Zinc Fingers and Thiol–Disulfide Oxidoreductase Activities of Chaperone DnaJ
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ABSTRACT: Chaperone DnaJ is a homodimer with each subunit containing 10 cysteine residues and two Zn(II) ions, which have been identified to form two zinc fingers, C144DVC147Zn(I)C197NK(II)C200 (Zn1) and C161PTC164Zn(II)C183PHC186 (Zn2), with C285 and C323 in reduced form. Guanidine hydrochloride at 6.4 M destroys only Zn1, which does not reform after refolding. p-Hydroxymercaptoacetic acid, but not ethylenediaminetetraacetic acid (EDTA) even at high concentrations, can remove two Zn(II) ions from DnaJ, but only Zn2 can be reconstituted. After removal of Zn(II) ions, only C144 and C147 in Zn1 are oxidation-resistant, and the other six cysteines are easily oxidizable. DnaJ shows reductase activity and oxidase activity but little, if any, isomerase activity. The reductase activity is reversibly inhibited by EDTA. Zn2 is important for the enzymatic activity, and only -C183PHC186, among the four motifs of -CXXC- functions as the active site of the enzyme. A C-terminal (Q181–R376) fragment shows a zinc finger of C183PHC186Zn(II)C197NK(II)C200 and full enzymatic activity of DnaJ. The N-terminal half sequence (M1–Q180) and Zn1 are not required for the enzymatic activity but are important for the chaperone activity of DnaJ.

Escherichia coli DnaJ, a primary Hsp40 homologue, collaborates specifically with DnaK, a Hsp70 protein, and GrpE, a nucleotide exchange factor, to participate in many cellular processes, such as protein folding, protein transport, degradation of misfolded proteins, and bacteriophage DNA replication (1, 2). DnaJ has also been reported to possess chaperone activity as revealed by its capacity to recognize nonnative proteins and prevent the aggregation of folding intermediates in vitro (3–5). In addition, DnaJ was reported to be able to catalyze the formation, reduction, and isomerization of disulfide bonds such as protein disulfide isomerase (PDI) (6); however, since the initial report, no more information about its enzymatic activities has been published.

Sequence analysis suggests that DnaJ homologues from various species all contain one or more domains originally identified from E. coli DnaJ. E. coli DnaJ is a homodimer, and each 41kDa subunit with 376 amino acid residues is composed of four successive domains from the N-terminus: a conserved J-domain responsible for stimulating the ATPase activity of DnaK, a glycine-rich region (G/F) in conjunction with J-domain for productive interaction with DnaK, a zinc finger-like domain, and a less conserved C-terminal domain for substrate binding as shown in Figure 1A (7). On the basis of the studies of recombinant fragments of (M1–G78), (M1–G104), (M1–E127), (M1–S209), (M121–R376), and (S209–R376), the zinc finger-like domain has been considered to be required for DnaJ to specifically recognize and bind with proteins in denatured states (4). The DnaJ subunit contains 10 cysteine residues and two Zn(II) ions. Apart from C285 and C323, the other eight cysteine residues in coordination with two Zn(II) ions have been proposed to form two zinc fingers of C144DVC147Zn(II)C197NK(II)C200 and C161PTC164Zn(II)C183PHC186, Zn(II)C197NK(II)C200, which closely resemble a C4-type zinc binding motif of some DNA-binding proteins as shown in Figure 1B (4). Recently the solution structure of the isolated cysteine-rich domain (G131–S209) solved by NMR has revealed a fold with an overall V-shaped extended B-hairpin topology and two symmetrical zinc fingers, C144DVC147, Zn(II)C197NK(II)C200 and C161PTC164Zn(II)C183PHC186, designated as Zn1 and Zn2, respectively (8). Coincidently, the eight cysteine 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). No three-dimensional structure of the entire DnaJ molecule has been reported so far.

To our knowledge, although the chaperone activity of DnaJ has been studied extensively (10, 11), its enzymatic activities and properties of the two zinc fingers are less explored. In this communication we have characterized for the first time the unusual topology and properties of the two zinc fingers in the intact DnaJ molecule, and we have shown that DnaJ
has EDTA-inhibited reductase activity but little, if any, isomerase activity.

EXPERIMENTAL PROCEDURES

**Materials.** Guanidine hydrochloride (GdnHCl), 4-hydroxy-mercuriphenylsulfonic acid (PMPS), 4-(2-pyridylazo)resorcinol, cyclic 2',3'-cytidine monophosphate, imidazole, β-mercaptoethanol, bovine serum albumin (BSA), bovine pancreatic trypsin inhibitor (BPTI), RNase A, and yeast RNA were Sigma products. Dithiothreitol (DTT), reduced glutathione (GSH), disulfide-linked glutathione (GSSG), and isopropyl 1-thio-β-D-galactoside were from Serva, proteinase K and iodoacetic acid were from Merck, Tris was from Promega, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was from Fluka, and molecular mass markers were from Amersham Pharmacia Biotech. Other chemicals were local products of analytical grade. Distilled water with resistivity of ≥ 18 MΩ cm obtained from 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 (12). 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 full-length *Escherichia coli* DnaJ gene was generously given by Dr. J. Buchner, Institut für Organische Chemie und Biochemie, TU Muenchen, Germany. DnaJ was expressed and purified mainly according to Zylicz et al. (13) and then stored with 10% glycerol at -80 °C or lyophilized. The purified DnaJ either frozen or lyophilized is soluble even in the absence of Zn(II) and showed one band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

