Red fluorescent protein eqFP611 and its genetically engineered dimeric variants

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Abstract. The red fluorescent protein (FP) eqFP611 from the sea anemone *Entacmaea quadricolor* shows favorable properties for applications as a molecular marker. Like other anthozoan FPs, it forms tetramers at physiological concentrations. The interactions among the monomers, however, are comparatively weak, as inferred from the dissociation into monomers in the presence of sodium dodecyl sulfate (SDS) or at high dilution. Analysis at the single-molecule level revealed that the monomers are highly fluorescent. For application as fusion markers, monomeric FPs are highly desirable. Therefore, we examine the monomer interfaces in the x-ray structure of eqFP611 to provide a basis for the rational design of monomeric variants. The arrangement of the four β cans is very similar to that of other green fluorescent protein (GFP-like) proteins such as DsRed and RTMS5. A variety of structural features of the tetrameric interfaces explain the weak subunit interactions in eqFP611. We produce functional dimeric variants by introducing single point mutations in the A/B interface (Thr122Arg, Val124Thr). By contrast, structural manipulations in the A/C interface result in essentially complete loss of fluorescence, suggesting that A/C interfacial interactions play a crucial role in the folding of eqFP611 into its functional form.
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1 Introduction

The green fluorescent protein from *Aequorea victoria* (avGFP) has become very popular in life science research as a protein label, marker of gene expression, and reporter of environmental conditions in living cells.[1–3] A large number of avGFP variants with modified properties have been created by site-directed and random mutagenesis. Efforts to create stable, red fluorescent variants from avGFP have not yet met with success. However, red-emitting fluorescent proteins (FPs) are highly desirable for fluorescent marker applications because of reduced cellular autofluorescence in the red spectral range, the possibility to apply less cytotoxic, longer wavelength excitation light, and their use in multicolor labeling or fluorescence energy resonance transfer (FRET) experiments.

In recent years, fluorescent avGFP homologs were discovered in nonbioluminescent anthozoa species living in a light-dependent symbiosis with zooxanthellae.[4–7] This observation suggested a photoprotective function for these FPs.[8–10] Lately, however, FPs were also found in azooxanthellate anthozoa living in habitats without light stress.[11] Some of the novel anthozoan FPs showed fluorescence in the red spectral region.[4–7] Their tendency to form obligate tetramers, however, hampers their applications as marker proteins.[12] For drFP583 (DsRed), the presently most popular red FP, a monomeric variant was obtained by exchange of 33 amino acids.[13] We have recently cloned and characterized eqFP611, a red FP from *Entacmaea quadricolor*.[14] This protein consists of 231 amino acids and has a molecular weight of ~26 kDa. With an excitation maximum at 559 nm and an emission maximum at 611 nm, it shows the most red-shifted emission and the largest Stokes shift (52 nm) of all nonmodified proteins in the FP family that are presently known. Moreover, its fast and essentially complete maturation and reduced oligomerization tendency makes eqFP611 a viable alternative to DsRed in a variety of applications.

Using site-directed mutagenesis, we have generated stable dimeric variants of eqFP611; the development of monomers is ongoing. In this work, we discuss the connections between the tetramerization tendency and the structural features of the protein.

2 Materials and Methods

2.1 *Mutagenesis*

Site-directed mutagenesis was performed using the QuikChange® Site-Directed Mutagenesis Kit according to the

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manufacturer's protocol (Stratagene, La Jolla, California). The clones were sequenced by a commercial facility (Microsynth, Balgach, Switzerland).

2.2 *Protein Preparation*

eqFP611 was cloned as described.[14] The drFP583 (DsRed) clone was a kind gift from Lukyanov (Russian Academy of Sciences, Moscow); the clone of the avGFP mutant S65T was obtained from Kaether [European Molecular Biology Laboratory (EMBL), Heidelberg]. All proteins were expressed in *E. coli* (BL21 DE3) and purified using a Talon metal affinity resin (BD Biosciences CLONTECH, Palo Alto, California) and gel filtration (Superdex 200, Äkta-System, Amersham Pharmacia, Little Chalfont, United Kingdom). Prior to determination of the apparent molecular weights of the eqFP611 mutants, the column was calibrated using carbonic anhydrase, bovine serum albumin, alcohol dehydrogenase, and blue dextran (all from Sigma-Aldrich, Steinheim, Germany) as standards.

