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Natural Animal Coloration Can Be Determined by a Nonfluorescent Green Fluorescent Protein Homolog

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The only known exception to this rule is green fluorescent protein (GFP) from Aequorea victoria (7). In contrast to other naturally occurring fluorescent proteins, the fluorescence of GFP is due entirely to an internal interaction between amino acids within the protein; no other cofactors or prosthetic groups are required. GFP owes its intrinsic fluorescence to a contiguous Ser-Tyr-Gly sequence centrally located within its primary structure. Upon folding, the protein modifies the fluorophore-forming sequence to produce an extended aromatic system (8–10), which imparts the characteristic green fluorescence to the mature protein. Due to these distinctive properties, GFP has enjoyed extensive use as a biological marker in vivo (11, 12). Recently we described six novel GFP-like fluorescent proteins (FP) from nonbioluminescent Anthozoa species (13). It therefore became clear that GFP-like proteins are not necessarily components of bioluminescent systems but may simply determine fluorescent coloration of animals.

In one particular case, we have shown that a GFP-like FP is responsible for the bright green fluorescence of the tentacle tips in the sea anemone Anemonia majano. However, in another sea anemone, Anemonia sulcata, we found that, although the tentacle tips do exhibit an intense purple color they are not significantly fluorescent (Fig. 1). The similarities of the color localization patterns and the close phylogenetic relationship of these two species led us to hypothesize that A. sulcata contains a purple nonfluorescent GFP homolog in its tentacles. In the present work, we describe the isolation of the cDNA for this protein and show that GFP-like proteins can determine nonfluorescent body coloration.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Mutagenesis of the asFP595 cDNA—Total RNA from the tips of tentacles of A. sulcata was isolated as described (14). cDNA synthesis, amplification of the cDNA fragment of interest using degenerate primers, and obtaining the full-length cDNA were performed as described for other Anthozoa FPs (13). The full-length coding region of asFP595 was cloned into pQE30 vector (Qiagen). The wild-type protein as well as its mutant variants were expressed in Escherichia coli with 6×His tag at the N terminus and purified using TALON metal affinity resin (CLONTECH). All preparations of the heterologous expression products were at least of 95% purity according to electrophoresis. Site-directed mutagenesis was performed by polymerase chain reaction with primers containing target substitution using the method described in Ref. 15.

Spectroscopy—To calculate the extinction coefficients at 280 nm for new protein using the average extinction coefficients of tryptophan, tyrosine, and cysteine, the model described in Ref. 16 was used. This value was then used to determine the concentration of protein and therefore the molar extinction coefficient in the visible band. Quantum yields for fluorescent mutants were determined relative to EGFP (CLONTECH). A Perkin-Elmer LS50B spectrometer was used for quantitative measurements. All samples were excited at 470 nm, absorbance at this wavelength was 0.02, and excitation and emission slits were 5 nm. The spectra were corrected for photomultiplier response and monochrometer transmittance, transformed to wave number, and integrated.

Expression in Eukaryotic Cells—The expression vector was developed from the pEGFP-N1 vector (CLONTECH) as follows. EGFP-coding region was removed and the asFP595 (T70A/A148S) mutant was inserted instead. Then, the protein kinase C β 1 subunit coding region was inserted in the multiple cloning site of this vector. As a result, the vector contained the continuous reading frame that encodes the fusion protein PKCβ-asFP595 under the control of the cytomegalovirus immediate-

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The tips of the tentacles are colored purple.

RESULTS AND DISCUSSION

Using a strategy previously described (13), cDNA for a novel GFP-like protein was amplified from RNA samples prepared from the tentacle tips of A. sulcata. An alignment (Fig. 2) of the amino acid sequences of the novel protein (named asFP595 according to nomenclature we suggested earlier) and other known FPs was manually constructed on the basis of our previous results (13). It appears that key secondary structure elements observed in GFP as well as amino acids that form the GFP chromophore (Tyr-66 and Gly-67) or that probably play a critical role in its formation (Arg-96 and Glu-222) are also present in asFP595. Overall, 20% of the asFP595 sequence is identical to GFP. When asFP595 is compared with Anthozoa FPs the identity level is 37–47%. Interestingly, among the Anthozoa species, the closest asFP595 homolog is drFP583 (47% identity), another red emitter that was cloned from Dis-cosoma sp., not amFP485, which derives from the more closely related A. majano (42% identity).

