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Molecular cloning, overexpression, and characterization of the ligand-binding D2 domain of fibroblast growth factor receptor

Kuo-Wei Hung,[a] T.K.S. Kumar,[b] Ya-Hui Chi,[a] Ing-Ming Chiu,[c,d] and Chin Yu[a,b,*]

[a] *Department of Chemistry, National Tsing Hua University, Hsinchu 300, Taiwan, ROC*
[b] *Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701, USA*
[c] *Department of Internal Medicine, Ohio State University, Columbus, OH 43210, USA*
[d] *Department of Molecular and Cellular Biochemistry, Ohio State University, Columbus, OH 43210, USA*

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Abstract

Fibroblast growth factors (FGFs) regulate a wide range of important cellular processes. The biological activities of FGFs are mediated by cell surface receptors (FGFRs). In the present study for the first time we report the cloning, expression, and characterization of the ligand (FGF)-binding D2 domain of human FGFR2. D2 domain is expressed in *Escherichia coli* in high yields (10 mg/L) as inclusion bodies. The protein is recovered by dissolving the inclusion bodies in 8 M urea and subsequently refolding on nickel affinity column. The protein is purified (to ~97% purity) to homogeneity using heparin–Sepharose affinity column. Far-UV circular dichroism data and chemical shift index plot based on ^{1}H-α, ^{13}C-α, ^{13}C-β, and ^{13}carbonyl group chemical shifts suggest that D2 domain is an all β-sheet protein consisting of 9 β-strands. Isothermal titration calorimetry and equilibrium urea unfolding experiments show that recombinant D2 domain is in a biologically active conformation and binds strongly to its ligand (FGF) and to the heparin analog, sucrose octasulfate (SOS). Using a variety of triple resonance NMR experiments, complete assignment of ^{1}H, ^{15}N, and ^{13}C resonances in D2 domain has been accomplished. The findings of the present study not only pave way for an in-depth investigation of the molecular mechanism(s) underlying the activation of FGF signaling but also provide avenues for the rational design of potent inhibitors against FGF-mediated pathogenesis.

Keywords: Growth factor; Receptor; Expression; NMR; β-Sheet

Fibroblast growth factors (FGFs) regulate a wide range of key cellular processes including angiogenesis, cell growth, embryogenesis, differentiation and, wound healing [1–4]. The diverse physiological activities of FGF are mediated by cell surface high affinity receptors (FGFRs) that belong to the tyrosine kinase receptor family [5–8]. The prototype FGFR consists of an extracellular ligand-binding domain that contains three immunoglobulin-like domains (D1, D2, and D3), a transmembrane domain, and a cytoplasmic domain that bears the tyrosine kinase activity [9,10]. The D2 and D3 modules of the extracellular ligand-binding domain are well conserved and are shown to be minimal unit sufficient for the specific ligand (FGF)-binding activity [7]. In addition, the D2 domain is believed to contribute residues that are crucial for ligand (FGF)-receptor binding [10]. Therefore, understanding the structural interactions governing the ligand–D2 domain binding is expected to pave way for the rational design of agonists and antagonists for the treatment of FGF-induced pathogenesis. In this context, in the present study for the first time we report the cloning, overexpression, and characterization of the D2 domain. In addition, we also demonstrate that recombinant D2 domain is in a biologically active conformation and exhibits strong binding affinity to its ligand (FGF).

Materials and methods

General protocol and reagents. *Taq* DNA polymerase, *Nde*I, and *Xho*I enzymes were purchased from Promega. *Escherichia coli*

* Corresponding author. Fax: 1-479-575-4049.
E-mail address: cyu@uark.edu (C. Yu).

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[BL21(DE3)pLysS] and pET20b(+) were purchased from Novagen. Heparin–Sepharose was obtained from Amersham–Pharmacia Biotech. Labeled $^{15}NH_4Cl$, [^{13}C]glucose, and D_2O were purchased from Cambridge Isotope Laboratories. Acetonitrile, urea, and imidazole were obtained from Sigma Chemical. All other chemicals used in this study were of high quality analytical grade.