2.3 *Determination of Optical Properties*

Absorbance spectra were recorded on a Cary 1 spectrophotometer (Varian, Darmstadt, Germany) to determine the extinction coefficients of eqFP611 mutants from the ratios of absorbances at 559 and 280 nm. The protein concentration was calculated from the latter absorbance, taking into account the known content of aromatic side chains.[15] Fluorescence excitation and emission spectra were measured on a SPEX Fluorolog II spectrofluorometer (Spex Industries, Edison, New Jersey), with the excitation linewidth set to 0.85 nm. The emission was recorded with 2.2-nm resolution. Fluorescence quantum yields were determined at low optical density (below 0.1 at 560 nm) to avoid reabsorption of the emitted fluorescence. Cresyl Violet (Fluka Chemie GmbH, CH-9471 Buchs, Switzerland) dissolved in methanol was used as a reference with a fluorescence quantum yield of 0.55.[16] Maturation times of the red chromophores were determined as described.[14]

2.4 *Single Molecule Analysis*

eqFP611 molecules were immobilized on glass coverslips coated with polyethylene glycol (PEG) polymer chains. The coverslips were silanized and amino-functionalized with Vectabond™ (Vector Laboratories, Burlingame, California) according to the manufacturer's protocol. Subsequently, they were incubated with a solution of 100 mg/ml PEGs in 50-mM Na_2CO_3 buffer (pH 8.2) for 90 min in the dark. The PEG chains carried a succinimidyl function on one end, which binds to the amino groups on the silanized glass surfaces. In 1% of the chains, the other end was biotinylated to obtain a sparse coverage with biotin anchors for attachment of proteins via a streptavidin-biotin linkage to the PEG-coated surface by using a mixture of 1% biotinylated PEG (Biotin-PEG-NHS MW 3400, Nektar Therapeutics, Huntsville, Alabama) and 99% PEG (mPEG-SPA MW 5000, Nektar Therapeutics). The x-ray structure of eqFP611 shows two surface-exposed cysteines.[17] They were biotinylated using biotin-maleimide (Sigma-Aldrich, Saint Louis, Missouri) according to standard labeling procedures (Hermanson, 1996). For protein attachment, the PEG surfaces were incubated with a 10-μg/ml streptavidin solution (Sigma-Aldrich) and afterward with a 100-pM solution of biotinylated eqFP611 for 10 min each. The protein solution was subsequently flushed out with plain buffer.

Single-molecule experiments were performed with a confocal microscope of our own design. Light from an Ar^+/Kr^+-ion laser (modified model 164, Spectra-Physics, Mountain View, California) was reflected by a dichroic mirror (Q525LP or 575DCXR, AHF, Tübingen, Germany) and focused on the sample with a water immersion objective (UPLAPO 60×1.2 W, Olympus, Hamburg, Germany) in an inverted microscope (Axiovert 35, Zeiss, Göttingen, Germany). The fluorescence emitted by the sample was collected by the same objective, passed through the dichroic mirror, and focused with a lens (f=150 mm) onto a confocal pinhole (80 μm diam). After passing through the pinhole, the light was split into two channels with a 50% beamsplitter and detected by two avalanche photodiodes (SPCM-AQR-14, PerkinElmer, Fremont, California). The observed spectral band was limited by a bandpass filter (HQ 665/170, AHF). The samples were mounted on a piezoelectric scanning stage (Tritor 102 Cap, Piezosysteme Jena, Germany). 18×18-μm^2 sized regions of the sample were scanned with a resolution of 128 ×128 pixels and an integration time of 5 ms/pixel. For the measurement of time trajectories, immobilized protein molecules were localized by scanning; their fluorescence emission was subsequently recorded with 625-ns bin width until photobleaching occurred.