After expressing asFP595 in E. coli, we measured the absorption and excitation-emission spectra of the purified protein (Fig. 3). With the exception of a slight shoulder at 530 nm, the protein displays a single absorption wavelength maximum, which occurs at 572 nm ($\varepsilon_{572} = 56,200 \text{ M}^{-1} \text{ cm}^{-1}$). As is evident from this analysis, asFP595 absorbs efficiently in the middle range of the visible spectrum but remains translucent when excited with blue or long wavelength red light. Consequently, to the observer, the protein appears intensely purple. Visual inspection of E. coli expressing asFP595 clearly shows that the protein confers a strong purple hue to its host (Fig. 4A).

The fluorescence of asFP595 is extremely weak (quantum yield < 0.001); nevertheless, the purple protein can be detected by spectrofluorimetry (Fig. 3B). When viewed by fluorescence microscopy, the novel protein shows an unexpected feature; although the fluorescence at 595 nm is virtually imperceptible at the start of observation, the emission intensity increases dramatically following a 10–20-s exposure to green light (Fig. 4B). This effect is reversible because in the absence of incident green light, the fluorescence capacity slowly decreases to the basal level. Even more surprisingly, asFP595 fluorescence can be quenched by a flash of blue light (Fig. 4C). This effect strongly depends on intensity of irradiation: the brighter the light the more pronounced increasing and quenching of the fluorescence. This phenomenon was observed in both eukaryotic and prokaryotic hosts expressing asFP595 as well as in samples of the protein purified from E. coli cell extracts. The spectral properties of recombinant asFP595 closely resemble those observed in vivo in the tentacle tips of the corresponding organism, A. sulcata. When examined by fluorescence microscopy, the purple-colored tentacles display a faint pinkish fluorescence that is enhanced when viewed under green light and quenched when irradiated with blue light. Collectively, these data suggest that the purple coloration of A. sulcata tentacles is determined by asFP595 due to differential light absorbance.

Based on their sequence similarities and fluorescent capacities, it is reasonable to speculate that asFP595 and other Anthozoa GFP-like fluorescent proteins evolved from a common ancestral fluorescent protein. However, asFP595 has lost the majority of its fluorescence capability. Using site-directed mutagenesis, we attempted to reconstruct the fluorescent antecedent of asFP595. The alignment of the nonfluorescent purple protein sequence with all other known FPs (Fig. 2) revealed a number of interesting sequence disparities that were evaluated experimentally. We paid particular attention to those residues surrounding the fluorophore. One of these stood out: residue 148 (numbering based on GFP). In GFP and all Anthozoa fluorescent proteins, position 148 is occupied by a polar residue (His in GFP and Ser in Anthozoa FPs). The equivalent site in asFP595 is occupied by a nonpolar residue (Ala). Because His-148 is in direct contact with the fluorophore in GFP, it seemed likely that it played a critical role in the protein’s fluorescence.

To test this hypothesis, we generated an A148S asFP595 point mutation which resulted in a protein with significantly reduced fluorescence (Fig. 4). This mutation confirmed that residue 148 is critical for the fluorescence observed in asFP595. Further analysis revealed that this fluorescence is red-shifted relative to GFP, with a peak emission at 595 nm, indicating that this amino acid substitution affects the emission of the chromophore.

In conclusion, the novel purple chromoprotein from A. sulcata, asFP595, shares significant sequence similarity with other Anthozoa GFP-like proteins and exhibits unique fluorescence properties not observed in previous FP variants. This protein offers a valuable tool for biological research, particularly in the study of marine organisms and their response to environmental stimuli.
First, it is the most red-shifted FP to date (emission maximum \( \lambda_{\text{max}} \approx 595 \) nm). Second, it matures much faster than another red FP-drFP583. Third, this mutant is reversibly quenched by blue light in a manner similar to that observed for the wild-type asFP595 (Fig. 3D). This quenching response can be used for discriminating between target and background red fluorescence.

