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Antoine Baudin, Anne Guichard, Gavin W Collie, Sabrina Rousseau, Stéphane Chaignepain, et al.. 1 H, 13 C, 15 N NMR Resonance Assignments and Secondary Structure Determination of the Extra-Cellular Domain from the Human Proapoptotic TRAIL-R2 Death Receptor 5 (DR5-ECD). *Biomolecular NMR Assignments*, 2018, 12 (2), pp.309-314. 10.1007/s12104-018-9828-1 . hal-02349452

**HAL Id: hal-02349452**

**<https://hal.science/hal-02349452>**

Submitted on 5 Nov 2019

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# **<sup>1</sup>H, <sup>13</sup>C, <sup>15</sup>N NMR Resonance Assignments and Secondary Structure Determination of the Extra-Cellular Domain from the Human Proapoptotic TRAIL-R2 Death Receptor 5 (DR5-ECD)**

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## **Abstract**

Death Receptors (DR) selectively drive cancer cells to apoptosis upon binding to the Tumor necrosis factor- $\alpha$ -Related Apoptosis-Inducing Ligand (TRAIL). Complex formation induces the oligomerization of the death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) and transduces the apoptogenic signal to their respective Death Domains, leading to Death Inducing Signaling Complex (DISC) formation, caspase activation and ultimately cell death. Several crystal structures of the ExtraCellular Domain from Death Receptor 5 (DR5-ECD) have been reported in complex with the TRAIL ligand or anti-DR5 antibodies, but none for the isolated protein. In order to fill this gap and to perform binding experiments with TRAIL peptidomimetics, we have produced isotopically labelled DR5-ECD and started a conformational analysis by using high-field 3D NMR spectroscopy. Herein, we present the first resonance assignment of a TRAIL receptor in solution and the determination of its secondary structure from NMR chemical shifts.

**Abbreviations:** DR5 Death Receptor 5; TRAIL Tumor necrosis factor- $\alpha$ -Related Apoptosis-Inducing Ligand; ECD Extra Cellular Domain; HSQC Heteronuclear Single Quantum Coherence transfer.

**Keywords:** DR5; TRAIL; Cancer; Apoptosis; NMR spectroscopy; Resonance Assignment; Secondary Structure.

## **Biological context**

Tumor necrosis factor- $\alpha$ -Related Apoptosis-Inducing Ligand (TRAIL/Apo2L/TNFSF10) is a type II transmembrane trimeric protein that induces apoptosis of cancer cells while sparing normal ones (Ashkenazi A and Dixit VM, 2008). Four membrane-bound TRAIL specific receptors have been described, comprising two Death Receptors (DR4/TNFRSF10A/TRAIL-R1 and DR5/TNFRSF10B/TRAIL-R2) that mediate the apoptogenic signal and two Decoy Receptors (DcR1/TNFRSF10C/TRAIL-R3 and DcR2/TNFRSF10D/TRAIL-R4) that bind the ligand but do not transduce the apoptogenic signal (MacFarlane M et al, 1997; Walczak H et al, 1997). TRAIL binding induces receptor oligomerization (Ashkenazi A et Dixit VM, 1998), transduction of the apoptogenic signal to the intracellular death domain (DD) of the receptors and DISC formation. Autocleavage of the initiator caspases results in activation of the death-executing caspase cascade, leading to cell death (Ashkenazi A and Dixit VM, 2008; Newsom-Davis T et al, 2009; Takeda K et al, Oncogene 2007; Merino D et al, 2007) Crystal structures of TRAIL

