Cracks in the β-can: Fluorescent proteins from *Anemonia sulcata* (Anthozoa, Actinaria)

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We characterize two green fluorescent proteins (GFPs), an orange fluorescent protein, and a nonfluorescent red protein isolated from the sea anemone *Anemonia sulcata*. The orange fluorescent protein and the red protein seem to represent two different states of the same protein. Furthermore, we describe the cloning of a GFP and a nonfluorescent red protein. Both proteins are homologous to the GFP from *Aequorea victoria*. The red protein is significantly smaller than other GFP homologues, and the formation of a closed GFP-like β-can is not possible. Nevertheless, the primary structure of the red protein carries all features necessary for orange fluorescence. We discuss a type of β-can that could be formed in a multimerization process.

Green fluorescent proteins (GFPs) were described for a number of bioluminescent cnidarians (1–6). Used as *in vivo* marker in cell, developmental, and molecular biology, the GFP cloned from the jellyfish *Aequorea victoria* became one of the most famous research tools of recent years (7–9). In addition to the use of GFP, many efforts were spent on engineering this protein. To broaden the field of use, one focal point of this research was the shift of the emission maximum toward longer wavelengths. These studies yielded a number of spectral variants from which the most red-shifted mutants emit in the yellow-green region of the spectrum (10, 11). Despite these efforts, the generation of a reddish emitting mutant was not achieved.

We decided to pursue another strategy. Therefore, we searched for naturally occurring sources of spectral variants that are suitable for comparative studies of the structural basis of fluorescence. GFP and its homologues generally were believed to emit greenish light and to serve as secondary emitters in bioluminescent cnidarians (12). Mainly green and orange fluorescence from unidentified pigments was described for various nonbioluminescent cnidarians (13–20). The green and orange fluorescent pigments we found in the color morphs of the Mediterranean sea anemone *Anemonia sulcata* revealed quite similar properties as GFP from *A. victoria*. Based on these findings, we hypothesized a widespread occurrence of GFP and related proteins with different colors in nonbioluminescent cnidarians (21). Most recently, this view was confirmed by the cloning of six fluorescent proteins from nonbioluminescent Anthozoa homologous to the GFP from *A. victoria* (22).

Our interest was focused on the morph var. *rufescens* of *A. sulcata* (23). The tentacles of this morph exhibit a bright green and orange fluorescence and a nonfluorescent reddish color in the tips of its tentacles. We assumed that these pigments protect this morph from bright sunlight and particularly from UV radiation (24).

Methods

Purification of Proteins from *A. sulcata* var. *rufescens*. Samples of tentacles containing one of the different colored proteins were obtained by dissection under UV light. Samples were homogenized with a Sonifier (Branson) in 0.1 M phosphate buffer at pH 6.8, incubated at 65°C for 2.5 min, and centrifuged at 100,000 $\times$ *g* for 1 h. The colored proteins in the supernatant were purified by adding isopropanol step by step. Ten percent of the original sample volume isopropanol was added per step. After each step, samples were centrifuged at 27,000 $\times$ *g* for 5 min. The presence of the colored proteins in the pellet was monitored visually under UV and visible light. AsFP595 and asCP562 precipitated mainly at a final concentration of 50% and asFP499 at a final concentration of 70% isopropanol. Pellets containing most of the pigments were dissolved in a small amount of phosphate buffer (pH 6.8), loaded on a sucrose gradient (40%/15% Saccharose in 0.1 M phosphate buffer), and centrifuged at 18°C for 12 h at 100,000 $\times$ *g*. Fluorescent/colored bands were soaked, and proteins were precipitated as described above. Pellets were dissolved in 0.1 M phosphate buffer containing 1% SDS, 20 mM β-mercaptoethanol, and 10% glycerol. Samples were loaded on an agarose gel (gel: 0.5% agarose, 0.5% SDS in 0.1 M phosphate buffer, pH 6.8; running buffer: 0.5% SDS in 0.01 M phosphate buffer, pH 6.8). Fluorescent/colored bands were cut out and transferred to a polyacrylamide gel (gel: 6% polyacrylamide, 0.5% SDS in 0.1 M phosphate buffer, pH 6.8; running buffer: 0.5% SDS in 0.01 M phosphate buffer, pH 6.8). Fluorescent/colored bands were cut out again and powdered in liquid nitrogen. Proteins were denatured completely by adding 0.625 M Tris·HCl, pH 6.8 supplied with 10% β-mercaptoethanol (14.3 mol/liter), 1% SDS, and 10% glycerol and heating at 99°C for 5 min. Afterward, SDS/PAGE was performed as described (25).

