A Green Fluorescent Protein Containing a QFG Tri-Peptide Chromophore: Optical Properties and X-Ray Crystal Structure

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Abstract

Rtms5 is an deep blue weakly fluorescent GFP-like protein (λmax, 592 nm; λmax, 630 nm; Φv, 0.004) that contains a Gln-Tyr-Gly chromophore tripeptide sequence. We investigated the optical properties and structure of two variants, Rtm5S-Y67F and Rtm5S-Y67F/H146S, in which the tyrosine at position 67 was substituted by a phenylalanine. Compared to the parent proteins the optical spectra for these new variants were significantly blue-shifted. Rtm5S-Y67F spectra were characterised by two absorbing species (λmax, 440 nm and 513 nm) and green fluorescence emission (λEm, 440 nm; λEm, 508 nm; Φv, 0.11), whilst Rtm5S-Y67F/H146S spectra were characterised by a single absorbing species (λmax, 440 nm) and a relatively high fluorescence quantum yield (Φv, 0.75; λmax, 440 nm; λEm, 508 nm). The fluorescence emissions of each variant were remarkably stable over a wide range of pH (3–11). These are the first GFP-like proteins with green emissions (500–520 nm) that do not have a tyrosine at position 67. The X-ray crystal structure of each protein was determined to 2.2 Å resolution and showed that the benzylidine ring of the chromophore, similar to the 4-hydroxybenzylidine ring of the Rtm5S parent, is non-coplanar and in the trans conformation. The results of chemical quantum calculations together with the structural data suggested that the 513 nm absorbing species in Rtm5S-Y67F results from an unusual form of the chromophore protonated at the acylamine oxygen. These are the first X-ray crystal structures for fluorescent proteins with a functional chromophore containing a phenylalanine at position 67.

Introduction

GFP-like proteins are valuable tools for use in molecular cell biology applications [1,2]. Extensive engineering has resulted in a range of proteins whose fluorescence emissions extend over the entire visible range. Many of the proteins have been cloned and developed from a limited number of naturally occurring fluorescent progenitors that include Aequorea victoria GFP (avGFP) [3] and DsRed isolated from Discosoma species [4]. Some non-fluorescent proteins such as hcCP, a chromoprotein isolated from Heteractis crispa have served as a valuable source of far-red fluorescent proteins that include HcRed [5].

Formation of the chromophore in GFP-like proteins is the result of a series of post-translational autocatalytic events involving a tripeptide motif. All naturally occurring GFP-like proteins isolated to date contain the tri-peptide X-Tyr-Gly, however the tyrosine can be substituted for other amino acids resulting in proteins with different optical properties. For example, substituting the chromophore tyrosine in avGFP with tryptophan or histidine resulted in blue-shifted fluorescent proteins (FPs) with cyan and blue fluorescence emissions, respectively [6]. A phenylalanine substitution results in FPs with the most blue-shifted emissions such as the avGFP166F (λmax, 442 nm) [7], and the more recently developed Sirius (λEm, 424 nm) [8].

A number of covalent modifications have been identified that further expand the range of optical properties including alternative chromophore structures [9]. For example, the red-shifted optical characteristics of DsRed and eqFP611 are the result of an acylamine linkage extending the chromophore conjugation system [10,11]. In addition to providing the appropriate environment to promote chromophore formation, contacts between the mature chromophore and the protein matrix determine the optical properties of these proteins. For instance, a Thr203Tyr substitution introduced to avGFP resulted in the first yellow fluorescent protein [12], whilst contacts with the acylamine oxygen...
are believed to contribute to the red-shifted properties of mPlum and Neptune [13,14].

Rtms5 is a deep blue weakly fluorescent GFP-like protein (Φf, 0.004; λmax, 592 nm) isolated from the coral Montipora efflorescens [15]. The X-ray crystal structure of Rtms5 suggests that its low fluorescence emission results from the trans non-coplanar configuration of the chromophore derived from an Gln-Tyr-Gly tripeptide [15]. An Rtms5H146S variant was significantly more fluorescent than Rtms5 particularly at high pH (Φf, 0.16 at pH 11.0; λEm, 630 nm), and the X-ray crystal structure showed evidence for a chromophore in a cis-coplanar configuration [16]. The chromophore in Rtms5 is extended by the presence of an acylimine linkage, and is in part responsible for the red-shifted optical properties of this protein [15–18].

Remarkably, there are few reports in the literature describing the properties of FPs with a phenylalanine in the chromophore tripeptide (i.e. X-Phe-Gly), and no X-ray crystal structures are available, other than those for proteins that do not have a correctly formed GFP-like chromophore [19]. Therefore, in this study we set out to investigate the optical properties and structure of Rtms5 and Rtms5H146S containing a Tyr67Phe substitution. The resulting proteins, Rtms5Y67F and Rtms5Y67F/H146S, have green fluorescence emission (λmax, 508 nm), and are the first FPs reported that have both green emissions (500–525 nm) and a phenylalanine in the chromophore tripeptide. The X-ray crystal structure of each of the variants was determined to 2.2 Å resolution. The structures show evidence for the presence of an acylimine linkage extending the chromophore conjugation system that contributed to the green fluorescence emission. The chromophores are in a trans non-coplanar conformation. To our knowledge, these are the first reported X-ray structures for GFP-like proteins containing a functional phenylalanine-substituted chromophore.

