\textbf{1H, 15N, 13C and 13CO assignments and secondary structure determination of basic fibroblast growth factor using 3D heteronuclear NMR spectroscopy}

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\section*{Summary}

The assignments of the 1H, 15N, 13CO and 13C resonances of recombinant human basic fibroblast growth factor (FGF-2), a protein comprising 154 residues and with a molecular mass of 17.2 kDa, is presented based on a series of three-dimensional triple-resonance heteronuclear NMR experiments. These studies employ uniformly labeled 15N- and 13N-13C-labeled FGF-2 with an isotope incorporation > 95% for the protein expressed in \textit{E. coli}. The sequence-specific backbone assignments were based primarily on the interresidue correlation of \textit{C}\textsuperscript{\alpha} and \textit{C}\textsuperscript{\beta} to the backbone amide 1H and 15N of the next residue in the CBCA(CO)NH and HBHA(CO)NH experiments and the intraresidue correlation of \textit{C}\textsuperscript{\alpha}, \textit{C}\textsuperscript{\beta} and 1H to the backbone amide 1H and 15N in the CBCANH and HNHA experiments. In addition, \textit{C}\textsuperscript{\alpha} and \textit{C}\textsuperscript{\beta} chemical shift assignments were used to determine amino acid types. Sequential assignments were verified from carbonyl correlations observed in the HNCO and HCACO experiments and \textit{C}\textsuperscript{\alpha} correlations from the HCANH experiment. Aliphatic side-chain spin systems were assigned primarily from H(CCO)NH and C(CO)NH experiments that correlate all the aliphatic 1H and 13C resonances of a given residue with the amide resonance of the next residue. Additional side-chain assignments were made from HCCH-COSY and HCCH-TOCSY experiments. The secondary structure of FGF-2 is based on NOE data involving the NH, H\textsuperscript{\alpha} and H\textsuperscript{\beta} protons as well as \textit{J}_{\text{HNHC}}\textsuperscript{\alpha} coupling constants, amide exchange and 13C\textsuperscript{\alpha} and 13C\textsuperscript{\beta} secondary chemical shifts. It is shown that FGF-2 consists of 11 well-defined antiparallel \beta-sheets (residues 30-34, 39-44, 48-53, 62-67, 71-76, 81-85, 91-94, 103-108, 113-118, 123-125 and 148-152) and a helix-like structure (residues 131-136), which are connected primarily by tight turns. This structure differs from the refined X-ray crystal structures of FGF-2, where residues 131-136 were defined as \beta-strand XI. The discovery of the helix-like region in the primary heparin-binding site (residues 128-138) instead of the \beta-strand conformation described in the X-ray structures may have important implications in understanding the nature of heparin–FGF-2 interactions. In addition, two distinct conformations exist in solution for the N-terminal residues 9-28. This is consistent with the X-ray structures of FGF-2, where the first 17–19 residues were ill defined.

\section*{Introduction}

Basic fibroblast growth factor (FGF-2) is a 17.2-kDa protein, consisting of 154 residues, which exhibits angiogenic and a variety of growth and differentiation activities. Its pleiotrophic nature suggests possible roles in tumor growth and wound healing and the protein is thus an attractive target for therapeutic drug development (Folkman and Klagsbrun, 1987; Baird and Böhlen, 1990; Basilico and Moscatelli, 1992). FGF-2 belongs to a nine-membered family that includes three oncogenes (FGF-3, FGF-4 and FGF-5), characterized by a high affinity toward heparin sulfate proteoglycans (HSPG) and a high, 30–55%, sequence identity (Miyamoto et al., 1993).

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Supplementary material available from the authors: tables containing the 1H, 15N, 13CO and 13C resonance assignments for the major and minor conformations of FGF-2.
interaction of FGF-2 with HSPG is required for binding to its cell-surface tyrosine kinase receptor (FGFR) and essential for mediating internalization and intracellular targeting (Yayon et al., 1991; Roghani and Moscatelli, 1992; Reiland and Rapraeger, 1993). Calorimetric binding studies and molecular modeling suggest that FGF-2 induces dimerization of FGFR, initiating transmembrane signaling (Pantoliano et al., 1994). Residues Lys\(^{128}\), Arg\(^{129}\), Lys\(^{134}\) and Lys\(^{138}\) have been identified as part of the heparin-binding domain of FGF-2 (Li et al., 1994; Thompson et al., 1994). Heparin has also been shown to protect FGF-2 from inactivation resulting from exposure to low pH, elevated temperature or proteases, and to restore bioactivity to inactive growth factor (Westall et al., 1983; Gospodarowicz and Cheng, 1986; Saksela et al., 1988; Sommer and Rifkin, 1989; Pineda-Lucena et al., 1994a).