Construction and expression of the D2 domain gene. The cDNA encoding the D2 domain contains 309 bp was amplified by polymerase chain reaction (PCR), using 5′-GATACATATGAACAGTAACAAC AAG-3′ and 5′-GTGCTCGAGAGAACCGCGTGGCACCAGCAC AACATCCAGGTG-3′ as primers. The former primer contains the *Nde*I site. The latter primer contains the *Xho*I site and encodes a thrombin-cutting site at C-terminal of the recombinant protein. The PCR product was digested with *Xho*I and *Nde*I, inserted into the vector pET20b(+). The authenticity of the clone construct was confirmed by nucleotide sequencing. *E. coli* cells (transformed with pET20b(+) containing the D2 domain insert were grown in 1 L Luria broth (LB) medium which contained 100 μg/mL ampicillin. Protein induction was achieved by the addition of IPTG (1 mM/L) when the OD_{600} of the growing culture had reached about 0.4. The culture was incubated at 37 °C for additional 4 h and harvested and lysed with the French pressure cell press. The expression and solubility of the D2 domain were checked by SDS–PAGE.

Recovery and purification of the protein from the inclusion bodies. The proteins expressed in the inclusion bodies were dissolved extracted in 20 mM phosphate (pH 7.5), containing 300 mM NaCl and 8 M urea at room temperature. Protein purification was achieved in two steps. Inclusion bodies dissolved in 20 mL of 8 M urea were loaded onto Ni–NTA nickel affinity column(s). D2 domain bound to the Ni–NTA column was washed with 10 bed volumes (~100 mL) of the elution buffer [20 mM phosphate buffer (pH 7.5) containing 300 mM NaCl, 20 mM imidazole, and 50 mM ammonium sulfate]. Finally, the refolded protein bound to the Ni–NTA column was eluted with the elution buffer containing 500 mM imidazole. Further purification of the protein was achieved using heparin–Sepharose affinity column(s). The refolded and partially pure protein obtained from the Ni–NTA column was loaded on a heparin–Sepharose column and washed with 20 mM phosphate buffer (pH 6.5) containing 20 mM NaCl and 50 mM ammonium sulfate to get rid of imidazole and contaminating proteins. D2 domain bound to the heparin–Sepharose column was eluted with 1 M NaCl.

High performance liquid chromatography. High performance liquid chromatography (HPLC) was performed on Hitachi HPLC (Model l–400). HPLC analysis was carried out on a C_{18} μ-Bondapak semi-preparative reverse phase column using a linear 0–60% gradient of acetonitrile containing 0.1% trifluoroacetic acid. The elution of the protein was monitored at 280 nm.

Equilibrium unfolding. Urea-induced equilibrium unfolding of D2 domain was performed at a protein concentration of 100 μg/mL and monitored by changes in the tryptophan fluorescence (at an emission wavelength of 350 nm). The excitation wavelength was set at 280 nm. The excitation and emission slit widths were set at 10 nm.

Circular dichroism. All circular dichroism (CD) measurements were carried out at room temperature (298 K) on a Jasco J720 spectropolarimeter using a quartz cell of 0.02 cm pathlength. Each spectrum was an average of 10 scans. The concentration of protein used for the CD measurements was 1 mg /mL.

Isothermal titration calorimetry measurements. The titration calorimetry measurements were performed with a Microcal VP titration calorimeter (Northampton, MA, USA). Samples were centrifuged prior to the titration and examined for precipitates, if any, after the titration. A typical titration consisted of injecting 5–10 μL aliquots of 1 mM ligand (FGF) solutions into 0.25–0.3 mM of D2 domain solution after every 3.5 min to ensure that the titration peak returned to the baseline prior to the next injection. Aliquots of more concentrated ligand solutions were injected into just the reaction buffer [20 mM phosphate buffer (pH 6.5) containing 50 mM NaCl and 50 mM ammonium sulfate] in separate isothermal titration calorimetry (ITC) runs in order to measure the heats of dilutions of the ligand.