complexed with the Extra Cellular Domain (residues 6-135 of our plasmid construct) of DR5 (DR5-ECD) unravel the mechanism for receptor oligomerization (Hymowitz SG et al, 1999: 1DOG.pdb; Mongkolsapaya J et al, 1999: 1D4V.pdb; Cha SS et al, 2000: 1DU3.pdb): The elongated DR5 receptors snuggle into long crevices between pairs of monomers of the homotrimeric TRAIL ligand, but do not interact with each other. The TRAIL/DR5 interface is divided into two distinct patches, one near the bottom of the complex close to the membrane surface (90S loop of DR5) and one near the top (50S loop of DR5). The elongated structure of DR5-ECD is arranged into three Cysteine-Rich Domains (CRD). CRD1 (32-48) adopts a beta hairpin conformation (B1B2) stabilized by the disulphide bridge 33-46 and is preceded by the N-terminal region in a disordered conformation. CRD2 (49-90) and CRD3 (91-135) possess two consecutive beta hairpins (B3-B6 and B7-B10 respectively) stabilized by three disulphide bridges (49-65, 68-81, 71-89 and 91-105, 108-122, 112-130 respectively). The 50S loop (residues S56 to R67) and 90S loop (residues E99 to R109) involved in TRAIL binding display similar conformations despite the absence of any sequence homology. The crystal structure of DR5-ECD has also been solved in complex with the human glycoprotein UL141 (Nemčovičova I et al, 2013: 4I9X.pdb) and with the Fab fragments of five anti-DR5 antibodies (Fellouse FA et al, 2005: 1ZA3.pdb; Li B et al, 2006: 2H9G.pdb; Adams C et al, 2008: 4OD2.pdb; Graves JD et al, 2014: 4N90.pdb; Tamada J et al, 2015: 3X3F.pdb). No structural characterization of the isolated DR5-ECD has yet been reported either by crystallography or NMR spectroscopy.

We report here for the first time the assignment of NMR resonances and the secondary structure determination of a TRAIL Death Receptor in solution, namely DR5/TRAIL-R2. Chemical shift assignment has been used to perform binding studies with TRAILmim peptidomimetics that bind to DR5 (Pavet V et al, 2010; Pulka-Ziach K et al, 2015; Beyrath J et al, 2016) and the results will be reported in a future publication.

## **Materials and Experiments:**

### **Sample preparation**

The DR5 extracellular domain (DR5-ECD) was cloned from a commercial pET23m plasmid containing the DR5 gene (Hye-Ji CHI, 2010) by using PCR amplification. The primers were designed to introduce NcoI and BamHI restriction sites, respectively, in order to insert the gene into a modified pET23-d plasmid containing an N-terminal Histidine<sub>(6)</sub>-tag followed by *E. coli* NusA protein and a tobacco etch virus (TEV) cleavage site. Correct cloning was confirmed by DNA sequencing. The production of the protein was carried out

by heat-shock transformation of the plasmid in BL21(DE3) *E. coli* bacteria (New England Biolabs), which were then cultured in 1 L of  $^{15}\text{N}/^{13}\text{C}$ ,  $^{15}\text{N}$  enriched minimal M9 medium, containing 1 g/L of  $^{13}\text{C}^{15}\text{N}$  Isogro® (Sigma Aldrich). Following bacteria growth, protein expression was induced at a bacterial  $\text{OD}_{600\text{nm}}$  of 0.8 with isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM, for 16 hours at 25 °C. The cells were then harvested by centrifugation and stored at -80°C. All purification steps were carried out with Fast Protein Liquid Chromatography (FPLC) on an AKTA Pure system (GE Healthcare). Cell pellets were thawed and resuspended in lysis buffer (50 mM Tris pH 7.5, 500 mM NaCl, 5 mM imidazole), in the presence of phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor, followed by cell lysis performed by sonication. Membranes and cell debris were centrifuged, and the supernatant was loaded on a 5 mL HisTrap HP column (GE Healthcare), previously equilibrated with the lysis buffer. The column was then washed with 10 column volumes of washing buffer (50 mM Tris pH 7.5, 500 mM NaCl, imidazole 25mM), and protein was eluted with a 50 mM Tris pH 7.5, 500 mM NaCl, imidazole 300mM buffer. Purity of the sample was enhanced by a step of size exclusion chromatography on a HiLoad 16/600 Superdex 200 prep grade column (GE Healthcare), previously equilibrated with appropriate buffer (20 mM Tris pH 7.8, 50 mM NaCl). TEV protease cleavage was then carried out overnight at room temperature, at a TEV:NusA-DR5 mass ratio of 1:100, after which the protein sample was loaded on a 5 mL HiTrap column (GE Healthcare), pre-equilibrated with the same buffer, for anion exchange chromatography. After elution at a NaCl concentration of 250 mM, the final DR5 protein consisted of 135 residues, including 3 N-ter residues ( $\text{Gly}_1\text{-Ala}_2\text{-Met}_3$ ) remaining from the TEV cleavage site. Sample purity was checked by SDS-PAGE (15 % gels). All NMR samples were buffer exchanged with 20 mM  $\text{Na}_2\text{HPO}_4$  pH 6.3 50 mM NaCl and either 10 % or 100 %  $\text{D}_2\text{O}$ , using PD-10 columns (GE Healthcare).  $^{13}\text{C}^{15}\text{N}$ -DR5 sample concentration was made to 300  $\mu\text{M}$  for assignment, using Amicon Ultra centrifugal 10 kD molecular weight cutoff filter units (Merck).

