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Mutants of *Discosoma* red fluorescent protein with a GFP-like chromophore

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Abstract The green fluorescent protein (GFP)-homologous red fluorescent protein (RFP) from *Discosoma* (drFP583) which emits bright red fluorescence peaking at 583 nm is an interesting novel genetic marker. We show here that RFP maturation involves a GFP-like fluorophore which can be stabilized by point mutations selected from a randomly mutated expression library. By homology modeling, these point mutations cluster near the imidazolidinone ring of the chromophore. Exciting the GFP-like absorption band in the mutant proteins produces both green and red fluorescence. Upon unfolding and heating, the absorption spectrum of the RFP chromophore slowly becomes similar to that of the GFP chromophore. This can be interpreted as a covalent modification of the GFP chromophore in RFP that appears to occur in the final maturation step.

Key words: Green fluorescent protein; Red fluorescent protein (drFP583); Mutagenic polymerase chain reaction; Maturation; Intermediate

1. Introduction

The fluorescent chromophore of *Aequorea victoria* green fluorescent protein (GFP) is formed autocatalytically by cyclization of the polypeptide backbone followed by oxidation [1]. A large variety of spectroscopically distinct GFP variants have been created by mutagenesis of the chromophore or the surrounding amino acids. The largest fluorescence red-shift observed in these variants is 17 nm, peaking at 527 nm. These variants are extensively used as in vivo markers of protein localization or gene expression (for a review, see [2]). In 1999, Matz and coworkers presented six new fluorescent proteins homologous to GFP which were cloned from Anthozoan organisms [3]. The red fluorescent protein (RFP) isolated from *Discosoma* sp. (drFP583) is most interesting from the application side. It's emission is red-shifted by 73 nm relative to GFP and this could be caused either by a different covalent structure of the fluorophore, or by different interactions with the highly structured protein matrix. It is known for GFP that the protonation state of the phenolic oxygen causes ~100 nm differences in absorption wavelength [4]; other mechanisms that have been implicated are ππ-interactions that occur in the so called yellow fluorescent proteins [5], the charge distribution around the chromophore and a twist in the chromophore plane [6]. Alternatively, the red fluorescing chromophore could be covalently different to that of GFP in a number of ways. Additional reactions with the surrounding residues, possibly including aromatic residues, may have occurred as well as an extension of the conjugated double-bond system further into the polypeptide backbone. To clarify by which mechanism the red fluorescence of RFP is achieved, we have tracked absorption and fluorescence during the maturation process and characterized the unfolded protein under various conditions. Finally, we performed a random mutagenesis of the RFP gene by polymerase chain reaction (PCR) and isolated variants with altered fluorescence properties.

2. Materials and methods

2.1. Vector system and protein expression

The drFP583 gene from Clontech vector pDsRed1-N1 was amplified in a two-stage PCR with the internal primer deleting the *Nco*I site and the C-terminal primer adding a terminal His_6-tag followed by an *Eco*RI site. The final PCR product was cleaved with *Nco*I and *Eco*RI and cloned into the vector pT7av (unpublished). The correct sequence was confirmed by DNA sequencing. In the resulting vector pt7RFPav (3500 bp) the RFP expression is controlled by the T7 promotor and resistance against ampicillin is provided by the gene for β-lactamase. Expression and purification of all proteins was carried out as previously described [4]. Expression temperature was 37°C for RFP and wild-type GFP, respectively, and 33°C for all RFP mutant proteins. Sequence numbering follows the wild-type protein sequence.

2.2. Spectroscopic measurements

Absorption (1 cm pathlength) and fluorescence spectra were determined on an Uvikon 943 spectrophotometer (Kontron Instruments) and a Hitachi 4500 fluorescence spectrometer, respectively (1 cm × 1 cm cuvettes). Excitation spectra were corrected and emission spectra were not corrected for instrumental response.

