Contributions of Cysteine Residues in Zn2 to Zinc Fingers and Thiol-Disulfide Oxidoreductase Activities of Chaperone DnaJ†

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ABSTRACT: *Escherichia coli* DnaJ, possessing both chaperone and thiol−disulfide oxidoreductase activities, is a homodimeric Hsp40 protein. Each subunit contains four copies of a sequence of -CXXCXGXG-, which coordinate with two Zn(II) ions to form an unusual topology of two C_4-type zinc fingers, $C^{144}DVC^{147}Zn(II)C^{197}NKC^{200}$ (Zn1) and $C^{161}PTC^{164}Zn(II)C^{183}PHC^{186}$ (Zn2). Studies on five DnaJ mutants with Cys in Zn2 replaced by His or Ser (C183H, C186H, C161H/C183H, C164H/183H, and C161S/C164S) reveal that substitutions of one or two Cys residues by His or Ser have little effect on the general conformation and association property of the molecule. Replacement of two Cys residues by His does not interfere with the zinc coordination. However, replacement of two Cys by Ser results in a significant decrease in the proportion of coordinated Zn(II), although the unique zinc finger topology is retained. The mutants of C183H, C186H, and C161S/C164S display full disulfide reductase activity of wild-type DnaJ, while C161H/C183H and C164H/183H exhibit severe defect in the activity. All of the mutations do not substantially affect the chaperone activity. The results indicate that the motif of -CXXC- is critical to form an active site and indispensable to the thiol−disulfide oxidoreductase activity of DnaJ. Each -CXXC- motif in Zn2 but not in Zn1 functions as an active site.

Escherichia coli DnaJ, a primary Hsp40 homologue, collaborates specifically with DnaK, an Hsp70 protein, and GrpE, a nucleotide exchange factor, to participate in many cellular processes, such as protein folding and transport, degradation of misfolded proteins, and bacteriophage DNA replication (*1*, *2*). DnaJ itself has also been reported to have autonomous DnaK-independent chaperone activity as revealed by its capacity to recognize nonnative proteins and prevent the aggregation of folding intermediates in vitro (*3*−*6*).

DnaJ is a homodimeric molecule, and each 41 kDa subunit with 376 amino acid residues is composed of four successive regions from the N-terminus representing potential functional domains: a J-domain, a glycine-rich region (G/F), a zinc finger-like domain, and a less conserved C-terminal domain as shown in Figure 1A (*7*). No three-dimensional structure of the entire DnaJ molecule has been reported so far. The zinc finger-like domain has been demonstrated to be involved in binding of unfolded polypeptide and preventing the aggregation of nonnative proteins (*4*). Apart from C^{265} and C^{323} in a reduced form, the other eight Cys residues in four repeated motifs of -CXXCXGXG- in the zinc finger-like domain have been identified to coordinate with two Zn(II) ions to form two C_4-type zinc fingers (*4*). The solution structure of the isolated zinc finger-like domain (G^{131}−S^{209}) solved by NMR has revealed a fold with an overall V-shaped extended β-hairpin topology and two symmetrical zinc fingers, $C^{144}DVC^{147}Zn(II)C^{197}NKC^{200}$ and $C^{161}PTC^{164}Zn(II)$-$C^{183}PHC^{186}$, designated as Zn1 and Zn2,[1] respectively (Figure 1B) (*8*). This is a novel zinc finger topology differing from that of all known cysteine-containing zinc-binding proteins in the Brookhaven Protein Data Bank. The unusual zinc finger topology in the isolated domain has been demonstrated to exist in the intact DnaJ molecule (*6*). Moreover, it has been shown that 6.4 M guanidine hydrochloride (GdnHCl) destroys only Zn1, which does not re-form after refolding; *p*-(hydroxymercuri)benzenesulfonate (PMPS), but not ethylenediaminetetraacetic acid (EDTA) even at high concentrations, can remove two Zn(II) ions from the molecule, but only Zn2 can be reconstituted (*6*).

As the eight Cys residues in two zinc fingers are all in a sequence of -CXXC-, which is a specific motif for the active site of thiol−disulfide oxidoreductases (*9*), we have demonstrated that DnaJ does show reductase activity and oxidase activity but little, if any, isomerase activity, and the reductase activity is reversibly inhibited by EDTA (*6*). It has also been suggested that Zn2 is important for enzymatic activity of

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[1] Abbreviations: Zn1, zinc finger $C^{144}DVC^{147}Zn(II)C^{197}NKC^{200}$; Zn2, zinc finger $C^{161}PTC^{164}Zn(II)C^{183}PHC^{186}$; GdnHCl, guanidine hydrochloride; PMPS, *p*-(hydroxymercuri)benzenesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PAR, 4-(2-pyridylazo)resorcinol; ANS, 8-anilino-1-naphthalenesulfonic acid; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase.