NMR spectroscopy. NMR data on the D2 domain were acquired at a protein concentration of 1.5 mM in 20 mM phosphate buffer (in 90% H_2O + 10% D_2O, pH 6. 5) containing 50 mM NaCl and 50 mM ammonium sulfate. A variety of triple resonance experiments were acquired at 298 K to accomplish complete assignment of ^{1}H, ^{13}C, and ^{15}N resonances in the protein. All spectra were referenced to TSP-d_4.

Results and discussion

Protein is expressed as inclusion bodies

The D2 domain construct used in the present study is 103 amino acids long (Fig. 1) spanning residues 147–249 of the full length human FGFR2 [7]. SDS–PAGE of the bacterial cells induced by IPTG revealed that most of the expressed protein is trapped as inclusion bodies (Fig. 2A). Less than 5% of the overexpressed protein is present in the supernatant. The pellet containing the protein (overexpressed as inclusion bodies) dissolved in 8 M urea shows an intense band and several other minor bands on SDS–PAGE (Fig. 1A). The intense band corresponds to a molecular mass of about 14 kDa and accounts for about 75–80% of the total protein content recovered from the bacterial cell lysate.

Purification of the D2 domain

The His_6-tag designed at the C-terminal of the protein facilitated the simultaneous purification and refolding of the protein. The overexpressed protein is observed to bind to the nickel affinity column quite strongly and the unbound contaminating proteins were eliminated by washing the affinity column elution with 10 bed volumes of the elution buffer [20 mM phosphate buffer (pH 7.5)] containing 300 mM sodium chloride and 20 mM imidazole]. Repeated washing of the column with the elution buffer also served as an effective protocol for refolding recombinant D2 domain on the affinity column. D2 domain eluted as a single peak in 500 mM imidazole and yielded an intense single band (with ~90% purity) on SDS–PAGE corresponding to a molecular mass of about 14 kDa (Fig. 2B).

M N S N N K R A P Y W T N T E K M E K R L H A V [24]

P A A N T V K F R C P A G G N P M P T M R W L K [48]

N G K E F K Q E H R I G G Y K V R N Q H W S L I [72]

M E S V V P S D K G N Y T C V V E N E Y G S I N H [97]

T Y H L D V V

Fig. 1. Amino acid sequence of the D2 domain of the human fibroblast growth factor receptor 2. Amino acids are indicated in single letter code. D2 domain comprises of 103 amino acids spanning residues, 147–249 of the full length FGFR sequence.

