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The C Terminus of σ^{32} Is Not Essential for Degradation by FtsH

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A key step in the regulation of heat shock genes in *Escherichia coli* is the stress-dependent degradation of the heat shock promoter-specific σ^{32} subunit of RNA polymerase by the AAA protease, FtsH. Previous studies implicated the C termini of protein substrates, including σ^{32}, as degradation signals for AAA proteases. We investigated the role of the C terminus of σ^{32} in FtsH-dependent degradation by analysis of C-terminally truncated σ^{32} mutant proteins. Deletion of the 5, 11, 15, and 21 C-terminal residues of σ^{32} did not affect degradation in vivo or in vitro. Furthermore, a peptide comprising the C-terminal 21 residues of σ^{32} was not degraded by FtsH in vitro and thus did not serve as a recognition sequence for the protease, while an unrelated peptide of similar length was efficiently degraded. The truncated σ^{32} mutant proteins remained capable of associating with DnaK and DnaJ in vitro but showed intermediate (5-amino-acid deletion) and strong (11-, 15-, and 21-amino-acid deletions) defects in association with RNA polymerase in vitro and biological activity in vivo. These results indicate an important role for the C terminus of σ^{32} in RNA polymerase binding but no essential role for FtsH-dependent degradation and association of chaperones.

The expression of heat shock genes in *Escherichia coli* is positively controlled at the transcriptional level by the product of the *rpoH* gene, the σ^{32} subunit of RNA polymerase (RNAP) (5, 8, 30). Stress treatment of the cells, such as a sudden temperature upshift, induces transient heat shock gene expression until the cells have adapted to the applied stress. This heat shock response is mediated by increases in the translation of *rpoH*, stabilization of σ^{32}, and activation of σ^{32} by sequestration of the DnaK chaperone and its DnaJ cochaperone from a complex with σ^{32}.

Under steady-state growth conditions, σ^{32} is an extremely unstable protein with half-lives at 30 and 42°C of less than 1 and 4 min, respectively (17, 23, 26). Heat shock treatment of the cells by transfer from 30 to 42°C increases the half-life of σ^{32} transiently to approximately 10 min (23). Degradation is mediated mainly by FtsH (HflB), an ATP-dependent metalloprotease associated with the inner membrane (10, 25, 27, 28). The interaction of σ^{32} with the RNAP core enzyme prevents degradation, indicating that FtsH and RNAP compete for binding to σ^{32} (28). The DnaK chaperone system plays an active but mechanistically unclear role in σ^{32} degradation, since σ^{32} is stabilized in *dnaK* and *dnaJ* mutant backgrounds (22, 24, 25, 28).

A conceptually interesting question with respect to σ^{32} degradation is how this biologically active, and hence seemingly folded, protein can be subject to such efficient proteolysis. Either σ^{32} carries specific degradation signals within its polypeptide, or it is thermodynamically unstable despite its biological activity. Earlier work showed that the in vivo half-life of fusions between N-terminal fragments of σ^{32} and β-galactosidase increased when a stretch of 23 residues (R122 to Q144), located between conserved regions 2 and 3 of σ^{32} and termed region C, is deleted or replaced by another reading frame (17). However, subsequent analysis of σ^{32} mutants altered in region C showed that this region is not essential for degradation but instead plays a role in σ^{32} binding to RNAP (1). Another study identified a role for the C terminus of σ^{32} in degradation by FtsH (3). In this previous study, *rpoH* genes with truncated 3′ ends were cloned into an expression vector such that fusion proteins between C-terminally truncated σ^{32} proteins (by 20 residues) and six amino acids from the vector sequence were generated. These fusion proteins were more stable than wild-type σ^{32} in *E. coli* cells and in vitro. However, this approach did not exclude the possibility that the vector-encoded extra residues at the C terminus artificially stabilize the fusion proteins.

In this study we investigated further the role of the C terminus of σ^{32} in the degradation process. Our analysis of C-terminally truncated σ^{32} proteins shows that the authentic C-terminal residues are not essential for degradation.

MATERIALS AND METHODS

Strains, plasmids, and media. Cells of strains C600 (*thr-1 leuB6 thi-1 lacY supE44 rfbD1 fhuA21*) and BB2019 [GW1000 *recA441 sulA11* Δ(*argF-lac*)*U169 supC*(Ts) *rpoH165*(Am) pDMI,1] (6) were grown aerobically at 30 or 42°C in Luria broth (LB) or in M9 minimal medium supplemented with glucose (0.2%; M9-Glu), thiamine (20 μg/ml), and appropriate amino acids (50 μg/ml). Growth media were further supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM), kanamycin (20 μg/ml), and ampicillin (50 μg/ml) when required. The wild-type *rpoH* gene cloned into plasmid pUHE21-2fdΔ12 (7) was used as template for construction of 3′-truncated *rpoH* alleles. These alleles (*rpoH*-Δ5aa,

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rpoH-Δ11aa, *rpoH*-Δ15aa, and *rpoH*-Δ21aa) were generated by PCR using appropriate oligonucleotides with *Hin*dIII and *Pst*I restriction sites for cloning of the coding sequences into pUHE21-2fdΔ12. For production of hexahistidine-tagged σ^{32} variants, wild-type and mutant alleles of *rpoH* were subcloned by inserting the *Hin*dIII-*Pst*I fragments into pUHE212-1 (to encode N-His$_6$-σ^{32}). For immunoblotting and pulse-chase experiments, the *rpoH* alleles encoding wild-type σ^{32}, N-His$_6$-σ^{32}, C-His$_6$-σ^{32} (7), and C-terminally truncated σ^{32} proteins were subcloned by inserting the respective *Eco*RI-*Hin*dIII fragments into pFN476 (24). Plasmid pDMI,1 carrying *lacI*^{q} and encoding kanamycin resistance (14) was used to provide cells with a Lac repressor.

