Characterization of the Photoconversion of Green Fluorescent Protein with FTIR Spectroscopy†

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ABSTRACT: Green Fluorescent Protein (GFP) is a bioluminescence protein from the jelly fish *Aequorea victoria.* It can exist in at least two spectroscopically distinct states: GFP_{395} and GFP_{480}, with peak absorption at 395 and 480 nm, respectively, presumably resulting from a change in the protonation state of the phenolic ring of its chromophore. When GFP is formed upon heterologous expression in *Escherichia coli,* its chromophore is mainly present as the neutral species. UV and visible light convert (the chromophore of) GFP quantitatively from this neutral- into the anionic form. On the basis of X-ray diffraction, it was recently proposed (Brejc, K. et al. (1997) *Proc. Natl. Acad. Sci. USA 94,* 2306−2311; Palm, G. J. et al. (1997) *Nat. Struct. Biol. 4,* 361−365) that the carboxylic group of Glu^{222} functions as the proton acceptor of the chromophore of GFP, during the transition from the neutral form (i.e., GFP_{395}) to the ionized form (GFP_{480}). However, X-ray crystallography cannot detect protons directly. The results of FTIR difference spectroscopy, in contrast, are highly sensitive to changes in the protonation state between two conformations of a protein. Here we report the first characterization of GFP, and its photoconversion, with FTIR spectroscopy. Our results clearly show the change in protonation state of the chromophore upon photoconversion. However, they do not provide indications for a change of the protonation state of a glutamate side chain between the states GFP_{395} and GFP_{480}, nor for an isomerization of the double bond that forms part of the link between the two rings of the chromophore.

Wild-type green fluorescent protein (GFP[1]) from the jellyfish *Aequorea victoria* functions as the secondary emittor of (green) chemiluminescence from this organism (*1*). The free energy for this luminescence is obtained via radiationless energy transfer, originating from oxyluciferin. Luminescence is emitted from the 4-hydroxybenzylidene-imidazolinone chromophore of GFP, which is formed by the cyclization of an internal Ser^{65}-Tyr^{66}-Gly^{67} tripeptide, followed by the 1,2 dehydrogenation of the tyrosine (for a review, see ref *2*).

GFP displays two major optical transitions in its visible absorption spectrum (*1*). Wild-type GFP, formed upon heterologous expression in *Escherichia coli,* is mainly present with its chromophore in the neutral form (with a λ_{max} at 395 nm); only a small fraction is in the anionic form (λ_{max} = 480 nm; ref *3*). Subsequent illumination, particularly with UV light (which was mentioned to be more efficient than visible light; ref *2*), converts nearly all chromophore into the anionic form. The distribution over these two species is altered in a number of mutant forms of GFP, developed for optimization of its expression level and/or fluorescence characteristics; in these either the neutral or the anionic form of GFP may fully dominate the visible absorption spectrum (*3*, *4*).

Since GFP can be (reversibly) photoconverted into distinct forms, it is a member of the family of photoactive proteins (e.g., refs *5* and *6*). Cubitt et al. (*2*) refer to the photoconversion process as "photoisomerization", without giving details as to whether this refers to the chromophore of GFP or to one or more of its amino acid side chains. Recently, it was proposed that the photoconversion of GFP has its molecular basis in an intramolecular proton-transfer event, initiated by deprotonation of the phenolic chromophore, which in turn leads to rearrangement of the hydrogen-bonding network in the protein, and eventually to protonation of the anionic Glu^{222} (*7*, *8*). This proposal is based on a detailed analysis of the crystal structure of wild-type GFP (in monomeric form) and its Ser65Thr variant (*7*), as well as a large number of genetically engineered variants of GFP (*8*). It was concluded that, in crystals of wild-type GFP, a 6:1 molar ratio of conformers with a neutral and an anionic chromophore, respectively, is present. In many of the mutant forms of GFP, one of these two conformers dominates.

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[1] Abbreviations: FTIR, Fourier transform infrared; GFP, green fluorescent protein; GFP_{395} and GFP_{480}, green fluorescent protein with the chromophore in the neutral and anionic form, respectively; GFP_{uv}, variant of wild-type GFP with identical spectroscopic, but improved solubility characteristics; PYP, photoactive yellow protein; pG and pB, ground-state and blue-shifted intermediates of PYP (which have their chromophore in the anionic and neutral form, respectively); mOD, absorbance at wavelength specified times 10^{-3}; pCA, *p*-coumaric acid (or 4-hydroxy-cinnamic acid).

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Photoactive proteins, like GFP and bacteriorhodopsin, have important scientific and technological applications (*5, 9*). It is therefore necessary to precisely understand the structural basis of the transitions in GFP, induced by illumination, and the pathway of the coupled proton transfer. For that reason we have characterized the photoconversion of GFP with Fourier transform infrared (FTIR) difference spectroscopy. This is a highly sensitive technique to detect changes (i) between different conformations of a protein, (ii) in the protonation and isomerization states of its amino acid side chains and chromophore(s), and (iii) in its secondary structure.

Here we report the first FTIR spectroscopic study of GFP. Our results unambiguously show that the protonation state of Glu^{222} is the same in GFP_{395} and GFP_{480}; most likely this residue is deprotonated in both forms. Because of a number of striking parallels between photocycle transitions in photoactive yellow protein (PYP, e.g., refs *10* and *11*) and photoconversion in GFP, some of the results obtained with GFP are compared between these two photoactive proteins (see also ref *12*).