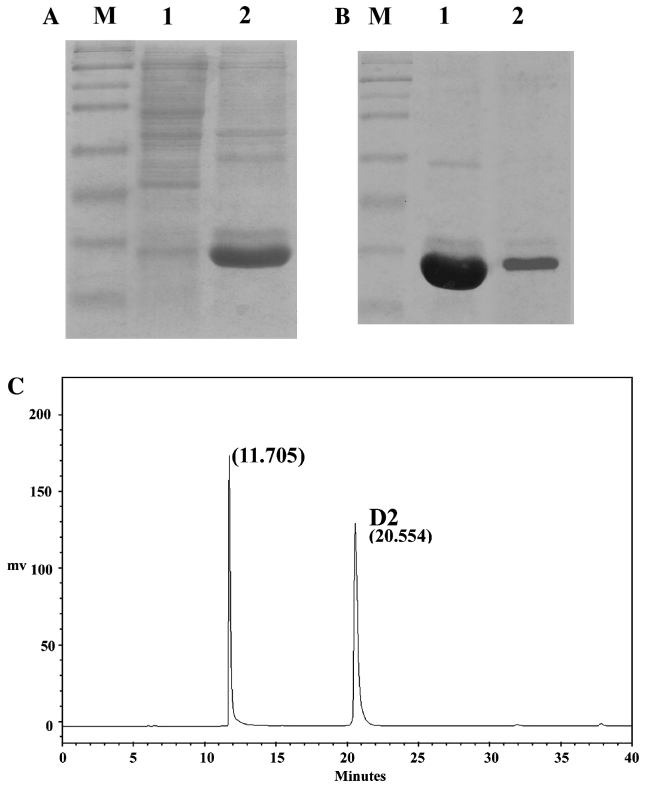

Fig. 2. SDS–PAGE analysis at different stages of purification of the D2 domain. (A) SDS–PAGE of the supernatant (lane 1) and pellet (lane 2) portions of the bacterial cell lysate. The protein (D2 domain) is mostly overexpressed as inclusion bodies. (B) SDS–PAGE of D2 domain purified on the Ni–NTA (lane 1) and heparin–Sepharose columns (lane 2). (C) HPLC profile of the purified fraction of the D2 domain. The numbers indicated in parentheses represent retention times of the fractions. The peak marked as D2 is the fraction containing the D2 domain. Lane M, in all the panels represents the molecular weight marker.

FGF receptor in its native state is known to bind to proteoglycans, including heparin. The high heparin binding of the receptor is facilitated by the presence of a positively charged "canyon" in FGFR, which is largely composed of residues from the D2 domain [10]. In this background, the completely refolded D2 domain of FGFR is also expected to show binding affinity to heparin. We extended this premise to achieve further purification of the protein using heparin–Sepharose affinity chromatography. As expected, D2 domain binds strongly to heparin–Sepharose implying that the protein is refolded to its native conformation. The protein elutes as a single peak in 1.0 M NaCl. Reversed phase HPLC analysis revealed that the purity of the protein is about 95% (Figs. 2B and C). ES-mass analysis yielded an expected molecular mass of 13,708 Da (data not shown) for the D2 domain.

Secondary structure of the D2 domain

The secondary structure of the protein was assessed by Far-UV circular dichroism (CD). Far-UV CD spectrum of the protein revealed a prominent negative CD band centered at around 225 nm and a positive ellipticity peak at about 205 nm (Fig. 3). These spectral features are reminiscent of an all β-sheet protein, with the constituent β-strands arranged into a β-barrel architecture [4,6]. These results are also consistent with the results of the secondary structure prediction employing the consensus secondary structure prediction algorithm [12], which predicts D2 domain of FGFR to be an all β-sheet protein with nine β-strands (data not shown). In addition, it is interesting to note that secondary structure estimation using ^{1}H-α, ^{13}C-α, ^{13}C-β, and ^{13}carbonyl group chemical shifts also yielded a similar conclusion on the secondary structure content in the D2 domain [13,14]. The near perfect agreement of the theoretical predictions with the experimental data strongly suggests that the D2 domain is an all β-sheet protein comprising 9 β-strands.

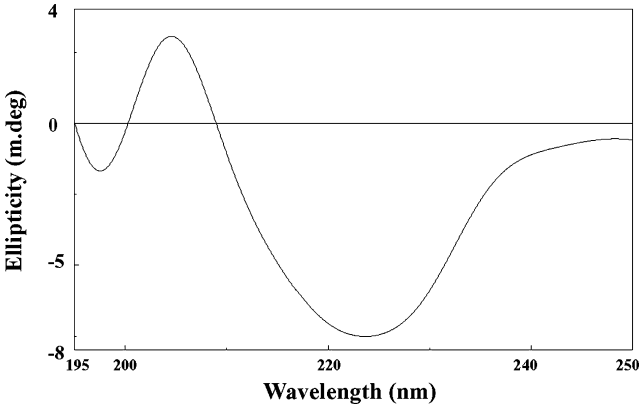

Fig. 3. Far-UV CD spectrum of the refolded recombinant D2 domain. The protein appears to be an all β-sheet protein with no helical segments.