Gel Filtration. Apparent molecular weights of the protein pigments were determined by using a SMART system and Superdex 75 column (Amersham Pharmacia) (0.05 M phosphate buffer, 100 mM NaCl, pH 7). Clarified raw extracts were diluted 1:50 (final protein concentration $<$0.5 mg/ml) in 0.05 M phosphate buffer, 100 mM NaCl, pH 7 (physiological conditions) or in 6 M guanidine hydrochloride (denaturing conditions). Target proteins were detected by measuring the absorption at 480, 562, and 574 nm.

Spectroscopy. Partially purified proteins in aqueous solutions were used for analysis. Excitation/emission spectra were determined with a fluorescence spectrometer (Spex Industries, Edison, NJ). Absorption spectra were measured with a spectro photometer (Cary 1, Varian).

Induction of Fluorescence. Partially purified proteins asFP595 and asCP562 were denatured completely, and SDS/PAGE was performed as described above. Proteins were transferred to a nitrocellulose membrane (NC 45, Serva) by using a modification of the semidry blotting procedure described by Khyse-Anderson (26). The buffers used for blotting were: anode buffer I (0.3 M Tris/10% methanol, pH 10.4); anode buffer II (0.025 M Tris/10% methanol, pH 10.4), and cathode buffer (0.025 M Tris/20%

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Abbreviation: GFP, green fluorescent protein.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF322221 and AF322222).

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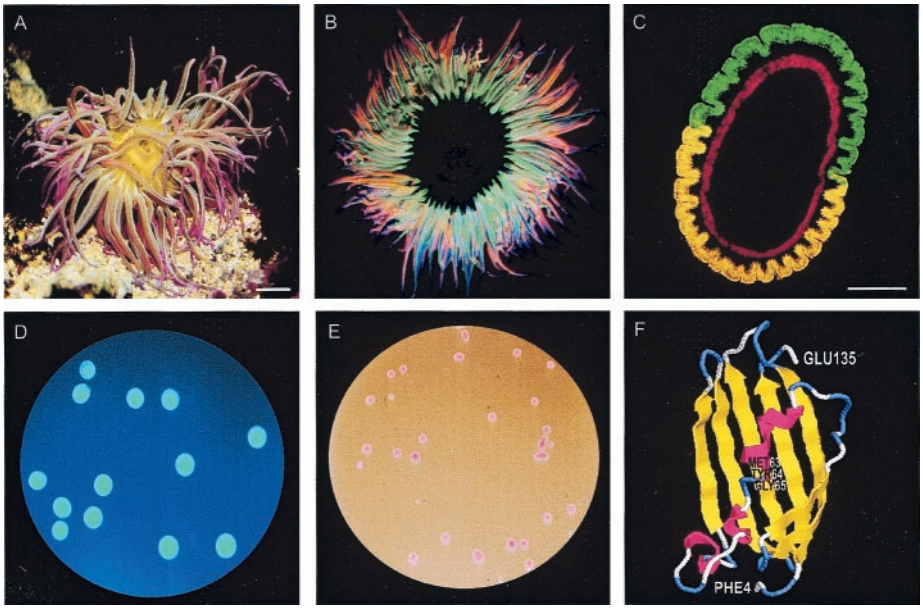