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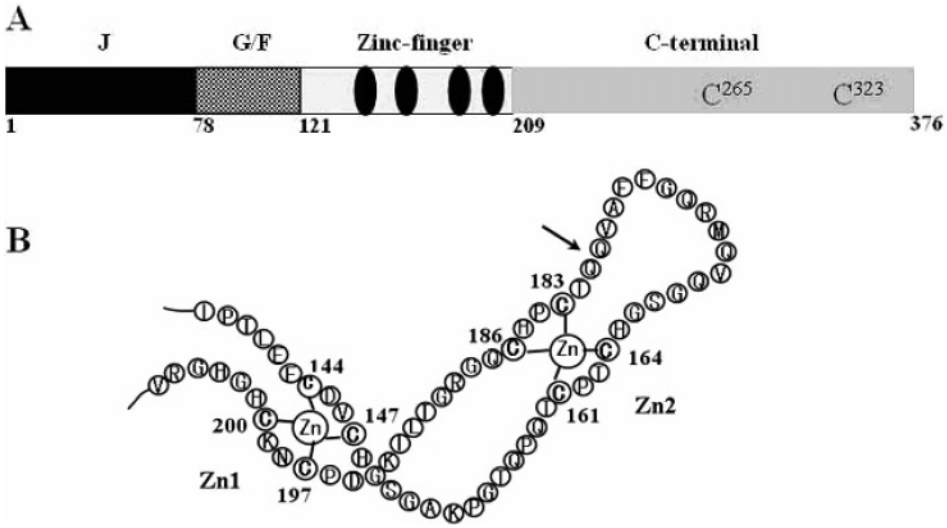

FIGURE 1: Schematic presentation of the domain structure and zinc finger topology of the *E. coli* DnaJ monomer. (A) Four consecutive domains: J-domain, glycine-rich region (G/F), zinc finger-like domain, and C-terminal domain. Black ovals represent the sequence of -CXXCXGXG- with C as Cys, G as Gly, and X as other amino acid residue (*7*). (B) Topology of the two zinc fingers in DnaJ. The arrow shows the cleavage site between Q^{180} and Q^{181} by proteinase K.

DnaJ, and -C^{183}PHC^{186}- among the four motifs of -CXXC- is sufficient for the enzyme function. The N-terminal half sequence (M^1−Q^{180}) and Zn1 are not required for the enzymatic activity but important for the chaperone activity of DnaJ (*6*).

In this report, we have prepared five DnaJ mutants with Cys in Zn2 replaced by Ser or His to further explore the contributions of Cys residues to zinc coordination and biological activities of DnaJ.

MATERIALS AND METHODS

Materials. GdnHCl, PMPS, 4-(2-pyridylazo)resorcinol (PAR), β-mercaptoethanol, 8-anilino-1-naphthalenesulfonic acid (ANS), and bovine serum albumin were Sigma products. pBluscript II SK(+) and *Escherichia coli* XL1-Blue strain were from Stratagene. Chelating Sepharose Fast Flow media and molecular mass markers were from Amersham Pharmacia Biotech, dithiothreitol (DTT) and isopropyl 1-thio-β-D-galactoside were from Serva, proteinase K and iodoacetic acid were from Merck, Tris was from Promega, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was from Fluka. Other chemicals were local products of analytical grade. Distilled water with resistivity of ≥18 MΩ/cm obtained using a Milli-Q water system was used for preparing buffer, which was then passed through a Chelex-100 column (Sigma) to remove adventitious metal contaminants (*10*). If not specified, 50 mM Tris-HCl buffer (pH 8.0) was used and referred to as Tris buffer.

Preparations. The expression plasmid pUHE21-2 containing the full-length *E. coli* DnaJ gene was generously donated by Dr. J. Buchner, Institut für Organische Chemie und Biochemie, TU München, Germany. The PCR fragments with *Bam*HI and *Pst*I sites at the 5′ and 3′ termini, respectively, were synthesized using pUHE-DnaJ as a template and ligated into pBluscript II SK(+). The full-length coding sequences of C161S/C164S, C183H, and C186H were confirmed with both strands and then subcloned into pUHE21-2 via the same restriction sites to create corresponding expression plasmids. The expression plasmids pQE701-C161H/C183H and pQE701-C164H/C183H were constructed by the same procedure as described above except using pUHE-C183H as the template for PCR fragment synthesis and pQE701 as the vector. The plasmid pQE701 is constructed from the original pQE70 by inserting a *Pst*I site. The overnight culture of the transformed XL1-Blue cells grown in 2 × YT media with 100 μg/mL ampicillin at 30 °C was diluted 100-fold and incubated for 6 h followed by addition of isopropyl 1-thio-β-D-galactoside to 0.1 mM and further incubation for 4 h. Wild-type DnaJ and the five mutant proteins were purified mainly according to Zylicz et al. (*11*). Refolded S-carboxymethylated DnaJ and refolded S-carboxymethylated C161S/C164S were prepared as previously described (*6*). All of the protein preparations showed one band on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and were stored with 10% glycerol at −80 °C. D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit muscle (*12*) was kindly provided by G. P. Ren in our group.