Heparin-binding affinity

Receptor dimerization is an obligatory event in FGF signaling and requires proteoglycans such as heparin [5,11]. Besides heparin, a number of chemically diverse, low-molecular weight sulfated sugars such as sucrose octasulfate (SOS) are known to mimic heparin action in supporting FGF-mediated cell proliferation activities in vitro [5,11,15]. As mentioned earlier, FGFR–heparin interactions are mediated by D2 domain [7,11]. We examined the proteoglycan(s)-binding affinity of the isolated recombinant D2 domain using SOS which is a structural analog of heparin. We preferred to use SOS instead of heparin due to problems in preparation of homogeneous heparin samples [6]. Energy parameters (characterizing ligand–protein interactions) estimated using non-homogeneous ligand preparations are reported to be unreliable [16].

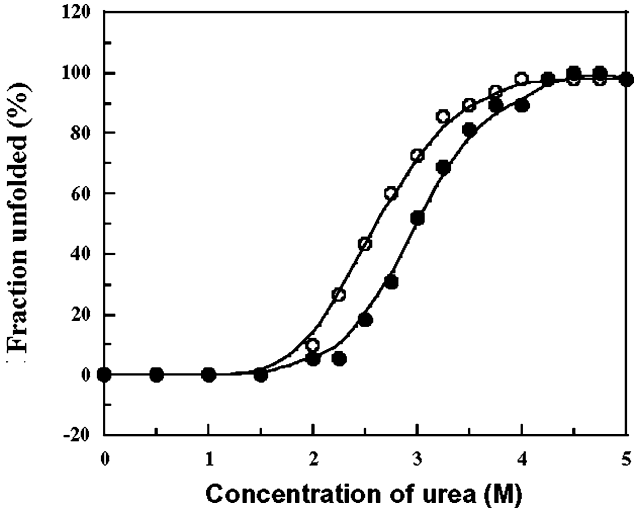

Fig. 4. Urea-induced unfolding of D2 domain in the presence (closed circle) and absence (open circle) of SOS. The ligand (SOS) binds to the D2 domain and stabilizes the protein.

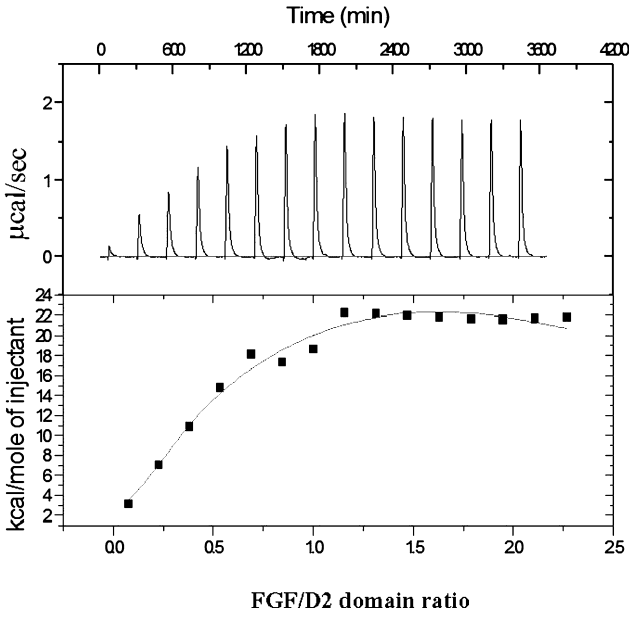

Fig. 5. Isothermal titration calorimetry of the interaction of the SOS with D2 domain. SOS binds to D2 domain in two phases, with binding constant values of 1.2×10^{-5} M (high affinity site) and 2.3×10^{-4} M (low affinity phase).

Urea-induced unfolding of D2 domain (monitored by changes in the tryptophan fluorescence) in the presence and absence of SOS is shown in Fig. 4. SOS is observed to bind and stabilize D2. The free energies of unfolding (ΔG_u) of D2 domain in the presence and absence of SOS are estimated to be 5.2 ± 0.07 and 4.0 ± 0.03 kcal mol^{-1}, respectively. These results suggest that recombinant D2 domain is in a biologically active conformation which facilitates clustering of the positively charged residues. SOS (or proteoglycans) can bind to this cluster of cationic residues and not only stabilizes the protein but also promotes dimerization of FGFR crucial for FGF-activation. Heparin-binding affinity of the D2 domain is also obvious from the strong binding of the protein to heparin–Sepharose (as discussed earlier).