Fig. 1. Pigments of *A. sulcata* var. *rufescens*. (*A*) Localization of GFPs in the upper side, orange fluorescent protein in the underside, and reddish protein in the tips of tentacles under daylight (scale bar: 1 cm) and UV (366 nm) (*B*). (*C*) Kryo-section (20 μm) of a tentacle fixed in seawater/4% paraformaldehyde irradiated with UV (365 nm) shows the ectodermal localization of the fluorescent proteins. The orange fluorescent protein located in the underside of the tentacle shows a yellow-shifted emission. This shift most likely is induced by the fixation. The red fluorescence of the entoderm is produced by chlorophyll of zooxanthellae (scale bar: 0.5 mm). (*D*) Agar plate with colonies of *E. coli* expressing asFP499 under UV light (366 nm). (*E*) *E. coli* expressing asCP562 under daylight. (*F*) Schematic model of asCP562 tertiary structure (residues 4–135) obtained from comparative protein modeling. Yellow: β-strands; red: helix; white/blue: loops. With a probability of 77%, the model describes a structure with a relative mean standard deviation lower than 5 Å compared with the corresponding control structure (37–39). The residues Met-63–Tyr-64–Gly-65 in the middle of the distorted helix stand for the putative chromophore. Figure was produced with RASMOL (40).

methanol/0.04 M ε-amino-*n*-caproic acid, pH 9.4). The fluorescent proteins on the nitrocellulose membrane were excited with broadband UV radiation. Nitrocellulose membranes were photographed by using a 610-nm long-pass filter.

Emission spectra of the induced fluorescence were examined directly on the nitrocellulose membrane with a fluorescence spectrometer (Spex Industries).

Construction and Screening of the cDNA Library. Tentacles were homogenized in TriPure (Roche Diagnostics), and total RNA was isolated as described by the manufacturer. Total RNA was DNase I (Roche Diagnostics)-digested for 0.5 h at 37°C, and mRNA was isolated by using an mRNA Isolation Kit (Roche Diagnostics). Purified mRNA was Dnase I-digested once again and phenol-extracted. The cDNA library was constructed by using a ZAP Express cDNA Synthesis Kit and Gigapack III Gold Cloning Kit (Stratagene) following the protocol. The phage library was transformed into a phagemid library by using the mass excision protocol of the supplier. Transformed *Escherichia coli* (XLOLR strain supplied with the kit) was plated on agar supplied with kanamycin and isopropyl β-D-thiogalactoside. Agarose plates were incubated overnight at 37°C and placed at 4°C for 48 h afterward. The plates were inspected visually on a UV screen and under visible light to pick fluorescent/colored colonies for further analyses.

Proteins were extracted from *E. coli* by repeated freeze and thaw cycles in phosphate buffer. Incubation of the samples at 4°C for 48 h increases the yield of dissolved protein. Cell debris was centrifuged down at 50,000 $\times$ *g* for 1 h. The clarified supernatant was used for further analysis.

Results and Discussion

Characterization of the Colored Proteins. *Localization.* Because of their content of protein pigments, the tentacles of *A. sulcata* var. *rufescens* exhibit three hues under daylight conditions. The upper side is green, the underside is orange, and the tips of tentacles show a vivid reddish color (Fig. 1*A*). The green and the orange pigments exhibit a bright fluorescence under irradiation with UV light at 366 nm (Fig. 1*B*). In a few specimens, two opposite spots at the mouth and the verrucae of the column also fluoresce in orange. The reddish protein of the tips is nonfluorescent. The protein pigments all are located in the ectoderm of the tentacles (Fig. 1*C*).

Spectral properties. The spectral properties are summarized in Table 1. The green fluorescence of partially purified protein solutions is characterized by an excitation spectrum with two maxima at 480 nm and 511 nm and a shoulder around 400 nm. A region of high excitation is found in the UV spectrum. The emission spectrum shows two maxima at 499 nm and 522 nm (Fig. 2*A*). The ratio between the emission maxima varies in dependence of the excitation wavelength. Such a variance also is found in samples derived from different specimens (data not shown). This points to the existence of two different forms of GFP in the tissue with excitation/emission maxima at 480 nm/499 nm and 511 nm/522 nm. The orange fluorescence distinguishes itself by three excitation maxima at 278 nm, 337 nm, and 574 nm and a single emission peak at 595 nm (Fig. 2*A*). In the visible region the maximum of absorption of the nonfluorescent red protein is at 562 nm (Fig. 2*C*).

According to the nomenclature introduced by Matz *et al.* (22) the fluorescent proteins from *A. sulcata* var. *rufescens* were named asFP499, asFP522, and asFP595. The nonfluorescent red protein from *A. sulcata* var. *rufescens* was named asCP562. In this context, CP stands for colored protein and the number identifies the major absorption maximum.