MATERIALS AND METHODS

Overproduction and Purification of Green Fluorescent Protein. The $pGFP_{uv}$ plasmid was obtained from Clontech (Palo Alto, CA). This construct encodes the *A. victoria gfp* gene, with the following "cycle 3" point mutations: Phe99Ser, Met153Thr, and Val136Ala. These replacements affect the hydrophobicity of the surface of GFP and increase its (water) solubility. In addition, several silent mutations were introduced (*13*). This GFP mutant is indistinguishable from the wild type with respect to its spectroscopic properties, but leads to higher expression levels in bacterial overexpression systems. The gfp_{uv} gene was inserted as a 793 bp *Kpn*I/*Stu*I fragment into the *Kpn*I/*Sma*I sites of the multiple cloning site of pQE31, an overexpression vector for N-terminally poly-histidine-tagged proteins (obtained from Quiagen, Hilden, Germany), in which GFP is expressed from a T5-promoter. The resulting vector was transformed into *E. coli* M15. Overproduction of GFP_{uv} was monitored by absorption measurements at 395 nm in cell-free extracts, using an extinction coefficient of 30 mM^{-1} cm^{-1} (*14*), and the product was purified on a Ni^{2+}-nitrilotriacetic acid (NTA) column, with a gradient of 0−500 mM imidazole in 50 mM sodium phosphate buffer, 300 mM NaCl, pH 8.0. After purification, the imidazole was removed by dialysis against 50 mM sodium phosphate buffer, pH 8.0. Purity of the sample was confirmed by SDS−PAGE analysis. The purity index of the final sample, that is, the ratio of the 280 nm over the 395 nm absorbance of this preparation (measured at an OD_{395} of 0.1), was 1.0 (*15*).

Overproduction and Purification of Photoactive Yellow Protein (PYP). Apo-PYP was expressed heterologously in *E. coli*, reconstituted with *p*-coumaric acid, and purified with Ni^{2+}/NTA affinity chromatography, as described in ref *16*. PYP was dissolved in 10 mM Tris/HCl, pH 7.0 (at a concentration of approximately 1 mM), and was subsequently dialyzed against the same buffer, adjusted to pH 5.0 with dilute HCl. A model compound, the methyl ester of *p*-coumaric acid, was kindly provided by H. Fennema (see also ref *16*).

Photoconversion. Photoconversion of GFP, either in a 1 cm quartz cell or in the IR cell (see below), was driven by illumination with a short-wavelength UV source [Universal UV lamp (Type TL-900) CAMAG, Muttenz, Switzerland; $\lambda_{max} = 254$ nm], with a dose of 0.1 mJ cm^{-2} s^{-1}. The conversion was followed spectroscopically from the interconversion of the 395 nm absorption band into the 480 nm absorption band (*2, 7, 15*), using an Aminco DW2000 UV/Vis spectrophotometer (SLM Instruments Inc., Urbana, IL), with a spectral resolution of 3 nm.

PYP was photoconverted from the ground-state pG into the intermediate pB (containing a protonated chromophore (*19*)), in the IR spectrometer, using a 150 W xenon lamp, fiber optics and a 440 nm interference filter (see also ref *17*).

Fourier Transform Infrared Spectroscopy. GFP samples were concentrated to about 2.5 mM, with a 30 kDa cutoff Millipore NMWL filter spin column, at room temperature, in 10 mM Tris, pH 8.0. Deuterated GFP was prepared by several subsequent concentration steps, each followed by dilution into 10 mM Tris/HCl in D_2O (pD 8.0), until the remaining amount of H_2O was below 5% (v/v). Samples were then incubated (in D_2O) for 12 h at room temperature, followed by 15 h at 4 °C. The residual amount of H_2O was quantitated with the use of FTIR absolute absorption spectra.

For FTIR measurements, GFP samples were sandwiched between two CaF_2 plates, using a 12 μm polyethylene spacer. FTIR spectra were measured using a BioRad FTS-60A infrared spectrophotometer (BioRad Analytical Instruments Group, Cambridge, MA), containing a mercury−cadmium−telluride detector. Spectra were only corrected for instrument drift and H_2O vapor, without further smoothing. Data manipulation was performed with software provided by the manufacturer. The spectral resolution obtained was 2 cm^{-1} (*17*). Each spectrum was averaged over multiples of 381 scans (usually 1524). The noise level in the baseline of these spectra was approximately 0.05 mOD in the region of the intense water line at 1650 cm^{-1} and less than 0.02 mOD in the regions well-separated from that line.

Absorption and Fluorescence Spectroscopy. UV/Vis absorption spectra were recorded with a model HP 8452A Hewlett-Packard diode array spectrophotometer (Portland, OR) and with an Aminco DW2000 dual beam spectrophotometer (SLM Instruments Inc., Urbana, IL). Fluorescence excitation spectra were taken with an Aminco Bowman Series 2 luminescence spectrometer (SLM-AMINCO, Rochester, NY) and were corrected for the lamp output spectrum with a photodiode.

RESULTS AND DISCUSSION

GFP_{395} to GFP_{480} Photoconversion by UV and Visible Light. The visible absorption spectrum of heterologously expressed GFP_{uv} (dissolved at low ionic strength and relatively low protein concentration, i.e., <0.1 mg/mL, or <3.5 μM) shows three maxima. Besides a band caused by the aromatic amino acids (at 280 nm), a dominant absorption band is present at 395 nm, presumably due to the neutral, that is, phenolic, form of its 4-hydroxybenzylidene-imidazolinone chromophore (see also below), as well as a second maximum in the visible part of the spectrum at 480 nm (due to the anionic, i.e., phenolate, form of the chromophore).

