



Solid-state NMR sequential assignments of the N-terminal domain of HpDnaB helicase

Thomas Wiegand, Carole Gardiennet, Francesco Ravotti, Alexandre Bazin, Britta Kunert, Denis Lacabanne, Riccardo Cadalbert, Peter Güntert, Laurent Terradot, Anja Böckmann, et al.

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Abstract	<p>We present solid-state NMR assignments of the N-terminal domain of the DnaB helicase from <i>Helicobacter pylori</i> (153 residues) in its microcrystalline form. We use a sequential resonance assignment strategy based on three-dimensional NMR experiments. The resonance assignments obtained are compared with automated resonance assignments computed with the ssFLYA algorithm. An analysis of the ^{13}C secondary chemical shifts determines the position of the secondary structure elements in this α-helical protein.</p>	
Keywords (separated by '-')	HpDnaB - Assignments - Solid-state NMR - Secondary chemical shifts - ssFLYA	
Footnote Information	T. Wiegand and C. Gardiennet have equally contributed to this work.	

2 Solid-state NMR sequential assignments of the N-terminal domain 3 of *HpDnaB* helicase

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17 mines the position of the secondary structure elements in
18 this α-helical protein.

20 **Keywords** *HpDnaB* · Assignments · Solid-state NMR ·
21 Secondary chemical shifts · ssFLYA

Biological context

DnaB helicases are bacterial ATP-driven enzymes which unwind double-stranded DNA in the presence of ATP during the fork movement in 5′–3′ direction in DNA replication (LeBowitz and McMacken 1986). Structurally, DnaB is a two-domain helicase with an amino-terminal domain and a carboxy-terminal domain separated by a linker region. The full-length protein forms ring-shaped hexameric assemblies which encircle single-stranded DNA (LeBowitz and McMacken 1986). The C-terminal domain supports the ATPase activity and is involved in the ring formation, while the N-terminal domain is forming an α-helical globule which has the function to activate the helicase. A fundamental step in DNA replication is the strand synthesis which is initiated by an interaction between the N-terminal domain of the helicase DnaB and the primase DnaG (Corn and Berger 2006).

This work focuses on the investigation of the N-terminus of DnaB extracted from *Helicobacter pylori* (*H. pylori*), a gram-negative microaerophilic spiral shaped bacterium which is, with a worldwide prevalence of approximately 50 %, responsible for the most common chronic bacterial infections, such as gastric ulcer diseases and gastric adenocarcinoma (Parsonnet et al. 1991; Peterson 1991). The replication system in *H. pylori* exhibits significant differences compared to other microorganisms which were investigated in detail, e.g. *Escherichia coli*. The most relevant differences are the absence of the *recF* gene, the presence of the *dnaA* gene ~600 kb away from the *dnaN*-*gyrB* genes and most importantly the absence of the *dnaC* gene (Soni et al. 2003). In *E. coli*, DnaC is essential for loading DnaB helicase at *oriC*, which is the origin of the chromosomal DNA replication, whereas in the case of *H. pylori* *HpDnaB* itself is able to take over the

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DnaC function (Soni et al. 2005). The crystal structure of the C-terminal domain (Stelter et al. 2012), as well as of the 121 N-terminal residues of *HpDnaB* were determined (Kashav et al. 2009). The latter one was found to consist of a dimer and two further degradation peptide fragments in the asymmetric unit (Kashav et al. 2009). This manuscript presents the solid-state NMR spectroscopic investigation of the N-terminus of *HpDnaB* as a step towards an integrated structural biology approach aiming at a detailed structural description of the full-length protein. In this context, solid-state NMR studies on a sedimented sample of the full-length protein have been described (Gardiennet et al. 2012). Previous biochemical investigations suggest that the N-terminal domain and the linker region play an important role in multimerisation, quaternary state transition and activity of *HpDnaB* (Kashav et al. 2009; Nitharwal et al. 2007).

Methods and experiments

Protein expression and purification, sample preparation

The DNA fragment corresponding to the N-terminal domain of the *H. pylori* helicase DnaB (strain 26695) was amplified by PCR (forward 5'-caccatggatcatttaagcattgcag-3' and reverse 5'-gcaccatagaaggcttaggaattag-3') from genomic DNA and inserted into the plasmid pET151/DTopo (Invitrogen™). The resulting vector was introduced into *E. coli* BL21(DE3) cells (One Shot® BL21 Star™ (DE3) Chemically Competent *E. coli*, Invitrogen™) and protein overexpression was performed in minimal M9 medium (Studier 2005) containing D-[U-¹³C]glucose 2 g L⁻¹ (Cambridge Isotope Laboratories, Inc. CLM-1396-PK) and ¹⁵NH₄Cl 2 g L⁻¹ (Sigma-Aldrich® 299251) as the only nitrogen and carbon sources. After cell lysis by a microfluidization process, ¹³C-¹⁵N-DnaB-Nter was purified by Ni²⁺-agarose affinity chromatography (Qiagen™). The pseudo-affinity tag was subsequently cleaved with the TEV (Tobacco Etch Virus) protease by dialysis. Six additional residues of the tag remain in the sequence (see Fig. 1).

