ELSEVIER

Computational analysis of Thr203 isomerization in green fluorescent protein

Andrew Warren and Marc Zimmer

Chemistry Department, Connecticut College, New London, CT 06320, USA

Green fluorescent protein (GFP) is an extensively used fluorescent tag. Photoisomerization between two spectroscopically distinct states in wild-type GFP is responsible for its two visible absorption bands at 398nm (A) and 475nm (B). We have used molecular mechanics and database analysis to support the suggestion of other researchers that the anionic form of the GFP chromophore is responsible for the B absorption band, while the phenol form is responsible for band A. The anionic ($^{-}O_{tyr}$, N_{imid}, $Glu_{222}H$) species is the only form that has a low energy pathway allowing for isomerization of Thr203 to a conformation where it stabilizes the phenolate form and is therefore the most likely species responsible for the B absorption band. The rotation of the Thr203 side-chain is restricted; this may be significant in the formation of the intermediate state which is central to the photoisomerization. Our calculations support the most commonly accepted mechanism for photoisomerization, and we have shown that the 201LSTQS205 sequence does not allow a g+ conformation for Thr203. © 2001 by Elsevier Science Inc.

Keywords: conformational analysis, fluorescent tag, molecular modeling, photochemistry

INTRODUCTION

Green fluorescent proteins (GFP) are of great interest due to their application to in situ monitoring of protein-folding, gene expression, protein movement, and cell development. GFP tagged proteins can be monitored noninvasively in living cells by flow cytometry, fluorescence microscopy, or macroscopic imaging methods.[1] Reasons for GFP's utility are that chromophore formation in GFP does not require any additional factors, the protein does not interfere with cell growth and function, and it is not toxic to cells. The crystal structures of wild-type GFP as a dimer[2] and a monomer,[3] as well as the solid state structures of several mutants[4,5] have been reported. The structure has been described as a "light in a can"; the chromophore is located in the center of a can consisting of 11 β sheets. The can is a nearly perfect cylinder with a height of 42Å and a radius of 12Å. The solid state structures of GFP provide an excellent starting point for computational analysis, since GFP fluoresces with similar spectral properties in the solid state and in aqueous solution.[6] This observation allows us to assume that the conformational changes between the solid state, in vitro and in vivo forms are minimal. Wild-type GFP has a major absorption at 398nm and a minor absorption 475nm[7] with a shoulder on the red edge.[8] There seems to be little doubt that a change in protonation is responsible for the different absorptions, however there is some controversy about the location of the acid labile site. A number of groups have suggested that the species responsible for the absorption at 398nm, species A, has a HO_y,N,Glu^{-} protonation state (see Figure 1 for description of the nomenclature used), while species B, which gives rise to the band at 475nm, is an $^{-}O_y$,N,GluH form, *vide infra.* Alternatively, semi-empirical calculations have been used to assign species A as a cationic form, HO_y,NH^{+}_{imid} and species B as a zwitterionic, $^{-}O_y$, NH^{+}_{imid}, form.[9] Based on the observation that the Y66H mutant only absorbs at 384nm,[10] changes in the absorption and fluorescence spectra that accompany other mutations, and the crystal structure of the Y66H mutant,[11] a mechanism, shown in Figure 2, for the photoisomerization of wild-type GFP was proposed.[3,5,10–12] The neutral form of the chromophore can convert to the anionic species (B) by going through the intermediate state (I). In going from the neutral chromophore (species A) to the charged chromophore (B), the Tyr66 phenolic proton is shuttled through an extensive hydrogen bonding network to the carboxylate oxygen of Glu222. The change from forms A to I is solely a protonation change, while the change from I to B is a conformational change, with most changes occurring at Thr203. Spectral hole-burning experiments have shown that the ground state of form I is higher in energy than both the ground states of A and B, and that it is

Corresponding author: Marc Zimmer, Connecticut College, Department of Chemistry, Box 5624, 270 Monegan Avenue, New London, CT 06320-4496, USA. Tel.: 860-439-2426; fax: 860-439-2477.

