Synergistic Binding of DnaJ and DnaK Chaperones to Heat Shock Transcription Factor σ^{32} Ensures Its Characteristic High Metabolic Instability

**IMPLICATIONS FOR HEAT SHOCK PROTEIN 70 (Hsp70)-Hsp40 MODE OF FUNCTION**

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**Background:** Interaction between chaperone and co-chaperone is crucial for chaperone binding to substrates.

**Results:** The reduced affinity of mutant σ^{32} for DnaJ reduces complex formation with DnaK.

**Conclusion:** The stable binding of σ^{32} to DnaK requires the exposure of σ^{32} sites to bind DnaJ.

**Significance:** This model can be applied to chaperone action in helping newly synthesized polypeptides to fold.

*Escherichia coli* heat shock transcription factor σ^{32} is rapidly degraded by ATP-dependent proteases, such as FtsH and ClpYQ. Although the DnaK chaperone system (DnaK, DnaJ, and GrpE) promotes σ^{32} degradation *in vivo*, the precise mechanism that is involved remains unknown. Our previous results indicated that σ^{32} mutants containing amino acid substitution in the N-terminal half of Region 2.1 are markedly stabilized *in vivo*. Here, we report the further characterization of these mutants by examining purified proteins. Co-precipitation and gel filtration analyses show that purified σ^{32} mutants *in vitro* is more susceptible to ClpYQ and FtsH proteases than wild-type σ^{32}, indicating that the stability of σ^{32} does not always reflect its susceptibility to proteases. Co-precipitation and gel filtration analyses show that purified σ^{32} mutants *in vitro* is more susceptible to ClpYQ and FtsH proteases than wild-type σ^{32}, indicating that the stability of σ^{32} does not always reflect its susceptibility to proteases. Co-precipitation and gel filtration analyses show that purified σ^{32} mutants *in vitro* is more susceptible to ClpYQ and FtsH proteases than wild-type σ^{32}, indicating that the stability of σ^{32} does not always reflect its susceptibility to proteases.

E. coli is much longer in *in vivo* degradation. We argue that the stable and effective interaction of heat shock protein 70 (Hsp70) with a substrate polypeptide may generally require the simultaneous binding of heat shock protein 40 (Hsp40) to distinct sites on the substrate.

Various cellular processes are controlled by the regulated degradation of key protein factors. *Escherichia coli* heat shock transcription factor σ^{32} (encoded by the *rpoH* gene), which is required for the heat shock response, is rapidly degraded with a half-life of 1–2 min during steady-state growth at 30 °C (1, 2). When *E. coli* cells are shifted from 30 to 42 °C, σ^{32} is transiently stabilized, and the translation of *rpoH* mRNA increases, which lead to much higher levels of σ^{32} and heat shock proteins (HSPs), including molecular chaperones and ATP-dependent proteases. After alleviating stress-induced damage caused by misfolded or unfolded proteins, σ^{32} becomes unstable and is rapidly degraded. However, because excess amounts of HSPs are toxic to the cell, the tight regulation of σ^{32} levels by the degradation machinery is crucial for sustaining growth under any circumstances.

Among the five ATP-dependent proteases (Lon, ClpAP, ClpXP, FtsH (HfB), and ClpYQ (HslU/V)) known in *E. coli* (3), FtsH is the major protease involved in σ^{32} degradation (4, 5). FtsH is a member of the AAA proteases (6) and is a membrane-bound metalloprotease with an active site that is exposed to the cytoplasm. It is known to degrade some cytoplasmic proteins as well as membrane proteins (7). Although FtsH is essential for growth, a ΔftsH strain can be isolated if the cell simultaneously contains an unusually high activity of R-3-3-hydroxyacyl-ACP dehydrase (encoded by the *fabZ* gene) (8). The major function of FtsH is the maintenance of the proper lipopolysaccharide/phospholipids ratio by the degradation of LpxC. Other proteases are cytosolic and generally target abnormal proteins as well as some other specific substrates. σ^{32} is stabilized in a mutant that is multiply deficient in Lon, ClpXP, and ClpYQ (9). Purified ClpYQ directly degrades σ^{32}; σ^{32} degradation by both FtsH and ClpYQ *in vitro* is promoted by higher temperatures (10).