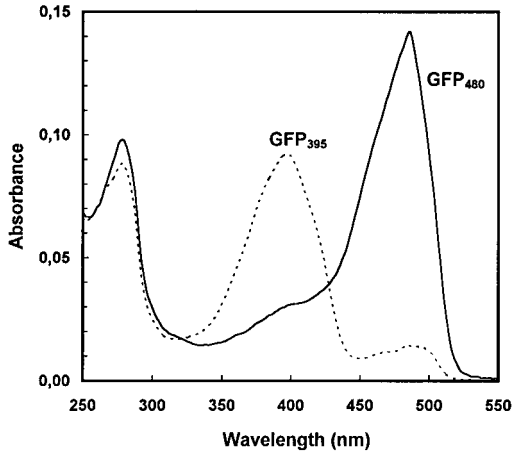

FIGURE 1: Visible absorption spectra of GFP, before (broken line) and after (continuous line) photoconversion with UV illumination. GFP_{uv} (as isolated) was concentrated to 2.5 mM in 10 mM Tris/HCl, pH 8.0. Absorbance spectra, corrected for the contributions of buffer and CaF_2, were recorded in the IR cell, with an optical path length of 10 μm, using a Hewlett-Packard diode array spectrophotometer. For UV illumination conditions, see Materials and Methods.

The ratio of the absorbance at 395 over 480 nm in such samples equals approximately 3. Taking into account the higher extinction coefficient of the latter (anionic) species, the molar ratio of the neutral form of the chromophore, over the anionic form, in the protein as isolated, equals approximately 6 (*18*).

By increasing the concentration of GFP to levels suitable for IR spectroscopy, a light-independent conversion of GFP into the form with the neutral (i.e., protonated) chromophore is initiated (*15*). When reaching a concentration of 2.5 mM, this molar ratio (i.e., neutral/anionic) has increased to 13 (see Figure 1), so that only a very small fraction of the anionic species remains in such samples.

Upon illumination of GFP with UV light, largely independent of the GFP concentration, a rapid photoconversion to the anionic species takes place, which can be driven almost to completion (see Figure 1 for the sample in H_2O). From this figure it can be seen that the extinction coefficients of the neutral and anionic form of the chromophore of GFP show a similar approximate 2-fold difference, as the corresponding forms in tyrosine (*19*) and in the chromophore of photoactive yellow protein (*10*). For these three compounds, deprotonation of the chromophore results in a red shift of their absorbance maximum of approximately 5000 cm^{-1}.This fully supports the presumed deprotonation of the chromophore of GFP upon photoconversion. This result also shows that photoconversion of GFP, under the conditions specified, takes place without appreciable photobleaching (ref *18*; but contrast ref *2*).

The rate of photoconversion, by the specific source of UV light used in this investigation, is given in Table 1. This rate, measured by following the optical spectra of the sample in time, in H_2O as well as in D_2O, is essentially identical in the two solvents: approximately 1.1×10^{-3} s^{-1}, independent of the protein concentration (data not shown). The return of the anionic species to the neutral form in the dark is extremely slow. An approximate rate in the H_2O sample of 1×10^{-6} s^{-1} has been determined.

We conclude that the conditions described above, in H_2O as well as in D_2O, are optimal for the study of the photoconversion of GFP, from GFP_{395} to GFP_{480}, like with FTIR difference spectroscopy. Precise information about the relative population of GFP in the neutral and anionic forms is not yet available for wild-type GFP in the crystalline state, nor for any of its mutant forms. In view of the concentration dependence of this distribution, noted above, it will be of interest to determine this ratio under those circumstances.

Table 1: UV-Induced Photoconversion Rates to the Neutral and Anionic Forms of GFP and Relative Quantum Yields of Fluorescence Emission in H_2O and D_2O[a]

	rate (N → A) (s^{-1})	rate (A → N) (s^{-1})	ϕ^{rel} neutral form	ϕ^{rel} anionic form	ϕ^{rel} UV abs
GFP−H_2O	1.1×10^{-3}	$\pm 1 \times 10^{-6}$	1	0.82	0.70
GFP−D_2O	1.1×10^{-3}	$\ll 1 \times 10^{-6}$	1	0.78	0.63

[a] Rates of photoconversion, at 1 J m^{-2} s^{-1} UV light, are measured by the relative change of absorbance at 480 nm during UV irradiation. Quantum yields (ϕ) are presented relative to the emission at 395 nm excitation (neutral form), calculated from normalized excitation and absorption spectra: A = anionic form; N = neutral form.

GFP_{480} − GFP_{395} FTIR Difference Spectra. X-ray diffraction, although very powerful in the overall resolution of protein structure, does not allow one to directly observe the state of protonation of individual amino acid carboxylates. FTIR spectroscopy (particularly in the mode of difference spectra), in contrast, is very sensitive to such structural changes in proteins, including protonation and deprotonation, chromophore isomerization, and perturbation of hydrogen bonding. We have therefore employed FTIR spectroscopy to analyze the photoconversion of GFP from GFP_{395} to GFP_{480}, while in parallel ascertaining the photoconversion in the IR cell to be as complete as in the UV/Vis experiment (Figure 1). Because this has been observed, and because the anionic state that is formed by UV illumination is stable on the time scale of hours (see Table 1), recording of a GFP FTIR spectrum before, and after, photoconverting illumination allows one to obtain GFP_{480} − GFP_{395} difference spectra.

Figure 2 shows, for a comparison, the absolute spectrum of GFP in water and the GFP_{480} − GFP_{395} difference spectra of GFP samples in H_2O and D_2O, in the mid infrared region, from 1800 to 1000 cm^{-1}. The absolute spectrum shows the typical features of a protein with intense amide I and II bands and less intense features in the fingerprint region, except for the intense band at approximately 1100 cm^{-1}. The photoconversion-induced FTIR difference spectra are shown in the lower part of Figure 2, in H_2O and D_2O. The latter solvent was used to facilitate the interpretation of these spectra and to obtain higher sensitivity in the range around 1650 cm^{-1}.

