Peptidase Activity of the *Escherichia coli* Hsp31 Chaperone*

Received for publication, July 22, 2004, and in revised form, November 17, 2004
Published, JBC Papers in Press, November 18, 2004, DOI 10.1074/jbc.M408296200

Abderrahim Malki†, Thérèse Caldas‡, Jad Abdallah‡, Renée Kern‡, Viola Eckey‡, So Jung Kim§, Sun-Shin Cha§, Hirotada Mori§, and Gilbert Richarme‡

*From ‡Stress Molecules, Institut Jacques Monod, Université Paris 7, 2 place Joussieu, 75005 Paris, France, the §Beamline Division, Pohang Accelerator Laboratory, Pohang, 790-784, Republic of Korea, and the ¶Nara Institute of Sciences and Technology, 8916-5 Takayama, Itohna, Nara 630-0101, Japan

Hsp31, the *Escherichia coli* hchA gene product, is a molecular chaperone whose activity is inhibited by ATP at high temperature. Its crystal structure reveals a putative Cys184, His185, and Asp213 catalytic triad similar to that of the *Pyrococcus horikosshi* protease PH1704, suggesting that it should display a proteolytic activity. A preliminary report has shown that Hsp31 has an exceedingly weak proteolytic activity toward bovine serum albumin and a peptidase activity toward two peptide substrates with small amino acids at their N terminus (alanine or glycine), but the physiological significance of this observation remains unclear. In this study, we report that Hsp31 does not display any significant proteolytic activity but has peptidolytic activity. The aminopeptidase cleavage preference of Hsp31 is Ala > Lys > Arg > His, suggesting that Hsp31 is an aminopeptidase of broad specificity. Its aminopeptidase activity is inhibited by the thiol reagent iodoacetamide and is completely abolished in a C185A mutant, which is consistent with Hsp31 being a cysteine peptidase. The aminopeptidase activity of Hsp31 is also inhibited by EDTA and 1,10-phenanthroline, in concordance with the importance of the putative His85, His125, and Glu90 metal-binding site revealed by crystallographic studies. An Hsp31-deficient mutant accumulates more 8–12-mer peptides than its parental strain, and purified Hsp31 can transform these peptides into smaller peptides, suggesting that Hsp31 has an important peptidase function both in vivo and in vitro. Proteins interacting with Hsp31 have been identified by reverse purification of a crude *E. coli* extract on an Hsp31-affinity column, followed by SDS-polyacrylamide electrophoresis and mass spectrometry. The ClpA component of the ClpAP protease, the chaperone GroEL, elongation factor EF-Tu, and tryptophanase were all found to interact with Hsp31, thus substantiating the role of Hsp31 as both chaperone and peptidase.

Every organism responds to a sudden increase in the environmental temperature by the overexpression of a set of highly conserved heat shock proteins (1, 2). Most of these heat shock proteins function either as molecular chaperones, assisting in protein folding and renaturation or as proteases which degrade proteins that are beyond rescue.

Hsp31, the hchA gene product (formerly known as YedU), is a heat-inducible homodimeric protein of 31-kDa subunits, which was recently shown to exhibit molecular chaperone activity (3, 4). It promotes the functional folding of citrate synthase, α-glucosidase, and alcohol dehydrogenase. It also prevents the aggregation at 43 °C of citrate synthase and alcohol dehydrogenase and interacts specifically with unfolded proteins (3, 4). Although Hsp31 does not exhibit any ATPase activity, some of its chaperone activities are partially inhibited by ATP (3, 4). The crystal structure of Hsp31 was solved at 1.6 Å resolution and revealed a system of hydrophobic patches, canyons, and grooves, which may stabilize partially unfolded protein substrates and explain its chaperone activity (5). A putative Cys-His-Asp catalytic triad that is only accessible through a small pocket with a length of 14 Å, and an entrance of about 4 Å was also revealed, suggesting that Hsp31 might present a protease or more probably a peptidase activity (5–8). Although initial attempts to determine whether Hsp31 functions as a protease or peptidase were unsuccessful (3, 4), it was recently reported that Hsp31 displays very weak protease activity and a peptidase activity against Ala and Gly conjugated to a fluorogenic group, but the physiological relevance of this observation remains unclear (7). In the present study, we show that Hsp31 is a broad specificity aminopeptidase with a preference for alanine and basic amino acids and that it is inhibited by cysteine protease inhibitors and by the metal ion complexing agents EDTA and 1,10-phenanthroline. Furthermore, we show that an Hsp31-deficient mutant accumulates increased amounts of 8–12-mer peptides, which can be processed in vitro to smaller peptides by purified Hsp31. Finally, we report that Hsp31 specifically interacts with ClpA, GroEL, EF-Tu, and tryptophanase. Our results suggest that, besides its chaperone activity, Hsp31 plays an active role in the downstream processing of peptides generated by the ATP-dependent proteases.