For crystallization, ¹³C-¹⁵N-DnaB-Nter was concentrated to 23.5 mg/ml using a centrifugal concentrator with a 10 kDa cut-off (Vivaspin® 20 VS2001 Sartorius), and the buffer was exchanged during the concentration step with the final buffer (50 mM Tris-HCl pH 6.5, 100 mM NaCl). Crystallization of the protein was performed by mixing an equal volume of protein and crystallization buffer (100 mM HEPES pH 7.0, 0.1 % (m/v) sodium azide, 10 % (v/v) polyethylene glycol 10000) in a nine-well glass plate with 2.3 M NaCl solution in the reservoir. Microcrystals

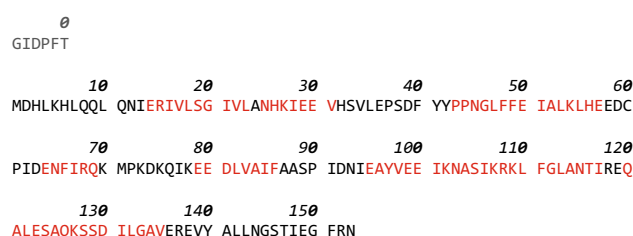


Fig. 1 Amino-acid sequence of the N-terminus of *HpDnaB* as extracted from the uniprot database (The UniProt Consortium-Activities at the Universal Protein Resource (UniProt) 2014). At the N-terminal domain a part of the tag (amino acid sequence GIDPFT) is still present after the cleavage of the tag with TEV protease (shown in grey). Residues highlighted in red are located in α -helices as determined from NMR secondary chemical shifts (see Fig. 9)

were obtained after 1 week at 20 °C and were harvested and centrifuged into the NMR rotor (25,000×g during 1 h at 4 °C) using a homemade device (Böckmann et al. 2009).

Solid-state NMR spectroscopy

Solid-state NMR spectra were acquired at 18.8 and 20.0 T static magnetic field strengths using 3.2 mm Bruker Biospin “E-free” probes (Gor’kov et al. 2007). The MAS spinning frequency was set to 17.5 and 17.0 kHz for the two fields, respectively. The 2D and 3D spectra were processed with the software TOPSPIN (version 3.2, Bruker Biospin) with a shifted (2.0–2.8) squared cosine apodization function and automated baseline correction in the direct dimension. The sample temperature was set to 278 K, for more details of the conducted experiments see Table 1. ¹³C and ¹⁵N resonance assignments were obtained by using a previously established assignment strategy based on a sequential walk applying 3D NMR spectra (Habenstein et al. 2011; Schuetz et al. 2010) which were analyzed with the software CcpNmr (Fogh et al. 2002; Stevens et al. 2011; Vranken et al. 2005). Many resonances were assigned applying conventional experiments, such as NCACB, NCACX, NCOCX and CANCO. In case of spectral overlap, it was essential to complement the assignment strategy with relayed NMR experiments, such as NcoCACB, CANcoCA and NcaCBCX. Although those experiments are less sensitive due to four polarization transfer steps, they strongly benefit from a larger spectral dispersion (Schuetz et al. 2010), and the signal/noise ratio achieved under the conditions used here (high field, full rotor, moderate-sized protein) is good. A selective C’-C α polarization transfer in those experiments was achieved by a modified band-selective homonuclear cross-polarization step (Chevelkov et al. 2013). The spectra used for assignment were all recorded on a single sample, whereas reproducibility was carefully checked by 2D measurements on samples from different preparations which yield

Table 1 Overview about experimental parameters of the performed solid-state NMR experiments

