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Per-deuteration and NMR experiments for the backbone assignment of 62 kDa protein, Hsp31

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Abstract Hsp31 protein is one of the members of DJ-1 superfamily proteins and has a dimeric structure of which molecular weight (MW) is 62 kDa. The mutation of DJ-1 is closely related to early onset of Parkinson's disease. Hsp31 displays Zn^{+2}-binding activity and was first reported to be a holding chaperone in *E. coli*. Its additional glyoxalase III active has recently been characterized. Moreover, an incubation at 60°C induces Hsp31 protein to form a high MW oligomer (HMW) *in vitro*, which accomplishes an elevated holding chaperone activity. The NMR technique is elegant method to probe any local or global structural change of a protein in responses to environmental stresses (heat, pH, and metal). Although the presence of the backbone chemical shifts (bbCSs) is a prerequisite for detailed NMR analyses of the structural changes, general HSQC-based triple resonance experiments could not be used for 62 kDa Hsp31 protein. Here, we prepared the per-deuterated Hsp31 and performed the TROSY-based triple resonance experiments for the bbCSs assignment. Here, detailed processes of per-deuteration and the NMR experiments are described for other similar NMR approaches.

Keywords Backbone chemical shift assignment, Hsp31, NMR, Per-deuteration, TROSY

Introduction

The increasing antibiotic resistant problem by commonly used antibiotics is a need for a novel antimicrobial agent which is not been exposed against microorganisms. Antimicrobial peptides (AMPs) are possible antibiotic candidates owing to they have broad spectrum antibacterial, antifungal and antiviral activities. To date more than 1500 AMPs have been discovered in all species, ranging from plants and insects to vertebrates.[1,2] They are being increasingly recognized as significant components of innate immunity in all living organisms.[3,4] Although the detailed mechanisms is not well understood, their mode of antibiotic action involves depolarization or permeabilization of the bacterial cell membrane, some AMPs can transverse intact membranes to interact with intracellular targets.[5-7]

Living cells including *Escherichia coli* (*E. coli*) express a set of heat shock proteins (Hsps) in response to environmental stresses (e.g. heat). Hsps can be classified according to their molecular weight (MW) and those with high MW such as Hsp60, 70, and 90 utilize ATP molecule for their activity. On the other hands, small Hsps (sHsps) do not have an ATPase activity and provide a molecular platform for the unfolded proteins to suppress the aggregation of

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damaged proteins, so called holding chaperone.[1,2] Hsp31 is the product of the gene, *hchA* (**h**eat-inducible **ch**aperone), and its holding chaperone activity under heat shock (50°C) is well characterized.[3] The expression of the *hchA* gene is under the control of dual promoters regulated by σ^D and σ^S factors, and thermal stress releases the H-NS mediated silencing of σ^D-dependent transcription.[4] The σ^S factor is also known to be the key regulator during the responses to general stresses including heat/cold-shock, high osmolality and starvation.[5] The presence of Hsp31 is also important for the acid resistance in stationary phase.[6]

Hsp31 belongs to the member of DJ-1 superfamily proteins, and the superfamily proteins are classified into three sub-groups (DJ-1/YajL, YhbO/PfpI and Hsp31/Ydr533C) in the aspect of the domain structures.[7] The structure of the Hsp31 monomer can be divided into A- and P-domain[8] and the presence of the additional P-domain is a distinct feature of the Hsp31/Ydr533C sub-group.[9] It has been known that various sHsps form an oligomeric structure for their holding chaperone activity.[1,10,11] We previously reported that Hsp31 also forms high MW (HMW) form at 60°C, which also displays a highly enhanced holding chaperone activity for citrate synthase. Major structural change in the P-domain during the transition to the HMW form was deduced from the changes of the intrinsic tryptophan fluorescence.[12]

The presence of glyoxalase III activity has recently been identified for Hsp31, and this activity seems to be conserved for various DJ-1 superfamily proteins. The glyoxalase III is able to detoxify highly reactive dicarbonyls (methyl glyoxal and glyoxal) without an additional co-factor such as NADPH and glutathione.[9,13,14] The stereospecific mechanism of the DJ-1 glyoxalase are recently reported for the D/L-lactate products based on the crystal structures mimicking the catalytic intermediate.[9] The presence of different biological function other than a holding chaperone activity is reported for many sHsps.[1] Interestingly, Hsp31 contains the Zn^{+2}-binding site consists of His86, Glu91, and His123,[15] and thus is capable of binding to Ni^{+2}-affnity column. It was reported that the presence of Zn^{+2} ion increases the holding chaperone activity of α-crystallin protein that is a prototype of α-crystallin domain (ACD)-type sHsps.[16]