In addition to proteases, the DnaK chaperone system consisting of DnaK (Hsp70), DnaJ (Hsp40), and GrpE (nucleotide exchange factor) is required for the rapid degradation of σ^{32} *in vivo* because the half-life of σ^{32} is much longer in *dnaK*, *dnaJ*, and *grpE* mutants (11, 12). σ^{32} directly interacts with DnaK or DnaJ and forms a stable ternary complex in the presence of ATP (13, 14, 15, 16, 17). Although this interaction with DnaK and DnaJ has been thought to induce a conformational change in σ^{32} and promote degradation by proteases, such DnaK chaper-

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This article contains supplemental Experimental Procedures and Figs. S1–S12.

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one system effects on \(\sigma^{32}\) degradation have never been observed in vitro (18, 19).

Hsp70 chaperone systems in most organisms are known to be involved in the folding of newly synthesized polypeptides, the refolding of denatured proteins, the dissociation of proteins from complexes, and the degradation of abnormal proteins (20–23). A canonical Hsp70 chaperone system consists of Hsp70, Hsp40, and a nucleotide exchange factor (Hsp70 has an ATPase activity). Although the ATP-bound form of Hsp70 has a low affinity for substrates due to its high substrate exchange rate, the ADP-bound form has a higher affinity for substrates with a low substrate exchange rate. Hsp40 acts as a co-chaperone and activates the Hsp70 ATPase, although it also acts as a chaperone and binds to unfolded polypeptides to prevent aggregation. Through an interaction with Hsp40, Hsp70 becomes an ADP-bound form and tightly binds substrates. A nucleotide exchange factor promotes the dissociation of ADP from complexes, and the degradation of abnormal proteins involves the refolding of denatured proteins, the dissociation of proteins from complexes, and the degradation of abnormal proteins (18, 19). A canonical Hsp70 chaperone system consists of Hsp70, Hsp40, and a nucleotide exchange factor (Hsp70 has an ATPase activity).

A model has been proposed in which Hsp40 first recognizes and binds substrate polypeptides and then transfers them to Hsp70. However, in some cases, DnaK itself recognizes and binds to substrate polypeptides independent of DnaJ, as in the case of \(\sigma^{32}\) (24). Because most of the substrates of the Hsp70 chaperone system are unfolded polypeptides that are structurally heterogeneous, a native form of \(\sigma^{32}\) that can directly interact with DnaK and DnaJ should provide a useful model to elucidate the function and mechanism of the Hsp70 chaperone system.

Studies with another native substrate, bacteriophage P1 RepA protein, have located DnaK- and DnaJ-binding sites in RepA (25).

To gain further insight into chaperone functions in \(\sigma^{32}\) degradation, we isolated many \(\sigma^{32}\) mutants that are stable in vivo (26). These mutants contain one or two amino acid substitutions in the N-terminal half of Region 2.1. In this study, we examined the affinity of some mutant \(\sigma^{32}\) for proteases and chaperones and obtained results indicating that mutations generally decrease the capacity of \(\sigma^{32}\) to form complexes with DnaK and DnaJ.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—KY1603 (MC4100 [F- araD139 (F− lacI5::Tn10 suhX401 (αF13-PrpoDsec-lacZ))]) (27), which is a MC4100 derivative that lacks the rpoH gene but is able to grow at 37 °C due to the overproduction of GroEL and GroES, was used for the purification of DnaK and DnaJ. Both DnaK and DnaJ were purified from HB101 cells harboring pKV1961::Tn10::kan (27) and applied to a HiPrep Sephacryl S-300 column (GE Healthcare). The flow-through fraction was repeatedly loaded onto an ATP-agarose (Sigma-Aldrich) column that was equilibrated with Buffer A, and proteins were eluted with Buffer A containing 100 mM NaCl and 5 mM MgCl2. The fractions containing DnaK were dialyzed against Buffer B (25 mM HEPES-NaOH (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol) and applied to a HiPrep Sephacryl S-300 column. The resulting lysate was centrifuged as in the purification of \(\sigma^{32}\). The supernatant was treated with ammonium sulfate. Proteins that were precipitated in a range of ammonium sulfate concentrations between 0.24 and 0.31 g/ml were dissolved, dialyzed against Buffer A, and loaded onto a HiTrap heparin column (GE Healthcare). The flow-through fraction was repeatedly loaded onto an ATP-agarose (Sigma-Aldrich) column that was equilibrated with Buffer A containing 100 mM NaCl and 5 mM MgCl2. Proteins were eluted with Buffer A containing 100 mM NaCl and 5 mM ATP. The eluate was loaded onto a HiTrap Q-Sepharose column (GE Healthcare) equilibrated with Buffer A, and proteins were eluted with a linear gradient of NaCl. The fractions containing DnaK were dialyzed against Buffer B (25 mM HEPES-NaOH (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, 100 mM NaCl) and applied to a HiPrep Sephacryl S-300 column (GE Healthcare). DnaK was concentrated and stored at −70 °C.