S-Carboxymethylated DnaJ was prepared by reduction of 34 μM DnaJ with 0.5 mM DTT in Tris buffer at 25 °C for 30 min and then alkylated at 30 °C for 90 min by addition of iodoacetic acid to 6.8 mM and adjustment of pH to 8.0. The reaction mixture was dialyzed thoroughly at 4 °C against buffer A (20 mM Tris-HCl with 0.15 M NaCl, pH 8.0) and then stored with 10% glycerol at -80 °C.

Zn(II)-free DnaJ [DnaJZn(-)] and Zn(II)-reconstituted DnaJ [DnaJZn(+)] were prepared basically according to Giedroc et al. (14). Native DnaJ was incubated with 8 equiv of mercurial reagent PMPS at 4 °C for 5 min and then with an additional 20 equiv of PMPS at 25 °C for 10 min to release Zn(II), which was then chelated by addition of EDTA to 1.5 mM. The PMPS–DnaJ complex was reversed to become DnaJZn(-) by addition of β-mercaptoethanol to 0.5 mM followed by thorough dialysis against buffer A containing 0.5 mM EDTA and 0.5 mM β-mercaptoethanol to remove PMPS and EDTA–Zn(II) complex. DnaJZn(+) was prepared by incubation of PMPS–DnaJ complex with 0.5 mM β-mercaptoethanol at 4 °C for 4 h in the absence of EDTA, allowing free Zn(II) to rebind to DnaJ, followed by dialysis against buffer A containing 0.5 mM β-mercaptoethanol and 0.1 mM EDTA. The preparations of DnaJZn(-) and DnaJZn(+) after dialysis were stored at -80 °C with 10% glycerol. The frozen preparation after thawing was loaded onto a fast desalting column HR 10/10 (Amersham Pharmacia Biotech) to remove EDTA and β-mercaptoethanol before use.

The C-terminal fragment (Q181–R376), fDnaJ, was prepared by limited digestion of DnaJ with proteinase K. DnaJ at 1.0

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**FIGURE 1:** Schematic presentation of domain structure and zinc finger topology of *Escherichia 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) Conventional model of the two C4-type zinc fingers (4). (C) New model of the two zinc fingers (8). The arrow shows the cleavage site between Q180 and Q181 by proteinase K to produce fDnaJ in shadow.
mg/mL was incubated with proteinase K (1.0 μg/mL) in 50 mM Tris-HCl (pH 7.5) containing 5 mM CaCl₂ and 10 mM EDTA at 25 °C for 60 min. After addition of phenylmethane-sulfonyl fluoride to 0.5 mM, the reaction mixture was loaded onto a buffer A-equilibrated Superose 12 HR10/30 column (Amersham Pharmacia Biotech), which had been calibrated with Bio-Rad gel filtration standard for the estimation of molecular size. Three main peaks were analyzed by SDS-PAGE (12%), and the fraction containing the fragment of 22 kDa, fDnaJ, was concentrated by Centricon and stored at −80 °C with 10% glycerol.

The plasmid coding for the Q¹⁸¹-R³⁷⁶ fragment of DnaJ was constructed by cloning a DNA fragment, amplified from the plasmid pUHE21-2 by use of primer I (5'-CGGGATCCTGGACGGTCGTC-3') and primer II (5'-AATGCAGTTAGCGGGTCAGGTGCGTCTG-3') with BamHI and PstI sites at the 5’ and 3’ termini (underlined) respectively, into BluescriptII/SK(+) vector (Stratagene). The construct was confirmed by DNA sequencing. The DNA fragment was then subcloned into pQE-30 vector (Qiagen) via the same restriction sites to create pQE-fDnaJ, which encodes the sequence of MRGSH6-Q¹⁸¹-R³⁷⁶ (re-fDnaJ). Transformed M15 [REP4] cells (Qiagen) were grown in 2× YT broth with 100 μg/mL of ampicillin and 25 μg/mL of kanamycin at 37 °C. The overnight culture was diluted 100-fold and cultured with vigorous shaking for 2–3 h followed by addition of isopropyl-β-D-galactoside to 0.1 mM and further incubation for 4 h.

The cell pellet was suspended in buffer B (20 mM phosphate buffer containing 0.5 M NaCl, pH 7.2) and sonicated. The pellet was washed with 1% Triton X-100 followed by treatment with 8 M urea in buffer B at room temperature for 1 h. The supernatant was loaded onto a 6.5 × 1 cm Ni²⁺-chelating Sepharose fast flow (Amersham Pharmacia Biotech) column equilibrated with buffer B containing 4 M urea and eluted with 20, 100, and 500 mM imidazole successively. Fractions eluted with 500 mM imidazole (re-fDnaJ) were dialyzed successively against Tris buffer containing 0.5 mM EDTA, 1 mM ZnCl₂, and 0.5 M NaCl and then 0.5 M NaCl, and the product was stored at −80 °C with 10% glycerol. Addition of ZnCl₂ to 150 μM to the culture at the time of induction does not improve the solubility of expressed re-fDnaJ. Zn(II)-free re-fDnaJ [re-fDnaJZn(−)] was prepared by the same protocol as for DnaJZn(−) except for the treatment with 4 equiv and then 10 equiv of PMPS.

