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Assignment of ^{1}H, ^{13}C, and ^{15}N signals of turkey ovomucoid third domain at pH 2.0*

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Biological context

Ovomucoid third domains are small (6 kDa), highly stable, globular proteins with potent inhibitory activity toward serine proteinases (Laskowski et al., 1987). The abnormal spectrophotometric and spectrofluorometric behavior of the phenolic groups in ovomucoids at low pH (Donovan, 1967), termed the 'Donovan transition', has been interpreted in terms of a limited conformational transition. The Donovan transition in avian ovomucoid domains involves protonation-induced disruption of the hydrogen bond between the side chains of the highly conserved Tyr31 and Asp27 residues located in the neighboring antiparallel strands of the β-sheet preceding the α-helix, as well as the electrostatic interaction between the ring of His52 and the carboxylate group at the C-terminus (March, 1980; Croll, 1982). The virtually complete sequence-specific assignments reported here for the 56-residue turkey ovomucoid third domain (OMTKY3) at pH 2.0 provide the basis for investigations of the physical origins of the observed Donovan transition, including comparisons with the NMR chemical shifts (Robertson et al., 1988) and structure (Krezel et al., 1994) performed in this laboratory at pH 4.0 and with the X-ray crystal structures of the complexes of OMTKY3 with serine proteinases at pH values above the Donovan transition (Fujinaga et al., 1987).

Methods and results

OMTKY3 was overproduced as a fusion protein with staphylococcal nuclease (SNase) in *Escherichia coli* strain BL21(DE3)/pLysS transformed with PTNOM3 plasmid by using the T7 promotor/T7 polymerase expression system (Hinck et al., 1993). Isopropyl thiogalactoside was used to induce expression of the fusion protein. Cells were grown at 37 °C in the presence of ampicillin and chloramphenicol in LB (Laura–Bertani) medium (unlabeled OMTKY3), in M9 medium with $^{15}NH_4Cl$ ([*U*-^{15}N]-OMTKY3), or in M9 medium with $^{15}NH_4Cl$ and ^{13}C [99.8% ^{13}C]-glucose ([*U*-^{15}N, ^{13}C]-OMTKY3). The cells were harvested by centrifugation, resuspended in Tris-HCl with ethylenediamine tetraacetic acid and phenyl methyl sulfonyl fluoride, frozen at −20 °C, and thawed at 37 °C. $CaCl_2$ was added to activate nucleases. Cell debris was removed by centrifugation. The supernatant was loaded onto a Q-Sephadex (Pharmacia Biotech, Piscataway, NJ, U.S.A.) column and washed with Tris-HCl at pH 9.2 (near the pI of SNase). Fractions that assayed for proteinase inhibitor activity were pooled; the pH of the combined fractions was adjusted to 7.6, and the solution was loaded onto a CM-Sephadex column equilibrated with Tris-HCl, pH 7.6. The SNase-OMTKY3 fusion protein was eluted by a salt gradient with Tris-HCl/NaCl. Fractions with proteinase inhibitor activity were pooled and concentrated by ultrafiltration (Amicon, YM 10 membrane, Millipore, Bedford, MA, USA). The fusion protein was then treated with CNBr/HCl to cleave at the Met residue between SNase and OMTKY3 (OMTKY3 has no Met residues). The lyophilized protein was dissolved in Tris-Cl, pH 8.0, loaded onto a Q-Sephadex column, eluted with a salt gradient (Tris-Cl/NaCl,

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pH 8.0) and desalted with a G-25 gel filtration column. Sample purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and enzyme activity assays. The yields of pure OMTKY3 were 10 mg per liter of LB medium and 8.5 mg per liter of culture in M9 medium.

NMR samples (0.5 ml) contained 2–3 mM unlabeled OMTKY3 for homonuclear proton NMR and ^{15}N uniformly labeled or $^{15}N/^{13}C$ double labeled OMTKY3 for heteronuclear NMR experiments. The protein was dissolved in 90% H_2O/10% D_2O, and the pH was adjusted to 2.0 with HCl. NMR data were collected at 25 °C on Bruker DMX-500 and Bruker DMX-600 spectrometers with Bruker $^{1}H,^{13}C,^{15}N$ triple-resonance gradient probes. Quadrature detection in the phase-sensitive mode was achieved by TPPI or States-TPPI. NMR data were processed on Silicon Graphics workstations with FELIX software (version 2.30 or 95 β, Molecular Simulations, Inc., San Diego, CA, U.S.A.). Time domain convolution was used to remove the residual H_2O signal during processing. Each spectrum was multiplied by a phase-shifted, skewed, sine-bell window function before Fourier transformation in each dimension. ^{1}H chemical shifts were referenced directly to internal 2,2-dimethylsilapentane-5-sulfonic acid (DSS); ^{13}C and ^{15}N chemical shifts were referenced indirectly to DSS by multiplying the spectrometer frequency corresponding to 0 ppm in the ^{1}H spectrum by the $^{13}C/^{1}H$ or $^{15}N/^{1}H$ frequency ratio.