DnaJ was purified from MC4100 cells harboring pKV1957 (pKV1142 trcp-dnaK). The cells were grown until the late log phase in L broth containing 50 μg/ml ampicillin at 30 °C, and DnaK synthesis was induced by 1 mM IPTG. Cells were harvested after 3 h, treated with lysozyme and sodium deoxycholate, and disrupted, and the resulting lysate was centrifuged as in the purification of \(\sigma^{32}\). The supernatant was treated with ammonium sulfate. Proteins that were precipitated in a range of ammonium sulfate concentrations between 0.24 and 0.31 g/ml were dissolved, dialyzed against Buffer A, and loaded onto a HiTrap heparin column (GE Healthcare). The flow-through fraction was repeatedly loaded onto an ATP-agarose (Sigma-Aldrich) column that was equilibrated with Buffer A containing 100 mM NaCl and 5 mM MgCl2. Proteins were eluted with Buffer A containing 100 mM NaCl and 5 mM ATP. The eluate was loaded onto a HiTrap Q-Sepharose column (GE Healthcare) equilibrated with Buffer A, and proteins were eluted with a linear gradient of NaCl. The fractions containing DnaK were dialyzed against Buffer B (25 mM HEPES-NaOH (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, 100 mM NaCl) and applied to a HiPrep Sephacryl S-300 column (GE Healthcare). DnaK was concentrated and stored at −70 °C.
Chaperone Binding to $\sigma^{32}$

RESULTS

**Purified Stable $\sigma^{32}$ Mutants Are Not Necessarily More Resistant to Proteases**—Among the $\sigma^{32}$ mutants that are stabilized in vitro (26), the two most stable variants, I54A $\sigma^{32}$ and L47Q/ L55Q $\sigma^{32}$, whose half-lives are 9-fold and more than 10-fold longer than that of the wild type, respectively, were examined in an in vitro degradation system using ClpYQ and FtsH proteases. As expected from high $\sigma^{32}$ activity in vivo (26), the purified $\sigma^{32}$ mutants were co-fractionated with core RNA polymerase like wild-type $\sigma^{32}$ using gel filtration (supplemental Fig. S1, A–C), indicating that large conformational changes did not occur due to amino acid substitution or during purification. When L47Q/L55Q $\sigma^{32}$ was incubated with ClpYQ at 37 or 42 °C, it was degraded much more slowly than wild-type $\sigma^{32}$ in accordance with its stability in vivo (Fig. 1, supplemental Fig. S2). In contrast, I54A $\sigma^{32}$ was degraded slightly faster than wild type at 37 °C and even faster at 42 °C (Fig. 1 and supplemental Fig. S2).
FIGURE 1. *In vitro* degradation of σ*32* by ClpYQ and FtsH proteases. σ*32* (2.4 μg) was incubated with ClpQ (1.0 μg) and ClpY (2.5 μg), or σ*32* (1.0 μg) was incubated with FtsH (2.0 μg), in a reaction mixture (60 μl) at 30, 37, or 42 °C. Aliquots (10 μl) were withdrawn at the indicated times and analyzed by SDS-PAGE followed by CBB staining (supplemental Fig. S2). Gel images were taken with LAS3000 image analyzer, and σ*32* bands were quantified with MultiGauge software. Percentages of σ*32* degraded are plotted against time. The mean values from three experiments are shown with S.E. (error bars). Circles, wild type; squares, I54A; triangles, L47Q/L55Q.