These difference spectra reveal the extent and nature of structural changes in GFP upon photoconversion, involving COOH groups (1760−1710 cm^{-1}), the protein backbone (amide I, 1680−1620 cm^{-1}; amide II, 1550−1530 cm^{-1}), and the chromophore (including bands peaking at 1580, 1497, and 1147 cm^{-1}). In the GFP sample in H_2O, well-resolved bands with positive amplitude (i.e., arising in the anionic form of GFP) are observed at 1580, 1538, 1497, 1342, 1316, and 1147 cm^{-1}. Of these, the vibrational modes at 1538, 1342, and 1147 cm^{-1} shift slightly, to 1539, 1343, and 1149 cm^{-1}, respectively, upon deuteration. The features that characterize the neutral species in GFP are much less well-

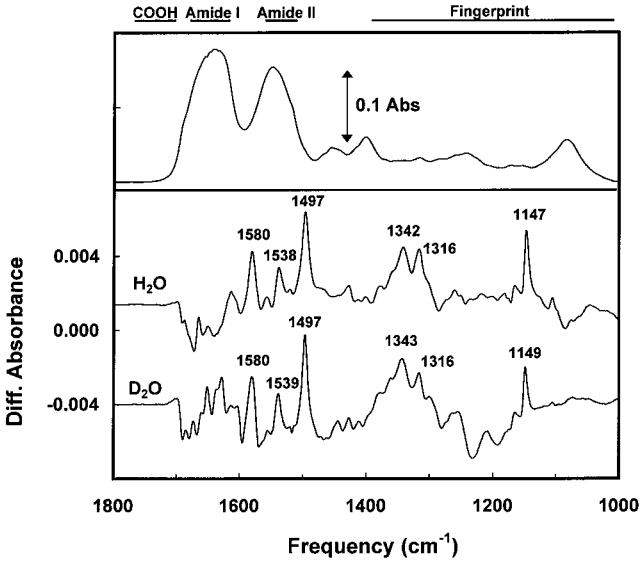

FIGURE 2: Absolute FTIR spectrum of GFP in water (upper panel) and FTIR difference spectra (lower panel) between neutral and anionic species of GFP (i.e., $GFP_{395} - GFP_{480}$) in H_2O (middle) and D_2O (bottom), showing the carboxyl, amide I, amide II, and fingerprint regions. First, an absolute spectrum (from 1800 to 1000 cm^{-1}) was obtained of the neutral form of GFP. The IR cell was subsequently removed from the spectrophotometer, to photoconvert the majority of the GFP into the anionic form. This was completed in approximately 15 min, using the conditions as described in Materials and Methods, and a visible absorption spectrum was recorded to check the degree of photoconversion of the sample (compare Figure 1). Sample conditions were identical to those described in the legend to Figure 1. The concentrated GFP_{uv} solution (2.5 mM), either in H_2O or in D_2O, was introduced into the 12 μm IR cell. The OD_{395} of the sample in H_2O was 0.093 (Figure 1). For further details, see Materials and Methods.

characterized. Bands at 1614 and 1105 cm^{-1} appear to be specific for the sample in H_2O, whereas the D_2O sample shows characteristic bands at 1628, 1445, and 1253 cm^{-1}.

Because the UV light-induced changes in the visible spectra allow an exact quantification of the amount of GFP that is photoconverted, the difference extinction coefficients of these transitions can be calculated. For example, the larger features characteristic for the anionic species (at 1497 and 1147 cm^{-1}) have infrared difference extinction coefficients of 1.0 and 1.5 mM^{-1} cm^{-1}, respectively. Characteristic bands are also observable in the amide I (1690−1620 cm^{-1}) and the amide II (~1530 cm^{-1}) regions of the difference spectra, both in H_2O and in D_2O. Particularly the latter are well-resolved in both solvents, which may indicate that changes in secondary structure accompany photoconversion. It must be kept in mind, however, that, to the bands observed in the amide II region, the chromophore may also contribute.

Identification of Chromophore Vibrational Modes. It has been extensively documented that the vibrational frequencies of a phenolic ring (like in tyrosine and in the anionic tyrosinate) are sensitive to ionization (i.e., deprotonation of the phenolate group). Vibrational modes, for instance, at 1518 and 1244 cm^{-1} of a protonated phenol ring are shifted to 1498 and 1270 cm^{-1} upon ionization (e.g., refs *20* and *21*). A stack plot of FTIR difference data has therefore been constructed of (1) $GFP_{480} - GFP_{395}$ of GFP, (2) ionization of the methyl ester of *p*-coumaric acid, (3) trans → cis photoisomerization of the methyl ester of *p*-coumaric acid, and (4) pB − pG intermediates of PYP (Figure 3). These data allow us to assign the most intense positive peaks from the fingerprint region of the difference spectra of GFP (at 1147 cm^{-1}) plus two additional positive bands (at 1580 and 1497 cm^{-1}) tentatively to deprotonation of (the phenolate part of) its chromophore, because very similar strong absorbance can be observed in the ionization difference spectrum of the methyl ester of *p*-coumaric acid, which evidently also resembles those in the absolute spectrum of PYP, in the difference spectra of its intermediates (ref *22* and Xie et al., unpublished experiments; see also Figure 3), and in the deprotonation-induced difference spectra of tyrosine (e.g., refs *20* and *21*). This fully confirms the interpretation of the UV/vis observations (Figure 1) and forms the most direct proof for deprotonation of the phenolate part of the chromophore as the molecular basis of photoconversion in GFP. In agreement with this, a corresponding vibrational mode is also observed in the Raman spectra of wild-type PYP (*23*), while these are not found in the *cis*−*trans* difference spectrum of the methyl ester of *p*-coumaric acid. The strong similarity between the GFP data and the data obtained with the ionized chromophore model compound further reveals that the ionized phenolic ring in GFP_{480} is not strongly strained.