D2 domain–FGF interaction

It is important to verify if the isolated D2 domain is in its biologically active conformation. In this context, substrate-/ligand-binding assay is an authentic test to ensure the "nativeness" of the protein conformation.

Isothermal titration calorimetry (ITC) is a useful and popular technique to measure binding affinity of a protein to its ligand [17,18]. In addition, ITC measurements also facilitate the estimation of binding stoichiometry of protein–ligand interactions [18]. Therefore, we examined the binding of the recombinant D2 domain (of FGFR) with its ligand [human FGF1 (hFGF-1)] using ITC. Isothermogram of the D2 domain/hFGF-1 titration is sigmoidal (Fig. 5). The titration curve representing the binding of D2 domain and hFGF-1 saturates at a protein to ligand ratio of 1:1. The binding of D2 domain to hFGF-1 is accompanied by the evolution of heat (Fig. 5). Best fit of the isotherm could be obtained with the assumption of two sites of binding for hFGF-1 on D2 domain. The binding constants of the high affinity and low affinity sites are estimated to be 1.2×10^{-5} and 2.3×10^{-4} M, respectively. The results obtained unambiguously suggest that recombinant D2 domain is indeed in its biologically active conformation capable of binding strongly to its natural ligand (FGF) in a 1:1 stoichiometry.

NMR assignment and its significance

FGF is involved in a wide range of pathogenesis. As mentioned earlier, FGFs manifest their biological activity by binding to its cell surface receptor. D2 domain in FGFR is known to be intricately involved in ligand (FGF) interaction. Therefore, development of inhibitors that block FGF-D2 domain interaction is believed to be an effective mechanism to inhibit FGF-mediated pathogenesis.

Structure–activity relationship monitored by NMR (SAR by NMR) is a useful technique for screening and rational design of drugs against a variety of protein-mediated diseases [19,20]. SAR by NMR technique mostly relies on the availability of a completely assigned ^{1}H–^{15}N HSQC spectrum of the target protein. The ^{1}H–^{15}N HSQC spectrum is a finger-print of the protein conformation and each crosspeak in the spectrum represents an amino acid acid residue in the protein [5,19]. Therefore based on the ^{1}H–^{15}N chemical shift perturbation (that occurs upon addition of a ligand/drug)

