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Verification of the interdomain contact site in the inactive monomer, and the domain-swapped fold in the active dimer of Hsp33 in solution

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ABSTRACT

Upon dimerization by oxidation, Hsp33 functions as a molecular chaperone in prokaryotes. Previously published structures of both the inactive and active species are of doubtful relevance to the solution conformations since the inactive (reduced) crystal structure was dimeric, while the active (oxidized) species was crystallized with a truncation of its regulation domain. The interdomain contact site of the inactive monomer, identified in this work, is consistent with that previously observed in the reduced dimer crystal. In contrast, fluorescence quenching of the active dimer contradicted the results expected from the domain-swapped fold observed in the truncated dimer crystal. The results of this study provide important new information concerning controversial issues in the activation process of Hsp33.
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1. Introduction

Hsp33 is a prokaryotic molecular chaperone that protect cells from severe oxidative stress [1,2]. While the expression of Hsp33 is regulated by heat-shock exposure, its activation is achieved at a post-translational level and depends upon the cellular redox potential. Activated Hsp33, in response to oxidative stress, exerts a holdase activity that binds the folding intermediates of substrate proteins and prevents their ultimate, irreversible denaturation. Under normal, reducing conditions, Hsp33 behaves as an inactive monomer and it adopts a dimerized structure upon oxidation. Mutational studies have suggested that dimerization is not a critical aspect in the process by which Hsp33 achieves chaperone functionality [3,4], but partially oxidized Hsp33, which retains in a monomeric state, is thought to be inactive [5,6]. Thus, the smallest unit of active, wild-type Hsp33 is an oxidized dimer, although oxidized Hsp33 can also form larger oligomers, which seem to exhibit more potent chaperone activity [7].

The activation process is triggered by oxidation of the C-terminal domain, a zinc-containing, redox switch [8]. Upon oxidation, four conserved cysteines that coordinate the zinc ion form two disulfide bonds, thereby releasing the zinc ion. Releasing the zinc ion results in the unfolding of the redox-switch domain [9], which is followed by dimerization of the entire protein and the exposure of a hydrophobic substrate-binding site [4,5]. The crystal structure of oxidized *Escherichia coli* Hsp33 (*Ec*Hsp33), in which the redox-switch domain had been truncated during crystallization, showed a domain-swapped dimer fold (Fig. 1A) [10,11]. The crystal structures of intact, reduced Hsp33 from *Bacillus subtilis* (*Bs*Hsp33) [12] and from *Thermotoga maritima* [13] provided important information on the interdomain contact site of the redox-switch domain. However, working models of the Hsp33 activation process are still the subject of much controversy, as the crystal structures of both active and inactive species contain features that conflict with biochemical findings in solution. First, the domain-swapped fold observed in the oxidized dimer crystal has not been observed in solution or in the intact protein crystal [14], and conflicts with a recent model for Hsp33 activation, which suggests that the domain-swapped region unfolds [5]. Second, the crystal structure of the reduced form was dimerized (Fig. 1B), whereas the protein is a monomer in its inactive state in solution [15,16]. Since a

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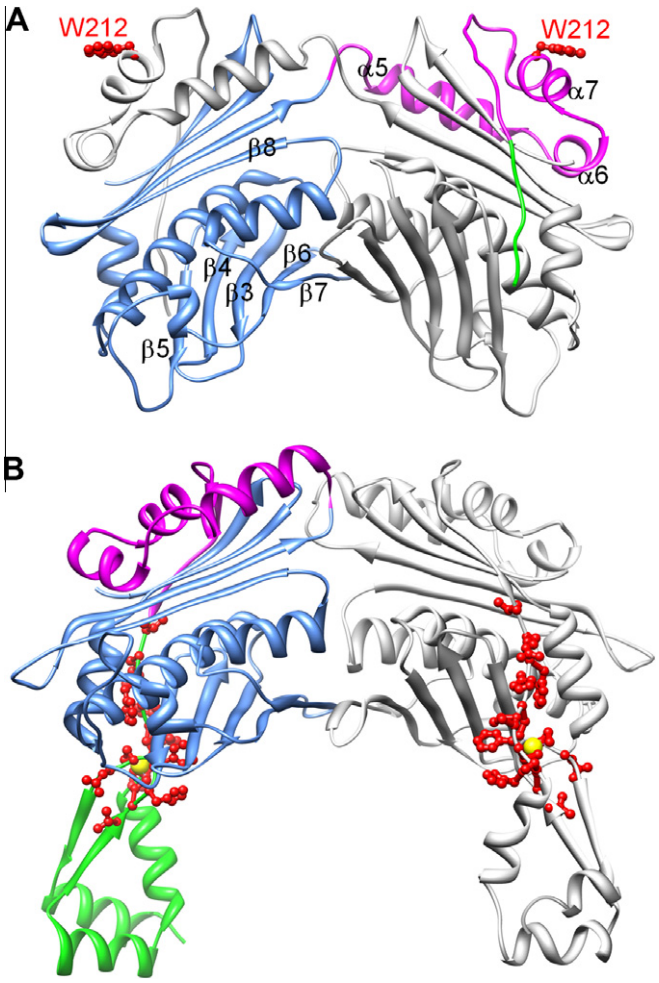

