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Molecular Dynamics of the C-Terminal Domain Mouse CDT1 Protein

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Abstract : The backbone molecular dynamics of the C-terminal part of the mouse Cdt1 protein (tCdt1, residues 420-557) was studied by high field NMR spectroscopy. The Secondary structure of this protein was suggested by analyzing of chemical shift of backbone atoms with programs TALOS and PECAN, together with NOE connectivities from 3D ^{15}N-HSQC-NOESY data. Measurement of dynamic parameters T_1, T_2 and NOE and limited proteolysis experiment provided information for domain organization of tCdt1(420-557). Analysis of the experimental data showed that the C-terminal part of the tCdt1 has well folded domain for residues 455-553. The residues 420-453 including α–helix (432-441) are flexible and probably belong to other functional domain in intact full length Cdt1 protein.
Keywords: protein structure, TALOS, PECAN, T_1 and T_2 relaxation, NOE.

INTRODUCTION

One important and critical question of the cell is how the cell ensures the complete and precise replication of the chromosomal DNA. In the normal cell, DNA replication

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occurs only once per cell cycle. The large details of the control mechanism of DNA replication process are still remained unknown for to day. However, it is known that eukaryotic DNA replication is initiated with an ordered assembly of protein complexes on replication origins to establish so called pre-replicative complex (pre-RC).[1] After DNA replication initiated, this pre-Replicate complex must be disassembled or transformed into the Replicate complex. And it is important to remark that secondary replication is impossible in normal cell. Cdt1 protein is one of important members of such licensing system to regulate the secondary DNA replication.

Cdt1 protein is the member of the sub proteins assembly in pre-RC. The major units of this pre-RC is origin recognition complex (ORC),[2] mini chromosome maintains (MCM) complex that contains six units MCM2-7,[3] and Cdt6 protein.[4] In the S phase of cell cycle the ORC loaded in to chromatin. The MCM complex is very important for the starting replication process. And two proteins Cdt6 and Cdt1 are responsible for loading MCM. In the work[5] with the Xenopus egg has been shown that Cdt1 could only license DNA replication and load Mcm2-7 onto DNA when it binds to chromatin that has already associated with Cdc6. For the inverse order associate Cdc6 and Cdt1 the loading MCM2-7 is impossible and DNA replication not happens. Maybe this moment is helpful for the finding answer on question how licensing replication system is working.

The authors of work [6] demonstrated that mammalians Cdt1 proteins can be divide in to three regions: an amino-terminal region, which is poorly conserved among eukaryotes; a central region (amino acids 177– 380), which is conserved among metazoans; and a carboxyl-terminal region (407–477), which is highly conserved among eukaryotes. These three regions of Cdt1 are responsible for the binding Cdt1 with DNA, Geminin protein and MCM complex. Our scientific interest is concentrated on the topic of finding spatial structure and understanding functional activity of Cdt1 protein. Here we are reporting our results of structural investigation C-terminal part of Cdt1(420-557) protein.

EXPERIMENTAL

Cloning, Expression and Purification

His-tagged tCdt1(420-557) was synthesized by PCR, and the products was digested with NdeI-XhoI and inserted into a PET28a vector. Protein was purified by a nickel column followed by a cation exchange (Mono-S column) and by gel filtration (Superdex 75 column) equilibrated with 50 mM Tris-HCl, 200 mM NaCl, 5 mM dithiothreitol (DTT), pH 6.8.

NMR spectroscopy

All spectra were acquired in 20mM MES hydrate (pH 6.7), 80mM NaCl, 10mM β-mercaptoethanol with 10% D_2O. Protein concentration was ~1 mM for T_1, T_2 and NOE measurement and ~0.2 mM for limited proteolysis experiment. The spectrum was recorded at 298 K on a Bruker DRX800 and DRX 900MHz spectrometer. All NMR spectra were processed with NMRPipe [7] and analyzed with SPARKY 3.110.[8] ^{1}H, ^{15}N, and ^{13}C resonance assignments for backbone atoms were obtained from the following 3D heteronuclear correlation experiments [9]: CBCA(CO)NH, CBCANH, HN(CA)CO, HNCO, HN(CO)CA, HNCA, HBHA(CO)NH. 3D ^{15}N-heteronuclear single quantum coherence (HSQC)-NOESY has been recorded with 100ms mixing time. Steady-state $^{15}N\{1H\}$-nuclear Overhauser effects (NOEs) of ^{13}C,^{15}N-labeled t-mCdt1(420–557) were measured following the methods in Farrow *et al.*. [10] AutoAssign 1.7.2 [11] and interactive method on Sparky has been used for starting and finishing assignment for backbone atoms signals. Spectra were referenced by external calibration on 2,2-dimethyl-silapentane-5-sulfonic acid (DSS), sodium salt.[12]

