Improved green fluorescence

SIR — The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has attracted widespread interest since the demonstration[1] that heterologous expression of the cloned gene[2] can generate striking green fluorescence. Despite the tremendous potential of recombinant GFP as a marker for gene expression or cell lineage or as an *in situ* tag for fusion proteins[3], the wild-type protein from *A. victoria* has several significant deficiencies. Its excitation spectrum (*a* in the figure) shows peaks at both 396 and 475 nm. The longer-wavelength excitation peak has the advantages of greater photostability[1] and better matching to standard fluorescein filter sets, but is relatively low in amplitude. Considerable improvement should be possible, because a closely related protein[4] from the sea pen *Renilla reniformis* has the same high quantum yield of emission (0.7–0.8), yet shows only one absorbance and excitation peak with an extinction coefficient per monomer more than 10 times that of the longer-

wavelength peak of *Aequorea* GFP[5,6]. We now report that simple point mutations in *Aequorea* GFP ameliorate its main problems and bring its spectra much closer to that of *Renilla*.

Serine 65 of the amino-acid sequence of *Aequorea* GFP becomes part of the *p*-hydroxybenzylideneimidazolinone chromophore. To test the hypothesis[7] that Ser 65 undergoes additional dehydration to form a vinyl side chain, we mutated that residue to Ala, Leu, Cys or Thr. If a vinyl group were formed by elimination of H_2O or H_2S, Ser and Cys should give identical spectra very different from Ala and Leu in which elimination is impossible. Serendipitously, all four mutants showed single excitation peaks, located at 470–490 nm, whose amplitudes were four- to sixfold greater than that of wild-type for equal numbers of molecules (*a* in the figure). These results exclude vinyl formation. The Ser 65→Thr mutant (S65T) was selected for further characterization because it had the longest wavelengths of excitation and emission (490 and 510 nm), which closely resembled those reported for *Renilla* GFP (498 and 508 nm). The crucial post-translational oxidation[8] to produce the fluorophore from the nascent polypeptide chain proceeded about fourfold more rapidly in S65T than in the wild-type protein (*b* in the figure). This acceleration ameliorates a poten-tially significant limitation in using GFP as a reporter protein for rapid gene inductions[5].

Mutations of Ser 65 to Arg, Asn, Asp, Phe, and Trp gave fluorescence intensities well below that of wild type. It remains unclear exactly how position 65 controls spectral properties or why *Aequorea* chose serine. Nevertheless, the greatly increased brightness and rate of fluorophore generation in mutants such as S65T should make them superior to wild-type *Aequorea* GFP for most experimental uses.

Note added in proof: GFP variants generated by combinatorial mutagenesis of positions 64–69 have excitation peaks near 490 nm, but their amplitudes and the kinetics of fluorophore formation have not been quantified[12].

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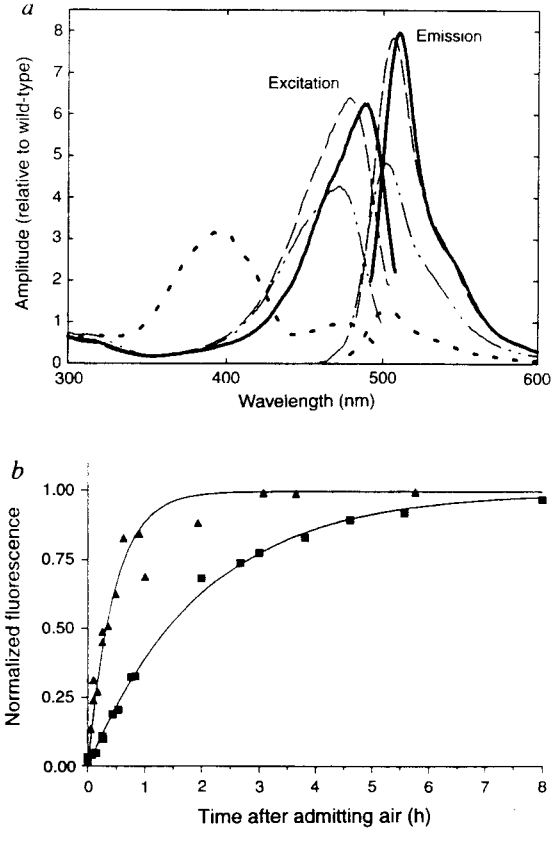

Comparison of recombinant wild-type and mutant green fluorescent proteins. *a*, Fluorescence excitation and emission spectra of wild-type protein (– – –), Ser 65→Ala (– · · –), Ser 65→Cys (– –) and Ser 65→Thr (—) mutants. The coding region of *Aequorea gfp* cDNA[2] (gift of D. Prasher) was cloned into the T7 expression vector $pRSET_B$ (Invitrogen), giving a polyhistidine-tagged fusion protein expressed in *Escherichia coli*, BL21(DE3)LysS (Novagen). Oligonucleotide-directed mutagenesis at the codon for Ser 65 was performed[9] on the same construct using the Muta-Gene Phagemid *in vitro* kit version 2 (Bio-Rad). All fusion proteins were purified on nickel-chelate columns (Qiagen). Spectra were obtained from equal concentrations of GFPs controlled by densitometry of denaturing gels stained with Coomassie blue. Excitation spectra were obtained collecting emission at the respective peak wavelengths (508, 503, 507, and 511 nm for wild-type, S65A, S65C and S65T) and were corrected by a quantum counter; emission spectra were likewise measured at the respective excitation peaks (475, 471, 479, and 489 nm) and were corrected using factors from the fluorometer manufacturer. The amplitude of the 475 nm excitation peak of wild-type GFP has been defined as 1.0. The sixfold greater peak amplitude of S65T arises from a 5.5-fold higher extinction coefficient (39,200 $M^{-1}cm^{-1}$ at 490 nm for S65T compared with 21,000 and 7,150 $M^{-1}cm^{-1}$ at 395 and 475-nm for wild-type), similar fluorescence quantum yield (0.68 versus 0.77), and slightly narrower emission spectrum. The extinction coefficients and quantum yields reported here for recombinant wild-type protein fused to a polyhistidine tag are in good agreement with literature[6] values for GFP extracted from *Aequorea* when corrected for the revised molecular mass[2]. Recombinant expression and polyhistidine tagging are known[1,10,11] not to affect the fluorescence spectra of wild-type GFP. *b*, Rates of autoxidative fluorophore generation in wild-type (■) and S65T (▲) GFP, measured by development of fluorescence after admission of air to *E. coli* cultures anaerobically grown in GasPak pouches (Becton-Dickinson) for 3 days. Air was readmitted while transferring the cells to phosphate-buffered saline containing 8 mM NaN_3 as a metabolic inhibitor. The time course of subsequent fluorescence development measured the final oxidation step in the protein's self-modification to generate its internal fluorophore[8]. Data from two independent runs normalized by their respective asymptotic fluorescence values were pooled for each protein. The smooth curves are exponential curve fits consistent with pseudo-first-order kinetics, with time constants of 2.0 and 0.45 h for wild-type and S65T, respectively. Previously reported time constants for wild-type GFP autoxidation[5] were rather longer, probably because the protein was held in bacteria for longer periods of anaerobic growth, which seems to slow subsequent oxidation.

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