Overproduction of σ^{32} proteins and ex vivo degradation assay. C600 cells carrying *rpoH* expression plasmids and pDMI,1 were grown at 30°C in 20 ml of LB containing ampicillin and kanamycin. IPTG (1 mM final concentration) was added to exponential-phase cultures (optical density at 600 nm [OD_{600}], 0.5 to 0.7) for 1 h, followed by harvesting and washing of the cells in buffer A (50 mM Tris-acetate [pH 8.0], 5 mM magnesium acetate, 2 mM β-mercaptoethanol, 50 mM KCl, 5% glycerol). The cell pellet was dissolved (OD_{600}, 50) in buffer A containing 1 mM phenylmethylsulfonyl fluoride and sonicated. The lysate was transferred to Eppendorf tubes and centrifuged at 2,500 × *g* for 2 min, and the supernatant was subjected to ultracentrifugation at 100,000 × *g* for 2 h. The protein content of the soluble cytoplasmic fraction was quantified by Bradford assay using bovine serum albumin as a standard. A 5-μg portion of the cytoplasmic fraction was mixed with FtsH reaction buffer (50 mM Tris-acetate [pH 8.0], 5 mM magnesium acetate, 2 mM β-mercaptoethanol, 50 mM KCl) to reach a final volume of 15 μl. Degradation assays were started by adding 1 μl of 100 mM ATP and 4 μl of 10 μM FtsH activated by the addition of Zn^{2+} (27) or buffer without FtsH as a control and incubated at 42°C for 1 h.

In vitro degradation of σ^{32}. Wild-type and C-terminally truncated N-His$_6$-σ^{32} proteins were purified and radiolabeled with *N*-succinimidyl[2,3-^{3}H]propionate (Amersham) as previously described (7, 28). These proteins (1 μM each) were incubated with purified FtsH (2 μM) and tested for degradation as described previously (1, 28). After precipitation with trichloroacetic acid (TCA; 10%) followed by centrifugation (15,000 rpm, 3 min), the radioactive peptides generated by proteolysis were quantified in the supernatant by liquid scintillation counting. For assaying degradation of peptides, the final volume of the reaction was 60 μl. The σ^{32}-derived peptides Q132-Q151-C (QRKLFFNLRKTKQRLG WFNQC) and A264-A284-C (AERVRQLEKNAMKKLRAAIEAC) (50 μM each) were mixed and incubated with FtsH (1). At various time points, aliquots of 18 μl were mixed with 92 μl of 0.5% trifluoroacetic acid to stop the reaction. Products were analyzed by reverse-phase chromatography using a 5-to-80% acetonitrile gradient in 0.1% trifluoroacetic acid.

Analysis of protein interactions. DnaK and RNAP core enzyme were purified as described previously (4, 15). Association of N-His$_6$-σ^{32} or C-terminally truncated N-His$_6$-σ^{32} with DnaK, DnaJ, and RNAP core was determined by gel filtration using a Superdex 200 column essentially as described previously (1, 7). To determine association of σ^{32} with RNAP core, N-His$_6$-σ^{32} (1 μM) was incubated with RNAP core (1.5 μM) for 10 min at 30°C in transcription buffer (20 μl, final volume). To determine association of σ^{32} with DnaK, DnaK (5 μM) was incubated for 2 h at 30°C in transcription buffer to disfavor oligomerization, mixed with N-His$_6$-σ^{32} (1 μM) in a final volume of 20 μl, and further incubated for 30 min at 30°C. These mixtures were placed on ice, adjusted to 100 μl by addition of transcription buffer, and loaded on a Superdex 200 column at 4°C. Labeled N-His$_6$-σ^{32} was detected in the elution fractions by liquid scintillation counting.

In vivo stability of σ^{32}. For determination of σ^{32} stability in vivo, C600 cells carrying *rpoH* expression plasmids were grown at 30°C in M9-Glu until mid-log phase. One milliliter of culture was labeled for 1 min with 70 μCi of [^{35}S]methionine followed by chase with unlabeled methionine (200 μg/ml, final concentration). After 30 s (time for synthesis of σ^{32}), aliquots of 200 μl were collected at various times, mixed with TCA (10%, vol/vol), and incubated for 15 min on ice. After centrifugation for 15 min at 14,000 rpm, the pellets were washed with acetone and resuspended in 50 mM Tris-HCl (pH 8.0), 1% sodium dodecyl sulfate (SDS), and 1 mM EDTA. Samples were subjected to immunoprecipitation using σ^{32}-specific rabbit antiserum as described previously (25, 29).

SDS-PAGE, immunoblotting, and quantifications. Polyacrylamide gel electrophoresis (PAGE) was carried out as described by Laemmli (13) using SDS–12% polyacrylamide gels and staining with Coomassie brilliant blue. Immunoblotting was carried out according to standard procedures, using rabbit antisera specific for the relevant proteins as primary antibodies, and blots were developed with a Vistra ECF fluorescence Western blotting kit (Amersham) or 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium color detection using alkaline phosphatase-conjugated anti-rabbit immunoglobulin G as the secondary antibody (Vector Laboratories, Inc.). Stained gels and developed immunoblots were scanned using a fluoroimager (FLA-2000) and quantified using MacBAS software (Fuji Film Co.).

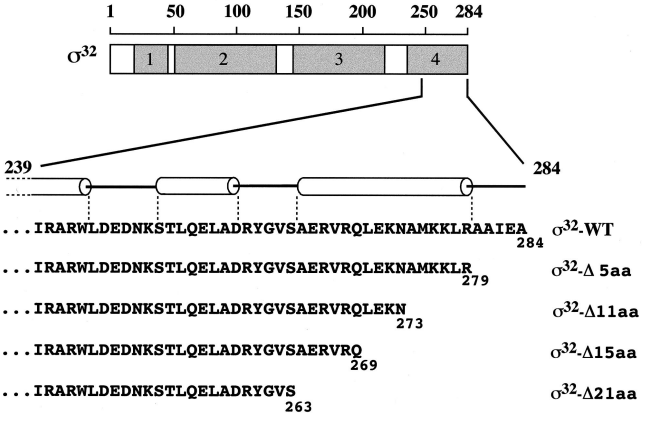

FIG. 1. Mutational alteration of σ^{32}. The locations of conserved regions 1 to 4 and details of the C-terminal region and its predicted secondary structure (using the PhD program) are shown. α-helices are shown as cylinders. The end points of the C-terminal truncations of the σ^{32} mutant proteins are indicated.