E-mail address: mzim@conncoll.edu

Journal of Molecular Graphics and Modelling 19, 297–303, 2001

655 Avenue of the Americas, New York, NY 10010

1093-3263/01/$–see front matter
PII S1093-3263(00)00057-7

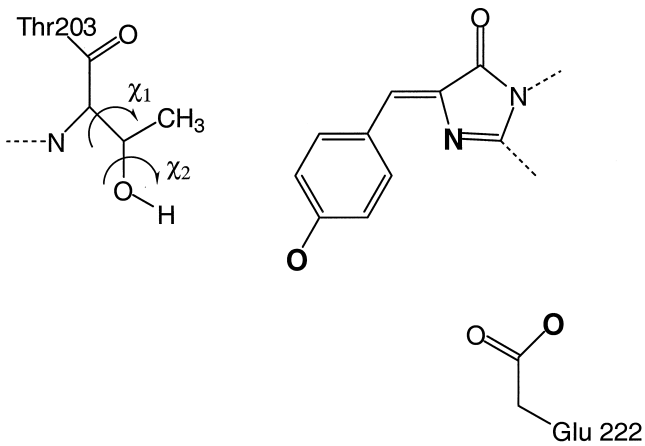

Figure 1. The GFP chromophore, and the χ_1 and χ_2 dihedral angles of Thr203. The protonation sites that were varied in all calculations are in bold, they are the phenolic oxygen (O_y), the oxygen on glutamic acid 222 (Glu) and the imidazolidinone nitrogen (N).

separated from them by energy barriers of several hundred wavenumbers. In the excited state, the barrier between A* and I* is low whereas that between I* and B* is at least 2000 cm^{-1}.[8] The mechanism shown in Figure 2 has been partially validated by the interpretation of the absorption and Stark spectra of the wild type, the S65T and Y66H/Y145F GFP mutants.[13] The electronic spectra show that the excitation of species A only involves a small charge displacement, while excitation of species B involves a significant change from the ground state. Since the intermediate state (I) is structurally similar to both the ground and excited state of species A and electronically similar to the ground and excited state of species B, the protein has to undergo structural changes in going from state A to state B. Additional evidence comes from the X-ray structure of S65T at low pH, which shows that there is no hydrogen bonding interaction between the side chain of Thr203 and the phenolic oxygen of the chromophore, while the side chain χ_1 dihedral rotates by 100° to form a hydrogen bond in the high pH structure.[14] Electrostatic calculations have been used to examine coupling of the ionization states of Thr203 and Glu222, and the related sidechain reorientations.[15] The calculations correctly reproduced the coupling between the protonation state of the chromophore and the side chain conformation of Thr203, which is shown in Figure 2. The first mutants that were designed on the basis of the S65T crystal structure are yellow emission mutants (YFP) that contain a T203Y mutation. The crystal structure of YFP shows that Tyr203 and the chromophore are coplanar and that π-π stacking occurs. The structure has a hydrogen bond between Glu222 and the chromophore nitrogen, suggesting a neutral imidazolidinone ring nitrogen.[16] Two studies have reported results that do not support the photoisomerization mechanism shown in Figure 2. An infrared study of both forms of GFP_{UV} found that photoisomerization involved a change in protonation, but that the protonation state of Glu222 remains unchanged, and that the Thr203 side chain was in the same conformation in both the A and B forms.[17] Good agreement between the predicted and observed absorption spectra were obtained in quantum mechanical calculations when the species A was modeled by a cationic (HO_Y,HN^+,O_X) form and species B by the zwitterionic (O_Y^-, HN^+,O_X), see Figure 1.[9,18] In this article we describe how we have used molecular mechanical calculations and database analysis to examine structural aspects of the photoisomerization observed in GFP and shown in Figure 2.

METHODOLGY

The coordinates of the wild-type GFP solid state structure (1GFL)[2] were obtained from the Protein Data Bank, hydrogen atoms were added to protein and solvent atoms using the HADD function of MacroModel v6.5 as required. Crystallographically determined water molecules were incorporated in the calculations. A "hot" sphere, in which all atoms were minimized without constraints, with a radius of 8Å from residues 65–67 and 203 was used in all conformational searches. The "hot" sphere was anchored by a secondary constrained sphere extending a futher 3Å. Constraints were introduced on all atoms in the secondary sphere with a harmonic restoring potential of 100kJ/$Å^2$. The AMBER* force field as implemented in MacroModel v6.5[19] was used for all molecular modeling. It uses a 6,12 Lennard Jones hydrogen bonding treatment and an improved protein backbone parameter set.[20] During the search procedure and generation of the Ramachandran plots minimization continued until convergence was reached, or until 5,000 iterations had been performed. The Polak-Ribiere conjugate gradient minimization mode was used with a derivative convergence criterion of 0.05kJ/mol. Ramachandran plots were generated by driving the χ_1 dihedral angles by 30° increments and χ_2 by 60° increments with 1000kJ/mol torsional constraints. A 5,000-step low mode conformational search[21] of the pentapeptide LSTQS was carried out in an aqueous GB/SA environment.[22] The low-mode conformational search works by exploring the low-frequency eigenvectors of the pentapeptide, which are expected to follow soft degrees of freedom, such as torsions. The MacroModel v6.5 default parameters were used in the search. No new conformations within 10kJ/mol of the lowest energy conformation were found in the last 2,000 steps. Small molecule searches were performed using the Cambridge Structural Database (CSD) version 5.17, which contains 197,481 structures and was last updated in April 1999.