RESULTS AND DISCUSSION

In our work, the secondary structure and their mobility of C-terminal domain of Cdt1(420-557) protein has been investigated. The ^{1}H-^{15}N HSQC spectrum of tCdt1(420-557) protein demonstrated good dispersion of backbone amide signals (Fig. 1). But some signals had higher intensity and concentrated in the center of the ^{1}H-^{15}N HSQC spectra. It means that the tCdt1(420-557) has good folded domain and also some part of the protein has disordered structure. After the resonance assignment for the backbone signals, the analysis

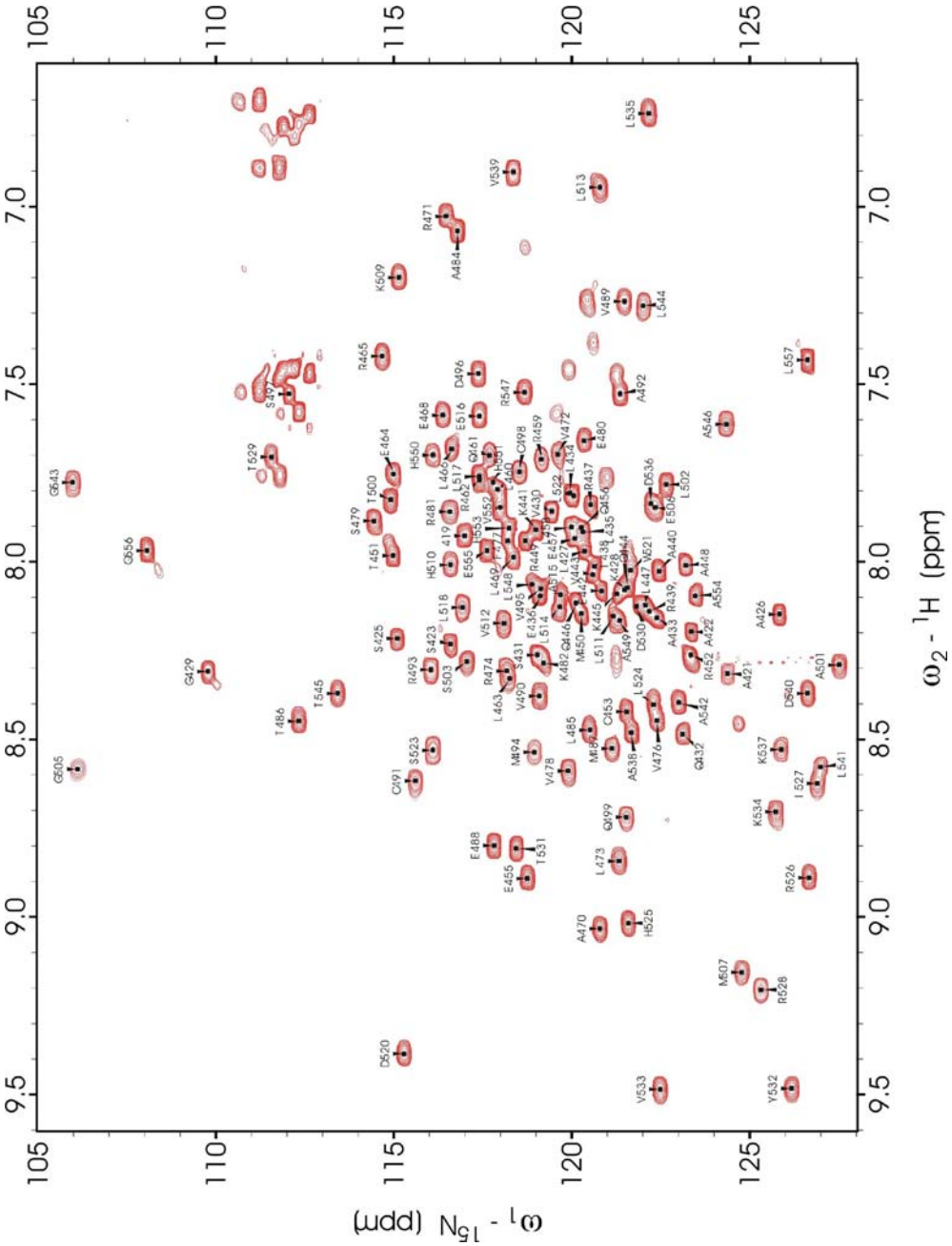

Fig. 1. Two-dimensional [^{1}H–^{15}N] HSQC spectrum of ^{15}N/^{13}C-labeled C-terminal Cdt1(420-557). The spectrum was recorded at 298K on a Bruker Avance 800 MHz spectrometer. The assignments of the well-resolved backbone HSQC peaks are indicated by the residue numbers.

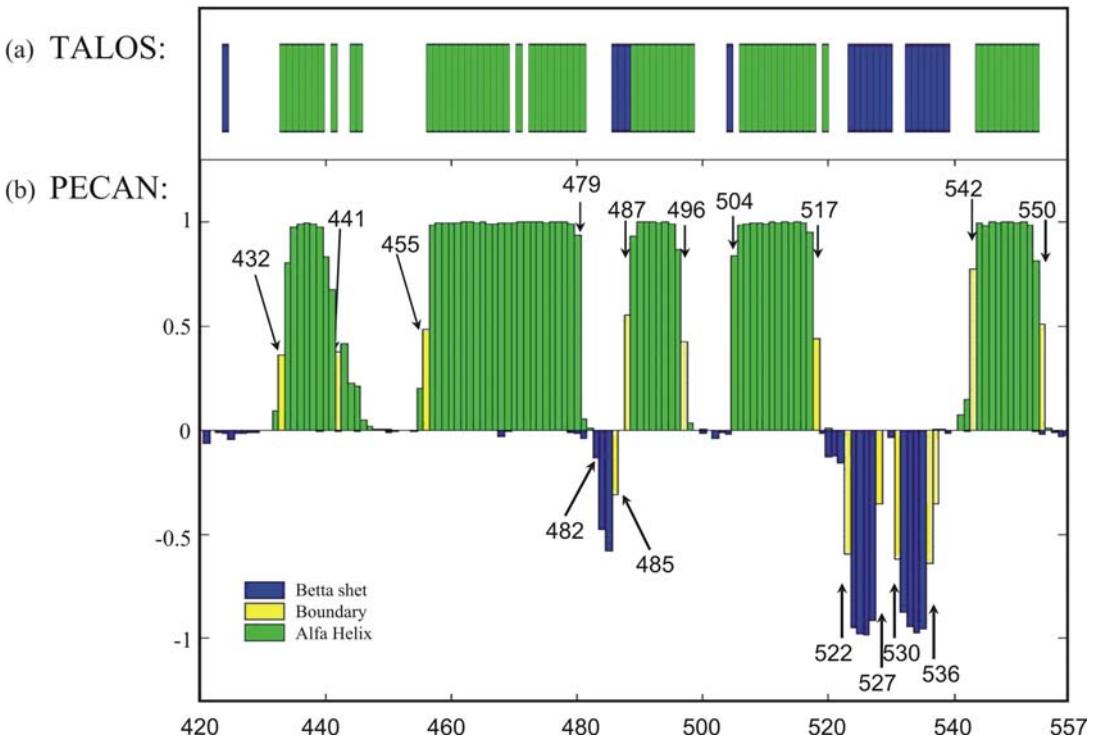

Fig. 2. Results of the secondary structure prediction obtained from backbone φ and ψ dihedral angles calculation: (a) the chemical shifts of the backbone ^{15}N, $^{13}C_a$, $^{13}C_b$, $^{13}C'$, H^{β}, and H^{α} calculation by TALOS program; (b) same previous chemical shifts data but calculated by PECAN algorithm.

of chemical shifts backbone atoms by TALOS [13] and PECAN [14] algorithms were carried out to determine the secondary structure (Fig. 2). These two analyses have resulted in the similar secondary structure to each other. The tCdt1(420-557) protein shows 5 α–helix and 3 β–strands. There was small difference between TALOS and PECAN in the helix 2 (residue 455- 479), which might be due to the proline residue (P467). Also there were some differences on the beginning and the ending secondary structure elements between these two predictions, especially for the α–helix 1.