Determinations. Concentrations of DnaJ and its five mutants were determined by the Bradford method with bovine serum albumin as a standard (*13*). Concentrations of bovine serum albumin and GAPDH were determined spectrophotometrically at 280 nm with absorption coefficients ($A_{1cm}^{0.1\%}$) of 0.66 and 0.98, respectively. Homotetrameric GAPDH and homodimeric DnaJ were considered as protomers in the calculation of molar ratios. Thiol groups were determined with DTNB (*14*). Intrinsic and 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescence spectra were measured in a Shimadzu RF-5301 PC spectrofluorometer at 25 °C with 280 and 380 nm for excitation, respectively. CD spectra from 200 to 250 nm were determined in a Jasco J720 spectropolarimeter.

Protein degradation was examined by incubation of proteins at 1.0 mg/mL with proteinase K of 1.0 μg/mL in 50 mM Tris-HCl (pH 7.5) containing 5 mM $CaCl_2$ and 10 mM DTT at 25 °C for 60 min and analysis of the reaction mixture by SDS−12% PAGE.

Zinc content of DnaJ and the five mutants was determined by atomic absorption spectrophotometry on an Instrument Laboratory ICAP-9000 spectrometer and by titration with

PMPS according to Hunt et al. (*15*). The Zn(II) released by PMPS titration was determined by use of $\epsilon_{500nm} = 6.6 \times 10^4$ M^{-1} cm^{-1} for a complex of one released Zn(II) with two metallochromic indicator molecules, PAR. Zn(II) released from GdnHCl-denatured proteins was determined by immediately reading the increase of absorbance at 500 nm of a 1 mL reaction solution containing 0.1 mM PAR upon addition of 17 μL of protein at 300 μM, which has been denatured with 6 M GdnHCl at 20 °C for 20 h. The Zn(II) remaining in the refolded proteins after dilution into the reaction solution was further determined by PMPS titration.

Determination of Enzyme and Chaperone Activities. Reductase activity was assayed by measuring the turbidity increase at 650 nm due to insulin reduction (*16*) and was expressed as a ratio of the slope of a linear part of the turbidity curve to the lag time (*17*). Aggregation during the refolding of denatured GAPDH upon dilution in the presence of DnaJ mutants was followed continuously by 90° light scattering at 488 nm in a Shimadzu RF-5301 PC spectrofluorometer at 25 °C for 30 min until it reached a maximal level according to Tang and Wang (*6*) to measure the chaperone activity of the mutants.

RESULTS AND DISCUSSION

Mutation Design. It has been shown in our previous work (*6*) that -C^{183}PHC^{186}- among the four -CXXC- motifs is functional as an active site for the enzymatic activity. To further understand the roles of the other -CXXC- motifs in the catalytic activity of DnaJ and the contribution of Cys residues to the zinc coordination, we have prepared four DnaJ mutants, two single-site mutants of C183H and C186H and two double mutants of C161H/C183H and C164H/C183H. His, a residue participating in the formation of C_2H_2- or C_3H-type zinc fingers, was used to replace Cys for the sake of minimizing possible perturbation on Zn2 structure. As Zn2 is more stable than Zn1 (*6*, *8*), and C^{183} and C^{186}, together with C^{197} and C^{200}, can also coordinate with one Zn(II) to form a zinc finger in a C-terminal fragment of Q^{181}—R^{376} (*6*), we were curious whether the zinc finger topology in the DnaJ molecule could be changed to a conventional style if the structure of Zn2 was loosened or damaged. One more double mutant of C161S/C164S, which should not affect the integrity and the activity of the -C^{183}PHC^{186}- motif, has thus been prepared to look into the possibility. Noncoordinating Ser was chosen as a replacement for Cys owing to the only substitution of an oxygen atom for a sulfur atom.

Conformation of DnaJ Mutants. The DnaJ molecule contains no Trp but ten Tyr residues with six located at both sides flanking the zinc finger-like domain. As shown in Figure 2A, the intrinsic fluorescence spectra of the mutants of C161S/C164S, C183H, C186H, and C161H/C183H are very similar to that of DnaJ. The ANS fluorescence spectra of the four mutants show insignificant red shift in emission maximum and slight decrease in intensity compared to that of the wild-type protein (Figure 2B). The CD spectra of the four mutants are almost the same as that of the wild-type DnaJ (Figure 2C). In addition, each mutant was eluted as a single peak at the same position as that of wild-type DnaJ on a Superose 6 HR10/10 column (data not shown). All the above suggest that mutations at Cys^{161}/Cys^{164}, Cys^{183}, Cys^{186}, and Cys^{161}/Cys^{183} to Ser or His substantially affect neither the spatial structure and the surface hydrophobicity nor the association property of the DnaJ molecule.