RESULTS AND DISCUSSION

There are currently sixteen GFP and GFP mutant crystal structures and the conformational differences between them are minor. One of the residues that does undergo a change in conformation is Thr203. In fact, as shown in Figure 2, the photoisomerization of species A to B is linked to the rotation of the χ_1 sidechain dihedral angle of Thr203. Table 1 lists the values of all the χ_1 dihedral angles, and the distance between the Thr203 alcoholic oxygen and the phenolic oxygen of Tyr66 in all the GFP crystal structures in the protein databank. Table 1 and Figure 3A show that Thr203 adopts two set conformations in GFP, one with a dihedral angle, χ_1, of $\sim -60°$ this is the g- conformation and the other of $\sim 180°$, the t conformation. It has been proposed that structures with a short interatomic distance between the oxygen of Tyr66 (O_y) and the side-chain oxygen of Thr203 correspond to the anionic form of the chromophore (O_y^-, species B and χ_1 = g-), while those with a longer distance are found for the phenolic form (HO_y,

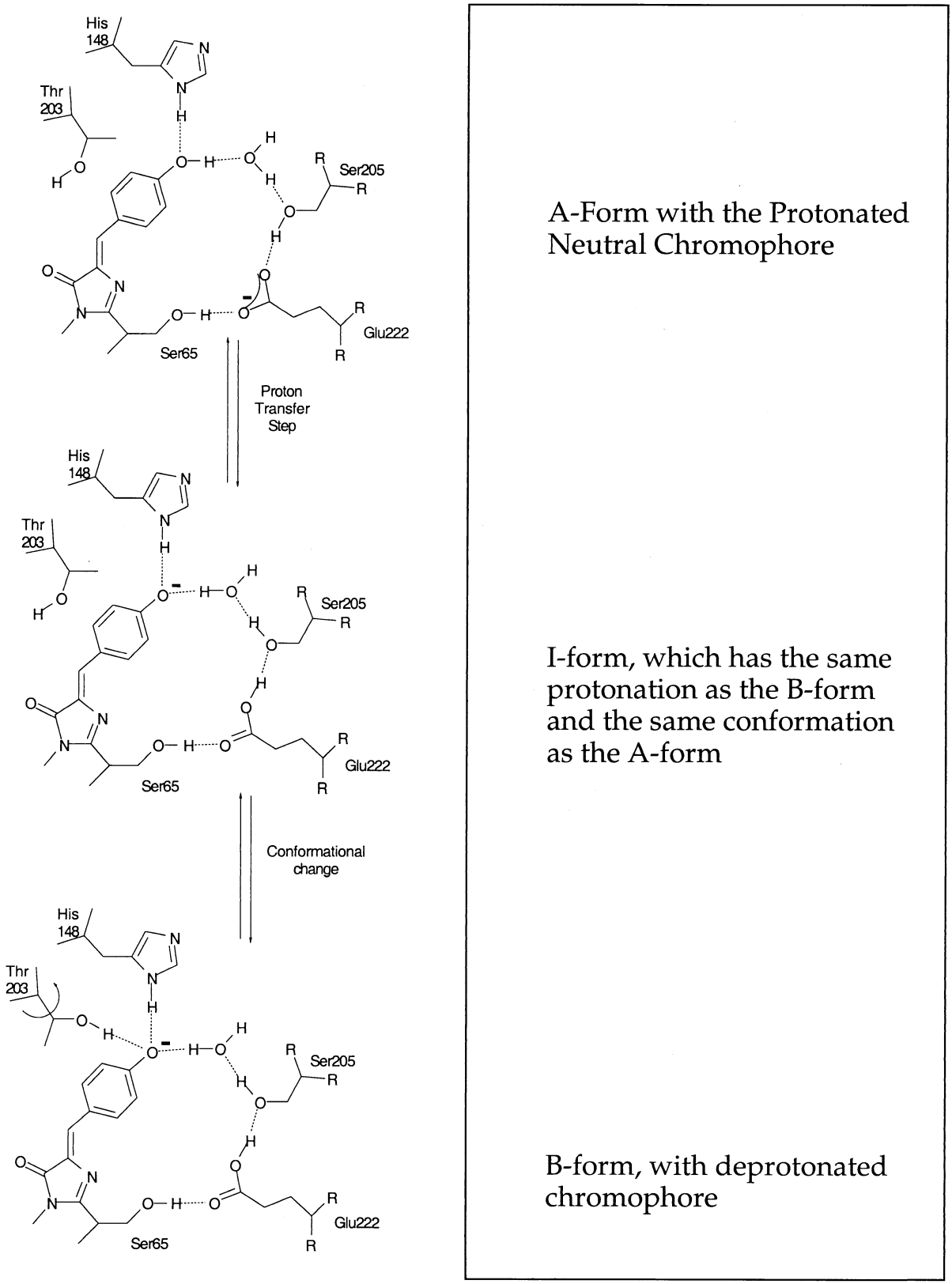

Figure 2. Proposed mechanism for the photoisomerization of wild-type GFP. The neutral form of the chromophore (A) can convert to the anionic species (B) by going through the intermediate state (I). In going from the neutral species (A) to the charged species (B) the Tyr66 phenolic proton is shuttled through an extensive hydrogen bonding network to the carboxylate oxygen of Glu222. The change from forms A to I is solely a protonation change, while the change from I to B is a conformational change with most changes occurring at Thr203.

Table 1. Key structural parameters of all GFP crystal structures in the Protein Databank. The distances listed are those between O_y of Tyr66 and the alcoholic oxygen of Thr203

PDB Reference Code	Distance Tyr66–Thr203 (Å)	χ_1 of Thr203 (°)
1emg[14]	2.59	−60.0
1ema[4]	2.66	−59.7
1emk[5]	2.71	−50.9
1eml[5]	2.77	−49.5
1emm[5]	2.97	−47.1
1emc[5]	3.06 (5.02)*	−50.7 (−179.8)
1emb[3]	4.32	−160.1
1gfl[2]	4.71	175.1
1eme[5]	5.04	−176.4
2emn[5]	3.20	−19.0
2emo[5]	3.23	−116.1
1bfp[11]	3.37	−44.8
1emf[5]	4.43	−163.7
2emd[5]	4.51	−173.3
1yfp[16]	Y203	Y203
2yp[16]	Y203	Y203

*Disordered T203 has two different conformations.

species A and $\chi_1 = t$).[5,11] Some of the crystal structures have been solved with different occupancies for the two conformations. It has been proposed that the structures with a g- conformation will have a deprotonated phenolic group, and that the dihedral rotation from the t conformation occurred so that a hydrogen bond can form between the phenolate anion and Thr203. To establish the effect of changing the protonation state of key residues on the conformation of Thr203 and to further examine the potential energy surface