2.5 *Determination of the Crystal Structure of eqFP611*

Crystals of eqFP611 were grown using the hanging-drop vapor-diffusion method. X-ray diffraction datasets were collected from eqFP611 crystals at 100 K using synchrotron radiation at a wavelength $\lambda=1$ Å at the Elettra synchrotron in Trieste, Italy.[17] The crystals diffracted out to 2.5 Å. As an initial model for molecular replacement, we used three polyalanine models of different dimers from the homotetrameric DsRed (pdb code 1GGX[18]), which bears 48.4% sequence identity with eqFP611. Good agreement for both rotation and translation functions for space group $P6_522$ was achieved using the dimer corresponding to the A/C assembly (shown later). The structure was subsequently refined using Refmac.[19] The final model agrees well with a structure of eqFP611 published recently.[20]

3 Results and Discussion

3.1 *Fluorescence Dynamics of Individual, Surface-Immobilized eqFP611 Molecules*

We have recorded the fluorescence emission from individual eqFP611 molecules that were immobilized on PEG surfaces by streptavidin-biotin linkage, using the Ar ion laser line at 514 nm for excitation (average rate 0.14 MHz). Figure 1(a) shows a confocal scan image of immobilized eqFP611 molecules. Typical examples of fluorescence time trajectories of individual molecules over several hundred milliseconds are shown in Fig. 1(c). The emission traces contain extended periods during which the FPs are completely nonemitting (background level) until they finally fall victim to photodestruction; the intensity drops to the background level in a single step, confirming (posthumously) that the emission derived from a

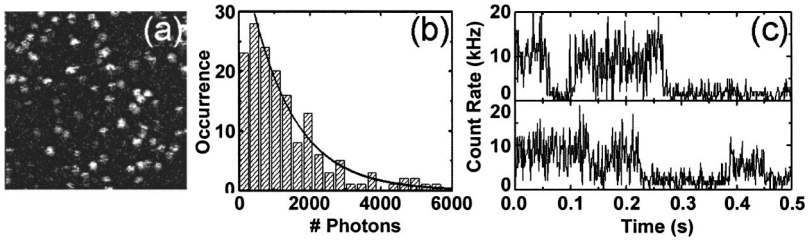

Fig. 1 (a) Confocal scanning microscopy image (128×128 pixels, field of view 18×18 μm^2, 5-ms integration time per pixel, excitation rate 0.14 MHz at 514 nm) of individual eqFP611 molecules attached to a PEG-coated surface. (b) Histogram of the total number of photons collected from 160 individual time traces before photodestruction. An exponential fit is included as the solid line. (c) Typical fluorescence time trajectories of individual eqFP611 molecules (traces recorded with 625 ns and plotted with 1-ms resolution).

single fluorophore. Single-step photobleaching clearly implies that the highly fluorescent, surface-immobilized eqFP611 molecules are monomeric, as observed earlier for eqFP611 immobilized in a PVA gel.[14] By contrast, Lounis and coworkers saw multistep bleaching in experiments with DsRed immobilized in agarose gel, implying that DsRed is oligomeric even under high-dilution conditions.[21] In DsRed, the tetramer can be disrupted by mutations that disturb the sidechain packing in the interfaces between the monomers. The fluorescence quantum yield decreases dramatically in this process, but can be recovered to a certain extent by introducing additional mutations.[13] Our single-molecule studies suggest that the fluorescence of eqFP611 should not markedly suffer from monomerization of the protein.

Figure 1(b) shows a histogram of the observed numbers of emitted photons from 160 eqFP611 molecules. The solid line is an exponential fit, yielding a 1/e decay at 1234±88 detected photons. Estimating the overall detection efficiency of the system as ~5%, this decay corresponds to 24,200 emitted photons. Considering a fluorescence quantum yield of 0.45 at room temperature,[14] we conclude that ~54,000 excitations occur on average before photodestruction, corresponding to a yield of photobleaching, $\Phi_B \approx 1.9\times10^{-5}$. This value is about five-fold larger than the one measured previously with eqFP611 embedded in poly(vinyl alcohol) (PVA).[14] A possible explanation for this discrepancy may be the lower oxygen permeability of PVA.[22]

We have measured the time dependence of the fluorescence emission from many individual eqFP611 molecules tethered to polyethylene glycol (PEG)-coated surfaces under low excitation conditions (0.14 MHz). From the analysis of the single-molecule autocorrelation functions, we have identified two distinct dynamic processes, a fast and a slow flickering process between bright and dark states on time scales of ~300 and ~10 ms, respectively.[22] The slow process is apparent from the extended dark periods in the time traces displayed in Fig. 1(c).

