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Triple isotope-[^{13}C, ^{15}N, ^{2}H] labeling and NMR measurements of the inactive, reduced monomer form of *Escherichia coli* Hsp33

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Abstract : Hsp33 is a molecular chaperone achieving a holdase activity upon response to a dual stress by heat and oxidation. Despite several crystal structures available, the activation process is not clearly understood, because the structure inactive Hsp33 as its reduced, zinc-bound, monomeric form has not been solved yet. Thus, we initiated structural investigation of the reduced Hsp33 monomer by NMR. In this study, to overcome the high molecular weight (33 kDa), the protein was triply isotope-[^{13}C, ^{15}N, ^{2}H]-labeled and its inactive, monomeric state was ensured. 2D-[^{1}H, ^{15}N]-TROSY and a series of triple resonance spectra could be successfully obtained on a high-field (900 MHz) NMR machine with a cryoprobe. However, under all of the different conditions tested, the number of resonances observed was significantly less than that expected from the amino acid sequence. Thus, a possible contribution of dynamic conformational exchange leading to a line broadening is suggested that might be important for activation process of Hsp33.

Keywords : Hsp33, zinc-binding, inactive monomer, redox-switch, triple labeling, conformational exchange

INTRODUCTION

The accumulation of reactive oxygen species (ROS), a condition termed oxidative stress, is

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deleterious to cells and organisms, due to unfavorable oxidation of most cellular macromolecules. A growing number of proteins have been identified that use the oxidation state to modulate their activity, thereby protecting cells from severe oxidative stress. One such protein is the redox-regulated molecular chaperone Hsp33.[1,2] The expression of Hsp33 is regulated by heat at transcriptional level but post-translationally, it exhibits a holdase activity upon response to oxidative stress. Hsp33's activation requires both heat and oxidative stress, or alternatively, the protein was found to be activated by certain oxidants, such as HOCl, even without elevating temperature.[3,4] The C-terminal redox-switch domain (residues 227 ~ 297) and its adjacent linker region (residues 179 ~ 226) of Hsp33 is responsible for sensing the dual stress. In particular, activation process upon oxidation is triggered by intramolecular disulfide bond formation of the four conserved cysteines, which bind a zinc ion at reduced states. Oxidation of the cysteines results in zinc release and concomitant unfolding of the redox-switch domain. Finally, Hsp33 becomes dimerized with exposing hydrophobic surfaces that bind folding intermediates as substrates. It has been also suggested that the activated Hsp33 can adopt high-order oligomers which have more potent chaperone activity than the dimeric form.[5] In addition, once it binds substrates, reduction of Hsp33 is not sufficient for its substrate release and dissociation into inactive monomers.[6] Thus, a reduced dimer species of Hsp33 can be stably formed until other chaperone systems start to act.[6,7]

The first crystal structures of *E. coli* Hsp33 were solved as a domain-swapped dimer, which lacks the C-terminal redox-switch domain, due to truncation during crystallization.[8,9] Then, the

structure of the redox-switch domain itself was determined by NMR.[10] Finally, intact form structures were solved on *B. subtilis* Hsp33 and *T. maritina* Hsp33, by crystallography.[7,11] However, the intact form structure showed a dimeric conformation, which would not be physiologically relevant. Unfortunately, the structure of the inactive Hsp33 monomer has not been solved yet, and there remain many uncertainties and controversial arguments regarding the activation process of Hsp33 at structural level. Thus, we have initiated structural investigation of the inactive Hsp33 monomer in solution by NMR. To overcome its high molecular weight (33 kDa), NMR study of Hsp33 requires stable isotope labeling, including deuteration, and use of high-field NMR machine. In this paper, we report the results of NMR sample preparation, biochemical characterization, and preliminary NMR measurements of the *E. coli* Hsp33.

EXPERIMENTAL

The expression plasmid pUJ30 encoding Hsp33, which was a generous gift from Dr. U. Jakob (Univ. of Michigan, USA),[2] was transformed into *E. coli* BL21(DE3)pLysS. To prepare isotope-enriched protein ([^{13}C, ^{15}N, ^{2}H]Hsp33) for NMR, the M9 minimal medium was supplemented with [^{15}N]NH_4Cl and [^{13}C]glucose, as the sole source of nitrogen and carbon, respectively, and D_2O was used as sole solvent of the medium. Prior to large culture, the bacterial cells were adapted to D_2O, by consecutive culture and inoculation into the M9 media containing increasing (0%~100%) contents of D_2O. Finally, the Hsp33-expressing cells were grown at 37 ℃ in the isotope-enriched M9 medium

and harvested 6 hr after induction with 1 mM IPTG. 1 mM $ZnSO_4$ was added in the medium at the time of induction, to produce zinc-bound Hsp33. The harvested cells were disrupted by sonication, and from the supernatant, Hsp33 was purified via the sequential application of anion-exchange, adsorption (hydroxyapatite column), and gel-permeation chromatography. Finally, the purified solution was concentrated and buffer-exchanged by ultrafiltration. To keep Hsp33 as an inactive form (reduced, zinc-bound momoner), 5 mM dithiothreitol (DTT) and 1 mM $ZnSO_4$ was always contained in every buffer used. The correct mass of the purified Hsp33 was identified by the MALDI-TOF mass spectrometry.