α-D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit muscle (15) and Escherichia coli DsbC, a periplasmic thiol-disulfide oxidoreductase (16), were kindly provided by J. Li and X. Cai of this laboratory. PDI was prepared from bovine liver (17). Reduced and denatured RNase A was prepared essentially according to Pigiet and Schuster (18), and scrambled RNase A was prepared according to Hillson et al. (19).

Determinations. Concentrations of DnaJ, fDnaJ, and re-fDnaJ were determined by the Bradford method with bovine serum albumin (BSA) as a standard (20). Concentrations of other proteins were determined spectrophotometrically at 280 nm with the following absorption coefficients (εₐ₄₅₄): 0.66 for BSA, 0.98 for GAPDH, 0.695 for native RNase A, 0.9 for PDI, and 0.7 for DsbC, and at 275 nm with ε₂₇₅₅₅ = 9200 M⁻¹ cm⁻¹ for fully reduced RNase A (18). Homotetrameric GAPDH and homodimeric DnaJ were considered as protomers in the calculation of molar ratios. Thiol groups were determined with DTNB (21).

The N-terminal amino acid sequence was determined on a MilliGen/Biosearch model 6600 ProSequence linked to a Waters high-pressure liquid chromatography system with simultaneous detection at 269 and 313 nm. The molecular mass was determined by electrospray ionization mass spectrometry on a Biflex III platform spectrometer. CD spectra from 200 to 250 nm were determined in a Jasco J720 spectropolarimeter. Fluorescence spectra were measured in a Shimadzu RF-5301 PC spectrophotometer. Zn content was determined by atomic absorption spectrophotometry on an Instrument Laboratory ICAP-9000 spectrometer.

Denaturation and Reactivation of DnaJ. DnaJ at 300 μM was denatured with 6 M GdnHCl and 2.0 mM DTT at 20 °C for 20 h. The denatured DnaJ was refolded by 25-fold dilution in Tris buffer and incubation for 17 h at 4 °C. The refolding solution was concentrated and dialyzed against Tris buffer containing 0.1 M NaCl to remove adventitious Zn(II). The denatured DnaJ was carboxymethylated at 30 °C for 90 min by addition of iodoacetic acid to 60 mM and adjustment of pH to 8.0, and then refolded by 25-fold dilution for 17 h at 4 °C. The refolded DnaJ or refolded S-carboxymethylated DnaJ at 2.0 mg/mL was digested with proteinase K (1.0 μg/mL) at 25 °C for 20 min, and a similar 22 kDa fragment was purified by the same protocol as described for fDnaJ.

Titration of DnaJ with PMPS. PMPS, a strongly sulfhydryl-dissociating reagent, was used to remove Zn(II) from DnaJ by forming mercaptide bonds with free thiols. The released Zn(II) was determined by use of ε₈₅₀₃ₕₐ₅ = 6.6 × 10⁴ M⁻¹ cm⁻¹ for a complex of one released Zn(II) ion with two metallochromic indicator molecules [4-(2-pyridylazo)-resorcinol] (22).

Activity Assay. Reductase activity was assayed by measuring the turbidity increase at 650 nm due to insulin reduction (23) and was expressed as a ratio of the slope of a linear part of the turbidity curve to the lag time (24). According to Lambert and Freedman (25), disulfide isomerase activity was assayed on the basis of the isomerization of scrambled RNase A, with both yeast RNA and cyclic 2',3'-cytidine monophosphate as substrates of renatured RNase A. Oxidase activity was assayed by the oxidative reactivation of reduced and denatured RNase A (18). Effects of DnaJ on the disulfide-coupled refolding reaction of reduced BPTI were determined basically according to Zapun and Creighton (26) except that the refolding reaction mixture was incubated at 25 °C for 20 min before the addition of BPTI to initiate the reaction (27). The acid-trapped samples were analyzed on a PepRPC HR 5/5 column (Amersham Pharmacia Biotech) with a gradient of 9–36% (v/v) acetonitrile in 50 mL of 0.1% trifluoroacetic acid.

Aggregation of Denatured GAPDH during Refolding. GAPDH at 125 μM was completely denatured in Tris buffer containing 3 M GdnHCl and 5 mM DTT at 4 °C overnight, and refolding was initiated by 100-fold dilution of the denatured enzyme into Tris buffer containing 0.1 M NaCl and 1 mM DTT in the presence or absence of different forms of DnaJ. Aggregation of GAPDH during refolding was followed continuously at 25 °C by 90° light scattering at 488 nm in a Shimadzu RF-5301 PC spectrophotometer for 30 min after dilution until it reached a maximal level (28).
the reductase activity of DnaJ (Figure 2B, inset). When EDTA was removed by extensive dialysis, the reductase activity was fully recovered, showing a reversible EDTA inhibition. DnaJ treated with a 250-fold excess of EDTA and dialyzed thoroughly still contains two zinc atoms measured by atomic absorption, confirming that EDTA inhibition of DnaJ activity results from the formation of a complex of the enzyme with EDTA via Zn(II) rather than the removal of Zn(II).

