters 1-5.

From the above mentioned results, we can assume that the tetrapyrrole ligands in phytochromes are flexible and can adopt many conformations, however their conformational space is limited/defined by the chemospacial characteristics of the protein cavity. The same should be true for the excited state of the ligand in IFP 1.4 and RpBphP3.

Dihedral space available to the excited state biliverdin IX α

Molecular mechanics and dynamics calculations have been used to examine the ground state conformations of tetrapyrroles in solution⁶¹ and in proteins.^{36,62} They are techniques that are based on classical physics and were designed to model structural and not electronic properties. Therefore, molecular mechanics and dynamics simulations of IFPs cannot examine the excited state of the chromophore. Quantum calculations are an excellent technique to determine the energy profiles of the ground and excited states of the chromophore. However, they are CPU intensive, and it would be cost prohibitive to do conformational searches on the excited state of the biliverdin within IFP 1.4. We have therefore, examined the conformational space available to biliverdin model systems, free biliverdin IX α , and biliverdin IX α within *Deinococcus* bacteriophytochrome and IFP1.4, using a freely rotating chromophore that is an approximation of the conformational space available to the chromophore in its excited state. We have previously used this method to examine the rotational freedom of the excited state chromophore in yellow, blue and green fluorescent proteins.⁶³

Hula-twists are volume conserving concerted two bond dihedral rotations, see Figure 1,^{26,30,64-67} to date no one has considered the possibility of a hula twist occurring in phytochromes. IFP1.4 has been designed to restrict the conformational freedom of the biliverdin,¹³ particularly its D-ring, therefore it may be a prime candidate for a space saving hula-twist.

In order to see whether the dihedral freedom of the tetrapyrrole backbone of biliverdin IX α depends on the steric bulk of its substituents, we conducted molecular dynamical simulations with freely rotating monomethine bridges, as described in the methods section. We only simulated freely rotating dihedral angles between the C and D rings because (1) IFP1.4 was designed to minimize the conformational freedom of the D-ring; (2) the conversion from the Pr to the Pfr state is thought to involve a Z to E isomerization of the C15-C16 bond between the C and D pyrrole rings;²¹⁻²³ and (3) rotations of the dihedral angles between rings A and B as well as between B and C will be volume demanding rotations due to leverage effects caused by the fact that the biliverdin is attached to Cys24 of the bacteriophytochrome.

Recent CASPT2 calculations have shown that dihedral deformations around C4-C5, C9-C10 and C10-C11 may also play an important role in the deactivation routes of all phytochromes.⁶⁸ We have not examined the free rotation of these dihedral angles.

Freely rotating biliverdin model compounds

As the steric bulk increases from the unsubstituted tetrapyrrole to the octasubstituted isopropyl tetrapyrrole (see Figure 3 for nomenclature used) the slope of the τ vs ϕ plot of the two freely rotating dihedrals approaches -1.00 ($r_{1-8}=\text{H}$ slope is -0.06; $r_{1-8}=\text{Me}$ slope is -0.10; $r_{1-8}=\text{Et}$ slope is -0.88; $r_{1-8}=\text{iPr}$ slope is -0.97). This means that the τ and ϕ dihedrals twist in a conrotatory fashion to avoid steric clashes. The steric bulk of the large substituents impose a strain on tetrapyrroles that can be relieved by a conrotatory twist of the τ and ϕ dihedrals - in the excited state this may predispose these substituted tetrapyrroles to a hula-twist.

Freely rotating biliverdin IX α

In order to confirm that the 10ns simulations with a freely rotating pair of dihedral angles was sufficient to sample the torsional space available to the freely rotating biliverdin a 50ns simulation was undertaken. Comparison of the ten τ vs ϕ plots for each of the 5ns stretches that comprise the 50ns simulation revealed that the τ_{min} , τ_{max} , ϕ_{min} , ϕ_{max} , dihedral range, area and intercept of the τ vs ϕ plot do not vary significantly between any of the 5ns stretches. Thereby, confirming that the 10ns simulations are sufficient to sample the dihedral space available to freely rotating biliverdins.