Experiment	DARR	NCA	NCACB	NCACX	NCOCX
<i>(a)</i>					
MAS frequency/kHz	17.0	17.0	17.5	17.0	17.0
Field/T	20.0	20.0	18.8	20.0	20.0
Transfer I	HC-CP	HN-CP	HN-CP	HN-CP	HN-CP
¹ H field/kHz	59.4	60.8	58.5	60.8	60.8
X field/kHz	40.9	43.1	44.0	43.1	43.1
Shape	Tangent ¹ H	Tangent ¹ H	Tangent ¹ H	Tangent ¹ H	Tangent ¹ H
¹³ C carrier/ppm	95	—	—	—	—
Time/ms	0.5	0.6	0.6	0.6	0.6
Transfer II	DARR	NC-CP	NC-CP	NC-CP	NC-CP
¹ H field/kHz	17.0	—	—	—	—
¹³ C field/kHz	—	6.1	10.0	6.1	6.1
¹⁵ N field/kHz	—	10.2	27.0	10.2	10.6
Shape	—	Tangent ¹³ C	Tangent ¹³ C	Tangent ¹³ C	Tangent ¹³ C
Carrier/ppm	95	60	56	60	178
Time/ms	10	6	4	6	4
Transfer 3	—	—	DREAM	DARR	DARR
¹ H field/kHz	—	—	—	25.1	25.1
¹³ C field/kHz	—	—	7.9	—	—
¹⁵ N field/kHz	—	—	—	—	—
Shape	—	—	Tangent ¹³ C	—	—
Carrier/ppm	—	—	56	178	178
Time/ms	—	—	4	60	30
t1 increments	2000	2000	80	108	108
Sweep width (t1)/kHz	100	66.7	5.7	6.0	6.0
Acquisition time (t1)/ms	10	15	7.1	9.0	9.0
t2 increments	2988	3072	116	136	116
Sweep width (t2)/kHz	100	100	10.9	8.6	6.4
Acquisition time (t2)/ms	14.9	15.4	5.3	8.0	9.0
t3 increments	—	—	1988	3072	3072
Sweep width (t3)/kHz	—	—	100	100	100
Acquisition time (t3)/ms	—	—	9.9	15.4	15.4
¹ H Spinal64 (Fung et al. 2000) decoupling power/kHz	89	89	90	89	89
Inter-scan delay/s	2.1	3	2.5	2.6	2.6
Number of scans	12	8	24	8	8
Measurement time/h	14	13	157	87	74
Experiment	CANCO	NcoCACB	CANcoCA	NcaCBCX	CCC
<i>(b)</i>					
MAS frequency/kHz	17.0	17.0	17.0	17.0	17.5
Field/T	20.0	20.0	20.0	20.0	18.8
Transfer I	HC-CP	HN-CP	HC-CP	HN-CP	HC-CP
¹ H field/kHz	59.4	60.8	58.4	58.4	66.0
X field/kHz	40.9	43.1	40.8	45.2	50.0
Shape	Tangent ¹ H	Tangent ¹ H	Tangent ¹ H	Tangent ¹ H	Tangent ¹ H
¹³ C carrier/ppm	95	—	103	—	58.5
Time/ms	0.5	0.6	0.5	0.6	0.7
Transfer II	CN-CP	NC-CP	CN-CP	NC-CP	DREAM

Table 1 continued

Experiment	CANCO	NcoCACB	CANcoCA	NcaCBCX	CCC
¹ H field/kHz	–	–	–	–	–
¹³ C field/kHz	6.0	6.1	6.0	5.9	5.4
¹⁵ N field/kHz	20.0	10.6	11.4	11.4	–
Shape	Tangent ¹³ C	Tangent ¹³ C	Tangent ¹³ C	Tangent ¹³ C	Tangent ¹³ C
¹³ C Carrier/ppm	60	178	60	60	56
Time/ms	4	4	5.5	5.5	4
Transfer 3	NC-CP	Mod. band-selective CP (Chevelkov et al. 2013)	NC-CP	DREAM	DARR
¹ H field/kHz	–	–	–	–	17.5
¹³ C field/kHz	6.0	8.5	6.0	7.6	–
¹⁵ N field/kHz	20.0	–	11.9	–	–
Shape	Tangent ¹³ C	Tangent ¹³ C	Tangent ¹³ C	Tangent ¹³ C	–
¹³ C Carrier/ppm	178	176	178	51	40
Time/ms	4		3.5	2	80
Transfer 4	–	DREAM	Mod. band-selective CP (Chevelkov et al. 2013)	DREAM	–
¹ H field/kHz	–	–	–	–	–
¹³ C field/kHz	–	7.7	8.3	7.6	–
¹⁵ N field/kHz	–	–	–	–	–
Shape	–	Tangent ¹³ C	Tangent ¹³ C	Tangent ¹³ C	–
¹³ C Carrier/ppm	–	51	178	26	–
Time/ms	–	2.75	5	4	–
t1 increments	130	62	150	68	172
Sweep width (t1)/kHz	8.5	3.4	8.6	4.3	15.7
Acquisition time (t1)/ms	7.6	9.0	8.8	7.9	5.5
t2 increments	98	154	80	156	172
Sweep width (t2)/kHz	6.03	8.6	4.3	12.8	15.7
Acquisition time (t2)/ms	8.1	9.0	9.3	6.1	5.5
t3 increments	3072	3072	3072	3072	1864
Sweep width (t3)/kHz	100	100	100	100	93.8
Acquisition time (t3)/ms	15.3	15.4	15.4	15.4	9.9
¹ H Spinal64 (Fung et al. 2000) decoupling power/kHz	89.0	89.0	91.1	91.1	90.0
Inter-scan delay/s	2.7	3	3	3	2.1
Number of scans	8	16	16	16	8
Measurement time/h	77	128	162	143	145

comparable spectra in all cases. Spectra were also recorded on samples with the His-tag, and showed differences large enough to discard the further use of this sample.

TALOS+ calculations were performed using version 3.8 (Shen et al. 2009). The DSSP algorithm (Kabsch and Sander 1983) was applied using the corresponding web interface (<http://www.cmbi.ru.nl/dssp.html>) with the 3D atomic coordinates extracted from the pdb file 3GXV (Kashav et al. 2009).

Solid-state FLYA calculations (Schmidt et al. 2013; Schmidt and Güntert 2012) were performed with CYANA version 3.97. The tolerance value for chemical shift

matching was set to 0.55 ppm for ¹³C and ¹⁵N. The calculations are based on experimental peak lists as obtained from the manual assignment procedure.