Results

Optical Properties of Rtms5Y67F and Rtms5Y67F/H146S

In order to investigate the effects of a tyrosine to phenyalanine substitution in Rtms5 and Rtms5H146S we determined the absorbance and fluorescence spectra for Rtms5 and Rtms5H146S at pH 8.0, and compared them to Rtms5 and Rtms5H146S, the parent proteins from which they were derived [15]. The absorbance spectrum for Rtms5Y67F/H146S showed a single species (λabs, 430 nm) whilst the absorbance spectrum for Rtms5Y67F showed two major species (λabs, 440 nm and 513 nm) and a shoulder at ~589 nm (Fig. 1a and b). The fluorescence excitation and emission spectra for Rtms5Y67F and Rtms5Y67F/H146S were similar (λEm, 508 nm) (Fig. 1), but compared to Rtms5 (Φf, 0.11) the fluorescence quantum yield for Rtms5Y67F/H146S (Φf, 0.75) was somewhat higher. No significant fluorescence emission was observed when the 513 nm species of Rtms5Y67F was excited. By comparison the tyrosine-containing chromophores of Rtms5 and Rtms5H146S show a single red-shifted absorbing species (Fig 1c and d; λabs, 592 nm and 588 nm, respectively) and very weak fluorescence emissions (Φf, 0.004 and 0.02 for Rtms5 and Rtms5H146S, respectively). The optical characteristics determined for proteins in this study are summarised and compared to those of other selected proteins in Table 1. Collectively these data indicate that a Tyr to Phe substitution results in Rtms5 variants that have significant blue-shifts in their optical spectra (~150 nm in λabs), and a significant increase in Φf.

Interestingly, compared to the phenylalanine-substituted chromophore of Sirius (λabs, 355 nm), a blue-emitting FP derived from

![Figure 1. Absorbance and fluorescence spectra for Rtms5Y67F, Rtms5Y67F/H146S, (A); Rtms5 and Rtms5H146S, (B); Rtms5, (C) and Rtms5H146S, (D) were determined in 20 mM Tris-HCl, pH8.0 and 300 mM NaCl. Absorbance spectra are normalised at 280 nm. Absorbance (solid line), excitation (dashed line), and emission (dotted line).](Image)
the extent of chromophore conjugation resulting from hydration of an acylimine linkage. The control proteins Rtms5 and Rtms5H146S which are known to contain an acylimine linkage [15,16] undergo a characteristic blue-shift (435 nm to 386 nm) in their absorbance spectra with an isosbestic point at 410 nm (Fig. 3c and d). Collectively, these data indicate that the Rtms5Y67F/H146S chromophore contains an acylimine linkage.

Changes in the absorbance spectrum for Rtms5Y67F incubated at pH 2.3 appeared more complex (Fig. 3a). At low pH a decrease in amounts of the 425 nm and 513 nm species was associated with a corresponding increase in the amount of the 349 nm species. These changes were irreversible as the 425 and 513 nm species did not reappear when the reaction mixture from the end point of the reaction was titrated back to pH 8.0. These results suggest that the 513 nm species of Rtms5Y67F exchange with the 349 nm species. The absence of a clear 390 nm suggests that both the 425 nm and 513 nm species contain an acylimine linkage, and that the 513 nm species of Rtms5Y67F exchange with the 349 nm species. The presence of a single isosbestic point at both the 513 nm and 425 nm chromophore species contain an acylimine linkage, and that the 513 nm species of Rtms5Y67F likely arises from alternate interactions of the chromophore with the protein matrix, and not a separate covalent modification of the Rtms5Y67F chromophore. Structural evidence presented later supports such a possibility.

In order to help exclude the possibility that exposure of proteins to low pH contributed to some change in chromophore structure, other than hydrolysis of the acylimine linkage, we investigated the chromophore at pH 8.0 in the presence of a protein denaturant. Guanidine HCl (GuHCl) promotes protein unfolding thereby exposing the chromophore acylimine linkage to the bulk solvent, and subsequent nucleophilic attack and hydration. We incubated Rtms5Y67F and Rtms5Y67F/H146S in 6 M GuHCl at pH 8.0, and determined the absorbance spectra at selected time points. For Rtms5Y67F the amounts of the 515 nm and 453 species decreased, leading to a corresponding increase in the 345 nm species (Fig. 4a). For Rtms5Y67F/H146S the amount of the 435 nm and 340 nm species decreased and increased, respectively (Fig. 4b). Collectively, these results together with those obtained at low pH suggest that all chromophore species in these proteins contain an acylimine linkage, and that the 513 nm species of Rtms5Y67F likely arises from alternate interactions of the chromophore with the protein matrix, and not a separate covalent modification of the Rtms5Y67F chromophore.

Finally, we investigated in further detail the effect of pH on the absorbance and fluorescence emission spectra of Rtms5Y67F and Rtms5Y67F/H146S. The absorbance and fluorescence emission for both Rtms5Y67F and Rtms5Y67F/H146S remained remarkably stable over the range pH 3–11 (pKa ~ 4.6 absorbance and emission) (Fig. 5a and b). Changes in absorbance and emission observed outside this pH range (<3 and >11) are likely the result of nucleophilic attack on the acylimine linkage and loss of chromophore conjugation as already discussed (Fig. 3). In comparison absorbance by Rtms5 and Rtms5H146S (pKa ~ 3.2 absorbance) decreases significantly below pH ~ 4 (pKu ~ 3.2 and 4.6 for Rtms5 and Rtms5H146S, respectively) (Fig. 5c and d) [17]. These proteins also show a significant increases in ΦF at pH > 10. The 4-hydroxybenzylidine moiety of the Rtms5 and Rtms5H146S chromophores titrates between an anionic form (zmax, ~ 392 nm) and neutral form (zmax, ~ 450 nm) [16], whereas the benzylidine moiety of the Rtms5Y67F and Rtms5Y67F/H146S chromophore, lacking a titratable group exists in a neutral form at all pH values (Fig. 2). Collectively these results indicate that the absorbance and fluorescence properties of Rtms5Y67F and in particular Rtms5Y67F/H146S are stable over a wider range of pH compared to their tyrosine-containing counterparts, Rtms5 and Rtms5H146S.
Structural Overview of Rtms5^{Y67F} and Rtms5^{Y67F/H146S}