Effects of Carboxymethylation on the Reductase Activity of DnaJ. As shown in Table 1 the two DTNB-detectable thiol groups of native DnaJ are those of C265 and C323, since the other eight thiols are in coordination with Zn(II) ions. S-carboxymethylated DnaJ prepared under non-denaturing conditions contains little free thiol but two zinc atoms determined by atomic absorption, indicating that modification occurs only at C265 and C323. The modified enzyme shows almost full reductase activity, indicating that the non-zinc-thiols are not required for the activity.

After denaturation, native DnaJ and S-carboxymethylated DnaJ exhibit six and four thiols, respectively, suggesting that 6.4 M GdnHCl destroys only one zinc finger and the other one is resistant to GdnHCl. One Zn(II) but only four thiols are evident in refolded DnaJ after dilution, suggesting that the destroyed zinc finger is not re-formed after refolding and two of the four thiols released in the destroyed zinc finger are oxidized. S-carboxymethylated DnaJ prepared under denaturing conditions contains no free thiol, indicating that the four cysteine residues in the GdnHCl-disrupted zinc finger were all carboxymethylated, and the modified DnaJ after refolding contains one Zn(II). Both refolded DnaJ and refolded S-carboxymethylated DnaJ are fully active. It appears, therefore, that the zinc finger destroyed by 6.4 M GdnHCl is not essential for activity, and DnaJ is enzymatically active regardless of whether these thiols are reduced, oxidized, or carboxymethylated.

Release and Reconstitution of Zn(II). PMPS has been used for titration of Zn(II) to further characterize the properties of the zinc fingers in DnaJ, DnaJZn(−), and DnaJZn(+). As shown in Figure 3 and Table 1, the absorbance at 500 nm increases linearly with increasing concentrations of PMPS until the ratio of PMPS to DnaJ reaches 8, indicating two Zn(II) ions are released from each DnaJ monomer. By PMPS titration, DnaJZn(−) releases 0.2 Zn(II) and DnaJZn(+) releases 1.2 Zn(II), close to the values of 0.11 and 1.13, respectively, determined by atomic absorption measurements. It is clear that PMPS is able to release both Zn(II) ions from DnaJ; however, only one zinc finger is reconstitutable. Both DnaJZn(+) and DnaJZn(−) exhibit only two thiols (non-zinc-finger thiols) that react with DTNB, as in the native molecule, and two more thiols upon denaturation with 6.4 M GdnHCl, suggesting that only two thiols in the unreconstituted zinc finger remain in reduced form and are exposed to react with DTNB only after denaturation, and all other six thiols in DnaJZn(−) are oxidized.

Conformation of DnaJ, DnaJZn(−), and DnaJZn(+). As shown in Figure 4A, the far-ultraviolet CD spectra of native DnaJ, DnaJZn(−), and DnaJZn(+) are identical with two typical negative peaks at 208 and 222 nm, respectively. The three DnaJ forms show similar intrinsic fluorescence spectra in terms of the emission maximum at 312 nm but with different emission intensity (Figure 4B). In addition, DnaJZn-
Zn Fingers and Enzymatic Activities of Chaperone DnaJ

Biochemistry, Vol. 40, No. 49, 2001

Table 1: Thiols, Zn(II), and Reductase Activity of DnaJ at Different Forms

<table>
<thead>
<tr>
<th>thiol</th>
<th>Zn(II) (mol/mol)</th>
<th>Reductase activity&lt;sup&gt;a&lt;/sup&gt; (&lt;xsup&gt;-10&lt;/xsup&gt;&lt;sup&gt;4&lt;/sup&gt; Δ&lt;sub&gt;A650nm&lt;/sub&gt; min&lt;sup&gt;-1&lt;/sup&gt;)</th>
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<tr>
<td></td>
<td>alone</td>
<td>in 6.4 M GdnHCl</td>
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<tr>
<td>DnaJ</td>
<td>1.9 ± 0.1</td>
<td>6.0 ± 0.4</td>
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<tr>
<td>S-carboxymethylated DnaJ</td>
<td>0.3 ± 0.1</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>refolded DnaJ</td>
<td>4.4 ± 0.3</td>
<td>0.85 ± 0.07</td>
</tr>
<tr>
<td>refolded S-carboxymethylated DnaJ</td>
<td>0.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>DnaJZn(−)</td>
<td>1.8 ± 0.2</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>DnaJZn(+)</td>
<td>2.0 ± 0.1</td>
<td>4.2 ± 0.2</td>
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<tr>
<td>fDnaJ</td>
<td></td>
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<tr>
<td>f[DnaJZn(+) + PMPS]</td>
<td></td>
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<tr>
<td>re-fDnaJ</td>
<td>2.4 ± 0.2</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>re-fDnaJZn(−)</td>
<td>1.8 ± 0.1</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>f(refolded S-carboxymethylated DnaJ)</td>
<td>2.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>f(refolded DnaJ)</td>
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<sup>a</sup>Proteins were prepared as described in the text. Data for each monomer are expressed as means ± SD (n ≥ 3). <sup>b</sup>The reductase activity was assayed by following the absorbance at 650 nm upon addition of 1 mM DTT into 0.1 M potassium phosphate, pH 6.6, containing 0.13 mM bovine insulin and 5 μM protein. <sup>c</sup>Average of two independent experiments.