Similar results were obtained when the same σ*32* mutants were examined with FtsH protease: L47Q/L55Q σ*32* degraded more slowly than wild type, whereas I54A σ*32* degraded much faster than wild-type at all temperatures (30, 37, and 42 °C) (Fig. 1 and supplemental Fig. S2). These results indicate that stability of σ*32* in vivo does not always reflect its direct susceptibility to proteases and that the high stability of I54A σ*32* in vivo is attributed to some intracellular state of σ*32*.

**Stable σ*32* Mutants Show Reduced Affinity for DnaJ**—The above results showed that other factors in addition to proteases contributed to some intracellular state of σ*32*. Because the DnaK chaperone system has been thought to be required for both inactivation and rapid degradation of σ*32* in vivo (11, 12, 35, 36, 37), and wild-type σ*32* is known to form complexes with DnaK and/or DnaJ (13, 14, 15, 16, 17), stable σ*32* mutants were strongly expected to have decreased affinity for DnaK and/or DnaJ chaperones. To test this hypothesis, co-immunoprecipitation experiments were performed using anti-σ*32* serum. Because the DnaK-σ*32* complex was not detected in the presence of ATP, and DnaK was precipitated even without σ*32* in the presence of ADP, these experiments were performed without adding nucleotide. When I54A σ*32* or L47Q/L55Q σ*32* was mixed with DnaK and immunoprecipitated with anti-σ*32* serum, the amounts of DnaK co-precipitated were similar to that obtained with wild-type σ*32* (Fig. 2A and supplemental Fig. S3A). In contrast, very little DnaJ was co-precipitated with mutant σ*32* compared with wild type (Fig. 2B and supplemental Fig. S3B), suggesting that the σ*32* mutants are specifically deficient in binding to DnaJ. Although an appreciable amount of DnaJ was precipitated with anti-σ*32* serum even without the σ*32* addition, as shown in Fig. 2B (control (−)), the effects of mutation on σ*32*-DnaJ interaction were clearly observed.

To otherwise examine the effects of mutation on σ*32*-DnaK or σ*32*-DnaJ interaction, we constructed His-tagged DnaK and DnaJ at the C terminus, and their activities were confirmed both by complementation of the dnaK/dnaJ mutant phenotype in vivo and by refolding activity of the unfolded luciferase *in vitro* (data not shown). In pull-down assays using Ni²⁺-NTA-agarose, more than 90% of the DnaK-His or DnaJ-His was recovered in the presence of ATP or ADP (data not shown). When wild-type σ*32* was mixed with DnaK-His, a significantly higher amount of DnaJ was co-precipitated with wild-type σ*32* (Fig. 3A and supplemental Fig. S4A). Two σ*32* mutants showed no detectable difference in the amount of σ*32* co-precipitated with DnaK-His compared with wild-type σ*32* (Fig. 3A and supplemental Fig. S4A). In contrast, similar pull-down assays using DnaJ-His revealed much less interaction with mutant σ*32* than with wild-type σ*32* (Fig. 3B and supplemental Fig. S4B). Taken together with the above results of the co-immunoprecipitation experiments (Fig. 2, A and B), these results clearly indicate that amino acid substitutions in Region 2.1 resulted in a reduced affinity for DnaJ specifically.
Stable $\sigma^{32}$ Mutants Cannot Efficiently Form Complexes with DnaK in the Presence of DnaJ and ATP—Although the above results show that each chaperone can bind to $\sigma^{32}$ independently, DnaJ is known to activate DnaK to form a stable complex with $\sigma^{32}$ in the presence of ATP (16, 17). Therefore, we examined the $\sigma^{32}$-DnaK interaction in the presence of DnaJ and ATP. The co-immunoprecipitation experiments first showed that the amount of DnaJ co-precipitated with mutant $\sigma^{32}$ was much less than that with wild-type $\sigma^{32}$ (Fig. 2C, supplemental Fig. S3C). This is consistent with the result obtained when $\sigma^{32}$ interacted with DnaJ alone (Fig. 2B). However, unlike the results seen when $\sigma^{32}$ interacted with DnaK alone (Fig. 2A), the amounts of DnaK co-precipitated with mutant $\sigma^{32}$ clearly decreased compared with the wild type in the presence of DnaJ (Fig. 2D and supplemental Fig. S3D). To examine the effects of mutation on $\sigma^{32}$-DnaK interaction in the presence of DnaJ and ATP, we constructed His-tagged $\sigma^{32}$ at the C terminus. In pull-down assays using Ni$^{2+}$-NTA-agarose, 60–80% of the $\sigma^{32}$-His was recovered. When wild-type $\sigma^{32}$-His was mixed with DnaK and DnaJ, a significantly higher amount of DnaK was co-eluted with $\sigma^{32}$-His in the presence of ATP than in the presence of ADP (supplemental Fig. S5). In the presence of ATP, there was no significant difference in the DnaK-$\sigma^{32}$ complex formation between wild-type $\sigma^{32}$-His and I54A $\sigma^{32}$-His, regardless of whether DnaJ was included or not (supplemental Fig. S5), suggesting that DnaJ has no effect on the $\sigma^{32}$-DnaK interaction in the presence of ADP. In the presence of DnaJ and ATP, a higher amount of DnaK was co-eluted with wild-type $\sigma^{32}$-His than with I54A $\sigma^{32}$-His (supplemental Fig. S5). These results suggest that the low affinity of mutant $\sigma^{32}$ to DnaJ leads to an unstable interaction between the altered $\sigma^{32}$ protein and DnaK. It is thus conceivable that the binding of DnaJ to wild-type $\sigma^{32}$ induces a stable interaction with DnaK.

