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Resonance assignments for the 21 kDa engineered fluorescein-binding lipocalin FluA

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Biological context

The lipocalins form a large family of extracellular proteins which serve for transport and storage of secondary metabolites such as lipids, pheromones or prostaglandins (Flower, 1996). In spite of large diversity at the sequence level, lipocalins are structural homologues: a single eight-stranded antiparallel β-barrel with an attached α-helix forms the distinct 'lipocalin scaffold'. One end of the barrel is opened to the solvent and contains a ligand binding site. A set of four loops connecting consecutive strands confer specificity for ligand binding. Recently, the lipocalin scaffold was used to engineer proteins with tailored specificity for non-natural ligands. Such designed lipocalins can be considered as antibody mimics and were thus named 'anticalins' (for a review, see Skerra, 2000). Starting with the bilin-binding protein (BBP) from *P. brassicae*, the anticalin 'FluA' with binding affinity toward fluorescein was created using a combinatorial protein design approach. Compared to the amino acid sequence of BBP, FluA contains 20 point mutations and binds fluorescein with high specificity and affinity (Beste et al., 1999). As a step toward exploring the structural basis of molecular recognition by anticalins and lipocalins in general, we have overexpressed and purified several stable isotope labeled samples of FluA(R95K). Here we report the nearly complete ^{15}N, ^{1}H and ^{13}C resonance assignments.

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Methods and experiments

FluA(R95K) protein containing a C-terminal *Strep*-tagII (Skerra and Schmidt, 2000) was overexpressed in *E. coli* KS474 using the plasmid pBBP21-FluA(R95K) essentially as described (Beste et al., 1999). Bacterial cell cultures were grown at 37 °C in (i) rich LB medium to express unlabeled FluA(R95K) (sample 1), in (ii) M9 minimal medium containing $^{13}C_6$-glucose and/or $^{15}NH_4Cl$ as sole carbon and nitrogen sources to produce either uniformly $^{13}C/^{15}N$-labeled (sample 2) or ^{15}N-labeled (sample 3) FluA(R95K), or in (iii) a M9 minimal medium containing $^{15}NH_4Cl$ in 30% H_2O/70% D_2O to synthesize ∼50% deuterated, uniformly ^{15}N-labeled FluA(R95K) (sample 4). In addition, a fraction of sample 1 was lyophilized and dissolved in D_2O yielding sample 1b. Sample purity (>95%) and stable isotope labeling were verified by SDS-PAGE and MALDI-TOF mass spectrometry. All NMR samples were prepared at 0.7 mM protein concentration in 90% H_2O/10% D_2O (150 mM NaCl, 10 mM Na-PO_4, 0.2 mM EDTA, 50 mM benzamidine, pH = 6.4).

NMR spectra were recorded at 25 °C on Varian INOVA NMR spectrometers operating at ^{1}H resonance frequencies of 600, 750 or 900 MHz. Resonance assignments were obtained by combining (i) 2D [^{15}N,^{1}H]-TROSY (Pervushin et al.,

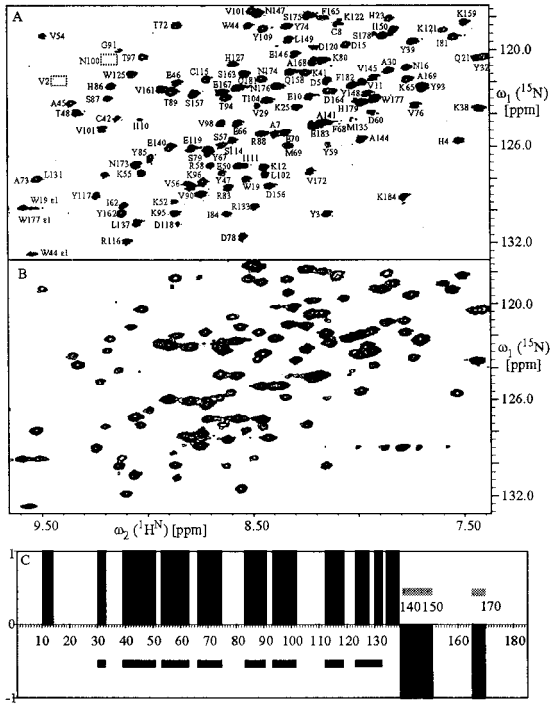

Figure 1. (A) 2D [^{15}N,^{1}H]-TROSY spectrum (Pervushin et al., 1997) recorded on a 900 MHz Varian INOVA spectrometer (25 °C) for uniformly ^{15}N-labeled and 50% deuterated FluA(R95K). The peaks are labeled using the one-letter code for amino acids. The peaks of Val 2 and Asn 100 are close to the noise level and their positions are indicated by boxes. (B) 2D [^{15}N,^{1}H]-HSQC spectrum recorded at 600 MHz with the same maximal evolution times as the spectrum shown in (A). (C) Chemical shift index (CSI) consensus plot (Wishart and Sykes, 1994) for identification of regular secondary structure elements. The eight β-strands forming the lipocalin β-barrel (black bars) as well as the α-helices (grey bars) are indicated.

1997; Figure 1) and a ^{15}N-resolved [^{1}H,^{1}H]-NOESY-[^{15}N,^{1}H]-TROSY acquired for sample 4 at 900 MHz, (ii) 2D [^{1}H,^{1}H]-TOCSY and NOESY acquired for both samples 1a and 1b at 900 MHz, (iii) 3D HNNCACB, CBCA(CO)NHN, HC(C)H COSY / TOCSY (Cavanagh et al., 1996) acquired for sample 2 at 600 or 750 MHz, (iv) reduced-dimensionality 3D $\underline{H}^{\alpha/\beta}\underline{C}^{\alpha/\beta}$(CO)NHN, 3D HNN<u>CAHA</u>, <u>HACA</u>(CO)NHN, 3D <u>HCCH</u> COSY, 2D <u>HBCB</u>(CGCD)HD, ^{1}H-TOCSY relayed <u>HCH</u> COSY (Szyperski et al., 1998; 2002) acquired for sample 2 at 600 and 750 MHz, and (v) 3D ^{13}C- and ^{15}N-resolved [^{1}H,^{1}H]-NOESY (Cavanagh et al., 1996) acquired, respectively, for samples 2 and 3 at 750 MHz.

Extent of assignments and data deposition

The combined use of double and triple resonance 3D spectra along with the 2D homonuclear spectra acquired at 900 MHz provided assignments (Figure 1) for 95% of the backbone and $^{13}C^{\beta}$, and for 91% of the side chain chemical shifts of FluA(R95K). The measurement of ^{15}N spin relaxation parameters (Szyperski et al., 1993) revealed that FluA(R95K) reorients with a correlation time of ∼10 ns at 25 °C (confirming that the protein is monomeric in solution). Thus, the use of TROSY (Pervushin et al., 1997) at 900 MHz dramatically improved the resolution of the 2D [^{15}N,^{1}H]-correlation map (Figures 1A,B), which facilitated the ^{15}N-$^1H^N$ spin system identification. Based on the chemical shift data, the strands forming the lipocalin β-barrel can be readily identified (Figure 1C). FluA(R95K) possesses a rather large number of aromatic residues (5 Phe, 15 Tyr, 7 Trp, 7 His), and the resonance assignment of the aromatic rings greatly benefited from employment of the traditional homonuclear approach (Cavanagh et al., 1996) at 900 MHz. The ^{1}H, ^{13}C and ^{15}N chemical shift data have been deposited in the BioMagResBank database (accession number 5756).

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