of Thr203 in GFP, we systematically varied the χ_1 and χ_2 dihedral angles of Thr203 in the following protonation states (HO_y, $^+$NH, GluH ; ^-O_y, $^+$NH, GluH; HO_y, N, GluH; HO_y, $^+$NH, $^-$Glu; ^-O_y, N, GluH; ^-O_y, $^+$NH, $^-$Glu; HO_y, N, $^-$Glu and ^-O_y, N, $^-$Glu). Table 2 lists the χ_1 and χ_2 dihedral angles of the lowest energy conformations for all the protonation states and the corresponding distance between the alcoholic proton of Thr203 and the phenolic oxygen of Tyr66. Figure 4 shows a Ramachandran plot generated for the ^-O_y, N, GluH protonation state, this plot is typical of all the plots generated for the phenolate forms of the chromophore. In this Ramachandran plot and in all other plots generated with an ^-O_y, except ^-O_y, N, $^-$Glu, the lowest energy conformation has the threonine side-chain in a g- conformation. According to the mechanism presented in Figure 2 there should be a low-energy pathway from the intermediate species I to species B, since both have the same protonation state and mainly differ in the orientation of the Thr203 side-chain. However, this type of low energy pathway is only found in the Ramachandran plot of the ^-O_y, N, GluH species, Figure 4. Minimizing this species from a starting χ_1 dihedral angle of 180° always results in a minimized structure with a g- conformation. Hole-burning experiments suggest that the barrier height between species I and B is a few hundred wavenumbers.[8] The zwitterionic ^-O_y, $^+$NH, $^-$Glu species adopts a g- conformation as its lowest energy structure; however, there is no low energy pathway connecting the lowest energy conformation to a t conformation. Although by no means definitive, especially since these are calculations of the ground state, these results seem to suggest that the anionic (^-O_y, N, GluH) form, and not the zwitterionic (^-O_y, $^+$NH, $^-$Glu) form is species B. Since there is no structural or titration evidence for a second labile proton on the chromophore,[14,23] it must therefore be assumed that species A is the neutral (HO_y, N, Glu$^-$) form and not the cationic (HO_y, $^+$NH, $^-$Glu) form. The lowest energy conformation of all HO_y structures is a t conformation, except the HO_y,N,$^-$Glu form. In all HO_y forms there are no low energy g- conformations available (The HO_y,N,GluH form has a χ_1 angle of 90°, but no low energy g- or t conformations). Our calculations show that GFP crystal structures with a g- conformation of the Thr203 side-chain can confidently be assigned a ^-O_y protonation, while the structures with t conformation have a HO_y. However one cannot make the assumption that all ^-O_y forms will be in the g- conformation, nor that all HO_y forms will be in the t conformation, as some of them adopt nonstandard conformations.