**EXPERIMENTAL PROCEDURES**

Hsp31 Expression and Purification—The *Escherichia coli* B/r strain AD494 (ara leu767 lac74 phoA phoR-malF3 F’[lac (lacF) pro] trxB::kan [DE3] pLYS3) was purchased from Novagen. It was transformed with plasmid pINTYEDU, which encodes the complete Hsp31/YedU protein fused to a chitin-binding domain tag at its N terminus (3). Cells were grown in LB broth in the presence of ampicillin and lysed by sonication (3). The recombinant Hsp31 protein fused to the chitin-binding domain was purified by affinity chromatography on a chitin affinity column (intermediated purification with an affinity chitin-binding tag kit from New England Biolabs, Inc.) as described previously (3). The eluted Hsp31 protein was pure as judged by SDS-polyacrylamide gel electrophoresis. It was stored at −20 °C in buffer (20 mM Tris, pH 7.4, 50 mM KCl, 0.5 mM diithiothreitol) supplemented with 30% DMSO.

* This work was supported by Grant CR 521090 from the Délegation Générale pour l’Armement (to G. R.). Work at Pohang Accelerator Laboratory was supported by a grant from the Biogreen 21 Project, Rural Development Administration, Republic of Korea. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 33-1-44-27-50-98; Fax: 33-1-44-27-57-16; E-mail: richarme@ccr.jussieu.fr.

‡ The abbreviations used are: Hsp, heat shock protein; HPLC, high performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; amc, 7-amino-4-methylcoumarin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
E. coli Hsp31 Chaperone/Peptidase

glycerol. The C185A mutant was constructed and purified as described previously (7).

Construction of the Hsp31-deficient Strain—The one-step inactivation of the hchA gene encoding Hsp31 was done using the phage λ recombination (Red) system (9). The strategy is analogous to the PCR-based gene deletion method in yeast, except E. coli cells carrying an easily curable, low copy number plasmid (pSC101 (replication temperature-sensitive derivative)-based plasmid pKD46 carrying the λ Red recombination genes (9)) expressing the Red system was used. The basic strategy is to replace a chromosomal sequence with a selectable anti-biotic resistance gene that is generated by PCR by using primers with 50-nucleotide extensions homologous to the adjacent upstream or downstream flanking regions of the target gene and 20-nucleotide 3′-end for the amplification of kanamycin (kan) resistance gene. Replacement is accomplished by λRed-mediated recombination in these flanking homologies (9); Strain BW25113 (lacF' rbsB3 ΔlacZΔ4787 hisdR514 ΔaraBAD567 Δ(harBAD)567 hsdR514 1) was used as the parent for construction of the hcha deletion mutant JW1950. Insertion of the kan resistance cassette in the hcha gene is not likely to have a polar effect on the expression of other genes, since the genes downstream of hcha (vedY, coding for a putative sensor-like histidine kinase and YedW, coding for a probable transcriptional regulatory protein) are transcribed in the opposite direction (genolist.pasteur.fr/Colibri/). The construction of the mutant is part of the systematic construction of E. coli single gene deletion mutants.2

Protease and Peptidase Assays—Autoysis of Hsp31 in test tubes was performed as described previously (3), and proteolysis products were analyzed by SDS-PAGE. Gelatin-PAGE and casein-PAGE in-gel proteolysis assays were performed as described in (3, 12); 2 μg of Hsp31 were electrophoresed onto 12% SDS-polyacrylamide gels containing either 0.25% gelatin or 0.2% casein. Individual bands with proteolytic activity was visualized by clearing zones resulting from gelatin or casein hydrolysis (12). Gelatin and casein zymography was also performed using electrophoresis under native conditions as described by Fukiaige et al. (13). Proteolysis of citrate synthase, α-casein, and β-casein was assayed by incubation of the reaction mixtures containing 2 μg of the protein substrate and 0.5 μg of Hsp31 at 37 °C in 20 mM Tris, pH 8, 5 mM MgCl₂. After incubation for appropriate periods, the reaction was stopped with 2% SDS, and the samples were subjected to SDS-PAGE. Proteolysis of insulin and insulin β-chain were measured by chromatographic analysis of proteolyis product on a C18 reverse phase HPLC column (Vydac C18 250 × 4.6 mm, Hewlett-Packard) equilibrated in 0.1% trifluoroacetic acid in water and eluted with a linear gradient of 0–100% acetonitrile containing 0.1% trifluoroacetic acid.

