Journal of Biomolecular NMR, **25:** 161–162, 2003.
KLUWER/ESCOM

Letter to the Editor: Backbone H^N, N, C^α and C^β assignment of the GFPuv mutant

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Received 26 September 2002; Accepted 6 November 2002

Key words: chromophore, deuteration, GFP, green fluorescent protein, NMR assignment

Biological context

Green fluorescent protein (GFP) a natural fluorescent protein from the jellyfish *Aequorea victoria*, has a primary sequence that contains a 4-(4-hydroxylphenyl) methylideneimidazol-5-on as a chromophore group (Chalfie and Kain, 1998). It originates from post translational autocatalytic cyclization of the peptide sequence –Ser65-Tyr66-Gly67- with a subsequent dehydrogenation of the α-β bond of the tyrosine in presence of molecular oxygen. In the jellyfish, this chromophore converts the blue chemiluminescence of the apoprotein aequorin into green fluorescent light. Fluorescence spectroscopic characterization indicate that GFPwt has a high absorbtion at 395 nm and a lower one at around 475 nm. Since the expression of GFP in bacteria and several other organisms is possible, it is widely applied in cell studies as fusion protein and in molecular biology techniques (FRET) as a marker. The x-ray structure of GFPwt revealed a regular β-barrel with 11 strands on the outside of a cylinder which dimerizes in the crystal. Inside the cylinder, well protected from solution, is a broken α-helix segment which contains the cyclicized backbone of the fluorophore. In order to understand the behaviour of the chromophore, crystallographic structure investigation were carried out on several mutants. Based on this and the fluorescence data, two theoretical models for the mechanisms of the fluorophore have been postulated. One assumes that the protonation of the nitrogen in the imidazol ring of His148 and the enclosed water is responsible for the neutral and anionic state of the chromophore. The second postulation centers on a permanent protonation of the Tyr66 nitrogen of the imidazolinone ring and the chromophore adopts a zwitterion and a cationic state. Until now however, the protonated ring of His148 and/or protonated Tyr66-nitrogen could not be structurally confirmed. Furthermore none of the model assumption took into account the dimerization at high concentrations, although this has been mentioned for years. In order to identify the protonation states of these two amino acids by NMR, the sequential backbone assignment of the GFPuv was carried out here. This mutant was assumed to improve solubility and/or reduce aggregation. However, this investigation reveals that GFPuv at NMR protein concentrations, behaves like an assymetrical dimer.

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Methods and experiments

The GFPwt and the GFPuv proteins encoding 244 residues were cloned into pt7GFPav vector at the N-terminus and in pRSETB vector at the C-terminus (Invitrogen). In comparaison to the wild-type, the GFPuv contains the following point mutations: F99S, M153T, V163A and at the N-terminus the point mutation A2G, a missing S3 followed by the random PCR mutation Q80R. Proteins were expressed as described previously in *Escherichia coli* strain BL21(DE3) (Georgescu, 2000) and grown in ^{15}N uniformly or selectively labelled media at 37 °C (Riesenberg et al., 1990; Senn et al., 1987). When recquierd, ^{13}C glucose was added to the medium. Protein was purified by Ni-NTA column (Qiagen) and dialysed against PBS (115 mM NaCl, 8 mM KH_2PO_4, 20 mM Na_2HPO_4) pH 7.0 before beeing concentrated on Centricon3 (Amicon). ^2H-^{15}N double and ^2H-^{13}C-^{15}N triple labelled GFPuv samples were obtained with