Restricted Rotation of Threonine 203 in GFP

Table 1, Table 2, Figure 3A, and the Ramachandran plots used to generate Table 2 show that the χ_1 dihedral angle of Thr203 in GFP does not adopt a g+ conformation in any of the protonation states examined. To establish whether this was a common occurrence we examined the conformations that the side chain of threonine adopts in other crystal structures. Such analyses of congeneric families are very useful as they can reveal the different conformations the structure can adopt in the different environments found in the crystals. This can provide information about conformations available to the side chain, how the conformers can interconvert, and the environmental factors that are responsible for certain conformations. Furthermore, if one assumes that the threonine will adopt a low energy conformation in most crystal structures, then the conformational space spanned by the crystal conformations can be equated and compared (qualitatively not quantitatively) with the potential energy surface of the residue itself.[24] A 1978 analysis of all the threonine χ_1 torsion angles in 19 well-resolved crystal structures showed that 48% adopted a g+ ($\chi_1 \sim 60°$) , 39% a g- ($\chi_1 \sim -60°$)and 13% a t ($\chi_1 \sim 180°$) conformation.[25] A second study using newer and more highly refined structures gave essentially the same results, 47.9% adopted a g+, 45.0% a g-, and 4.7% a t conformation.[26] To establish whether the χ_1 torsion of threonine is commonly found in a bimodal distribution with little or no g+ conformations, as observed in the GFP structures, or in a trimodal (g- $\sim$ g+ $>$ t) distribution as mentioned above, the crystal structures of all the threonine containing compounds in the CSD were examined. These are all small molecules with less than 500 atoms. Figure 3B is a polar plot of the χ_1 torsion of all the crystal structures in the CSD containing a threonine substructure. The dihedral angles are clustered around the g-, t, and g+ positions and the distribution is roughly the same as that found in the Janin[25] and Ponder[26] studies. There are two possible reasons that Thr203 adopts g- and t, but not g+ conformations in GFP crystal structures. The first is that interactions with adjacent residues favor the g- and t conformations, and the