Endopeptidase and aminopeptidase activities were assayed by monitoring the production of T-aminoethylcoumarin (amc) from the fluorescent amine acid or peptides substrates, succinyl-LLVY-amc, t-butyloxycarbonyl-LAR-amc, Ac-YVAD-amc, AAF-amc, ALK-amc, Ac-A-amc, t-Ala-amc, t-Arg-amc, t-Asp-amc, t-Asn-amc, t-Gly-amc, t-Leu-amc, t-Lys-amc, t-Met-amc, t-Phe-amc, t-Pro-amc, t-Thr-amc, t-Tyr-amc, t-Val-amc, t-Ser-amc, t-Cys-amc, t-His-amc, t- Glu-amc as described previously (3, 10). One μg of Hsp31 was incubated at 37 °C for appropriate times (1–20 h, in conditions in which the appearance of product is linear as a function of time) with 500 μM substebte in 50 mM Tris, pH 8, 5 mM MgCl₂, 1 mM diithiothreitol, 1% dimethyl sulfoxide, in a total volume of 100 μl, and amc fluorescence (excitation at 380 nm, emission at 460 nm) was measured after arrest of the reaction with 900 μl 1% SDS. Carboxypeptidase dase activity was assayed by measuring the hydrolysis of hippuryl-l-1% SDS. The eluted proteins were then separated by SDS-PAGE, and the individual bands that were not retained by two control columns were analyzed by MALDI-TOF mass spectrometry. One of these two control columns was made with the chitin-binding domain alone, and the other with the chitin-binding domain fused to Ybho, an E. coli peptidase of 20 kDa under study in our laboratory.

MATERIALS—Citrate synthase (from porcine heart), α-casein, β-casein, insulin, insulin β-chain, and all other chemicals were from Sigma and were of the highest grade. The molecular weight marker used for gel filtration chromatography was from Sigma. The fluorogenic peptides were from either Bachem or Sigma.

RESULTS

Absence of Proteolytic Activity of Hsp31—As reported previously (3), we could not detect any proteolytic activity of Hsp31 toward gelatin or casein when using a polyacrylamide in-gel assay similar to that used for the detection of PP1 protease activity (12), in which the substrate was incorporated uniformly in the gel (these negative results were obtained with both SDS-polyacrylamide gels and native gels (data not shown)). Several other protein substrates, such as α-casein, β-casein, insulin, and insulin β-chain, were not hydrolyzed by Hsp31 in a test tube assay, in several types of experimental conditions (0.01–0.05% SDS– or Triton-containing buffers, partial thermal unfolding (15), presence of 1 mM ATP and 5 mM magnesium chloride), suggesting that Hsp31 does not function as a broad specificity protease (data not shown). We could not detect any significant autolytic activity of Hsp31 in a test tube assay as described under “Experimental Procedures.” Hsp31 underwent proteolysis in an in-gel assay consisting of a polyacrylamide gel loaded with purified Hsp31, run in SDS, subsequently transferred for 90 min into phosphate buffer containing 2% Triton-X-100, and incubated overnight in the same buffer without detergent and finally colored with Coomassie Blue (data not shown). However, such proteolysis was also observed with the C185A mutant (deficient in the nucleophilic cysteine, see below), suggesting that it results from a contaminating in-gel proteolytic activity.

Aminopeptidase Activity of Hsp31—We studied the cleavage specificity of Hsp31 for aminoacyl-amc fluorescent substrates. Hsp31 hydrolyzed five out of the 18 aminoacyl-amc substrates tested. Alanine, arginine, lysine, and histidine substrates were hydrolyzed with a decreasing efficiency in that order (Fig. 1A). This suggests that Hsp31 is a broad specificity aminopeptidase.

2 T. Baba, T. Ara, Y. Okumura, M. Hasegawa, Y. Takai, M. Baba, K. A. Davisenko, T. Oshima, M. Tomita, B. L. Wanner, and H. Mori, submitted for publication.
Hsp31 was assayed for aminopeptidase activity toward aminomethyl-aminocarbonyl (amc) derivatives (0.5 mM) as described under "Experimental Procedures." The free amc released after substrate hydrolysis by Hsp31 was measured in a spectrofluorimeter. The legend on the right represents the single letter code for the amino acids that are present at the amino terminus of the peptide bond hydrolyzed. "1000 arbitrary units" represents a specific activity of 2120 nmol/h/mg Hsp31.