RESULTS

Construction of truncated σ^{32} mutant proteins. To investigate whether C-terminal residues of σ^{32} constitute a destabilizing element, we generated σ^{32} mutant proteins with truncations of their C termini by 5, 11, 15, and 21 residues (σ^{32}-Δ5aa, σ^{32}-Δ11aa, σ^{32}-Δ15aa, and σ^{32}-Δ21aa) (Fig. 1). The truncations were chosen such that the new termini are at two positions within (σ^{32}-Δ11aa and σ^{32}-Δ15aa), N-terminal to (σ^{32}-Δ21aa), or C-terminal to (σ^{32}-Δ5aa) an α-helix predicted by a secondary-structure-predicting algorithm (Fig. 1). PCR fragments of the appropriately truncated *rpoH* genes were cloned into the expression vector pUHE21-2fdΔ12 (7) such that a stop codon immediately follows the last codon, thus ensuring that no vector-encoded amino acids are fused to the σ^{32} mutant proteins. The C termini of the truncated σ^{32} mutant proteins do not resemble the consensus sequence of destabilizing C termini, as described by Sauer and coworkers (21), are not particularly hydrophobic, and do not constitute predicted DnaK binding sites (19).

In vivo activity of truncated σ^{32} mutant proteins. To determine the in vivo activity of the truncated σ^{32} mutant proteins, we first tested the ability of pUHE21-2fdΔ12-borne *rpoH* mutant alleles to complement the temperature-sensitive growth of *rpoH165*(Am) mutant cells on LB agar plates. The *rpoH165*(Am) mutant cells carry a temperature-sensitive amber suppressor mutation which is active and supports growth at 30°C. Only the *rpoH*-Δ5aa mutant allele allowed complementation of growth at 42°C to an extent similar to that allowed by wild-type *rpoH* (data not shown). Even in the absence of IPTG, a condition in which read-through expression of the *rpoH* mutant alleles produced σ^{32} levels only approximately fivefold above wild-type levels, σ^{32}-Δ5aa complemented the growth defects of *rpoH*(Am) mutants at 42°C (data not shown).

We then determined the cellular levels of the σ^{32} mutant proteins after IPTG-induced overproduction in *rpoH165*(Am) mutant cells. We observed that wild-type σ^{32} was poorly over-

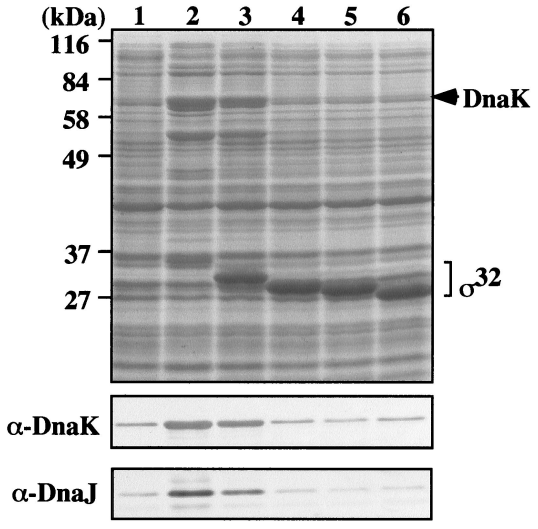

FIG. 2. In vivo activity of C-terminally truncated σ^{32} proteins. Cells of strain BB2019 [*rpoH165*(Am)] were transformed either with pUHE212-1 expressing wild-type or 3′-truncated *rpoH* alleles (producing N-His_6-σ^{32} proteins) or with pUHE212-1 control vector. Cultures of these cells were induced with IPTG, followed by SDS-PAGE of equal amounts of total protein and staining of the gels with Coomassie brilliant blue (top) or immunoblotting using DnaK- and DnaJ-specific sera (bottom). Lanes: 1, vector control; 2, N-His_6-σ^{32}; 3, N-His_6-σ^{32}-Δ5aa; 4, N-His_6-σ^{32}-Δ11aa; 5, N-His_6-σ^{32}-Δ15aa; 6, N-His_6-σ^{32}-Δ21aa.

produced, while σ^{32}-Δ5aa was overproduced to intermediate levels and σ^{32}-Δ11aa, σ^{32}-Δ15aa, and σ^{32}-Δ21aa were overproduced to high levels (Fig. 2).

Overproduction of wild-type σ^{32} led to strong increases in DnaK and DnaJ levels, overproduction of σ^{32}-Δ5aa led to intermediate increases, and overproduction of the other σ^{32} mutant proteins did not lead to increases in DnaK or DnaJ levels. The changes in DnaK and DnaJ levels were the result of the biological activity of σ^{32} in transcribing heat shock genes, including *dnaK* and *dnaJ*, and of the loss of this activity in the case of the σ^{32} mutant proteins with C-terminal truncations. Thus, through its ability to promote production of heat shock proteins, including FtsH and DnaK, the active σ^{32} triggers its own degradation and hence prevents stronger overproduction. Together, these results indicate that deletion of the ultimate C-terminal five residues (280 to 284) do not lead to complete loss of activity, whereas the segment between residues 264 and 279 is essential for the activity of σ^{32}.