We have determined the X-ray crystal structure of Rtms5^{Y67F} and Rtms5^{Y67F/H146S}. The crystallography and structural statistics are reported in Table 2. Each of the protomers in Rtms5^{Y67F} and Rtms5^{Y67F/H146S} consist of the same 11-stranded β-can motif (Fig. 6a) typical of members of the GFP-superfamily of proteins. Located at the core of the barrel is the circularised tri-peptide QFG chromophore maintaining covalent links to Cys65 and Ser69 of the main-chain. Within the asymmetric unit of Rtms5^{Y67F} there are 2 tetramers with 222 non-crystallographic symmetry (Fig. 6b) which both match the biological unit predicted by analysis using PISA [21] and the biological unit observed for Rtns. Rtms5^{Y67F/H146S} is also predicted to form a tetramer with 222 non-crystallographic symmetry in the biological unit. The greatest rmsd value between protomer A and its 7 non-crystallographically symmetry related protomers of Rtms5^{Y67F} was 0.134 Å and, as such, the protomers are considered identical. Clear electron density for the Rtms5^{Y67F} chromophore was observed in each protomer with clear links to Cys65 and Ser69 while the density for the Rtms5^{Y67F/H146S} chromophore was more ambiguous.

Figure 3. The effect of low pH on the absorbance spectra of Rtms5^{Y67F} and Rtms5^{Y67F/H146S}. Rtms5^{Y67F} (A) and Rtms5^{Y67F/H146S} (B) at a protein concentration of 0.25 mg/ml in 0.1 M potassium phosphate, pH 2.3 were incubated at 21 uC and the absorption spectra determined at selected time points. Rtns (C) and Rtns^{H146S} (D) at a protein concentration of 0.30 mg/ml in 0.1 M potassium phosphate, pH 2.3 were included as controls. The first absorbance scan of the incubation mixture (t0) is indicated. Relative trends (decrease or increase) in the absorbance spectra at different positions are indicated by arrows. The kinetics for changes in amount of individual absorbing species for each protein are shown (inset).
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In the following section we describe the chromophore structure and environment of Rtms5Y67F and Rtms5Y67F/H146S in relation to the parent protein Rtms5 [15]. Rtms5 Y67F and Rtms5Y67F/H146S each contain a benzylidine imidazolinone chromophore derived from the tripeptide Gln-Phe-Gly (Fig. 2). In each variant the Gln66 Cα, originally in the sp3 hybrid conformation is planar and sp2 hybridised as observed for other Rtms5 structures [15,16,17]. This arrangement is consistent with the formation of an acylimine linkage extending the π-bonding system of the chromophore as suggested by the red-shifted spectral data (Fig. 3; Fig. 2).

Contacts between the Rtms5 Y67F chromophore (glutaminyl, imidazolinone and glycyl moieties) and the protein matrix are similar to those observed for Rtms5 (Table 3; Fig. 7; [15]). However, contacts between the respective protein matrix and the 4-hydroxybenzylidine of Rtms5 or benzylidine of Rtms5Y67F are different. The 4-hydroxybenzylidine of Rtms5 is stabilised by a total of 17 van der Waals (vdw) interactions (Fig. 7b) whereas the benzylidine moiety of Rtms5Y67F is stabilised by only 14 vdw interactions. The vdw interactions in Rtms5 Y67F are contributed by His146, Arg197, Asn161, Glu148, Arg97 and Phe177 (Fig. 7a; Table 3).

The 4-hydroxybenzylidine moiety of Rtms5 is stabilised by a water-mediated (W310) H-bond with Thr179 and an H-bond with Asn161 (Fig. 7b). However, in the absence of a hydroxyl group the benzylidine moiety of Rtms5Y67F lacks such contacts. As a consequence the side-chain of Asn161 of Rtms5Y67F is rotated around the Cα, and extends towards the 4-hydroxybenzylidine moiety of the Rtms5 chromophore, where O82 maintains a water-mediated H-bond with Oτ1 of Thr179, whilst N82 forms an H-bond with Nδ1 of the imidazole ring of His146 (Fig 7a). Since the chromophores in both Rtms5Y67F and Rtms5 are non-coplanar it can be concluded that contact with Thr179 does not contribute to stabilisation of this conformation.

Two waters (W292 and W1092) not observed in Rtms5 or Rtms5Y67F/H146S, contribute to differences in hydrogen bonding around the chromophore of Rtms5Y67F (Fig. 7a). The Ne2 of His146 forms a water-mediated H-bond with Oe1 of Glu215 through water molecule W1092. Notably, this water is within 2.1 Å of Cβ2 of the chromophore methine bridge (Fig. 7a). It is possible that the proximity of W1092 to the methine bridge contributes to the observed red-shift in the absorbance spectrum of Rtms5Y67F compared to that of Rtms5Y67F/H146S (Fig. 1; Table 1) by coordinating increased electron pair density on the bridge of the chromophore [22]. A water-mediated H-bond is maintained between Oe2 of Glu148 and Ne of Arg197 through water A292. Additionally, the Glu215 carboxyl Oe1 H-bonds to N2 of the chromophore imidazolinone ring, while Glu215 Oe2 maintains water-mediated H bonds with Oγ Ser217 and Oγ1 Thr73 through water W292, and a water-mediated H-bond to N2 of the chromophore imidazolinone ring through water W247.