To exclude the possibility that small amounts of contaminating pepN were responsible for these aminopeptidase activities, we purified Hsp31 further on a DEAE-Sepharose column. The alanine and arginine aminopeptidase activities were detected in the same fractions as the Hsp31 protein peak, suggesting that Hsp31 is responsible for these activities (Fig. 1B). Similar results were obtained when purified Hsp31 was analyzed on a hydroxylapatite column (data not shown).

Inhibitors of Hsp31 Aminopeptidase Activity and Mutational Test of the Nucleophilic Activity of Cys185—We tested the aminopeptidase activity of Hsp31 in the presence of several inhibitors known to affect cysteine proteases (iodoacetamide and dithiothreitol), serine proteases (phenylmethylsulfonyl fluoride), and metalloproteases (EDTA and 1,10-phenanthroline) (Fig. 2). Iodoacetamide completely blocks the ability of Hsp31 to degrade Ala-amc. Hsp31 is also severely inhibited by 1 mM dithiothreitol (80% inhibition). In contrast, the serine protease inhibitor PMSF had little effect on Hsp31. More importantly, the aminopeptidase activity of Hsp31 was completely abolished in a C185A mutant, suggesting that, in accordance with crystallographic data, Cys185 is the active nucleophile of Hsp31. Metalloprotease inhibitors strongly inhibit Hsp31: 5 mM EDTA and 5 mM 1,10-phenanthroline provoke a 95% inhibition of the Hsp31 aminopeptidase. This suggests that the putative Hsp31 His122z and Glu180 metal-binding site of Hsp31 revealed by crystallographic studies (6) is important for its aminopeptidase activity. Hsp31 inhibitors are different from pepN inhibitors. PepN is strongly inhibited by PMSF but not by EDTA nor by iodoacetamide (16–17).

Effect of Temperature, pH, Divalent Cations, and ATP on the Hsp31 Aminopeptidase—Since Hsp31 is a heat shock protein and displays chaperone activity, we suspected that its aminopeptidase activity might be revealed at high temperatures, in a manner reminiscent of that of the temperature-dependent switch from chaperone to protease of DegP/HtrA (this switch...
occurs at temperatures ranging between 30 °C and 50 °C (18).

The dependence of the Hsp31 aminopeptidase activity on temperature within the range of 20–45 °C displays a classical curve with a $Q_{10}$ of around 2, suggesting that, unlike DegtP/HtrA, there is not an exceptional activation of Hsp31 by heat shock temperatures (Fig. 3A).

The aminopeptidase activity of Hsp31 was optimal around pH 8 and was greatly decreased below pH 6 and above pH 9 (Fig. 3B).

In crystallographic studies of Hsp31, a zinc ion was observed, coordinated by His$^{85}$, Glu$^{90}$, and His$^{122}$. Consistent with this observation, Hsp31 is almost completely inhibited by 5 mM $o$-phenanthroline, which has a particularly high affinity for zinc (Fig. 2). Furthermore, in the presence of 2 mM $o$-phenanthroline and 50 μM zinc, manganese, magnesium, calcium, iron, or cobalt divalent cation (90 min at 25 °C), and its activity was assayed at 37 °C with 0.5 mM Ala-amc as described under “Experimental Procedures.” 100% represents the Hsp31 activity without added phenanthroline or cation, and the Hsp31 activity in the presence of 2 mM $o$-phenanthroline alone was 8% of the control.

Since ATP interacts with Hsp31 and inhibits some of its chaperone properties (4), we tested the aminopeptidase activities of Hsp31 as described in the above sections, in the presence of 5 mM ATP and 2 mM MgCl$_2$, and found that ATP was without any effect (data not shown).

**Peptide Accumulated during Growth of an Hsp31-deficient Mutant**—To test the hypothesis that the absence of Hsp31 leads to peptide accumulation during bacterial growth, the following experiment was performed. The Hsp31-deficient and parental strains were grown for several generations at 37 °C in the presence of [3H]leucine. The cells were harvested and washed, and the material that was soluble in 1 M acetic acid

![FIG. 3. Temperature and pH dependence of the aminopeptidase activity of Hsp31.](image)

A. The activity of Hsp31 (1 μg) was assayed between 30 °C and 50 °C with 0.5 mM Ala-amc as described under “Experimental Procedures.” An activity of 100 represents 490 nmol/h/mg of protein.