In vivo stability of truncated σ^{32} mutant proteins. We determined the in vivo half-lives of two of the C-terminally truncated σ^{32} mutant proteins (σ^{32}-Δ11aa and σ^{32}-Δ15aa) by pulse-chase experiments followed by immunoprecipitation of σ^{32}. Such experiments were complicated by our finding that most of the C-terminally truncated σ^{32} mutant proteins lack activity in vivo. Consequently, the synthesis of heat shock proteins is lower in cells producing the inactive mutant proteins from plasmids than in cells producing wild-type σ^{32} from plasmids (Fig. 2). These heat shock proteins include DnaK and DnaJ, which are limiting for degradation of σ^{32} (24, 25, 28). In the cells expressing the inactive σ^{32} mutant proteins, σ^{32} should be less subject to chaperone-mediated degradation. When the σ^{32} proteins are overexpressed, the efficiency by which σ^{32} mutant

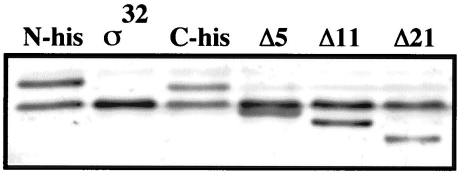

FIG. 3. Cellular levels of plasmid-encoded σ^{32} mutant proteins. Cells of strain C600 transformed with plasmid pFN476 expressing the appropriate *rpoH* allele were grown at 30°C in LB medium. Aliquots of exponential-phase cultures (OD_{600}, 0.5 to 0.7) were collected, and equal amounts of total protein were analyzed by SDS-PAGE. Levels of σ^{32} were determined by immunoblotting.

proteins are degraded in vivo will therefore be influenced by their biological activity. This complication led us to perform the σ^{32} half-life determinations in cells of strain C600 expressing low levels of plasmid-encoded σ^{32} proteins. Cells were transformed with plasmids expressing wild-type or mutant *rpoH* alleles under transcriptional control of a promoter specific for T7 polymerase. Since C600 cells lack T7 polymerase, only very little read-through from the T7 promoter occurs, just enough to produce approximately wild-type levels of σ^{32} (Fig. 3). This fact allowed us to perform the half-life determinations of the σ^{32} proteins under almost physiological conditions.

Only minor differences existed between the half-life of wild-type σ^{32} (approximately 60 to 100 s) and those of σ^{32}-Δ11aa and σ^{32}-Δ15aa (Fig. 4). This result indicates that the C-terminal truncations do not affect significantly the half-life of σ^{32} in vivo provided that the levels of DnaK and DnaJ are kept constant.

In vitro degradation of truncated σ^{32} mutant proteins by FtsH. We investigated the susceptibility of N-His_6-tagged wild-type and C-terminally truncated σ^{32} mutant proteins to degradation by FtsH in vitro. These degradation assays were per-

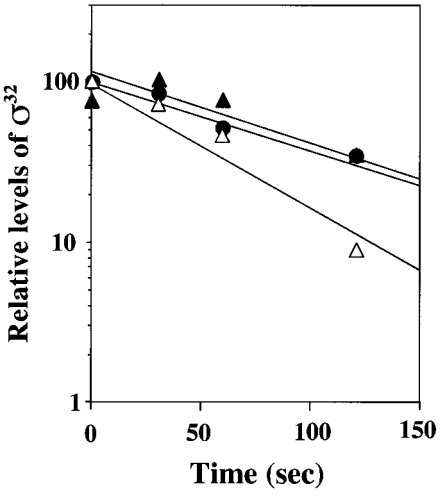

FIG. 4. In vivo stability of σ^{32} mutant proteins at 30°C. Cells of strain C600 which harbor pFN476 expressing *rpoH*-Δ11aa, *rpoH*-Δ15aa, or wild-type *rpoH* were grown at 30°C, pulse-labeled with [^{35}S]methionine, and chased with unlabeled methionine. Aliquots were taken at the indicated times, followed by immunoprecipitation of σ^{32} and quantification of the precipitated proteins relative to the largest value. Closed circles, wild-type σ^{32}; closed triangles, σ^{32}-Δ11aa; open triangles, σ^{32}-Δ15aa.