To further evaluate the extent of $\sigma^{32}$-DnaK (or $\sigma^{32}$-DnaJ) interaction, mixtures of $\sigma^{32}$, DnaK, and DnaJ were analyzed by gel filtration. DnaJ-His was used instead of DnaJ because of its higher purity. DnaK concentration was varied from 0.8 to 3.2 $\mu$M, whereas $\sigma^{32}$ and DnaJ-His were kept constant at 0.4 $\mu$M. As shown in Fig. 4 and supplemental Fig. S6, $\sigma^{32}$ was mainly eluted in three peaks: Fractions 21–24, 25–28, and 31–35. The $\sigma^{32}$ found in Fractions 31–35 is free $\sigma^{32}$. The faster moving complexes (Fractions 21–24) contain two or more DnaK and one or less DnaJ molecule per $\sigma^{32}$ (supplemental Fig. S7). Judging from the standard curve, Fractions 25–28 appear to be a mixture of (DnaK)-$\sigma^{32}$-(DnaJ)$_x$, DnaK-$\sigma^{32}$, and (DnaJ)$_x$-$\sigma^{32}$. Wild-type $\sigma^{32}$ moved faster (from Fractions 31–35 to Fractions 25–28 and 21–24) as DnaK concentration increased (Fig. 4, A–D, circles). Although DnaJ was eluted over a wide range, distinctive peaks of eluted DnaJ were detected and corresponded with those of $\sigma^{32}$ in the case of wild-type $\sigma^{32}$, whereas in the case of mutant $\sigma^{32}$, no significant DnaJ peak was observed, and most DnaJ molecules were eluted in Fractions 17–19 (aggregated DnaJ) (supplemental Fig. S8).
The fraction (percentage) of free wild-type $\sigma^{32}$ decreased with increasing DnaK; it was 32% at 0.8 $\mu$M DnaK, 15% at 1.6 $\mu$M DnaK, 4% at 2.4 $\mu$M DnaK, and 3% at 3.2 $\mu$M DnaK. Similar results were obtained when authentic DnaJ was used instead of DnaJ-His (supplemental Fig. S9). At 1.6 $\mu$M or higher concentrations of DnaK, most wild-type $\sigma^{32}$ was detected in high molecular weight regions. In contrast, 39% of I54A $\sigma^{32}$ and 37% of L47Q/L55Q $\sigma^{32}$ remained free even when incubated with 3.2 $\mu$M DnaK. Because $\sigma^{32}$ mutants have a reduced affinity for DnaJ, we expected that the percentage of free $\sigma^{32}$ mutant might decrease if the concentration of DnaJ-His was elevated. However, even when a 3-fold higher concentration of DnaJ-His (1.2 $\mu$M) was used with fixed concentrations of $\sigma^{32}$ (0.4 $\mu$M) and DnaK (0.8 $\mu$M), the percentage of free $\sigma^{32}$ did not change, and most mutant $\sigma^{32}$ remained in Fractions 31–35 (supplemental Fig. S10). Because it was difficult to analyze an effect of even higher amounts of DnaJ on complex formation by gel filtration due to its self-aggregation, pull-down assays were performed with His-tagged $\sigma^{32}$. Even when a higher amount of DnaJ was used (in this case, authentic DnaJ was used), the level of DnaK co-eluted with I54A $\sigma^{32}$-His did not change (supplemental Fig. S11). All of these results together with those of co-immunoprecipitation and pull-down experiments (Figs. 2 and 3) suggest that much higher concentrations of DnaJ are required to overcome the low affinity of mutant $\sigma^{32}$ for DnaJ in forming DnaK-$$\sigma^{32}$-(DnaJ) ternary complex under these conditions.