B. The activity of Hsp31 (1 μg) was assayed at 37 °C between pH 6 and 9 with 0.5 mM Ala-amc, 5 mM MgCl$_2$, 1 mM dithiothreitol, 1% dimethyl sulfoxide in a total volume of 100 μl. The pH values were made with 50 mM phosphate or Tris buffers and were normalized to Tris buffer. An activity of 100 represents 2220 nmol/h/mg of protein.

C. Reactivation of Hsp31 by Zn$^{2+}$. Hsp31 (1 μg) was incubated in 100 μl of 50 mM Tris, pH 8.0, in the presence of 2 mM $o$-phenanthroline and 50 μM zinc, manganese, magnesium, calcium, iron, or cobalt divalent cation (90 min at 25 °C), and its activity was assayed at 37 °C with 0.5 mM Ala-amc as described under “Experimental Procedures.” 100% represents the Hsp31 activity without added phenanthroline or cation, and the Hsp31 activity in the presence of 2 mM $o$-phenanthroline alone was 8% of the control.

![FIG. 4. Analysis of accumulated peptides in an Hsp31-deficient strain and a control strain.](image)

**Fig. 4.** HPLC C18 reverse phase chromatography of peptides from the Hsp31-deficient strain JW1950 and from its parental strain BW25113. Cells were labeled during growth at 37 °C with [3H]leucine, washed by centrifugation, and extracted with 1 M acetic acid. The acetic acid extracts of cultures were first chromatographed on a TSK 2000 HPLC column, and the low molecular mass peak (100–3000 Da) was lyophilized and chromatographed on a Vydac C18 reverse phase HPLC column equilibrated in water containing 0.1% trifluoroacetic acid. The column was eluted with a linear gradient of 0–100% acetonitrile containing 0.1% trifluoroacetic acid at a rate of 0.3 ml/min, and fractions of 0.3 ml were collected.

A. The chromatograms represent the absorbance at 215 nm of extracts from the wild-type strain (thin line) and the hcha-deficient strain (thick line). 100 arbitrary units represents an absorbance of 0.084 at 215 nm.

B. Fractions from the hcha-deficient strain extract, whose chromatogram is shown in Fig. 6A (thick line), were tested for fluorescamine reactivity before (open circles) and after (filled circles) hydrolysis in base and for [3H]leucine radioactivity (triangles). 10 arbitrary units represents 11 nmol of N-terminal (fluorescamine detection) or 120 cpm ([3H]leucine radioactivity). The straight line starting at tube 10 represents the 0–100% acetonitrile gradient.
and leucine (131 Da), respectively. Circles mass peak (100–3000 Da) of the TSK 2000 HPLC column loaded with phy on a Bio-Gel P-2 column (2 ml bed volume) of the low molecular weight peak of Dextran Yellow (20,000 Da), vitamin B₁₂ (1350 Da), and leucine (131 Da), respectively.

was extracted (14). The acetic acid-soluble fractions from the two cultures were subjected to gel filtration on a HPLC TSK 2000 SW gel permeation column. Both cultures displayed a similar peak of radioactivity with a molecular mass of around 100–3000 Da (not shown). Peak fractions were pooled, concentrated by lyophilization, and subjected to reverse phase chromatography on a C18 HPLC column equilibrated with water containing 0.1% trifluoroacetic acid and eluted with a linear 0–100% acetonitrile gradient in 0.1% trifluoroacetic acid. As shown in Fig. 4A, the elution profile of peptides from the Hsp31-deficient strain is different from that of the wild-type strain. The Hsp31-deficient strain accumulates a higher quantity of peptides eluting between 50 and 80% acetonitrile (8–12-mers, see below) and fewer peptides eluting between 0 and 30% acetonitrile (3–5-mers, see below). The quality of peptides accumulating in the Hsp31-deficient strain was studied by the following procedure: fractions eluting from the C18 column were assayed for [³H]leucine radioactivity, fluorescamine reactivity (NH₂ terminus determination), and fluorescamine reactivity after peptide hydrolysis in base, as described in (14) (Fig. 4B).

The results of this experiment indicate that (i) the radioactive material applied on the C18 column is heterogeneous, and (ii) all fractions contain fluorescamine positive material, (iii) the fluorescamine detection is severalfold increased by prior hydrolysis in base. These data indicate that the material present in the pepD-deficient strain is a heterogeneous mixture of peptides. The ratio of fluorescamine values before and after hydrolysis in base gives an estimation of the average length of peptides in each peak. Peptide length ranges from 3–5-mers (in fractions eluting between 0–30% acetonitrile) to 8–12-mers (in fractions eluting between 50 and 80% acetonitrile). Similar results were obtained for peptides of the control strain (not shown), except that, as shown in Fig. 7A, there was a higher amount of 3–5-mer peptides and a lower amount of 8–12-mer peptides.