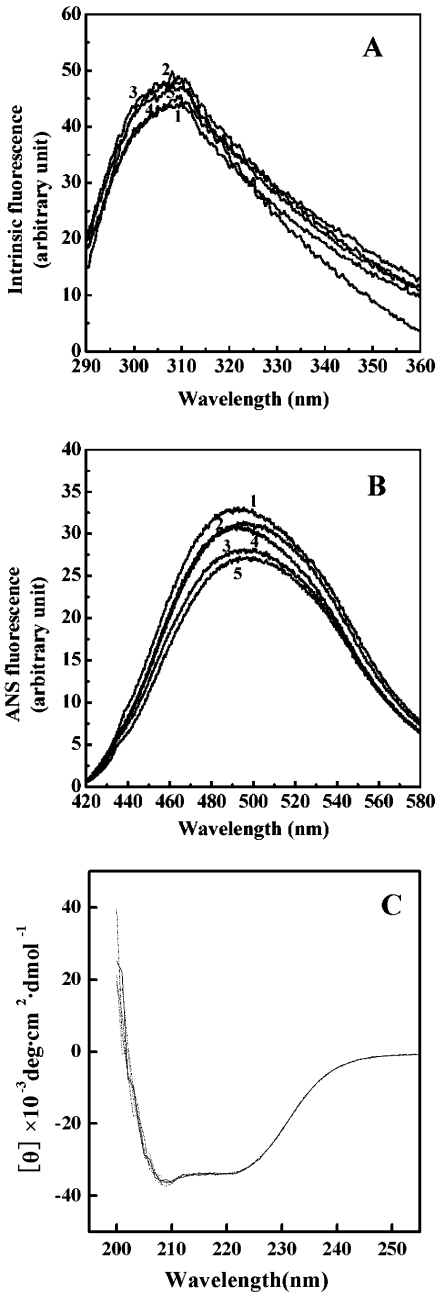

FIGURE 2: Conformation of DnaJ mutants. Intrinsic fluorescence spectra (A) and ANS fluorescence spectra (B) with excitation at 280 and 380 nm, respectively, were measured at 25 °C. Curve 1 is for wild-type DnaJ, and curves 2, 3, 4, and 5 are for C161S/C164S, C183H, C186H, and C161H/C183H, respectively. CD spectra (C) of DnaJ (solid line) and mutants were determined at 25 °C. Protein concentrations were 0.20 mg/mL in panels A and B and 0.6 mg/mL in panel C.

By proteinase K limited hydrolysis of DnaJ we identified a main cleavage site between Q^{180} and Q^{181} (*6*), and this site is located in the middle of strand β4 (*8*) just next to the Zn2. Therefore, limited hydrolysis by proteinase K was used to examine possible changes of the zinc fingers in the mutants. The digestion profiles of C161S/C164S, C183H, C186H, and C161H/C183H by proteinase K under the same conditions are very similar to that of wild-type DnaJ (data not shown), further indicating that substitution of one or two zinc-coordinated Cys residues in Zn2 by His or Ser has little effect

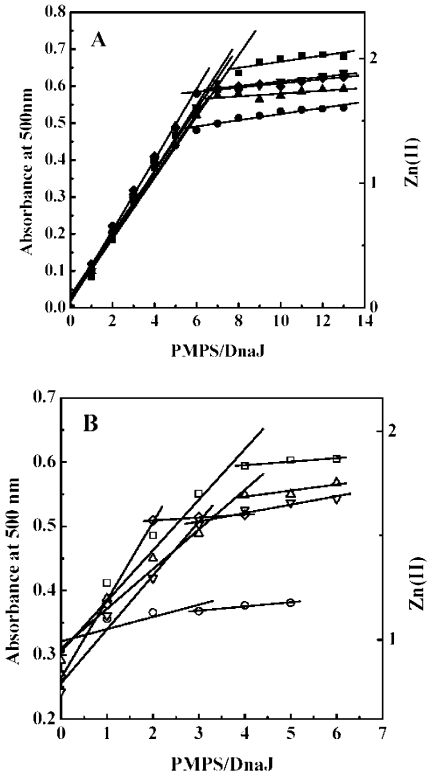

FIGURE 3: Determination of Zn(II) with PMPS. (A) Zn(II) determination in DnaJ and the mutants. The release of Zn(II) was monitored by following the increase of absorbance at 500 nm of a 1 mL reaction solution containing 0.1 mM PAR and 5.0 μM DnaJ (■), C161S/C164S (●), C183H (▲), C186H (▼), or C161H/C183H (◆) with stepwise addition of 1.0 mM PMPS in portions of 5 μL. (B) Determinations of Zn(II) released from GdnHCl-denatured proteins and remaining in refolded proteins (corresponding open symbols). The assay was carried out as described in the text. The first point of each line without addition of PMPS is for the Zn(II) released from GdnHCl-denatured protein. The right ordinate indicates the number of released Zn(II) calculated by use of the absorbance value at the end of the linear part of the titration curve and $\epsilon_{500nm} = 6.6 \times 10^4$ M^{-1} cm^{-1} for a complex of one released Zn(II) with two metallochromic indicator molecules, PAR.

on either the global conformation of the molecule or the local conformation of the Zn2.