The imidazole ring of His146 in Rtms5Y67F is rotated around Cβ towards the benzylidine ring and contributes to a significant increase in the non-coplanarity of the Rtms5Y67F chromophore compared to the Rtms5 chromophore (Fig. 7a). The benzylidine moiety of Rtms5Y67F is twisted out of plane with respect to the imidazolinone ring with tilt and twist angles of −178° and 53°, respectively averaged across all eight protomers (Table 4) whereas...
the 4-hydroxybenzylidine ring of Rtms5 is twisted out of plane with respect to the imidazolinone ring with tilt and twist angles of 170° and 43°, respectively [15].

The different constraints imposed by the protein matrix upon the Rtms5 Y67F and Rtms5 chromophores are reflected in the average angle for the Cα2-Cβ2-Cc2 bond of the methine bridge (Fig. 2). The average angle of 121° for the Cα2-Cβ2-Cc2 bond in Rtms5 Y67F is close to the ideal angle for this bond, compared to angles of 139° and 140° observed in Rtms5 and Rtms5 H146S, respectively (Table 4).

### Table 2. Rtms5Y67F and Rtms5Y67F/H146S data collection and refinement statistics.

| Parameter | Rtms5Y67F | Rtms5Y67F/H146S |
|-----------|-----------|-----------------|
| Beamline   | APS IMCA-CAT | Australian Synchrotron MX-01 |
| Resolution range (Å) | 54.8-2.2(2.3-2.2)** | 50.0-2.2(2.3-2.2) |
| Space group | C222,1 | P4,22 |
| α, β, γ (°) | 150.3, 186.1, 185.2 | 93.1, 93.1, 76.9 |
| Total reflections | 974,663 | 118,881 |
| Unique reflections | 130,962 | 17,639 |
| Multiplicity | 7.4(1.5) | 6.7(6.6) |
| Mean I/σ(I) | 7.1(1.6) | 6.7(6.6) |
| Completeness (%) | 100(100) | 99(99) |
| Rmerge** (%) | 8.9(45.6) | 8.2(63.5) |
| Refinement | | |
| Resolution Range (Å) | 117.0-2.2(2.3-2.2) | 36.6-2.2(2.3-2.2) |
| Completeness (%) | 99.97(100) | 100(100) |
| Reflections | 124,333(9,125) | 16,740(1,187) |
| Rfactor (%) | 15.42 | 19.68 |
| Rfree (%) | 19.77 | 23.99 |
| Non-Hydrogen atoms | | |
| Protein | 13,898 | 1,693 |
| Chromophore | 184 | 23 |
| Water | 1,416 | 144 |
| R.m.s. deviations | | |
| Bond lengths (Å) | 0.024 | 0.022 |
| Bond angles (°) | 2.02 | 1.97 |
| Ramachandran plot | | |
| Most favored regions (%) | 98.6 | 98.1 |
| Allowed regions (%) | 1.4 | 1.4 |
| B factors | | |
| Avg. main-chain (Å²) | 26.87 | 52.38 |
| Avg. side-chain (Å²) | 29.65 | 54.14 |
| Avg. water (Å²) | 35.93 | 55.22 |
| Avg. chromophore (Å²) | 34.87 | 82.52 |
| *Values in parentheses refer to the highest resolution shell. |
| **Rmerge = Σ|IhkI–<|IhkI>|Σ|IhkI|. |

Compared to Rtms5Y67F, the structure of the Rtms5Y67F/H146S chromophore is less well defined with B-factors higher than the side-chains of the surrounding residues. A simulated annealing omit map shows that compared to Rtms5Y67F, the Rtms5Y67F/H146S chromophore is not well-defined in the electron density (Fig. 8). This effect may result from a reduced number of chromophore contacts as observed for Rtms5Y67F when compared to Rtms5, together with the additional His146Ser substitution. Nevertheless, sufficient electron density exists to enable the modelling of a trans, non-coplanar Rtms5Y67F/H146S chromophore. As a result of the His146Ser substitution, a pocket exists in Rtms5Y67F/H146S with the potential to accommodate the chromophore in a cis conformation (Fig. S1). In order to investigate the possibility that the Rtms5Y67F/H146S chromophore is mobile and is able to adopt alternate conformations, the trans and cis chromo-
phore conformations were modelled at different occupancies. The difference maps showed increasing amounts of negative density in the position corresponding to the cis conformation as the occupancy of the cis chromophore approaches 1 (Fig. S2). The analysis suggests that the trans conformation of the Rtms5Y67FH146S chromophore is favoured.

Quantum Chemical Calculations

In order to guide the assignment of the absorbance bands of the Rtms5Y67F variants investigated in this study, we performed quantum chemical calculations of the electronic excitation energies of a truncated model of the chromophore. The chemical structure of the chromophore model is shown in Figure 9. The model is truncated at a level consistent with earlier studies of acylimine-substituted FP chromophore models, and includes all atoms that contribute to the π-electron system [23,24]. We examined four distinct protonation states of the model: an unprotonated neutral form, and three singly protonated forms with the proton bound to the imidazoline nitrogen site (ImNH⁺), the imidazolinoine oxygen site (ImOH⁺), and the acylimine oxygen site (AcOH⁺). The excitation energies and dipole observables associated with the S₀–S₁ transition of the Rtms5Y67F chromophore model are listed in Table 5.