Molecular masses. The fluorescence or color of the proteins were used as an indicator for the presence of the pigments in the process of purification. All proteins were purified to near homogeneity. Both asFP499 and asFP522 could not be separated by the purification steps. The molecular masses, determined by

Table 1. Properties of the pigments

Protein name	Determined molecular mass, kDa	Calculated molecular mass, kDa	Number of amino acids	Absorption/excitation maxima, nm	Emission maxima, nm
asFP499/522	26.2*/23.0†/66.0‡	—	—	<280/≈400/480/511	499/522
asFP499 (cloned)	26.2*	25.4	228	278/403/480	499
asFP595	19.1*/66.0†‡	—	—	278/337/574	595
asCP562	19.1*/66.0†‡	—	—	562	595§
asCP562 (cloned)	19.1*/66.0†‡	16.5	148	562	595§

*Determined by SDS/PAGE.
†Determined by gel filtration under denaturing conditions.
‡Determined by gel filtration under physiological conditions.
§After a denaturation-renaturation process.

SDS/PAGE, are 26.2 kDA for the mixed fraction of asFP499/asFP522 and 19.1 kDa for both asFP595 and asCP562 (Fig. 3 *A* and *B*). In gel filtration experiments under physiological conditions all protein pigments show apparent molecular masses of 66 kDa (Fig. 3.1 and 3.3). For the GFPs this indicates a natural occurrence as dimers or trimers (Fig. 3.3). Under denaturing conditions these oligomers can be partially split into monomers with apparent molecular masses of 23 kDa (Fig. 3.4). Stable dimers also are reported for GFP from *Renilla* and some other pennatularians (12, 27).

Stability. All colored proteins show a remarkable stability after treatment with heat, detergent, chaotrop, reducing agent, and pH extremes (Table 2). Thermostability of asFP595 fluorescence is clearly increased compared with that of asFP499/asFP522. In contrast, fluorescence of asFP499 is more stable when the solutions are exposed to 1% SDS, 8 M urea, and pH 11. Fluorescence of asFP499/asFP522 and asFP595 is also stable when the proteins are treated with 4% paraformaldehyde. Overall, the stability of the pigments from *A. sulcata* is comparable to that of the GFPs from *Aequorea* and *Renilla* (27–30).

Reversible denaturation. Partially purified extracts of the proteins asFP595 and asCP562 were heat-denatured in the presence of 2% SDS and 10% β-mercaptoethanol. Proteins were separated by SDS/PAGE and transferred to a nitrocellulose membrane by semidry blotting. On the membrane, the band corresponding to asFP595 appears red under daylight conditions and exhibits orange fluorescence with an emission maximum at 595 nm under UV light (Fig. 4 *A* and *C*). The fluorescence is stable and can even be detected after passage of the renatured protein through the membrane because of extended blotting. Renaturation with full restoration of fluorescence in the presence of β-mercaptoethanol has been described for heat-denatured GFP (31). Surprisingly, asCP562, which is nonfluorescent *in vivo*, behaves in the same manner and shows identical fluorescence as renatured asFP595 (Fig. 4 *A* and *C*).

Cloning of the Proteins. A cDNA library was constructed from mRNA derived from tentacles of *A. sulcata* var. *rufescens.* The deduced phagemid library was expressed in *E. coli*, and about 10^6 clones were visually screened for the target proteins. On average, one colony of 700 showed green fluorescence under UV light (Fig. 1*D*). This ratio reflects the high content of GFPs in the tentacles of the sea anemone. By examination of the agar plates under daylight, 17 nonfluorescent red colonies could be detected (Fig. 1*E*). The low yield most easily can be explained by the less-sensitive screening method. The excitation spec-