Gel-permeation chromatography to analyze the apparent molecular weight of the purified Hsp33 was performed as described elsewhere.[12,13] Chaperone activity of the purified Hsp33 was checked by light scattering measurement during thermal aggregation of citrate synthase in the presence and absence of Hsp33, as described by Jakob *et al*.[2,14] Circular dichroism (CD) measurement was conducted on a Jasco J-715 spectropolarimeter at 20 °C, using a 0.2 cm path-length cell, with a bandwidth of 1 nm and a response time of 4 s. NMR spectra were acquired on a Bruker Biospin Avance 900 spectrometer equipped with a cryoprobe. 2D-[^{1}H, ^{15}N]TROSY and the following TROSY-based triple resonance spectra were recorded on the 0.6 mM of [^{13}C, ^{15}N, ^{2}H]Hsp33: HNCA, HN(CO)CA, HNCACB, HN(CO)CACB, HNCO, and HN(CA)CO. All NMR spectra were processed using NMRPipe/NMRDraw software and analyzed with NMRView program.

RESULTS AND DISCUSSION

The main purpose of this study was to obtain NMR spectra of Hsp33 at its inactive state, which corresponds to a reduced, zinc-bound, monomeric form. Upon oxidation or without binding zinc, Hsp33 undergoes significant extent of unfolding. Thus, to make sure the inactive monomer state of our Hsp33 sample, we first checked its far-UV CD spectrum (Figure 1A), which indicated a well-folded conformation. Then, the apparent molecular weight, analyzed by gel-permeation chromatography, was approximately 38 kDa, indicative of monomeric state (Figure 1B). Finally, the purified Hsp33 showed no significant chaperone activity against thermal denaturation of citrate synthase (Figure 1, inset). Taken all together, our purified Hsp33 could be concluded as an inactive, monomeric form.

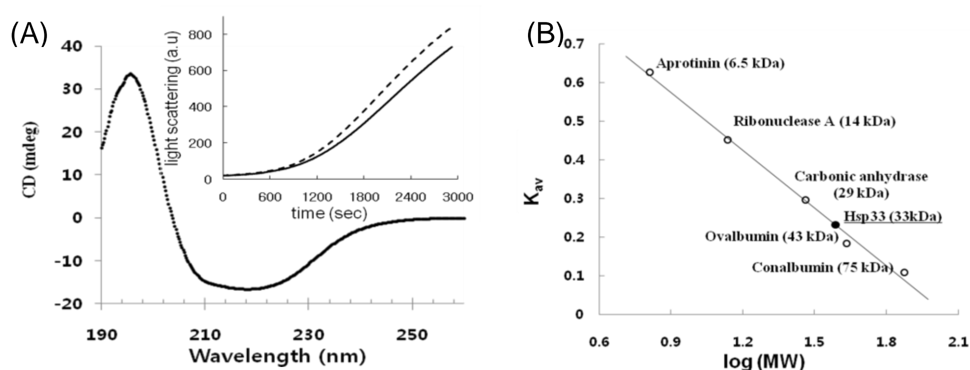

Figure. 1. (A) Far-UV CD spectrum of the 5 M of purified Hsp33, in 20 mM Tris-HCl buffer at pH 7.4. Inset: Light scattering during thermal denaturation of citrate synthase at 43 ℃, in the presence (solid line) and in the absence (dashed line) of the purified Hsp33. (B) Gel-permeation

chromatography results assessing apparent molecular weight (open circles for protein standards used and the filled circle for Hsp33).

Despite high molecular weight (33 kDa) of Hsp33, good quality of NMR spectra could be obtained for its deuterated sample, on a 900 MHz NMR machine with a cryoprobe. Initial measurements in a phosphate buffer required relatively long duration of high power (90°) pulse, while under Tris-HCl buffer condition it could be shortened. Since both the two buffer conditions produced similar quality of spectra (data not shown), we employed the Tris-HCl as a standard buffer system for NMR measurements. Then, 2D-[^{1}H, ^{15}N]TROSY spectra of the [^{13}C, ^{15}N, ^{2}H]Hsp33 were measured at two different temperatures (298K and 313K) and at two different pH (pH 7.4 and 6.5). Spectral dispersion was not significantly affected by change pH and temperature (Figure 2).