The computational results were obtained for the truncated model in gas phase and any effects of the protein environment, both steric and electronic, are neglected. For this reason, the confidence that one can place on assignments based on these data is determined by the relative separation of the distinct absorbance bands in the proteins and the separation of excitation energies for different states of the model. Fortunately, the excitation energies of most of the states used in the calculations are quite distinguishable. However, we note that in all cases the optimized geometries of the models are planar. Non-planar distortions of the methine bridge are expected to provide a modest red-shift (on the order of 0.1 eV) [24]. Non-planarity of the acylimine linkage is expected to affect the absorbance to a smaller extent, because the conjugation through the imine nitrogen can occur even with significant twisting [23].

Rtms5Y67F but not Rtms5Y67F/H146S has an absorbance band at 513 nm (Fig. 1). The calculated excitation energy of the state protonated at the acylimine oxygen (AcOH⁺) is significantly redder than the neutral chromophore (368 nm) (Table 5). This suggests that the absorbance band near 513 nm, characteristic of Rtms5Y67F should not be attributed to an unprotonated chromophore species. Instead, this band is more reasonably assigned to a species that is protonated at the acylimine oxygen. A difference in the position of the side-chain of Ser69 in Rtms5Y67F compared to Rtms5Y67F/H146S lends support to this idea. The O of Ser 69 and OOH of Tyr 14 in Rtms5Y67F/H146S are within H-bonding distance of the acylimine oxygen (Fig. 10). Rotation of the Ser69 side-chain and repositioning of the acylimine oxygen in Rtms5Y67F place them beyond hydrogen bonding distance suggesting a change in the charge associated with the acylimine oxygen.

Discussion

This is the first report describing an FP with green fluorescence emission (λ_em, 500–520 nm) that does not have tyrosine as the aromatic amino acid in the chromophore tripeptide. Only two other FPs, the cyan emitting mBlueberry 2 (λ_em, 467 nm) and mBlueberry 1 [25], are presumed to contain the same chromophore structure as Rtms5Y67F and Rtms5Y67F/H146S. mBlueberry 2 was derived from the acylimine-containing red fluorescent mCherry by introduction of number of amino acid substitutions including a Tyr to Phe substitution at position 67. In the absence of an X-ray crystal structure for mBlueberry 2 the reasons for the marked difference in emission maxima (∼40 nm) between mBlueberry 2 and the Rtms5Y67F variants (Table 2) are unclear but presumably arise from altered contacts of the chromophore with the surrounding amino acid side-chains. It is known that subtle changes in chromophore contacts can generate significant differences in the emission spectra. For example, the position of the positively charged side-chain of Arg197 relative to the 4-hydroxy benzylidene moiety is, in part, believed to be responsible for producing the significantly red-shifted spectra of mNeptune (λ_em, 655 nm) [14]. In Rtms5Y67F the same side-chain of...
Arg197 is within vdw distance of the benzylidine ring (Table 3; Fig. 7a; Fig. S1) whereas in mBlueberry1 and mBlueberry2 the charged side chain of Arg197 is substituted by the non charged side-chain of isoleucine [25], a change that would be consistent with the blue-shifted spectra observed for the mBlueberry variants. The weak fluorescence emission observed for both Rtms5 and Rtms5$^{H146S}$ ($\Phi_F$, 0.004 and 0.02, respectively) has been attributed previously to their trans non-coplanar chromophores [15]. A significant increase in fluorescence emission (20-fold; $\Phi_F$ 0.16) observed for Rtms5$^{H146S}$ at alkaline pH (see Fig. 5d) was accompanied by an increased proportion of a cis-coplanar chromophore as observed in the X-ray crystal structure [16]. Since Rtms5$^{Y67F}$ and Rtms5$^{Y67F/H146S}$ are considerably more fluorescent ($\Phi_F$, 0.11 and 0.75, respectively) compared to their Rtms5 parents, we were surprised by the lack of evidence for a cis-coplanar chromophore in their structures. The poor electron density corresponding to the chromophore in Rtms5$^{Y67F/H146S}$ suggests it is mobile, and may adopt alternate conformations. However, the difference maps for trans and cis Rtms5$^{Y67F/H146S}$ chromophore conformations under different occupancies indicated that the trans conformation is favoured (Fig. S2) leaving no clear explanation for the increased $\Phi_F$ of these proteins.

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whereas in the case of the coplanar chromophore, stabilisation is provided by H-bonds mediated through W329, W319 and W282 (Fig. S3c and d). How and when movement of the Arg197 side-chain takes place in Rtms5Y67F/H146S is not clear. However, repositioning of the side-chain of a histidine at the same amino acid location is a key feature of the molecular mechanism for photoswitching of DronPA [28]. On illumination with excitation light repositioning of His197 in DronPA promotes isomerisation of the chromophore from a trans non-coplanar, non-fluorescent form to a cis coplanar, brightly fluorescent form. If our model is correct Rtms5Y67F/H146S and Rtms5 belong to very small group of FPs that have a fluorescent trans chromophore conformation. Further studies of Rtms5Y67F/H146S are required to investigate the validity of this model.

