# Symposium-in-Print: Green Fluorescent Protein and Homologs

# The Family of GFP-Like Proteins: Structure, Function, Photophysics and Biosensor Applications. Introduction and Perspective

### Rebekka M. Wachter\*

Department of Chemistry and Biochemistry, Arizona State University, Tempe, AZ 85287

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## ABSTRACT

In this issue, we offer a symposium-in-print that is focused on several new advancements in fundamental research related to the family of GFP (green fluorescent protein)-like proteins. A few applied aspects are also included to illustrate the impact this amazing set of colored proteins has made on our understanding of cell biology at the molecular level. The six articles presented here cut across several disciplines ranging from biological function to protein structure to photophysical aspects. These highly original pieces of work include both experimental and computational approaches, and will provide the reader with significant insight into current, state-of-the-art research activities in this very dynamic and fast-paced field. In the first part of this perspective, I will give a brief overview of the history and salient features of GFPs, cite some examples that illustrate their impact on biotechnology, and provide a brief review of the structural and chemical features that lend these proteins their fascinating appearance. In the second part, I will introduce each of the peer-reviewed contributions of the participating authors.

## INTRODUCTION

Green fluorescent protein (GFP) is the founding member of a new class of proteins, the family of GFP-like proteins (1). This homologous group of colored proteins consists of a small, compact, 11-stranded  $\beta$ -barrel that spontaneously generates its own fluorophore (2,3) (Fig. 1). Due to their bright colors ranging from cyan to green to red, these proteins are used extensively as research tools in molecular and cell biology (1). The various colors originate from a fluorescent entity that is generated in the protein's interior by covalent modification of three buried amino-acid residues that are part of the protein's polypeptide chain and are consecutive in sequence (Fig. 2). The chromophore is generated only under conditions permissive of protein folding; that is, the polypeptide must be able to obtain its native three-dimensional structure to become visibly fluorescent.

The existence of GFP in the Pacific Northwest jellyfish Aequorea Victoria, in close association with the luciferase

aequorin, was first reported by Shimomura (4). The gene was cloned from the jellyfish cDNA (5) and the fluorophore chemically characterized (6–8). The protein's three-dimensional structure was determined in 1996 by X-ray crystallography (2,3), and the biochemical and physical properties of its chromophore have been characterized in detail over the last decade (1). A few years ago, a series of GFP-like proteins with low sequence homology to GFP were isolated from reef-building corals (9–11). Some of these homologues contain a chromophore  $\pi$  system that is further modified from the green stage (12–14), suggesting that GFP may comprise one of the simplest systems with the least number of chemical steps leading to chromophore maturation. To gain a more comprehensive understanding of the molecular properties of GFP and GFP-like proteins, the reader is referred to several recent reviews (1,11,15,16).

### Biotechnological and biomedical impact of GFP-like proteins

The family of GFP-like proteins has a profound impact on the versatility of techniques available to cell biologists studying diseases ranging from cancer to developmental disabilities to genetic defects (1). The large variety of gene-based fluorescent probes now available is providing researchers with very powerful tools to explore cellular events, especially when used in conjunction with highly sensitive fluorescence microscopes (17). Not surprisingly, close to 200 new publications on GFP-related research have appeared in the PubMed database each month, and many biomedical applications for GFP as a non-invasive visual reporter can be enumerated. To give just one impressive example, a recent article has reported on the implantation of embryonic stem cells derived from GFP-transgenic mice into genetically engineered mice that constitute good mouse models for specific disease states. With this powerful technique, the fate of implanted donor cells can be monitored visually, and different strategies for cell-implantation therapies can be devised and evaluated (18).

An increasing number of GFPs have been engineered for specific biosensor applications to monitor biochemical changes in the intracellular environment (1). For example, redox-sensitive GFPs have been devised to determine mitochondrial redox potential (19), and therefore real-time dynamic imaging of redox changes within mammalian cells has become feasible (20). The use of GFP variants as intracellular pH sensors or chloride sensors was described several years ago (21–23). Exciting new applications in

<sup>\*</sup>Corresponding author email: RWachter@asu.edu (Rebekka M. Wachter) © 2006 American Society for Photobiology 0031-8655/06



**Figure 1.** Ribbon diagram of the GFP  $\beta$ -can fold, generated from the X-ray structure of GFP-S65T (2). The chromophore is buried in the protein's interior, and shown in ball-and-stick representation.

various labeling experiments, such as the use of GFP-like proteins as fluorescent timers in developmental biology (24), have been reported, and some members of this family have been developed into novel imaging agents for multicolor and FRET labeling experiments (16,25).