Zinc Fingers of DnaJ Mutants. As shown in Figure 3A and Table 1, by PMPS titration the absorbance at 500 nm increases linearly with increasing concentrations of PMPS until the ratio of PMPS to DnaJ reaches 8, 7 for C183H and C186H, and 6 for C161H/C183H, indicating that almost two Zn(II) ions (1.9, 1.7, 1.8, and 1.8, respectively) are released from one subunit of each mutant. Similar values of Zn(II) content were also determined by the method of atomic absorption. Taking the measured data of DnaJ as two Zn(II) ions, the calibrated Zn(II) number is 1.8 and 2.0 for C183H; 1.9 and 1.8 for C186H; and 1.9 and 2.0 for C161H/C183H by PMPS titration and by atomic absorption, respectively, indicating that one or even two Cys residues in the Zn2 can be replaced by His without weakening the coordination of Zn(II). However, markedly less Zn(II), 1.4 and 1.3, was measured in the mutant C161S/C164S by the two methods, respectively, suggesting that the replacement of two Cys residues by noncoordinating Ser results in much weaker zinc coordination in Zn2. Szabo et al. reported only 0.7 Zn(II) for the same mutant of C161S/C164S by PMPS titration and ascribed it to the damage of the N-terminal zinc finger according to the conventional topology of zinc fingers (*4*).

As shown in Table 1 the refolded S-carboxymethylated DnaJ contained 1.1 Zn(II) (Figure 4A), while the refolded modified C161S/C164S showed only 0.2 Zn(II) (Figure 4B). If the zinc coordination in the C161S/C164S mutant changed to so-called "conventional" topology as an N- and a C-terminal zinc finger, the refolded S-carboxymethylated C161S/C164S should contain one Zn(II) at least (Figure 4C). The present results indicate that the C161S/C164S mutant will still adopt the same zinc finger topology as in the wild-type DnaJ although only two Cys residues, C^{183} and C^{186}, coordinate with Zn(II) ion to form a much weaker Zn2.

Direct reaction with the metallochromic indicator reagent PAR showed that 1.0 Zn(II) was released from GdnHCl-denatured DnaJ, and 1.2, 1.1, 0.9, and 1.0 Zn(II) from the denatured forms of C161S/C164S, C183H, C186H, and C161H/C183H, respectively (Figure 3B and Table 1). Further PMPS titration of the refolded proteins showed that the refolded forms of DnaJ, C183H, C186H, and C161H/C183H all contain one Zn(II), while refolded C161S/C164S contained only 0.2 Zn(II). It is noticed that in each individual experiment such as the one shown in Figure 3B the sum of the values of Zn(II) released from denatured protein and detected in the refolded protein is always close to that determined in the corresponding native protein. In the three His-substituted mutants, as in the wild-type DnaJ, the Zn2 is more stable than Zn1 and is not destroyed by 6 M GdnHCl (Table 1). However, the weakly coordinated Zn(II) in the Zn2 of the C161S/C164S mutant seemed to be released by 6 M GdnHCl to a certain extent so that only 0.2 but not 0.4 Zn(II) was detected in the refolded mutant (Figure 3B and Table 1).

Like the wild-type DnaJ (*6*), C183H, C186H, and C161H/C183H exhibit two thiol groups and six (four from Zn1 and two from the non-zinc-finger thiols, C^{265} and C^{323}) when denatured with 6.4 M GdnHCl detected by DTNB (Table 1). The mutant C161S/C164S with a weaker Zn2 also shows six but not more thiol groups when denatured with 6.4 M GdnHCl. Two possibilities might be involved in this process: (1) part of C^{183} and C^{186} coordinates with Zn(II) so that not detected by DTNB, that is somehow similar to the case of wild-type DnaJ; (2) C^{183} and C^{186} were oxidized to form a disulfide bond if not coordinated with Zn(II) (refer to Figure 4B). It has been demonstrated (*6*) that C^{144} and C^{147} are more resistant to oxidation than the other six Cys residues, which are oxidized to form disulfides following the release of Zn(II).

A mutant of HIV-1 nucleocapsid protein, which contains a conventional N- and a C-terminal C_3H-type zinc finger in the same -$CX_2CX_4HX_4C$- motif, with one Zn(II)-coordinating residue in the C-terminal zinc finger substituted by a noncoordinating Ser or Ala, was reported to bind Zn(II) with the same high affinity, but the retained tetrahedral coordination of the zinc finger was suggested to be through a four-coordinate geometry with the vacant ligand position occupied by water molecules (*18*). It has been reported that the replacement of His23 by Cys (*19*) or Cys28 by His (*20*) in the N-terminal zinc finger of HIV-1 nucleocapsid protein caused large structural changes around the metal atom and the proximity of the two fingers. The protein became a stable and highly constrained structure from an unfolded molecule when Zn(II) is rebound (*21*). Differently, the secondary

