Translocation, Folding, and Stability of the HflK/C Complex with Signal Anchor Topogenic Sequences*

(Received for publication, July 20, 1998, and in revised form, August 15, 1998)

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HflK and HflC are plasma membrane proteins of *Escherichia coli*, each having a large C-terminal domain exposed to the periplasmic space and an N-terminally located transmembrane segment, which should act as a signal anchor sequence for their biogenesis. They form a complex, HflKC. We studied in *vivo* processes of biogenesis of this pair of membrane proteins. Translocation of the C-terminal domains across the membrane, as assessed by their accessibility to externally added protease, was completed within 1 min after the synthesis in wild-type cells as well as in the secB mutant cells or in the FtsY-depleted cells. In contrast, translocation of these domains was retarded markedly when sodium azide was added to inhibit SecA ATPase and blocked almost completely in secY- or secD-defective mutant cells. Thus, although targeting of these membrane proteins depends neither on the SecB chaperone nor on the SRP pathway, their translocation occurs exclusively via the Sec translocase complex. Translocated HflK molecules were then folded into a partially protease-resistant conformation, taking a few minutes, and this folding was induced upon association with HflC. Singly expressed HflK and HflC were unstable in *vivo* and periplasmic proteases DegP and Prc were involved in the degradation of the HflK subunit. We characterized several hflA alleles isolated in early studies; they alter the HflK or the HflC sequence and destabilize the HflK/C complex.

A signal sequence induces translocation of the C-terminally adjacent mature portion of a secretory protein. Similar topogenic function is assigned for a class of transmembrane sequences of membrane proteins. They are called signal anchor sequences and are thought to have a similar role in translocation of the following polypeptide segment as an ordinary signal sequence. Additionally, they function as a transmembrane anchor; they are not cleaved and become stably embedded in the lipid phase of the membrane. Membrane proteins with a single signal anchor sequence are called type II integral membrane proteins (1). Although functions of signal anchor equivalents in *Escherichia coli* have been studied in several artificial constructions (2, 3), biogenesis of native type II membrane proteins has not been studied in detail. In this work we followed in *vivo* the processes of translocation, folding, and assembly of a pair of type II membrane proteins (4), HflK and HflC, which form a complex (5).

Secretory protein translocation across the plasma (cytoplasmic or inner) membrane of *E. coli* is mediated by the Sec proteins (for reviews, see Refs. 6 and 7). SecB, a secretory protein-specific chaperone, maintains a preprotein in a translocation-competent conformation (8). The SecA ATPase drives protein translocation through its insertion-deinsertion cycles (9, 10). Integral membrane components, SecY, SecE, and SecG, form a heterotrimERIC complex, providing a channel-like pathway (6, 7, 11). SecD and SecF are also membrane proteins with large periplasmic domains and are required for full translocation activity (12–14).

Involvement of the SecA-SecYEG translocase system has been documented for insertion/translocation of some authentic membrane proteins (15–17) or of fusion protein derivatives of membrane proteins (2, 18). Other studies suggest that a class of membrane proteins or some particular domains of membrane proteins integrate without the aid from the Sec system (19–22). A secY-dependent but secA-independent integration was also reported (23). Thus, different membrane protein segments may use the Sec translocase to different extents. However, mutational effects should be interpreted carefully, since different genetic systems could give different conclusions about the Sec factor requirement for integration of a membrane protein (17, 24). Recent studies suggest that Ffh, 4.5 S RNA and FtsY, the components of the *E. coli* version of SRP, are involved in targeting of some membrane proteins (25–29), presumably to the Sec translocase on the membrane (30). Finally, in addition to the above mentioned role of facilitating translocation, SecYEG complex may also have a role in the “stop transfer” mode of integration processes (31, 32).

Each of the HflK and HflC proteins contains a single transmembrane region located at the N-terminal region, while their remaining domains are exposed to the periplasmic space (4). They form a complex, HflK/C (5), and affect the stability of CII protein and thereby control the lysogenization frequency of this phage. Historically, identification of these proteins goes back to the isolation of the hflA class of *E. coli* mutations, which cause high frequency λ lysogenization (33). Sequencing studies later revealed that this genetic locus contains three genes, *hflX* of unknown function, *hflK*, and *hflC* (34). Although it was proposed that HflKC is a protease that degrades the CII protein (5), we showed that this is not the case (4). Instead, FtsH is the protease against CII (4, 35) and HflKC binds to FtsH (36) and seems to modulate its activities against different substrates (4, 37). HflKC directly binds to a membrane-embedded substrate of FtsH as well (37).