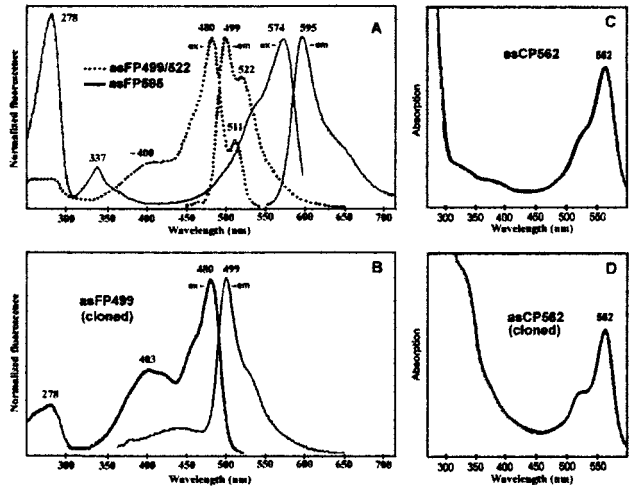

Fig. 2. Excitation (ex)/emission (em) spectra of fluorescent proteins isolated from *A. sulcata* (*A*) and cloned in *E. coli* (*B*). Absorption spectra of the red protein isolated from the animal (*C*) and cloned in *E. coli* (*D*).

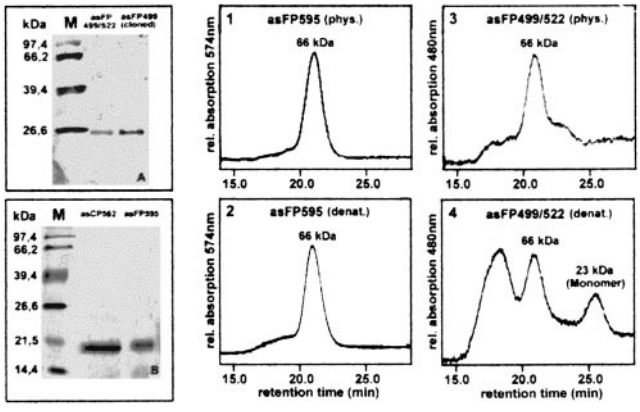

Fig. 3. Molecular masses of the proteins. (*A*) The purified GFPs asFP499 and asFP522 isolated from *A. sulcata* migrate as one band in SDS gels (silver staining). Cloned asFP499 has the same molecular mass. (*B*) The nonfluorescent red protein asCP562 and the orange fluorescent protein asFP595 show identical molecular masses. (Part 1) Separated chromatographically on a Superdex 75 column, the proteins asFP595 and asCP562 isolated from the animal and cloned asCP562 exhibit identical elution profiles. The elution profiles of asFP595 is representative for these proteins. All proteins show apparent molecular masses of 66 kDa under both physiological conditions (0.05 M phosphate buffer, 100 mM NaCl, pH 7) and denaturating conditions (6 M guanidine hydrochloride) (part 2). (Part 3) The proteins of asFP499/522 show an apparent molecular mass of 66 kDa under physiological conditions, which indicates a natural occurrence of dimers or trimers. (Part 4) Under denaturing conditions three peaks can be observed. The first peak contains high molecular aggregations. The 66-kDa peak corresponds to oligomers of asFP499/522. The fraction with an apparent molecular mass of 23 kDa indicates asFP499/522 monomers.

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Table 2. Stability of color and fluorescence of the pigments isolated from *A. sulcata*

Treatment	65°C/5 min	80°C/5 min	95°C/2.5 min	1% SDS	8 M Urea	pH 5.5	pH 11	200 mM β-Mercaptoethanol	Mineral oil	4% Paraformaldehyde
asFP499/522*	+++++	+	+	+++ (502 nm)†	+++++	+++	++ (509 nm)†	+++++	++++	(d.)
asFP595*	+++++	++++	++	++	++++ (600 nm)†	+++	+	+++++	+++++	(d.)
asCP562	(d.)	(d.)	(d.)	(d.)	(d.)	(d.)	(n.d.)	(d.)	(d.)	(d.)

(d.), Color detectable; (n.d.), color not detectable.

*Values are given as % of the peak maximum as compared to untreated samples: +++++ = >90%; ++++ = 75–79%; +++ = 30–35%; ++ = 15–20%; + = <10%.

†Shifts of emission maximum caused by the treatment, points of reading.

trum of the cloned GFPs are characterized by maxima at 278 nm, 400 nm, and 480 nm. The emission spectrum shows a single peak at 499 nm (Fig. 2*B*). These maxima also are found in the corresponding spectra of asFP499/asFP522 isolated from the animal (Fig. 2*A*). The cloned asFP499 migrates with the same mobility in SDS gels as asFP499/asFP522 derived from the sea anemone (Fig. 3*A*).