There is overall similarity between the difference spectra of the neutral-minus-anionic forms of GFP and PYP, in the spectral range from 1400 to 1000 cm^{-1} (Figure 3). Its dominant band (i.e., at 1147 cm^{-1} in GFP and at 1162 cm^{-1} in PYP) may be due to in-plane bending of the aromatic hydrogens of a phenolate chromophore and/or to the C8−C9 stretching mode. The in-plane bending modes of neutral phenolic hydrogens generally are very weak, but this is not so in the *p*-coumaric acid model compounds. In the pB − pG difference spectra of PYP, not only protonation but presumably also isomerization is involved (*24*). Whether a similar isomerization also plays a role in photoconversion of GFP cannot be excluded, nor claimed, yet. Such a chromophore photoisomerization, involving a ring flip around the double bond, could be hidden by the ionization signals, which are stronger than those of isomerization of neutral chromophore (see Figure 3). We would expect that photoisomerization of the chromophore will strongly perturb the interaction between the chromophore and Arg^{96}, and possibly also with Gln^{183}, modulations which have not been identified in the difference spectra. Such interactions would make these difference spectra sensitive to ^{15}N labeling in the region between 1700 and 1650 cm^{-1}.

The amide I and II bands are spectral markers for protein conformational changes. In the $GFP_{480} - GFP_{395}$ FTIR difference spectrum, both the amide I and amide II bands are rather small, indicating that there are only small changes in the protein conformation, with respect to secondary structure and tertiary fold, upon photoconversion of GFP. It is therefore likely that only local structural changes are needed to stabilize the buried charge in GFP_{480} after photoconversion.

Is Glu^{222} the Proton Acceptor for Chromophore Ionization in the GFP_{395} to GFP_{480} Transition? FTIR difference spectra, in the region between 1760 and 1710 cm^{-1} are uniquely contributed to by the C═O stretching modes of COOH groups. Structural perturbation of COOH groups will yield shifted peak positions and/or changes in spectral bandwidths, resulting in a combination of positive and

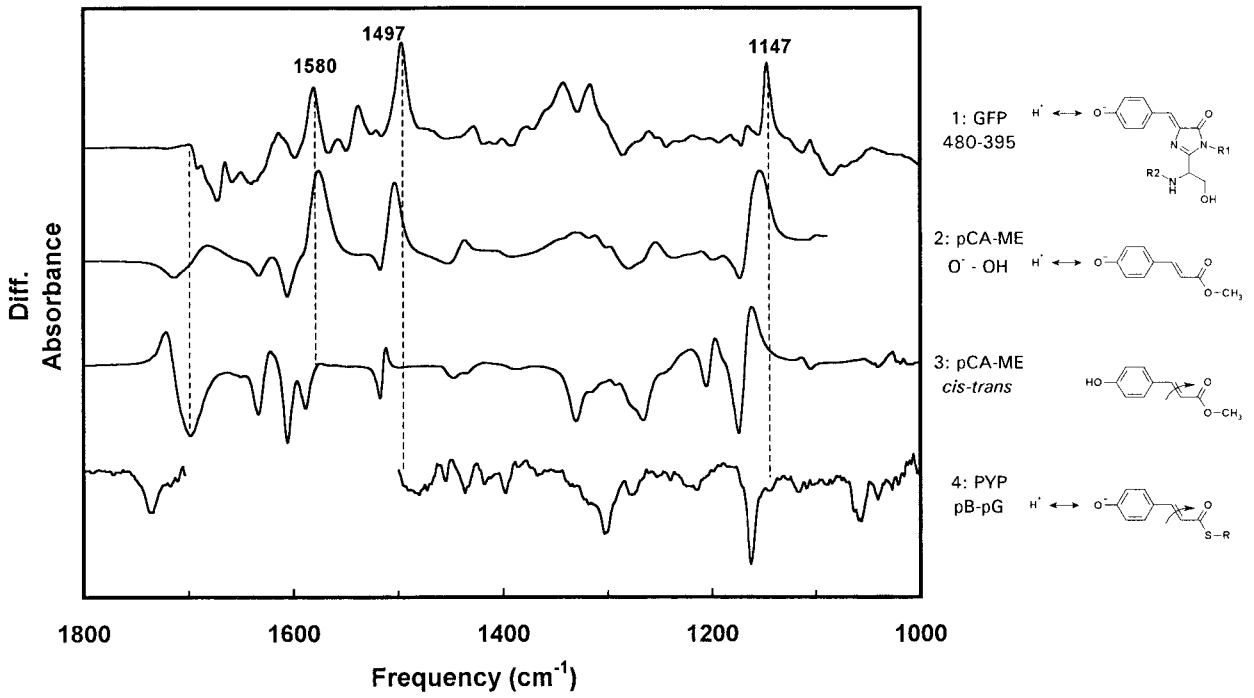

FIGURE 3: Comparison of FTIR difference spectra of GFP and PYP, before and after photoconversion, with a model compound (methyl-*p*-coumarate). For difference spectra of GFP (before and after illumination; spectrum 1) and PYP (pB-pG; spectrum 4), see the legends of Figures 2 and 4, respectively. Difference spectra of the model compound, the methyl ester of *p*-coumaric acid, dissolved at approximately 1 mM in 10 mM Tris/HCl, pH 7.0, were obtained by measuring spectra before and after (i) alkalization to pH = 9.5 (spectrum 2), and (ii) illumination with UV light (spectrum 3).