Table 1: Thiols, Zn(II), and Reductase Activity of DnaJ and the Mutants[a]

protein	thiol		Zn(II) (mol/mol)				reductase activity ($\times 10^{-4}\Delta A_{650nm}$·min^{-2})
			PMPS titration			atomic absorption, native	
	alone	in 6.4 M GdnHCl	native	released from GdnHCl-denatured proteins	refolded proteins		
DnaJ	1.8 ± 0.2	6.2 ± 0.2	1.9 ± 0.2 (2.0)	0.8[b] (1)	1.0[b]	1.74[b] (2.0)	1.5 ± 0.2
C161S/C164S	2.2 ± 0.2	5.8 ± 0.1	1.3 ± 0.3 (1.4)	1.0[b] (1.2)	0.2[b]	1.09[b] (1.3)	1.4 ± 0.2
C183H	1.7 ± 0.1	6.1 ± 0.2	1.7 ± 0.2 (1.8)	0.9[b] (1.1)	0.8[b]	1.73[b] (2.0)	1.5 ± 0.2
C186H	2.1 ± 0.2	5.9 ± 0.3	1.8 ± 0.2 (1.9)	0.7[b] (0.9)	0.8[b]	1.53[b] (1.8)	1.4 ± 0.2
C161H/C183H	1.8 ± 0.2	5.6 ± 0.1	1.8 ± 0.1 (1.9)	0.8[b] (1)	0.8[b]	1.73[b] (2.0)	0.03 ± 0.01
refolded S-carboxymethylated DnaJ		0.2 ± 0.1	1.1[b]				
refolded S-carboxymethylated C161S/C164S		0.3 ± 0.1	0.2[b]				

[a] Data for each monomer are expressed as means ± SD ($n \geq 3$). [b] Average of two independent experiments. The calibrated Zn(II) numbers taking the measured Zn(II) of DnaJ as 2 are shown in parentheses.

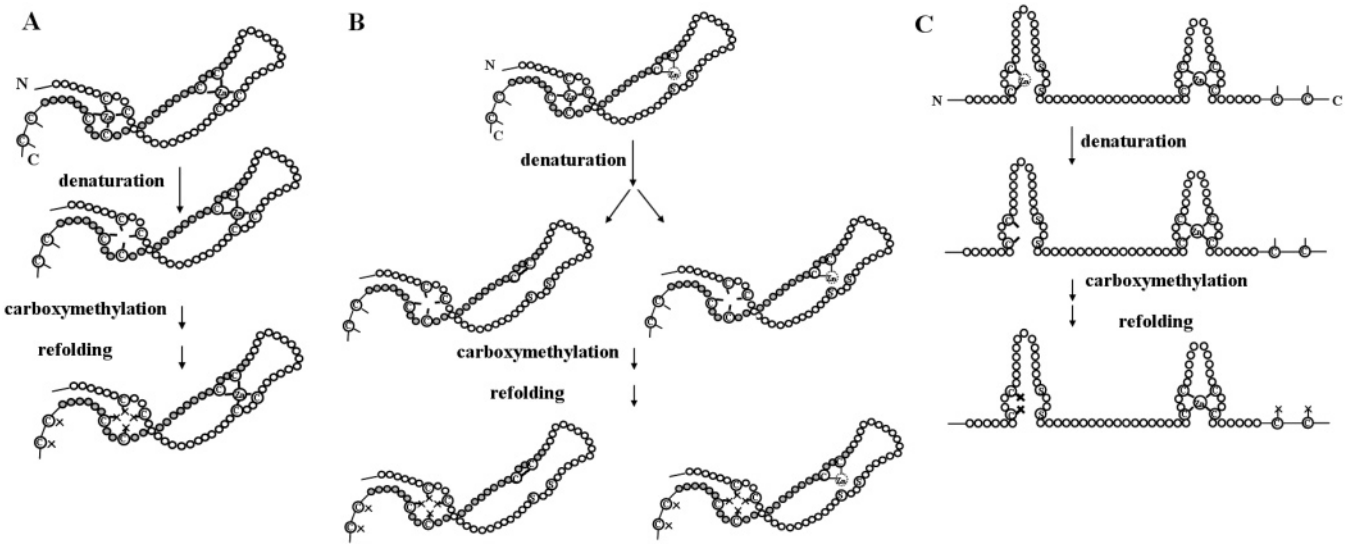

FIGURE 4: Identification of the topology of the two zinc fingers in the C161S/C164S monomer. DnaJ (A) or C161S/C164S (B) at 300 μM was denatured with 6 M GdnHCl and 2.0 mM DTT at 20 °C for 20 h and then carboxymethylated at 30 °C for 90 min by addition of iodoacetic acid to 60 mM and adjustment of pH to 8.0. The denatured and modified proteins were refolded for 17 h at 4 °C by 25-fold dilution and then concentrated and dialyzed against Tris buffer containing 0.1 M NaCl to remove adventitious Zn(II). The results were expressed using the current model of zinc fingers (*8*) as shown in Figure 1B (A and B) and the conventional model (*4*) (C). X represents the S-carboxymethylated group. The weakly coordinated Zn(II) in Zn2 of C161S/C164S is enclosed with a dotted circle (B).

structure, the tertiary structure, and the association property of DnaJ do not change when one or even two Zn(II) ions are removed (*6*), and the Zn2 with two Cys replaced by Ser coordinates with Zn(II) much more weakly but keeps the unique topology of the original Zn2 with little effect on either the global conformation of the molecule or the local conformation of the Zn2.