To confirm the secondary structure, the 3D ^{15}N-HSQC-NOESY spectrum has been recorded and assignments for backbone signals have been completed. Using the assignment

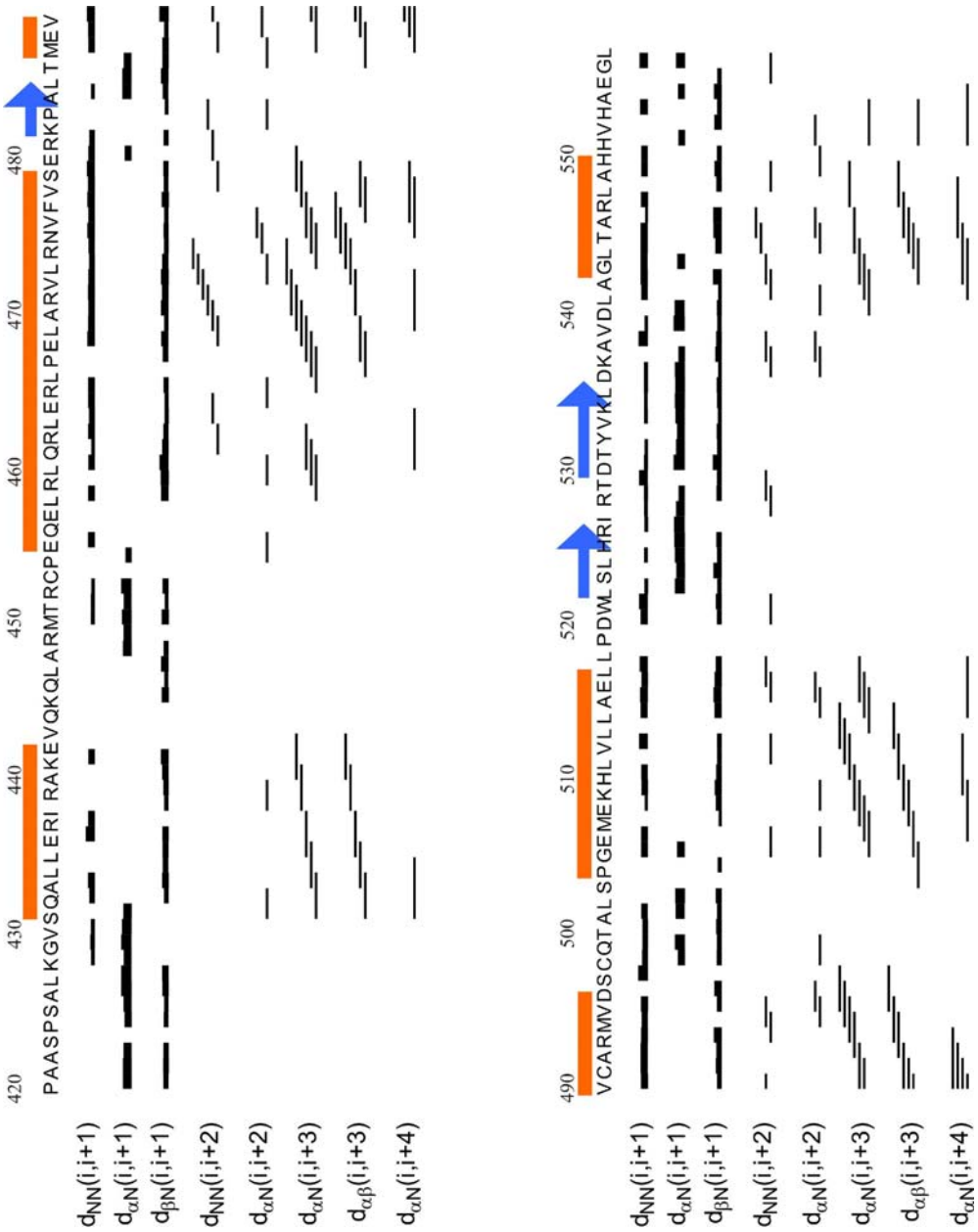

Fig. 3. Primary sequence of tCdt1(420-557) with the summary of the NMR data. For the short and medium-range NOEs the line thickness is approximately proportional to NOE intensity (classified as weak, medium or strong).

