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ARTICLE

Solid-state NMR sequential assignments of the C-terminal oligomerization domain of human C4b-binding protein

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Abstract The complement 4 binding protein (C4bp) plays a crucial role in the inhibition of the complement cascade. It has an extraordinary seven-arm octopus-like structure with 7 tentacle-like identical chains, held together at their C-terminal end. The C-terminal domain does oligomerize in isolation, and is necessary and sufficient to oligomerize full-length C4bp. It is predicted to form a seven-helix coiled coil, and its multimerization properties make it a promising vaccine adjuvant, probably by enhancing the structural stability and binding affinity of the presented antigen. Here, we present the solid-state NMR resonance assignment of the human C4bp C-terminal oligomerization Domain, hC4pbOD, and the corresponding secondary chemical shifts.

Keywords C4-binding protein · Oligomerization domain · Solid-state NMR · Assignments · Secondary structure

Biological context

C4bp is part of the complement system, which is a key component of the innate immune system (Blom et al. 2004). Three major pathways can activate the complement cascade: the so-called classical, alternative, and lectin pathways. Activation of immune reactions has to be tightly controlled, as they can also be destructive to the organism. C4bp is a plasma-circulating complement inhibitor of the classical and lectin pathways of the complement cascade, and it suppresses the activity of the C3-convertases. Those are crucial enzymatic complexes in all three pathways of complement, whose functions are to activate the major complement protein C3, resulting in the assembly of the membrane attack complex. C4bp is a high-molecular-weight glycosylated protein (MW around 570 kDa) composed of identical subunits (MW = 70 kDa) linked by disulfide bonds (Dahlback et al. 1983). It exists in several isoforms having 6–8 α- and a single β-chains, the most common with seven identical α-chains (Hillarp and Dahlback 1990). The overall structure of C4bp in solution was suggested to be a bundle of seven extended arms (the α-chains) held together at their C-termini (Perkins et al. 1986). The β-chain is not required for oligomerization. The C-terminal oligomerization domain (hC4bpOD) consists of 57 amino acids, the corresponding domain in mice, 54 amino-acid residues. It has been shown that this domain is necessary (Kask et al. 2002) and sufficient for the

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oligomerization of C4bp. It contains 2 cysteines, which stabilize the interaction between the chains by intermolecular disulfide bonds; however, these SS-bonds are not required for the oligomerization of the protein (Kask et al. 2002). The C-terminal domain of C4bp was also shown to be able to oligomerize even in the presence of other proteins fused to it, with the potential to increase the half-lives or activity of the fusion protein (Libyh et al. 1997; Shinya et al. 1999; Oudin et al. 2000; Dervillez et al. 2006). Furthermore it has been observed that the murine and avian C-terminal domains of C4bp can act as an adjuvant for vaccines against malaria and tuberculosis when fused to the antigen (Draper et al. 2008; Ogun et al. 2008; Spencer et al. 2012).

The seven C-terminal helices in the C4bp oligomer are predicted to form a coiled-coil structure. Interestingly, the only other protein that has been shown to form a coiled-coil heptameric arrangement experimentally is a mutant form of the GCN4 leucine zipper, and its structure has been determined recently (Liu et al. 2006).

In the following we present the solid-state NMR resonance assignments and secondary structure analysis of the crystalline form of a heptameric protein corresponding to the oligomerization domain of human C4bp, here designated as hC4bpOD, in its crystalline form. We compare the obtained information with structure predictions for coiled–coiled states of the protein, and show that these predictions do not correspond to the actual helical core observed by NMR.