The NMR structure analysis of the isolated zinc finger-like domain (*8*) clearly showed that the fold of the domain is zinc-dependent. Under reducing condition the domain is unfolded in the absence of Zn(II). As zinc is added, the Zn2 folds first and independently of the Zn1. The unique and conserved topological structure of the zinc finger domain is encoded by the highly conserved sequence, so that the domain forms an stable autonomous folding unit, which is maintained in the isolated domain even though there are no longer any interactions with other domains of the molecule. Nevertheless, the two zinc fingers in the isolated zinc finger-like domain appear to be less stable than in the intact molecule. Zn(II) titration can be readily reversed upon the addition of excess EDTA in the former while Zn(II) in the latter cannot be removed by an excess of EDTA. The isolated zinc finger-like domain is completely unstructured in the absence of Zn(II), while both the conformation and the association properties of DnaJ do not change substantially after the removal of two Zn(II) ions (*6*) or the replacement of two Cys residues by Ser to make a weakly coordinated Zn2. The presence of the other three domains in the intact DnaJ molecule obviously enhances the stability of the zinc fingers and the whole molecule.

Enzymatic Activity of DnaJ Mutants. The zinc finger domain of DnaJ contains four -CXXC- motifs, among which -C^{183}PHC^{186}- and -C^{161}PTC^{164}- are identical to the active site of DsbA (*22*) and DsbE (*9*), respectively. Only -C^{183}PHC^{186}- was suggested to function as the active site of the enzyme, as the other three were demonstrated to be not required (*6*). It has been suggested that both cysteine residues in the -CXXC- active site motif are required for thiol−disulfide oxidoreductases to catalyze reduction or oxidation of disulfide (*23*). Moreover, mutations at either one of the two Cys residues in -CGHC- for PDI and in -CGYC- for DsbC have been reported to decrease the activity of catalyzing reduction and isomerization of the disulfide bond (*24*−*26*). Therefore, it was unexpected that the mutants of C183H and C186H

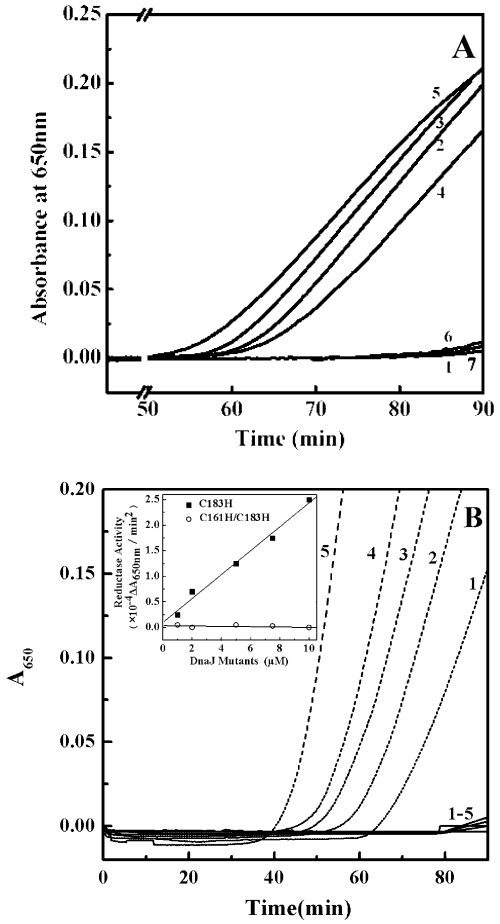

FIGURE 5: Thiol−disulfide reductase activity of DnaJ mutants. (A) The assay was carried out by addition of 1 mM DTT into 0.1 M potassium phosphate, pH 6.6, containing 0.13 mM bovine insulin in the absence (curve 1) and presence of DnaJ (curve 2), C161S/C164S (curve 3), C183H (curve 4), C186H (curve 5), C161H/C183H (curve 6), or C164H/C183H (curve 7) of 5 μM, and the absorbance at 650 nm resulting from the reduction of insulin was followed immediately. The data represent one assay. (B) Dependence of reductase activity on the concentration of C183H (dashed line) and C161H/C183H (solid line). The assay was carried out in the same way as described in (A) except the concentration of proteins was 1 μM (curve 1), 2 μM (curve 2), 5 μM (curve 3), 7.5 μM (curve 4), or 10 μM (curve 5), respectively. The inset shows the linear dependence of the activity on protein concentration.