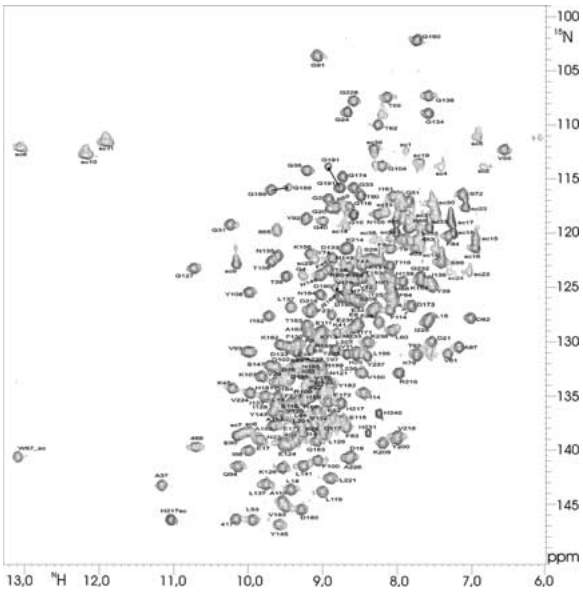

Figure 1. The ^{1}H-^{15}N-HSQC spectrum of GFPuv mutant in PBS buffer, pH 7.0, at 310 K. Backbone amide peaks are indicated by residue (one letter code) and number. The arrows indicate some of the residues near the dimer interface.

1 g/l of $^{15}NH_4Cl$ and 1 g/l ^{13}C glucose as sole nitrogen and carbon source. The cells were expressed in BL21(DE3) pLysE strain and grown in 100% D_2O and 70% D_2O enriched minimal media respectively for 4 hours after induction. The degree of deuterium incorporation in the purified sample was determined by MALDI-TOF mass spectrometry. The incorporation was found to be up 93% for the ^{2}H-^{15}N and 67% for the triple labelled. For both cultures the yield was about 50 mg protein/1 l culture. Final samples for NMR analysis were prepared in PBS buffer at pH 7.0 containing 0.02% sodium azide and 10% D_2O. Final protein concentration was about 1.5 mM. For the H-D exchange experiment, ^{15}N labelled sample was liophylised and dissolved in 100% D_2O.

NMR spectra were acquired at 310 K on Bruker DRX 600 MHz and DMX 750 MHz spectrometers equipped with triple resonance probeheads and pulsed field gradient units. The backbone resonances were assigned using ^{15}N-HSQC, HNCA, HNCO, HN(CA)CO, CBCA(CO)NH (Muhandiram and Kay, 1994) tripel resonance spectra recorded on the the $^{1}H/^{2}H$-^{15}N and $^{1}H/^{2}H$-^{15}N-^{13}C labelled samples. The used pulse sequences were modified version of CT-HNCA and CT-HNCO experiments (Grzesiek and Bax, 1992a, b) and were acquired using the WATERGATE sequence. Further $^{1}H/^{2}H$-^{15}N heteronuclear NOE (Farrow et al., 1994) were carried out to increase the reliability of the assignment. All the data were processed and residues were assigned using a home written software (ccnmr and glxcc, Cieslar et al., 1993). Proton chemical shifts were reported with respect to DSS. The ^{15}N and ^{13}C chemical shifts were referenced indirectly respectively to ammonia and TMS with internal dioxane.

Extent of assignments and data deposition

^{N}H and ^{15}N assignment of the 2D ^{15}N-HSQC backbone peaks were obtained for 211 (including prolines) of the expected 239 residues (245 minus 6 polyhistidine tag). Assignments for $^{13}C\alpha$, ^{13}CO and $^{13}C\beta$ were obtained for all of them. The only exception was formed by the amino acids involved in the interface of the assymetrical dimer which is represented by the four β-sheets (7, 8, 10 and 11). Since the protein sample contains a mixture up to 40% dimer and 60% monomer in the sample, and a tendency for futher dimerization, shifts in backbone resonances were observed (Figure 1). Whereas other residues could not be identified at all. The mentioned chemical shifts, including Hα resonances have been deposited in the BioMagResBank database under the accession number 5514 (http://www.bmrb.wisc.edu).

Acknowledgements

This work has been supported by grants from Sonderforschungsbereich *SFB* 533.

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