2.3. Chromophore maturation

Escherichia coli BL21 DE3 transformed with the vector pt7RFPav were grown at 37°C to an $OD_{600\,nm}$ of 0.8. Expression was induced with IPTG (0.5 mM) and performed at 37°C for 8 h. Cells were stored overnight in −80°C, lysed with a French press and by brief sonification. Purification of the protein in the supernatant after centrifugation at 45 000 × *g* was performed with a Ni-NTA column [4]. The protein was eluted with 300 mM imidazole in 300 mM NaCl, 50 mM Na_2HPO_4, pH 8.0 and the absorption was measured immediately and at later time points. Samples were stored and measured at 20°C. Aliquots of the sample were diluted 100-fold after the first absorption measurements and fluorescence scans were performed immediately after each absorption scan. To show that there is no influence of the imidazole present in the elution buffer, a sample dialyzed against PBS (phosphate-buffered saline solution; 4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl, pH 7.4) was compared and found to possess spectral properties identical to the native protein (not shown).

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2.4. Random mutagenesis

Mutagenic PCR of the full length RFP gene was performed according to [7]. A 100 μl PCR mix contained 10 mM Tris pH 8.3, 10 μg bovine serum albumin, 50 mM KCl, 7 mM $MgCl_2$, 2 mM GTP, 2 mM ATP, 10 mM TTP, 10 mM CTP, 3 nM of each primer, 42 ng pt7RFPav template DNA, 1.25 mM $MnCl_2$ and 5 U Taq polymerase. To avoid contaminations with the template DNA the reverse primer deleted a *Eco*RI site. Cycling was performed with a paraffin overlay in a Landgraf thermocycler with 1 cycle 94°C for 60 s and 30 cycles 94°C for 60 s, 45°C for 60 s, 72°C for 60 s. The PCR product was cleaved with *Nco*I, *Hin*dIII and *Eco*RI and cloned back into the vector pT7av.

The DNA was transformed into *E. coli* XL1 Blue, prepared and electroporated into *E. coli* BL21 DE3 which were plated on ampicillin containing LB agar plates. Screening of 1.5×10^5 colonies was carried out with a handheld UV lamp (365 nm) in the dark after different time points. From approximately 45 bright red colonies the DNA was isolated, transformed in *E. coli* XL1 Blue and prepared again. Brightness of the colonies was confirmed with a fluorescence microplate reader. The DNAs of the 16 brightest (A) red fluorescing variants and a subset of two others (B) were pooled and taken as templates for the second mutagenic PCR which was performed as described above. Now 6×10^4 colonies derived from each templates A and B were screened. Five clones of yellow and five of orange color were selected. DNA was isolated as above, retransformed to *E. coli* BL21 DE3 and the phenotype was confirmed. Colonies of each clone were suspended in PBS pH 7.4 to a final $OD_{600\,nm}$ of ~0.1 and fluorescence spectra were measured. The orange and yellow clones and both template DNAs of set B were sequenced. A subset of these proteins were expressed and further characterized. For the absorption measurements, protein solutions (PBS pH 7.4) with identical maximal absorptions of 0.25–0.27 were prepared and 10-fold diluted for fluorescence measurements.

2.5. Protein denaturation

Proteins (GFP, RFP, Ao1) were buffered in 1 mM imidazole, 1.5 mM Tris, 1 mM glycine, 0.15 mM EDTA and 100 mM NaCl, pH 9.0 in protein concentrations adjusted to similar absorption of the unfolded proteins. 630 μl buffer (6.67 M guanidinium chloride, 1.5 mM Tris, 1 mM glycine, 0.15 mM EDTA; adjusted to pH 6.7, pH 8.1, pH 10.8 with HCl/NaOH) was added to 70 μl protein solution and mixed gently. Samples were heated for 2 min at 85°C in the waterbath with interspersed brief pulses of mixing. Absorption scans were performed immediately. Measurements were performed at 20°C if not otherwise specified. Time trace measurements demonstrated that no significant visible changes occurred during storage at 20°C. To get further information about changes due to the temperature treatment and about the stability of the chromophore, samples were heated two times for 1 h to 60°C and measured as before.