Figure 3: Determination of Zn(II) in DnaJ, DnaJZn(+), and DnaJZn(−) with PMPS. The release of Zn(II) was monitored by following the increase of absorbance at 500 nm of 1 mL of reaction solution containing 0.1 mM 4-(2-pyridylazo)resorcinol and 4.0 mM native DnaJ (●), DnaJZn(+)(△), or DnaJZn(−)(◆) with stepwise addition of 1.0 mM PMPS in portions of 4 μL. The right ordinate indicates the number of Zn(II) released.

(+ ) and DnaJZn(−) show the same elution position as that of native DnaJ on a Superose 12 HR10/10 column (data not shown). It appears that the lack of one or even two Zn(II) does not substantially affect the secondary structure, the tertiary structure, and the association properties of DnaJ.

Enzymatic Activity of DnaJ, DnaJZn(--), and DnaJZn(+-) DnaJ (5 μM) shows clear reductase activity in the absence of EDTA with an activity of 1.42 × 10<sup>-4</sup> Δ<sub>A650nm</sub> min<sup>-1</sup>, which is 8% and 12% that of thioredoxin and DsbC, respectively (Figure 2B). DnaJZn(−) retains only 30% of the reductase activity of native DnaJ. DnaJZn(+) with only one Zn(II) coordinated shows 80% activity (Table 1). As shown in Figure 5, DnaJ exhibits significant ability to stimulate the oxidative refolding of denatured and reduced RNase A, but at a lower rate and extent compared to DsbC, and DnaJZn(+) and DnaJZn(−) have even lower activity, similar to their reductase activity. With yeast RNA (Figure 6A,B) and cyclic 2′,3′-cyclic monophosphate (Figure 6C,D) as substrates of RNase A under the same conditions as used by de Crouy-Chanel et al. (6), PDI at a ratio to substrate of 1:80 shows an isomerase activity of 496 unit/g (Figure 6A); however, the RNase A activity recovery in the presence of DnaJ at an equivalent amount to the substrate is always the same as in the control sample of spontaneous refolding of RNase A even over an extended period of 3 h, which is comparable to the level of spontaneous refolding of RNase A under the same redox conditions as reported by Pigiet and Schuster (18). The kinetics of the disappearance of reduced BPTI and the appearance of native three disulfide-bonded proteins catalyzed by DnaJ have also been compared to that by DsbC to check further the isomerase activity of DnaJ (Figure 7). In the absence of catalyst, the half-time for the disappearance of reduced BPTI, 5.8 min, decreases to 3.2

Figure 4: Conformation of DnaJ, DnaJZn(+), and DnaJZn(−). CD spectra (A) and intrinsic fluorescence spectra with excitation at 280 nm (B) of native DnaJ (solid line), DnaJZn(+) (dashed line), and DnaJZn(−) (dotted line) were measured at 25 °C. Protein concentrations were 0.60 mg/mL in panel A and 0.16 mg/mL in panel B.
min in the presence of DnaJ and further to 2.2 min in the presence of DsbC, indicating that DnaJ catalyzes the oxidation of the thiols in the reduced BPTI as does DsbC, but at a lower rate. In contrast to DsbC, which catalyzes 50% reactivation of BPTI in 16.0 min, DnaJ shows no effect on the spontaneous formation of native BPTI even over a prolonged time period (also see the inset of Figure 7), confirming the lack of isomerase activity of DnaJ.

Limited Digestion of DnaJ by Proteinase K.

Limited digestion of DnaJ with proteinase K has been performed to dissect the activity and zinc coordination of the resultant fragments. As shown in Figure 8, under the conditions used, proteinase K digestion results in a main fragment of approximately 22 kDa, which has been purified and named as fDnaJ. The N-terminal six amino acid residues of fDnaJ have been determined to be QTCPHC, indicating that fDnaJ results from the cleavage of DnaJ between Q180 and Q181 (Figure 1C). Determined by mass spectrometry, fDnaJ has a mass of 21 325 Da, which is in agreement with the
calculated value of 21 322 Da for the fragment Q^{181-1 R^{376}}. On a Superose 12 HR10/10 column, DnaJ and fDnaJ were eluted at positions corresponding to apparent masses of 68 and 72 kDa, respectively, suggesting that the former exists as a dimer and the latter as a tetramer. It is noticed that both of the apparent mass values of DnaJ and fDnaJ are lower than the expected values of 82 and 85.3 kDa, respectively.