To analyze further the action of Hsp31 on these natural peptides, the acetic acid-soluble fraction (the 500–3000 Da peak from the TSK 2000 SW gel filtration HPLC column) from the Hsp31-deficient strain was subjected to gel filtration on a Bio-Gel P-2 permeation column, before and after incubation with purified Hsp31. As shown in Fig. 5, the [³H]leucine radioactivity of peptides from the Hsp31-deficient strain elutes as a broad peak, corresponding to 200–1500-Da molecular mass peptides (2–12-mers; the size of these peptides is in accordance with that obtained above by fluorescamine detection experiments). After incubation for 2 h in the presence of purified Hsp31, there is a decrease in the amount of high molecular weight peptides and a concomitant increase in the amount of low molecular weight peptides (Fig. 5), suggesting that Hsp31 exerts a peptidase effect on these natural peptides. Longer incubation times did not significantly increase peptide hydrolysis, which implies that the combined action of several peptidases is required in vivo for their complete hydrolysis (data not shown).

One can postulate, from the results presented in this section, that Hsp31 is involved in the processing of 8–12-mer peptides (approximately the size of the peptides produced by ATP-dependent endopeptidases) both in vitro and in vivo.

The growth phenotype of the Hsp31-deficient strain was similar to that of its parental strain, either in minimal or rich medium (data not shown). Furthermore, there was no increase in the lag time (before resuming exponential growth) of the mutant after shift from nutrient broth to minimal glucose medium. These negative results are not surprising, since only multiple peptidase mutants (pepN, pepA, pepB, pepD) display a slow growth phenotype or an increased lag time after nutritional downshift (the increased lag time results from a deficiency in the degradation of intracellular proteins to amino acids required for the synthesis of new proteins) (14).

Proteins Interacting with Hsp31—We used affinity purification and mass spectrometry to characterize the proteins that specifically interact with Hsp31. A chimera protein of Hsp31 fused to a chitin-binding domain was bound on a chitin affinity column as described under “Experimental Procedures.” An E. coli crude extract was loaded onto this Hsp31 affinity column in the presence of 0.5 M NaCl, and the column was extensively washed with 0.5 M NaCl. After the NaCl wash, the remaining proteins were eluted with SDS. They were then separated by denaturing gel electrophoresis; individual protein bands were digested by trypsin, analyzed by MALDI-TOF mass spectrometry, and identified by data base algorithms. Hsp31 specifically retained four proteins (Table I), which were characterized as the ATPase component ClpA of the ClpAP protease (19), the chaperone GroEL (2), translation elongation factor EF-Tu (20–22), and tryptophanase (an enzyme that degrades tryptophan to indole and pyruvate) (23). None of these proteins was retained on a control chitin affinity column or on a YhbO affinity column (YhbO is another E. coli peptidase under study in our laboratory; data not shown). The interaction observed between Hsp31 and ClpA is in accordance with its function as an aminopeptidase involved in the downstream processing of peptides produced by endopeptidases such as ClpAP or Lon. The interaction between Hsp31 and GroEL is consistent with the chaperone and peptidase function of Hsp31, since the GroEL chaperone has also been implicated in proteolysis (2). The interaction between Hsp31 and EF-Tu can be accounted for by the recently discovered chaperone functions of EF-Tu (20–22). We cannot provide a straightforward explanation, however, for the interaction of Hsp31 with tryptophanase, although it is noteworthy that tryptophanase is involved in E. coli protection against alkali stress (see “Discussion” and Ref. 23).

**DISCUSSION**

We show in this study that Hsp31 does not display any significant proteolytic activity but displays an aminopeptidase activity that is specific against peptide substrates with alanine or basic amino acids at N terminus and is inhibited by iodoacetamide, EDTA, and 1,10-phenanthroline. Furthermore, an Hsp31-deficient strain accumulates higher quantities of 8–12-mer peptides than its parental strain, and Hsp31 can process these peptides to smaller peptides, which suggests that it is involved in the processing of 8–12-mer peptides both in vitro and in vivo.
The proteolytic activity of Hsp31 is negligible with several protein substrates frequently used for protease studies, such as gelatin, α- or β-casein, insulin, and insulin β-chain. Furthermore, no proteolytic activity could be detected in the presence of low amounts of detergents, upon partial unfolding of the protein substrate by temperature increase, or by other types of treatments that have the potential to stimulate the activity of various proteases. This is consistent with previous results, which concluded that Hsp31 does not display any proteolytic activity (3, 4) (however, a very weak proteolytic activity of Hsp31 toward bovine serum albumin has been reported in (7)).