Assignment and data deposition

The solid-state NMR spectra of the N-terminal domain of *HpDnaB* (residues 1–153) reveal significant spectral overlap as expected for a protein of 153 amino acids, even though also many isolated, well resolved signals are detected as can be seen in the 2D dipolar correlation NMR

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signal-to-noise ratio which allows also to acquire 3D
assignment NMR spectra even with four polarization-
transfer steps. The assignment was mainly achieved by
using a combination of two strategies. The first one is based

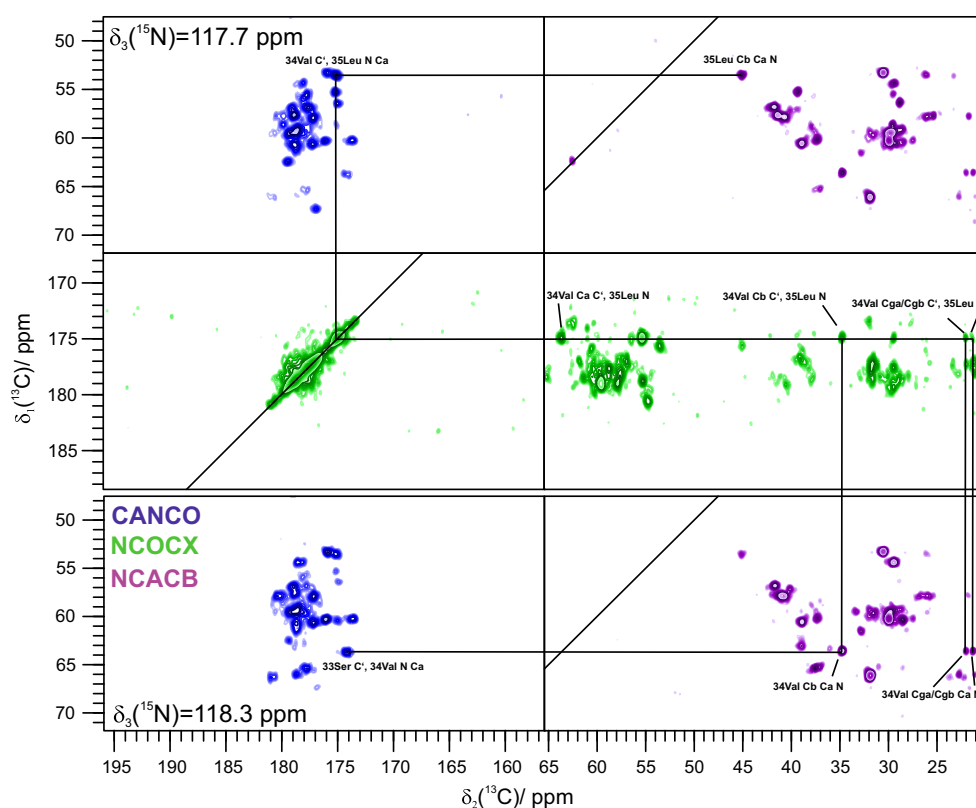


Fig. 3 Example for the sequential walk along the protein backbone (from the C- to the N-terminus) by using NCACB (negative peaks are shown in *magenta*), CANCO (positive peaks are illustrated in *blue*) and NCOCX (positive peaks are shown in *green*) spectra

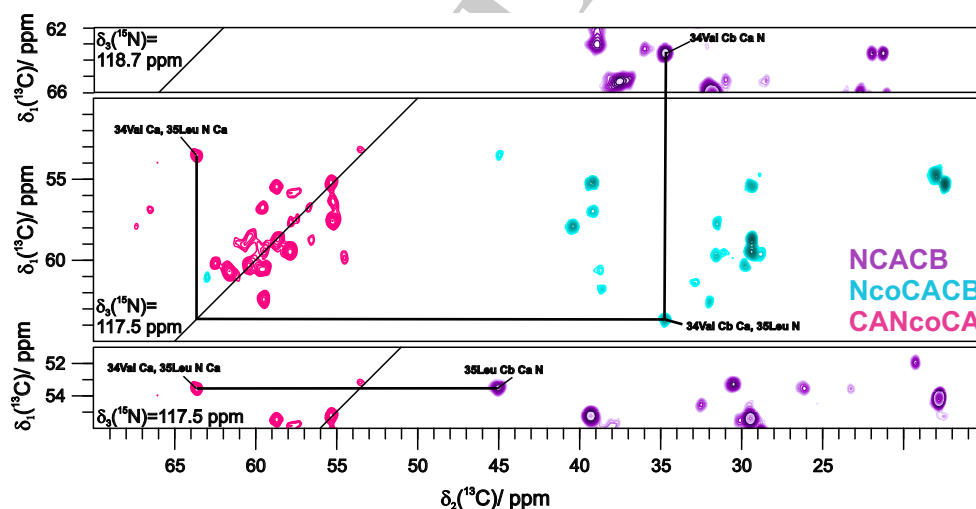


Fig. 4 Example for the sequential walk along the protein backbone (from the N- to the C-terminus) of the N-terminus of DnaB by using NCACB (negative peaks represented in *magenta*), NcoCACB