We were intrigued as to the source of the 513 nm species in Rtms5Y67F. The results of chemical quantum calculations suggest this species may arise from a protonation event involving the acylimine oxygen (AcOH$^+$; Scheme 2). This idea is given additional support by the structural data that suggests a change in the position of the side-chain of Ser 69 in Rtms5 compared to Rtms5Y67F/H146S (Fig. 9). Interaction of the acylimine oxygen with the protein matrix appears to be important for generating a red-shift in the spectra of other FPs [29]. A hydrogen bond between the side-chain of Glu16 and the acylimine carbonyl has

Figure 7. The chromophore environment of Rtms5Y67F and Rtms5. Stereoviews are shown comparing the chromophore environments and H-bonding for Rtms5Y67F (A) and Rtms5 (B). Chromophores are shown in orange (Rtms5Y67F) or blue (Rtms5). H-bonding is indicated by broken lines (corresponding distances are shown in Table 3). Waters are shown as red spheres. Two waters (W1092 and W2932) present in Rtms5Y67F but not Rtms5, that contribute to differences in H-bonding are labelled. The distance between W1092 and Cβ of the methine bridge of the Rtms5Y67F chromophore is 2.2 Å and highlighted by a red broken line. H-bonds between the 4-hydroxybenzylidene moiety of Rtms5 and Thr179 (water mediated) and Asn161 are not present in Rtms5Y67F.
The data in Table 5 also suggest that the absorbance band near 440 nm (Fig. 1), characteristic of both Rtms5Y67F and Rtms5Y67F/H146S should not be attributed to an unprotonated chromophore species. Instead, this band is more reasonably assigned to a species that is protonated at either the nitrogen (ImNH$^+$) or oxygen site (ImOH$^+$) on the imidazolinone ring. Although the excitation

Table 4. Measured angles for the chromophores of Rtms5 variants and selected fluorescent proteins.

| Protein      | Methine Bridge angle (°) | Tilt (°) | Twist (°) |
|--------------|--------------------------|----------|-----------|
| Rtms5$^{Y67F}$ | 121 (±2)                 | -178 (±2) | 53 (±3)   |
| Rtms5$^{Y67F/H146S}$ | 133                   | -178     | 43        |
| Rtms5$^{Y67F}$ | 139                     | 170      | 43        |
| Rtms5$^{H146S}$ | 140                    | 169      | 42        |
| mCherry$^{(3)}$| 134                     | 26       | -13       |
| mNeptune$^{(4)}$ | 122                    | 5        | -9        |

*The measured angle between the C$\alpha$-C$\beta$ and C$\beta$-C$\gamma$ bonds of the chromophore. PDB files analysed in this table include.

Figure 8. Simulated annealing omit maps for the chromophores of Rtms5Y67F and Rtms5Y67F/H146S. Alternate views are shown for the non-coplanar chromophores of Rtms5Y67F (A and B; orange) and Rtms5Y67F/H146S (C and D; green). Nearby waters (numbered red spheres) were included in the omit map calculation. The omit map calculation for the Rtms5Y67F/H146S chromophore included a nearby chloride (green sphere). The omit map indicates that the Rtms5Y67F chromophore is in the trans conformation whilst the Rtms5Y67F/H146S chromophore omit map is more ambiguous. The mesh representing the omit maps is contoured to 2.5σ. Difference maps showing the trans and cis Rtms5Y67F/H146S chromophore conformations under different occupancies are shown in Fig. S2.

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Figure 9. The chromophore model used for quantum chemical calculations. The chromophore model is truncated at a level consistent with earlier studies of acylimine-substituted FP chromophore models. The neutral unprotonated form is shown. The protonation sites for each of the three singly protonated forms are indicated.

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The data in Table 5 also suggest that the absorbance band near 440 nm (Fig. 1), characteristic of both Rtms5$^{Y67F}$ and Rtms5$^{Y67F/H146S}$ should not be attributed to an unprotonated chromophore species. Instead, this band is more reasonably assigned to a species that is protonated at either the nitrogen (ImNH$^+$) or oxygen site (ImOH$^+$) on the imidazolinone ring. Although the excitation
energy calculated for the ImOH\textsuperscript{+} model is closer to the experimentally measured energy gap, the weight of precedent favors assignment to a nitrogen-protonated ImNH\textsuperscript{+} species. Protonation at either of these two positions might be expected be reflected in altered chromophore/protein matrix contacts. Although differences exist in the H-bond network around the chromophore in the Rtms\textsubscript{Y67F} variants compared to their tyrosyl-containing counterparts (Rtms5 and Rtm5\textsuperscript{H146S} (Fig. 7) in each case an H-bond exists between N2 and O2 of the imidazolinone ring and the Glu215 carboxyl and Arg95, respectively (Fig. 7). We have previously reported that the Rtms5 chromophore is not protonated \[16,30\].

There are few reports in the literature of FPs containing a phenylalanine in position 67 of the chromophore. The phenylalanine substituted variants reported here represent an alternative platform on which to develop fluorescent proteins with green emissions (500–520 nm) and superior pH stability. These proteins may also have a fluorescent \textit{trans} chromophore conformation. Rtms\textsubscript{Y67F} and Rtm5\textsubscript{Y67F}/H\textsubscript{146S} have the most pH-stable (pK\textsubscript{a}, 3.5) green emissions of any FPs (500–520 nm) reported to date. The green emissions of Sapphire FPs having been reported previously to be the most stable to pH (pK\textsubscript{a} 4.9) \[31\]. This feature of Rtms\textsubscript{Y67F} and Rtms\textsubscript{Y67F}/H\textsubscript{146S} can be attributed to the benzylidine moiety which lacks a titratable group. The chromophore of the pH-stable blue-emitting Sirius contains the same benzylidine moiety, and has a pK\textsubscript{a} \textless 3.0 \[8\]. The Sapphire chromophore contains a 4-hydroxy benzylidine moiety \[31\]. The ability of Rtms\textsubscript{Y67F} and Rtm5\textsubscript{Y67F}/H\textsubscript{146S} to fluoresce with little attenuation down to \textasciitilde pH 3.5, may prove useful for engineering new improved biosensors for monitoring autophagy in live cells \[32\]. Autophagy is an important cellular process characterised by the delivery of material to the acidic (pH 4.8) lumen of the lysosome for degradation.

