Crystallographic Evidence for Isomeric Chromophores in 3-Fluorotyrosyl-Green Fluorescent Protein

Jae Hyun Bae, Prajna Paramita Pal, Luis Moroder, Robert Huber, and Nediljko Budisa*[a]

The chromophore (4-(*p*-hydroxybenzylidene)imidazolid-5-one) of green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* (*av*) is completely encoded in its amino acid sequence.[1] It is autocatalytically formed in the post-translational modification reaction between the side chains of residues Ser65-Tyr66-Gly67, with oxygen being the only external requirement.[1,2] The presence of an aromatic amino acid at position 66 is crucial for the formation of fluorescent *av*GFPs. However, the standard genetic code limits the number of possible amino acids at this position to the canonical residues tryptophan, tyrosine, histidine and phenylalanine. Further direct chromophore redesign is possible only by the use of an expanded amino acid repertoire. This was demonstrated in recent reports about the design of novel classes of tailor-made GFP variants with a variety of Trp, Phe and Tyr analogues and surrogates.[3]

Our method of selective pressure incorporation (SPI) is based on the traditional use of auxotropic strains that exploits the absence of absolute substrate specificity of aminoacyl-tRNA synthetases, which are crucial enzymes in the interpretation of the genetic code.[4] This approach enables a residue-specific mode of noncanonical amino acid incorporation, in this case, the replacement of Tyr side chains with 3-fluorotyrosine, (3-F)Tyr. Fluorine, as the most electronegative element in the context of a C–X bond, dramatically changes the residue's electronic properties when compared with a C–H bond, that is, the dipole moment is opposite to that of a C–H bond. The isosteric replacement of hydrogen with fluorine in organic compounds is often accompanied by profound and unexpected changes in their properties.[5]

Correspondingly, fluorination of the Tyr66 as integral part of chromophore (Cro66) of *av*GFP by (3-F)Tyr incorporation should endow it with unusual spectral properties. As a model protein, we used the commercially available "enhanced green fluorescent protein" EGFP (Phe64Leu/Ser65Thr), which is a variant of wt-*av*GFP with the phenolate ion in the Cro66 (absorption: $\lambda_{max} = 488$ nm; fluorescence $\lambda_{em} = 510$ nm).[1a,b] It contains 11 Tyr residues,[6] which were quantitatively replaced by the use of the SPI approach, as confirmed by mass spectrometric

[a] *Dr. J. H. Bae, P. Paramita Pal, Prof. Dr. L. Moroder, Prof. Dr. R. Huber, Dr. N. Budisa*
Max-Planck-Institut für Biochemie, Abt. Strukturforschung
Am Klopferspitz 18a, 82152 Martinsried (Germany)
Fax: (+49) 89-8578-3516
E-mail: budisa@biochem.mpg.de

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

 © 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim DOI: 10.1002/cbic.200300818

analyses.[7] The presence of traces of Tyr-containing protein cannot be excluded.

Interestingly, the basic spectral features of EGFP are not dramatically changed upon global fluorination of all Tyr residues. The absorbance in the Tyr + Trp and Cro66 spectral regions was slightly blue-shifted (wt-EGFP: 277 nm and 488 nm; (3-F)Tyr-EGFP: 274 nm and 484 nm) at neutral pH. Conversely, the fluorescence emission maximum of (3-F)Tyr-EGFP (λ_{max} = 514 nm) is red-shifted by 4 nm with decreased intensity of about 10% when compared with native EGFP (λ_{max} = 510 nm)[7] (Figure 1). Single absorption and emission peaks clearly indicate an anionic state of the Cro66. There is no doubt that the observed marginal red shift in the (3-F)Tyr-EGFP Cro66 fluorescence was due to its fluorination.[7] A lowering of the pK_a of tyrosine –OH (10.05) upon fluorination by about 1.5 pH units is due to an inductive effect exerted by the fluorine atom (pK_a = 8.5 of (3-F)Tyr –OH group in solution).[8] Indeed, pH titration of (3-F)Tyr-EGFP Cro66 revealed a decrease of its pK_a value for about 0.5 pH units.[7]

