X-ray Diffraction and Time-resolved Fluorescence Analyses of Aequorea Green Fluorescent Protein Crystals*

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The green fluorescent proteins (GFPs)

The most striking feature of the protein is its bright green fluorescence, which has been extensively studied (1, 4, 5, 10, 11). Both crystal forms fluoresce almost identically to the protein in solution (see below), suggesting that the conformation of the protein in the crystal does not vary significantly from that in solution. Moreover, the fluorescence may serve as a useful probe for the structure and dynamics of the crystalline protein (12-14).

MATERIALS AND METHODS

Specimens of Aequorea victoria were hand collected at the University of Washington’s Friday Harbor Laboratories, Friday Harbor, WA during the late summer. The methods of Blinks et al. (15) were used to isolate the protein in the form of ammonium sulfate pellets. This preprocessed material was later purified using gel filtration and ion exchange chromatography as described elsewhere (4, 5). All preparations yielded absorbance ratios $A_{488}/A_{505}$ greater than 1.0 to separate isoproteins, selected samples were further purified by anion exchange chromatography, using an analytical Pharmacia LKB Biotechnology Inc. Mono-Q fast protein liquid chromatography column, eluted with a 0-0.25 M NaCl salt gradient in 50 mM BisTris, pH 5.8.

Large single crystals from several purified GFP samples were prepared using “hanging drop” vapor diffusion techniques (16). Hexagonal and monoclonic crystals were prepared by equilibrating 10-$\mu$l drops consisting of 1.0 M cation phosphate, pH 7.0, 5.0 mg/ml GFP, over a 1.0-ml reservoir of 2.0 M phosphate, pH 7.0, at 23 ± 5°C. Crystal density was measured using Ficoll density gradients by the

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1 The abbreviations used are: GFPs, green fluorescent proteins; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

2 $\alpha = 77.5, c = 370 \AA$, and no more than three molecules per asymmetric unit. Monoclinic parallel-epipeds have been obtained in the C2 space group with $\alpha = 93.3, b = 66.5, c = 45.5 \AA$, $\beta = 108^\circ$, and one molecule per asymmetric unit. The monoclinic form is better suited for use in a structure determination, and a data set was collected from the native crystal. Time-resolved fluorescence measurements of large single crystals are possible due to the unique, covalently bound chromophore present in this molecule. Fluorescence emission spectra of Aequorea green fluorescent protein in solution and from either the hexagonal or monoclonic single crystal show similar profiles suggesting that the conformations of protein in solution and in the crystal are similar. Multifrequency phase correlation analysis will aid in the elucidation of this novel protein’s structure-function relationship.

The green fluorescent proteins (GFPs) are a unique class of chromoproteins found in many bioluminescent hydrozoan and anthozoan coelenterates (1). These proteins have been characterized best from the sea pansy Renilla reniformis (2) and the hydromedusan jellyfish Aequorea victoria (3-5), where they serve as the in vivo bioluminescent emitters. In Renilla, energy is transferred from the single excited state of an oxylyuciferin monoanion to the GFP by a radiationless process. In contrast, in Aequorea the evidence seems to favor radiative transfer to GFP from the photoprotein aequorin.

The well characterized GFPs from both species have been purified to homogeneity and found to be acidic, globular proteins of molecular mass 27,000–50,000 daltons. These molecules are amazingly resistant to denaturing conditions and have been shown to be stable in 8 M urea (6). Even in vitro fluorescence is unaffected by prior treatment in 5 M guanidine HCl, 8 M urea, or 1% sodium dodecyl sulfate (3). In addition, GFP is very resistant to a variety of proteases (7).

The fluorescent and bioluminescent characteristics of GFP result from a covalently bound chromophore. The Aequorea GFP chromophore has been proposed by Shimomura (8) to be a cyclic tripeptide apparently derived from the primary structure of the protein. Because the chromophore in Renilla GFP is thought to be identical, the large difference in absorption spectrum maxima of Renilla and Aequorea GFPs (103 nm) is believed to result from differences in noncovalent interactions between the chromophore and other regions of the protein (3). Fluorescence polarization and oxygen quenching measurements suggest that the chromophore is held rigidly within a conformationally inflexible domain (9). However, changes in pH, ionic strength, and protein concentration do perturb the spectral properties of the protein (10).

We have prepared two crystal forms of Aequorea GFP suitable for structure analysis by x-ray diffraction. A structural model of GFP based on a single crystal x-ray diffraction analysis will be crucial to the resolution of a number of questions concerning the nature of the energy transfer mechanism, the notable stability of the molecule, and details of the structure and environment of the unique chromophore.
method of Westbrook (17). Optical photographs were taken on a Bausch and Lomb Stereozoom 7 microscope with an AX-1 camera system. Exposures were taken using the automatic mode on Kodak Panatomic-X film.

Precession photographs of both crystal types were taken at beamline X23-B, National Synchrotron Light Source, Brookhaven National Labs, on a Charles Supper precession camera with a crystal-to-film distance of 100 mm, precession angle $\mu$ of 15°, and monochromatic x-radiation with a wavelength of 1.63 Å. A data set of the native crystal form was collected on a Nicolet Imaging Proportional Counter system (Xentronics). The data were corrected and processed as described elsewhere. For all diffraction analyses, crystals were mounted in quartz capillaries that had previously been silanated by immersing in a 3% dichlorodimethylsilane/toluene solution, followed by sequential rinsing with toluene, ethanol, and water.