(negative peaks shown in *turquoise*) and CANcoCA (positive peaks represented in *pink*) spectra

on 3D NMR spectra such as NCACB, NCACX, NCOCX and CANCO (Schuetz et al. 2010). A representative example for the backbone walk using this “classical” strategy is given in Fig. 3. The spectral overlap observed in the NMR spectra requires the largest possible spectral

dispersion in all dimensions, which is in terms of 3D NMR spectra given by circumventing the detection of the CO-dimension which possesses the smallest chemical shift dispersion. For fulfilling this objective, an assignment strategy based on NCACB, NcoCACB and CANcoCA

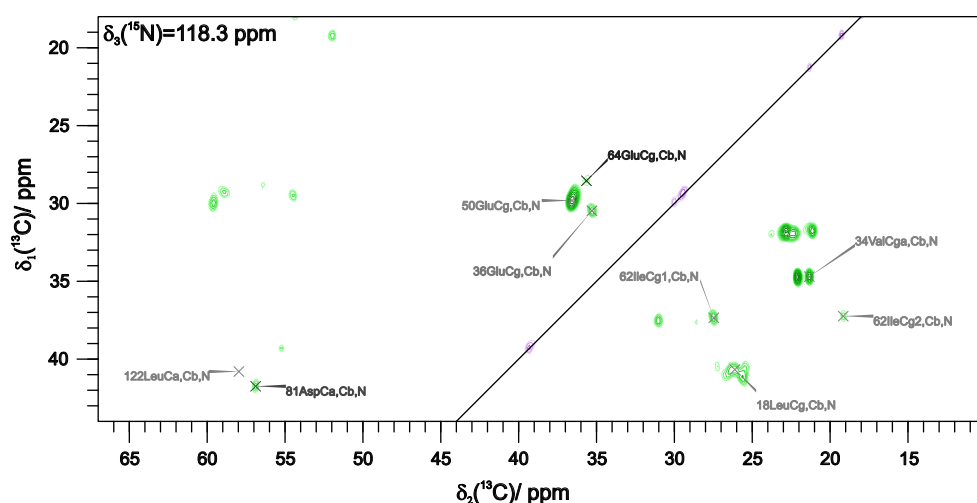


Fig. 5 Example of a representative plane extracted from the 3D NcaCBCX spectrum ($\delta(^{15}\text{N}) = 118.3$ ppm) used for side-chain assignment

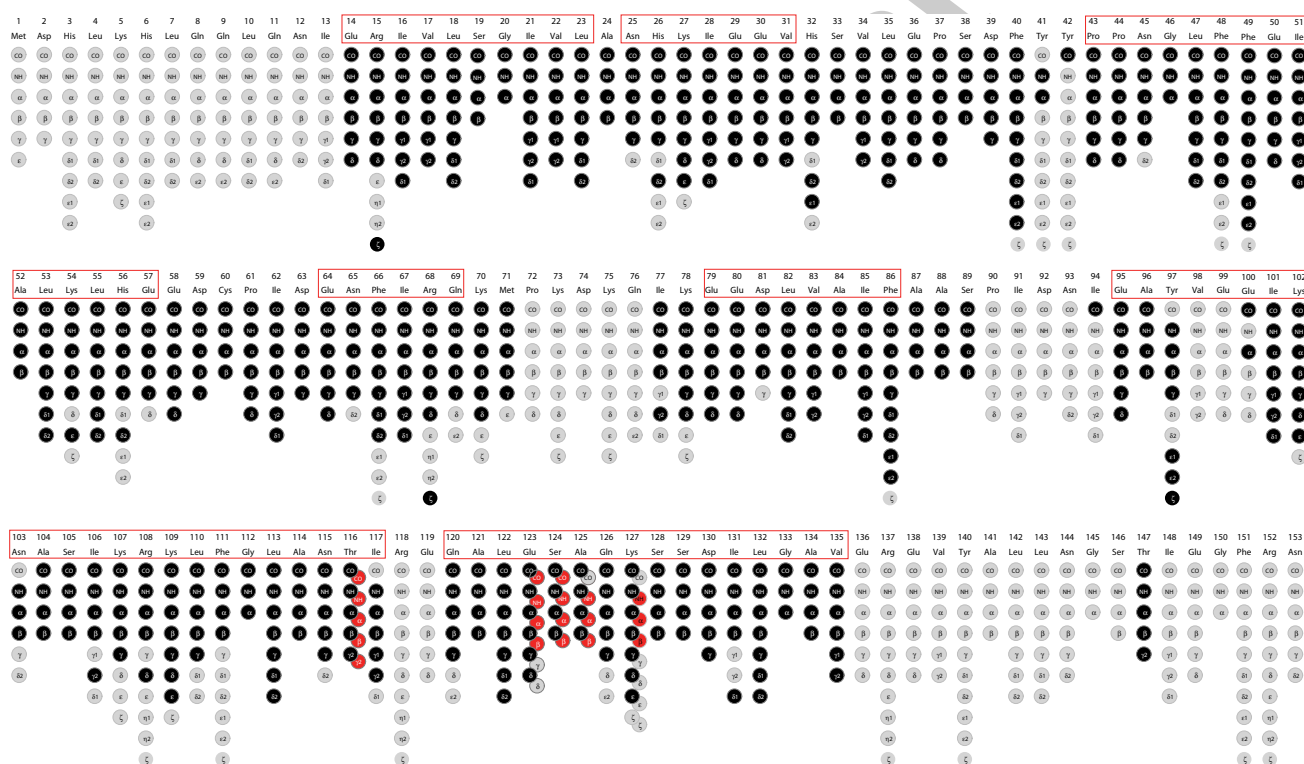


Fig. 6 Assignment graph of the N-terminus of *HpDnaB* created using the CcpNmr software. Residues marked in red show significant peak doubling. Black or red dots indicate assigned spins, grey dots