A variety of exciting examples demonstrate how photolabeling techniques based on GFPs can be used to monitor the dynamics of cellular events in real time. Photoactivatable GFPs have been used to track protein movement in living cells (17), photoswitchable cyan-to-green fluorescent proteins have been used to study trafficking of human dopamine transporter in live cells (26), and "kindling" nonfluorescent proteins have been shown to become fluorescent upon irradiation with light of a specific wavelength (27). A technique termed protein highlighting was recently used to observe shuttling of signaling molecules between the nucleus and the cytoplasm, employing a fluorescent protein with reversible photochromic properties (28). Though these cell-biological experiments are intrinsically fascinating and allow us to visualize and study biology with truly revolutionary precision in both space and time, they all require that a chromophore-bearing probe be already present in the cell when dynamic changes are initiated.

### **Outline of GFP chromophore biosynthesis**

Chromophore formation in GFP-like proteins is a highly unusual posttranslational modification that spontaneously follows protein folding and requires molecular oxygen. It has been demonstrated that the polypeptide precursor synthesized by chemical means is capable of developing fluorescence upon folding under aerobic conditions (29). The autocatalytic mechanism (Scheme 1) is initiated by an intrachain ring closure that leads to the formation of a cylopentyl group from the backbone atoms of the original polypeptide (Ser65, Tyr66 and Gly67 in wild-type GFP) (30,31). Full chromophore maturation, and hence the development of visible fluorescence, requires further processing steps that follow spontaneously. In all GFP-like proteins characterized to date, these steps include at least one oxidation event. To generate the green chromophore present in mature GFP (Fig. 2a), the mechanism, in outline, must contain a cyclization, dehydration and oxidation step



**Figure 2.** Chemical structures of chromophores of GFP-like proteins: (a) GFP, (b) DsRed, (c) Zoanthus yellow, (d) Kaede.

(Scheme 1) (30). The fully conjugated chromophore is known to form only in the presence of molecular oxygen (31).

Regardless of the final color of the particular protein, an internal peptide backbone cyclization appears to play a pivotal role in chromophore biosynthesis. This condensation reaction occurs early in the process of chromophore formation, initiating the covalent modification process, and appears to be common to all GFP-like proteins. Although a sequence motif has yet to be identified that codes for this posttranslational modification, significant progress has been made in understanding the structural preorganization of the protein and the roles of specific catalytic groups in generating the chromophore (32–37).

# Chemical diversity of chromophores found in GFP homologues

A series of GFP homologues distantly related to GFP have been cloned over the last few years, primarily from reef-building corals and sea anemones (9–11). These proteins are brightly colored, varying from cyan to green, yellow, orange, red and purple, and are responsible for the bright pigmentation of coral reefs (38). Most of these GFP-like proteins are vividly fluorescent under UV or visible light, and constitute promising new tools in molecular biology. As predicted from the sequence, several crystal structures have demonstrated that the fold of all GFP-like proteins is that of the GFP  $\beta$ -can, an 11-stranded  $\beta$ -barrel with a helix threaded through the center. A self-modification process similar to that in GFP leads to covalent alterations of an analogous internal tripeptide (Fig. 2). Though the first of the three residues may vary (Gln, His, Lys, etc.), the second and third are consistently a tyrosine and glycine residue, equivalent to Tyr66 and Gly67 in GFP. Other 100% conserved residues are the structural equivalents of Arg96 and Glu222 that play important catalytic functions in the self-modification process (36,37).

During the last few years, many reports have described GFP-like chromoproteins in more detail. For example, a green-fluorescent homologue was isolated from the stony coral and was shown to exist as a tight tetramer in its native form, similar to other coral proteins (39,40). A related protein from Pocilloporin, termed *Rtms5*, was reported to be essentially nonfluorescent, apparently because this protein contains a nonplanar GFP-like chromophore (41). Another chromoprotein, asCP, isolated from the sea anemone Anemonia sulcata, is initially nonfluorescent, but converts to a green-fluorescent form upon irradiation with green light (27). Redshifted GFP-like proteins contain a chromophore  $\pi$  system that is further modified from the green stage (Fig. 2), which appears to be an intermediate in these proteins (12-14,42). In drFP583 (DsRed) from Discosoma (13) and eqFP611 (43), the additional chemical modification includes a further oxidation step that may be correlated with the formation of a *cis* peptide bond (44). The result is an acylimine extension of the chromophore  $\pi$ -system along the peptide backbone that is directly adjacent and bonded to the imidazolinone ring (Fig. 2b).