Fig. 1. Domain organization in the crystal structures of Hsp33 from *E. coli* (A) and from *B. subtilis* (B) (PDB code 1I7F and 1VZY, respectively; drawn using UCSF Chimera program [22]). Each domain of one polypeptide chain in a dimer is colored differently: blue, magenta, and green for the N-terminal core domain, middle linker domain, and the C-terminal redox-switch domain, respectively. The zinc ion is depicted as a yellow ball. Sidechains of W212 residues (A) and the interdomain contact site of the redox-switch domain (B), identified in this study, are presented in red.

reduced, but active dimer species can be formed *in vivo* via the reduction of substrate-bound Hsp33 [12,17], the domain contact site in the reduced Hsp33 dimer crystal cannot be conclusively said to be representative of the inactive monomer in solution. In this study, we aimed to clarify these doubtful aspects of the crystal structures of active and inactive Hsp33: the interdomain-contact site in the inactive monomer and the domain-swapped fold in the active dimer. The present results in solution validate those found previously in regard to the inactive monomer but contradicted those found in regard to the active dimer, providing useful information to inspect current working models of the activation process of Hsp33.

2. Materials and methods

2.1. Protein preparation

Recombinant plasmids for protein expression [2,9] were generously provided by Drs. Ursula Jakob (University of Michigan, USA) and H. Jane Dyson (The Scripps Research Institute, USA). Both the redox-switch domain (residues 227–287) and full-length (residues 1–294) *Ec*Hsp33 were prepared in their reduced, zinc-bound forms as described previously [9,16]. After oxidation by H_2O_2, the three kinds of oxidized *Ec*Hsp33 species were separated and identified (refer to the Supplementary material): an oxidized oligomer, an oxidized dimer, and a half-oxidized monomer. For NMR, isotope-enriched proteins were also prepared in their reduced, zinc-bound forms, as described previously [9,16]: [^{15}N] *Ec*Hsp33(227–287) and [^{2}H, ^{13}C, ^{15}N] *Ec*Hsp33(1–294).

2.2. NMR spectroscopy

NMR spectra of 0.6 mM protein dissolved in a 20 mM Tris–HCl buffer (pH 7.4) containing 50 mM NaCl, 5 mM DTT, and 50 μM $ZnSO_4$ were obtained at 298 K on a Bruker Biospin Avance 900 spectrometer equipped with a cryoprobe. Conventional 2D-[^{1}H, ^{15}N]-TROSY spectra were recorded for both the full-length *Ec*Hsp33 and *Ec*Hsp33(227–287). 3D-HNCA and HN(CO)CA spectra of the full-length *Ec*Hsp33 were measured in a TROSY type. Chemical shifts were referenced directly to DSS for ^{1}H and indirectly for ^{15}N and ^{13}C atoms using the chemical shift ratios suggested in the BMRB (http://www.bmrb.wisc.edu). Chemical shift perturbations were evaluated as their weighted average variations (Δ_{AVE}), using the $^{1}H^{N}$ ($\Delta\delta_H$) and $^{15}N^{\alpha}$ ($\Delta\delta_N$) chemical shifts [18]: $\Delta_{AVE} = [(\Delta\delta_H^2 + \Delta\delta_N^2/25)/2]^{1/2}$.

2.3. Fluorescence spectroscopy

Fluorescence of 5 μM *Ec*Hsp33 dissolved in 40 mM HEPES-KOH buffer (pH 7.5) was monitored using a Varian Cary Eclipse fluorometer at 25 °C. An excitation wavelength of 295 nm was used with a 5 nm excitation bandwidth. Emission scans were taken from 300 to 450 nm, with a 10 nm emission bandwidth and a 0.5 nm data interval. Fluorescence emission was then quenched by the progressive addition (1 μl at a time) of a 5.5 M acrylamide to the 0.5 ml sample solution, to a final concentration of 0.1 M. Emission intensities at λ_{max} were acquired as a function of quencher concentration ([Q]) and fit to the Stern–Volmer equation [19]: $F_0/F = 1 + K_{SV}[Q]$, where F_0 and F are fluorescence intensities in the absence and presence of the quencher, and K_{SV} is the dynamic (Stern–Volmer) quenching constant.

3. Results

3.1. The interdomain contact site of the inactive monomer

The NMR structure of the isolated redox-switch domain, Hsp33(227–287) [9] is consistent with the crystal structures of full-length Hsp33. In this study, the 2D-[^{1}H, ^{15}N]-TROSY spectrum of *Ec*Hsp33(227–287) was compared with that of full-length (1-294) *Ec*Hsp33 (Fig. 2A). Resonances from the *Ec*Hsp33(227–287) were assigned according to previous assignment data (BMRB accession number 6332). Each of the shifted peaks in the full-length *Ec*Hsp33 spectrum was then traced through confirmation of the $^{13}C^{\alpha}$ resonance connectivity in the 3D-HNCA and HN(CO)CA spectra of full-length *Ec*Hsp33. Finally, except for the two N-terminal residues of the isolated redox-switch domain, D227 and V228, which could not be verified in the full-length *Ec*Hsp33 spectrum, all of the resonances from the redox-switch domain were unambiguously assigned in both constructs. Chemical shift perturbations (CSPs) were calculated for the assigned resonances and plotted along the amino acid sequence, as shown in Fig. 2B. It was reasonable to expect large CSPs at the N- and C-termini of *Ec*Hsp33(227–287) due to differences in the sizes of the two constructs: 227–287 vs. 1–294 residues. However, in addition to the terminal residues, we identified two specific regions with appreciable CSP (0.06 ppm or more): residues F230 to S235 and H264 to G269. The observed CSPs

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