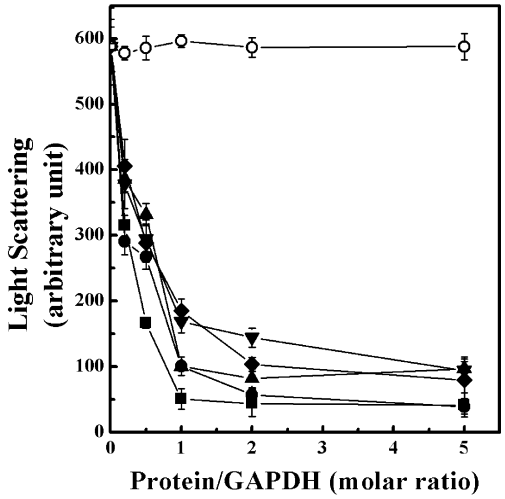

FIGURE 6: Effects of DnaJ mutants on the eventual level of aggregation of denatured GAPDH during refolding. GAPDH at 125 μM was denatured overnight in Tris buffer containing 3 M GdnHCl and 5 mM DTT at 4 °C. The refolding was initiated by 100-fold dilution of the denatured enzyme into 50 mM Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 1 mM DTT at 25 °C in the presence of DnaJ (■), C161S/C164S (●), C183H (▲), C186H (▼), C161H/C183H (◆), and bovine serum albumin as a negative control (○) at different molar ratios. Aggregation during refolding of denatured GAPDH was followed continuously by 90° light scattering at 488 nm for 30 min after dilution. The data were expressed as the mean ± SD (n = 3).

retain full reductase activity of DnaJ (Figure 5A and Table 1). The present results suggest two possibilities: first, the motif of -C^{161}PTC^{164}- could function as an active site independently; second, C^{183} or C^{186} with either C^{161} or C^{164} could reconstitute a thiol pair to function as a new active site. Mutant C161H/C183H with Cys in different -CXXC- motif mutated was thus prepared to test the above two possibilities. The result that C161H/C183H exhibits little reductase activity (Figure 5A and Table 1) favors the first possibility, i.e., not only the 183/186 but also the 161/164 thiol pair can function as an activity site. The two Cys residues not in the -CXXC- motif are unable to form an active site. To make further confirmation, we prepared one more mutant, C164H/C183H, which also showed little reductase activity as C161H/C183H does. In these two mutants of C164H/C183H and C161H/C183H, both of the -CXXC- motifs were destroyed, and the mutants showed little reductase activity, demonstrating that the remaining C^{164} and C^{186} in C161H/C183H and C^{161} and C^{186} in C161H/C183H do not form the active site of oxidoreductase. The motif C^{183}XXC^{186} in C161S/C164S and the motif C^{161}XXC^{164} in C183H and C186H is still intact and functions as an active site indeed, and the oxidoreductase activity of these three mutants was therefore not affected. The above led to the conclusion that the -CXXC- motif of DnaJ is critical to form an active site and indispensable for the oxidoreductase activity, but two Cys not in the -CXXC- motif do not form an active site.

Moreover, the little reductase activity of C161H/C183H and C164H/C183H mutants indicated that either one -CXXC- motif in Zn1 does not function as an activity site.

As shown in Figure 5B the reductase activity of mutants (taking C183H as an example for a fully active mutant and C161H/C183H for an inactive mutant) at five different concentrations in a range of 1−10 μM increased proportionally with increasing protein concentration, showing the validity of the turbidimetric assay at the protein concentrations used in this work.

Chaperone Activity of DnaJ Mutants. The zinc finger-like domain has been suggested to be involved in the binding of unfolded polypeptides (*4*). The NMR structure of the zinc finger-like domain revealed that the domain adopts an extended V-shaped structure, with two separate zinc-binding modules, one in each "wing" of the domain. A pronounced groove within the base of the "V", near the Zn1 binding site, has been suggested to represent a potential binding site for unfolded peptides or proteins (*8*). As shown in Figure 6 the presence of wild-type DnaJ at a molar ratio to GAPDH of 1 efficiently suppresses the strong aggregation of denatured GAPDH during refolding. C183H, C186H, C161S/C164S, and C161H/C183H show a similar capacity of the wild type in preventing the aggregation. Bovine serum albumin as a negative control has no ability to prevent the

aggregation of GAPDH. The above indicate that mutations at Cys^{161}/Cys^{164}, Cys^{183}, Cys^{186}, or Cys^{161}/Cys^{183} do not substantially affect their chaperone activity. However, Szabo et al. reported a 10-fold decreased capacity of the mutants C161S/C164S and C183S/C186S to prevent rhodanese aggregation during refolding compared to that of the wild-type DnaJ (*4*).

The conserved sequence of -CXXCXGXG- encodes the highly conserved and specific structural motif of DnaJ-like chaperones that must be related to special biological functions of this multifunctional DnaJ protein (*8*). DnaJ shows not only DnaK-dependent chaperone activity and autonomous DnaK-independent chaperone activity as well but also thiol−disulfide oxidoreductase activity (*6*). The Zn1 is important for the DnaK-independent chaperone activity and is not required for enzyme activity (*6*), while the Zn2 is essential for in vivo function in the DnaJ/DnaK/GrpE system (*27*) with the -CXXC- motif necessary for the enzyme activity.

CONCLUSION

(1) The replacement of two Cys residues by noncoordinating Ser in Zn2 results in much weaker zinc coordination but does not destroy the zinc finger. The unusual structure of the DnaJ zinc fingers is very stable.

(2) The two Cys residues only in the -CXXC- motif function as an active site of DnaJ.

(3) Both $-C^{183}PHC^{186}-$ and $-C^{161}PTC^{164}-$ in Zn2, but not -CXXC- motifs in Zn1, function as an active site independently.

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