A yellow fluorescent protein from Zoanthus, zFP538, has been shown to contain a novel three-ring chromophore (42), where the third ring leads to an extension of the  $\pi$  system by an imine bond (Fig. 2c). The third ring is generated by cyclization of a lysine side chain, apparently via a transimination reaction that leads to adjacent peptide backbone cleavage in the protein's interior. A green intermediate state was observed during maturation, which was shown to convert to the yellow species, likely via the red-emitting acylimine as a second intermediate (42). An orange-fluorescent protein from the tube anemone Cerianthus has recently been crystallized, though its chromophore structure is as yet unknown (45). A photoconversion process from the green-emitting to the red-emitting form has been characterized in a related protein termed Kaede (46). This process also incorporates a peptide backbone cleavage adjacent to the chromophore, though the mechanism must be different form zFP538. The red form of Kaede contains an extension to the  $\pi$ system that is generated by modification of a histidine side chain (Fig. 2d). The acylimine form present in DsRed does not appear to be an intermediate in the photoconversion process of Kaede.

Several proteins with cyan emission are also part of the accessible color spectrum of the GFP-like family of proteins. A recent study reporting a high-resolution crystal structure and mutational analysis of the cyan protein from *Anemonia majano* concluded that the spectral shift from green to cyan has its primary origin in the positioning of positively charged side chains close to the chromophore phenolate (47). Compared to the GFP chromophore, additional covalent modifications were not observed (Fig. 2a).

Regardless of final color, a GFP-like chromophore appears to be an intermediate state in all chromoproteins homologous to GFP that have been studied so far. Clearly, a chemical entity very similar to that present in the jellyfish-derived GFP must be synthesized first, to generate the broad spectrum of chromophore colors accessible to Anthozoa GFPs (Fig. 2).

# Improved biophysical properties by directed evolution of GFP-like proteins

Throughout the literature, there are many examples of directed evolution experiments applied to GFP and its homologues. To il-



**Scheme 1.** Mechanism of GFP chromophore formation. Peptide cyclization is followed by loss of water and oxidation by molecular oxygen, which may occur in either sequence. The mature green fluorescent chromophore exists in two different charge states in the intact protein.

lustrate the overwhelming success of this approach, a few examples are summarized here. In work aimed at knocking out the tetramer interface of DsRed while conserving its red color, a monomeric red fluorescent protein was identified after many rounds of mutagenesis and selection (48). In this tour-de-force example of directed evolution, a total of 33 substitutions were necessary to rescue red fluorescence from a monomeric precursor derived from DsRed but arrested at the green stage. In another astounding piece of work, improved monomeric red, orange and yellow fluorescent variants were subsequently selected by additional extensive directed evolution experiments (25).

On another front, researchers have attempted not only to vary color and aggregation state of GFPs, but also to accelerate the overall maturation process. In a tremendously successful recent study, variants of DsRed were isolated that exhibited a 10-15-fold reduction in the time required for green-to-red conversion (49). The best variant identified by error-prone PCR was reported to mature with a half-time of only 0.7 h, whereas wild-type DsRed requires more than 24 h to reach the red-fluorescent state from the green intermediate. In this work, colony screening was primarily carried out by flow cytometry, assaying for strong red fluorescence and reduced green fluorescence. In a related study aimed at accelerating the oxidation step in the maturation of the green chromophore (Scheme 1), some novel yellow GFP variants were identified by directed evolution (50) that were reported to exhibit very fast reoxidation rates after chemical reduction of the mature chromophore (51).

The sensitivity of yellow fluorescence to chloride concentrations (23) was enhanced dramatically by randomly mutating residue pairs in the vicinity of a previously characterized halide binding site (52). On the other hand, the chloride effect turned out to be a nuisance when accurate recording of cellular pH was attempted. Therefore, error-prone PCR was used to identify a mutation (Q69M) that reduces the sensitivity of yellow GFPs to halides, thus facilitating

the use of this variant as a pH sensor (53). A recent study was aimed at modifying the GFP fluorescence decay lifetime for multiprobe imaging. Here, error-prone PCR was employed to select for variants with altered lifetimes, and their utility in time-resolved imaging using a single detector channel was demonstrated (54).

# CONTRIBUTIONS TO THE SYMPOSIUM-IN-PRINT

The symposium-in-print presented in this issue addresses a variety of different areas of intense interest in GFP research, ranging from the ecological effects of coloration in reef-building corals, to the structural basis of the rich GFP photophysical properties, to the development of novel biosensors. Both experimental and computational work is included.