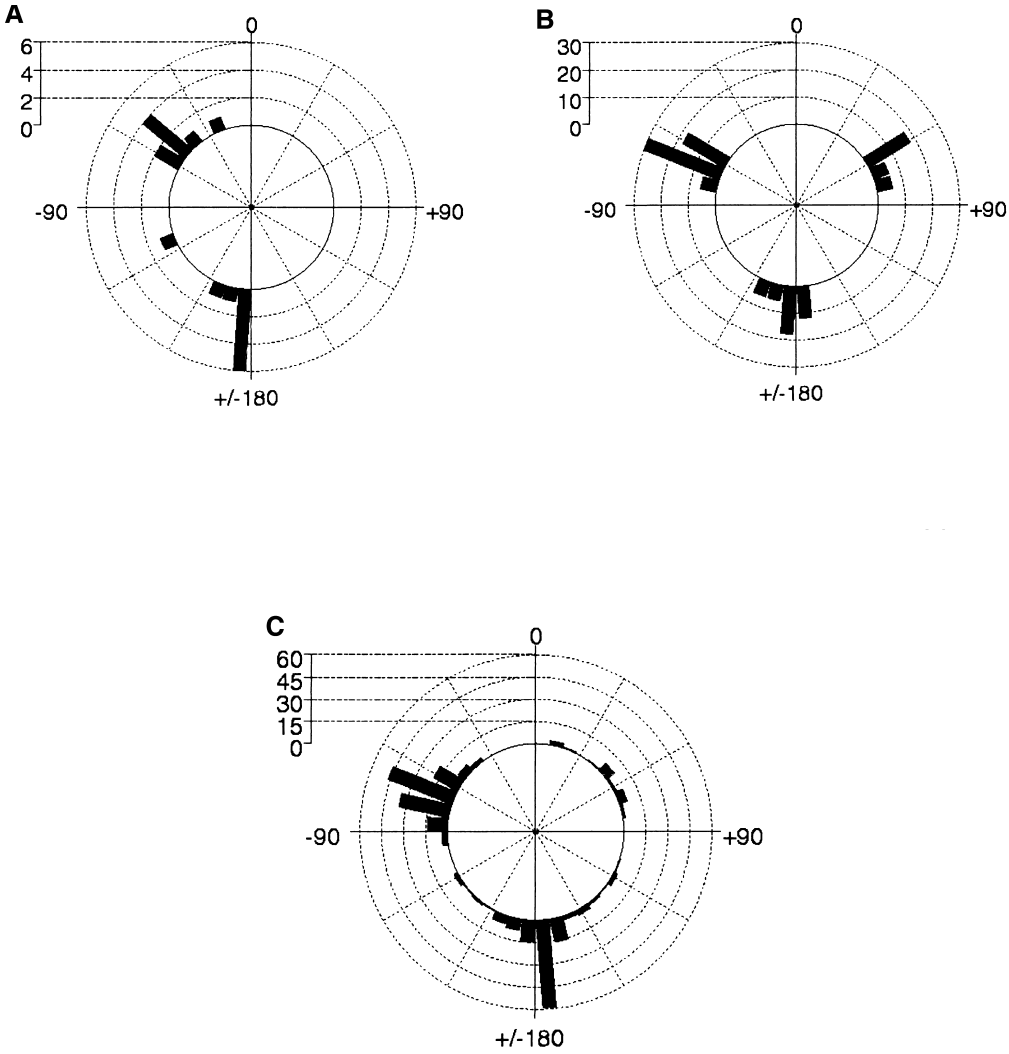

Figure 3. Distribution of the χ_1 angles in (A) all crystal structures of GFP in the pdb; (B) all crystal structures containing a threonine substructure in the CSD; (C) all crystal structures containing a STQ sequence in the pdb.

second is that hydrogen bonding and steric interactions with the remainder of the protein are responsible for g- and t being energetically favored over g+. The hydrogen bond formed between the phenolate anion and Thr203 is an example of the latter reason. In wild-type GFP Thr203 is located in a LSTQS fragment. A search of the pdb found 1 protein that has a LSTQS stretch and 275 that have a STQ stretch. Figure 3C is a polar plot of the Thr χ_1 torsion in all the STQ tripeptide fragments found in the PDB. The majority of the pdb structures containing the STQ stretch adopt the g- and t conformations. Both the Janin study and our own search of the CSD show that threonine favors the g+, g-, and t conformations. When Thr is part of a STQ sequence, however, the g^+ conformation is hardly ever found, it must be a high energy conformation in STQ tripeptide sequences. A low mode conformational search of the pentapeptide LSTQS fragment was conducted to confirm this observation. An analysis of all structures within 50 kJ/mol of the lowest energy structure revealed 66% and 32% adopted the t and g- conformations respectively. While the g+ conformation was adopted by only 1.5% of the structures. These findings show that Thr203 does not adopt a g^+ conformation because it is located in a LSTQS pentapeptide fragment, and the remainder of the GFP protein environment is not responsible for this observation. An analysis of the individual interactions responsible for the difference in energy between the g- and g+ conformations in both the LSTQS fragment and in the GFP crystal structures revealed that the electrostatic interactions between alcoholic oxygen of the threonine and the carbonyl oxygens of the threonine itself and the preceding serine residue are much less favorable in the g+ conformation than in the g- and t conformations. Similar results were found when Thr203 was constrained to a g+ conformation in GFP. The restricted rotation of Thr203 is a steric consequence of the LSTQS sequence and should not be affected by the excitation of the chromophore. To establish the effect of the chromophore phenol(ate) on the conformations available to Thr203, Tyr66 was mutated to a Gly and the χ_1 dihedral angle of Thr203 was rotate in 10° steps and minimized after each rotation. The results show that the g+ conformation of Thr203 is also higher in energy than the g- and t conformations in the absence of the phenolic group at position 66. To establish the effect of the chromophore phenol(ate) on the conformations available to

Table 2. Key structural parameters of the lowest energy conformations found in the Ramachandran plots for all the different protonation states of GFP examined in this study. The distances listed are thos between O_y of Tyr66 and the alcoholic oxygen of Thr203

	Lowest Energy Conformation		
Protonation state	Thr203–Ty66 (Å)	χ_1	χ_2
HO_y, $^+$NH, GluH	5.2	180	60
HO_y, $^+$NH, $^\pm$Glu	5.9	−180	0
HO_y, N, GluH	5.5	−180	0
HO_y, N, $^-$Glu	4.6	−90	−60
^-O_y, $^+$NH, GLuH	3.1	−61	−59
^-O_y, $^+$NH, $^-$Glu	3.1	−60	−60
^-O_y, N, GluH	2.6	−60	120
^-O_y, N, $^-$Glu	2.6	−3	−179

Glu$^-$ refers to the anionic form of Glu222 while GluH is the neutral form.