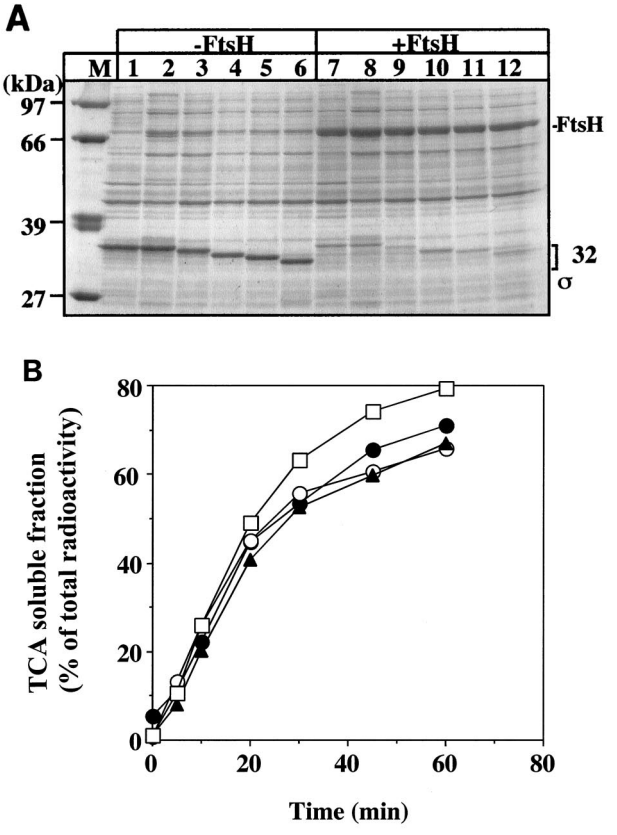

FIG. 5. (A) FtsH-dependent degradation of overproduced σ^{32} in cytoplasmic fractions. Cells of strain C600 which harbor plasmid pUHE21-2fdΔ12 expressing wild-type or truncated *rpoH* alleles mutant σ^{32} or the control plasmid were grown at 30°C with or without (lanes 1 and 7) IPTG. The extracts of cells grown without IPTG served as a control to ensure that the endogenous chaperones, whose cellular concentrations increase upon IPTG-induced production of plasmid-encoded wild-type σ^{32}, do not interfere with the degradation of σ^{32} in the extracts. Cytosolic extracts were prepared and assayed for degradation by exogenously added FtsH. Lane M, molecular weight marker. All other lanes show extracts of cells producing plasmid-encoded wild-type σ^{32} supplemented with 2 μM purified wild-type σ^{32} (since wild-type σ^{32} could not be overproduced to large amounts) (lanes 1, 2, 7, and 8) or plasmid-encoded mutants σ^{32}-Δ5aa (lanes 3 and 9), σ^{32}-Δ11aa (lanes 4 and 10), σ^{32}-Δ15aa (lane 5 and 11), and σ^{32}-Δ21aa (lanes 6 and 12). The extracts were incubated with (+FtsH) or without (−FtsH) added protease. (B) In vitro degradation of σ^{32} mutant proteins by FtsH. ^{3}H-labeled N-His$_6$-σ^{32} proteins were incubated with FtsH in the presence of 5 mM ATP, followed by TCA precipitation at the indicated times. The curves represent the percentage of radioactivity in the supernatants which contain the proteolytic fragments. Closed circles, N-His-σ^{32}; open circles, N-His$_6$-σ^{32}-Δ5aa; closed triangles, N-His$_6$-σ^{32}-Δ11aa; open squares, N-His$_6$-σ^{32}-Δ21aa.

formed only at 42°C, since FtsH is known to be poorly active at 30°C when tested in vitro in the presence of detergent (12). We first determined whether exogenously added FtsH is capable of degrading the σ^{32} proteins present in extracts of C600 cells after IPTG-induced overproduction. The rationale for this experiment was that for these ex vivo degradation assays we could use authentic σ^{32} mutant proteins without histidine tags, ruling out any contribution by the tag. All truncated and wild-type σ^{32} proteins were degraded by FtsH in presence of ATP to similar extents (Fig. 5A).

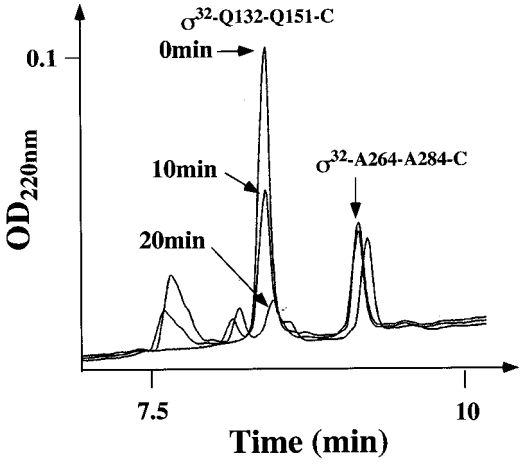

FIG. 6. FtsH-mediated degradation of peptides derived from σ^{32}. Peptides from region C (σ^{32}-Q132-Q151-C) and the C terminus (σ^{32}-A264-A284-C) of σ^{32} were mixed and incubated with FtsH. Degradation of the peptides at various times after mixing is shown as high-pressure liquid chromatography–reverse-phase chromatography data.

We then determined with purified components the efficiency by which FtsH degrades the N-His$_6$-tagged σ^{32} mutant proteins. All σ^{32} proteins were soluble after overproduction in *E. coli* cells and had wild type-like elution profiles during nickel-nitrilotriacetic acid and ion-exchange chromatography. Furthermore, they were indistinguishable from wild-type protein with respect to the proteolysis pattern obtained by partial proteinase K and trypsin digestion (data not shown). Therefore, there is no indication of changes in their overall tertiary structures. All C-terminally truncated σ^{32} mutant proteins were degraded by FtsH in the presence of ATP, with kinetics similar to that of wild-type σ^{32} (Fig. 5B). Thus, the C-terminal truncations did not affect the efficiency of σ^{32} degradation by FtsH in vitro.

C-terminal residues of σ^{32} do not constitute a substrate for FtsH. As an independent experimental approach to investigate the possibility that the C-terminal residues of σ^{32} constitute a destabilizing element for FtsH-dependent degradation, we tested whether the C-terminal sequences themselves provide a substrate motif for FtsH. We designed a peptide comprising the C-terminal 21 residues of σ^{32} and an additional cysteine at the C-terminal end (AERVRQLEKNAMKKLRAAIEAC) and tested whether it is a substrate for FtsH. The length of this 22-mer peptide was well above the minimal length of approximately 15 residues required for FtsH to degrade peptides (T. Tomoyasu and B. Bukau, unpublished results). As a positive control, we included a peptide of the same length and carrying a cysteine at the C terminus, derived from region C of σ^{32} (QRKLFFNLRKTKQRLGWFNQC). This peptide has been shown previously to be a good substrate peptide for FtsH (1).

In one experiment, both peptides were mixed at a 1:1 molar ratio and incubated with FtsH and ATP. Aliquots taken at 0, 10, and 20 min were analyzed for peptide degradation by high-pressure liquid chromatography. While the region C peptide was efficiently degraded with a half time of approximately 10 min, the C-terminal peptide was stable within the duration of the experiment (Fig. 6). This result was verified in experiments

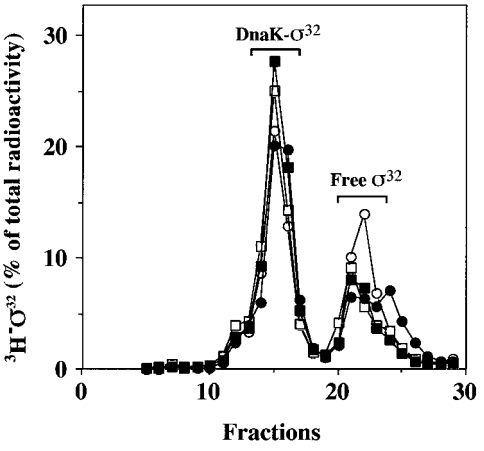

FIG. 7. Binding of σ^{32} mutant proteins to DnaK. ^{3}H-labeled N-His$_6$-σ^{32}, N-His$_6$-σ^{32}-Δ5aa, N-His$_6$-σ^{32}-Δ11aa, and N-His$_6$-σ^{32}-Δ21aa were incubated with DnaK, followed by gel filtration of the reaction product. Labeled protein was quantified in the elution fractions. Open circles, wild-type σ^{32}; closed circles, σ^{32}-Δ5aa; open squares, σ^{32}-Δ11aa; closed squares, σ^{32}-Δ21aa.