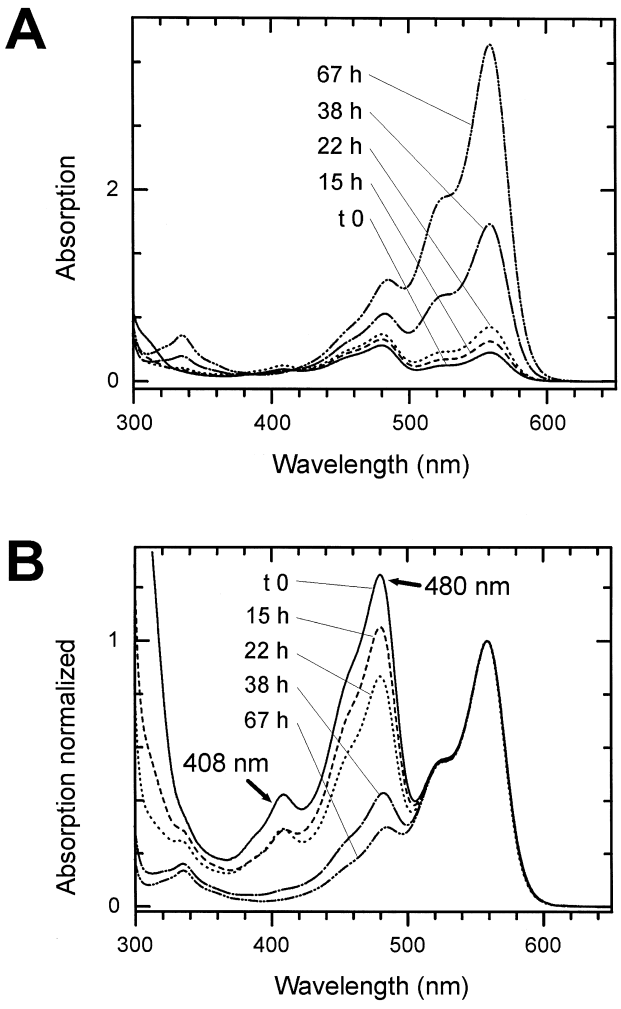

Fig. 1. Absorption spectra of freshly expressed and purified RFP at different time points after the first measurement (*t* 0). A: Original absorption curves. B: Same curves as in A, but normalized to the final absorption peak.

3. Results

3.1. Chromophore maturation

Freshly expressed RFP shows additional absorption peaks at 408 nm and 480 nm. (Fig. 1). After maturation, the 408 nm absorption is no longer observed. Apparently, fluorescence cannot be efficiently excited at this wavelength since the peak is not detectable in excitation scans at any time point. In contrast, the absorption peak at 480 nm persists in the final absorption spectrum. Its excitation results in weak green fluorescence, peaking at 500 nm (Fig. 2C), which decreases in the later stages of the maturation process and the typical red emission at 583 nm, increasing over time. The absolute increase of absorption at 480 nm over time is due to overlap with the major peak of the matured spectrum at 559 nm which increases in amplitude. The changing peak ratios are visualized by normalizing to the 559 nm absorption (Fig. 1B); the absolute amplitude of the 480 nm band decreases in the late maturation stages. This demonstrates that the peak is characteristic of an intermediate of the maturation process. Neither the excitation and emission bands nor the absorption bands change their shape during maturation. Thus the composite spectra can be interpreted as mixtures of discrete populations with distinct spectral properties.

In a second experiment with shorter expression and purification times and additional dialysis against PBS pH 7.4 buffer, we apparently observed later time points of the maturation processes. This points to a dependence of the maturation process on the precise conditions of protein expression.

3.2. Random mutagenesis

To further define the maturation process of RFP, we attempted to isolate RFP variants with altered spectral properties. Five yellow and five orange clones (see Section 2) were isolated from a library of randomly mutated genes and characterized. The amino acid mutations in both B template genes (referring to the RFP sequence) and in the mutant proteins are given in Table 1. All yellow phenotypes of the B set derive from template B2; one of these reverted the K166R mutation. Because B2 itself shows the wild-type RFP fluorescence, the additional mutations K83R present in four yellow clones and P37S in the remaining clone seem to cause the yellow phenotype. The same is true for the N42H and the T217S mutations