The absorption maximum at 562 nm of the red protein, expressed in *E. coli*, matches that of asCP562 isolated from *A. sulcata* (Fig. 2 *C* and *D*). Fluorescence of cloned asCP562 on nitrocellulose membranes with an emission maximum at 595 nm can be induced as described for asFP595 and asCP562 isolated from the sea anemone (Fig. 4 *A* and *C*). Furthermore, Fig. 4*A* shows that cloned asCP562 has the identical molecular mass as asFP595 and asCP562. With 66 kDa under both physiological and denaturing conditions, the apparent molecular mass also is the same as determined for asFP595 and asCP562 (Fig. 3.1 and 3.2). Because of uncertainties in purification and folding of the recombinant proteins, reliable extinction coefficients and quantum yields are not yet available.

Primary Structure. Based on the primary structure, asFP499 consists of 228 aa and has a calculated molecular mass of 25.4 kDa. With 148 aa and a calculated molecular mass of 16.5 kDa asCP562 is significantly smaller than other GFP homologues, which range between 25.4 and 30.4 kDa (22). The protein sequences were aligned with GFP and its homologues (7, 22) by using the CLUSTAL method (32) (Fig. 5). Based on this alignment, the identity of the new proteins with GFP reaches from 18.0% (asFP499) to 24.8% (asCP562), identifying them as distant homologues. The N termini of both asFP499 and asCP562 are 3 aa shorter than GFP and its homologues. The relationship of all known GFP homologues including asFP499 and asCP562 evaluated by an multiple alignment suggests that colors of fluorescence evolved independently from one fluorescent ancestor protein (see supplemental material and Fig. 6, which are published as supplemental data on the PNAS web site, www.pnas.org). Key secondary structure elements of the β-can fold of GFP (10, 22, 33) seem to be well conserved. This conservation includes altering β- and turn regions and stretches forming the caps (positions 82–91 and 129–140). Compared with GFP, the region of asFP499 from the N terminus up to position 140 is better conserved than the rest of the sequence. The sequence of asCP562 from the N terminus up to residue 135 also is well conserved. In contrast, it totally lacks the positions 136–214, which comprise the β-strands 7–10. However, the C-terminal region corresponds to the C terminus of GFP including β-strand 11. Based on the alignment and the putative tertiary structure obtained from comparative protein modeling (Swiss model; refs. 37–39), we assume that the protein asCP562 forms a kind of "semi-β-can" with at least six β-strands (Fig. 1*F*). For asFP499 the "classic" β-can fold can be expected. In GFP, the tripeptide Ser-65–Tyr-66–Gly67 forms the fluorophore (41–43). A catalytic function in fluorophore formation is attributed to Arg-96 (10). The residues 66 and 67 are conserved in GFP homologues, whereas Ser-65 is replaced by Gln, Lys, or Arg (22). Some mutational changes of Ser-65 in GFP also are tolerated (43, 44). In asFP499 and asCP562 the strict conservation of Tyr-66, Gly-67, and Arg-96 can be confirmed. Position 65 is altered to Gln or Met. Deduced from the alignment and the functional expression in *E. coli*, formation of the asFP499 fluorophore and the asCP562 chromophore basically seems to be the same as in GFP.

One of the features that allows GFP-like proteins to be used as *in vivo* markers is the ability to form their fluorophores autocatalytically in absence of other factors except molecular oxygen (8, 45). Our results suggest that additional mechanisms can be involved in the formation of the fluorescent state of GFP