Rtms\textsubscript{Y67F} and Rtm5\textsubscript{Y67F}/H\textsubscript{146S} are obligate tetramers. However, we recently described a monomer of Rtms5 called Ultramarine \[33\] that represents a starting point to develop monomer forms of the phenylalanine-substituted FPs, thereby allowing them to be used as fusion partners with other proteins of interest.

### Materials and Methods

**Mutagenesis, Protein Expression and Purification**

Expression vectors encoding Rtm5\textsubscript{Y67F} or Rtm5\textsubscript{Y67F}/H\textsubscript{146S} were constructed by site-directed mutagenesis (QuickChange, Invitrogen) using the primer pair 5’-caccacagtgattcgaagcagcagcact-3’ and 5’-gaatggtatgcttccgaactgacactgtggtg-3’ and expression vectors pQE10:Rtms5 or pQE10:Rtms5\textsubscript{H146S} as templates.
**Cryocrystallography**

Rtms5<sup>V67F</sup> and Rtms5<sup>V67F/H146S</sup> proteins were expressed in the NovaBlue (DE3) strain of *E. coli* (Novagen) and purified by NiNTA chromatography as described [15]. For cryocrystallography purposes proteins were subjected to chromatography on a S200 size exclusion column equilibrated in 20 mM Tris-Cl, pH 8.0, 300 mM NaCl. Fractions containing Rtms5<sup>V67F</sup> or Rtms5<sup>V67F/H146S</sup> tetramer were pooled and concentrated to 15 mg.ml<sup>-1</sup> ready for crystallization trials by the hanging drop vapour diffusion technique.

**Spectrometry**

Fluorescence spectra were determined using a Varian Eclipse fluorescence spectrophotometer (Melbourne, Australia). Φ<sub>ps</sub> values were determined for proteins (in 20 mM Tris-Cl (pH 8.0), 300 mM NaCl) at 25°C as described [15,34] using solutions of Rhodamine 101 (Φ<sub>ps</sub>, 1.0) in buffer as standard. Absorbance spectra were determined using a Varian Cary 50 spectrophotometer. For pH titrations, proteins in 20 mM Tris-Cl (pH 8.0) were diluted (~100-fold) as required into selected 0.1 M buffers [16,17]. Absorbance spectra were recorded at 24°C after 30 sec gentle mixing. Sample pH was monitored using a micro-pH probe. Data from a single a determination are presented.

**Cystallization and Structural Determination**

Crystals of Rtms5<sup>V67F</sup> and Rtms5<sup>V67F/H146S</sup> that appeared brown or pale green, respectively were obtained at 20°C via the hanging drop method. Protein (15 mg.ml<sup>-1</sup>) in 20 mM Tris, 300 mM NaCl, pH 8.0 was mixed 1:1 or 1:2 with crystallization buffer. Rtms5<sup>V67F</sup> crystals (0.1–0.2 mm in length) were obtained using a crystallization solution composed of 22% PEG 3350 and 0.34 M KI buffered with 0.2 M Tris-HCl pH 8.5 in 3 μl hanging drops (1:2 protein/crystallization solution ratio). Rtms5<sup>V67F/H146S</sup> crystals 0.1–0.2 mm in length were obtained using a crystallization solution with 21% PEG 3350, 0.36 M KI, and 25% glycerol buffered with 0.2 M Tris pH 8.5 in 3 μl hanging drops (1:2 protein/crystallization solution ratio). Rtms5<sup>V67F</sup> crystals were flash frozen prior to data collection using 30% (v/v) glycerol in the precipitant as cryoprotectant. Crystals were transferred stepwise (5% increments) into increasing amounts of glycerol over a time period of 2 h. Rtms5<sup>V67F/H146S</sup> crystals were dipped (5% increments) into increasing amounts of glycerol over a time period of 2 h. Rtms5<sup>V67F/H146S</sup> were collected at the MX-1 beamline of the Australian Synchrotron, respectively. Rtms5<sup>V67F</sup> and Rtms5<sup>V67F/H146S</sup> were predicted using PISA (51-53). All calculations were carried out using the MOLPRO software package (http://www.molpro.net) [54].

**Quantum Chemical Calculations**

For each protonation state examined, we optimized the geometry of the model using Moller-Plesset 2nd order perturbation theory [47] and a cc-pvdz basis set [48] (MP2/cc-pvdz). At these geometries, we calculated the excitation energies, transition dipole and difference dipole moments using multi-state multi-reference 2nd order perturbation theory [49,50] on a four-electron, three-orbital two-state averaged complete active space self-consistent field wavefunction, again with a cc-pvdz basis set [48] (SA2-CAS(4,3)*MS-MRPT2/cc-pvdz). This protocol has previously been used to study the halochromism of GFP chromophore models [51,52,53]. All calculations were carried out using the MOLPRO software package (http://www.molpro.net) [54].