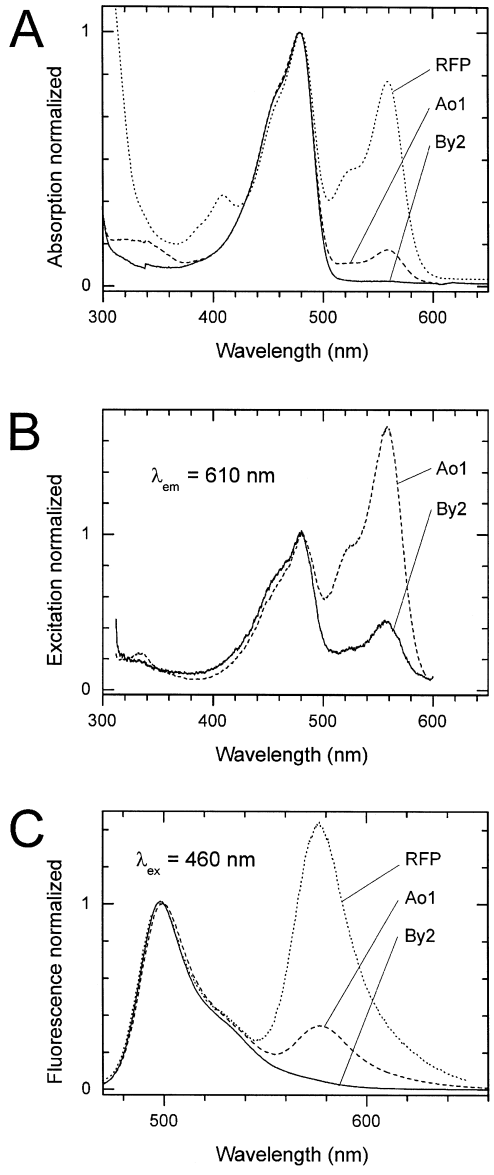

Fig. 2. Absorption and fluorescence properties of the maturing wild-type RFP (*t* 0) and mutated proteins. Just two representatives are shown since the spectra of the investigated orange proteins were similar. A: Absorption spectra normalized to the GFP-like peak at 480 nm. B: Excitation scans of the red fluorescence detected at 610 nm (corrected for instrumental response). Curves are normalized to the GFP-like peak. C: Fluorescence spectra upon excitation at 460 nm (not corrected for instrumental response). Curves are normalized to the green emission.

in the orange phenotypes. In Ao2, T217S is the only mutation present.

All proteins except for Ay2 and By3 were expressed and purified. Expression yields were low for Ay1, By1 and Bo2 so only the remaining proteins (Table 1) were further characterized. Absorption scans show an absorption peak at 480 nm for all proteins which is similar to that of the wild-type RFP maturation intermediate. The mature RFP absorption peak at 559 nm is observed to varying extents (Fig. 2A). Excitation scans of red emission reproduce the 480 nm absorption peak in the mutant proteins (and slightly in the maturing wild-type) in addition to the 559 nm absorption (Fig. 2B). Emission scans of the 480 nm excitation line again show green emission peaking at 500 nm for all proteins and, to varying extents, red fluorescence (Fig. 2C). Just as in the maturation process of wild-type RFP, the observed differences in absorption or fluorescence spectra are additive effects of a limited set of spectroscopically distinct populations and do not affect curve shapes or peak positions.

3.3. Chromophore absorption after denaturation

In order to establish which effects are due to non-covalent interactions of the fluorophore with the protein matrix and which may be due to covalent modifications, we characterized absorption spectra of the unfolded fluorophore for GFP, RFP and the Ao1 mutant. The absorption spectra of the proteins denatured by short heat treatment and guanidinium chloride at different pH are shown in Fig. 3. After 2 min at 85°C the fluorescence of all samples is totally lost and GFP exhibits absorption spectra similar to those published [8] including an isosbestic point at 406 nm (not shown). The spectra of RFP mutant Ao1 are nearly indistinguishable from GFP including the isosbestic point (not shown); only at pH 10.8 a small additional peak around 339 nm is visible. After 2 h of heat treatment, both absorption maxima show a decrease of $8 \pm 2\%$ at pH 6.7 and to $18 \pm 1\%$ at pH 10.8, showing that the two fluorophores are not only spectroscopically similar but also similarly sensitive to alkaline conditions. In comparison the absorption spectra of the denatured RFP are significantly different from GFP and Ao1 since they are red-shifted by 5 nm and 20 nm at low and high pH, respectively. With time, these absorption peaks become more similar to GFP but in contrast to Ao1, differences remain. After 2 h of heat treatment, the RFP absorption maxima show a 22% and a 74% decrease at pH 6.7 and pH 10.8, respectively.