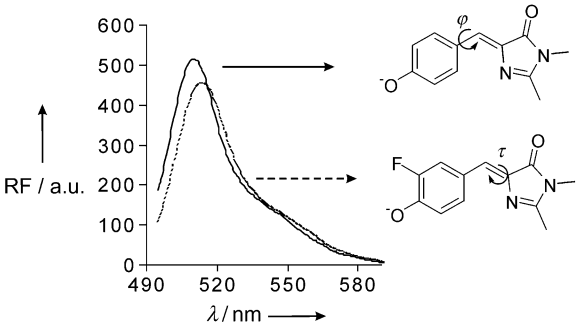

Figure 1. *Fluorescence emission spectra of wt-EGFP and (3-F)Tyr-EGFP in neutral aqueous buffered solution (50 mM Na-phosphate pH 7.6, 100 mM NaCl; 25 °C) upon excitation at absorption maximum wavelengths. The (3-F)Tyr-EGFP chromophore has a slightly red-shifted emission fluorescence (λ_{max} = 514 nm) when compared with the parent EGFP (λ_{max} = 510 nm). Note the positions of dihedral angles φ and τ.*

Crystallographic evidence suggests that the fluorine atoms are capable of acting as hydrogen-bond acceptors from both –NH and –OH donors.[9] This was fully confirmed in the analysis of the crystal structure of glutathione transferase M1-1 globally substituted with (3-F)Tyr.[10] We made similar observations in the crystal structure of (3-F)Tyr-EGFP at a resolution of 2.1 Å.[7] A closer inspection of this structure revealed various crystallographically distinct microenvironments where different types of interactions of the fluorine atoms can be mapped. For example, partially exposed Tyr143 shows two conformations but has no detectable distances that might indicate its involvement in a particular interaction. Conversely, the fluorine of Tyr145 has numerous contacts to neighbouring residues, for example to Pro58 (amide oxygen, 2.54 Å), His169 (ring nitrogen, 3.27 Å) and water molecule S14 (2.99 Å). As expected, all other (3-F)Tyr residues involved in interactions with the environment were characterised by only one conformation (e.g. Tyr39, 106, 182, 200). Detailed analyses of the fluorinated tyrosines will be reported elsewhere.

The most interesting observation from the available structure of (3-F)Tyr-EGFP is the existence of two conformers of the fluorinated Tyr66, in the context of chromophore. Rather rigid internal architecture in the crystalline state of the fluorinated Cro66 (Figure 2) and its surrounding residues is reflected in the average crystallographic B factors for atoms in Cro66 of 15 Å^2, while the B factors of the surrounding residues are from 5 to 25 Å^2. In such a rigid environment, (3-F)Tyr145 has only one conformer; this is plausible, since it is involved in interactions with other residues and the solvent water. The crystallographic occupancy (i.e. intensities of electron densities) is higher (about 60%) for one conformer (assigned as "major") than for the other (40%, "minor") (Figure 2). The hydroxyl groups of both (3-F)Tyr-Cro66 conformers interact directly with Thr203 and His148 through hydrogen bonds. When fitted in the