It is also consistent with the restricted accessibility of the Cys-His-Asp catalytic triad of Hsp31, through a small pocket 14 Å long with an entrance of about 4 Å in diameter (5–7) that appears to confine the substrate spectrum of Hsp31 to peptides rather than protein substrates.

Hsp31 functions as a broad specificity aminopeptidase, which cleaves alanine and basic amino acid substrates better than others. The specific aminopeptidase activity of Hsp31 (2120 nmol/h/mg of protein), which is of the same order of magnitude as PepD (1260 nmol/h/mg of protein (24)), is considerably lower than that of PepN (2 × 10^5 nmol/h/mg of protein), the major E. coli aminopeptidase (16, 17). We have shown that the aminopeptidase activities assigned to Hsp31 are not contaminated by PepN by extensive purification of Hsp31 and by a different inhibitor sensitivity of Hsp31 and PepN; in contrast with Hsp31, PepN is not inhibited by EDTA or iodoacetamide but is, however, inhibited by PMSF (16–17). Hsp31 does not display any significant endopeptidase activity, since it does not cleave Ac-alanine-amc and other peptides blocked at their N terminus nor does it possess any carboxypeptidase activity, as reported previously (3). The hydrolysis of Ala-amc and Gly-amc has been reported by Lee et al. (7) but not that of Arg-amc, Lys-amc, and His-amc.

The aminopeptidase activity of Hsp31 is inhibited by the cysteine protease inhibitor iodoacetamide and is completely abolished in the C185A mutant. This is in accordance with crystallographic results suggesting that Cys185 is the active nucleophile of Hsp31 (5). Metalloprotease inhibitors (EDTA and 1,10-phenanthroline) also inhibit Hsp31. This suggests that the putative His^85, His^122, and Glu^90 metal-binding site revealed by crystallographic studies is important for Hsp31 activity (6). Zn^{2+} is the only divalent metal ion that is able to reactivate Hsp31 after inhibition by o-phenanthroline, suggesting that it is the biologically relevant cofactor of Hsp31, as suggested in crystallographic studies (6).

Hsp31 shows a classical kinetic dependence on temperature, in contrast with DegP/HtrA which displays a temperature-dependent switch from chaperone to protease between 30 and 40 °C, with an impressive increase in proteolytic activity in that temperature range (18).

It seems likely that Hsp31 plays an important physiological function in peptide degradation, since an Hsp31-deficient strain accumulates higher amounts of 8–12-mer peptides than its parental strain, and since purified Hsp31 can process these peptides to smaller peptides in vitro (this study). Several peptidase-deficient mutants of Salmonella typhimurium (a pepN pepP pepB pepD mutant (14), a pepP mutant, and a pepQ mutant (25)) also accumulate small peptides during exponential growth in minimal medium. It is generally assumed that 5–15-mer peptides are produced by ATP-dependent proteases involved in the intracellular regulated protein breakdown (ClpAP, ClpXP, Lon, HslUV, and FtsH in E. coli (26) and the proteasome in eucaryotes (10)), and both the processing of 8–12-mer peptides by Hsp31 and its interaction with ClpA strengthens the hypothesis of its involvement in the processing of peptides resulting from intracellular proteolysis. The accumulation of 8–12-mer peptides in the Hsp31-deficient strain, despite the presence of more efficient peptidases such as PepN, suggests that Hsp31 can degrade several classes of peptides that are not easily cleaved by other peptidases or that it helps in the degradation of peptides by other peptidases.

