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Letter to the Editor: ^{1}H, ^{13}C and ^{15}N sequence-specific resonance assignments of the two-domain thrombin inhibitor dipetalin

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Biological context

The proteinase inhibitor dipetalin, isolated from the Mexican blood-sucking bug *Dipetalogaster maximus*, belongs to the class of Kazal-type inhibitors. It consists of two protein domains connected by a flexible peptide linker. Thrombin is the cognate target of the proteinase inhibitor dipetalin. This key enzyme of the blood coagulation cascade is involved in pathological processes like e.g., thrombosis. The two-domain protein (Dip-I + II) strongly inhibits thrombin ($K_i = 7.8 \times 10^{-11}$) and its inhibition potency is comparable to that of hirudin. The canonical thrombin binding site (Bode et al., 1989) is located in the first domain and previous structural investigations on the dipetalin-I domain have shown that the residues R10–A11 (Schlott et al., 2002) are involved in the inhibitor binding at the respective positions. The isolated N-terminal domain of dipetalin (Dip-I) shows a significantly lower inhibition potency ($K_i = 7.5 \times 10^{-9}$) and the isolated second dipetalin domain (Dip-II) lacks any thrombin inhibition. However, from X-ray studies of the complex of the homologous protein rhodniin with thrombin a contribution of the second domain was proposed (van de Locht et al., 1995). In order to elucidate the structural basis of the contribution of the second domain of dipetalin in the binding process and to understand the similarities and differences between the two homologous Kazal-type domains in structure and function, we have assigned the backbone ^{1}H, ^{13}C and ^{15}N chemical shifts as a first step towards determination of the solution structure and binding motifs of dipetalin-I + II.

Methods and experiments

Dipetalin-I + II was subcloned into the pET-15b vector (Novagen) and expressed in *Escherichia.coli* strain BL21(DE3) by a fusion protein approach. The fusion protein contained a His_6-tagged wild-type staphylokinase Sak42D at the N-terminus (Behnke and Gerlach, 1987; Schlott et al., 1994, 1997) followed by the amino acids Gln–Leu, a coagulation factor Xa (FXa) cleavage site and the two dipetalin domains at the C-terminus. The cultivation and preparation of cell lysates for the purification of unlabelled dipetalin-I + II followed a procedure described earlier (Schlott et al., 1994). Uniformly ^{15}N- or $^{13}C/^{15}N$-labelled proteins were expressed under modified culture conditions using M9 minimal medium as detailed in Ohlenschläger et al. (1997). Cleavage of the N-terminal part of the fusion protein was performed with factor Xa (New England Biolabs). The purification involved Ni–NTA column (Qiagen) and ÄKTA®/FPLC® chromatography (Amersham Pharmacia Biotech) steps. Concentration of the NMR samples was accomplished by Amicon ultrafiltration concentrators (Millipore).

The purity of the sample was verified by SDS-PAGE and the protein identity was analysed by MALDI-TOF mass spectrometry. The isotope

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labelled samples for collection of NMR-spectra were conditioned as described earlier (Ohlenschläger et al., 1997, 1998). The NMR samples had a concentration of 1.1 mmol/l [U-^{15}N]-Dip-I + II and 1.3 mmol/l [U-^{13}C,^{15}N]-Dip-I + II, respectively, in phosphate buffer at a total volume of 600 µl with 93% H_2O/7% D_2O (Cambridge Isotopes) in sealable NMR tubes.

NMR data were acquired on Varian $^{\text{UNITY}}$*INOVA* 600 MHz or $^{\text{UNITY}}$*INOVA* 750 MHz four channel NMR spectrometers equipped with pulsed field gradient accessories and triple resonance probes with actively shielded Z-gradient coils. The ^{1}H carrier was set at 4.86 ppm. Measurements were performed at a temperature of 288 K. Chemical shifts were referenced as described previously (Ohlenschläger et al., 1997). The NMR data were processed with VNMR (Varian) and analysed with the program XEASY (Bartels et al., 1995) on SGI workstations. The data set used for assignment comprised 2D ^{1}H–^{15}N HSQC, ^{1}H–^{13}C HSQC as well as 3D HNCACB, CBCA(-CO)NH, CC(CO)NH, HCC(CO)NH, HCCH-TOCSY, HNCO and HNHA spectra.

Extent of assignments and data deposition

Figure 1 shows the well-resolved 2D ^{1}H–^{15}N HSQC spectrum of the 108 residue protein dipetalin-I + II. 91% of $^1H^{\alpha}$, 90% of $^1H^{\beta}$, 81% of $^{13}C'$, 81% of N′ and 80% of $^1H^{N}$, 91% of $^{13}C^{\alpha}$ and 90% of $^{13}C^{\beta}$ chemical shifts were assigned. The assignments for residues Cys6–Cys8 and Glu50–Glu56, the latter corresponding to residues in the linker region between the two Kazal-type domains, were not attainable under the outlined experimental conditions. The HCCH-TOCSY allowed the identification of five additional spin systems which however could not be linked to the unassigned residues mentioned due to missing NH correlations. In total, assignments for 93% of the residues in the canonical Kazal-type domains could be established and the assignment of their aliphatic side chain protons could be completed to 100%.

The secondary structural data acquired indicates that the two dipetalin domains both fold into Kazal-type structures with a β1–β2–α1 motif. The α1-helices of both domains are predicted by the consensus chemical shift index (Wishart and Sykes, 1994) to involve eight residues each. As in the case of free dipetalin-I (Schlott et al., 2002) the prediction of the β-sheets is inconclusive due to the expected short length of the strands (~3 residues).

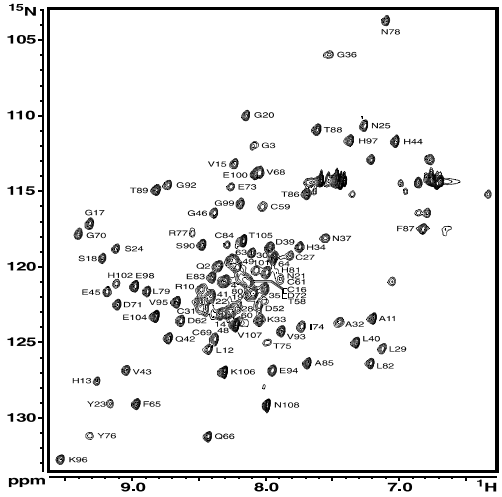

Figure 1. 2D ^{1}H-^{15}N HSQC spectrum of dipetalin-I + II at 750 MHz, 288 K. The annotations correspond to the residue numbers.

The assignment of the ^{1}H, ^{13}C and ^{15}N chemical shifts of dipetalin-I + II described here, have been deposited in the BioMagResBank under the accession number BMRB–6242.

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