The high resolution NMR technique is an effective and a unique method to trace the structural changes and the binding events of a target protein that occur in solution state. Various 2D NMR experiments implementing the HSQC (Heteronuclear Single-Quantum Correlation) module can be used to measure many solution events of the protein including the dynamic motions of each residue. However, the presence of the backbone chemical shifts (bbCSs) of the protein is a prerequisite for these NMR experiments. Dimeric Hsp31 is a 62 kDa protein and its T2-relaxation time is very short due to its very long rotational correlation time. The general triple resonance NMR experiments using the $^{13}C/^{15}N$-labeled protein are not practical for the backbone assignment. Therefore, the per-deuterated Hsp31 protein ($^{2}H/^{13}C/^{15}N$-labeled) was prepared to increase the T2-relaxation time and then we performed the TROSY-based triple resonance experiments for the bbCSs assignment.[17] We reported detailed processes of the per-deuteration and TROSY-based NMR experiments required for the bbCSs assignment of the $^{2}H/^{13}C/^{15}N$-labeled Hsp31 protein.

Experimental Methods

Per-deuteration of Hsp31- The *E.coli hchA* gene was cloned into pET-21b using the Nde I and Xho I restriction enzymes, and the stop codon (TAA) was added before the Xho I site to produce an intact form of Hsp31 protein. The constructed plasmid was transformed into *E.coli* BL21 (DE3) strain and then several colonies with an average size were inoculated into LB media supplemented with 100 μg/ml ampicillin. The glycerol was added to the culture (OD_{600} 0.4~0.6) to be 25%, and then this expression cell stock was stored in -70°C refrigerator.

In the morning, the expression cell stock (0.2 ml) was inoculated into 70% D_2O-LB media (5 ml) supplemented with 50 μg/ml ampicillin and then was

grown at 37°C for 9-h. The media of the culture (1 ml) was removed by centrifugation and then the cell pellet was inoculated into 99% D_2O-M9 minimal media (50 ml) supplemented with 100 μg/ml ampicillin. After overnight culture, the seed was added into 1-liter of 99% D_2O-M9 media supplemented with 50 μg/ml ampicillin that was pre-equilibrated in the incubator at 37°C at least for 30 min. The IPTG-induction (1 mM) was done when the OD_{600} of the culture became to be ~0.7. The cultured cell was harvested by centrifugation after 5 h more incubation. All materials were prepared in 99% D_2O solution. The contents of the used M9 minimal media are summarized in Table 1. Detailed conditions of the M9 media were selected from the several previous reports on the protein labeling techniques and auto-inducing media.[18-21]

Purification of Hsp31- The harvested cell was re-suspended in a lysis buffer (pH 8.0, 50 mM HEPES, 500 mM NaCl, and 10 mM 2-mercaptoethanol) and then was disrupted by sonication. Hsp31 protein was first purified by Ni^{+2}-affinity column chromatography using the Histrap FF column (GE healthcare). Non-specific binding proteins were washed with a buffer (pH 8.0, 25 mM HEPES, 500 mM NaCl, 10 mM imidazole, and 10 mM 2-mercaptoethanol), and then the protein elution was done with a buffer (pH 8.0, 50 mM HEPES, 500 mM NaCl, 250 mM imidazole, and 10 mM 2-mercaptoethanol). 5 mM dithiothreitol (DTT) was additionally added into the protein fraction and then was concentrated by using Amicon Ultra centricon (Millipore) for the size exclusion chromatography using the HiLoad 16/60 Superdex 75 column (GE healthcare) with a buffer (pH 8.0, 10 mM HEPES, 150 mM NaCl, 1 mM DTT, and 1 mM EDTA). The pH of the used DTT stock solution (1.0 M) was pre-adjusted to be 7.0 by adding an appropriate amount of concentrated NaOH solution. The Hsp31 solution was concentrated to be 1.0 mM and then was frozen in liquid nitrogen for the storage in -70°C refrigerator. Any further buffer exchange was done with micro-dialysis using Float-A-Lyzer G2 (Spectrum laboratories).