**Moderately Stabilized $\sigma^{32}$ Mutants in Vivo Show Intermediate Affinity for Chaperones**—To clarify the relationship between the in vivo stability of $\sigma^{32}$ and the affinity for chaperones, we examined two other $\sigma^{32}$ mutants, A50S and K51E, that exhibit moderately increased in vivo stability (half-life approximately 4 times longer than that of wild type) (26). The purified $\sigma^{32}$ mutants were co-fractionated with core RNA polymerase like wild-type $\sigma^{32}$ using gel filtration (supplemental Fig. S1, D and E). When these $\sigma^{32}$ mutants were incubated with FtsH protease at 42 °C, K51E $\sigma^{32}$ was slightly more stable, but A50S $\sigma^{32}$ was more susceptible to FtsH than the wild-type, much like I54A $\sigma^{32}$ (Fig. 5A and supplemental Fig. S12A). This again indicates that in vivo stability does not directly reflect susceptibility to FtsH protease. We next analyzed the interaction of these $\sigma^{32}$ mutants with DnaK-His and DnaJ-His by gel filtration. Although DnaK-His was used in this experiment, the results were similar to those with authentic DnaK; in other words, the percentage of wild-type $\sigma^{32}$ and I54A $\sigma^{32}$ in its free form (Fractions 31–35) at 1.6 $\mu$M DnaK-His was 10.8 and 62.3%, respectively (Fig. 5B and supplemental Fig. S12B), and 9.2 and 38.6% at 2.4 $\mu$M DnaK-His (Fig. 5C and supplemental Fig. S12C), indicating that the His tag does not appreciably affect the efficiency of complex formation (Table 1). In the case of A50S $\sigma^{32}$ and K51E $\sigma^{32}$, the percentage of free $\sigma^{32}$ was 40.4 and 59.4% at 1.6 $\mu$M DnaK-His and 26.5 and 18.5% at 2.4 $\mu$M DnaK-His, respectively (Fig. 5, B and C, and supplemental Fig. S12, B and C). When the percentage of free $\sigma^{32}$ was plotted against relative half-lives in vivo, a clear correlation was evident between these

**TABLE 1**

| Type of $\sigma^{32}$ | Authentic DnaK | DnaK-His |
|----------------------|---------------|----------|
|                      | 1.6 $\mu$M | 2.4 $\mu$M | 1.6 $\mu$M | 2.4 $\mu$M |
| WT                   | 14.7% | 4.1% | 10.8% | 9.2% |
| I54A                 | 47.3% | 40.4% | 62.3% | 38.6% |
| A50S                 | 40.4% | 26.5% |
| K51E                 | 59.4% | 18.5% |

*Data from the experiment shown in Fig. 4, B and C.

Data from the experiment shown in Fig. 5, B and C.

**FIGURE 6. Correlation between the percentage of $\sigma^{32}$ that remains free in gel filtration analysis and the stability of $\sigma^{32}$ (half-life) in vivo.** Gel filtration experiments were repeated 2–4 times, and the percentages of free $\sigma^{32}$ (Fractions 31–35) at 2.4 $\mu$M DnaK-His were calculated: wild-type $\sigma^{32}$ (closed circles), 9.2, 5.0, 9.7, and 13.6%; I54A $\sigma^{32}$ (open squares), 38.6 and 56.6%; A50S $\sigma^{32}$ (closed squares), 26.5 and 27.0%; K51E $\sigma^{32}$ (closed triangles), 18.5, 31.3, and 25.6%. Half-lives in the obsccissa are shown relative to the wild type.
two parameters (Fig. 6), strongly suggesting that the effective interaction of DnaK and DnaJ with \( \sigma^{32} \) is critical for sustaining the characteristic instability of \( \sigma^{32} \) in vivo.