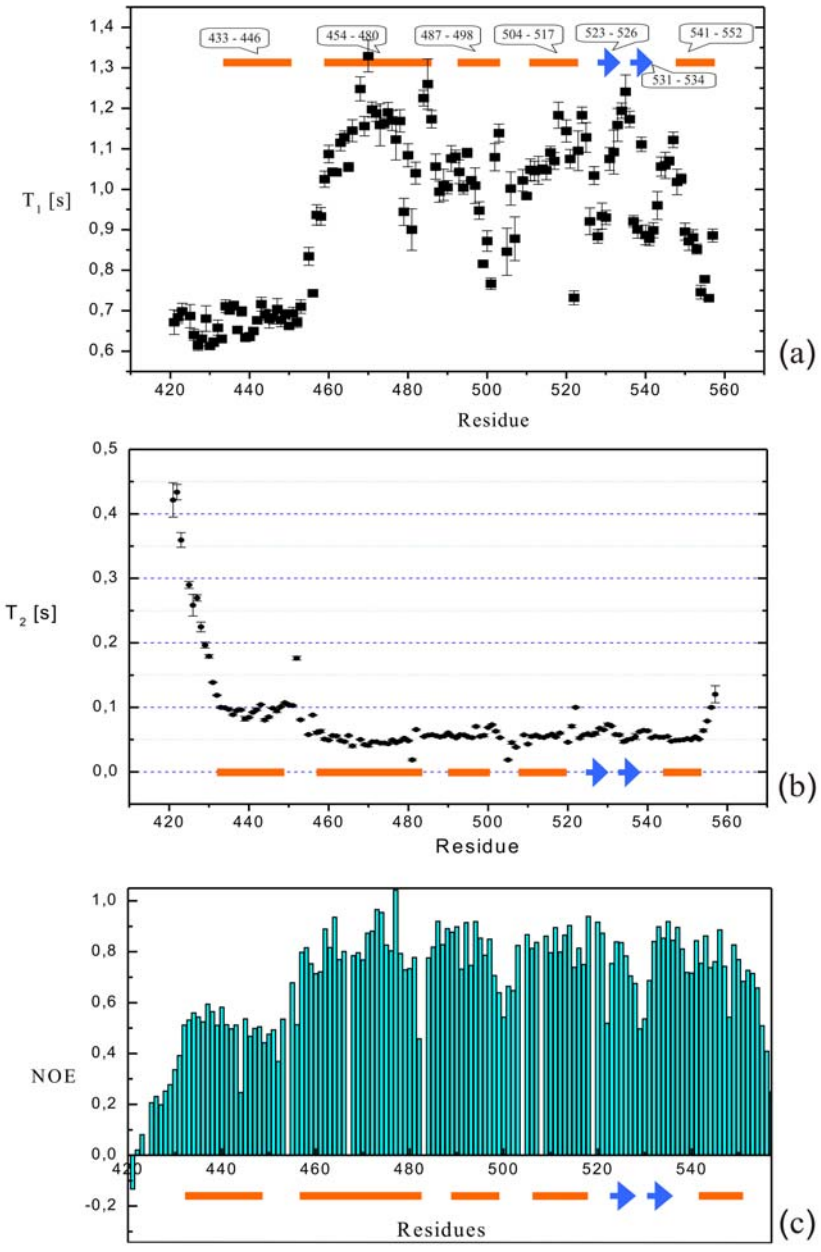

Fig. 4. (a) ^{15}N NMR relaxation data of the ^{15}N/^{13}C -labeled C- terminal Cdt1: (a) ^{15}N longitudinal relaxation time (T_1); (b) ^{15}N transverse relaxation time (T_2); and (c) heteronuclear Overhauser effects between proton and the ^{15}N nuclei of the amide ^{15}NH group.

of NOE signal, we constructed NOE connectivity plot by Cyana [15,16] program (Fig.3). We could observe a good agreement between NOE connectivity data and secondary structure prediction by TALOS and PECAN. The Fig. 3 clearly shows the the typical pattern of α helices along the residues in tCdt1, including the helix 1 (431-442).