**Supporting Information**

**Figure S1** The chromophore cavities of Rtms5<sup>V67F</sup> and Rtms5<sup>V67F/H146S</sup>. Orthogonal cutaway views are shown for Rtms5<sup>V67F</sup> (A and B) and Rtms5<sup>V67F/H146S</sup> (C and D). The sidechain of His146 stabilises the trans conformation of the Rtms5<sup>V67F</sup> chromophore. The His146Ser substitution (C) creates a pocket with the potential to accommodate an Rtms5<sup>V67F/H146S</sup>-chromo-
phore with a cis conformation. The non-coplanar conformation of the chromophores in both Rtm5Y67F and Rtm5Y67F/H146S is stabilised by the side-chains of Arg96 and Arg197 (C and D). Waters are shown as red spheres. (TIF)

Figure S2 Difference maps showing the trans and cis Rtm5Y67F/H146S chromophore conformations under different occupancies. Occupancy ratios (trans/cis) are 0.0/1.0, (A); 0.25/0.75, (B); 0.5/0.5, (C); 0.75/0.25, (D) and 1.0/0.0 (E). The positive (green mesh) and negative (red mesh) difference maps are contoured to +2.5σ and −2.5σ, respectively. The trans chromophore conformation is favoured in Rtm5Y67F/H146S. A nearby chloride ion (green sphere) was omitted from the map calculation. (TIF)

Figure S3 A model showing the chromophore cavity of Rtm5Y67F/H146S with a hypothetical trans-coplanar chromophore. Orthogonal views of the trans Rtm5Y67F/H146S chromophore in a trans non-coplanar as suggested by the X-ray structure (A and B), and modelled in a trans coplanar conformation (C and D) are shown. The conformation of the Arg197 residue, which contacts the benzylidene moiety of the chromophore (pink dashed lines, distances in Å numbered in pink) restricts the possibility of a trans coplanar chromophore (A). The conformation of Arg197 is stabilised by H-bonds (black dashed lines, distances in Å shown numbered in black) to two nearby water molecules (red spheres, numbered in red) and to Glu140 [B]. Repositioning of the Arg197 side chain (C) creates a space in which a trans coplanar chromophore could be accommodated. The side-chain of Arg197 in is stabilised by different contacts (D). A nearby chloride is shown (green sphere). The hypothetical model was created in WinCoot, avoiding major clashes with nearby atoms, and only the rearrangement of the Arg197 side chain has been considered. (TIF)

Figure S4 Hypothetical resonance structures for the chromophore model. (TIF)

Text S1 (DOCX)

Scheme S1 (TIF)

Scheme S2 (TIF)

Author Contributions
Conceived and designed the experiments: JB MP SO DT. Performed the experiments: JB DT EB SO. Analyzed the data: JB DT MP EB JR MW SO. Contributed reagents/materials/analysis tools: MP JR MW. Wrote the paper: JB DT RD MW MP.

References
1. Shcher NC, Patterson GH, Davidson MW (2007) Advances in fluorescent protein technology. J Cell Sci 120: 4247–4260.
2. Chudakov DM, Matz MV, Lukyanov S, Lukyanov KA (2010) Fluorescent proteins and their applications in imaging living cells and tissues. Physiol Rev 90: 1105–1163.
3. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. Science 263: 802–805.
4. Matz MV, Fridkov AF, Labas YA, Zaraiskaya AG, et al. (1999) Fluorescent proteins from nonbioluminescent Anthozoa species. Nat Biotechnol 17: 969–973.
5. Gurskaya NG, Fridkov AF, Terskikh A, Matz MV, Labas YA, et al. (2001) GFP-like chromoproteins as a source of far-red fluorescent proteins. FEBS Lett 507: 16–20.
6. Heim R, Prasher DC, Tsien RY (1994) Wavelength mutations and post-translational autoxidation of green fluorescent protein. Proc Natl Acad Sci U S A 91: 12501–12504.
7. Cubitt AB, Heim R, Adams SR, Boyd AE, Green LA, et al. (1993) Understanding, improving and using green fluorescent proteins. Trends Biotechnol 20: 448–455.
8. Tomosugi W, Masuta S, Tani T, Nemoz T, Kotera I, et al. (2009) An ultramarine fluorescent protein with increased photo-stability and pH in-sensitivity. Nat Methods 6: 351–353.
9. Wachter RM, Watkins JL, Kim H (2010) Mechanistic diversity of red fluorescence acquisition by GFP-like proteins. Biochemistry 49: 7417–7427.
10. Gross LA, Baird GS, Hoffman RC, Baldrige KK, Tsien RY (2000) The structure of the chromophore within DsRed, a red fluorescent protein from coral. Proc Natl Acad Sci U S A 97: 11990–11995.
11. Wiedenmann J, Schenk A, Roeder C, Girod A, Spindler KD, et al. (2002) A far-red fluorescent protein with fast maturation and reduced oligomerization tendency from Entacmaea quadricolor. Biochemistry. 41: 11646–11651.
12. Cubitt AB, Woollenweber LA, Heim R (1999) Understanding structure-function relationships in the Aquorea victoria green fluorescent protein. Methods Cell Biol 50: 39–50.
13. Shu X, Wang L, Colip L, Kallio K, Remington SJ (2008) Unique interactions between the chromophore and glutamate 16 lead to far-red emission in a red fluorescent protein. Protein Sci 17: 969–973.
14. Lin MZ, McKewin MR, Ng H, Aguiler TA, Shancer NC, et al. (2009) Autofluorescent proteins with Excitation in the Optical Window for Intravital Imaging in Mammals. J. Chrom. Biobel. 116: 1169–1179.
15. Prescott M, Ling M, Beddoes T, Oakley AJ, Dove S, et al. (2003) The 2.2 Å crystal structure of a polcoplin pigment reveals a nonplanar chromophore conformation. Structure. 11: 273–284.
16. Battad JM, Wilkman PG, Olsen SC, Byres E, Smith SC, et al. (2007) A Structural Basis for the pH-Dependent Increase in Fluorescence Efficiency of Chromoproteins. J Mol Biol 368: 998–1010.