negative bands. Protonation of a COO^- group will lead to an entirely new band, since the COO^- stretching vibrations are beyond this region. The relatively flat spectrum in the COOH region of the GFP difference spectra already suggests that there are no net changes in the protonation state of carboxylic groups in GFP during photoconversion. In contrast, a well-resolved COOH stretching band is observed in the pG − pB FTIR difference spectrum of PYP, due to the protonation of Glu[46] in the latter protein (*11*). The amplitude of the features in the region of these spectra, where the carbonyl groups absorb in GFP and PYP, can now be mutually scaled on the basis of the features resulting from the (de)protonation of the phenolate parts of their chromophore (this scaling gives results identical to those of the scaling based on visible absorption spectra; data not shown). The resulting FTIR difference spectrum of the two proteins is displayed in the region from 1800 to 1700 cm^{-1} in Figure 4. Since in PYP a single glutamic acid residue is deprotonated in pB (Glu[46]; ref *11*), and assuming identical extinction coefficients for the C═O bands of Glu[222] from GFP and Glu[46] from PYP, this allows one to unambiguously conclude from these difference spectra (with the signal-to-noise ratio obtained) that less than 0.1 glutamate residue of GFP protein changes its state of protonation upon photoconversion. These results argue against the model proposed by Brejc et al. (*7*) and Palm et al. (*8*), based on their X-ray analyses.

Nevertheless, since the FTIR results clearly confirm the change in protonation state of the GFP chromophore upon photoconversion, it is relevant to try to identify alternative proton acceptors in the vicinity of the chromophore. His[148] is one of the candidates for this. The crystal structure of the Ser65Thr mutant of GFP, containing the ionized form of the chromophore, shows that the imidazole group of His[148] is hydrogen bonded to the phenolate oxygen of the chromophore, whereas in the neutral form of wild-type GFP Brejc et al. (*7*) assume it not to be. This is in contrast to the model of Palm et al. (*8*) for wild-type GFP, which supposes that both forms are in hydrogen-bonding contact with His[148]. Further work (e.g., with ^{15}N labeling) will have to reveal the precise role (if any) of His[148] in the photoconversion of GFP.

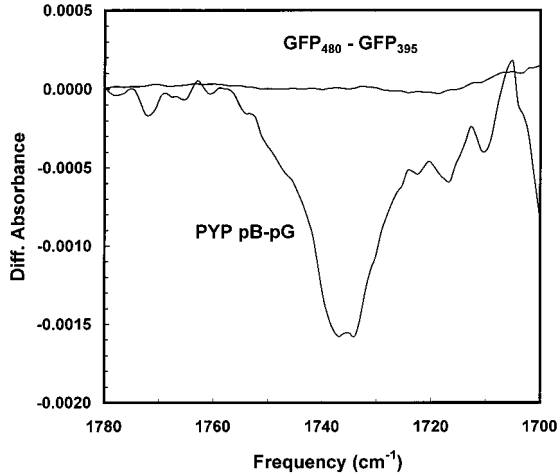

FIGURE 4: Comparison of photoconversion-induced difference spectra of GFP and PYP in the carboxyl region. Comparison of the light-induced difference spectra from 1780 to 1700 cm^{-1} for (i) $GFP_{395} - GFP_{480}$ (upper spectrum) and (ii) pB-pG of photoactive yellow protein (lower spectrum). The PYP difference spectrum has been scaled to GFP, on the basis of the complete photoconversion of GFP (see Figure 1) and a steady-state accumulation of 30% pB, as calculated from parallel UV/vis difference spectra of PYP. For the details of the photoconversion of the pG form of PYP into the pB form, see Materials and Methods.

It may also be that Glu[222] (only) functions transiently as a proton acceptor. The crystal structure of the Ser65Thr

mutant form of GFP shows that, of the 16 Glu and 18 Asp side chains in GFP, Glu^{222} is the only one that has its γ-carboxylate group buried inside the hydrophobic core of the protein. However, a chain of water molecules (numbers 374, 395, 383, and 320 in the PDB file 1EMA of Ser65Thr-GFP; ref *25*) at approximate hydrogen-bonding mutual distance (i.e., between 2.5 and 3.2 Å) form a hydrogen-bonding chain, starting from Glu^{222} and extending out into the direction of the bulk water. Our data therefore could also be interpreted by assuming that Glu^{222} is deprotonated in both the GFP_{395} and the GFP_{480} forms, while the proton coming from the chromophore would only very transiently reside on Glu^{222}, on its way to the bulk water. This is in line with the X-ray crystallographic structure of the GFP variants, as reported by Wachter et al. (*26*), and the results of random mutagenesis studies, as reported by Ehrig et al. (*27*).

Besides effects due to Glu^{222} protonation, significant changes in the IR characteristics of Thr^{203} are also expected upon photoconversion. According to the model of Brejc et al. (*7*), the involvement of this amino acid in the hydrogen-bonding network of the chromophore shifts from the backbone C═O to its side chain OH (hydrogen-bonded to the phenolate oxygen of the chromophore) in this transition. Also this rearrangement (expected at approximately 1050 cm^{-1} for the C−O stretch vibration) is not evident in the FTIR difference spectra. These results therefore lead to the conclusion that, under the assumption that GFP_{uv} is sufficiently similar to wild-type GFP, published models for photoconversion of GFP fail to explain the structural data as recorded by FTIR.