### Mass spectroscopy

The average mass of intact DR5 was measured on a MALDI-TOFTOF mass spectrometer (Ultraflex III, Bruker) used in linear mode with sinapinic acid (Sigma-Aldrich) as a matrix [10 mg/mL solution in 50% acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA)]. The dried droplet method was used for sample loading on a stainless steel target. The instrument was calibrated using an external calibration with a mixture of four standard proteins: Insulin ( $[\text{M}+\text{H}]^+_{\text{average}} = 5734.51$ ), Ubiquitin ( $[\text{M}+\text{H}]^+_{\text{average}} = 8565.76$ ), Cytochrom C

( $[M+H]^+$ <sub>average</sub>= 12360.97), Myoglobin ( $[M+H]^+$ <sub>average</sub>= 16952.30) (Bruker). Mass spectrometry (MS) study was performed to measure the average mass of the produced DR5 in order to assess its integrity. This analysis demonstrated the presence of one major form corresponding to an average mass  $M=14998$  Da (mean of four individual analysis). The mass difference between the theoretical mass of the recombinant protein calculated from the sequence (15020.34 Da, taking into account all disulfide bonds in oxydized form) and the experimental mass is in accordance with the mass tolerance accepted for this measurement.

### **NMR spectroscopy**

Spectra were acquired at 303 K on a Bruker Avance III 700 MHz and 800 MHz spectrometers, equipped with a TXI triple resonance probe and cryoprobe, respectively. All data were processed using NMRPipe (Delaglio et al, 1995) and analysed by using CCPNMR analysis 2.4 software (Skinner et al, 2016). Chemical shifts were referenced to TriMethylSilylPropanoic acid (TMSP) inside the samples (Wishart et al., 1995). The  $^1H$ ,  $^1H\alpha$ ,  $^{13}C\alpha$ ,  $^1H\beta$ ,  $^{13}C\beta$  and  $^{15}N$  backbone resonances of DR5-ECD were derived by sequence specific triple resonance 3D NMR spectra, namely HNCO, HN(CA)CO, HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH, HNHA and HBHA(CO)NH. Side-chain resonance assignment was partially achieved by 3D NMR spectra, namely HC(C)H-TOCSY, (H)CCH-TOCSY, H(C)CH-COSY,  $^{15}N$ -HSQC-NOESY (120 ms mixing time) and  $^{15}N$ -HSQC-TOCSY (60 ms mixing time), as well as by 2D NMR spectra in 100%  $D_2O$ , namely aliphatic and aromatic  $^1H^{13}C$ -HSQC, CBHD, CBHE,  $^1H^1H$ -TOCSY and  $^1H^1H$ -NOESY (Sattler et al, 1999).

### **Extents of assignments and data deposition**

Analysis of the 3D sequential NMR experiments allowed us to assign 111 out of the 125 expected  $^{15}N$  and  $^1H$  backbone resonances (135 residues less 9 prolines and the N-terminal glycine). The backbone assignment is nearly complete (89%), except for 14 residues (A2, Q11, S40, D54, D61, C68, V76, T87, V88, C91, F96, R97, R106, K107). Ten peaks of the  $^1H^{15}N$  HSQC spectrum however remain unassigned (Figure 1). The presence of additional peaks corresponding to a minor conformation was observed for residues located in the N-terminal flexible region (S5, L7, I8, T9, D12, A14, A20, K24, S26, S27, S29) and for the tryptophan residue W125. Such minor conformations probably result from the surrounding proline residues in a cis conformation (P15, P21, P28 and P124). Slow conformational exchange has also been observed in the loop of the first beta hairpin