4. Discussion

4.1. RFP chromophore maturation occurs via GFP-like intermediates

During the RFP maturation, two intermediates with absorptions peaking at 408 nm and 480 nm (I408 and I480) are observed (Fig. 1). These resemble the two ground states

Table 1
Mutations in the RFP gene

RFP		V	N	T	P	N	K	N	K	L	K	V	P	Q	Y	T
aa #		0	0	0	0	0	0	0	1	1	1	1	1	1	2	2
		0	0	2	3	4	8	9	3	5	6	7	8	8	1	1
		1a	6	1	7	2	3	8	9	7	6	5	6	8	4	7
B1									R							
B2			D	S							R					
By1			D	S	S						R					
By2	→		D	S			R				/					
By3			D	S			R				R					
Ay1				S			R									
Ay2				S			R	I								
Bo1	→		D	S					R		/	A	S			S
Bo2			D	S						Q	R			P	C	
Bo3	→	M				H			R							
Ao1	→			S												S
Ao2	→															S

The name of the mutant proteins derives from their templates in the mutagenesis (A or B) and their phenotype (yellow or orange). B1 and B2 represent all B templates. The numbering of the amino acid positions is according to the RFP sequence. →: proteins that were studied in detail; /: most likely reverted mutations.

of GFP that possess a protonated and deprotonated phenolic oxygen, respectively (RH and R^-, [9]). Only I480 emits green fluorescence at 500 nm, reminiscent of GFP variants in which the RH state has a very poor quantum yield due to fast internal conversion (our unpublished results).

4.2. Intermediates of chromophore maturation are stabilized by single point mutations

The single point mutations P37S, K83R, N42H and T217S (Table 1) cause the maturation process to terminate at phenotypes with absorption and fluorescence properties which are identical to that of the I480 intermediate. Just as in the wild-type protein red fluorescence develops slowly, but with a strongly decreased amplitude (Fig. 2A). After maturation the absorption spectra remain unchanged for months indicating that the mutations do not introduce a kinetic barrier, but change the equilibrium constant of conversion of the GFP-like fluorophore to the RFP fluorophore.

4.3. The mutations cluster in the folded protein

The sequence alignment of GFP and RFP [3] was used to map the location of the mutated residues to their respective positions on the GFP structure [10] (Fig. 4). At this level of sequence similarity, the precise alignment of loop residues may be unreliable, but the secondary structure elements can be confidently assigned, especially due to the relatively low mutability of core residues. In GFP, the residues A37, N42, F84 and V224 correspond to the RFP mutations P37S, N42H, K83R and T217S, respectively. The resulting structure shows a clustering of the four single point mutations around the C-terminal region of the central α helix near the imidazolidinone ring of the chromophore.

4.4. The intermediate chromophore is GFP-like

The GFP-like spectroscopic properties of I480 in the native state become evident if the chromophores are investigated in the unfolded proteins. The pH dependent absorption spectra (Fig. 3) of the mutant Ao1 are nearly indistinguishable from those of GFP. We observe that the Ao1 protein is completely unfolded after 2 min at 85°C and that the adjacent amino acids do not significantly influence the absorption of the chromophore in the unfolded state. Thus Ao1 is a model with a stabilized intermediate I480 of the RFP maturation and this intermediate is indistinguishable from the GFP fluorophore. This is consistent with the conservation of the chromophore Tyr-Gly motif and of arginine 96 of GFP which is assumed to be essential for the formation of the GFP chromophore [11].