**DISCUSSION**

Several specific amino acid residues in the N-terminal half of Region 2.1 of \( \sigma^{32} \) are intimately involved in the rapid degradation of \( \sigma^{32} \) (26). Here, we demonstrate that amino acid substitutions in this region lead to reduced affinity of \( \sigma^{32} \) for DnaJ (Figs. 2B and 3B), which in turn leads to unstable interaction of \( \sigma^{32} \) with DnaK in the presence of DnaJ and ATP (Figs. 2D and 4). The close correlation found between the efficiency of complex formation of \( \sigma^{32} \) with DnaK/DnaJ and in vivo stability (Fig. 6) strongly suggests that the defective interaction of \( \sigma^{32} \) with DnaK and DnaJ causes stabilization of \( \sigma^{32} \) in vivo. Thus, binding of DnaK and DnaJ to \( \sigma^{32} \) most probably exerts conformational change on \( \sigma^{32} \), which then promotes degradation by the proteases.

Obrist et al. selected \( \sigma^{32} \) mutants (L47Q, A50V, I54F, and I54T) based on their resistance to proteases (38). They argue that these mutations do not affect their affinity for DnaK or DnaJ and that Region 2.1 is involved in the interaction with FtsH protease (39, 40). The present finding that I54A \( \sigma^{32} \) and A50S \( \sigma^{32} \) are more sensitive to ClpQY and FtsH, whereas L47Q/L55Q \( \sigma^{32} \) and K51E \( \sigma^{32} \) are less sensitive (Figs. 1 and 5A), also suggests that Region 2.1 could be recognized directly by these proteases. Yura et al. (41) isolated \( \sigma^{32} \) mutants (A50D, K51E, I54N, and I54T) based on resistance to feedback inhibition of \( \sigma^{32} \) activity by DnaK and DnaJ or simply on increased \( \sigma^{32} \) activity. They showed that these mutants are defective in chaperone-mediated feedback control, in addition to having increased \( \sigma^{32} \) stability. However, analysis of the most defective mutant (I54N) exhibits chaperone-mediated inactivation in vitro and only a 2-fold higher dissociation constant \( (K_D) \) value for DnaJ using surface plasmon resonance. This is insufficient to account for the strong in vivo phenotype. Although the reasons for these differences remain unclear, our gel filtration results examining the interaction of \( \sigma^{32} \) with DnaK in the presence of DnaJ and ATP led to the finding that the \( \sigma^{32} \) mutants tested are clearly defective in chaperone-\( \sigma^{32} \) complex formation (Figs. 4 and 5). Analyses of I54N \( \sigma^{32} \) gave results very similar to those obtained for I54A \( \sigma^{32} \) (data not shown). Although the reduced affinity of the \( \sigma^{32} \) mutants for DnaK and DnaJ can account for their stability in vivo, detailed mechanisms of chaperone-promoted \( \sigma^{32} \) degradation remain unsolved. In this connection, we note that recent results suggest that the bacterial signal recognition particle plays a critical role in the chaperone-mediated feedback inhibition/degradation of \( \sigma^{32} \).

As for the role of the N-terminal half of Region 2.1 of \( \sigma^{32} \) in DnaJ binding, neighboring residues 57–66 of \( \sigma^{32} \) were recently reported to be a DnaJ-binding site (42). A \( \sigma^{32} \) mutant with three amino acid substitutions at positions 60, 62, and 63 showed reduced affinity for DnaJ. However, the fact that various amino acid substitutions at positions 47, 50, 51, 54, and 55 lead to \( \sigma^{32} \) stabilization primarily due to defects in DnaJ binding suggests

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\(^3\) T. Yura, personal communication.
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the importance of this region in DnaJ binding. We propose that an expanded region of $\sigma^{32}$, residues 47–66, affects DnaJ-binding directly.