Freely rotating biliverdin IX α in *Deinococcus* bacteriophytochrome and IFP1.4

Although the photochemistry and sequence of IFP1.4 is known,¹³ its crystal structure has not been reported. In order to obtain a starting structure for our molecular dynamics simulations we graphically mutated the *Deinococcus* bacteriophytochrome structure (pdb code = 2O9C¹⁵) so that it has the same primary sequence as the reported IFP1.4¹³ sequence and undertook a combined Monte Carlo torsional variation and low mode^{37,38} conformational search. The lowest energy structure obtained in the search was further subjected to a large scale low mode conformational search.^{40,41} The IFP1.4 structures obtained in this way were used to initiate molecular dynamics (MD) simulations with freely rotating $\text{N}_\text{C}-\text{C}_{14}-\text{C}_{15}-\text{C}_{16}$ and $\text{C}_{13}-\text{C}_{14}-\text{C}_{15}-\text{C}_{16}$ dihedrals (for τ_{CD}), and $\text{C}_{14}-\text{C}_{15}-\text{C}_{16}-\text{N}_\text{D}$ and $\text{C}_{14}-\text{C}_{15}-\text{C}_{16}-\text{C}_{17}$ dihedrals (for the ϕ_{CD}). Since the photochemistry of IFP1.4 is similar to that of the wild-type bacteriophytochrome we assume that active site of IFP1.4 is intact and that there are no large scale structural differences between wild-type bacteriophytochrome and IFP1.4. The conformational search found no low energy conformations of IFP1.4 with large protein backbone deviations from the wild-type bacteriophytochrome structure. Therefore, we felt confident in using the lowest energy IFP1.4 found as the starting structure for the free-rotating molecular dynamics.

Table 1 lists all the metrics that describe the conformational space spanned by a freely rotating methine bridge between the C and D rings in all systems examined. The area containing 80% of the τ vs ϕ space is the most useful indicator of the dihedral space available to the excited biliverdin.

We expect that the conformational freedom of free biliverdin in solution will be larger than that of biliverdin in phytochrome since its conformation space will be restricted by its ligation to Cys24, and by the steric constraints imposed by the surrounding phytochrome protein matrix. It is therefore, not surprising that Table 1 and Figure 5 show that the free rotation of both the ϕ_{CD} and τ_{CD} dihedral angles of biliverdin IXa is restricted when the chromophore is in either wild-type bacteriophytochrome or IFP1.4.

IFP1.4 was designed to restrict the conformational space available to the D ring of biliverdin IXa in bacteriophytochrome. A comparison of the areas covered by the 80% convex hull, which is a good reflection of the dihedral freedom of the biliverdin IXa D ring in wild-type bacteriophytochrome and IFP1.4, reveals that the mutations made to wild-type bacteriophytochrome restrict a freely rotating D ring in all protonation states examined – that is all IFP1.4's have smaller 80% areas than the corresponding wild-type forms (2o9c).

In order to determine the effect different charge distributions around the D ring will have on the conformational space that is available to an excited biliverdin we have performed the freely rotating dihedral simulations with the oxygen on the D ring in the enol, enolate and keto forms.

Figure 6 and Table 1 show that the C and D pyrroles adopt different conformations in IFP1.4 vs wild-type *Deinococcus radiodurans* bacteriophytochrome. Not only do the mutations in IFP1.4 restrict the dihedral freedom around the methine bridge between the C and D rings, but they also change the dihedral space occupied by the excited biliverdin. The different protonation states were used to model the variation possible in charge distribution. Figure 6 confirms that the protonation state will influence the available conformational space, but that the overall trends stay the same. As we go from the enolate to the keto form the dihedral space available to both wild-type *Deinococcus* bacteriophytochrome and IFP1.4 moves from higher τ and ϕ values to lower values.

The further the freely rotating dihedral angles are from the crystal structure the less complementary the chromophore cavity is with the conformation adopted by the biliverdin in the crystal structure. The conformation adopted by the C and D pyrroles in the crystal structure of wild type *Deinococcus* bacteriophytochrome (yellow circle in Figure 5 and 6) is at the confluence of all the freely rotating simulations, but at the edge of all of them. Implying that both the wild-type and the IFP1.4 chromophore cavities are non-complementary with the ground state conformation and that in both cases excitation will result in a new conformation that is able to minimize the strain. The dihedral space available to a freely rotating IFP tends to be closer to planarity than that of the wild-type *Deinococcus* bacteriophytochrome (i.e. closer to the red circle in Figure 6).

The pyrrole C and D moieties of biliverdin IXa cannot adopt a planar conformation in any of our freely rotating simulations i.e. none of the dihedral angles of the conformations shown in Figure 5 approach the planar ring C and D fragment, which would have the dihedrals represented by the red circle (-180° , 0°). This is not surprising since a planar C and D fragment is not found in either the tetrapyrrole structures in the CSD and PDB (see Figure 4) nor in the crystal structure of wild type *Deinococcus radiodurans* bacteriophytochrome (yellow circle in Figure 5 and 6).