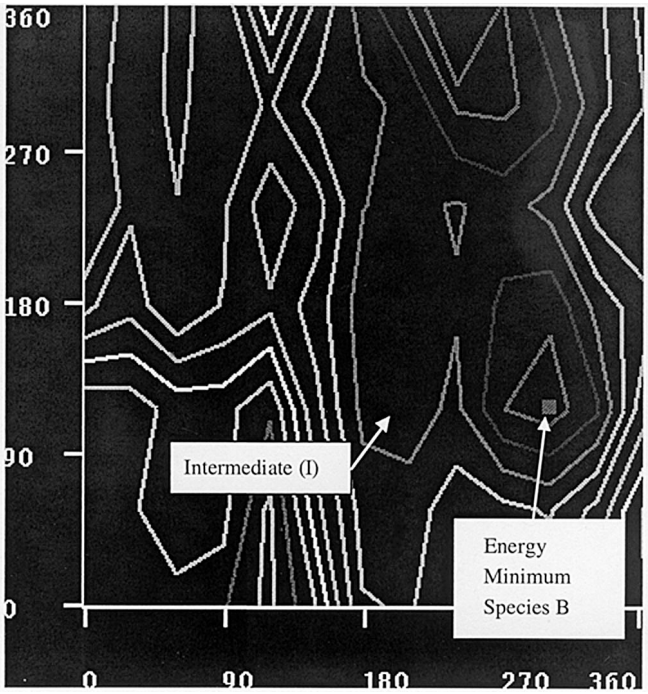

Figure 4. Ramachandran plot of the χ_1 vs χ_2 dihedral angles of Thr203 in GFP with a ^-O_y,N,GluH protonation state. See Figure 1 for the definition of the dihedral angles. Energy contours increase from purple the eanergy minimum, each contour line corresponds to an energy difference of 14 kJ/mol. A possible low energy pathway from the low energy t conformation ($\chi_1 \sim 195°$) to the lowest energy g- conformation ($\chi_1 \sim -60°$) exists.

Thr203, Tyr66 was mutated to a Gly and the $^{x}{}_1$ dihedral angle of Tyr66 was rotate in 10° steps and minimized after each rotation. The results show that the g+ conformation of Thr203 is higher in energy than the g- and t conformations in the absence of the phenolic group at position 66 and therefore that the chromophore does not sterically prevent the g+ conformation. The rotational movement of the side chain of Thr203 is restricted and the 201LSTQS205 sequence does not allow a g+ conformation, this maybe a significant factor in the photoisomerization mechanism shown in Figure 2. The photoisomerization of GFP is an excellent example of how the protein environment can modify the absorption properties of a biological pigment. A mechanism for the photoisomerization has been proposed, on the basis of a number of crystal structures. We have used computational methods to support the protonation state of the GFP chromophore and thereby the photoisomerization mechanism shown in Figure 1. We have also shown that the rotation of the Thr203 side chain is restricted due to its location in a LSTQS sequence. This knowledge can be used to design GFP mutants with freely rotating Thr203 side chains.

ACKNOWLEDGMENTS

We thank the NIH (Grant GM59108-01) and the Research Corporation for financial support. MZ is Henry Dreyfus Teacher-Scholar.

REFERENCES

1 Chalfie, M., and Kain, S. Eds. *Green Fluorescent Proteins: Properties, Applications and Protocols*. Wiley-Liss, New York, 1998
2 Yang, F., Moss, L.G. , and Phillips, G.N. Jr. The molecular structure of green fluorescent protein. *Nat. Biotech.* 1996, **14**, 1246–1251
3 Brejc, K., Sixma, T.K., Kitts, P.A., Kain, S.R., Tsien, R.Y., Ormoe, M., and Remington, S.J. Structural basis for dual excitation and photoisomerization of the Aequprea victoria green fluorescent protein. *Proc. Nat. Acad. Sci.* 1997, **94**, 2306–2311
4 Ormoe, M., Cubitt, A.B., Kallio, K., Gross, L.A., Tsien, R.Y., and Remington, S.J. Crystal structure of the *Aequorea victoria* green fluorescent protein. *Science* 1996, **273**, 1392–1395
5 Palm, G., Zdanov, A., Gaitanaris, G.A., Stauber, R., Pavlakis, G.N., and Wlodawer, A. The structural basis for spectral variations in green fluorescent protein. *Nat. Struct. Bio.* 1997, **4**, 361–365
6 Perozzo, M.A., Ward, K.B., Thompson, R.B., and Ward, W.W. X-Ray diffraction and time-resolved fluorescence analyses of *Aequorea* green fluorescent protein crystals *J. Biol. Chem.* 1988, **263**, 7713–7716
7 Morise, H., Shimomura, O., Johnson, F.H., and Winant, J. Intermolecular energy transfer in the bioluminescent system of *Aequorea*. *Biochemistry* 1974, **13**, 2656–2662
8 Creemers, T.M.H., Lock, A.J., Subramaniam, V., Jovin, T.M., and Voelker, S. Three photoconvertible forms of green fluorescent protein identified by spectral hole-burning *Nat. Struct. Bio.* 1999, **6**, 557–560
9 Voityuk, A.A., Michel-Beyerle, M.-E., and Roesch, N. Quantum chemical modeling of structure and absorption spectra of the chromophore in green fluorescent proteins. *Chem. Phys.* 1998, **231**, 13–25
10 Heim, R., Prasher, D.C. ,and Tsien, R.Y. Wavelength mutations and posttranslational autoxidation of green