Further mutagenesis was performed on the basis of the mutant A148S. Additional substitutions of amino acids probably adjacent to chromophore (e.g. S68A, W94Y, S165V, E201A/E201V/E201L, and H203S) resulted in the appearance of an additional green emission peak at 514 or 523 nm, depending on the mutant (Fig. 3D). In these mutants, the nature of the particular amino acid substitution affected the ratio between the green and red emission intensities as well as brightness of the protein but only slightly affected the position of the emission maximums. The congruency of these outcomes may indicate that the green and red fluorescence represent distinct fluorophores in a heterogeneous population of green and red fluorescent protein molecules. This idea is supported by the observation that the red and green emissions can be stimulated independently; excitation of the mutants by 338 nm light produces only red fluorescence, not green. It is tempting to speculate that the red fluorophore may be produced due to either alternative or additional protein backbone modification that extents the conjugated \( \pi \)-electron system in comparison with the green fluorophore.

For some of the mutants that displayed dual-color emission spectra (e.g. T70A/A148S/S165V), the red emission peak oc-
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curred at 610 nm instead of 595 nm. Although these mutant proteins are of no practical use as fluorescent markers (due to slow maturation rates, low quantum yields, and dual-color emissions), they demonstrate that long wavelength fluorescence is attainable in GFP-like fluorescent proteins.

To evaluate the usefulness of asFP595 for biotechnological applications, we tested its fluorescent variant in a eukaryotic expression system. Fusion of protein kinase C β1 subunit (PKCβ) and asFP595 fluorescent mutant T70A/A148S was constructed and expressed in a human cell line. Using fluorescence microscopy, we were able to monitor the real-time translocation of red fluorescence from the cytoplasm to the plasma membrane upon activation of the PKC pathway with phorbol 12-myristate 13-acetate (Fig. 5).

Vivid coloration is perhaps the most impressive and eye-catching feature of reef-associated animals, from sponges to fish. However, very little is known about the chemical identity and function of coloration, especially in invertebrates. For cnidarians, some pigments were identified as carotenoproteins, proteins containing porphyrin or copper and low molecular pigments (1–6). In many studies, pigments were identified on the basis of indirect data only (e.g. similarity of spectra). Because carotenoproteins present colors ranging from blue to red it was generally inferred that they were the agents responsible for the diversity of colors in these animals. The idea that an apoprotein, a protein devoid of ligands and prosthetic groups, could possess chromogenic qualities was not considered. In this work, we show that coloration can also be determined by a nonfluorescent A. victoria GFP homolog, asFP595.

Existing evidence suggests that GFP-like chromoproteins are widely distributed in nature. In 1987, Blanquet and Phelan (17) described an unusual blue-colored protein from the jellyfish Cassiopea xamachana. The blue protein contained a covalently bonded chromophore of unknown chemical composition and appeared not to be associated with any metal ions. In 1995, Dove et al. (18) described similar features for pink, purple, and blue chromoproteins that were isolated from tissues of reef-building corals. The authors concluded that the unique spectral characteristics of these proteins depended on an unidentified covalently attached chromophore and not on the presence of any particular transition metals. In addition to their shared biochemical properties, these proteins have certain key physical traits in common with asFP595: they possess comparable molecular masses (28,000) and display similar absorbance spectra.

Due to its self-contained fluorescence, rapid maturation rate, stability, and negligible cytotoxicity, GFP has been recruited for use in many research applications. The novel GFP-homolog described here displays these same desirable properties but also possesses others that could be particularly beneficial for future research and industrial applications. Those researchers attempting to resolve two or more fluorescent tags within the same cell may profit from the use of the T70A/A148S asFP595 fluorescent variant, which extends the emission range covered by existing FPs to nearly 600 nm. Wild-type asFP595, which displays a highly distinctive color, could be used to mark an individual organism to distinguish it from others in its group. Careful monitoring of animals in their natural environments is more conveniently done with nonfluorescent markers because the label can be easily detected in the field without the need for special instruments. Chromoproteins such as asFP595 could also serve as convenient visual aids for the selection of transgenic organisms. In plant research, for example, during the early stages of seed germination and prior to the production of chlorophyll, the expression of a colored protein in developing acrospires should be very conspicuous. In an industrial setting, transgenic sheep carrying and expressing a particular chromoprotein could simplify and detoxify the process of producing colored clothing by eliminating the need for noxious chemical dyes.

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