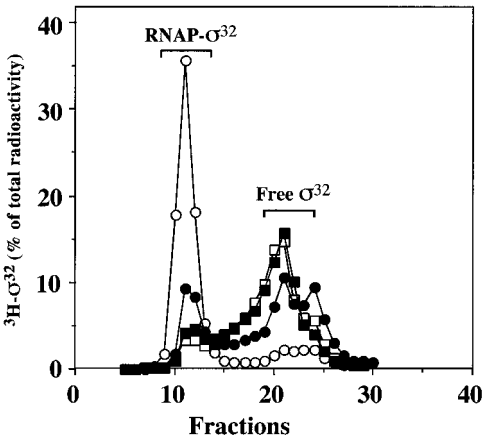

FIG. 8. Binding of σ^{32} mutant proteins to RNAP. ^{3}H-labeled N-His$_6$-σ^{32} proteins were incubated with RNAP, followed by gel filtration. The amount of labeled protein was quantified. Open circles, wild-type σ^{32}; closed circles, σ^{32}-Δ5aa; open squares, σ^{32}-Δ11aa; closed squares, σ^{32}-Δ21aa.

in which each peptide was tested individually for degradation by FtsH (data not shown). These results show that at the peptide level, the 22 residues of the C terminus of σ^{32} do not provide a recognition site for degradation by FtsH.

DnaK and DnaJ bind to truncated σ^{32} mutant proteins in vitro. We tested whether DnaK binding to σ^{32} is impaired by the C-terminal truncations. ^{3}H-labeled wild-type σ^{32} and mutant N-His$_6$-σ^{32} were incubated with DnaK followed by gel filtration to separate DnaK-σ^{32} complexes from free σ^{32} (Fig. 7). Under the conditions used, approximately 75% of wild-type [^{3}H]σ^{32} was recovered in complex with DnaK (eluting in fractions 12 to 17). The ^{3}H-labeled C-terminally truncated N-His$_6$-σ^{32} mutant proteins (σ^{32}-Δ5aa, σ^{32}-Δ11aa, and σ^{32}-Δ21aa) showed similar efficiencies of complex formation. Earlier work established that the interaction of DnaK with σ^{32} does not occur through the histidine tag (6). Furthermore, no defects in chaperone binding were observed when the N-His$_6$-σ^{32} mutant proteins were incubated with DnaK together with DnaJ in the presence of ATP (data not shown). These data indicate that the authentic C terminus of σ^{32} is not essential for interaction with DnaK and DnaJ.

RNAP binding to truncated σ^{32} mutant proteins in vitro. We considered the possibility that the truncated C-terminal segments of σ^{32} are involved in the interaction with the RNAP core enzyme. We determined the efficiency of association of ^{3}H-labeled N-His$_6$-σ^{32} with RNAP by gel filtration. The relative amounts of all truncated σ^{32} proteins (σ^{32}-Δ5aa, σ^{32}-Δ11aa, σ^{32}-Δ15aa, and σ^{32}-Δ21aa) recovered in association with RNAP (eluting in fractions 8 to 15) were lower than that of wild-type σ^{32}, with σ^{32}-Δ5aa showing the highest affinity for RNAP among all truncated proteins (Fig. 8). These results indicate a role for the C terminus of σ^{32} in the binding to RNAP.

Involvement of N-terminal segments of σ^{32} in degradation by FtsH in vitro. Given our finding that the C terminus of σ^{32} is not an essential degradation signal, we considered that perhaps N-terminal sequences play such a role. However, N-terminal sequences of σ^{32} are not amenable to mutational analysis, since the efficiency of translation of *rpoH* mRNA drops dramatically upon mutational alteration of the downstream box located at the 5′ end of the coding sequence (18).

We therefore restricted our efforts to an analysis of fragments of authentic σ^{32}, N-His$_6$-σ^{32}, and C-His$_6$-σ^{32} which we observed to accumulate during FtsH-dependent proteolysis in vitro. Although these fragments may be dead-end products, as they did not chase into smaller peptides, they may provide a tool for dissecting the degradation process. The immunologically detectable fragments, ranging from approximately 10 to 20 kDa, were identical for authentic σ^{32} and N-His$_6$-σ^{32}, which indicates that the N-terminal His$_6$ extension of the N-His$_6$-σ^{32} protein is missing in these fragments (Fig. 9). In contrast, the C-His$_6$-σ^{32} fragments showed a similar pattern but had higher molecular weights than the σ^{32} and N-His$_6$-σ^{32} fragments, which indicates that the C-terminal extension of the C-His$_6$-σ^{32} protein was still attached. Together these findings suggest that the degradation of σ^{32} proceeds through cuts in the N-terminal segment of σ^{32}, although they do not indicate whether the N terminus of σ^{32} is involved in the recognition by FtsH.

DISCUSSION

The aim of this study was to investigate the role of the C terminus of σ^{32} in the degradation by FtsH. C termini frequently constitute the degradation determinants of naturally unstable proteins, which target them to proteolysis by AAA proteases (9, 21). Such a role has been also proposed for the C terminus of σ^{32} on the basis of an analysis of C-terminal variants of σ^{32} (3).