(residues 41-44) and may arise from conformational heterogeneity of the disulphide bridge 33-46 or protein oligomerization. We were able to extend the assignment up to 94, 93 and 87% for the CO, C $\alpha$  and C $\beta$  chemical shifts, respectively. H $\alpha$ , C $\alpha$ , C $\beta$  and CO chemical shift values allowed us to assess the secondary structure of the protein in solution by using random coil values (Wang Y and Jardetsky O, 2002) and TALOS-N software (Yang S and Bax A, 2013). We found 8 of the 10 expected strands from crystal structures of DR5 complexes and one additional strand at the N-terminus (Figure 2), namely  $\beta_0$ [residues 32:33],  $\beta_1$ [37:39],  $\beta_2$ [46:48],  $\beta_3$ [54:55],  $\beta_4$ [69:70],  $\beta_5$ [75:79],  $\beta_6$ [86:91],  $\beta_9$ [116:120] and  $\beta_{10}$ [129:132]. An excellent agreement (Figure 3) has been found for the protein in solution with the X-ray structures of DR5-ECD bound to TRAIL (1DOG.pdb, 1DU3.pdb and 1D4V.pdb), to FAB fragments (1ZA3, 2H9G, 3X3F, 4OD2 and 4N90) and to the glycoprotein UL141 (4I9X.pdb). Most of the resonances for the residues of the third expected  $\beta$ -hairpin  $\beta_7$ [94:96]- $\beta_8$ [104:106] were not found in the 3D NMR spectra. All the unassigned residues seem to be clustered in the vicinity of the beta sheets ( $\beta_1$  : residue S40 ;  $\beta_3$  : D54 ;  $\beta_4$  : C68 ;  $\beta_5$  : V76,  $\beta_6$  : T87 and V88,  $\beta_7$  : F96 and R97 ;  $\beta_8$  : R106 and K107). These residues have not yet been assigned because peaks are missing or are too weak in intensity, probably arising from an excessive linebroadening beyond detection due to the presence of a conformational exchange on the intermediate millisecond timescale. The backbone and side-chain  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{15}\text{N}$  chemical shift values of DR5-ECD have been deposited at BioMagResBank under the accession number 27400.

## Acknowledgments

We thank the structural biology platform at the Institut Européen de Chimie et Biologie (UMS 3033) for access to NMR spectrometers and technical assistance. Antoine Baudin was supported by a French PhD fellowship afforded to the University of Bordeaux by the Ministère de la Recherche (MNERT) and a fourth year PhD extension by the Ligue nationale Contre le Cancer (LCC). The project was funded by the Ligue Contre le Cancer de la Gironde. We thank Dr Yong-Sung Kim from the Ajou University of the Republic of Korea for providing us the plasmid containing the gene coding for DR5-ECD. AL and MB acknowledge the H2020 program (ERC-2015-StG GA no. 639020).

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## Figures with titles and legends

### **Figure 1:**

#### **Annotated $^1\text{H}$ - $^{15}\text{N}$ HSQC spectrum of DR5-ECD**

The spectrum was acquired at 303K on a Bruker Avance III 800 MHz spectrometer equipped with a 5 mm TXI  $^1\text{H}/^{13}\text{C}/^{15}\text{N}/^2\text{H}$  cryoprobe. The  $^{15}\text{N}/^{13}\text{C}$  labelled protein was at a concentration of 300  $\mu\text{M}$  in 20 mM  $\text{Na}_2\text{HPO}_4$  pH 6.3 50 mM NaCl in a mixture of 90%  $\text{H}_2\text{O}$ /10%  $\text{D}_2\text{O}$ . The assignment of the peaks is indicated with the amino acid letter followed by the residue number. For clarity, a more detailed view of the central region is displayed separately in the top left corner of the spectrum. Unassigned peaks are labelled by the letters na. Assigned peaks corresponding to the major (65%) and minor (35%) conformations for the same residue are respectively colored in black and cyan.

### **Figure 2:**

#### **Secondary structure of DR5-ECD in solution**

Assignment of secondary structure results from the TALOS prediction based on the backbone resonance values. Chemical Shift Indexes (CSI) are calculated with chemical shift values of  $^{13}\text{C}\alpha$ ,  $^{13}\text{C}\beta$ ,  $^{13}\text{CO}$  and  $^1\text{H}\alpha$  resonances. The ten strands of the consecutive five beta hairpins are sequentially annotated by the letter beta followed by the corresponding number.

### **Figure 3:**

#### **Comparison of DR5-ECD secondary structures in crystals and in solution**

Alignment of the DR5-ECD protein sequences used in crystallography and NMR spectroscopy. Crystal structures of the DR5 bound to TRAIL or Fab fragments of anti-DR5 antibodies are indicated on the left of the figure with the pdb code and the subunit. Visible and missing residues are respectively in black and in grey. Residues in the beta conformation are in blue. Disulphide bridges are indicated. The three Cysteine Rich Domains (CRD) are represented schematically by rectangles in different colors.