In the next step of our research, we obtained and analyzed dynamic properties Cdt1 protein by the measurement relaxation parameters T_1, T_2 and steady-state NOE. The results of these measurements present on Fig. 4. As we can see on this figure it is possible to separate signals from amino acid residues into three groups. To the first group, we included residues 420-432 from N-terminal part and residues 454-557 from C-terminal. The second group is consist of amino acids 432-453 and third is 455-553. The residues from the first group are characterized by the high values of T2 (>), and low values of NOE (<). And the range of changing value is considerable. For example T_2 is changed from 0.42±0.02 sec (residue 421) to 0.1187±0.0009 sec (residue 432) and NOE is changed from -0.13±0.4 to 0.51±0.17 for same amino residues. Such behavior of T_2 and NOE like for the first group residues usually corresponded unfolded part of proteins. And this conclusion is consistent with the result of secondary structure calculation by TALOS and PECAN where all signals of first group are pertaining random coil. The T_2 relaxation times of the third group backbone nitrogen have approximately same value about 0.055±0.011 sec and also NOE value lay in the range between 0.7 and 1.0. Also average T_1 relaxation times of ^{15}N nuclear from third group are distinct from T_1 for the first and the second groups (Fig. 4 a). The high value of NOE and same T_1 and T_2 allow concluding that amino acids of third group form well folded functional domain of Cdt1 protein. Amino acid residues from the second group are indicated as α-helical structure based on the TALOS and PECAN analysis. But relaxation and NOE measurement is distinct from those of random coil (the first group) or well folded part (the third group). The average T_2 value of this region is 0.098±0.020 sec, which is higher then those of well folded domain and lower then those of unstructured region. Also NOE values are between 0.4 and 0.7, which is lower than those of the third group. But T_1 is similar for the first group (see Fig. 4). Together with the PECAN, Talos, and backbone NOE connectivity data, we can conclude that residues of the second group forms α–helix structure, though it has higher mobility than the folded

domain. It is suggested that this region is belong to other domain of Cdt1 protein that was unfolded due to the truncation in the middle of the domain.

The flexible nature of the N-terminal part of tCdt1(420-557) was confirmed by limited proteolysis experiment. After the partial digestion during the incubation at 4°C, we were able to separate two protein peaks from the gel filtration chromatography. One was exactly same like tCdt1(420-557), and the other has lower molecular mass (about 10kDa, determined by the electrophoresis). We found that, in the lower band protein, the NMR signals from the N-terminal 35 residues disappeared in the ^{1}H-^{15}N HSQC spectrum (Fig. 5). We concluded that the unfolded residues (420-432) and α–helix 1 with neighboring residues (432-453) had been cut off due to the degradation of the flexible part.

CONCLUSION

The Cdt1 protein is an essential protein in the DNA replication and it plays a role in the replication licensing system under tight control through the cell cycle. In present work we investigated the C-terminal part (residues 420-557) of the Cdt1 mouse protein which is responsible for binding with MCM complex and subsequently loading MCM on to chromatin and starting replication. Using high field NMR spectroscopy the secondary structure and backbone dynamics of tCdt1(420-557) were investigated. The analyses of chemical shifts of backbone atoms by TALOS and PECAN programs suggested that tCdt1(420-557) has 5 α-helices and 3 β–strands. The secondary structure was confirmed by the NOE connectivity obtained from 3D ^{15}N-HSQC-NOESY spectrum. Also the relaxation parameters such as T_1, T_2, and ^{1}H-^{15}N heteronuclear NOE and limited proteolysis experiment demonstrated that residues 455-557 form well folded functional domain. In the N-terminal flexible region, the residues 432-453 have α helical structure and is probably belong to other domain. We hope that our data will helpful for further structure investigation and understanding functional mechanism of Cdt1 protein.