We show here that a series of C-terminally truncated σ^{32} proteins are as unstable as wild-type σ^{32} in vivo, in cell extracts supplemented with FtsH and ATP, and in a purified ATP-dependent degradation system with FtsH and σ^{32} as the sole protein components. Furthermore, we show that the C-terminal truncations do not compromise the ability of σ^{32} to associate with DnaK in vitro. The C-terminal truncations were chosen such that the local predicted secondary structures are

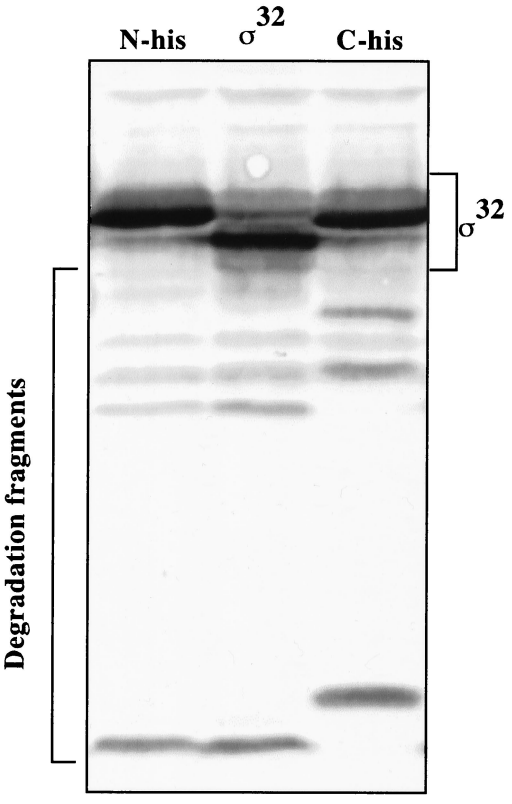

FIG. 9. Determination of in vivo degradation intermediates of σ^{32}. Cells of strain C600 which harbor plasmids expressing IPTG-regulated *rpoH* alleles encoding N-His-σ^{32}, C-His-σ^{32}, or authentic σ^{32} were grown at 30°C in LB medium. Aliquots of exponential-phase cultures (OD_{600}, 0.5 to 0.7) were collected, and equal amounts of total protein were analyzed by SDS-PAGE. The degradation intermediates were determined by immunoblotting using σ^{32}-specific antisera.

considered and that the novel C termini do not constitute predicted DnaK binding sites (16, 19) and do not resemble the consensus sequence of protease targeting sites (9). Taken together, our results do not provide any evidence for an essential role of C-terminal sequences of σ^{32} in the FtsH-dependent degradation process or in chaperone binding. Our findings agree well with the results of a recent study of truncated σ^{32} proteins and hybrids of stable *Bradyrhizobium japonicum* and unstable *E. coli* proteins (2). That study mapped a region of 85 residues, located between residues 36 to 122 of σ^{32}, as being responsible for degradation of σ^{32}.

We do not know the reason for the discrepancy with a previous study claiming an essential role for the C terminus of σ^{32} in degradation (3). It is possible that the additional six residues encoded by the vector which were fused to the C-terminally truncated σ^{32} proteins in the former study generated fusion proteins which became stabilized for unknown reasons. Our results are, however, in agreement with the findings that (i) class I fusions between N-terminal segments of σ^{32} and β-galactosidase exhibit normal shutoff of the heat shock response and σ^{32} instability (17) and (ii) blocking the N terminus, but not the C terminus, of σ^{32} by fusion with green fluorescent protein resulted in stabilization of the fusion protein, indicating the importance of sequences at or near the N terminus of σ^{32} in its degradation by FtsH (T. Tatsuta and T. Ogura, unpublished results). At this point we cannot exclude an involvement of internal sequences of σ^{32} in degradation. Furthermore, our conclusion is supported by our finding that fragments of σ^{32} proteins which were generated by FtsH-dependent degradation in vitro lack N-terminal segments but not C-terminal segments of σ^{32}. Although this finding should still be interpreted with caution, it suggests important roles for N-terminal segments of σ^{32} in the degradation process. Further genetic and biochemical dissection is required to identify such a role.

Our analysis provided evidence for a role of the C-terminal sequences of σ^{32} in association with RNAP. Only the mutant protein lacking five C-terminal residues remained partially proficient in binding to RNAP and in in vivo activity, whereas all mutant proteins with longer truncations showed no significant RNAP binding or in vivo activity. The molecular basis for this role of the C terminus of σ^{32} in the association with RNAP is unclear. However, on the basis of several criteria (solubility and wild type-like partial proteolysis pattern), the C-terminally truncated σ^{32} proteins are not perturbed in their overall structure. Thus, either local conformational changes induced by the truncations or the lack of C-terminal residues directly involved in the association process may account for the observed loss in affinity. In this respect it is interesting that many regions within the polypeptide chain of σ^{32}, including the regulatory region C and the conserved regions 2.1, 2.2, 3, and 4, have been implicated in the binding of RNAP (1, 3, 11, 20, 31). Structural analysis seems essential to solve this obvious complexity of the interaction between σ^{32} and RNAP.

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REFERENCES

1. **Arsène, F., T. Tomoyasu, A. Mogk, C. Schirra, A. Schulze-Specking, and B. Bukau.** 1999. Role of region C in regulation of the heat shock gene-specific sigma factor of *Escherichia coli*, σ^{32}. J. Bacteriol. **181:**3552–3561.
2. **Bertani, D., A. B. Oppenheim, and F. Narberhaus.** 2001. An internal region of the RpoH heat shock transcription factor is critical for rapid degradation by the FtsH protease. FEBS Lett. **493:**17–20.
3. **Blaszczak, A., C. Georgopoulos, and K. Liberek.** 1999. On the mechanism of FtsH-dependent degradation of the σ^{32} transcriptional regulator of *Escherichia coli* and the role of the DnaK chaperone machine. Mol. Microbiol. **31:**157–166.
4. **Buchberger, A., A. Valencia, R. McMacken, C. Sander, and B. Bukau.** 1994. The chaperone function of DnaK requires the coupling of ATPase activity with substrate binding through residue E171. EMBO J. **13:**1687–1695.
5. **Bukau, B.** 1993. Regulation of the *E. coli* heat shock response. Mol. Microbiol. **9:**671–680.
6. **Gamer, J., H. Bujard, and B. Bukau.** 1992. Physical interaction between heat shock proteins DnaK, DnaJ, GrpE and the bacterial heat shock transcription factor σ^{32}. Cell **69:**833–842.
7. **Gamer, J., G. Multhaup, T. Tomoyasu, J. S. McCarty, S. Rüdiger, H.-J. Schönfeld, C. Schirra, H. Bujard, and B. Bukau.** 1996. A cycle of binding and release of the DnaK, DnaJ and GrpE chaperones regulates activity of the *E. coli* heat shock transcription factor σ^{32}. EMBO J. **15:**607–617.
8. **Gross, C. A.** 1996. Function and regulation of the heat shock proteins, p. 1382–1399. *In* F. C. Neidhardt et al. (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
9. **Herman, C., D. Thévenet, P. Boulox, G. C. Walker, and R. D'Ari.** 1998.