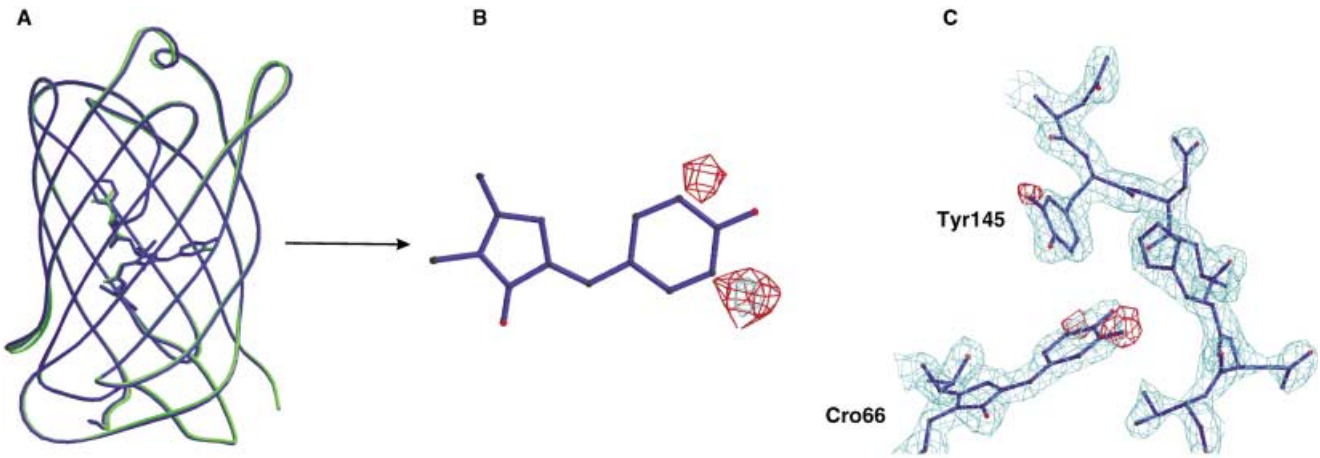

Figure 2. *A) Crystal structure of (3-F)Tyr-EGFP (C_α traces are blue) is indistinguishable from that of parent EGFP (C_α traces are green). The chromophore (Cro66) and residues 64–68 are represented as sticks in the same colours. B) Difference Fourier maps (Fo–Fc; contouring levels: 2.5 and 3.5 σ) of (3-F)Tyr-Cro66 in (3-F)Tyr-EGFP. C) Portion of the continuous electron densities (2 Fo–Fc; contouring levels 1 σ) for residues Cro66, Asn144, Tyr145, Asn146, Ser147, His148 Asn149 and Val150 of wt-EGFP superimposed onto the Fo–Fc difference electron density map (with the same contouring levels as above) of (3-F)Tyr-EGFP. Note the single conformer state of (3-F)Tyr145.*

 © 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

"minor" (3-F)Tyr-Cro66 conformer, fluorine is in hydrogen-bond contact (2.82 Å) with the hydroxyl of Ser205 and conserved solvent water (S316, 2.74 Å). On the other hand, Cγ2 of Thr203 is 3.87 Å away from the fluorine of the "major" conformer; this indicates a possible weak -CF···HC- hydrogen bond. In the light of these observations, it is difficult to explain the higher crystallographic occupancy of the fluorine atom position in the "major" conformation.

The crystal structure of (3-F)Tyr-EGFP enabled "visualisation" of the positions of some Cro66 hydrogen atoms due to their replacement by fluorine atoms. Does this mean that, at least in part, the wt-EGFP chromophore structure flips in its cavity? Modelling and molecular-dynamics simulation studies indicate that *av*GFPs have a fairly large central cavity in the protein core that allows the planar Cro66 to have some rotational freedom, especially in the φ dihedral angle.[2] Electronic excitation ($S_0 \rightarrow S_1$) was found to alter the conformation of the Cro66.[1] It was speculated that in the excited state, responsible for characteristic fluorescence, the conformation may be twisted relative to the ground state.[2] The *cis–trans* photoisomerisation cannot occur by a 180° rotation of the τ dihedral angle (Figure 1).[2] The phenolic oxygen of Tyr66 is less basic in the excited state than in the ground state.[2] In this context, it is possible to draw at least two canonical structures of Cro66. In one of these, mesomeric structures the rotation around φ dihedral angle (Figure 1) should be difficult because of double-bond character.[7] Thus, it might well be that the observed conformers represent Tyr-flipping "caught" or "frozen" by the chromophore formation and maturation. Other alternatives include the possibility that the measured electron difference maps indeed represent an isomerisation of a mature chromophore.