### Ecology

Coral colors and fish ecology. The symposium begins with an article by Mikhail Matz and coworkers (page 345) that addresses the biological significance of the colorful appearance of reefbuilding corals (class Anthozoa), which is determined by members of the GFP-like family of proteins. Though the biological function for at least some of these protein pigments is thought to be that of photoprotection toward endosymbiotic algal species (55), the adaptive evolutionary advantage of the large diversity of coral colors is as yet poorly understood. The contribution by Matz et al. very nicely addresses the ability of coral reef fishes to perceive a variety of coral colors. For three different fish species, color vision in the fishes is modeled on the basis of absorption spectra of individual photoreceptors, and compared to coral spectra that were collected in situ by SCUBA divers at the Great Barrier Reef in Australia and in coastal waters off Long Key, Florida. The authors raise the tantalizing possibility that the evolution of coral color diversity may have been the driving force behind the specialization of the fish visual systems.

### Protein structure and optical properties

Photoconversion and cellular imaging. On page 351, Ulrich Nienhaus and coworkers describe a green fluorescent protein from a stony coral that is photoconvertible to a red-fluorescent form. In this and related work, the authors demonstrate convincingly that irradiation with near-UV light initiates a photochemical process that results in the cleavage of a main-chain covalent bond near the chromophore. The  $\pi$ -system of the fluorescent entity is extended by introduction of an additional double-bond between the  $\alpha$  and  $\beta$  carbons of a histidine residue, which results in redshifting of the spectrum (Fig. 2d) (56). In addition to a description of protein structural parameters obtained via X-ray crystallography, several novel applications are presented in the areas of cellular imaging, developmental biology, and high-throughput screening.

Anion binding as modulator of optical properties. On page 359, Jamie Rossjohn and coworkers describe structural studies of a purple nonfluorescent chromoprotein called *Pocilloporin*. By lowering the solution pH, color transitions are observed from blue to yellow, indicative of protonation of the anionic chromophore. As confirmed by X-ray crystallography, the observed optical transitions are clearly linked to the binding of several halide ions in the chromophore-bearing interior cavity, somewhat reminiscent of the GFP-based halide sensors described some years ago (23,57).

#### The protein matrix and photophysical properties

Steric restriction of the protein matrix. On page 367, Marc Zimmer and coworkers use molecular mechanics simulations to explore the role of the protein matrix in restricting the movement of the intrinsic chromophore. The computer simulations indicate that amino-acid residues are placed around the chromophore in such a way as to favor hula-twist motions of the chromophore because of its volume-conserving nature. The authors conclude that the hula twist may be the preferred mechanism for cis-*trans*-isomerization of the GFP chromophore embedded in the protein's interior. An analysis of a number of X-ray structures of GFP-like proteins indicates that none of these proteins contain interior cavities structurally complementary to a planar chromophore. However, the authors also note that not only steric but also electronic restraints play a role in the restriction of chromophore movements.

*Excited-state proton transfer*. On page 373, Steven Meech and coworkers present a study aimed at extending our knowledge of the tremendously rich photophysical properties of wild-type GFP. Time-resolved emission spectra collected via Kerr-gate spectroscopy are used to better understand the excited-state proton transfer operational in wild-type GFP (58,59). Time-resolved spectra are compared for the chromophore excited state in wild-type GFP and the T203V mutant, and the absence of any spectral shifts in either protein is interpreted in terms of a lack of structural reorganization upon creation of the excited chromophore. This conclusion is consistent with a highly rigid protein environment in which reorientation of polar and polarizable amino acids is severely restricted within the excited-state lifetimes.

These results are interesting in light of a recent study aimed at identifying mutants with altered fluorescence decay lifetimes (60). In this study, no variants could be found with increased fluorescence lifetimes; only mutants with shorter lifetimes were identified. A plausible explanation is that amino-acid substitutions close to the chromophore lead to interior packing perturbations that may result in increased flexibility of the protein matrix, allowing the chromophore to exist in a wider range of conformational states.

Biophysics of emission heterogeneity in photoactive proteins. The final paper in the series (page 380) by John Kennis and coworkers provides a broader perspective on photoactive proteins by presenting a comparative biophysical study of bacteriorhodopsin, photoactive yellow protein and GFP. The authors present a nice story demonstrating emission heterogeneity for all three proteins, and successfully place the photophysics of GFP into the context of other light-responsive proteins. Although the three proteins differ in their biological function, the time-dependent response of the protein matrix to excitation of the chromophore is a theme of more general interest in the role of protein groups in modulating optical properties. The observation of a dynamic Stokes shift in the time-resolved fluorescence spectra on subpicosecond timescales is of broad general interest in expanding our knowledge of protein solvent effects on the photodynamics of protein-bound chromophores.

In summary, we thank all the authors for the submission of manuscripts on highly original research, in an area that has captured the attention of a large number of academic investigators as well as the biotechnology industry.

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