4.5. Denaturation alone does not convert the RFP chromophore into the GFP chromophore

Heat denaturation of RFP in 6 M guanidinium chloride results in two absorption bands with shape and titration behavior similar to that of wild-type GFP. Nevertheless RFP shows significant red-shifts with respect to GFP (5 nm to 20 nm). Further heat treatment reduces the red-shift but simultaneously the peaks decrease and additional peaks appear. Apparently the RFP chromophore is unstable to heat and basic pH. If the maturation process from I480 to RFP comprises

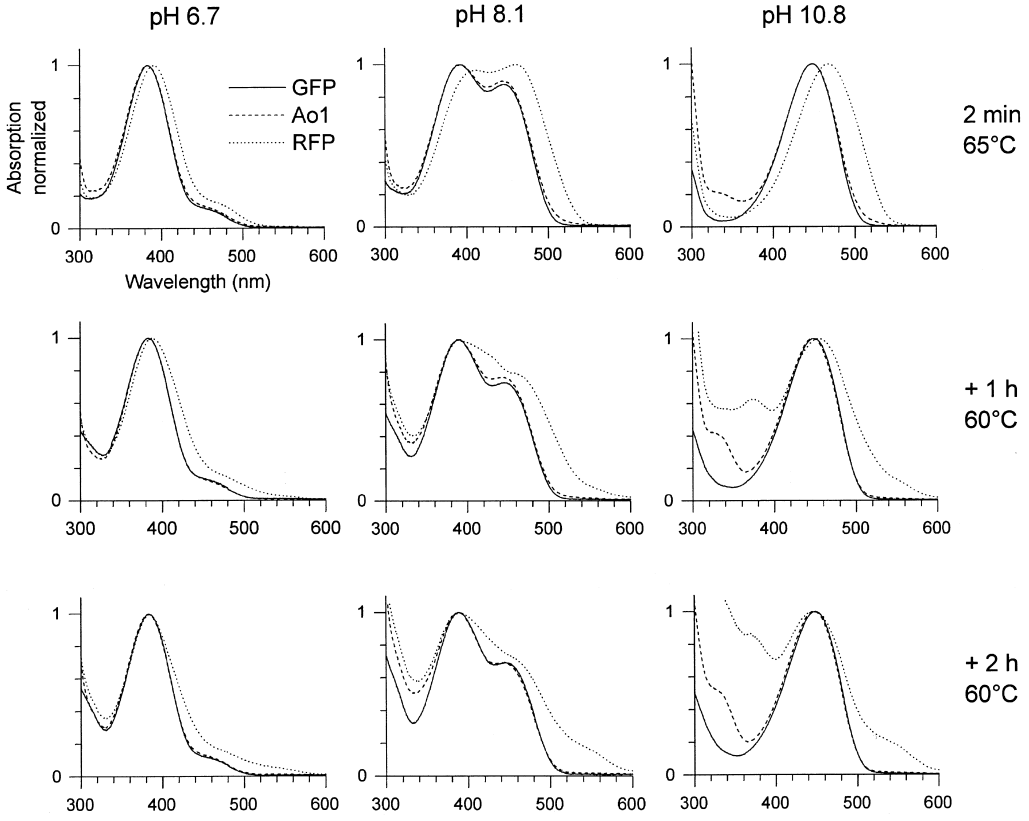

Fig. 3. Normalized absorption spectra of the unfolded proteins GFP, Ao1 and RFP at different pH in 6.0 M guanidinium chloride. Denaturation was performed with short heat treatment (2 min at 85°C). The samples were then heated to 60°C repeatedly.