unassigned spins. Residues highlighted by red rectangles are located in α -helices as determined from NMR secondary chemical shifts (see Fig. 9)

experiments (Schuetz et al. 2010) was applied, a representative example of a sequential walk is given in Fig. 4. This assignment procedure could clarify many assignments which remained ambiguous after the firstly described assignment strategy. The selective $C'-C\alpha$ polarization transfer step in the relayed experiments (Chevelkov et al. 2013) yields a good signal-to-noise ratio also for these

types of experiments (see Fig. 4). Nevertheless, it has to be noted that a small number of resonances could not be assigned, mainly due to the spectral overlap as especially visible in the leucine, glutamic acid and glutamine region. The sidechains are mainly assigned by analyzing CCC (DREAM and DARR transfer) and NcaCBCX (two DREAM transfers) spectra (see Fig. 5). On basis of the

Table 2 Statistics of the manually performed peak assignments

Category	Assigned/%
C	69.3
CA	70.6
CB	69.4
CG	61.7
CD	54.5
CE	34.5
CZ	17.6
N	69.9
Residue Ala	91.7
Residue Arg	50.0
Residue Asn	55.6
Residue Asp	62.5
Residue Cys	100.0
Residue Gln	42.9
Residue Glu	72.2
Residue Gly	66.7
Residue His	60.0
Residue Ile	81.3
Residue Leu	68.8
Residue Lys	72.7
Residue Met	50.0
Residue Phe	85.7
Residue Pro	66.7
Residue Ser	88.9
Residue Thr	100.0
Residue Tyr	75.0
Residue Val	75.0

Data created with the CcpNmr software

conclusion. The C α and C β chemical shifts of these two threonine residues already reveal that Thr116 is located in an α -helix, whereas Thr147 is most probably located in a loop in agreement with the observed flexible character. The chemical shifts have been deposited in the BMRB database under the accession number 26548.

As indicated in Fig. 6, for some resonances in the amino-acid region Thr116-Lys127 a peak doubling is observed which is exemplarily illustrated by analyzing the unique serine-alanine amino acid pair (residues Ser124 and Ala125). The sequential backbone walk for these residues using NCACB, NcoCACB and CANcoCA spectra is illustrated in Fig. 7 and clearly demonstrates the presence of two sets of resonances. Pronounced spectroscopic differences are mainly observed for the ^{15}N frequencies. The observed peak doubling might indicate crystallographically distinct molecules in the asymmetric unit, which would agree with the previously published crystal structure for the residues 1–121 consisting of a dimer in the asymmetric unit (Kashav et al. 2009).

The results of the manual assignment procedure are validated by automated peak assignments as implemented in the solid-state FLYA algorithm (Schmidt et al. 2013). Figure 8 illustrates the good agreement between the manually assigned residues and the assignments obtained by FLYA calculations based on the peak lists from the manual assignment procedure. Only a few significant differences (e.g. for the two prolines Pro43 and Pro44, as well as for Thr147, and the C' resonances of Ser89 and Val135) were observed and in those cases the manually assignment was carefully rechecked and its resonances were taken for the final assignment as they unambiguously result from the performed sequential walk.

Secondary structure

Secondary chemical shifts were obtained by subtracting the random-coil shifts (Wang and Jardetzky 2002) from the observed solid-state NMR chemical shifts (Wishart et al. 1992) and are visualized in Fig. 9. These data clearly illustrate the dominant α -helical character of the N-terminus of *HpDnaB*. Seven α -helices are identified in total, the longest one is reaching from residue 95 to 117. Our results indicate an interruption after the sixth α -helix and a succeeding seventh α -helix with positive secondary chemical shifts observed for residues 120–135. It is reasonable to assume that the C-terminal part of the protein is mainly flexible, since most resonances are not detected in the performed NMR experiments. The visible residue Thr147 shows C α /C β chemical shifts more typical for a loop or extended conformation than an α -helical arrangement (Wang and Jardetzky 2002). The secondary chemical shifts

acquired spectra and the applied assignment strategies, approximately 70 % of the backbone carbon and nitrogen atoms could be assigned (see Fig. 6; Table 2). 77 % of the residues for which ^{15}N , $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ chemical shifts were assigned are located in α -helices as indicated by the NMR results (vide infra). The resonances of most of the unassigned residues could not be detected in the 3D NMR spectra, most probably because they are located in flexible parts of the protein (those resonances were also not detected in ^{13}C , ^1H INEPT and ^{15}N , ^1H HSQC spectra indicating intermediate dynamics in this protein). Notably, the resonances of Thr147 are clearly detected in the 2D DARR spectrum (see Fig. 2a), but appear only very weakly in the 3D NCACB and CCC spectra and are even absent in the other 3D NMR spectra. Since the second threonine residue (Thr116) present in the N-terminus of *HpDnaB* could be assigned on the basis of the before described assignment strategies, the remaining Thr signal can be assigned to Thr147, and weak correlations visible in the 2D DARR spectrum at long mixing times support this