As shown in Table 1, fDnaJ monomer contains one zinc atom (0.79 ± 0.02) measured by atomic absorption, suggesting a zinc finger of C^{183}PHC^{186}Zn(II)C^{197}NKC^{200}. The fragment of 22 kDa prepared from the protease K digestion of DnaJZn(+) under the same condition as for DnaJ, f[DnaJZn(+)], is supposed to be fragment Q^{181-1 R^{376}} of DnaJZn(+) since the conformation and proteinase K digestion profile of DnaJZn(+) are very similar to that of DnaJ, f[DnaJZn(+)] also contains one zinc atom (0.78 ± 0.04), probably the zinc finger of C^{183}PHC^{186}Zn(II)C^{197}NKC^{200}. fDnaJ shows full activity of the intact molecule (Table 1 and Figure 5), indicating that the N-terminal (M^{1-1 Q^{180}}) sequence is not required for DnaJ to display enzymatic activity.

Recombinant fDnaJ. As the amount of fDnaJ prepared from protease K digestion is limited, a recombinant C-terminal (Q^{181-1 R^{376}}) fragment of DnaJ (named as re-fDnaJ) has been overexpressed and purified for further characterization. re-fDnaJ possesses one zinc atom (1.09 ± 0.07) and two thiols (non-zinc-finger thiols) in either the native or denatured state, indicating a GdnHCl-resistant zinc finger C^{183}PHC^{186}Zn(II)C^{197}NKC^{200}. re-fDnaJ shows full enzymatic activity, as do fDnaJ and intact DnaJ (Table 1 and Figure 5). However, in contrast to fDnaJ, which is a tetramer, re-fDnaJ is a dimer (data not shown). Like re-fDnaJ, re-fDnaJZn(−) prepared with PMPS contains two thiols in denatured form, indicating that the four thiols of C^{183}, C^{186}, C^{197}, and C^{200} are in the oxidized state. Also in contrast to the intact molecule, treatment with 250-fold excess EDTA does release the Zn(II) from re-fDnaJ, showing that the zinc finger of C^{183}PHC^{186}Zn(II)C^{197}NKC^{200} in the C-terminal half of the molecule is different in stability compared to the zinc fingers in the intact protein.

Effects of DnaJ, DnaJZn(−), DnaJZn(+), and re-fDnaJ on the Aggregation of GAPDH during Refolding. The ability of a protein to prevent aggregation of other proteins during folding or unfolding has been widely taken as a measurement of its chaperone activity (3, 28). As shown in Figure 9, the presence of native DnaJ efficiently decreases the strong aggregation of denatured GAPDH during refolding and suppresses the aggregation when the concentration increases to a molar ratio to GAPDH of 1. Like the native protein, both DnaJZn(−) and DnaJZn(+) with only one zinc finger prevent the aggregation of GAPDH, but to a lower extent, and suppress the aggregation at a molar ratio of 5. re-fDnaJ, the C-terminal half-molecule with one zinc finger, shows an even lower capacity in preventing the aggregation of GAPDH, indicating that the N-terminal half of DnaJ, Met^{1-1 Gln^{180}}, contributes to peptide binding.

Zinc Coordination. Recently Martinez-Yamout et al. (8) have reported that the two Zn(II) ions in a cysteine-rich domain, the fragment G^{31-1 S^{39}} (309), coordinate with cysteine residues to form Zn1 and Zn2 in a manner different (Figure 1C) from that proposed by Szabo et al. (4) shown in Figure 1B. Therefore it is necessary to reinspect the coordination of the two Zn(II) ions in the intact DnaJ molecule, as proposed by Szabo et al. for the DnaJ molecule (4) or identified by Martinez-Yamout et al. (8) for the isolated cysteine-rich fragment, since the structure of a fragment, lacking interactions with other parts of the molecule, is not necessarily the same as that when it is in the intact molecule. The following experiments have been carried out to examine the coordination of the two Zn(II) ions in the intact DnaJ molecule (Figure 10). DnaJ denatured by GdnHCl with six thiols was carboxymethylated and then refolded by dilution. Panels A and B of Figure 10 show one of the possibilities, as at this stage we were not sure which zinc finger was destroyed by GdnHCl, and it is not critical for identification of the topology by this stratagem. The refolded modified DnaJ, containing no free thiol (0.1 ± 0.1) but one zinc atom and full activity (Table 1), was then digested with proteinase K. The fragment of approximately 22 kDa was purified and determined to have a mass of 21 600, which is in agreement with the calculated value of 21 554 for the fragment Q^{181-1 R^{376}} with four cysteine residues carboxymethylated. As shown in Table 1, this fragment, f(refolded S-carboxymethylated DnaJ), shows very little zinc but 2.4 thiols (Figure 10A), while the fragment purified from the proteinase K digestion of the refolded DnaJ without carboxymethylation, f(refolded DnaJ), has 0.69 zinc (Figure 10B). The above suggests that the two Zn(II) ions in the intact DnaJ molecule adopt the coordination as shown in Figures 10A and 1C to form Zn1 and Zn2, which are the same as in the isolated cysteine-rich domain. If the zinc coordination in DnaJ were in a conventional way as shown in Figures 1B and 10C, the N- and C-terminal zinc fingers would correspond to the Zn1 and Zn2, respectively, on the basis of the fact that fDnaJ and re-fDnaJ show full activity of DnaJ, and therefore the fragment purified from proteinase K digestion of the refolded S-carboxymethylated DnaJ should contain the C-terminal zinc finger but no free thiol (Figure 10C).