In order to better understand the mode of action of FGF-2 and in particular its interaction with HSPG and its cell surface receptor, we initiated a structural program to determine the three-dimensional structure of FGF-2 in solution by NMR spectroscopy. Here, we report the nearly complete \(^1H\), \(^{15}N\), \(^{13}CO\) and \(^{13}C\) assignments of the spectrum of FGF-2 from a series of 3D triple-resonance experiments. These assignments have led to the determination of the solution secondary structure for FGF-2, based on NOE data involving the NH, H\(^\alpha\) and H\(^\beta\) protons as well as \(^3\)J\(^{HNH\alpha}\) coupling constants, amide exchange and \(^{13}C\) secondary chemical shifts. Two crystal structures have been reported for FGF-2, where the protein is described as a \(\beta\)-sheet barrel of six antiparallel \(\beta\)-strands with a base of six additional \(\beta\)-strands (Zhu et al., 1983; Ago et al., 1991; Eriksson et al., 1991; Zhang et al., 1991). Comparison between the NMR secondary structure and the refined X-ray structure indicates clearly similar structures; however, some distinct differences exist, particularly at the heparin-binding site.

**Materials and Methods**

**Production of recombinant \(^{15}N\)- and \(^{13}C\)-\(^{15}N\)-labeled FGF-2**

The 154-residue form of Glu\(^{3,5}\) Ser\(^{78,96}\) human recombinant FGF-2 was expressed in *E. coli* essentially as described before (Seddon et al., 1991), except that cultures were grown in M9 minimal media containing \([^{15}N]\)ammonium chloride (1.25 g/l) and \([^{13}C]\)glucose (2 g/l) as the sole nitrogen and carbon sources. Cells from a 1 liter culture were harvested by centrifugation, resuspended in 30 ml of 50 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA and 0.6 M NaCl and disrupted by sonication (6 x 30 s pulses). About 50% of the expressed protein was in a soluble form and was purified as follows. After centrifugation (10000 \(\times\) g; 20 min), the supernatant solution was incubated with 25 ml of hydrated heparin sepharose (Pharmacia Biotech, Piscataway, NJ) at 4 \(^\circ\)C for 1 h under constant rotation. The resin was isolated by centrifugation and washed extensively with 10 mM Tris-HCl, pH 7.4, containing 0.6 M NaCl; bound protein was eluted with Tris buffer containing 2 M NaCl. The 2 M NaCl eluent was diluted 10-fold with 50 mM sodium phosphate buffer (pH 7.2) and loaded onto a Mono S column (HR 5/5; Pharmacia Biotech). Elution of bound material was monitored at 280 nm and was accomplished using a linear salt gradient (0.1–1.0 M NaCl in 60 min) at a flow rate of 1.0 ml/min.

Labeled FGF-2 was also obtained from the insoluble fraction. The pellet was resuspended in 10 mM Tris (pH 7.8) containing 8 M urea, 10 mM DTT (30 ml/pellet from 1 l of cells) and incubated at room temperature for 30 min. Insoluble material was removed by centrifugation (27000 \(\times\) g; 20 min) and the supernatant solution was loaded onto an S-sepharose column (25 ml), equilibrated with 10 mM Tris (pH 7.4) containing 8 M urea, 50 mM NaCl and 1 mM DTT at pH 7.4. After extensive washing, bound protein was eluted with the same buffer containing 0.35 M NaCl. Elution was monitored at 280 nm and fractions containing FGF-2 were pooled and diluted to 20 \(\mu\)g/ml with elution buffer and dialyzed (6 kDa cutoff) overnight against 18 l of 10 mM Tris buffer (pH 7.6) containing 1 mM DTT and 0.6 M NaCl at 4 \(^\circ\)C. Insoluble protein was removed by centrifugation and the supernatant solution was loaded onto a heparin sepharose column (25 ml), equilibrated with 50 mM Tris (pH 7.4) containing 0.6 M NaCl; the bound protein was eluted with buffer containing 2 M NaCl. The final purification step was accomplished by Mono S anion exchange chromatography as described above.

These procedures typically yielded 8 mg and 6 mg of FGF-2 per liter of bacterial culture from the soluble and insoluble fractions, respectively. FGF-2 prepared from the soluble or insoluble fractions were indistinguishable by electrospray mass spectrometry, reversed-phase HPLC, and NMR spectroscopy. Analyses by N-terminal sequence analysis and electrospray mass spectrometry were consistent with the 154-residue form of FGF-2 and indicated essentially complete removal of the N-terminal methionyl residue (introduced for expression of the mature protein). Isotope enrichments were >95% by electrospray mass spectrometry. Stock solutions of \(^{13}C\)-\(^{15}N\)- and \(^{15}N\)-labeled FGF-2 at 1 mg/ml were stored in 50 mM sodium phosphate buffer (pH 7.2) containing 0.5 M NaCl at –45 \(^\circ\)C.

**NMR sample preparation**

NMR samples of \(^{13}C\)-\(^{15}N\)- and \(^{15}N\)-labeled FGF-2 in 90% H\(_2\)O/10% D\(_2\)O were prepared by concentration and buffer exchange of FGF-2 stock solutions using Centricon...