Fluorescence emission and corrected excitation spectra of the protein in solution and in crystals were obtained on a Spex Fluorolog variable frequency phase fluorimeter as described previously by sequential rinsing with toluene, ethanol, and water. The fluorescence lifetime was determined on an ISS Fluorimeter. The fluorescence lifetime was determined on an ISS variable frequency phase fluorimeter as described previously (19, 20). For all measurements single crystals were mounted in glass capillaries that exhibited minimal fluorescence even when excited at ultraviolet wavelengths. Further details of the experiments are given in the figure legends (Figs. 3 and 4).

RESULTS AND DISCUSSION

Crystals grew in 4–7 days. Crystal growth rate and crystal size were significantly increased when protein samples were further purified using ion exchange fast protein liquid chromatography. Hexagonal plates increased in thickness, and monoclinic parallelepipeds increased in all dimensions. No great increase was seen in $A_{\text{obs}}/A_{\text{calc}}$ ratio, indicating that the improved quality of crystals was due only to better separation of GFP isoproteins. Average crystal dimensions were $0.1 \times 0.1 \times 0.8 \text{ mm}$ for the monoclinic parallelepipeds and $0.4 \times 0.4 \times 0.1 \text{ mm}$ for the hexagonal plates (Fig. 1).

Principal net precession photographs of both crystal types appear in Fig. 2. Hexagonal plate crystals exhibit symmetry of the space group $P6_322$ or $P6_22$ with $a = b = 77.5, c = 337 \text{ Å}$. Based on crystal density measurements of 1.15 g/cm$^3$ and calculated water volume, there are no more than three molecules per asymmetric unit. Still diffraction photographs show reflections beyond 3.9-Å resolution. Analysis of the monoclinic parallelepiped crystal data show C$\bar{2}$ space group symmetry with $a = 93.3, b = 66.5, c = 45.5 \text{ Å}$ and $\beta = 108°$. Crystal density is 1.15 g/cm$^3$, there is one molecule per asymmetric unit and 39% calculated solvent content. Still diffraction photographs show reflections well beyond 2.2-Å resolution.

Based on resolution and the number of molecules in the asymmetric unit, the monoclinic crystal form is the preferred form for a structure determination. In addition, the large lattice constant in the hexagonal crystal makes it unwieldy to work with, although all reflections can be resolved easily with the well collimated radiation of a synchrotron source. Therefore, a native data set was collected from monoclinic crystals. Of the 14,682 reflections within 2.2 Å, 10,267 were collected. Of these, 8,582 were observed to be greater than 2$\sigma$ above background level. A search for isomorphous heavy atom derivatives is in progress.

Fig. 3 depicts the fluorescence emission spectra of Aequorea GFP in solution and from a single hexagonal crystal; the monoclinic form exhibited a similar spectrum. The minor apparent increase in intensity on the red edge of the crystal spectrum can be attributed to some reabsorption of the blue side emission by the highly concentrated protein in the crystal; the longest wavelength excitation band is superimposed to make this apparent. The similarity of the crystal and solution spectra suggests that, under these conditions, the conformation of the protein in solution differs little from that in the crystal.

The emissive lifetime of protein fluorescence can also provide important information about the fluorophore and its dynamics (13, 14). The lifetime of the fluorescence from the monoclinic crystal form is extremely similar to that found in solution; the best fit to the phase data in Fig. 4 gives a single lifetime of 3.298 ± 0.090 ns with a $\chi^2$ of 7.0 and a fractional intensity greater than 96%. By comparison, the protein in solution exhibits a lifetime of 3.150 ns.$^3$

Preliminary polarized excitation spectra taken from single crystals in different, fixed orientations (and corrected for the

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$^2$ A. J. Howard (1986) A Guide to Data Reduction for the Nicolet Imaging Proportional Counter, unpublished.

$^3$ F. G. Prendergast, unpublished observations.
wavelength variations of polarized light transmission by the excitation monochromator) show variations in the relative intensities of the excitation bands.\(^4\) Such a result is expected from a lattice of fluorophores because, as the crystal is reoriented with respect to the incident beam, the transition dipoles within the fluorophore corresponding to the excitation (absorption) bands assume different orientations with respect to the polarized exciting light. Thus it should be possible to determine the orientation of the transition dipoles with respect to the crystal lattice and the protein molecule by fluorescence-detected linear dichroism, in a manner analogous to

\(^4\) R. B. Thompson, M. A. Perozzo, and K. B. Ward, manuscript in preparation.
the polarized absorption spectrophotometric method employed for heme protein crystals (21, 22).

Fluorescence spectroscopy is a widely used tool for studying protein structure and dynamics that often provides data complementary to x-ray crystallographic methods. Although Weber found that lysozyme exhibited a fluorescence lifetime in the crystal similar to that in solution, we know of no other fluorescence lifetime data collected from protein crystals. The fact that the emission spectra and fluorescence lifetime of Aequorea GFP in the crystal are nearly identical to those in solution suggests that the protein conformation under these circumstances is the same. Because of its strong absorption band, high quantum yield and photostability, Aequorea GFP represents a favorable case for studying protein structure dynamics using both fluorescence and x-ray methods. Finally, x-ray crystallography will provide the structure of the fluorescent moiety and especially its relation to the rest of the protein, which in turn will elucidate the structural basis of its remarkable fluorescent properties. A single crystal x-ray diffraction structure analysis is in progress.

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