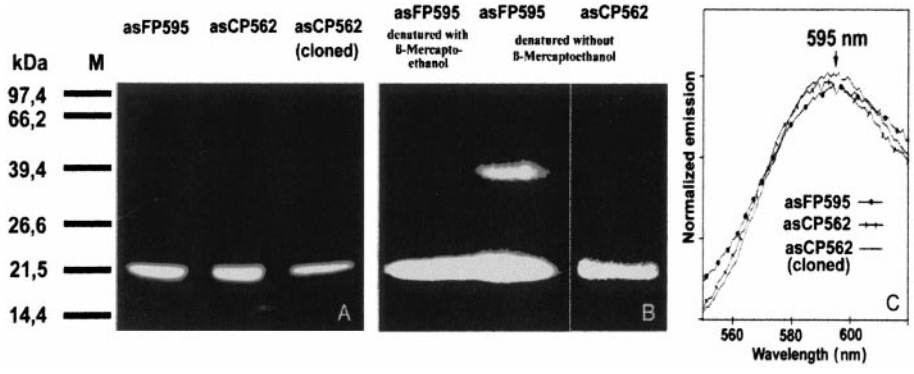

Fig. 4. (*A*) The proteins asFP595 and asCP562 isolated from *A. sulcata* and cloned asCP562 exhibit orange fluorescence when they are blotted on a nitrocellulose membrane. (*B*) A second fluorescent band at twice the molecular mass of asFP595 becomes visible if the protein is denatured without β-mercaptoethanol. This band is not detectable for asCP562 denatured without β-mercaptoethanol. The proteins were excited with broadband UV radiation. Nitrocellulose membranes were photographed by using a 610-nm long-pass filter. (*C*) All fluorescent proteins on the membrane have an emission maximum at 595 nm.

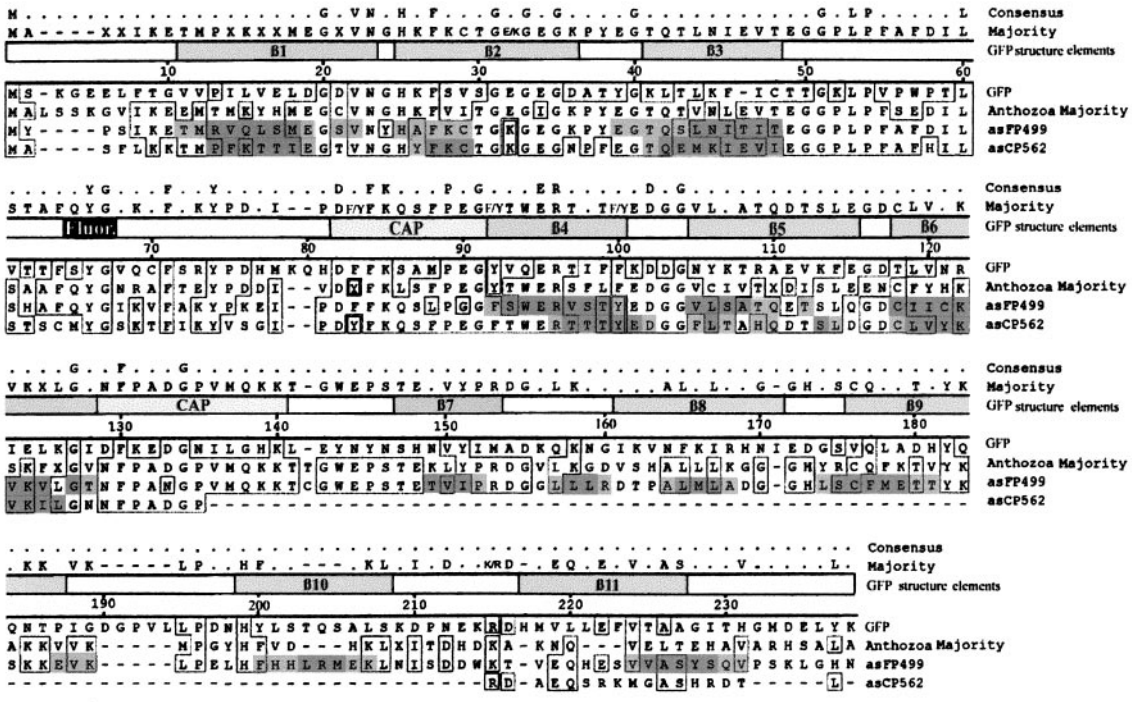

Fig. 5. Multiple alignment of the proteins asFP499 and asCP562 with GFP of *A. victoria* and a majority sequence obtained from an alignment of six Anthozoa fluorescent proteins. Boxes mark the matching residues. The numbering of the alignment is based on GFP of *A. victoria*. The schematic diagram in the third line shows key structure elements of GFP of *A. victoria* as β-strands, cap regions, and the fluorophore. The secondary structure of the regions corresponding to the β-strands of GFP was predicted for the proteins by using PHD prediction (34–36). Predicted β-strands are shaded with light gray (reliability >62.1%) and dark gray (reliability >78.5%).

