The Conserved Carboxyl Terminus and Zinc Finger-like Domain of the Co-chaperone Ydj1 Assist Hsp70 in Protein Folding*

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Ydj1 is a member of the Hsp40 (DnaJ-related) chaperone family that facilitates cellular protein folding by regulating Hsp70 ATPase activity and binding unfolded polypeptides. Ydj1 contains four conserved subdomains that appear to represent functional units. To define the action of these regions, protease-resistant Ydj1 fragments and Ydj1 mutants were analyzed for activities exhibited by the unmodified protein. The Ydj1 mutant proteins analyzed were unable to support growth of yeast at elevated temperatures and were found to have alterations in the J-domain (Ydj1 H34Q), zinc finger-like region (Ydj1 C159T), and conserved carboxyl terminus (Ydj1 G315D). Fragment Ydj1 (1–90) contains the J-domain and a small portion of the G/F-rich region and could regulate Hsp70 ATPase activity but could not suppress the aggregation of the model protein rhodanese. Ydj1 H34Q could not regulate the ATPase activity of Hsp70 but could bind unfolded polypeptides. The J-domain functions independently and was sufficient to regulate Hsp70 ATPase activity. Fragment Ydj1 (179–384) could suppress rhodanese aggregation but was unable to regulate Hsp70. Ydj1 (179–384) contains the conserved carboxyl terminus of DnaJ but is missing the J-domain, G/F-rich region, and a major portion of the zinc finger-like region. Ydj1 G315D exhibited severe defects in its ability to suppress rhodanese aggregation and form complexes with unfolded luciferase. The conserved carboxyl terminus of Ydj1 appeared to participate in the binding of unfolded polypeptides. Ydj1 C159T could form stable complexes with unfolded proteins and suppress protein aggregation but was inefficient at refolding denatured luciferase. The zinc finger-like region of Ydj1 appeared to function in conjunction with the conserved carboxyl terminus to fold proteins. However, Ydj1 does not require an intact zinc finger-like region to bind unfolded polypeptides. These data suggest that the combined functions of the J-domain, zinc finger-like region, and the conserved carboxyl terminus are required for Ydj1 to cooperate with Hsp70 and facilitate protein folding in the cell.

Hsp40 proteins make up an essential family of molecular chaperones that function to specify the cellular processes facil-

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between the J-domain and other regions in Hsp40 proteins (4). In the case of DnaJ, the G/F-rich region is required in conjunction with the J-domain in order for productive interactions with E. coli Hsp70 to occur (42, 43). However, because several Hsp40 family members lack the G/F-rich region and still interact with Hsp70, this conserved motif is not required for the function of all Hsp40 proteins (13, 14). Cysteine-rich repeats that resemble C4 zinc binding domains comprise the zinc finger-like domain and play a role in polypeptide binding by Hsp40 family members (44, 45). Fragments of DnaJ that contain the zinc finger-like region are capable of suppressing protein aggregation (45). However, family members that lack the zinc finger-like region can form complexes with cellular proteins and appear to function as molecular chaperones (1–3). These data suggest that other regions of Hsp40 are also involved in interactions with non-native proteins. The conserved region in the carboxyl terminus of DnaJ lies adjacent to the zinc finger-like region and is typically present in Hsp40 proteins that bind polypeptides (1–5). However, a role for this domain in the function of Hsp40 family members has not been determined.

To further define the mechanism of action for Hsp40 proteins in cellular protein metabolism, we have carried out a structure-function analysis of Ydj1. The functional analysis of protease-resistant fragments and mutant proteins has identified subdomains in Ydj1 responsible for its regulatory and chaperone activities. The J-domain functions independent of domains responsible for chaperone functions and is sufficient for regulation of Hsp70 ATPase activity. The conserved carboxyl terminus was identified as a region in Ydj1 that plays a major role in binding unfolded polypeptides. The zinc finger-like region was found to function in protein folding, but was not observed to be essential for stable binding of denatured model substrates. These data demonstrate that Ydj1 contains independent functional units that act via a coordinated mechanism to assist Hsp70 in interactions with cellular proteins.

MATERIALS AND METHODS

**Purification of Hsp70 and Ydj1**—Hsp70 8sDa protein, termed Hsp70 for the remainder of the text, was overexpressed in yeast strain MW141 and purified by ATP-agarose, DEAE, and hydroxyapatite chromatography by established techniques (46). The open reading frames for Ydj1, Ydj1 H34Q (39), and Ydj1 G315D (24) were subcloned into pET9d, and purified by ATP-agarose, DEAE, and hydroxyapatite chromatography as described previously (46). Ydj1 C159T was tagged with six histidine residues at its amino terminus and purified by metal chelate chromatography (21). In experiments with Ydj1 C159T, a histidine-tagged version of Ydj1 was utilized as a control.

Ydj1 H34Q and Ydj1 G315D both behaved similarly to Ydj1 on ion-exchange columns, and these proteins all ran as dimers on gel filtration columns (data not shown). Therefore, it does not appear that the mutant forms of Ydj1 we have purified are grossly misfolded. Histidine-tagged Ydj1 was capable of suppressing the temperature-sensitive growth phenotype of Δydj1 strains and appeared to function with the same efficiency as Ydj1 (data not shown). Additionally, purified His-Ydj1 behaved identically to the nontagged version in assays for regulatory and chaperone function described below (data not shown). Ydj1 C159T behaved similarly to His-tagged Ydj1 in all purification steps and ran as a dimer on gel filtration columns.

**Limited Proteolysis of Ydj1**—Ydj1 (0.5 mg/ml) was digested with trypsin or proteinase K (PK)1 at the concentrations indicated in digestion buffer (150 mM KCl, 25 mM Hepes, pH 7.4, and 10 mM DTT) for 1 h at 25 °C. Digestions were terminated by the addition of phenylmethylsulfonyl fluoride to a final concentration of 1 mM. Proteinase-resistant fragments of Ydj1 liberated were then analyzed by SDS-PAGE on 12.5% or 16% acrylamide gels for the trypsin and PK digestions, respectively.

**Amino-terminal Sequencing of Proteolytic Fragments from Ydj1**—Digested Ydj1 (4.0 μg) was run out on SDS-PAGE gels to resolve the products of the proteolytic digest from each other. Gels were then soaked for 20 min in transfer buffer (10 mM 3-cyclohexylamino-1-propanesulfonic acid made in 10% methanol). Fragments were then electropheroretically transferred to polyvinylidene fluoride membranes in a Bio-Rad Mini-Wet blot cell blower for 60 min with the power supply set at 50 V constant voltage. Membranes were then soaked in water for 1 min to remove the transfer buffer. Membranes were run over with 0.1% Bio-Rad-250 to illuminate the positions of the transferred protein fragments. Fragments were excised from the membranes, and the identity of the first six amino acid residues in them were determined with a Perkin-Elmer Applied Biosystems Microsequencing Apparatus.

**Molecular Weight Determination of Ydj1 Fragments by Mass Spectrometry**—The molecular weights of proteins liberated from Ydj1 by limited proteolysis were determined on a Perseptive Biosystems (Framingham, MA) Voyager Elite MALDI-TOF mass spectrometer. Aliquots of digested material (5 μl) were mixed with a saturated solution of α-cyano-4-hydroxy-cinnamic acid in water:acetonitrile (50:50) mixture acidified with 0.1% trifluoroacetic acid. A 1-μl aliquot of this mixture was spotted onto a gold plate target. Ionization of the sample was then carried out with a nitrogen laser operating at 337 nm. A delayed extraction method was used in the determination of molecular weight. Measurement of ion flight times through the drift region of the mass spectrometer were carried out with a Tektronix (Beaverton, OR) TDS784A oscilloscope. The instrument was calibrated with external molecular weight standards.

**Preparation of Protease-resistant Fragments from Ydj1 for Functional Analysis**—To analyze the function of Ydj1 (1–383), Ydj1 was digested with 2 μg/ml of trypsin for 30 min at 25 °C. The trypsin was then inactivated with 0.5 mM phenylmethylsulfonyl fluoride, and the activity of Ydj1 (1–383) was analyzed. At this trypsin concentration, Ydj1 is almost completely converted to Ydj1 (1–383) and contains less than 1% contamination with Ydj1 or other fragments (Fig. 1, lane 3). The levels of contaminants in this preparation were calculated to be insufficient to account for the observed activity of Ydj1 (1–383).

To generate Ydj1 (102–394), Ydj1 (179–384), and Ydj1 (1–90) for functional analysis, digestions of Ydj1 with two different concentrations of PK were carried out. Then, the different Ydj1 fragments generated were separated from each other by high performance liquid chromatography gel filtration chromatography, concentrated, and immediately assayed for activity. To generate Ydj1 (102–394) virtually free of Ydj1 (179–384) and Ydj1 (1–90), 1 mg of Ydj1 (1 mg/ml) was digested with 1–2.0 μg/ml PK for 45 min at 25 °C. The concentration of PK utilized for this digestion was adjusted so that Ydj1 was efficiently converted to Ydj1 (102–394), but only minimal conversion to Ydj1 (179–384) could occur (see Fig. 1, lane 3). To separate Ydj1 (102–394) from Ydj1 (1–90) generated in protease digestions, reaction mixtures loaded onto a Bio-Rad Bio-Select G-250 gel filtration column. The column was then developed with a mobile phase consisting of 150 mM KCl, 20 mM Hepes, pH 7.4, and 2 mM DTT that was pumped at 0.6 ml/min with a back pressure of 850 psi. Fractions (0.5 ml) were collected with a Bio-Rad Biologic medium pressure chromatography system and analyzed by SDS-PAGE to identify peaks. Ydj1 (102–394) and Ydj1 (179–384) eluted at 0 and 8 ml, respectively, at 9.0 ml of fraction collected. Ydj1 fragments in this step was typically 40% of the starting material. Gel filtration typically resolved the majority of Ydj1 (1–90) from Ydj1 (102–394), but a 3% contamination of this fragment remained, and Ydj1 (179–384) was present as a 5% contaminant. The quantity of Ydj1 (179–384) and Ydj1 (1–90) present in the Ydj1 (102–394) preparations were calculated to be insufficient to account for the activity of these fragments in functional assays.

To prepare Ydj1 (179–384) free of Ydj1 (102–394), Ydj1 was digested under the conditions listed above, with 7.5 μg/ml PK. Ydj1 fragments liberated in a representative digestion are exhibited in Fig. 1C, lane 5. To separate Ydj1 (179–384) from Ydj1 (1–90) and the unidentified lower molecular weight fragments generated, these samples were loaded onto on a Bio-Rad Bio-Select G-250 column and treated as described above. Ydj1 (179–384), eluted at 8.0 ml of mobile phase, and Ydj1 (1–90) eluted at 9.0 ml. The Ydj1 (179–384) fraction was routinely 95% pure with a 4% contamination of Ydj1 (1–90) and a 3% contamination of unidentified proteolytic products. The level of Ydj1 (1–90) and other contaminating bands present in this preparation were calculated to be insufficient to account for the activity of the fragments in functional assays.

To generate a preparation of Ydj1 (1–90) that was free of larger Ydj1 fragments, digestions were carried out with 2.0 μg/ml as described above. Ydj1 (1–90) was then resolved from the Ydj1 (102–394) and Ydj1 (179–384) generated by the PK digestion (Fig. 1C, lane 3) by gel filtration as described above. The Ydj1 (1–90) peak fraction was about 95% pure and contained a 5% contamination with Ydj1 (102–394) and Ydj1 (179–384). Ydj1 (1–90) ran as a broad band on SDS-PAGE gels, but

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1 The abbreviations used are: PK, proteinase K; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.
microsequence analysis indicated that the majority of protein in this band had an amino terminus that corresponded to residues 1–6 of Ydj1 (Fig. 1D) and to the J-domain of Ydj1. Analysis of the peptides in the Ydj1 band by mass spectrometry indicated that the major fragment of Ydj1 present in this band was 9596 Da in size and corresponded to Ydj1 (1–90). We have calculated that the levels of Ydj1 (102–394) and Ydj1 (179–384) that contaminate the Ydj1 (1–90) preparation are too low to account for the functional activity attributed to this fraction.

**Assay for Hsp70 ATPase Activity**—Purified Hsp70 was incubated in a reaction mixture containing 10 mM Hepes, pH 7.4, 80 mM KCl, 10 mM DTT, 1 mM MgCl2, and 50 μM ATP (25°C) for 0 min at 30 °C. Reactions were then placed on ice, and duplicate 2-μl aliquots were assayed for ADP formation by thin layer chromatography on PEI cellulose plates (46). Spontaneous ADP formation was assayed and subtracted prior to calculations for rates of ATP hydrolysis. The kinetics of Hsp70 ATPase activity were linear for at least 20 min under these experimental conditions (46).

**Rhodanese Aggregation Assay**—Rates of rhodanese aggregation were determined by light scattering as described previously (31). Briefly, bovine rhodanese (50 μM; Sigma) was denatured for 1 h at 25 °C in 6 M guanidinium-HCl buffered with 20 mM Hepes, pH 7.4, and fresh 10 mM DTT. Denatured rhodanese was diluted 100-fold into 300 μl of reaction buffer composed of 20 mM Hepes, pH 7.4, 80 mM KCl, and 10 mM DTT. When present, respective chaperone proteins were added prior to rhodanese. Rates of rhodanese aggregation were determined by monitoring increases in light scattering over time with a spectrophotometer set at 320 nm at 25 °C.

**Luciferase Folding Assays**—Refolding of firefly luciferase (Promega) by Ydj1 and Hsp70 was carried out as described previously (47). Luciferase (15 mg/ml) was diluted 40-fold into denaturation buffer (25 mM Hepes, pH 7.4, 50 mM KCl, 5 mM MgCl2, 6 M guanidinium-HCl, 5 mM DTT). The denaturation reaction was allowed to proceed for 40 min at 25 °C, and then a 1-μl aliquot was removed and mixed with 125 μl of refolding buffer (25 mM Hepes, pH 7.4, 50 mM KCl, 5 mM MgCl2, 1 mM ATP) and incubated at 30 °C. Aliquots of 1 μl were removed from the refolding buffer at indicated times and mixed with 60 μl of luciferase assay reagent (Promega). Luciferase activity was then measured with a Turner TD-20/20 luminometer. Ydj1 (1.6 μM, the Ydj1 mutants (1.6 μM), and Hsp70(0.8 μM) were added to reactions prior to luciferase. The level of luciferase activity observed when Ydj1 and Hsp70 were present in reaction mixture was 17-fold higher than that activity observed when these chaperone proteins were omitted, which is consistent with previous reports (47).

**Measurement of Complex Formation between Ydj1 and Luciferase**—Firefly luciferase (7.5 μM) in a 100 μM stock that was denatured in 6 M guanidinium-HCl, 10 mM Hepes, pH 7.4, and 10 mM DTT was added to reactions prior to luciferase. The numbers indicate residues where YDJ1 was cleaved by trypsin or PK. H34, C159, and G315 denote the positions of residues in YDJ1 that are mutated in YDJ1 H34Q, YDJ1 C159T, and YDJ1 G315D, respectively (see below). B, digestion of YDJ1 (0.5 mg/ml) with indicated concentrations of trypsin at 25 °C for 1 h. C, digestion of YDJ1 with indicated concentrations of PK at 25 °C for 1 h. D, characterization of the protease-resistant fragments from YDJ1. In panel D, A, B, C, and D correspond to bands labeled as such on SDS-PAGE gels in panels B and C. Molecular mass of individual fragments was determined by mass spectrometry. The amino-terminal sequence of the respective Ydj1 fragments was determined by microsequencing. The column titled amino acid residues denotes the position in YDJ1 to which the respective protease-resistant fragments correspond (see under “Materials and Methods” for details).

**RESULTS**

To determine its domain structure, Ydj1 was digested with limited concentrations of trypsin and PK, and the fragments that were liberated were analyzed by SDS-PAGE (Fig. 1). Amino-terminal amino acid sequencing and mass spectrometry was then utilized to determine the origin of the fragments liberated by these treatments. This analysis revealed that Ydj1 contains several protease-resistant subdomains that might correspond to independent functional units. Trypsin cleaved a 2602-Da peptide from the carboxyl terminus of Ydj1 to generate band A, which corresponded to amino acids 1–383 (Fig. 1, B and D). Digestion of Ydj1 with PK converted Ydj1 into bands B, C, and D, which were 32268, 22800, and 9596 Da in size and corresponded to Ydj1 (102–394), Ydj1 (179–384), and Ydj1 (1–90), respectively. Ydj1 (102–394) and Ydj1 (1–90) were prominent when digests were carried out with 1 μg/ml PK, whereas Ydj1 (179–384) was observed at higher protease concentrations (Fig. 1C). Ydj1 (1–90) contains the complete J-domain of Ydj1 and a small portion of G/F region (Fig. 1A). Ydj1 (102–394) is missing the J-domain and G/F region, but contains the zinc finger-like region and the conserved carboxyl terminus of DnaJ. Ydj1 (179–384) contains two of the four cysteine-rich repeats that make up the zinc finger-like region and the entire conserved carboxyl terminus.

To determine the function of the different subdomains in Ydj1 that were defined by limited proteolysis, several unsuc-
successful attempts were made to express corresponding fragments of Ydj1 in E. coli (data not shown). Fragments of Ydj1 that contained just the J-domain appeared to be toxic to the cell and were expressed at levels too low to allow for purification to homogeneity. Ydj1 (102–394) and Ydj1 (179–384) were not toxic to cells but did not accumulate to high levels in E. coli. Histidine-tagged versions of the Ydj1 fragments could be purified, but these proteins appeared to be misfolded and were inactive. Therefore, we chose to purify the different protease-resistant Ydj1 fragments by gel filtration and analyze them for activity in reactions that are catalyzed by the full-length protein (see under “Materials and Methods” for details).

To identify regions in Ydj1 responsible for interaction with Hsp70, Ydj1 (1–90), Ydj1 (1–383), Ydj1 (102–394), and Ydj1 (179–384) were tested for their ability to stimulate Hsp70 ATPase activity (Fig. 2). Ydj1 (1–90) and Ydj1 (1–383) were able to stimulate the ATPase activity of Hsp70 to the same degree and in the same concentration range as Ydj1. Conversely, Ydj1 (102–394) and Ydj1 (179–384) appeared unable to interact with Hsp70. The J-domain of Ydj1 contains sufficient information for high affinity recognition by Hsp70.

To define regions in Ydj1 responsible for interactions with unfolded polypeptides (31), the aforementioned Ydj1 fragments were tested for their ability to suppress the aggregation of denatured rhodanese (Fig. 3). Ydj1 reduced rhodanese aggregation in a dose-dependent manner with maximal suppression occurring at a 10:1 molar ratio of chaperone to substrate (Fig. 3). Ydj1 (1–90) was completely inactive, whereas Ydj1 (1–383) suppressed rhodanese aggregation in a manner nearly identical to Ydj1. Ydj1 (102–394) and Ydj1 (179–384) were also capable of suppressing rhodanese aggregation, but a 2-fold higher concentration was required.

These data suggest that a region capable of binding unfolded proteins is present within Ydj1 (179–384). Ydj1 (179–384) is missing the J-domain, G/F region, and two of the four cysteine-rich repeats present in the zinc finger-like region (Fig. 1A) but contains the conserved carboxyl terminus of DnaJ. Ydj1 (102–394) contains the complete zinc finger-like region and the conserved carboxyl terminus of Ydj1 but is no more effective than Ydj1 (179–384) in suppressing rhodanese aggregation. Loss of half of the zinc finger-like region, therefore, does not alter the ability of Ydj1 to suppress protein aggregation. The portion of the zinc finger-like region that remains in Ydj1 (179–384) may contribute to the polypeptide binding activity of this fragment. Attempts to generate a fragment of Ydj1 that contained only the conserved carboxyl terminus were unsuccessful (data not shown). Nonetheless, these data support the conclusion that regions in the carboxyl terminus of Ydj1 participate in the binding of unfolded polypeptides.

Ydj1 and Hsp70 synergistically cooperate to suppress protein aggregation in a process that requires hydrolysis of ATP (31). To determine what regions of Ydj1 are required for this synergism to occur, the ability of the Ydj1 fragments to aid Hsp70 in the suppression of protein aggregation was measured (Fig. 4A). When added at concentrations where Hsp70 (0.5 μM) or Ydj1 (1.0 μM) alone had no influence on aggregation of 0.5 μM rhodanese, the combination of these two chaperone proteins suppressed this reaction by over 80%. When Ydj1 (1–90) was added at concentrations at which it saturates the ATP regulatory site on Hsp70, it could not enhance the action of Hsp70, whereas Ydj1 (1–383) suppressed protein aggregation by over 70%. Ydj1 (102–394) and Ydj1 (179–384) were also unable to assist Hsp70 in the suppression of protein aggregation. Similar results were observed when the combination of Ydj1 (1–90) and Ydj1 (102–394) was added to reaction mixtures with Hsp70. Thus, the regulatory or chaperone activity of Ydj1 alone is insufficient to assist Hsp70 in the suppression of protein aggregation. The coordinated action of the regulatory and chaperone domains on Ydj1 assist Hsp70 in facilitating cellular protein folding.

Regions within the J-domain that are recognized by Hsp70 are not clearly defined. Sequence analysis and structural data suggest that helix II and the HPD tripeptide may be specifically recognized by Hsp70 (35–37). Tsai and Douglas (39) have reported that a 20-residue peptide, Ydj1 (21–40), which corresponds to helix II and the HPD motif, blocks interactions between Ydj1 and Hsp70. However, whether Hsp70 recognizes other regions of the J-domain that contain the HPD tripeptide remains unclear. Therefore, we compared the ability of Ydj1 (1–90), Ydj1 (21–40), and YDJ1 (33–52) to interact with Hsp70. Ydj1 (33–52) contains the HPD motif, which is flanked by helix III instead of helix II. To assay for interactions between these peptides and Hsp70, their ability to block the cooperative suppression of protein aggregation by Ydj1 and Hsp70 was determined (Fig. 4B). Ydj1 (1–90), which contains the HPD tripeptide flanked by both helix II and helix III, blocked Ydj1-Hsp70 interactions at a 10:1 molar ratio of Ydj1 (1–90) to Ydj1. At concentrations up to 250 μM, a control peptide, Ydj1 (1–20), which contains helix I of the J-domain, had very little influence on Ydj1-Hsp70 interactions. Ydj1 (21–40) inhibited Ydj1-Hsp70 interactions, with maximal inhibition occurring at 100 μM peptide. Ydj1 (33–52) could also block Ydj1-Hsp70 interactions but was 2.5-fold less effective than Ydj1 (21–40) and 25-fold less effective than Ydj1 (1–90). When the ability of Ydj1 or Hsp70 to suppress protein aggregation was assayed independently, none of the aforementioned peptides were inhibitory (data not shown).

Helix II and the HPD motif appear to interact with Hsp70 with higher affinity than HPD-helix III. However, Ydj1 (21–40) and Ydj1 (33–53) were both an order of magnitude less effective than Ydj1 (1–90) at blocking interactions between Ydj1 and Hsp70. Therefore, Hsp70 can recognize HPD-helix II and HPD-helix III peptides, but with a much lower affinity than Ydj1 (1–90).

To further understand the role the J-domain, the zinc finger-like region, and the conserved carboxyl terminus play in Ydj1 function, Ydj1 H34Q, Ydj1 C159T, and Ydj1 G315D were puri-
fied and tested for functions exhibited by Ydj1. Ydj1 H34Q has a point mutation in the HPD motif that hinders the ability of Ydj1 to interact with Hsp70 (39). Ydj1 C159T contains a mutation in a conserved cysteine found in the first of two putative zinc binding domains in Ydj1 (Fig. 1A) and is unable to support protein translocation into mitochondria (21). Ydj1 G315D has a mutation in a highly conserved glycine in the carboxyl terminus and is not capable of supporting Hsp90 mediated signal transduction to the nucleus (24). The respective Ydj1 mutants can support growth of yeast at 30 °C and therefore appear to fold properly in vivo, but strains become nonviable at 37 °C (21, 24, 39). All three mutant proteins were purified by standard techniques and ran as dimers on gel filtration columns, which suggested that they did not misfold during overexpression in E. coli or purification by column chromatography (data not shown).

Ydj1 C159T and Ydj1 G315D were both able to stimulate the ATPase activity of Hsp70 approximately 6–7-fold (Fig. 5A). The shapes of titration curves for stimulation of Hsp70 ATPase by Ydj1 C159T and Ydj1 G315D were identical to those of Ydj1 (data not shown; Fig. 2). As reported by Tsai and Douglas (39), Ydj1 H34Q was unable to regulate the ATP hydrolytic cycle of Hsp70 (Fig. 5A). These data demonstrate that mutations in the zinc finger-like region and the conserved carboxyl terminus do not hinder the ability of Ydj1 to regulate Hsp70 ATPase activity and that Ydj1 C159T and Ydj1 G315D are functional proteins.

Next, the ability of the different forms of Ydj1 to function as molecular chaperones and suppress protein aggregation was examined (Fig. 5B). Ydj1 H34Q and Ydj1 C159T behaved similarly to Ydj1 and were both able to suppress the aggregation of rhodanese in a dose-dependent manner. Maximal suppression of protein aggregation by Ydj1, Ydj1 H34Q, and Ydj1 C159T was observed at a 10:1 molar ratio of chaperone to substrate. These data suggest that the J-domain and residue Cys-159 in the zinc finger-like region are not essential for interactions between Ydj1 and unfolded polypeptides to occur. On the other hand, mutation of the conserved carboxyl terminus caused severe defects in Ydj1 chaperone function. Ydj1 G315D at 10:1 and 20:1 molar ratios to rhodanese was inefficient at suppressing protein aggregation (Fig. 5C). In three independent experiments, 5 μM Ydj1 suppressed the aggregation of 0.5 μM rhodanese and average (± S.D.) of 77 ± 3.2%, whereas 5 μM Ydj1 G315D reduced aggregation by 7.5 ± 7.0%. It therefore appears that Gly-315 is required for efficient interactions between Ydj1 and unfolded proteins. These data support the previous inter-

**Fig. 3. Suppression of protein aggregation by different subdomains of YDJ1.** Bovine rhodanese (50 μM) was denatured in 6 M guanidinium-HCl and was allowed to aggregate after a 100-fold dilution into buffer that contained no denaturant. When present, different forms of YDJ1 were added to reactions prior to unfolded rhodanese. Protein aggregation was monitored by light scattering at 320 nm at 25 °C for the indicated times. Protein aggregation is expressed as a percentage of the total amount of light scattering observed in the absence of chaperones after a 10-min incubation (see under “Materials and Methods” for details).
pretation that the conserved carboxyl terminus of Ydj1 functions in polypeptide binding.

Tested next was the ability of the respective Ydj1 mutants to cooperate with Hsp70 in the suppression of rhodanese aggregation. Ydj1 H34Q was unable to cooperate with Hsp70, whereas Ydj1 C159T was almost as effective as Ydj1 in assisting Hsp70 in the suppression of protein aggregation (Fig. 5C). Interestingly, Ydj1 G315D was able to partially suppress protein aggregation when added in combination with Hsp70. In six separate trials, the combination of Ydj1 G315D and Hsp70 reduced rhodanese aggregation to 65±11% of control levels as compared with 29±9% for Ydj1. Analysis of these data by Student’s t test indicates that the activity of the Hsp70/Ydj1 G315D co-chaperone pair is significantly different from the control and the Hsp70/Ydj1 pair to a confidence level of greater than 99%. Therefore, the data presented in Fig. 5 demonstrate that Ydj1 G315D exhibits a reduced level of polypeptide binding activity and is less efficient than Ydj1 at assisting Hsp70 in the suppression of protein aggregation.

If Ydj1 C159T can regulate the ATPase activity of Hsp70 and suppress the aggregation of protein folding intermediates, then what is the defect in its function? One possibility is that Ydj1 C159T can bind unfolded proteins efficiently but is inefficient at folding them. To address this issue, we examined the ability of Ydj1 C159T to refold the model protein luciferase, which had been denatured with guanidinium-HCl and allowed to refold at 25 °C (Fig. 6A). The molar ratios of Hsp70 and Ydj1 to luciferase in these reactions were optimized to allow efficient refolding of luciferase to occur (data not shown). The combination of Ydj1 and Hsp70 enhanced the folding of luciferase 17-fold over spontaneous rates and 8–9-fold over rates observed when these chaperone proteins were added independently. Substitution of Ydj1 H34Q for Ydj1 reduced folding rates to those observed

![Fig. 4. Suppression of protein aggregation by Ydj1 and Hsp70.](image)

![Fig. 5. Biochemical characterization of YDJ1 mutants. A, regulation of Hsp70 ATPase activity by YDJ1 H34Q, YDJ1 C159T, and YDJ1 G315D.](image)
Ydj1 Assists Hsp70 in Protein Folding

A

B

C

FIG. 6. Analysis of Ydj1 mutants in luciferase refolding assays. A, refolding of guanidinium-HCl denatured luciferase (0.04 μM) at 25 °C by Hsp70 (0.8 μM) and YDJ1 (1.6 μM) (see under “Materials and Methods” for details). When added, the mutant YDJ1 proteins were also present at 1.6 μM. Activity of refolded luciferase was quantitated in a Turner TD 20/20 luminometer and expressed in arbitrary units. B, inhibition of luciferase refolding by YDJ1 mutants. Reactions contained Hsp70 (0.8 μM), YDJ1 (1.0 μM), and the YDJ1 mutant (indicated at 10 μM). Luciferase activity after 20 min of incubation was measured with a luminometer. Values are expressed as the percentage of luciferase activity observed in the presence of YDJ1 and Hsp70, which was equivalent to 7200 units. C, complex formation between Ydj1 and denatured luciferase. Complex formation between Ydj1 and unfolded luciferase was measured by gel filtration chromatography as described under “Materials and Methods.” Complex formation is expressed as a percentage of the total quantity of luciferase (2.5 μM) bound to the different forms of YDJ1 (5.0 μM). YDJ1 bound luciferase in a 1:1 molar ratio.

when just Hsp70 was present, which demonstrated that a functional J-domain is required for protein folding by Ydj1. The combination of Ydj1 G315D and Hsp70 was also inactive in refolding luciferase. Ydj1 C159T exhibited severe defects in its ability to cooperate with Hsp70 and refold luciferase but was partially active. Over the first 30 min of the folding reaction, Ydj1 C159T cooperated with Hsp70 to refold luciferase at rates that were 4–5-fold slower than with Ydj1.

Ydj1 C159T and Ydj1 G315D might refold luciferase inefficiently because their J-domains are not accessible to Hsp70 under these assay conditions. To exclude this possibility, the ability of Ydj1 C159T and Ydj1 G315D to block protein refolding by the Hsp70-Ydj1 co-chaperone pair was determined (Fig. 6B). Addition of Ydj1 C159T and Ydj1 G315D to incubations at 10:1 molar ratio over Ydj1 reduced the level of luciferase folding by greater than 80%. Addition of Ydj1 H34Q at similar concentrations had no influence on the ability of Ydj1 and Hsp70 to refold luciferase. Thus, it appears that defects in the chaperone functions of Ydj1 C159T and Ydj1 G315D are the reason for their inability to fold proteins.

Inability of Ydj1 to form complexes with unfolded luciferase might explain why Ydj1 C159T and Ydj1 G315D exhibit defects in protein folding. To test this possibility, complex formation between Ydj1 and luciferase was measured by monitoring the co-migration of these proteins on a gel filtration column (see under “Materials and Methods” for details). Shown in Fig. 6D is the quantitation of such a binding assay, which demonstrated that Ydj1 C159T is capable of forming tight complexes with protein folding intermediates. The level of complex formation between luciferase and Ydj1 C159T was routinely the same as that observed with Ydj1 and corresponded to approximately 10% of the total luciferase added to reactions. Ydj1 C159T was also able to form ternary complexes with unfolded luciferase and Hsp70 with an efficiency similar to that of Ydj1 (data not shown). Levels of complex formation between Ydj1 G315D and unfolded luciferase were approximately 90% lower than that observed for Ydj1, but they were detectable.

These data demonstrate that mutation of Cys-159 in the zinc finger-like region does not grossly alter the ability of Ydj1 to form stable complexes with unfolded luciferase, whereas mutation of the conserved carboxyl terminus severely reduces polypeptide binding activity. Defects in formation of stable complexes with unfolded polypeptides are likely to account for the inability of Ydj1 G315D to suppress the aggregation of rhodanese (Fig. 3) and refold luciferase (Fig. 6). Defects in the function of Ydj1 C159T appear more complex and are discussed below.

DISCUSSION

To fold polypeptides, Hsp70 and Hsp40 form complexes with folding intermediates to prevent them from entering nonproductive pathways that lead to aggregation (5). Polypeptides released from this ternary complex are often in non-native conformations and must be rebound by Hsp70 and Hsp40 several times before they fold properly. Data presented herein indicate that the combined action of the J-domain, the zinc finger-like region, and the conserved carboxyl terminus is required for Ydj1 and Hsp70 to efficiently bind polypeptides at different stages of folding and promote their passage to the native state.

Defective chaperone function of Ydj1 C159T and Ydj1 G315D appears to be the mechanism attributable to their inability to support growth of yeast at elevated temperatures (21, 24). Inability of Ydj1 C159T to interact productively with nascent polypeptides would explain why mitochondrial precursor proteins accumulate in import-incompetent conformations in strains that harbor this allele of Ydj1 (21). Reduced complex formation between Ydj1 G315D and hormone receptors has
been observed (24). Our data suggest that a defect in the polypeptide binding activity of Ydj1 is the cause of this result, and it leads to diminished signal transduction to the nucleus in strains that harbor Ydj1 G315D (24).

The J-domain of Ydj1 appears sufficient for regulation of Hsp70 ATPase activity, but the sequence within it that is recognized by Hsp70 is not defined. Based on the NMR structure, it has been suggested that helix II-HPD is the minimal unit of the J-domain that is recognized by Hsp70 (35–37). Consistent with this proposal, synthetic peptides that correspond to helix II and the HPD tripeptide are recognized by Hsp70. However, this J-domain peptide is one-tenth as efficient as fragment Ydj1 (1–90) at blocking interactions between Ydj1 and Hsp70. Perhaps the helix II-HPD sequence is the minimal sequence recognized by Hsp70, but the presence of adjacent regions in the J-domain, such as helix III, is required to stabilize it in a high affinity conformation. This might explain why Ydj1 (1–90) is 10–25-fold more effective than the helix II-HPD and helix III-HPD peptides as an inhibitor of interactions between Ydj1 and Hsp70.

Data from domain swap experiments carried out by the Silver group support the interpretation that the helix II-HPD helix III region plays an important role in J-domain function (8). When the J-domain from a cytosolic Hsp40 protein was substituted for this same region in an endoplasmic reticulum-localized family member cells became inviable. Through conservatively mutating two amino acid residues in helix II and one residue in helix III in the J-domain of the cytosolic Hsp40 protein, these investigators were able to convert this inactive J-domain to one that was capable of interacting with endoplasmic reticulum Hsp70 and supporting cell growth. When made independently, the alterations in helix II and helix III were insufficient to generate a functional J-domain (8). Thus, information in helices II and III is required for the formation of a functional J-domain.

How Hsp40 proteins interact with non-native polypeptides and function as molecular chaperones is not understood at the mechanistic level. To address this issue, we have examined which region of Ydj1 is responsible for its polypeptide binding activity. We present data that strongly argue that the conserved carboxyl terminus of Ydj1 participates in the binding of unfolded polypeptides. This conclusion is based on two observations: 1) a 22-kDa internal fragment of Ydj1, Ydj1 (179–384), can suppress rhodanese aggregation with similar efficiency as the full-length protein. This fragment lacks the J-domain, the G/F region, and the first zinc binding clef but contains the conserved carboxyl terminus of DnaJ. 2) Ydj1 G315D contains a mutation in the conserved carboxyl terminus and exhibits severe defects in polypeptide binding as evidenced by its inability to suppress rhodanese aggregation and to form complexes with denatured luciferase.

The portion of Ydj1 that is referred to as the conserved carboxyl terminus corresponds to amino acid residues 206–380. This region contains a series of seven highly conserved glycine residues that are present in Hsp40 family members demonstrated to function as polypeptide-binding proteins (1–5, 22). Substitution of Gly-315 with Asp in this region could diminish polypeptide binding activity by altering the conformation of the polypeptide binding fold of Ydj1. Such alterations in Ydj1 structure are predicted to result from restrictions in allowed backbone conformations caused by the G315D amino acid substitution. Glycine can adopt a ϕ/ψ angle about the α-carbon, and residues (such as aspartate) that contain a β-carbon cannot form this angle. Alternatively, chaperone function of Ydj1 G315D may be compromised because it has a charged hydrophilic side chain projecting into a region of the protein that is predicted to interact with hydrophobic portions of unfolded proteins.

The zinc finger-like region also appears to participate in interactions between Ydj1 and unfolded polypeptides, but the action of this domain does not appear essential for the binding of the model substrate proteins we examined in this study. Proteolytic removal of the first zinc binding domain from Ydj1 did not significantly diminish the ability of Ydj1 (179–384) to suppress rhodanese aggregation. The Ydj1 C159T mutant was able to bind proteins in a manner that was similar to the wild type protein. However, Ydj1 C159T exhibited severe defects in its ability to cooperate with Hsp70 and refold the denatured luciferase. These data suggest that the first zinc binding cleft and possibly the whole zinc finger-like domain of Ydj1 are dispensable for binding unfolded proteins. However, the zinc finger region appears to play a critical role in the folding polypeptides once they are bound to Ydj1.

Consistent with our studies on Ydj1, the zinc finger-like region of DnaJ has been shown to participate in interactions with unfolded proteins (44, 45). However, the relative importance of the zinc finger-like region in polypeptide binding reactions carried out by different Hsp40 family members appears to require further clarification. Szabo et al. (45) reported that site-directed mutagenesis of conserved cysteines in the zinc finger-like region of DnaJ caused severe defects in its ability to suppress rhodanese aggregation. This result suggested that the zinc finger-like region is critical for polypeptide binding by DnaJ. Zylicz and colleagues (44) recently reported that deletion of the zinc finger-like region of DnaJ reduced the binding of some but not all substrates. Therefore, these investigators concluded that the zinc finger-like region helped determine the substrate specificity of DnaJ. Thus, although it is clear that the zinc finger-like region helps Hsp40 proteins interact with unfolded polypeptides, its exact role in the binding and folding of proteins requires further study.

How do the conserved carboxyl terminus and the zinc finger-like region cooperate to facilitate protein folding by Ydj1? In order for Hsp70 and Ydj1 to fold proteins, they must bind and release them in an efficient manner. The zinc finger-like region may act at either or both of these steps in the Hsp70 reaction cycle. At early stages of protein folding, the conserved carboxyl terminus of Ydj1 might be competent for binding unfolded polypeptides that are in unordered conformations. However, the conserved carboxyl terminus may require the assistance of the zinc finger-like region to bind late stage folding intermediates that have adopted secondary or tertiary structure. Inability of Ydj1 C159T to interact productively with molten globule intermediates of protein folding pathways could be why it is unable to efficiently refold denatured polypeptides.

Zylicz and colleagues (44) have suggested the zinc finger-like domain of DnaJ functions to alter the conformation of Hsp70 in a manner that alters its affinity for unfolded proteins. The zinc finger-like region of Ydj1 may have a similar function, and this may be abrogated in Ydj1 C159T, which causes a decrease in its efficiency in protein folding reactions. Ongoing studies are focused on investigating how the zinc finger-like region of Ydj1 functions to increase the efficiency of Hsp70 action in the cell.

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