One of the mutations undertaken to create a fluorescent form of *Deinococcus* bacteriophytochrome is D207H, this clearly an important mutation that has significant consequences on the photophysics and conformations available to biliverdin IX α , as the amide oxygen of Asp207 (or Asp216 in the *Rhodospseudomonas palustris* numbering) hydrogen bonds to the A, B and C pyrroles in both the *Deinococcus* and *Rhodospseudomonas palustris* bacteriophytochrome crystal structures. In fact Kennis et al. propose that in RpBphP3 biliverdin fluorescence is quenched by the “excited state decay to the Pr ground state through proton transfer from the ring A, B or C pyrrole nitrogen to the main-chain carbonyl of Asp-216.”²⁴ In all the simulations of the wild type *Deinococcus* bacteriophytochrome (2O9C) the only hydrogen bonding interactions that the A, B or C pyrrole nitrogen undergo are those with the amide oxygen of Asp207 – the same ones observed in RpBphP3. In the enolate form of IFP1.4, which has the D207H mutation, the ring A, B or C pyrrole nitrogen's form hydrogen bonds to the amide oxygen of His207, while the C pyrrole ring also forms occasional hydrogen bonds with the imidazole side chain of His260. In the enol form of IFP1.4 both the carboxyl oxygen of His207 and the imidazole of His260 form hydrogen bonds with the A, B and C ring nitrogens. In the keto simulations the imidazole of His207 occasionally forms hydrogen bonds with the amide oxygen of ring A.

Mathies³² et al have shown that in GFP photoexcitation triggers a chromophore wagging motion that optimizes the geometry of the chromophore for ESPT. The wagging motion is attributed in part to the chromophore cavity being non-complementary with a planar ground state chromophore conformation.³¹ Our calculations show that like RpBphP3, *Deinococcus* bacteriophytochrome may exhibit the opposite behavior – in the non-fluorescent wild-type bacteriophytochrome the chromophore cavity is not complementary with its ground state geometry and excitation leads to rapid adoption of an excited state geometry that is optimized for ESPT, which efficiently leads the bacteriophytochrome into the photocycle. While the chromophore cavity of IFP1.4 has different physico/structural properties, which lead to a different dihedral space being accessed upon excitation, reducing fluorescence quenching ESPT.

In RpBphP3 D ring rotation is constrained by hydrogen bonds from His299, Lys183 and Ser297, which is responsible for the extended excited-state lifetime and decreased Lumi-R quantum yield observed in fluorescent RpBphP3 (vs. non-fluorescent RpBphP2).²⁴ In our simulations the conformational freedom of the D ring is most constrained for IFP1.4 if it is in the enol form. Examination of the structures used in the generation of Figure 6B revealed that this due to the fact that the D ring in this simulations forms the most stable hydrogen bonds. The hydrogen bond between the oxygen of the D ring and the imidazole of His290 is present through out the whole simulation. It is the only hydrogen bonding interaction that is maintained for any of the complete 10ns freely rotating simulations. The D ring oxygen also forms occasional hydrogen bonds with the imidazoles of His207 and His260. The enolate form of IFP1.4 also forms a strong hydrogen bonding interaction with the imidazole of His207 that is maintained for most of the simulation. The enol form of the wild type *Deinococcus* bacteriophytochrome forms hydrogen bonds between the D ring and the side chains of Asp207 and His290. However, these interactions are not as sustained or as strong as those found for the corresponding form of IFP1.4. The enolate form of wild type *Deinococcus* bacteriophytochrome does not form any hydrogen bonds with the D ring.

Conclusions

The conformational space available to the excited state chromophore in IFP1.4 can be evaluated using freely rotating molecular dynamics simulations. This technique is particularly useful in examining the photophysics of fluorescent proteins as the protein

environment is crucial to the emission and absorption spectra, the method is a supplement to quantum mechanical calculations, since QM can't always model absorption spectra of FPs due to the influence of the protein environment.⁶⁹ Phytochromes have evolved to minimize their fluorescence quantum yield and to maximize their entry into the photocycle. In order to maximize its fluorescence IFP1.4 was designed to have a restricted rotation around the C and D rings. Our simulations show that the excited state of IFP1.4 will have a slightly restricted dihedral space in comparison to the wild-type structure, and that perhaps more importantly it has a different area of dihedral space available to it – an area of dihedral space that limits access to fluorescence quenching ESPT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