Methods and experiments

Protein expression and purification; sample preparation

The protein was transformed with a plasmid consisting of a pRSET backbone under the control of the T7 promoter as described previously (Ogun et al. 2008). A tag (MASMNHKGS) was added at the N-terminal of the protein. Expression was carried out in C41(DE3) cells in M9-complete medium /Amp after the induction with 0.5 M IPTG at 37 °C for 4 h. Cells were harvested at 6,000 g and lysed by sonication in 50 mM Tris–HCl, pH 8.0, 5 mM MgCl2, 10 % glycerol, 1 mM PMSF, 5 mg lysozyme, 1000U Benzonase-Nuclease® (Sigma Aldrich). After centrifugation at 6,000 g for 15 min, the lysate supernatant was fractionated by ion-exchange chromatography on DEAE (DEAE F.F 16/10, GE Healthcare) eluting with a 0 to 50 % gradient of 1 M NaCl (in 10 mM Tris, pH 7.0). The fractions containing hC4bpOD were pooled and contaminating bacterial proteins were precipitated by 50 mM NaAc pH 4.5 overnight. The supernatant was further purified by cation exchange chromatography (HiTrap S.P.F.F., GE Healthcare) by eluting with a gradient from 0 to 70 % 1 M NaCl (20 mM NaAc pH 4.5). Incubation overnight in 50 mM Tris–HCl at pH 8.6 allowed disulfide-bond formation. The protein was loaded on a Superdex S75 26/60 column (GE Healthcare) and separated from residual contaminating proteins (50 mM Tris, pH 7.2, 2.4 ml/min, elution at 54 min). hC4bpOD crystallization occurs after several days in sitting drops prepared by adding equal amounts (150 µl) of crystallization buffer (30 % 2-methyl-2,4 pentanediol (MPD) /20 % EtOH/ 50 % H_2O) and protein solution (15 mg/ml in 50 mM Tris, pH 7.2) at 4 °C. Crystals are temperature sensitive and dissolve when transferred to room temperature, and were thus directly centrifuged at 4 °C into the NMR rotor (Böckmann et al. 2009). The rotor was stored at −20° C and quickly transferred into the precooled NMR probe.

NMR spectroscopy

We used a suite of 2D and 3D experiments, namely 2D DARR and DREAM, and 3D NCACB, NCACX, NCOCA, CANCO and CCC experiments to perform the assignment as described in detail in references (Habenstein et al. 2011; Schuetz et al. 2010). Each spectrum took 2–4 days to be recorded. Full experimental details are given in Table S1. The sequential walk was achieved by connecting resonances from NCACB/NCACX, CANCO and NCOCA spectra. Side-chain assignments were done using NCACB, NCACX and CCC spectra. Spectra were recorded on either of two different preparations which, as judged from 2D spectra recorded under identical conditions, yielded identical spectra.

All spectra were recorded on a Bruker Avance II 600 MHz spectrometer operating at a static field of 14.1 T. A 3.2 mm Bruker triple-resonance MAS probe was used. The spectra were recorded at a sample temperature of -12 ± 2 °C. The pulse sequences were implemented as recently reported (Schuetz et al. 2010). All spectra were processed using TopSpin 2.1 (Bruker Biospin) with zero filling to the next power of 2 of acquired points, but a minimum of once. The time domain signals were apodized with a squared cosine function, shifted by values between 0.2 and 0.3. Spectra were analyzed and annotated using the CcpNmr Analysis package (Fogh et al. 2002; Vranken et al. 2005).

Assignment and data deposition

Mass spectroscopy and SDS-PAGE analyses of purified cC4bpOD under reducing and non-reducing conditions confirmed its heptameric association and formation of intermolecular disulphide bonds (Ogun et al. 2008). The crystals of hC4bpOD give rise to well-resolved 2D DARR

Fig. 1 2D DARR spectrum of hC4bpOD with the sequentially assigned residues labeled. The spectrum was acquired using a Bruker 600 MHz WB spectrometer at 12.5 kHz MAS frequency. Spectral analysis has been performed using the CcpNmr software (Fogh et al. 2002; Vranken et al. 2005)

spectra, which display only little spectral overlap in both the aliphatic and carbonyl regions. The 10 ms 2D DARR spectrum with the resonance assignments of hC4bpOD is shown in Fig. 1. The vast majority of the cross peaks are explained by our assignments. Two additional spin systems could be identified, probably as E or Q. They show weaker resonances in the DARR spectrum, and could not be assigned sequentially as they lack resonances in some of the 3D assignment spectra. They might belong to the stretch between 39E and 47S, which is unassigned and contains two Q and one E residues. Some rather broad peaks could not be assigned as well.

To illustrate sequential assignments, a representative plane of the 3D NCACB, NCACO, CANCO and NCOCA experiments is shown in Fig. 2. The observed line width in the spectra is between 0.5 and 1 ppm, which allows for an assignment using the afore-mentioned 3D methods. The chemical shifts have been deposited in the BMRB under the accession number 18731.