homologues. Two green fluorescent pigments can be distinguished by their spectral properties in the tentacles of *A. sulcata* var. *rufescens* although they could not be separated (Fig. 2*A*). This indicates that the two proteins have identical molecular weights or that they reflect two states of the same protein. The second hypothesis is supported by the construction of a cDNA library. It resulted in the cloning of several hundred GFPs of the asFP499 type. As the content of asFP499 and asFP522 in the tissue of tentacles is comparably high, one would expect that this ratio should be detectable in the library as well. We therefore assume the existence of a second stable conformation of asFP499, with red-shifted fluorescence, which can be formed only in the expressing cells of the sea anemone.

Also the orange fluorescent protein asFP595 and the nonfluorescent red protein asCP562 most likely represent two states of the same protein. Both proteins have the same molecular weight. A striking argument for their relationship is the finding that after a denaturation-renaturation process both asFP595 and asCP562 exhibit orange fluorescence with an emission maximum that matches that of asFP595. Therefore, the primary structure of asCP562 also must carry all features necessary for orange fluorescence. The putative semi-β-can structure of asCP562 can give a possible explanation of these phenomena. For GFP fluorescence the β-can structure is essential. The β-can surrounds the fluorophore by 360°, providing a wide range for possible protein side-chain interactions and protects it from quenching agents (28, 45). Such an environment cannot be produced by the putative semi-β-can of asCP562. Nevertheless, the stability of asFP595 fluorescence and the color of asCP562 are comparable to that of GFP-like proteins, so a β-can-like environment must be present at least in asFP595. We propose the formation of a β-can-like structure in a multimerization process. This type of β-can consists of at least two molecules of asCP562. Together they could form a β-can with at least 12 β-strands surrounding at least two stretches homologous to the fluorophore region of GFP. Our hypothesis is supported by the finding that both asCP562 and asFP595 show apparent molecular masses of 66 kDa (Fig. 3.1 and 3.2). This molecular mass corresponds exactly to a tetramer consisting of four molecules asCP562 (4 × 16.5 kDa) (Table 1). Under the conditions necessary for renaturation of asFP595 a second fluorescent band can be observed if the samples are denatured without the presence of β-mercaptoethanol (Fig. 4*B*) The second band migrates at twice the molecular mass of asFP595. This result confirms the hypothesis that asFP595 consists of at least two asCP562 monomers. Unlike GFP dimers, in asFP595 disulfide bonds seem to be involved in dimer interactions as a complete splitting is only possible under reducing conditions. A possible position of a disulfide bond in the assumed β-can is between the two Cys-64 in the direct neighborhood of the fluorophore homologues. It might create a steric environment necessary for fluorophore formation or stabilization. We assume that the formation of the chromophore of asCP562 is comparable to fluorophore formation in GFP. However, to obtain the fluorescent state, it seems likely that a β-can-like structure is formed in which at least two molecules of asCP562 are linked with a disulfide bond. With the exceptions of *Aequorea* GFP and its recombinant forms, which exist as monomers in dilute aqueous solutions, all studied GFPs are stable, nondissociable dimers (12). Nevertheless, these exceptions suggest that dimerization is not generally involved in formation of the fluorescent state.

Application of asFP499 as an *in Vivo* Marker. The protein asFP499 was tested for applicability as *in vivo* marker in eukaryotic systems. Expressed in *Drosophila melanogaster* cells and in protoplasts of *Nicotiana tabaccum* and *Arabidopsis thaliana*,

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asFP499 exhibits bright green fluorescence (see supplementary material and Figs. 7–10, which are published as supplemental data on the PNAS web site). The fluorescence of asFP499 expressed in protoplasts of *N. tabaccum* seems to be brighter than one of the optimized variants for plant expression of *Aequorea*-GFP (smGFP) (46). When expressed in protoplasts of *A. thaliana*, asFP499 appears to be 3–4 times more fluorescent than smGFP (46), when the latter is excited at its minor absorbance peak. The bright fluorescence of asFP499 and the functional expression in prokaryotic, plant, and insect cells suggests that it will have a general utility for biological studies in a wide range of organisms.

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