Degradation of carboxy-terminal-tagged cytoplasmic proteins by the *Escherichia coli* protease HflB (FtsH). Genes Dev. **12:**1348–1355.
10. **Herman, C., D. Thévenet, R. D'Ari, and P. Bouloc.** 1995. Degradation of sigma 32, the heat shock regulator in *Escherichia coli*, is governed by HflB. Proc. Natl. Acad. Sci. USA **92:**3516–3520.
11. **Joo, D. M., A. Nolte, R. Calendar, Y. N. Zhou, and D. J. Jin.** 1998. Multiple regions on the *Escherichia coli* heat shock transcription factor σ^{32} determine core RNA polymerase binding specificity. J. Bacteriol. **180:**1095–1102.
12. **Kanemori, M., H. Yanagi, and T. Yura.** 1999. Marked instability of the σ^{32} heat shock transcription factor at high temperature. J. Biol. Chem. **274:** 22002–22007.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
14. **Lanzer, M.** 1988. Ph.D. thesis. University of Heidelberg, Heidelberg, Germany.
15. **Lowe, P. A., D. A. Hager, and R. R. Burgess.** 1978. Purification and properties of the σ subunit of *Escherichia coli* DNA-dependent RNA polymerase. Biochemistry **18:**1344–1352.
16. **McCarty, J. S., S. Rüdiger, H.-J. Schönfeld, J. Schneider-Mergener, K. Nakahigashi, T. Yura, and B. Bukau.** 1996. Regulatory region C of the *E. coli* heat shock transcription factor, σ^{32}, constitutes a DnaK binding site and is conserved among eubacteria. J. Mol. Biol. **256:**829–837.
17. **Nagai, H., H. Yuzawa, M. Kanemori, and T. Yura.** 1994. A distinct segment of the σ^{32} polypeptide is involved in DnaK-mediated negative control of the heat shock response in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **91:** 10280–10284.
18. **Nagai, H., H. Yuzawa, and T. Yura.** 1991. Regulation of the heat shock response in *E coli*: involvement of positive and negative cis-acting elements in translation control of σ^{32} synthesis. Biochimie **73:**1473–1479.
19. **Rüdiger, S., L. Germeroth, J. Schneider-Mergener, and B. Bukau.** 1997. Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. EMBO J. **16:**1501–1507.
20. **Sharp, M. M., C. L. Chan, C. Z. Lu, M. T. Marr, S. Nechaev, E. W. Merritt, K. Severinov, J. W. Roberts, and C. A. Gross.** 1999. The interface of σ with core RNA polymerase is extensive, conserved, and functionally specialized. Gene Dev. **13:**3015–3026.
21. **Smith, C. K., T. A. Baker, and R. T. Sauer.** 1999. Lon and Clp family proteases and chaperones share homologous substrate-recognition domains. Proc. Natl. Acad. Sci. USA **96:**6678–6682.
22. **Straus, D., W. Walter, and C. A. Gross.** 1990. DnaK, DnaJ, and GrpE heat shock proteins negatively regulate heat shock gene expression by controlling the synthesis and stability of σ^{32}. Genes Dev. **4:**2202–2209.
23. **Straus, D. B., W. A. Walter, and C. A. Gross.** 1987. The heat shock response of *E. coli* is regulated by changes in the concentration of σ^{32}. Nature **329:** 348–350.
24. **Tatsuta, T., D. M. Joo, R. Calendar, Y. Akiyama, and T. Ogura.** 2000. Evidence for an active role of the DnaK chaperone system in the degradation of σ^{32}. FEBS Lett. **478:**271–275.
25. **Tatsuta, T., T. Tomoyasu, B. Bukau, M. Kitagawa, H. Mori, K. Karata, and T. Ogura.** 1998. Heat shock regulation in the *ftsH* null mutant of *Escherichia coli*: dissection of stability and activity control mechanisms of sigma32 *in vivo*. Mol. Microbiol. **30:**583–593.
26. **Tilly, K., J. Spence, and C. Georgopoulos.** 1989. Modulation of stability of the *Escherichia coli* heat shock regulatory factor σ^{32}. J. Bacteriol. **171:**1585–1589.
27. **Tomoyasu, T., J. Gamer, B. Bukau, M. Kanemori, H. Mori, A. J. Rutman, A. B. Oppenheim, T. Yura, K. Yamanaka, H. Niki, S. Hiraga, and T. Ogura.** 1995. *Escherichia coli* FtsH is a membrane-bound, ATP-dependent protease which degrades the heat shock transcription factor σ^{32}. EMBO J. **14:**2551–2560.
28. **Tomoyasu, T., T. Ogura, T. Tatsuta, and B. Bukau.** 1998. Levels of DnaK and DnaJ provide tight control of heat shock gene expression and protein repair in *E. coli*. Mol. Microbiol. **30:**567–581.
29. **Yano, R., H. Nagai, K. Shiba, and T. Yura.** 1990. A mutation that enhances synthesis of sigma 32 and suppresses temperature-sensitive growth of the *rpoH15* mutant of *Escherichia coli*. J. Bacteriol. **172:**2124–2130.
30. **Yura, T., and K. Nakahigashi.** 1999. Regulation of the heat-shock response. Curr. Opin. Microbiol. **2:**153–158.
31. **Zhou, Y. N., W. A. Walter, and C. A. Gross.** 1992. A mutant sigma 32 with a small deletion in conserved region 3 of sigma has reduced affinity for core RNA polymerase. J. Bacteriol. **174:**5005–5012.