Excluding the N-terminal 6 amino acids added to improve protein expression, 71 % of all amino acids could be assigned sequentially. More specifically, the assignment comprises: 74.5 % of the nitrogen backbone, 72.7 % of the carbonyl, 76.4 % of the Cα and 75.5 % of the Cβ resonances. However, all strong signals in the dipolar-transfer based spectra could be assigned. The assigned residues are located in three amino-acid stretches, comprising V9-K13, M16-E39 and S47-L57. The corresponding signals were present in all NCACO/CX, CANCO and NCOCA/CX spectra used for sequential assignment, and overlap did not impede the sequential walk. P3 could be assigned due to the fact that it was the only residue left of its type, and no polymorphism was observed in the sample. Three regions, spanning residues E1 to Q8, R14-L15 and Q40-D46, are not or only

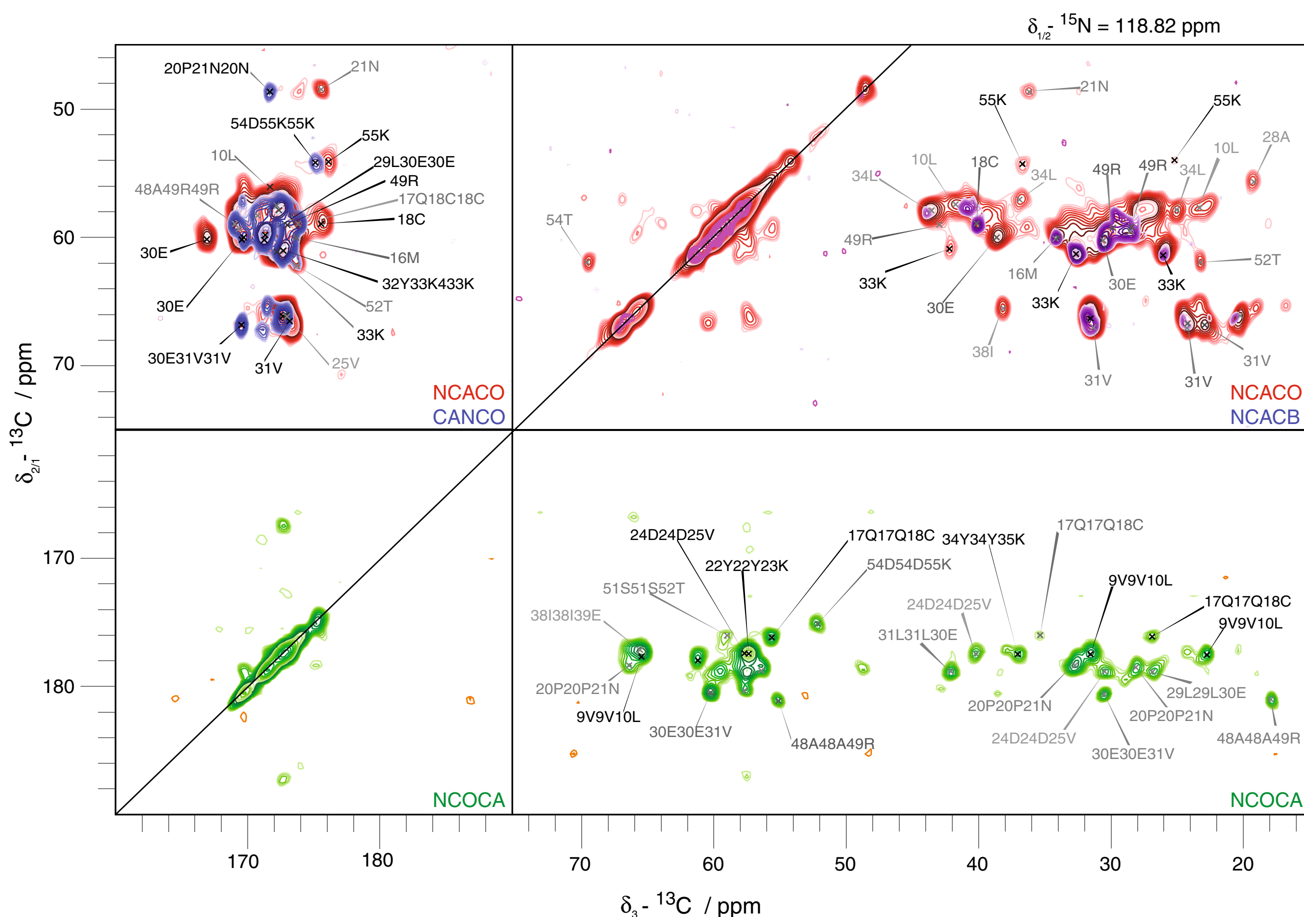

Fig. 2 Representative plane of the 3D NCC assignment spectra with the corresponding assignments. The spectra were acquired with a Bruker 600 MHz WB spectrometer at 12.5 kHz MAS frequency. Spectral analysis has been performed using the CcpNmr software (Fogh et al. 2002; Vranken et al. 2005). NCACB spectra are represented in *purple* (negative signals) and *pink* (positive signals), NCACO spectra in *red*, CANCO spectra in *blue* and NCOCA spectra in *green*. *Grey* assignments have a slightly different ^{15}N chemical shift as the represented plane