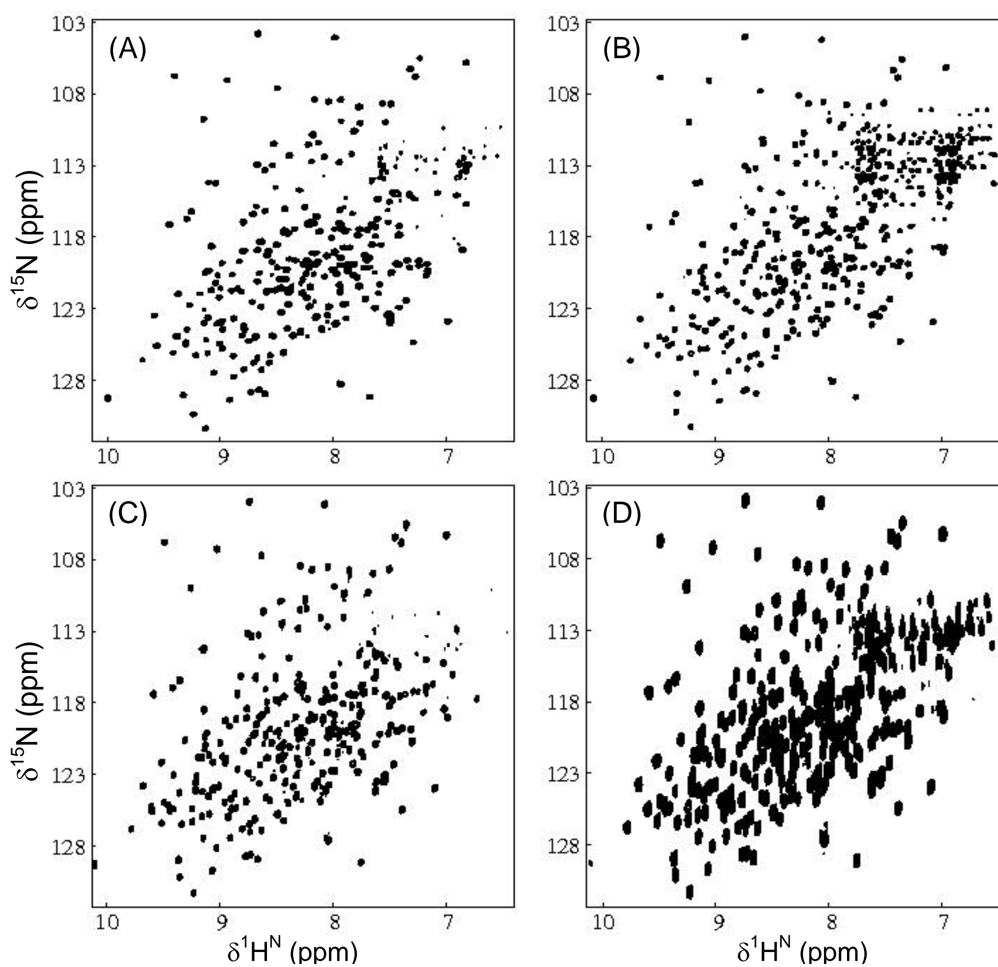

Figure. 2. 2D-[^{1}H, ^{15}N]TROSY (A-C) and 3D-HNCO (D) spectra of the [^{13}C, ^{15}N, ^{2}H]Hsp33 measured on a 900 MHz NMR machine with a cryoprobe. (A) at pH 7.4 and at 298 K; (B) at pH 7.4 and at 313 K; (C, D) at pH 6.5 and at 313 K. HNCO spectrum (D) is shown as a 2D projection along the ^{13}C axis.

Table 1. Number of backbone amide resonances observed in NMR spectra of [^{13}C, ^{15}N, ^{2}H]Hsp33.

pH	temperature	Number of backbone amide resonances observed	
		2D TROSY	3D HNCO
7.4	298 K	248	235
7.4	313 K	250	241
6.5	313 K	253	248

Then, the approximate number of backbone resonances detected was counted on the 2D-TROSY and the most sensitive triple resonance spectrum, 3D-HNCO (Table 1). Since Hsp33 possesses 294 amino acids including 14 prolines and the N-terminal residue, total 279 backbone amide resonances are expected from the spectra. Elevating temperature and/or lowering pH resulted in a slight increase of the number of peaks from backbone amide, in particular on the 3D triple resonance spectra. However, under all of the conditions tested, the number of peaks observed was quite less than that expected from the amino acid sequence of Hsp33; *i.e.* more than 25 possible signals are missing.

Although Hsp33, in respect of NMR, might be regarded as a huge (33 kDa) molecule with a short transverse relaxation time (T_2), we could expect that the size limitation, such as overall line broadening, was overcome by protein deuteration and the use of ultra-high magnetic field (900 MHz) and a cryoprobe. Indeed, judging from overall quality (dispersion, resolution, and linewidth) of the spectra, the missing resonances were not attributable to the large molecular size. Thus, alternatively, we postulate that a certain motional effect at specific regions would be attributable to a significant line broadening of their corresponding resonances. Conformational exchanges at the intermediate time scale (s ~ ms), thereby resulting in disappearance of NMR signals, are often found in many

proteins, particularly at specific regions of inactive form.[15,16] Recently, it has been suggested that a conformational equilibrium at linker domain of Hsp33 would be involved for kinetic response in the middle of activation process.[4,17] Similar to, but different with this model, our results imply that certain parts of inactive Hsp33 monomer in solution would be already undergoing a conformational exchange at the intermediate time scale. As an innate property of inactive Hsp33, such a motional dynamics would be thermodynamically favorable for dual sensing of heat and redox status, to drive conformational change. We expect that backbone NMR assignments using the present data set, which is currently in progress, could eventually identify the dynamic regulatory region of Hsp33.

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