3.2 *Analysis of the Quaternary Structure*

We determined the crystal structure of eqFP611 (Ref. 17). As shown in Fig. 2, eqFP611 exhibits the typical fold of GFP-like proteins.[18,23,24] The monomer is formed by an 11-stranded βcan, with the central helix being interrupted by the fluorophore (Met-Tyr-Gly). The overall structures of the A/B and A/C interfaces linking the monomers are similar to those found in DsRed[25] (Fig. 3). However, differences both in the extension and the composition of the interfaces of eqFP611 explain their unique monomeric functional state at high dilutions. The A/B interface is weakened by the lack of interactions between Glu19, Glu26, and Lys123 (Glu16, Gln23, and Lys120 in eqFP611). The missing salt bridge between the residues corresponding to Glu26 and Lys123 in DsRed is also observed for the blue chromoprotein RTMS5.[24] This protein appears to be predominantly monomeric after a modification of the A/C interface, which suggests weak A/B interactions.

As in DsRed, the A/C interface of eqFP611 is more extended than the A/B interface. The C terminus of the polypeptide chain embraces the neighboring polypeptide chain, which has a stabilizing effect on the A/C interface. Four consecutive amino acids had to be modified in the C-terminal tail of the A/C interface (His222-Leu-Phe-Leu225) during monomerization of DsRed.[13] This region is less extended and modified in eqFP611 (Cys222-Asp-L224) (Fig. 3) and thus may contribute to a lesser extent to interactions stabilizing the A/C interface. A large number of water molecules can be found between the amino acids forming the interface, many of which are involved in mediating hydrogen bonds between the monomers. With Tyr148, Tyr157, Tyr169, Phe192, and Phe194, there is even one more aromatic residue involved in the A/C interface of eqFP611 than in DsRed. However, only three of them (Tyr148, Phe192, and Phe194) are found in locations corresponding to aromatic amino acids in DsRed. To create a monomeric DsRed, the positively charged Arg153 was replaced by the negatively charged Glu residue. Notably, nonpolar residues are present in eqFP611 (Ala150, Tyr169) in place of charged/polar interface residues in DsRed (Arg 153, His 172). These substitutions are likely responsible for the weaker interactions between monomers A and C in eqFP611. The protein surface of the DsRed tetramer is predominantly negatively charged, except for a cluster of positive charges at the N terminus ranging from residue 1 to 20 (isoelectric point pI=11.05). Elimination of these charges by site-directed mutagenesis led to reduced aggregate formation, presumably by preventing basic-acidic surface interactions between monomers.[13] In eqFP611, with pI=6.4 for the corresponding first 17 residues, the N-terminal charge is smaller at physiological pH than in DsRed, which may have the same effect as the artificial charge reduction in other anthozoan FPs.[26]

3.3 *Mutagenesis*

A weakly fluorescent, tetrameric avGFP-homolog from the sea anemone *Heteractis crispa*, has been reported earlier.[27] A