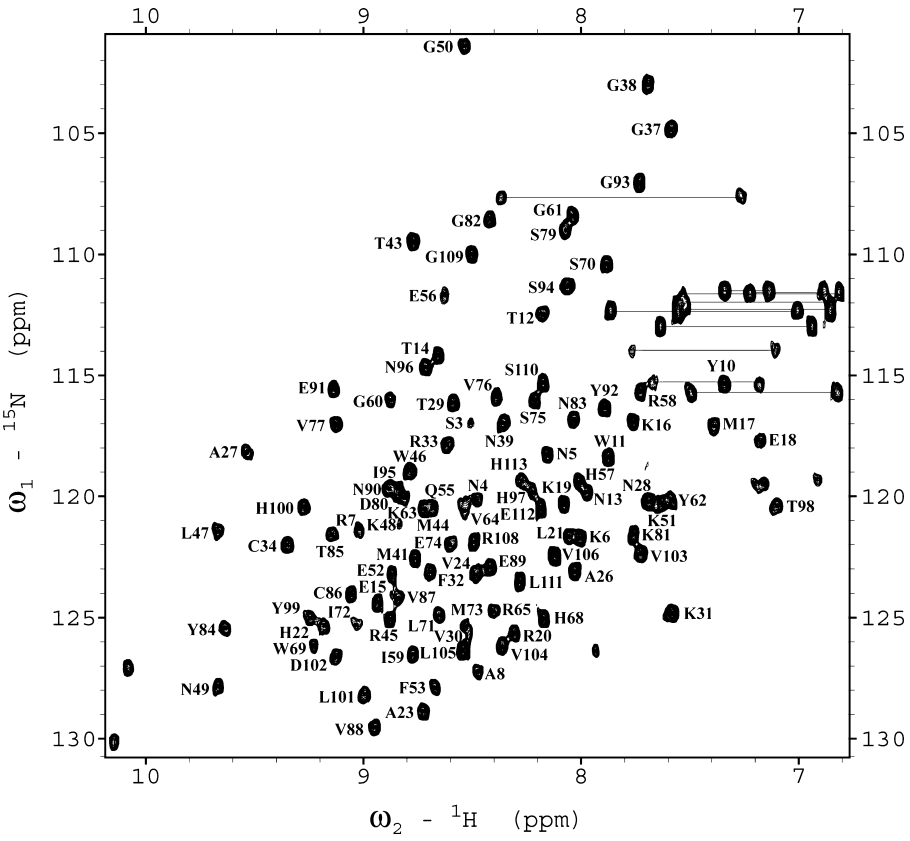

Fig. 6. ^{1}H–^{15}N HSQC spectrum of the D2 domain of the fibroblast growth factor receptor obtained at pH 6.5 and 298 K. Side chains of NH_2 resonances of asparagines and glutamines are connected by horizontal lines.

observed in ^{1}H–^{15}N HSQC spectrum, the binding site(s) of the ligand/drug on the target protein can be conveniently mapped [19,20].

All the resonances (^{1}H, ^{13}C, and ^{15}N) in the protein have been unambiguously assigned using isotope enriched (^{15}N and ^{13}C) recombinant D2 domain. Fig. 6 shows the completely assigned ^{1}H–^{15}N HSQC spectrum of the D2 domain. The spectrum is well dispersed implying the presence of structured elements in the protein (Fig. 6). Chemical shift index (CSI) plot (of the D2 domain), which identifies secondary structural elements based on the ^{1}H-α, ^{13}C-α ^{13}C-β, and ^{13}carbonyl group chemical shifts reveals that D2 domain of FGFR is an all β-sheet protein consisting of 9 β-strands (Fig. 7). The 9 β-strands are uniformly spread in the sequence of the protein. The results of the CSI plot corroborate the conclusions drawn from the secondary structure predictions (using the consensus algorithm) and the Far-UV CD data (Fig. 3).

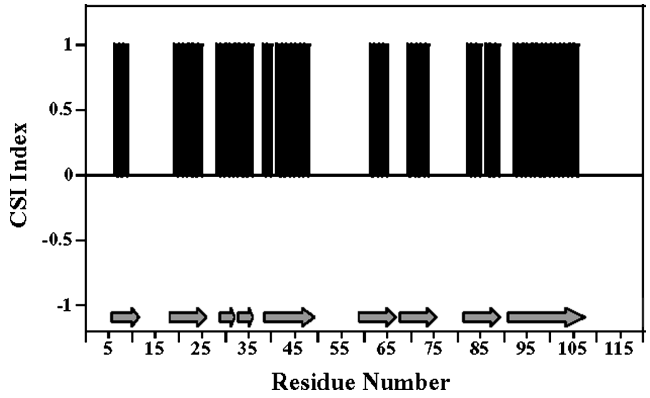

Fig. 7. Consensus chemical shift index plot of the D2 domain. The secondary structural elements in the protein include nine β-strands (indicated by arrows at the bottom of the figure).

This is the first report of the cloning, expression, and characterization of the isolated D2 domain of FGFR. The findings of the present study are expected to trigger more research towards understanding the molecular mechanism(s) underlying the regulation of the FGF-mediated biological activities. In addition, the availability of complete resonance assignment data of the D2 domain would generate new initiatives on the rational design of potent therapeutic principles against FGF-mediated pathogenesis.

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