Supplementary Information

Chromophore Orientations in the Crystal. The direction cosines associated with the chromophore of wild-type GFP and of the Ser65Thr variant were determined from the X-ray crystal models 1EMB.pdb (wild-type GFP; (*29*)), 1EMA (Ser65Thr variant; (*4*), 1EMG, and 1C4F (Ser65Thr variant at pH 8.0 and 4.6, respectively; (*12*). In all cases these crystals correspond to the orthorhombic space group $P_{2_12_12_1}$. First, vectors between pairs of benzyledineimidazolinone group atoms that define the plane of the chromophore (*e.g.*, CA1, C1, N2, N3, C2, O2, CA2, CB2, CG2, CD1, CD2, CE1, CE2, and CZ; see Figure 1) were determined from their coordinates as reported in the protein data bank files. The cross products of different combinations of these vectors were calculated and averaged to determine the equation of the line normal to the chromophore plane (***n***). The equations of the unit vectors are summarized in Table S1. Note, however, that because of space group symmetry, one of the four chromophores in the crystallographic unit cell can be described with a normal vector where all three direction cosines have positive values. For simplicity, therefore, the absolute values were used in the analysis of the crystal absorption dichroism.

Comparison of the dichroism of wild-type GFP and Ser65Thr variant crystals. Orthorhombic crystals of wild-type GFP exhibit greatest absorption of light when the electric vector of incident plane-polarized light is aligned with the crystallographic *c*-axis. This observation also applies to crystals of the Ser65Thr variant. However, the polarization ratio associated with crystals of the variant protein at 480 nm is smaller ($\frac{OD_{\parallel c}}{OD_{\perp c}}$ = 3.0) than it is for crystals of wild-type GFP ($\frac{OD_{\parallel c}}{OD_{\perp c}}$ = 4.7). The difference in

the polarization ratios of the two proteins stems in large part from the differences in the crystal packing. Figure S1 illustrates how plots of Equations 4 and 5 shift with respect to the angle θ to compensate for subtle orientation differences in the crystal lattice (*i.e.*, differences in the direction cosines of the chromophore).

Supplementary Information Figure Legend

Figure S1. Comparison of the polarization ratio expected as a function of the angle between the reference axis χ and the transition dipole moment of the anionic form of the chromophore in wild-type GFP (bold curves; from 1EMB) and the Ser65Thr variant (light curves; from 1EMA, 1EMG, 1C4F). Each profile was determined as in Figure 3 with Equations 4 and 5 and the direction cosines as summarized in Table S1.

Table S1. Orientations of the chromophore in the crystal structures of wild-type GFP and the Ser65Thr variant.

Protein	Coordinate file	pH	Resolution (Å)	Equation of the line normal to the chromophore plane
wild-type GFP	1EMB.pdb	3.8	2.13	$\boldsymbol{n} = 0.461\boldsymbol{i} - 0.880\,\boldsymbol{j} + 0.11\boldsymbol{k}$
	1EMA.pdb	8.2	1.90	$\boldsymbol{n} = 0.48\boldsymbol{i} - 0.856\,\boldsymbol{j} + 0.20\boldsymbol{k}$
Ser65Thr variant	1EMG.pdb	8.0	2.00	$\boldsymbol{n} = 0.46\boldsymbol{i} - 0.869\,\boldsymbol{j} + 0.18\boldsymbol{k}$
	1C4F.pdb	4.6	2.25	$\boldsymbol{n} = 0.45\boldsymbol{i} - 0.867\,\boldsymbol{j} + 0.21\boldsymbol{k}$

Rosell and Boxer, Figure S1.