There is more data available from the literature that is not easily reconciled with the models of Brejc et al. (*7*) and Palm et al. (*8*). Thr^{203}, for instance, can be exchanged for a Phe (or Tyr), which causes only a 15−20 nm red shift of the anionic form (*25*). On the other hand, Thr203Ile and Ser205Phe mutant forms of GFP fully stabilize the neutral form (*3*, *27*), as expected. More detailed analysis of difference spectra, in combination with the assignment of the various bands, using isotope enrichment and site-directed mutagenesis, will be required to resolve the structural changes accompanying photoconversion in GFP. In addition to that, it will be very instructive to study photoconversion of GFP in crystals, using X-ray diffraction, preferably in combination with microspectrophotometry. Such studies will also have to reveal whether in the photoconversion of GFP isomerization of the double bond (between the phenolate and the dihydroimidazole rings of the chromophore) is also involved. Particularly the mutual orientation of the two rings and the position of the C═O group of the dihydroimidazole ring are important in this respect (*28*). Considering the similarities between GFP and PYP, it is very interesting to resolve this point.

Fluorescence Quantum Yields. More than two intermediates are involved in the photoconversion of GFP. Dickson et al. (*5*) reported transitions of single molecules between three ground states, two of which are photoactive. Chattoraj et al. (*18*) observed that, after excitation of the neutral form of GFP, a deprotonation into the anionic form takes place before significant 510 nm fluorescence is emitted. The fluorescence lifetime of GFP is 3.3 ns (*29*), whereas the deprotonation occurs within the picosecond time scale (*18*). Comparison of fluorescence excitation and electronic absorption spectra shows that, both in H_2O and in D_2O, the quantum yield of the fluorescence of GFP_{395} is higher than that of GFP_{480} (Table 1). This indicates that separate excited states are populated, from both species, which recombine to their respective ground state with different rates. Photoconversion can proceed from the excited state of both forms of GFP. The fluorescence decay measured at 460 nm is slowed significantly in deuterated samples, indicating that the proton on the chromophore can be exchanged for a deuteron. This kinetic isotope effect is so large that one must assume that multiple H/D exchanges affect this transition (*18*). The fluorescence lifetime of the excited state of the neutral form is no more than a few percent of the fluorescence lifetime of the 508 nm emission. Therefore, in deuterated samples, neither the quantum yield of fluorescence nor the rate of photoconversion is expected to change, which is in agreement with our observations (see Table 1). The fluorescence quantum yield of both forms of GFP is very high, while the quantum yield of photoconversion of GFP_{395} is very small. This suggests that the light-induced ionization of the chromophore is largely reversible (i.e., the excited state of GFP_{395} must return to the ground state rapidly) and only a small fraction is converted to the long-lived GFP_{480} state. This suggests that this latter structure must be different from that of the short-lived (excited) red-shifted state.

The Role of Aromatic Amino Acid Residues in the Photoconversion of GFP. To analyze the contribution of its multiple absorbance bands to fluorescence emission, GFP was diluted into 10 mM Tris, pH 8.0, both in H_2O and in D_2O, to a final OD_{395} of 0.08. The sample was partially photoconverted, to obtain an approximately 1:1 molar ratio of the neutral and anionic species of the chromophore, as estimated from the absorption spectrum. Absorption and corrected excitation spectra were then recorded, with the emission wavelength set at 508 nm, and used for the calculation of relative quantum yields of fluorescence. Table 1 shows these for the 280 and 480 nm absorption maxima, relative to the quantum yield of the neutral form, which is highest. The relatively high efficiency of fluorescence excitation at 280 nm is striking.

GFP_{uv} contains 12 Phe, 10 Tyr, and 1 Trp residues, which are more or less randomly distributed over the surface and the inside of the GFP barrel, with a minimal distance to the chromophore of 6, 4.5, and 11 Å for the three types of aromatic residue, respectively (*25*, *30*). The majority of the absorbance in the 280 nm region originates from the Trp, Tyr, and Phe residues, because chromophore-containing peptides do not appreciably absorb at this wavelength (*31*) and the total absorbance of GFP at this wavelength closely matches the sum of the contributions of the three aromatic amino acids (*32*). Because the quantum yield of fluorescence emission, upon 280 nm excitation, is appreciable (i.e., at least 0.65), this implies that excitons are transferred efficiently from the aromatic amino acids to the chromophore. On the basis of the lifetime and quantum yields of fluorescence, the spectral overlap, and mutual distance, it can be calculated that this resonance energy transfer should be possible with high efficiency (*33*). Surprisingly, replacement of Phe^{64}, which is by far the closest aromatic side chain to the chromophore, by a leucine residue, did not have a detectable

effect on the excitation spectrum of the protein (data not shown).

Photoconversion of GFP can be driven by the absorption of photons of sufficient energy, including light of 395 and 480 nm (*2*). However, these latter wavelengths are much less efficient than short-wavelength UV light. In contrast to the efficient photoconversion achieved with our UV source (see Materials and Methods), the use of a 250 W Xenon light source, in combination with a broad-band 400 nm interference filter, caused only minimal photoconversion within an hour of illumination (i.e., had a low quantum yield), whereas photobleaching of GFP was significant under these conditions and the extinction coefficient of GFP_{395} is comparable at 280 and 400 nm. The high quantum yield of fluorescence, emitted upon UV illumination, indicates that the aromatic amino acids form a connected system, from which resonance energy is transferred to the chromophore efficiently. This may lead to a vibrationally excited protein, which is more ready for photoconversion than a form of GFP in which exclusively the chromophore is electronically excited.