In bacteriophytochrome the biliverdin IX α chromophore is covalently bound to Cys24 through the C3 vinyl side chain of pyrrole ring A. The τ_{CD} (N_C -C₁₄-C₁₅-C₁₆) and ϕ_{CD} (C₁₄-C₁₅-C₁₆-N_D) dihedral angles are shown above. In ϕ_{CD} one-bond-flips (ϕ -OBF) the dihedral rotation occurs around the ϕ_{CD} torsional angle, while in a negatively correlated hula-twist (-HT) the ϕ_{CD} and τ_{CD} dihedral angles concertedly rotate in opposite directions as shown. A plot of the ϕ_{CD} and τ_{CD} dihedrals for a perfectly correlated negative HT will have a slope of -1. A ϕ -OBF will result in a slope of 0 and a τ -OBF = ∞ . If the chromophore cavity is complementary with co-planar C and D rings then the ϕ_{CD} and τ_{CD} best fit line will pass through the origin and all the ϕ_{CD} and τ_{CD} angles will be centered around the origin. In our calculations the oxygen on the D-ring was modeled in the keto, enolate and enol forms.

Figure 2.
Active site of IFP1.4 showing the biliverdin IX α (ball and stick) and the residues mutated (vdWals spheres) in order to produce IFP1.4 from bacteriophytochrome.

Figure 3.

CSD small molecule search done with R₁-R₈=any substituent. PDB search done with R₁= C ; R₂= C₂ ; R₃= C ; R₄= C₂CO₂ ; R₅= C₂CO₂ ; R₆= C ; R₇= C ; R₈= C₂. In both searches any combination of double and single bonds leading to a conjugated tetrapyrrole was used. In the text R₁-R₈ = H referred to as the tetrapyrrole backbone, R₁- R₈ = Et referred to as octaethyl tetrapyrrole.

Figure 4. Average structures from each of the nine most significant clusters. First number is the cluster number, and the number of members in that clusters is in parenthesis. The biliverdin IX α in the *Deinococcus* bacteriophytochrome crystal structure (2o9c) that is the basis for IFP1.4 is in cluster 7.

Figure 5.

Plot of the τ_{CD} vs ϕ_{CD} dihedral angles (see Figure 1 for nomenclature) for the *Deinococcus* bacteriophytochrome crystal structure (pdb = 2O9C) and the 2083 structures obtained in each freely rotating molecular dynamics simulations of 2O9C and IFP1.4. Table 1 quantifies the dihedral space covered by the different simulations.

Figure 6.

Plot of the τ_{CD} vs ϕ_{CD} dihedral angles (see Figure 1 for nomenclature) for the *Deinococcus* wild-type and IFP1.4 molecular dynamics simulations with ring D modeled as **A** enolate, **B** enol and **C** keto. Red circle – planar biliverdin; yellow circle – biliverdin in 2O9C crystal structure; blue triangles – 2O9C simulations; brown squares – IFP1.4 simulations.

Table 1

Slopes and metrics describing the dihedral space spanned from plots of the τ vs ϕ dihedral angles ($\tau_{CD} = N_C-C_{14}-C_{15}-C_{16}$ and $\phi_{CD} = C_{14}-C_{15}-C_{16}-N_D$) for the two thousand and eighty three biliverdin IX α , biliverdin IX α in bacteriophytochrome (pdb code 2o9c) and biliverdin IX α in IFP1.4 structures obtained from the freely rotating molecular dynamics simulations.

	Slope	Area	80% Area
Free Biliverdin	-0.688	11970	3490
IFP1.4-keto	-1.335	6920	1520
2o9c-keto	-0.628	5720	1610
IFP1.4-enol	-0.827	3340	754
2o9c-enol	-0.916	8170	2630
2o9c-enolate	-0.520	7070	1350
IFP1.4-enolate	-1.02	6720	1330

	τ min	τ max	τ range	τ ave	ϕ min	ϕ max	ϕ range	ϕ ave
Free Biliverdin	-252	-116	136	-184	4.8	161	156	101
IFP1.4-keto	-234	-112	121	-184	-21	152	173	91
2o9c-keto	-172	-66	106	-114	-41	70	110	5
IFP1.4-enol	-168	-101	67	-132	-19	71.2	90.2	15.6
2o9c-enol	-242	-121	121	-173	20.5	181	161	92.6
2o9c-enolate	-214	-92.8	121	-134	31.9	136	104	70.9
IFP1.4-enolate	-241	-125	116	-177	53.8	186	132	119