NMR experiments and data processing- 17 μl of 99.9% D_2O solution was added to 323 μl of 0.8 mM $^{2}H/^{13}C/^{15}N$-labeled Hsp31 protein solution that was prepared in a buffer (pH 7.0, 50 mM HEPES, 50 mM NaCl, 5 mM DTT), and then was transferred to Shigemi NMR tube. The sample in the NMR tube was degassed by using low vacuum pump, and then was tightly sealed using Parafilm for the long-term

Table 1. Composition of the D_2O-M9 minimal media.

M9 salts	Na_2HPO_4 (6.8 g, 47.76 mM), KH_2PO_4 (3.0 g, 22.04 mM), NaCl (0.5 g, 8.56 mM), citric acid (0.1 g, 0.52 mM) • Can be dumped in as a powder.
Stable isotopes	1.5 g ^{15}N-NH_4Cl, 2.5 g $^{2}H/^{13}C$-D-glucose, 0.5 g $^{2}H/^{13}C/^{15}N$-IsoGro (SIGMA) or Celtone (CIL) powder • Can be dumped in as a powder.
Metals	1 ml $MgSO_4$ (MW 120.37, 2.0 M), 0.1 ml $CaCl_2$ (MW 110.98, 1.0 M)
Trace metals	$CoCl_2 \cdot 6H_2O$ (MW 998.73, 2.00 g, 2.0 mM), $CuSO_4 \cdot 5H_2O$ (MW 833.60, 1.67 g, 2.0 mM), $FeCl_3 \cdot 6H_2O$ (MW 270.30, 13.5 g, 50.0 mM), H_3BO_3 (MW 61.834, 0.62 g, 2.0-**10.0** mM), $MnSO_4$ (MW 151.00, 1.51 g, 10.0 mM), $Na_2MoO_4 \cdot 2H2O$ (MW 831.91, 4.16 g, **2.0**-5.0 mM), $Na_2SeO_3 \cdot 5H_2O$ (MW 263.03, 0.53 g, 2.0 mM), $ZnSO_4 \cdot 7H_2O$ (MW 867.46, 4.34 g, 5.0-**10.0** mM) for 1-liter. • To complete dissolve, add about 5 ml of concentrated HCl solution. To prepare D_2O stock solution, lyophilize and re-dissolve this using D_2O solution. Add 0.2 ml into 1-liter LB/M9 culture.
Vitamin stock solution (A or B)	Stock-A. Biotin (0.5 g), choline chloride (**0.1**-0.4 g), cobalamin (0.4 g), folic acid (**0.1**-0.4 g), myo-inositol (**0.4**-0.8 g), nicotinamide (0.4 g), pantothenic acid (0.4 g), pyridoxal-HCl (0.4 g), riboflavin (**0.1**-0.4 g), thiamine-HCl (0.5 g), for 1-liter • Add 1.0 ml into 1-liter M9 culture. Stock-B. MEM Vitamin Solution (×100, M6895 SIGMA) • To prepare D_2O stock solution, lyophilize & re-dissolve this using D_2O. Add 10 ml into 1-liter M9 culture.

NMR experiments at 40°C. All the TROSY-version of NMR experiments including 2D-TROSY (trosyetf3gpsi.2),TROSY-HNCACB(trhncacbgp2h3d), TROSY-HNCOCACB (trhncocacbgp2h3d), TROSY-HNCACO(trhncacogp2h3d), TROSY-HNCO (trhncogp2h3d), and TROSY-HNCA (trhncagp2h3d2) were carried out using 900 MHz NMR (Bruker Avance II) equipped with the cryo-probe head. Additional 2D-TROSY spectra were recorded after each 3D experiment to check the status of the sample. The NMR data were processed using the NMRpipe program[22] and then were analyzed using the Sparky program.[23]

Results and Discussion

Composition of 99% D2O-M9 minimal media for an optimal growth of E. coli- The amount of di-basic salt was slightly increased as 6.8 g Na_2HPO_4 and 3.0 g KH_2PO_4, which resulted in the pH 7.2 of the media. The cost of the D_2O-M9 minimal media is very high,