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REFERENCES

1. Morise, H., Shimomura, O., Johnson, F. H., and Winant, J. (1974) *Biochemistry 13*, 2656−2662.
2. Cubitt, A. B., Heim, R., Adams, S. R., Boyd, A. E., Gross, L. A., and Tsien, R. Y. (1995) *Trends Biochem. Sci. 20*, 448−455.
3. Heim, R., Prasher, D. C., and Tsien, R. Y. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12501−12504.
4. Delagrave, S., Hawtin, R. E., Silva, C. M., Yang, M. M., and Youvan, D. C. (1995) *Bio/Technology 13*, 151−154.
5. Dickson, R. M., Cubitt, A. B., Tsien, R. Y., and Moerner, W. E. (1997) *Nature 388*, 355−358.
6. Hellingwerf, K. J., Hoff, W. D., and Crielaard, W. (1996) *Mol. Microbiol. 21*, 683−693.
7. Brejc, K., Sixma, T. K., Kitts, P. A., Kain, S. R., Tsien, R. Y., Ormö, M., and Remington, S. J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 2306−2311.
8. Palm, G. J., Zdanov, A., Gaitanaris, G. A., Stauber, R., Pavlakis, G. N., and Wlodawer, A. (1997) *Nat. Struct. Biol. 4*, 361−365.
9. Birge, R. R. (1995) *Sci. Am. 272*, 90−95.
10. Hoff, W. D., Van Stokkum, I. H. M., Van Ramesdonk, H. J., Van Brederode, M. E., Brouwer, A. M., Fitch, J. C., Meyer, T. E., Van Grondelle, R., and Hellingwerf, K. J. (1994) *Biophys. J. 67*, 1691−1705.
11. Xie, A., Hoff, W. D., Kroon, A. R., and Hellingwerf, K. J. (1996) *Biochemistry 35*, 14671−14678.
12. Hellingwerf, K. J., Van Thor, J. J., Hoff, W. D., and Hendriks, J. (1998) in *Nanotechnology* (ten Wolde, A., Ed.) Stichting Toekomstbeeld der Techniek, Den Haag, The Netherlands (in press).
13. Crameri, A., Whitehorn, E. A., Tatae, E., and Stemmer, W. P. C. (1996) *Nat. Biotechnol. 14*, 315−319.
14. Ward, W. W. (1997) *Abstract*, Annual Meeting of the American Society for Photobiology, pp 81S.
15. Ward, W. W., Prentice, H. J., Roth, A. F., Cody, C. W., and Reeves, S. C. (1982) *Photochem. Photobiol. 35*, 803−808.
16. Kroon, A. R., Hoff, W. D., Fennema, H. P., Gijzen, J., Koomen, G. J., Verhoeven, J. W., Crielaard, W., and Hellingwerf, K. J. (1996) *J. Biol. Chem. 271*, 31949−31956.
17. Kooter, I. M., Pierik, A. J., Merkx, M., Averill, B., Mogulevsky, N., Bollen, A., and Wever, R. (1997) *J. Am. Chem. Soc. 119*, 11542−11543.
18. Chattoraj, M., King, B. A., Bublitz, G. U., and Boxer, S. G. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 8362−8367.
19. Rava, R. P., and Spiro, T. G. (1985) *J. Phys. Chem. 89*, 1856−1861.
20. Dollinger, G., Eisenstein, L., Lin, S.-L., Nakanishi, K., and Termini, J. (1986) *Biochemistry 25*, 6524−6533.
21. Rothschild, K. J., Roepe, P., Ahl, P. L., Earnest, T. N., Bogomolni, R. A., Das Gupta, S. K., Mulliken, C. M., and Herzfeld, J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 347−351.
22. Imamoto, Y., Mihara, K., Hisatomi, O., Kataoka, M., Tokunaga, F., Bojkova, N., and Yoshihara, K. (1997) *J. Biol. Chem. 272*, 12905−12908.
23. Kim, M., Mathies, R. A., Hoff, W. D., and Hellingwerf, K. J. (1995) *Biochemistry 34*, 12669−12672.
24. Kort, R., Vonk, H., Xu, X., Hoff, W. D., Crielaard, W., and Hellingwerf, K. J. (1996) *FEBS Lett. 382*, 73−78.
25. Ormö, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., and Remington, S. J. (1996) *Science 273*, 1392−1395.
26. Wachter, R. M., King, B. A., Heim, R., Kallio, K., Tsien, R. Y., Boxer, S. G., and Remington, S. J. (1997) *Biochemistry 36*, 9759−9765.
27. Ehrig, T., O'Kane, D. J., and Prendergast, F. G. (1995) *FEBS Lett. 367*, 163−166.
28. Perman, B., Šrajer, V., Ren, Z., Teng, T.-y., Pradervand, C., Ursby, T., Schotte, F., Wulff, M., Kort, R., Hellingwerf, K. J., and Moffat, K. (1998) *Science 279*, 1946−1950.
29. Perozzo, M. A., Ward, K. B., Thompson, R. B., and Ward, W. W. (1988) *J. Biol. Chem. 263*, 7713−7716.
30. Yang, F., Moss, L. G., and Phillips, G. N. (1996) *Nat. Biotechnol. 14*, 1246−1252.
31. Cody, C. W., Prasher, D. C., Westler, W. M., Prendergast, F. G., and Ward, W. W. (1993) *Biochemistry 32*, 1212−1218.
32. Mach, H., Middaugh, C. R., and Lewis, R. V. (1992) *Anal. Biochem. 200*, 74−80.
33. Förster, T. (1965) in *Modern Quantum Chemistry, Part III, Action of light and organic molecules* (Sinanoglu, O., Ed.) pp 93−137, Academic Press, New York.

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