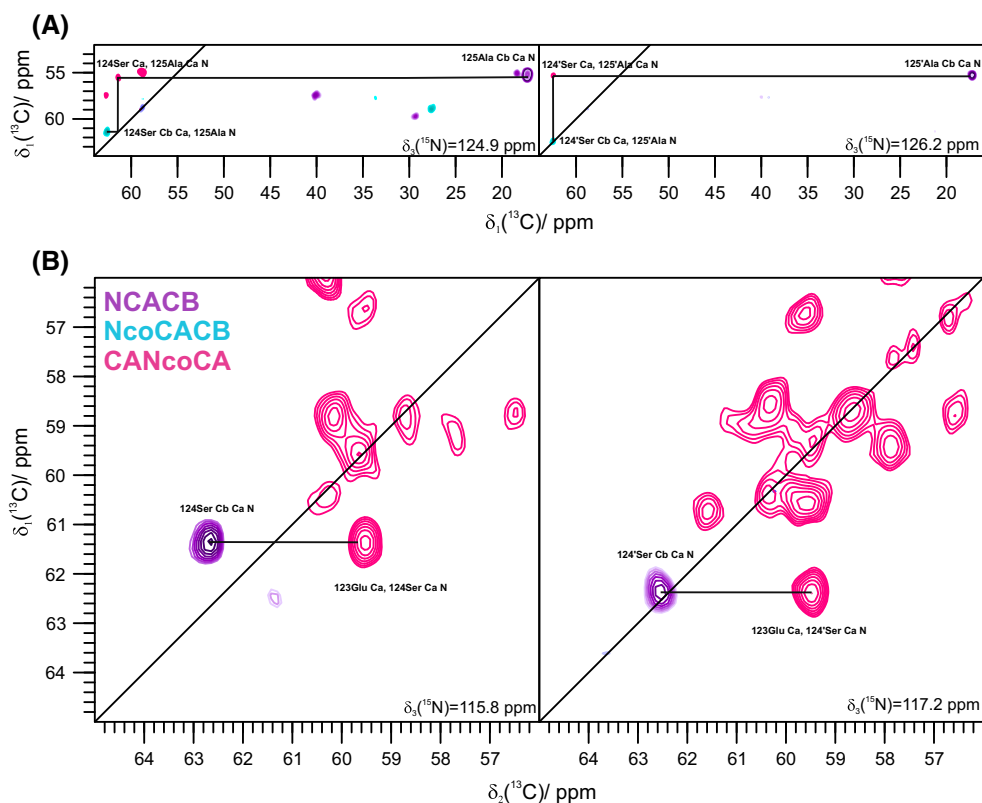


Fig. 7 Illustration of peak doubling for a unique Ser-Ala amino acid pair (124Ser-125Ala). **a** The sequential walk along the protein backbone based on NcoCACB (negative peaks are illustrated in turquoise), CANcoCA (positive peaks are shown in pink) and

NCACB (negative peaks are shown in magenta) spectra is shown for the two sets of resonances. **b** Correlation between the residues Ser124 and Glu123 in the NCACB and CANcoCA spectra, for the color code see (a)