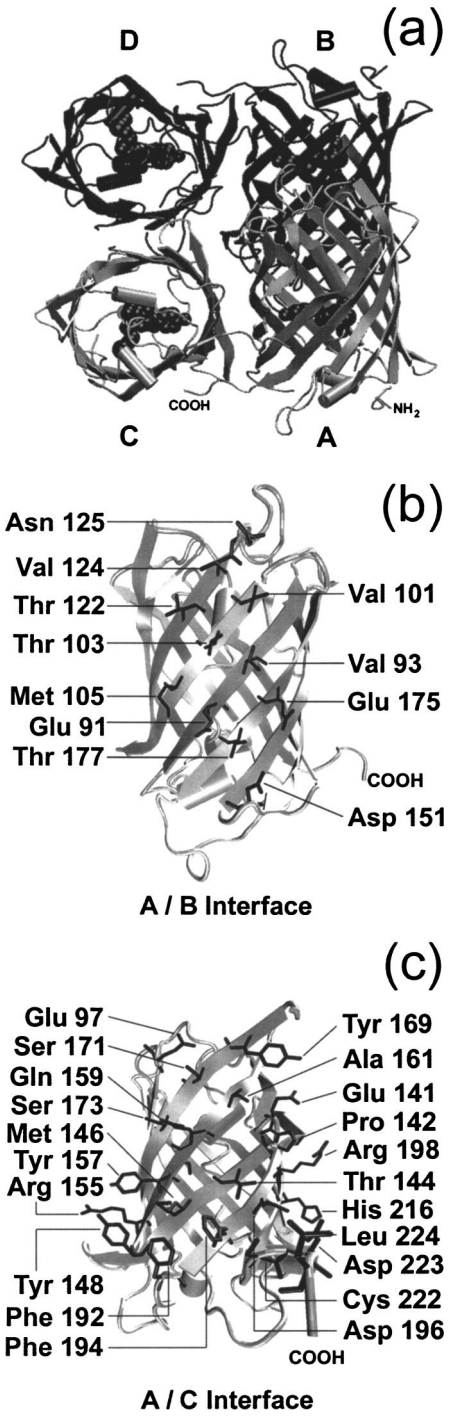

Fig. 2 Structure of eqFP611. (a) Tetrameric organization shown as ribbon diagrams. Monomers are labeled A through D according to Ref. 22. (b) View of the A/B interface, with the residues building the interface displayed in stick representation. (C) View of the A/C interface.

	dsRed	eqFP611
A / B Interface	E19	E16
	E26	Q23
	E94	E91
	V96	V93
	V104	V101
	T106	T103
	T108	M105
	K123	K120
	I125(R)	T122(R)
	V127(T)	V124(T)
	N128	N125
	D154	D151
	K178	E175
	I180	T177
A / C Interface	E100	E97
	E144	E141
	A145	P142
	T147	T144
	R149	M146
	Y151	Y148
	R153(E)	A150
	K158	R155
	E160	Y157
	H162(K)	Q159
	A164(R)	A161
	H172	Y169
	L174(D)	S171
	E176	S173
	Y192(A)	F192
	Y194(K)	F194
	D196	D196
	K198	R198
	R216	H216
	E218	V218
	H222(S)	C222
	L223(T)	D223
	F224(G)	L224
	L225(A)	P225

Fig. 3 Alignment of interface residues involved in the formation of tetramers of the red fluorescent proteins dsRed and eqFP611. The numbering of the amino acids equals the position in the amino acid sequence specific for each protein. Interacting residues in the respective protein are underlayed in gray. Data for dsRed are taken from Ref. 24. For eqFP11, residues within a distance of 3.6 Å to the adjacent monomer are assumed to participate in the tetrameric interactions.

dimeric variant, commercially available as HcRed, was generated by exchanging a leucine by a histidine in the A/B tetrameric interface. This residue corresponds to Ile125 in DsRed.[18,23,25] Along with the mutation Val127Thr, the substitution Ile125Arg helped create a dimeric form of DsRed. In view of the comparatively weak interactions in the A/B interface of eqFP611, we anticipated that introducing these mutations at the corresponding sequence positions in eqFP611 should suffice to obtain a dimeric variant. Indeed, either of the two mutations (Thr122Arg, Val124Thr) yielded highly fluorescent proteins. They migrated in size exclusion chromatography experiments with apparent molecular weights of ~40 kDa, which is intermediate between the values determined for

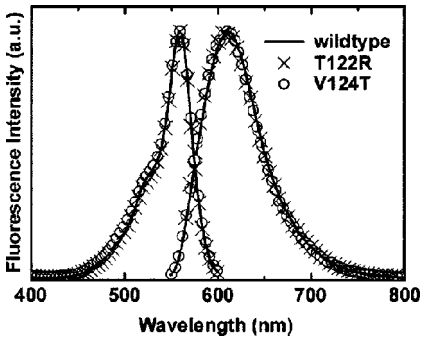

Fig. 4 Excitation (left) and emission (right) spectra of the wild-type eqFP611 and the dimeric mutants T122R and V124T. The excitation spectrum was measured by recording the emission at 611 nm; the emission spectra were taken with excitation at 559 nm.