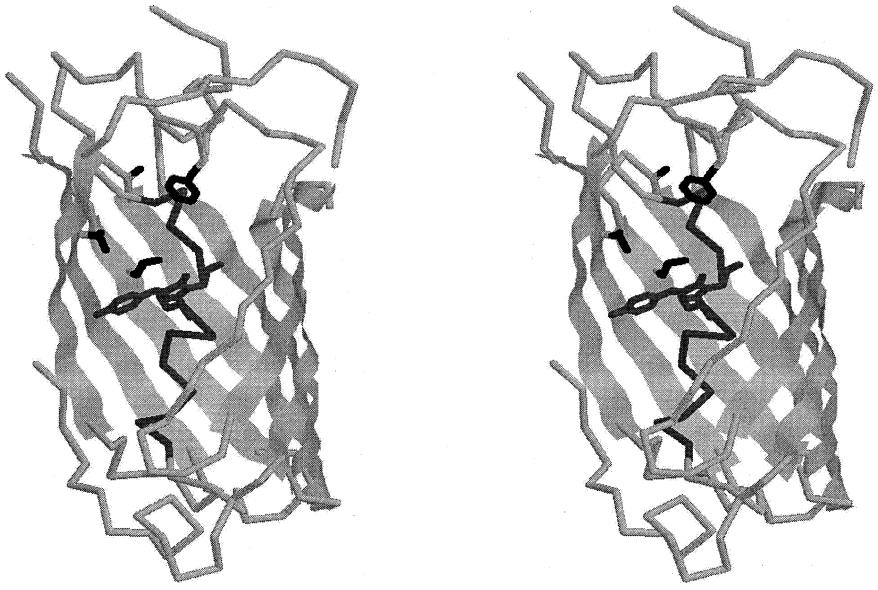

Fig. 4. Stereo view of the GFP structure (1gfl; [9]). The chromophore and the amino acid side chains (at corresponding positions) likely causing the GFP-like phenotypes in RFP are colored black. The central α helix is shaded and residues 147–187 have been removed for visualization.

only additional non-covalent interactions, these interactions would need to persist under the chosen unfolding conditions to explain our observations. By comparison to GFP, the Ao1 denaturation is complete under the experimental conditions chosen. The explanation that the P37S/T217S mutations severely destabilize Ao1 with respect to RFP and thus a partially folded RFP protein matrix may persist under the same unfolding conditions is not consistent with a mutation which merely deletes a single methyl group [12]. Thus the observed difference in absorption spectra of GFP and Ao1 to RFP is most likely explained by a covalent modification that persists (at least partially) in the denatured state and which is chemically unstable to heat and basic pH. In addition, non-covalent interactions with the folded protein matrix may cause a major part of the red-shift, as compared to GFP.

4.6. FRET in heterooligomers of mutant proteins

In all RFP-derived proteins with yellow and orange phenotype, excitation of the 480 nm peak leads not only to green but also to the red emission identical to that of the wild-type 559 nm peak. There are two possible explanations of this phenomenon: excited state proton transfer, such as seen in GFP [9], or fluorescence resonance energy transfer (FRET) from the GFP-like chromophore to the acceptor, the final RFP chromophore [13]. FRET between different GFP variants has been shown [15] and was used to measure the calcium concentrations in cells [16]. (A third possibility of a second fluorophore within the protein seems unlikely from homology considerations and is not further discussed.) Evidence for FRET in maturing RFP has been experimentally demonstrated by kinetic fluorescence measurements and deuteration of the proteins (submitted). For efficient FRET a donor–acceptor distance smaller then the Förster distance (typically 100 Å to 60 Å) is needed [14]. Therefore we predict heterooligomers of the proteins in which both chromophores are present. Since the protein concentrations in the fluorescence measurements were low (0.7–1.3 μM) we expect that the mutant proteins and likely also the wild-type RFP have strong tendencies to oligomerize.

Note added in proof

While this manuscript was under review, further studies on the nature of the RFP fluorophore have been published (Baird, G.S., Zacharias, D.A. and Tsien, R.Y. (2000) Proc. Natl. Acad. Sci. USA 97, 11984–11989; Gross, L.A., Baird, G.S., Hoffman, R.C., Baldridge, K.K. and Tsien, R.Y. (2000) Proc. Natl. Acad. Sci. USA 97, 11990–11995). Recently, the high-resolution structure of dsRFP has been made available (Wall, M.A., Socolich, M. and Ranganathan, R. (2000) Nat. Struct. Biol. 7, 1133–1138. PDB accession code 1GGX).

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