Reverse purification of a crude E. coli extract on an Hsp31 affinity column led to the detection of four proteins, ClpA, GroEL, EF-Tu, and tryptophanase, which specifically interact with Hsp31. None of these proteins interacted with two control columns containing either the chitin-binding domain used for Hsp31 purification or the E. coli peptidase YhbO. The interaction between Hsp31 and the GroEL chaperone suggests that Hsp31, which might function as a holding chaperone (4), could deliver unfolded proteins to the GroEL/ES chaperone machine for refolding. Recent work reported a possible cooperation between Hsp31 and the DnaK chaperone machine (27), and it is possible that a cooperation also occurs with the GroEL chaperone machine. The interaction between Hsp31 and elongation factor EF-Tu can be explained in light of the recently discovered function of elongation factors as chaperones and nascent chain-binding proteins (20–22); it has been suggested that eEF-1A (the eukaryotic homolog of EF-Tu) functions as a key component of the protein quality control mechanism (22), and a cooperation between Hsp31 and EF-Tu could be envisioned for such a function. The interaction between Hsp31 and ClpA strengthens the hypothesis that Hsp31 is involved in the intracellular protein/peptide degradation pathway. ClpA/ClpP is one of the ATP-dependent proteases exerting a function in intracellular protein degradation, which produce peptides of 5–15 amino acids that are cleaved further by peptidases such as PepN, PepB, PepD, PepE, PepQ, and PepT, and since purified Hsp31 can process these peptides to smaller peptides in vitro (this study). Several peptidase-deficient mutants of Salmonella typhimurium (a pepN pepP pepB pepD mutant (14), a pepP mutant, and a pepQ mutant (25)) also accumulate small peptides during exponential growth in minimal medium. It is generally assumed that 5–15-mer peptides are produced by ATP-dependent proteases involved in the intracellular regulated protein breakdown (ClpAP, ClpXP, Lon, HslUV, and FtsH in E. coli (26) and the proteasome in eucaryotes (10)), and both the processing of 8–12-mer peptides by Hsp31 and its interaction with ClpA strengthens the hypothesis of its involvement in the processing of peptides resulting from intracellular proteolysis. The accumulation of 8–12-mer peptides in the Hsp31-deficient strain, despite the presence of more efficient peptidases such as PepN, suggests that Hsp31 can degrade several classes of peptides that are not easily cleaved by other peptidases or that it helps in the degradation of peptides by other peptidases.
formic acids, both of which neutralize excess alkali. The Hsp31 chaperone might specifically protect tryptophanase against denaturation under these stress conditions.

Acknowledgment—We thank A. Kropfinger for help with the English language.

REFERENCES
1. Lindquist, S., and Craig, E. A. (1988) Annu. Rev. Genet. 22, 631–677
2. Hartl, F. U. (1996) Nature 381, 571–579
3. Malki, A., Kern, R., Abdallah, J., and Richarme, G. (2003) Biochem. Biophys. Res. Commun. 301, 430–436
4. Sastry, M. S., Korotkov, K., Brodsky, Y., and Baneyx, F. (2002) J. Biol. Chem. 277, 46026–46034
5. Quigley, P. M., Korotkov, K., Baneyx, F., and Hol, W. G. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 1363–1374
6. Zhao, Y., Liu, D., Kaluarachchi, W. D., Bellamy, H. D., White, M. A., and Fox, R. O. (2003) J. Biol. Chem. 278, 22260–22270
7. Cho, J. H., Kim, D. H., Lee, K. J., Kim, D. H., and Choi, K. Y. (2001) Biochemistry 40, 10197–10203
8. Blumentals, I. I., Robinson, A. S., and Kelly, R. M. (1999) Appl. Environ. Microbiol. 56, 1092–1099
9. Fukiage, C., Nakajima, E., Ma, H., Azuma, M., and Shearer, T. R. (2002) J. Biol. Chem. 277, 20678–20685
10. Yen, C., Green, L., and Miller, C. G. (1980) J. Mol. Biol. 143, 21–33
11. Kim, K. I., Park, S. C., Kang, S. H., Cheong, G. W., and Chung, C. H. (1999) J. Mol. Biol. 294, 1363–1374
12. Caldas, T. D., El Yaagoubi, A., and Richarme, G. (1998) J. Biol. Chem. 273, 11478–11484
13. Kudlicki, W., Coffman, A., Kramer, G., and Hardesty, B. (1997) J. Biol. Chem. 272, 32206–32210
14. Smith, D. H., Nakamura, T., and Wiedmann, M. (2002) J. Biol. Chem. 277, 18545–18551
15. Blankenhorn, D., Phillips, J., and Slonczewski, J. L. (1999) J. Bacteriol. 181, 2209–2216
16. Schröder, U., Henrich, B., Fink, J., and Flapp, R. (1994) FEMS Microbiol. Lett. 123, 153–160
17. Miller, C. G., and Green, L. (1983) J. Bacteriol. 153, 350–356
18. Wickner, S., Maurizi, M. R., and Gottesman, S. (1999) Science 286, 1888–1893
19. Muñoz, M., Bader, M. W., and Baneyx, F. (2003) Mol. Microbiol. 51, 849–859