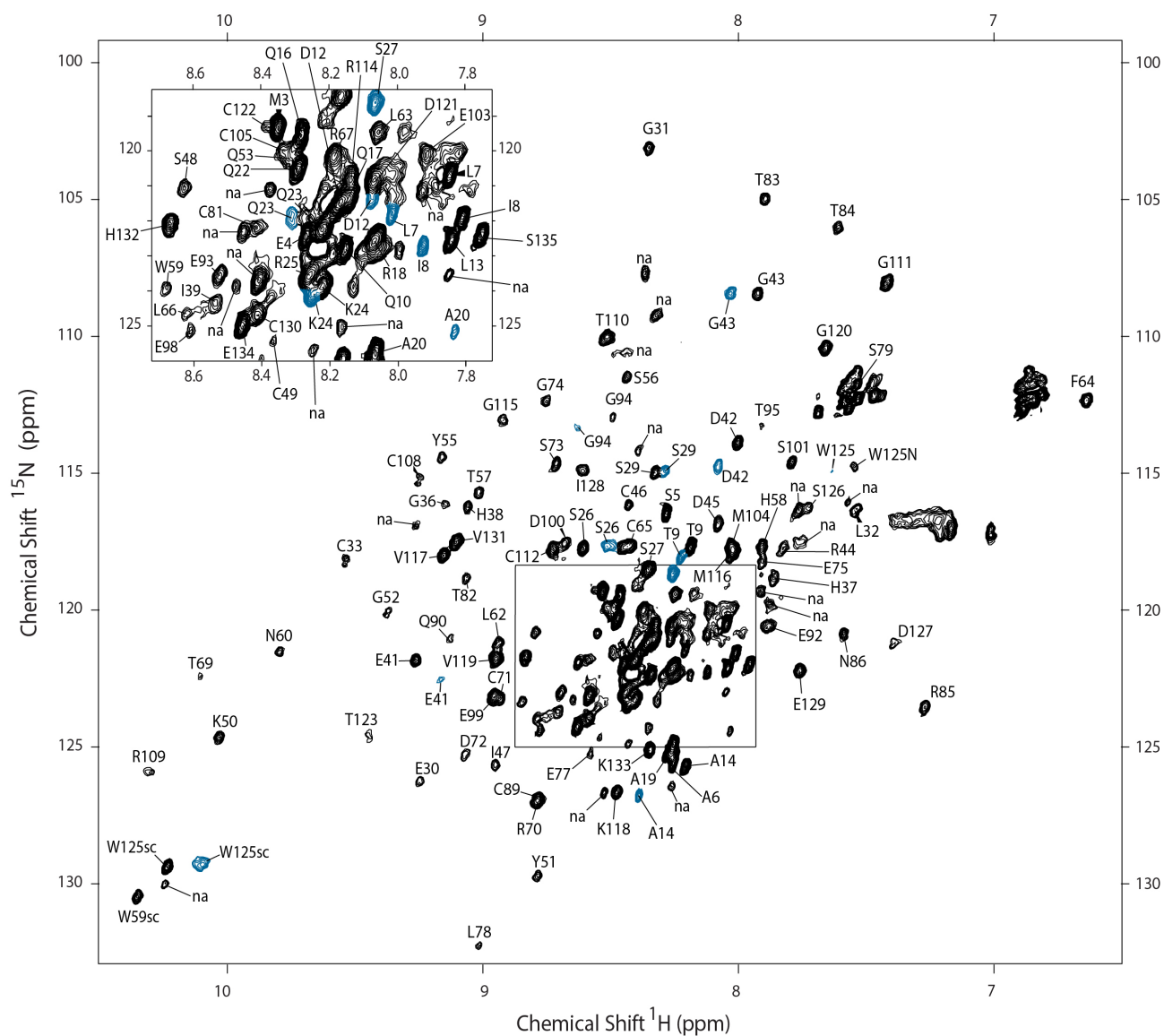


Figure 1

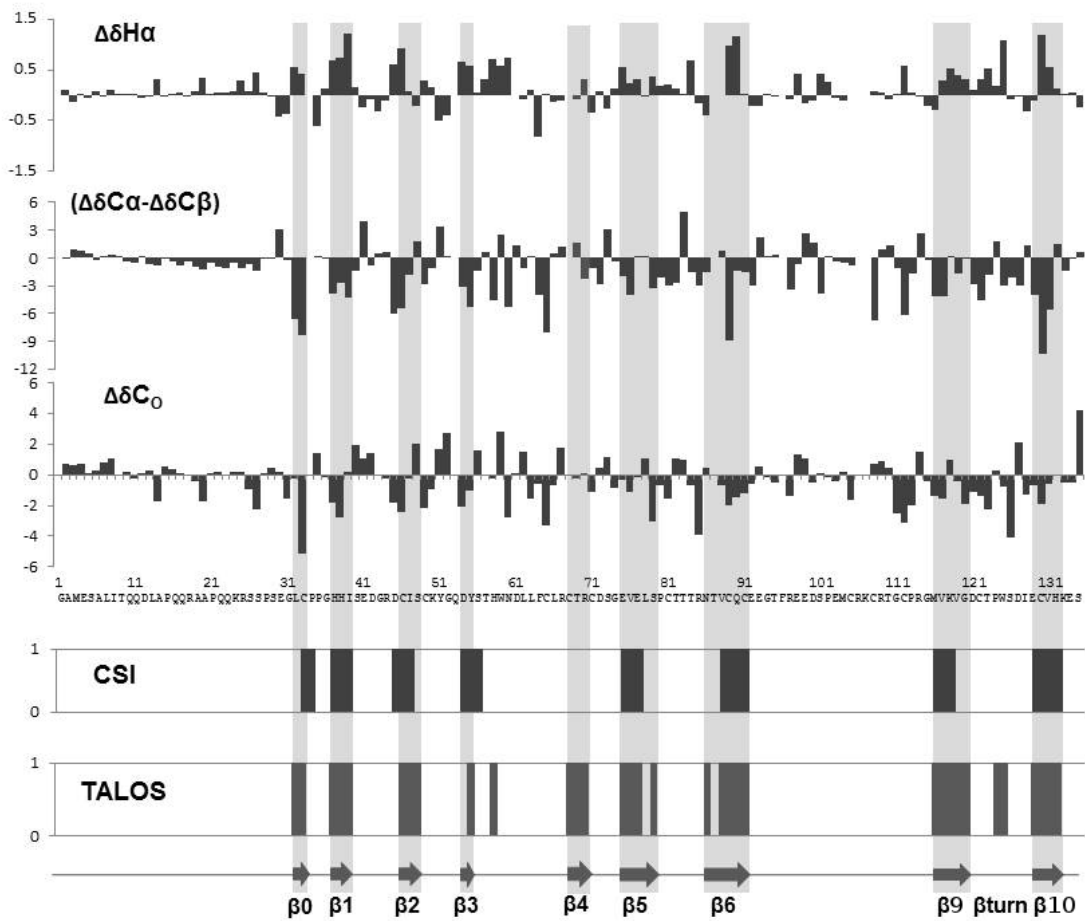