**DISCUSSION**

It has been found in recent years that both enzyme and chaperone activities reside in the same molecule of eukaryotic
PDI (28) and prokaryotic DsbA (29), DsbC (15), and DsbG (30), catalyzing the formation of native disulfide bonds. The ATP-dependent proteases (31), catalyzing the degradation of misfolded proteins, also have intrinsic chaperone activity. On the other hand, DnaJ and trigger factor (32), well-known E. coli chaperones, show thiol–disulfide oxidoreductase activity and peptidyl–prolyl cis/trans isomerase activity, respectively. The above may be the result of evolution to increase molecular efficiency (33).

Zinc Fingers of DnaJ. It is now confirmed that each DnaJ monomer contains two Zn(II) ions as determined by the methods of PMPS titration and atomic absorption and two free thiols detected by DTNB. Each of the eight thiols is coordinated with Zn(II) to form two C4-type zinc fingers (4). EDTA, a typical metal chelator, at a high concentration, in 250-fold excess, is unable to remove Zn(II) from DnaJ. The two Zn(II) ions can be released by PMPS modification of the thiol groups, but only one of the two zinc fingers can be reconstructed. Although it was reported that denaturation of DnaJ by 8 M urea exposes all 10 cysteine residues to DTNB (34), in our hands, 6.4 M GdnHCl destroys only one of the two zinc fingers to release four free thiols, and the destroyed zinc finger does not re-form after refolding by removal of the denaturant. It is clear that the two zinc fingers of DnaJ are markedly different in stability. However, it is found that the plot of $\Delta A_{500nm}$ is a linear function of PMPS added during titration but not in two phases as would be expected. The linearity of the release of Zn(II) has been explained as the rate-limiting step in Zn(II) release of mercurial attack on the first of the four tetrahedrally zinc-coordinated thiols in one zinc finger (22). Once the first of the four thiols reacts with PMPS, the other three rapidly form mercaptides with the reagent. It is therefore suggested that the first thiol attacked in the two zinc fingers of DnaJ is indistinguishable in reactivity with PMPS.

As cysteine residues that act as zinc ligands have been found to generally group quite closely in the sequence, the coordination of eight cysteine residues with two Zn(II) ions in the DnaJ molecule was naturally proposed as shown in Figure 1B (4). We have now demonstrated for the first time that in the intact DnaJ molecule the two zinc fingers are C144-DVC147-Zn(II)-C197-NKC200 and C161-PTC164-Zn(II)-C183-PHC186, designated as Zn1 and Zn2 respectively, indicating that the very conserved sequence in this cysteine-rich domain determines its highly conserved structure so that the same folding topology of the two zinc fingers can be maintained in the isolated domain (8) even though there are no longer any interactions with other domains of the molecule. The folding topology of the zinc fingers in the DnaJ molecule differs from that of all known cysteine-containing zinc-binding proteins in the Brookhaven Protein Data Bank (8) and must be related to its special multifunction. Nevertheless, the two zinc fingers in the isolated cysteine-rich domain appear to be less stable than in the intact DnaJ molecule, as 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 excess of EDTA. Moreover, the isolated cysteine-rich 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 as measured by different methods (also see ref 34). The presence of the other three domains in the intact DnaJ molecule obviously enhances the stability of the zinc fingers and the whole molecule.

Martinez-Yamout et al. (8) have reported that the Zn2 module can assemble at low concentrations of Zn(II), but Zn1 assembles only at higher Zn(II) concentrations. That Zn2 assembles before the Zn1 module has been ascribed to the lower entropic cost for the Zn2 folding and the ligating cysteine residues are closer in the amino acid sequence. According to the above, it appears that Zn2 is the more stable zinc finger. Therefore, it is suggested that the zinc finger destroyable by 6.4 M GdnHCl is Zn1, which is not re-formed after refolding and is not required for the enzymatic activity.

**Figure 10:** Identification of the topology of the two zinc fingers in DnaJ monomer. Experiments were carried out as described in the text with the model of zinc fingers as shown in Figure 1C (panels A and B) and the model in Figure 1B (panel C). X represents an S-carboxymethylated group. In this experiment only one possibility of destroyed Zn1 was presented.
of DnaJ; and the zinc finger reconstituted to form DnaJzn- (+) is Zn2. The new zinc finger formed in re-fDnaJ is destructible by excess EDTA but not GdnHCl.