weakly visible in our spectra, indicating that they show dynamic behavior. Indeed, resonances belonging to Ala, Asp, Glu, Leu, Lys, Met, Ser and Thr residues can be observed in the INEPT spectrum shown in Fig. 3. Additional signals from the crystallization buffer are observed. According to the amino-acid distribution in the primary sequence, and taking into account the sequentially assigned residues of the rigid part of the molecule, the resonances likely arise from the N-terminal tag, as well as from unassigned stretches E1–Q8 and Q40–D46. Notably, the tag alone could not explain the observed signals, supporting that the unassigned stretches are, at least partly, dynamic.

Figure 4 displays the secondary chemical shifts of the assigned residues (Luca et al. 2001). More than three positive values in a row indicate α-helical conformation, three or more negative ones a β-strand (Wishart and Sykes 1994). Most of the assigned residues are found in α-helical conformation (compare to blue residues below the plot). In the first stretch, V9 to K13 show a α-helical structure element, followed by what might be a flexible turn, as indicated by the absence of assignments for residues R14 and L15. Amino acids E23–E39 clearly show a continuous α-helical conformation. The first part of the last stretch comprising S47–L55 might form a short α-helix, followed by a turn and some residues in β-strand conformation. Green letters in the second amino-acids sequence underneath the plot highlight a possible consensus coiled-coil region in hC4bpOD predicted by different programs including PCOILS (Lupas et al. 1991), MARCOIL (Delorenzi and Speed 2002) and Multicoil (Wolf et al. 1997); see Figure S1 for details. The programs predict the stretch from roughly A28 to L53 to be α-helical (marked with green letters in Fig. 4), in disagreement with our experimental data that find a long helix from E23-E39.