Figure 2

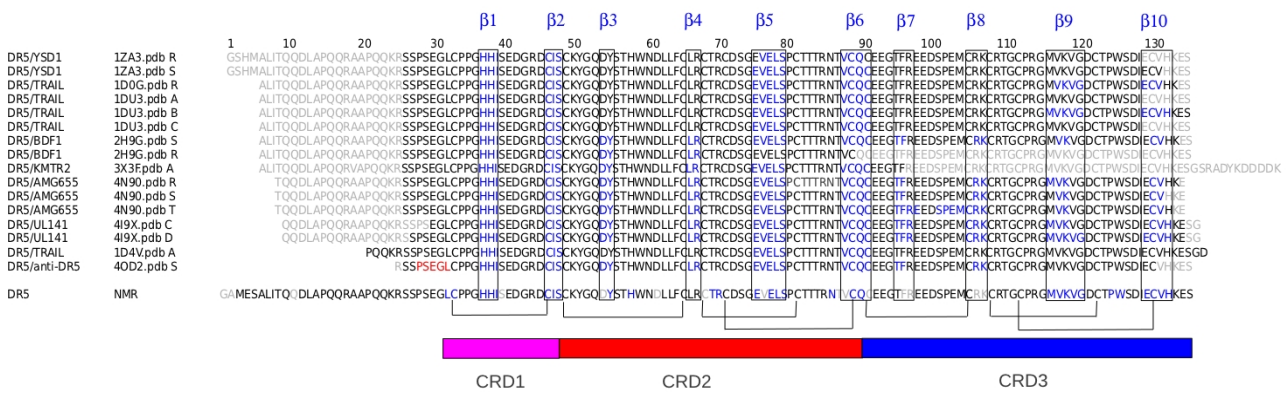


Figure 3