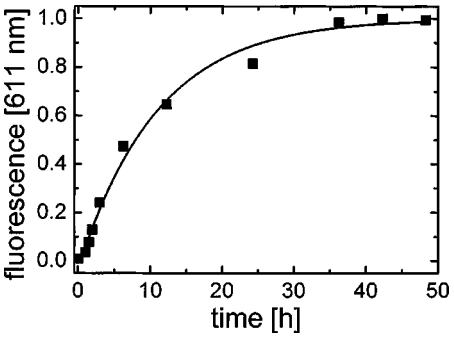

Fig. 5 *In-vitro* maturation of dimeric mutant eqFP11 V124T. After ~7.5 h at 21°C, the red fluorescence has increased to 50% of its maximum amplitude.

the tetrameric DsRed and eqFP611 (~66 kDa) on the one hand and the monomeric avGFP (~27 kDa) on the other hand. Therefore, we inferred that both mutants are true A/C dimers. Note that the apparent molecular weights of the dimeric and the tetrameric forms are smaller than expected (dimer ~50 kDa, tetramer ~100 kDa). This behavior may arise from the dense packing of the monomers within the quaternary assembly (Fig. 2). Absorption and fluorescence spectra were determined for wild-type eqFP611 and the dimeric mutants T122R and V124T. Figure 4 shows excitation (left) and emission (right) spectra. The positions of the peaks, extinction coefficients, and quantum yields are compiled in Table 1. Also included are the maturation half-times of the red chromophore. In Fig. 5, we present a typical increase in fluorescence at 611 nm as a function of time during maturation of a purified protein sample in a spectrometer cuvette. It is apparent from Table 1 that the spectroscopic parameters are not appreciably affected by dimerization. In contrast, the dimeric DsRed mutant Ile125Arg became poorly red fluorescent and took more than 10 days to fully mature.[28] It required a total of 17 mutations to generate the improved variant dimer2 (Table 1).[13] Considering the similar interface structures of DsRed and eqFP611, we assumed that the A/C interface mutations established in the monomerization of DsRed would guide us toward a monomeric eqFP611. Thus, we introduced the following mutations into the A/C interface of the eqFP611 tetramer to test their efficacies in producing A/B dimers: Phe192Ala, Phe194Ala, Ala161Arg, Gln159Arg, and Cys222Ser. Moreover, a variant with a truncated and modified C terminus was generated: Phe221His-Cys222Ser-Asp223Gly -Leu224-Pro225del-Ser226del-Lys227del-Leu228 del-Gly229del-Arg230del-Leu231del (del denotes residues removed from the sequence). As eqFP611, in contrast to other anthozoan FPs, can exist as a fully functional monomer under mildly denaturing conditions or at high dilutions,[14] we expected that a monomerization based on interface modification should yield fluorescent monomers. To our surprise, however, all manipulations in the A/C interface resulted in essentially complete loss of fluorescence. Only on extended incubation of the expressing bacteria at low temperature (2 to 3 weeks at 4°C) could weak fluorescence be observed from these proteins.

Possible explanations for the catastrophic decrease in the fluorescence could be destabilization of the fluorophore environment within the monomers or the lack of stabilizing interactions between neighboring monomers. However, these scenarios appear unlikely for several reasons. Subunit

Table 1 Spectroscopic properties of dsRed, eqFP611, and their mutants.