A final and unambiguous answer, as to whether the *av*GFP chromophore flips, as well as about its possible ring-flipping rates, will come from dynamic ^{19}F NMR studies. For example, slow exchange rates of the ECFP Cro66 were documented recently by the use of ^{19}F NMR.[11] For that purpose a position-specific fluorination of chromophore Tyr66 compared to other unlabelled Tyr-residues of the *av*GFP structure is necessary. Other approaches might include molecular dynamic simulations, quantum calculations and even time-resolved X-ray crystallography. Undoubtedly, the crystal structure of (3-F)Tyr-EGFP and possibly the structures of other fluorinated *av*GFP will offer a framework for a possible structural model capable of correlating the chromophore environment configuration, dynamic behaviour and its spectroscopic function. Thus, we anticipate that fluorinated *av*GFP-chromophores will become valuable and general tools to assess chromophore dynamics of the green fluorescent and related proteins.

Acknowledgements

We thank Gregor Jung and Tad A. Holak for a critical reading of the manuscript. This work was supported by SFB533 of the Deutsche Forschungsgemeinschaft.

Keywords: chromophores · fluorotyrosine · green fluorescent protein · isomerization · selective pressure incorporation

[1] a) R. Y. Tsien, *Annu. Rev. Biochem.* **1999**, *67*, 509–544; b) G. J. Palm, A. Wlodawer, *Methods Enzymol.* **1999**, *33*, 378–394.
[2] A. A. Voityuk, M. E. Michel-Beyerle, N. Rösch, *Chem. Phys.* **1998**, *231*, 13–25.
[3] J. H. Bae, M. Rubini, G. Jung, G. Wiegand, M. H. J. Seifert, M. K. Azim, J. S. Kim, A. Zumbusch, T. A. Holak, L. Moroder, R. Huber, N. Budisa, *J. Mol. Biol.* **2003**, *328*, 977–1202.
[4] a) N. Budisa, B. Steipe, P. Demange, C. Eckerskorn, J. Kellermann, R. Huber, *Eur. J. Biochem.* **1995**, *230*, 788–796; b) N. Budisa, C. Minks, S. Alefelder, W. Wenger, F. Dong, L. Moroder, R. Huber, *FASEB J.* **1999**, *13*, 41–51; c) C. Minks, S. Alefelder, R. Huber, L. Moroder, N. Budisa, *Tetrahedron* **2000**, *56*, 9431–9442; d) N. Budisa, M. Rubini, J. H. Bae, E. Weyher, W. Wenger, R. Golbik, R. Huber, L. Moroder, *Angew. Chem.* **2002**, *114*, 4238–4242; *Angew. Chem. Int. Ed.* **2002**, *41*, 4066–4069.
[5] a) C. Minks, R. Huber, L. Moroder, N. Budisa, *Biochemistry* **1999**, *38*, 10649–10659; b) E. Neil, G. March *Chem. Biol.* **2000**, *7*, R153–R157; c) C. Minks, R. Huber, L. Moroder, N. Budisa, *Anal. Biochem.* **2000**, *284*, 29–34.
[6] a) M. Ormo, A. B. Cubitt, K. Kallio, L. A. Gross, R. Y. Tsien, S. J. Remington, *Science* **1996**, *96*, 1392–1395; b) F. Yang, L. G. Moss, G. N. Phillips, *Nat. Biotechnol.* **1996**, *14*, 1246–1251.
[7] More details on the experimental set-up of fermentation experiments, UV absorption, mesomeric structures, molecular mass difference, pH titration of both proteins, crystallisation, data collection, structure elucidation and refinement statistics are available in the Supporting Information. The structure reported here has been deposited in RCSB Protein Data Bank (accession code: 1RRX).
[8] B. Brooks, R. Philips, W. F. Benisek, *Biochemistry* **1998**, *37*, 9738–9742.
[9] G. Xiao, J. F. Parsons, R. N. Armstrong, G. L. Gilliland, *J. Am. Chem. Soc.* **1997**, *119*, 9325–9326.
[10] G. Xiao, J. F. Parsons, K. Tesch, R. N. Armstrong, G. L. Gilliland, *J. Mol. Biol.* **1998**, *281*, 323–339.
[11] M. H. J. Seifert, D. Ksiazek, P. Smialowski, M. K. Azim, N. Budisa, T. A. Holak, *J. Am. Chem. Soc.* **2002**, *124*, 7932–7942.

Received: November 11, 2003 [Z818]

 © 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim