Cooperation of the molecular chaperone Ydj1 with specific Hsp70 homologs to suppress protein aggregation

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Abstract **Ydj1p, a cytosolic DnaJ homolog from *Saccharomyces cerevisiae*, is demonstrated to function as a molecular chaperone. Purified Ydj1p formed complexes with non-native polypeptides and suppressed protein aggregation. Ydj1p cooperated with Ssa Hsp70 proteins in the prevention of protein aggregation, but not with the Ssb Hsp70 proteins. Cooperation between these different molecular chaperones was only observed in the presence of hydrolyzable ATP and correlated with the ability of Ydj1p to stimulate the ATPase activity of the Hsp70 homolog with which it was paired. The regulatory and chaperone activities of a eukaryotic DnaJ homolog thus act together to assist Hsp70 in modulating the conformation of proteins.**

Key words: DnaJ; Hsp70; Molecular chaperone; Protein folding

1. Introduction

Eukaryotic DnaJ homologs are a family of proteins that specifically interact with different Hsp70 proteins to facilitate protein folding, assembly, translocation across membranes and degradation (reviewed in [1]). Hsp70 proteins act as molecular chaperones to mediate these events by transiently binding and releasing polypeptides in an ATP dependent cycle [2,3]. DnaJ-like proteins appear to regulate this cycle through modulating the ATPase activity of Hsp70 [4,5]. In *S. cerevisiae,* five gene families encode nine different Hsp70 homologs; SSA1–4, SSB1–2, SSC1, SSD1/KAR2 and MSI3 [6,7]. The Ssa and Ssb families of Hsp70 proteins are localized to the cytosol where they play roles in facilitating protein translocation across membranes [8] and protein synthesis [9], respectively. Two of the eight DnaJ homologs so far identified in *S. cerevisiae* are also localized in the cytosol [1]. One of these DnaJ homologs is Ydj1p, a protein that is essential for growth at elevated temperatures and helps facilitate protein translocation across ER and mitochondrial membranes [10, 11]. Purified Ydj1p strongly stimulates the ATPase activity of Ssa1p and Ssa2p [5], but has little effect on the ATPase activity of a purified mixture of Ssb1/2p [12]. This observation suggests that defined pairs of Hsp70 and DnaJ homologs interact in the eukaryotic cell.

E. coli DnaJ can function as a molecular chaperone as well as a regulatory molecule [2,13]. The ability of eukaryotic DnaJ homologs to act as molecular chaperones is being investigated currently. To demonstrate the chaperone function of purified Ydj1p, its ability to interact with partially folded polypeptides was examined. Ydj1p acted as a molecular chaperone and prevented the aggregation of protein folding intermediates by forming stable complexes with them. Ydj1p cooperated with Ssa2p, but not Ssb1/2p, to prevent protein aggregation. An eukaryotic DnaJ homolog was demonstrated directly to function with specific Hsp70 proteins as a regulatory chaperone.

2. Materials and methods

2.1. Protein purification

Ydj1p was overexpressed in *E. coli* and purified as previously described [5]. Ssa2p and a mixture of Ssb1 and Ssb2 proteins, which are 99% identical and termed Ssb1/2p, were purified as previously described [12]. All protein solutions (2.0–3.0 mg protein/ml) were of greater than 95% purity and were stored in 10 mM HEPES/KOH, pH 7.0, 50 mM NaCl, 10% glycerol and 10 mM DTT at −80°C until use. Protein concentrations were determined using the BioRad Bradford assay kit with Bovine serum albumin as the standard.

2.2. Protein aggregation assays

The aggregation of rhodanese was determined by light scattering as previously described [14]. Briefly, bovine rhodanese (50 μM; Sigma Chemical Co.) was denatured for precisely 1 h at 25°C in 6 M guanidinium-HCl buffered with 10 Tris-HCl, pH 7.4, and 10 mM DTT. Denatured rhodanese was diluted 100-fold into 200 μl of reaction buffer composed of 10 mM Tris-HCl, pH. 7.4, 25 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 [14].

2.3. Determination of complex formation between [^{3}H]rhodanese and Ydj1p

Bovine rhodanese was labeled with [^{3}H]sodium borohydride as previously described by standard methods [15]. [^{3}H]Rhodanese (50 μM) was denatured as described above. Denatured [^{3}H]rhodanese was diluted 100-fold upon addition to reaction mixtures containing Ydj1p under buffer conditions identical to those described for the protein aggregation assays. Reactions were incubated at 25°C for 15 min and placed immediately on ice. Complexes that formed between partially folded [^{3}H]rhodanese and Ydj1p during the incubation period were then determined by native gel electrophoresis on 6% acrylamide gels as previously described [5,12]. Ydj1p does not form detectable complexes with native proteins under these conditions [5]. Up to 45% of the [^{3}H]rhodanese migrated in an U-shaped band with a mobility coincident to that of Ydj1p, indicating the two proteins had formed a complex. When non-radioactive rhodanese was denatured and added to reaction mixtures, a significant reduction in binding of [^{3}H]rhodanese to Ydj1p was observed. This demonstrated that the Ydj1p–[^{3}H]rhodanese complex detected was specific (data not shown). Quantitation of complex formation between [^{3}H]rhodanese and Ydj1p is expressed in the % of total [^{3}H]rhodanese. Values were determined by laser densitometry of autoradiographed gels [16].

3. Results and discussion

3.1. Purified Ydj1p functions as a molecular chaperone

Molecular chaperones interact with polypeptides in non-native conformations to assist in events such as protein folding

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Abbreviations: Hsp70, heat shock protein 70.

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and suppression of protein aggregation. Rhodanese is a mitochondrial protein that rapidly aggregates after dilution out of denaturant [17]. Previous work has established the utility of monitoring the influence of heat shock proteins on rhodanese aggregation to measure their chaperone activity [18]. To test if Ydj1p behaves as a molecular chaperone, the influence of this heat shock protein on rhodanese aggregation was determined (Fig. 1A). When unfolded rhodanese was added to reaction mixtures at a final concentration of 0.5 μM it aggregated over time. Inclusion of Ydj1p in reaction mixtures decreased the rate of rhodanese aggregation in a dose-dependent manner. In the presence of 2.5 μM Ydj1p, rhodanese aggregation was reduced by over 85%. Addition of similar quantities of BSA to reaction mixtures had no detectable effect on rates of rhodanese aggregation (data not shown). Thus, Ydj1p possesses an activity that allows it to retard the aggregation of proteins.

Proteins with chaperone functions act through forming transient, but stable, complexes with their substrate polypeptides. If Ydj1p prevents rhodanese aggregation via a mechanism consistent with it being a chaperone, then complex formation between Ydj1p and rhodanese should parallel suppression of rhodanese aggregation. This was indeed found to be the case when [^{3}H]rhodanese was allowed to refold after dilution out of denaturant in the presence of Ydj1p (Fig. 1B). Ydj1p and [^{3}H]rhodanese formed a stable complex that could be detected on native polyacrylamide gels. The quantity of the Ydj1p–rhodanese complex detected was dependent on the concentration of Ydj1p in reaction mixtures. Approximately 45% of [^{3}H]rhodanese present in reactions was detected in a complex with Ydj1p at a concentration (2.5 μM) where 85% suppression of protein aggregation was observed. It is likely that higher levels of rhodanese were not detected in a complex with Ydj1p because some of the complex dissociated during analysis. Nonetheless, these data demonstrate that formation of a complex between Ydj1p and unfolded polypeptides results in the suppression of protein aggregation (Fig. 1). Ydj1p behaved similarly when incubated with the denatured form of a chimeric mitochondrial protein that consisted of amino acids 1–167 of the cytochrome B_2 precursor fused to amino acids 51–325 of the β subunit of the F_1-ATPase from *Neurospora crassa* (data not shown). The activities of Ydj1p therefore fit the criteria for its classification as a molecular chaperone.

3.2. *ATP-dependent cooperation of Ydj1p with Ssa2p to prevent protein aggregation*

Ydj1p has been shown to participate in pathways that require Ssa2p to modulate the conformation of polypeptides, but whether Ydj1p acts alone or in combination with Ssa2p in this process is unknown [10,11]. To investigate this possibility, the influence of Ydj1p on rhodanese aggregation in combination with Ssa2p was examined (Fig. 2A,B). For these experiments, Ssa2p and Ydj1p were used at levels that prevented rhodanese aggregation by 20 and 30%, respectively, when added alone (Fig. 2A). Ydj1p and Ssa2p added together in the absence of ATP had only additive effects on rates of protein aggregation, reducing it by about 50%. ATP addition did not significantly reduce protein aggregation over that observed with Ssa2p alone (Fig. 2B). However, the combination of Ssa2p, Ydj1p and ATP almost completely suppressed rhodanese aggregation (Fig. 2A). As observed with purified *E. coli* DnaJ and Hsp70, the combination of these chaperones suppressed rhodanese aggregation

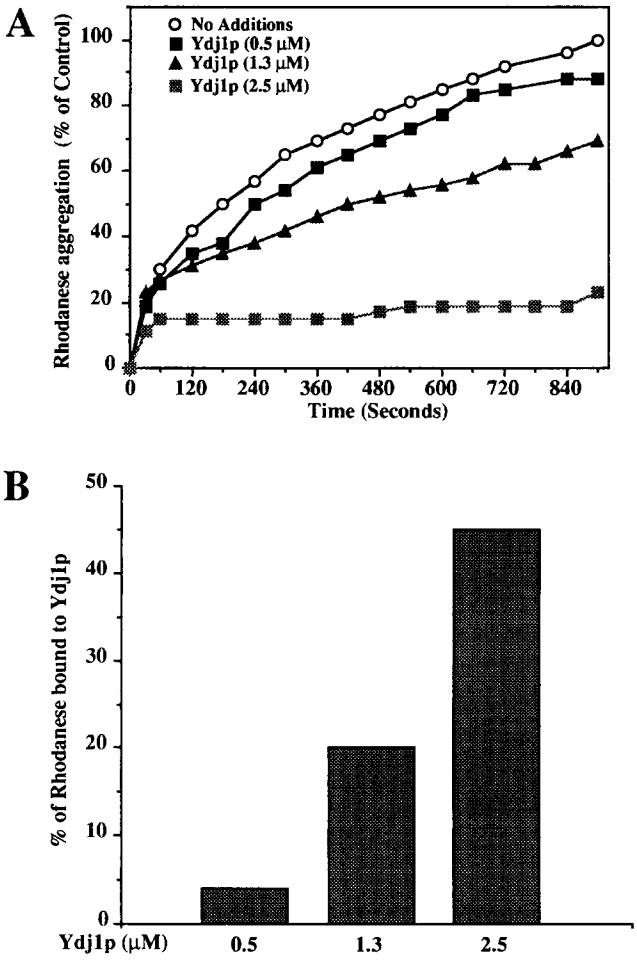

Fig. 1. Ydj1p forms complexes with non-native polypeptides to prevent protein aggregations. (A) Suppression of rhodanese aggregation by Ydj1p. Bovine rhodanese was denatured in 6 M guanidinum-HCl and was allowed to refold after a 100-fold dilution, to a final concentration of 0.5 μM, into a buffer containing 10 mM Tris-Cl, 25 mM KCl and 10 mM DTT and Ydj1p as indicated. The aggregation of rhodanese was monitored at 320 nm at 25°C for the indicated time. After 15 min incubation over 90% of added rhodanese had aggregated. Values for aggregation are expressed as percentage of total aggregation that took place at the 15 min time point when no Ydj1p was added to reaction mixtures. (B) Complex formation between Ydj1p and [^{3}H]rhodanese. [^{3}H]Rhodanese was denatured as described above and added to a final concentration of 0.5 μM to reaction mixtures identical to those used in the protein aggregation assays. After incubation for 15 min at 25°C, complexes formed between Ydj1p and [^{3}H]rhodanese were determined by native gel electrophoresis (see section 2 for details). Values are expressed as the % of total [^{3}H]rhodanese that was found in a complex with Ydj1p.

through the formation of a ternary complex with rhodanese ([18]; data not shown). This team work between Ydj1p and Ssa2p appeared to require ATP hydrolysis, since substitution of ATP with AMP-PNP, a non-hydrolyzable ATP analog, abolished the synergistic effects of this Hsp70:DnaJ chaperone pair on rhodanese aggregation (Fig. 2B, lane 5 vs. 6). These data directly demonstrate that eukaryotic Hsp70 and DnaJ homologs can function together to modulate the conformation of a substrate protein in an ATP dependent manner.

Since two different subfamilies of Hsp70 are localized to the cytosol, it was asked next if Ydj1p could cooperate with the Ssb

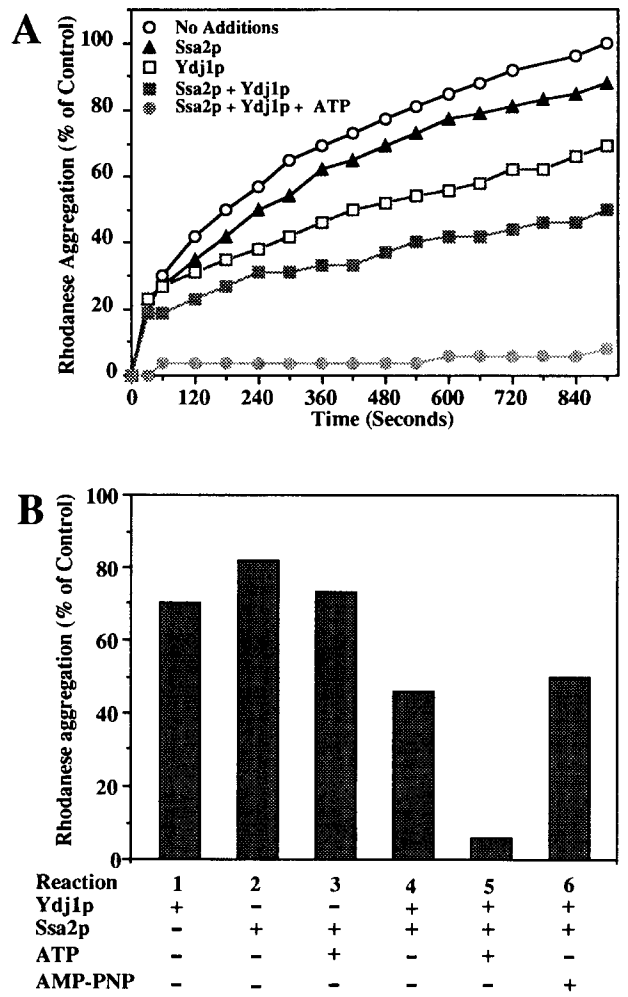

Fig. 2. Ydj1p and Ssa2p cooperate to synergistically prevent protein aggregation. (A) Kinetics of Rhodanese aggregation. Bovine rhodanese was denatured in 6 M guanidinium-HCl and its aggregation at 25°C after 100-fold dilution from denaturant to 0.5 μM was monitored as described in the legend to Fig. 1. Where indicated, Ydj1p (1.3 μM), Ssa2p (0.5 μM) and Mg-ATP (1 mM) were added to reactions prior to addition of rhodanese. (B) The presence of hydrolyzable ATP is required for Ydj1p and Ssa2p to cooperate in preventing rhodanese aggregation. Where indicated, Mg-AMP-PNP was 1 mM. Values are expressed in % of total rhodanese aggregation that was observed after 15 min of incubation when no additions were made to reaction mixtures.

proteins in addition to Ssa protein family members in suppressing protein aggregation. Ssb1/2p at 0.5 μM suppressed rhodanese aggregation by 25%; addition of ATP did not increase the efficiency of this reaction (Fig. 3B). At several-fold higher concentrations Ssb1/2p was observed to suppress the majority of protein aggregation (data not shown). Ydj1p in combination with Ssb1/2p had only additive effects on protein aggregation. Addition of ATP did not promote any further reduction of protein aggregation by Ssb1/2p and Ydj1p (Fig. 3B, lane 4 vs. 5). This result was in contrast with results obtained with Ssa2p and was consistent with the earlier finding that Ydj1p does not detectably stimulate the ATPase activity of Ssb1/2p [12]. These data also strongly suggest that the synergistic effects of Ssa2p and Ydj1p on protein aggregation observed in the presence of ATP result from specific cooperation between this particular pair of DnaJ and Hsp70 homologs.

3.3. Concluding remarks

This study presents data that provide new information on a number of issues concerning the functions of eukaryotic DnaJ homologs in protein metabolism. (1) Ydj1p can suppress protein aggregation by forming a complex with polypeptides in non-native conformations and can therefore be classified as a molecular chaperone. The mammalian DnaJ homolog Hsp40 has also been observed to form complexes with polypeptides [19]. Thus, data are emerging to support the statement that multiple members of the family of eukaryotic DnaJ-like proteins have the conserved ability to function as chaperones. However, not all DnaJ homologs are expected to act as chaperones. Some contain only the conserved J domain thought to be responsible for regulation of the ATP hydrolytic cycle of Hsp70 [1,20,21]. The polypeptide binding site of DnaJ homologs has not been identified, but it is thought to lie in a region independent of the J domain. (2) This study and others demonstrates that DnaJ-like proteins can bind polypeptides independent of Hsp70 [13,18,19,22]. This feature of DnaJ-like proteins suggests they can act individually as chaperones in certain situations. It will be important to determine the mechanism for substrate binding and release by DnaJ-like proteins to further understand the cellular processes they facilitate. (3) DnaJ-like proteins exhibit specificity in the substrates they recognize. They bind partly folded polypeptides, but not linear model proteins [5,18]. The aspect of secondary protein structure recognized by DnaJ-like proteins needs to be elucidated in order to learn the basis of their chaperone function. (4) Two direct observations now suggest that DnaJ and Hsp70 homologs act in specific pairs. First, Ydj1p is a strong stimulator of ATPase activity of the Ssa Hsp70 proteins, but not that of the Ssb Hsp70 proteins [12]. Second, Ydj1p cooperates with the Ssa Hsp70, but not the Ssb Hsp70, proteins to prevent protein aggregation. There is also indirect evidence that Hsp70 homologs specifically recognize DnaJ-like proteins [24]. The factors that determine the specificity of these observed interactions need to be elucidated to investigate the possibility that DnaJ-like proteins act as specificity factors that target Hsp70 proteins to different substrate proteins in the eukaryotic cell [1,22]. (5) The ability of Ydj1p to bind non-native proteins does not appear sufficient to enhance the ability of Hsp70 proteins to prevent protein aggregation in the absence of its regulatory activities. Hydrolyzable ATP must be present in order for synergism between Ydj1p and Ssa2p to be observed. Furthermore, if Ydj1p was unable to stimulate the ATPase activity of the Hsp70 homolog with which it is paired, team work in preventing rhodanese aggregation was not observed. The ATP bound state of Hsp70 appears to be the form of the molecule that binds polypeptides [2], but it has a relatively low affinity for substrate protein compared to the ADP bound state [25]. Up regulation of the ATP hydrolytic cycle by Ydj1p converts Hsp70 to the ADP bound high affinity state which, combined with the ability to form a stable Hsp70–DnaJ–polypeptide complex [18], appears required in order for DnaJ-like proteins to assist Hsp70 in the prevention of protein aggregation.

In some situations the ternary complex between these chaperones and polypeptides is not formed because the substrate protein is not recognized and bound by the DnaJ-like

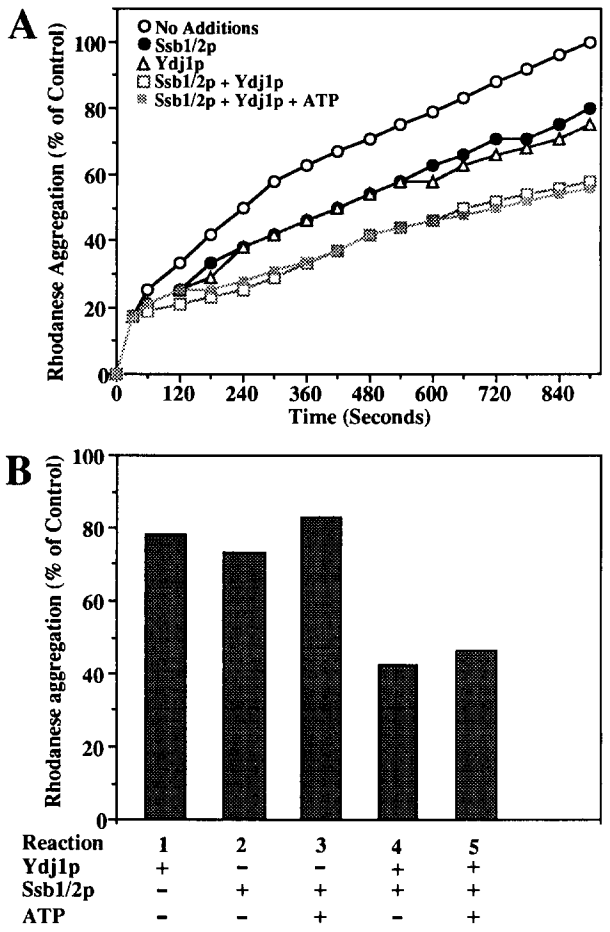

Fig. 3. Ydj1p and Ssb1/2p do not cooperate in the suppression of protein aggregation. (A) Kinetics of rhodanese aggregation. Bovine rhodanese was denatured in 6 M guanidinium-HCl and its aggregation at 25°C after 100-fold dilution from denaturant to 0.5 μM was monitored as described in the legend to Fig. 1. Where indicated, Ydj1p (1.3 μM), Ssb1/2p (0.5 μM) and Mg-ATP (1 mM) were added to reactions prior to addition of rhodanese. (B) Quantitation of rhodanese aggregation at a single time point. Values are expressed in % of total rhodanese aggregation that was observed after 15 min of incubation when no additions were made to reaction mixtures.

protein. In these instances, DnaJ-like proteins promote the dissociation of complexes between Hsp70 and polypeptides by increasing the rate of the ATP hydrolytic cycle [5,12,23]. Regulation of ATP hydrolysis by DnaJ-like proteins, in the absence of chaperone function, was recently demonstrated to be sufficient to assist Hsp70 homologs in some cellular reactions [20,21].

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