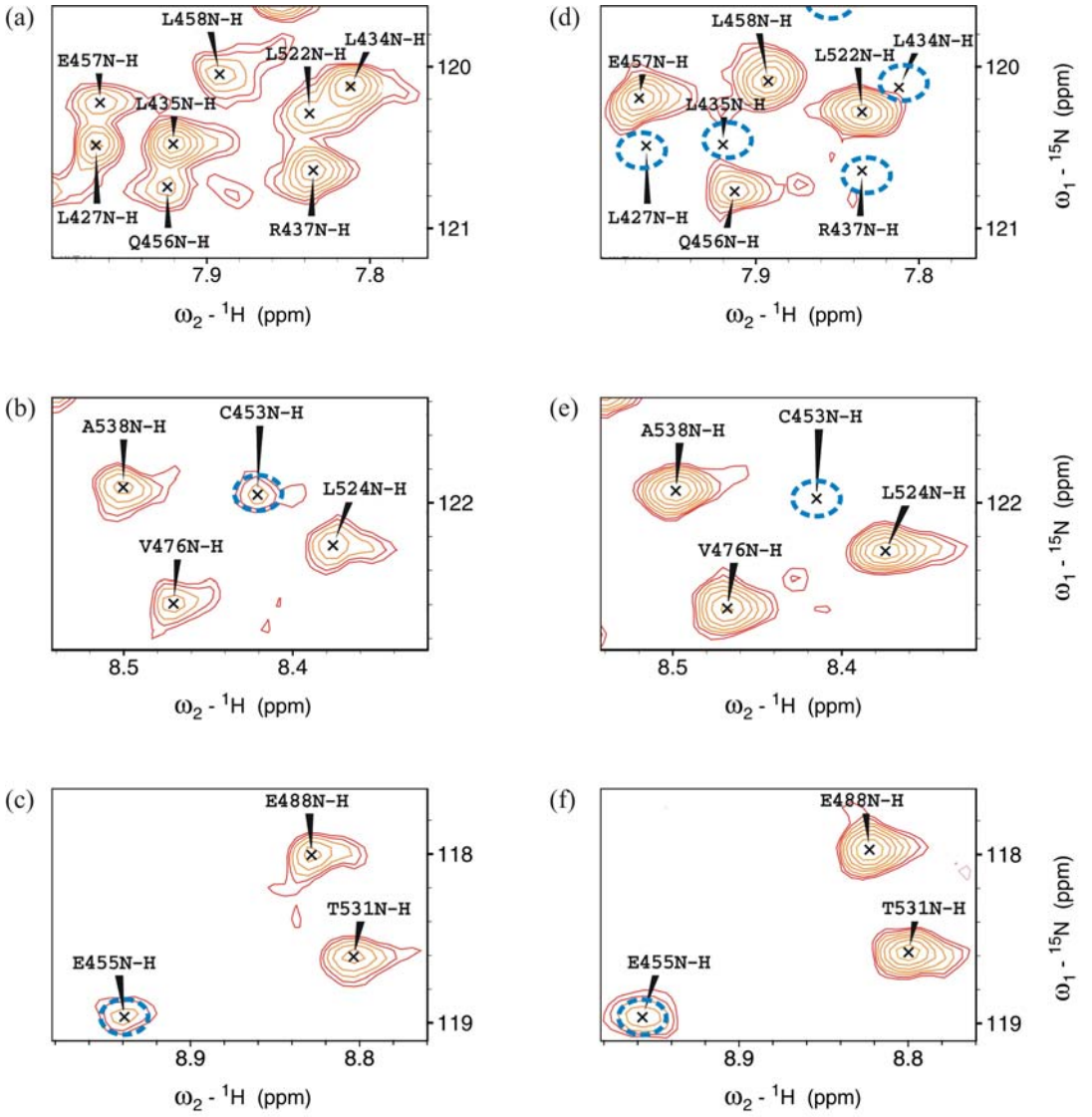

Fig. 5 [^{1}H–^{15}N] HSQC spectra of ^{15}N/^{13}C-labeled C-terminal Cdt1(420-557) showing result of limited proteolysis experiment: (a)-(c) full tCdt1-C (420-557) with flexible amino residues 420-454; (d)-(f) tCdt1-C (455-557) only well folded domain.

Acknowledgment

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