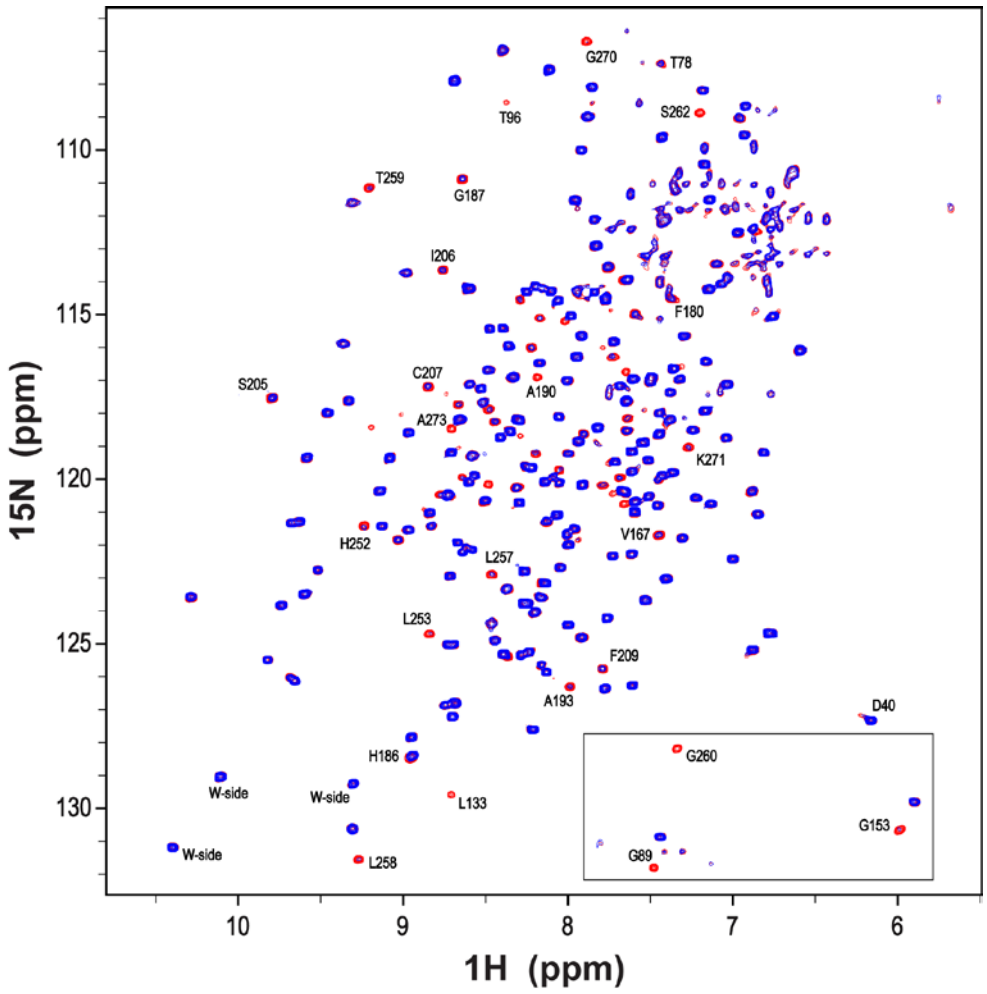

Figure 1. TROSY spectrum of $^{2}H/^{13}C/^{15}N$-labeled Hsp31 protein. The initial TROSY spectrum (blue) is overlaid to the same TROSY spectrum (red) that was recorded after several days of NMR experiment at 40°C. The different TROSY peaks between blue and red originated from the residues of which amide protons did not completely exchange with water molecules during the purification. The folded peaks that are originally located in the up-field of the 15N-axis are indicated with a rectangular box.

and ^{15}N-NH_4Cl is relatively cheap component. Although 1 g of ^{15}N-NH_4Cl (18.3 mM) is sufficient for 1-liter M9 minimal media, it has been reported that 25 mM NH_4^+ significantly reduces the protein expression in the auto-induction media.[21] Therefore, we used 1.5-times higher amount of ^{15}N-NH_4Cl during the per-deuteration of Hsp31. The presence of the iron ion is critical for the maximal growth of *E. coli*,[21] and thus we added 50 mM $FeCl_3$ and 0.1 g citrate into the trace metal stock and in M9 salts, respectively. Both Fe^{+2} and Fe^{+3} ion are used in the each different recipe for the M9 minimal media. Gram-negative bacteria uptake ferric (Fe^{+3})-siderophore (ion carrier) complex *via* outer membrane receptors[24], and it has recently been reported that FecB, a periplasmic ferric-citrate transporter of *E. coli*, can bind to diverse metal-free and metal-loaded tricarboxylic acids.[25] Cobalamin (vitamin B12) and biotin (vitamin B7) were additionally added to the previous recipe of the vitamin stock[20], and the amounts of the components were further adjusted for general usages. The presence of cobalamin is critical for the Se-Met labeling using *E. coli* BL21 (DE3) strain.[21] However, we recently started to use the MEM vitamin solution (SIGMA) for the convenience.