Judging from the prevailing thought that Hsp40 first recognizes substrate polypeptides and then transfers them to Hsp70, the low affinity of mutant $\sigma^{32}$ for DnaK could be explained by assuming that the $\sigma^{32}$ mutant with low affinity for DnaJ is not recognized directly by DnaK. However, DnaK alone can recognize and bind $\sigma^{32}$ without the help of DnaJ (Figs. 2A and 3A) (13, 14, 16, 17), and DnaK appears to bind to $\sigma^{32}$ at a site different from that which binds to DnaJ (42). A truncated DnaJ mutant lacking the substrate-binding domain can induce the stable interaction of DnaK with $\sigma^{32}$ by stimulating ATPase activity of DnaK, although excess amounts are required compared with wild-type DnaJ (16). Furthermore, $\sigma^{32}$ and DnaJ synergistically stimulate the ATPase activity of DnaK (43). So far, chaperone mutants have been used in most previous experiments to examine chaperone-substrate interaction, whereas substrate mutants were used in the present study. These data complement each other and suggest that the major function of DnaJ is stimulation of DnaK ATPase activity in the DnaK chaperone cycle and that the most efficient stimulation occurs when both DnaJ and DnaK simultaneously bind to the same $\sigma^{32}$ molecule. We propose the following model for $\sigma^{32}$-chaperone interaction (Fig. 7A). DnaJ interacts with $\sigma^{32}$ at DnaJ-binding sites, and ATP-bound DnaK independently interacts with $\sigma^{32}$ at DnaK-binding sites. When DnaK-binding sites are located near the DnaJ-binding sites, ATP bound to DnaK is promptly hydrolyzed through a transient DnaK-DnaJ interaction. The resulting ADP-bound DnaK could stably and effectively bind $\sigma^{32}$. Chaperone binding would induce conformational change on $\sigma^{32}$, which then promotes degradation by the proteases. Without the proper interaction of DnaJ with DnaK on the same $\sigma^{32}$ molecule, the hydrolysis of ATP bound to DnaK would be slow, and the affinity of DnaK for $\sigma^{32}$ would remain low. As a result, weak interaction between mutant $\sigma^{32}$ and DnaJ could lead to high amounts of free $\sigma^{32}$.

It has been demonstrated that DnaK and DnaJ can independently bind to other substrates (oligopeptides and denatured proteins) and that DnaJ efficiently stimulates the ATPase activity of DnaK when DnaJ and DnaK bind to different segments of the same polypeptide chain (24). In a mammalian Hsp70 chaperone system, it was also recently shown that Hsp70 and Hsp40 bind independently to an unfolded protein (44). Thus, we can expand the above model to explain the general function of the Hsp70 chaperone system (Fig. 7B). The present results suggest that a polypeptide with low affinity for Hsp40 cannot be an active substrate for Hsp70. This scheme can be applied to the folding processes of unfolded polypeptides, such as newly synthesized polypeptide chains. In the process of repeated association with and dissociation from an unfolded polypeptide, Hsp70 molecules interacting with Hsp40 on the same polypeptide could stably bind to the substrate polypeptide. Only this stable binding by ADP-bound Hsp70 could effectively modulate the folding process of substrate polypeptides. Given that DnaJ catalytically activates DnaK functions (16, 24, 43), Hsp40 appears to promptly dissociate from Hsp70-substrate complexes. After Hsp70 dissociation, if Hsp40-binding sites on the substrate are sequestered inside the polypeptide chain, the polypeptide can no longer form a stable complex with Hsp70, although it retains a high affinity for Hsp70. Hsp40-binding sites appear to play an important role in stable Hsp70-substrate interaction. To effectively and stringently regulate Hsp70-substrate interaction, the number of Hsp40-binding sites per substrate polypeptide may be inherently fewer than that of the Hsp70-binding sites (44), or Hsp40-binding sites may be folded into the inside of the substrate polypeptide early in the folding process. In at least some proteins, such as $\sigma^{32}$ and RepA, Hsp40-binding site(s) remain on their surface even after completing the folding steps and can be used as regulatory domains (Fig. 7A). An important question is why Hsp40 binding dominates Hsp70 binding. Major Hsp40s function in a dimer form. The binding of Hsp70 to a substrate through an interaction with Hsp40 is expected not only to raise substrate specificity but also to raise cooperativity among Hsp70 molecules for binding to a substrate.

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