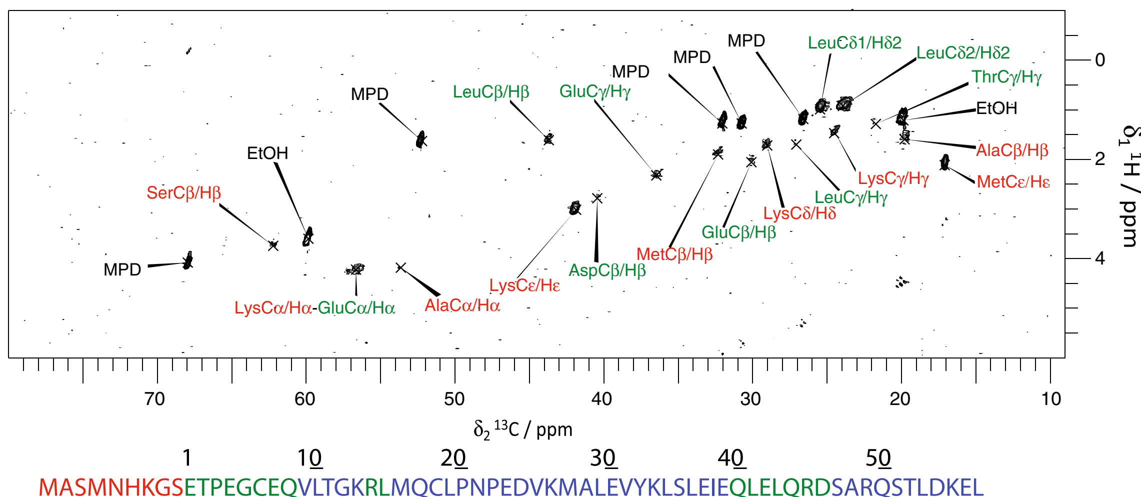

Fig. 3 INEPT spectrum. Solvent signals are labeled accordingly EtOH or MPD. Signals which likely stem from the N-terminal tag are labeled in *red*, those attributed to unassigned residues are labeled in *green*. The sequence is shown with the corresponding color codes, and assigned residues in *blue*

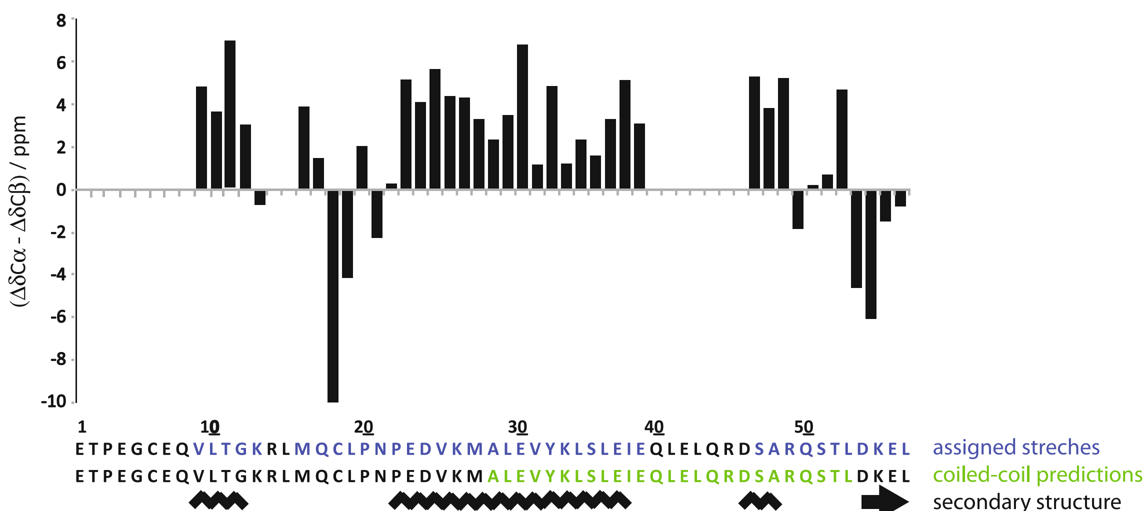

Fig. 4 Secondary chemical shifts for hC4bpOD. Three or more negative values in a row suggest β-strand conformation, 3 positive values helical conformation. Isolated positive or negative residues suggest turn- or random coil-conformation. Of glycines, only the ΔδCα are plotted. Below the plot, the amino-acid sequence (without tag) of hC4bpOD is shown. Assigned residues are marked in *blue*. The *green* residues in the sequence below belong to a possible consensus coiled-coil region predicted by different programs (summary of the results from PCOILS, MARCOIL, Multicoil; see Figure S1). The secondary structure elements found in this study are indicated in the *lowest row* (zick-zack for α-helical and arrow for β-sheet)

Conclusion

The vast majority of all hC4bpOD resonances visible in dipolar-based spectra could be assigned sequentially by using 3D assignment strategies (Habenstein et al. 2011; Schuetz et al. 2010). Most of the assigned residues show chemical shifts corresponding to α-helical conformations; a long and continuous stretch reaches from E23 to E39. Coiled-coil bioinformatics predictions proposed a possible coiled coil region for a different region, including residues A28 to T52. This stretch includes residues which are not observed in the cross-polarization NMR spectra of hC4bpOD, indicating that they are flexible. This is supported by the observation of the corresponding residue types in INEPT spectra. According to our sequential assignments, the coiled-coil region is probably restricted to the stretch between amino acid residues 23–39. The present sequential assignment is the first step towards the structure determination of hC4bpOD, which is mandatory to the biophysical characterization of this efficient vaccine adjuvant (Draper et al. 2008; Ogun et al. 2008; Spencer et al. 2012).

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