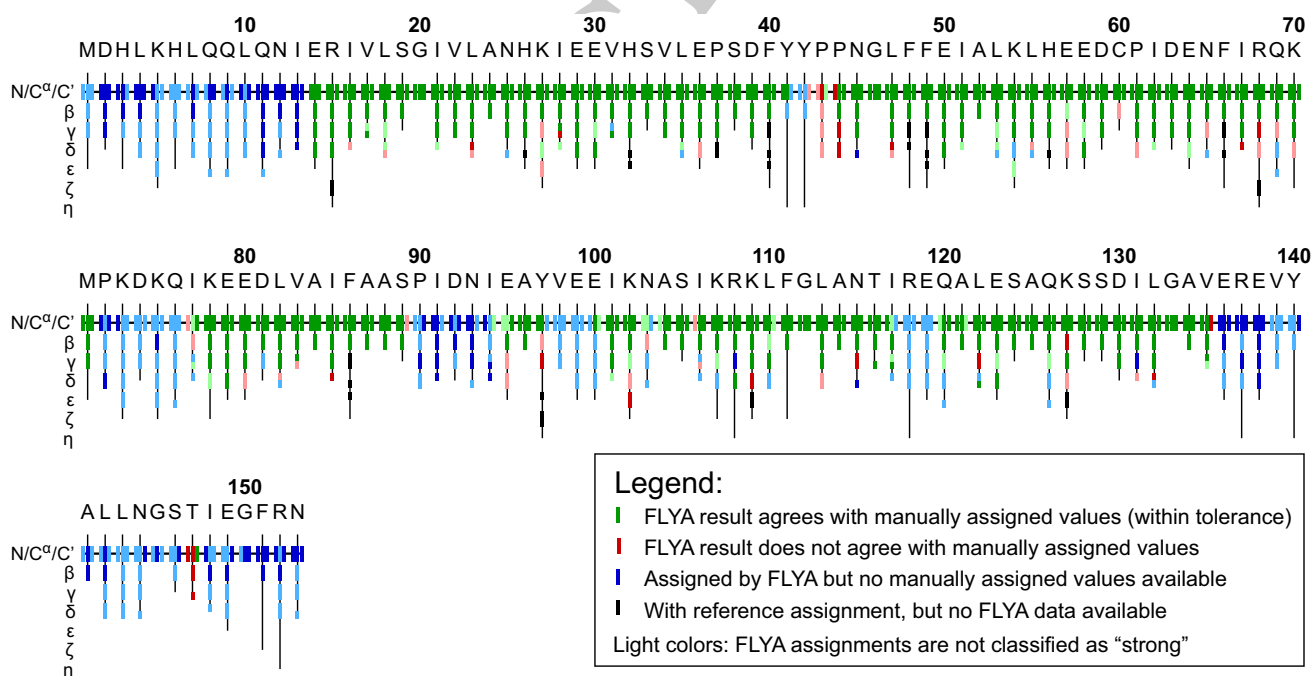


Fig. 8 Result of an automated peak assignment using ssFLYA calculations on the basis of the manually created peak lists (which were used for the sequential assignment) extracted from NCACB, CANCO, NCOCX, NCACO, CANcoCA, NcoCACB and NcaCBCX spectra

Fig. 9 Secondary ^{13}C chemical shifts (obtained by subtracting the random-coil shifts (Wang and Jardetzky 2002) from the observed chemical shifts). Glycines are marked in *black* (and $\Delta\delta\text{C}\alpha$ is plotted) and residues for which significant peak doubling was observed are highlighted with a+

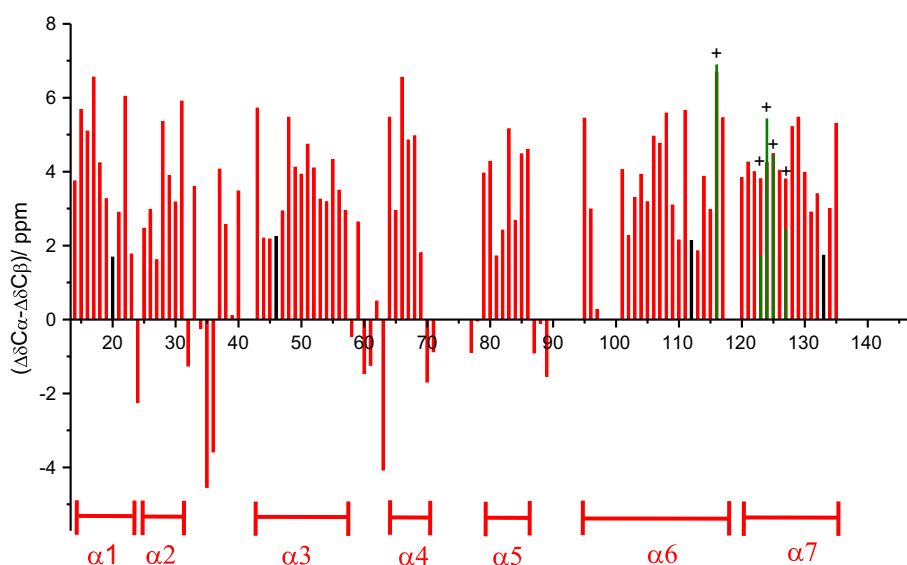


Table 3 α -Helices determined from the secondary chemical shifts and from the 3D atomic coordinates deposited in the pdb file 3GXV using the DSSP algorithm (Kabsch and Sander 1983) via the corresponding web interface

α -Helix	NMR	TALOS+ ^a	DSSP ^b
1	Glu14-Leu23	Arg15-Leu23	His3-Leu23
2	Asn25-Val31 ^c	His26-His32	Ile28-His32 ^d
3	Pro43-Glu57	Pro44-Glu57	Pro43-Glu57
4	Glu64-Gln69	Glu64-Gln69	Glu64-Gln69
5	Glu79-Phe86	Glu79-Phe86	Glu79-Phe86
6	Glu95-Ile117	Ile101-Ile117	Glu95-Gln120
7	120Gln-135Val	120Gln-135Val	–

^a Calculated from the solid-state NMR chemical shifts described in this work using the TALOS+ software (Shen et al. 2009)

^b Data for chain A are given

^c Data do not allow a distinction between 3/10 helix and α -helix

^d A 3/10 helix is predicted for residues Asn25-Lys27

TALOS+ backbone torsion angle calculations (Shen et al. 2009)] and the X-Ray structure is observed (see Table 3).

Conclusions

We describe the sequential resonance assignment of the N-terminal domain of *HpDnaB* (residues 1–153) based on 3D solid-state NMR experiments leading to a site-specific chemical shift assignment of approximately 70 % of the backbone resonances. Those manually obtained peak assignments are validated by solid-state FLYA calculations. Seven α -helices were identified by a secondary chemical shift analysis which mostly agrees with the single crystal structure published for the residues 1–121 (PDB 3GXV). The observed peak doubling might point to crystallographically distinct molecules in the asymmetric unit.

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