The H^{α}, H^{N}, and ^{15}N resonances of non-proline spin systems were identified from the fingerprint regions of 2D DQF-COSY and 2D $^{1}H,^{15}N$ SE-HSQC spectra in H_2O. Data from 2D HOHAHA (τ_m = 40 ms and 58 ms with the DIPSI-2 mixing sequence) and 3D $^{1}H,^{15}N$ HSQC-TOCSY (DIPSI-2 mixing time of 48 ms) allowed the extension of spin systems from the backbone to side chains. Results from 2D NOESY in H_2O (τ_m from 50 to 180 ms) and 3D $^{1}H,^{15}N$ HSQC-NOESY (τ_m = 126 ms and 150 ms), with GARP decoupling during the proton evolution and acquisition, were used to complete the analysis of intraresidue spin systems and to extend the analysis to neighboring residues through NOEs connecting the backbone and β-protons of a given residue with the amide proton of the next residue in the sequence. Most of the ambiguities in interpreting 2D data arising from spectral overlap were resolved in 3D spectra through dispersion in the ^{15}N dimension. Proline spin systems were deduced from DQF-COSY and HOHAHA spectra and sequentially assigned on the basis of NOEs connecting proline δ-protons with the backbone and β-protons of the previous residue in the sequence. 2D $^{1}H,^{13}C$ HSQC was used to extend the assignments to C^{α} and C^{β}. Side-chain carbon resonances were deduced from a 3D HCCH-TOCSY spectrum. The sequential assignments were confirmed and extended to carbonyl carbons by 3D triple-resonance HNCO, HN(CO)CA, CBCA(CO)NH, HNCACB, C(CO)NH, and HBHACONH spectra.

Extent of assignments, pH dependence, and data deposition

Probably because of its mobility, the resonances of Leu1 were not observed, and it remains the only unassigned residue. Backbone ^{1}H, ^{13}C, and ^{15}N resonances were all assigned, with the exception of ^{15}N in Ala2 and $^{13}C'$ in Tyr11, Lys13, Arg21, Cys38, Asn45, and Lys55. The only non-aromatic side-chain protons that remain unassigned are the H^{γ} of Pro22. A number of methylene and isopropyl groups displayed degeneracy. The C^{β} carbons of all non-glycine residues were assigned, with the exception of those from Ser26 and Thr30. All non-aromatic side-chain carbons beyond the β-position were assigned except C^{γ} and C^{δ} in Pro12, C^{γ} in Pro22, and $C^{\gamma 2}$ in threonines 30 and 49. Single carbon resonances were observed and assigned in isopropyl groups of Val4, Val42, and Leu48. Aromatic proton assignments are complete for Tyr20 and Phe53 but partial for Tyr11, Tyr31, and Phe37. Assignments were made for the labile side-chain protons of one arginine, one histidine, five asparagines, and Lys55. The heterogeneity displayed by the δ-protons and nitrogens of asparagine were symptomatic of slow exchange between different conformations. Signals from the exchangeable H^{ζ} of Lys13 and Lys34 were not observed.

Comparison of proton and nitrogen chemical shifts at pH 2.0 with the assignments determined previously at pH 4.0 (Robertson et al., 1988; Hoogstraten, 1995) suggests substantial variations in torsion angles, structural rigidity, hydrogen bonding patterns, and electrostatic interactions induced by the change of pH. In particular, the Chemical Shift Index analysis (Wishart and Sykes, 1994) implies that some of the secondary structural elements present in OMTKY3 at pH 4.0 are disrupted at pH 2.0. These observations justify further efforts toward determining the solution structure of OMTKY3 at pH 2.0.

The ^{1}H, ^{13}C, and ^{15}N chemical shifts for OMTKY3 at pH 2 and T = 298 K have been deposited in BioMagResBank under BMRB accession number 4068.

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