fluorescent protein. *Proc. Natl. Acad. Sci. USA* 1994, **91**, 12501–12504
11 Wachter, R.M., King, B.A., Heim, R., Kallio, K., Tsien, R.Y., Boxer, S.G., and Remington, S. J. Crystal structure and photodynamic behavior of the blue emission variant Y66H/Y145F of green fluorescent protein. *Biochemistry* 1997, **36**, 9759–9765
12 Chattoraj, M., King, B.A., Bublitz, G.U., and Boxer, S.G. Ultra-fast excited state dynamics in green fluorescent protein: multiple states and proton transfer *Proc. Natl. Acad. Sci.* 1996, **93**, 8362–8367
13 Bublitz, G., King, B.A., and Boxer, S.G. Electronic structure of the chromophore in green fluorescent protein (GFP). *J. Am. Chem. Soc.* 1998, **120**, 9370–9371
14 Elsliger, M.-A., Wachter, R.M., Hanson, G.T., Kallio, K., and Remington, S.J. Structural and spectral response of green fluorescent protein variants to changes in pH. *Biochemistry* 1999, **38**, 5296–5301
15 Schnargl, C., Raupp-Kossmann, R., and Fisher, S.F. Molecular basis for pH sensitivity and proton transfer in green fluorescent protein: Protonation and conformational substrates from electrostatic calculations. *Biophysical J.* 1999, **77**, 1839–1857
16 Wachter, R.M., Elsiger, M.-A., Kallio, K., Hanson, G.T., and Remington, S. J. Structural basis of spectral shifts in the yellow emission varients of green fluorescent protein. *Structure* 1998, **6**, 1267–1277.
17 Van Thor, J.J., Pirik, A.J., Nugteren-Roodzant, I., Xie, A, and Hellingwerf, K.J. Characterization of photoconversion of green fluorescent protein with FTIR spectroscopy. *Biochemistry* 1998, **37**, 16915–1692
18 Voityuk, A.A., Michel-Beyerle, M.-E., and Roesch, N. Protonation effects on the chromophore of green fluorescent protein. Quantum chemical study of the absorption spectrum. *Chem. Phys. Lett.* 1997, **272**, 162–167
19 Mohamadi, F., Richards, N.G.F., Guida, W.C., Liskamp, R., Lipton, M., Caulfield, C., Chang, G., Hendrickson, T., and Still, W.C. MacroModel—an integrated software system for modeling organic and bioorganic molecules using molecular mechanics *J. Comp. Chem.* 1990, **11**, 440–467
20 McDonald, Q., and Still, W.C. AMBER* Torsional parameters for the peptide backbone *Tetrahedron Lett.* 1992, **33**, 7743–7746
21 Kolossvary, I., and Guida, W.C. Low mode search. An efficient, automated computational method for conformational analysis: Application to cyclic and acyclic alkanes and cyclic peptides. *J. Am. Chem. Soc.* 1996, **118**, 5011–5019.
22 Still, W.C., Tempczyk, A., Hawley, R.C., and Hendrickson, T. Semianalytical treatment of solvation for molecular mechanics and dynamics. *J. Am. Chem. Soc.* 1990, **112**, 6127–6129
23 Bell, A.F., He, X., Wachter, R.M., and Tonge, P.J. Probing the ground state structure of the green fluorescent protein chromophore using raman spectroscopy. *Biochemistry* 2000, **39**, 4423–4431
24 Dunitz J.D., and Buergi H.-B. Can statistical analysis of structural parameters from different crystal environments lead to quantitative energy relationships? *Acta Cryst.* 1988, **B44**, 445–448
25 Janin, J., Wodak, S., Levitt, M., and Maigret, B. Conformation of amino acid side-chains in proteins *J. Mol. Biol.* 1978, **125,** 357–386
26 Ponder, J.W., and Richards, F.M. Tertiary templates for proteins. Use of packing criteria in the enumeration of allowed sequences for different structural classes. *J. Mol. Biol.* 1987, **193**, 775–791