Protein	Absorption λ_{max} [nm]	Emission λ_{max} [nm]	Extinction coefficient [M^{-1} cm^{-1}]	Quantum yield	Maturation half-time $t_{0.5}$ [h]
dsRed (Ref. 13)	558	583	57.000	0.79	~10 (37.0°C)
dsRed dimer 2 (Ref. 13)	552	579	60.000	0.69	~2 (37.0°C)
dsRed mRFP1 (Ref. 13)	584	607	44.000	0.25	<1 h (37.0°C)
eqFP611	559	611	78.000	0.45	4.5 (24.5°C)
eqFP611 T122R	559	611	84.000	0.39	7.5 (21.0°C)
eqFP611 V124T	559	611	74.000	0.42	7.5 (21.0°C)

interactions are already rather weak in eqFP611, which leads to spontaneous dissociation into monomers at high dilution, as is shown, for example, in our studies of immobilized eqFP611.[14] The bright fluorescence from these monomers clearly proves that the fluorophore environment is not *eo ipso* altered by monomerization. The detergent SDS (1%) also induces dissociation of the eqFP611 tetramer into its subunits, yet the monomers are still highly fluorescent even though SDS is known to destabilize proteins.[14] We note that the β can of eqFP611 is a thermodynamically particularly stable fold. From these findings, we believe that properly folded, functional monomers of eqFP611 are stable proteins, comparable to avGFP. Consequently, the observed loss of fluorescence during mutagenesis of the A/C interface is most likely the result of the inability of the polypeptide chains to assume their proper, functionally competent native structure. The chromophoric properties of the A/B dimers are essentially identical to those of the natural protein, and therefore, the A/C interactions could play an important role in the folding dynamics of the monomers. Therefore, our current work focuses on identifying mutations that facilitate proper folding of the polypeptide chain.

3.4 *Biological Significance and Evolution of the Tetrameric Structure*

The data presented here suggest that tetrameric interactions, especially A/C interactions, assist in the proper folding of eqFP611. The participation of these comparatively weak interactions in folding might also be indicated by the finding that functional eqFP611 expression in mammalian cells is only possible up to 30°C:[14] with increasing temperature, the already weak interfacial interactions will be further weakened for entropic reasons. If these interactions are indeed crucial for proper folding, expression of functional eqFP611 will become impossible above a certain temperature. For the sea anemone *Entacmaea quadricolor*, from which eqFP611 was isolated, thermosensitivity is probably without biological significance, as temperatures around 30°C are rarely exceeded in its habitats in the Red Sea and the Indopacific.[29]

A variant of the weakly dimerizing GFP from *Aequorea victoria* from the colder, Northwestern Pacific waters was transformed by altering a few amino acids into a true monomer that folds properly at 37°C.[30] This proves that the proper β-can fold can, in principle, be obtained without the assistance of subunit interactions also at higher temperatures. Given the great sequence variability of GFP-like proteins found in nature,[31] a monomeric structure could have easily evolved by natural mechanisms. The majority of nonbioluminescent, anthozoan GFP-like proteins, however, exist as tight tetramers.[25] One may, therefore, speculate that this feature evolved because it can fulfill a special biological function. Campbell et al. proposed that the chromophores in the tetramers are better protected from photobleaching under strong tropical light.[13] This assumption is in agreement with a photoprotective function of these proteins that was suggested earlier for anthozoans living in symbiosis with unicellular algae (zooxanthellae).[8,9] However, red fluorescent *Discosoma* species, from which the strongly tetramerizing DsRed was isolated, settle typically in lower light habitats in nature and, furthermore, suffer from high light intensities in captivity.[32,33] Moreover, we demonstrated recently that tetrameric GFP-like proteins also occur in temperate, azooxanthellate anthozoa species living in habitats with low or no light stress.[11] Therefore, no clear picture exists concerning the biological function(s) of GFP-like proteins and the mechanisms of how, for example, a photoprotective effect is achieved in some cases. The tetrameric structure, however, probably plays an important role.

4 Conclusions

The overall structures of the interfaces between monomers within the eqFP611 tetramer are similar to those observed in DsRed. Several characteristic differences in the interfacial amino acid residues explain the weak tetrameric interactions of eqFP611, which greatly facilitate the generation of functional dimeric variants by the exchange of single amino acids (Thr122Arg, Val124Thr). As of yet, attempts to break up the A/C interface by amino acid replacement have resulted in essentially nonfluorescent variants. We suggest that A/C interfacial interactions in eqFP611 play a crucial role in the proper folding of the β can structure. Further amino acid substitutions will have to be introduced to increase the yield of properly folded monomers. These studies are currently under way; they will likely be helpful for developing general strategies of monomerization of other GFP-like proteins.

Acknowledgments

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