The TROSY spectrum of 2H/13C/15N-labeled Hsp31- The Hsp31 is stable even at 40°C, and any structural change of the Hsp31 was not identified from the TROSY spectra recorded at temperatures from 30 to 40°C (data not shown). The line-broadening of the TROSY peaks became narrow as the temperature increased from 30 to 40°C, and thus we performed the TROSY-based triple resonance experiments at 40°C for the bbCSs assignment. Interestingly, many TROSY peaks (~35) appeared again as the NMR experiments progressed, which means that the corresponding amide protons did not completely exchange with water molecules during the purification of the ^{2}H/^{13}C/^{15}N-labeled Hsp31 (Fig. 1). The number of the TROSY peaks after the complete exchange of the amide protons was about 240, and was much less compared to the number of the Hsp31 amino acids (283 residues). It is likely that the

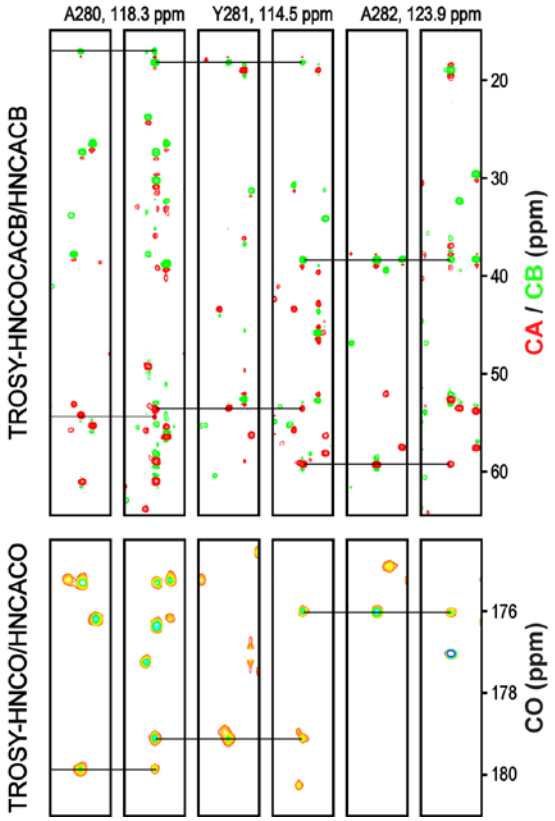

Figure 2. Strip plots of TROSY-HNCOCACB/HNCACB and TROSY-HNCO/HNCACO spectra. The sequential connectivity from A279 to A282 is clearly shown in the triple resonance NMR spectra of ^{2}H/^{13}C/^{15}N-labeled Hsp31 protein.

presence of the local dynamic motion of the Hsp31 resulted in this missing TROSY peaks of at least 40 residues. Additionally, the intensities of many peaks were much lower than those of other well resolved peaks, which also indicated the corresponding residues likely had a dynamic property (Fig. 1).

Partial bbCSs assignment of the Hsp31- Although we did not complete the assignment of the bbCSs, the TROSY-based triple resonance spectra of the ^{2}H/^{13}C/^{15}N-labeled Hsp31 shows the clear connectivity between the sequential residues (Fig. 2). The manual assignment of the bbCSs is time-consuming process[26] and becomes more difficult as the size of a protein increases due to higher spectral complexity. Although several programs such as the AutoAssign[27] and the Pine[28]

that enable the automatic backbone assignment has been developed, their performance was limited to the well resolved data set, and a higher spectral complexity inevitably results in the incomplete and inaccurate auto-assignment. Recently developed program, the RASPnmr[29] accomplishes the highly confident auto-assignment of the bbCSs in the presence of the PDB coordinate, because it is able to utilize the structural information (the reference CSs, rCSs) for the auto-assignment. The rCSs can be calculated from the PDB coordinates using the Sparta+[30] and the ShiftX2[31] programs with relatively high accuracy. The homologous PDB models can also be used for the calculation of the rCSs, and the RASPnmr program indeed completes the bbCSs assignment of the Ube2g1 (E2 enzyme) using the rCSs resulting from the PDB models.[32]

The complete assignment of the backbone CSs will make it possible to probe any structural change or the binding events of Hsp31 protein in the presence of possible solution effector such as a heat, metal, and reactive dicarbonyls. The innate dynamic properties of the Hsp31 backbone that probably response to a heat is also interesting, since the structural transition to the HMW form likely correlates with the dynamic property of Hsp31 protein.

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