Peptide segments around the nick sites by proteinase are usually flexible and exposed loop or mobile linker region between domains (35). The peptide segment H165→I182 appears as a flexible and exposed loop to provide a nick site between Q186 and Q181 for proteinase K. It is interesting to note that the proteolysis results in the destruction of the coordination of the two very stable zinc fingers in DnaJ molecule so as to form the fragment fDnaJ, which contains one Zn(II). Since f(refolded S-carboxymethylated DnaJ) has little Zn(II) and f(refolded DnaJ) contains 0.69 Zn(II), it is suggested that the Zn(II) is not coordinated only with C183 and C186 in fDnaJ but very likely with four thiols to form a new zinc finger, C183PHC186 Zn(II)C197NKC200. Compared to the dimeric intact molecule, fDnaJ exists as a tetramer as shown by size-exclusion chromatography, suggesting that the removal of the N-terminal M′→Q180 sequence and the formation of a new zinc finger may result in more exposed hydrophobic surface, leading to increased self-association.

The presence of six histidine residues at the N-terminus of re-fDnaJ molecule might alleviate the association of re-fDnaJ dimers for the repulsion of the positive charges exposed.

As mentioned above, both DnaJzn (+) and refolded DnaJ contain only Zn2 and show four thiols after denaturation, suggesting that one of the two -CXXC- in Zn1 is oxidized. re-fDnaJzn (–) exhibits only two thiols detected by DTNB either in the native or denatured state, suggesting that C183, C186, C197, and C200 are all oxidized. In addition, DnaJzn(–) contains two and four free thiols in the native and denatured states, respectively. All the above demonstrate that C144 and C147 are more resistant to oxidation than the other six cysteine residues, which are oxidized to form sulfides following the release of Zn(II). Although the release and reconstitution of Zn(II) appear not to affect substantially the general structure of DnaJ, DnaJzn (+) shows 80% activity compared to the refolded DnaJ. It is suggested that the conformation of Zn2 in DnaJzn (+) maybe somehow changed during the removal and reconstitution of Zn(II) compared to the refolded molecule with the original Zn2, which is important for activity.

The zinc finger-like domain has been suggested for unfolded substrate binding (4). The recent solution structure of the domain suggests a pronounced groove within the base of the V, near the Zn1 binding site as a potential binding site for unfolded peptides or proteins (8). The present results, that DnaJzn (–) and DnaJzn (+) show weaker ability to suppress aggregation of GAPDH during refolding than that of the native molecule, provide further evidence that Zn1 is important for peptide binding although not required for enzymatic activity. The fact that re-fDnaJ shows even lower ability to suppress the aggregation of GAPDH supports the hypothesis that the other domains are also required for DnaJ to function as a chaperone (5, 8). A similar fragment, Q179→R184 of yeast DnaJ homologue, Ydj1, has been reported to suppress rhodanese aggregation but to a lower extent than the intact Ydj1 does (36).

Enzymatic Activities of DnaJ. It was first reported by de Crouy-Chanel et al. (6) that DnaJ possessed activities that catalyze the formation, reduction, and isomerization of disulfide bonds. However, we were surprised to find that DnaJ showed no reductase activity under the same conditions described by these authors (6) because of the presence of high concentration of EDTA. We have further identified that the inhibition of DnaJ activity by EDTA is reversible and appears to result from the formation of an inactive complex of EDTA with DnaJ via Zn(II) rather than the removal of Zn(II) from the zinc fingers. Similar action of EDTA has been observed for horse liver alcohol dehydrogenase (37) and carbonate dehydratase (38).

The present data have also shown that, in contrast to the previous observation (6), DnaJ shows little, if any, isomerase activity. We have noticed that the redox agents in buffer could affect the activity determination of thiol–disulfide oxidoreductase (18, 39); therefore the isomerase activity of DnaJ was assayed in the present work under the same redox conditions as previously reported (6) with not only RNA but also cyclic 2',3'-cytidine monophosphate as substrates for the activity assay of RNase. The lack of isomerase activity of DnaJ has been further confirmed by one more method well established for measuring the disulfide-coupled refolding reaction of reduced BPT1 (26).

The -XX- dipeptide in the active site of thiol–disulfide oxidoreductases has been empirically suggested as the main determinant of the redox properties of these enzymes (40). It is noticed that in DnaJ, -C183PHC186- and -C161PTC164- are the same as the active sites of DsbA (26) and DsbE (9), respectively, while the other two -CXXC- sequences are not found in all thiol–disulfide oxidoreductases characterized so far (41), and their contributions to the oxidoreductase activity are not known. Since fDnaJ and re-fDnaJ exhibit the full activity of the intact molecule, the N-terminal M′→Q180 sequence is not necessary for the activity of DnaJ, and it appears that -C144DYC147- and -C161PTC164- in the N-terminal part do not function as the active sites. The non-zinc-finger C265 and C323 are also not involved in the enzyme activities. Moreover, the refolded S-carboxymethylated DnaJ with C144, C147, C197, and C200 modified exhibits full reductase activity. The above indicate that -C183PHC186- is the only functional active site. In this respect DsbA shows low isomerase activity compared to its oxidase activity (26). As DnaJzn (–) and re-fDnaJzn (–) have only 30% the activity of native DnaJ or fDnaJ, the zinc finger structure is important for -C183PHC186- to exert its function, possibly through maintaining a necessary structure.

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