In this work, we studied in *vivo* processes of translocation and assembly of HflK and HflC. It was found that they do not depend on the SecB or the SRP targeting system, but their translocation absolutely depends on the membrane compo-

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* This work was supported by grants from the Ministry of Education, Science and Culture, Japan, Human Frontier Science Program Organization, and from CREST, JST (Japan Science and Technology Corporation). 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.

‡ Supported by Japan Society for the Promotion of Science (JSPS) Research Fellowship for Young Scientists.

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RESULTS

Proteinase K Digestion Patterns of HflK and HflC—The HflK and HflC proteins have their major domains exposed to the periplasmic side of the plasma membrane (4). Thus, these proteins in the intact spheroplasts were digested with a high concentration of proteinase K (Fig. 1A, lane 5 for HflK (4)). Treatment of spheroplasts with a lower concentration (50 μg/ml) of proteinase K converted HflK into a fragment of apparent molecular mass of 42 kDa (Fig. 1A, lane 3, band P1). Identity of this fragment was shown by its reactivity with the anti-HflK serum. Proteinase K treatment after solubilization of the membrane with Triton X-100 yielded a fragment that was further shortened to 38 kDa (Fig. 1A, lane 3, band P2). It is reasonable to assume that the N-terminal cytoplasmic and membrane anchor portions became proteinase K-accessible in the presence of the detergent. It is thus suggested that the C-terminal end of HflK is degraded by proteinase K while the remaining periplasmic domain is folded into a partially protease-resistant conformation (Fig. 1C). In contrast, HflC is fully sensitive to the low concentration of proteinase K (data not shown). Similar results were obtained for spheroplasts from cells with overproduced HflK and HflC (Fig. 1A, lanes 1–3). When HflK alone was overproduced, however, it was degraded without producing the P1 or P2 fragments (Fig. 1B, lanes 5 and 6). It is suggested that folding of HflK into the partially protease-resistant conformation can occur only in the presence of HflC. It should be noted that HflK, once folded, can remain so even out producing the P1 or P2 fragments (Fig. 1B, lanes 1–3) or 2 h (lanes 4–6). Samples for lanes 3 and 6 received Triton X-100 (Triton) before proteinase K. Proteins were separated by SDS-PAGE, and HflK and HflC were visualized by immunoblotting using anti-HflK antiseraum. P1 and P2 represent the proteinase K resistant fragments of HflK. The dot in lane 3 indicates a nonspecific background. B, cells of AK1316 (hflK-hflC-λ) carrying PK258 (plac-hflK’-hflC’) (lanes 1–3) or PK288 (plac-hflK-hflC’) (lanes 4–6), pregrown in L medium supplemented with 50 μg/ml ampicillin and 0.2% glucose, were further grown at 37°C in L medium containing 50 μg/ml ampicillin and 0.1 mM cyclic AMP for induction of plasmid-encoded HflK and HflC for 2 h. Spheroplasts were prepared and treated with or without 50 μg/ml proteinase K and 1% Triton X-100 as indicated. After incubation at 0°C for 30 min, proteins were analyzed by SDS-PAGE and immunoblotting using anti-HflK, C, a schematic representation of HflK disposition in the membrane. The coiled region represents the proposed proteinase K-resistant domain.

ice. Cells were collected by centrifugation, resuspended in 30 mM Tris-HCl (pH 8.1), 20% sucrose, followed by addition of 0.1 volume of 1 mg/ml lysozyme dissolved in 0.1 mM EDTA (pH 8.0) and incubation on ice for 30 min. Spheroplasts thus prepared were treated with proteinase K (50 μg/ml or 1 mg/ml) in the presence or absence of 1% Triton X-100 at 0°C for 30 min, followed by termination of the digestion with 1 mM phenylmethylsulfonyl fluoride and precipitation of proteins with trichloroacetic acid. Samples were then subjected to immunoprecipitation. Unlabeled cells were similarly processed and visualized by immunoblotting.

Cloning and Sequencing of the hflA Mutations—KpnI fragments from the chromosomal DNA of strains Y1089 (with an insertion of Mu phage within hflA) (50) and SY798 (hflC1) were ligated with KpnI-treated pMW119 (a pSC101-based lac promoter vector from Nippon Gene) and a 5′-AmpR transformants were selected on AK1290 (purA::kan) and AK1588 (AK1129, degP::kan) using SY798 as a donor. AK1839 (AK1129, degF::kan) was constructed by cloning a 1.3-kb KpnI fragment of pHflA100 (5) into pTWV228 (Takara Shuzo). pKH289 (pBR322-derived cloning a 2.9-kb EcoRI fragment of pHflX-hflK-hflC-purA promoter vector obtained from Nippon Genetic Engineering) was constructed by cloning a 1.2-kb SalI–I fragment of pKH276 (within Mu) fragment of pKH276 (pHflX-hflK-hflC::kan) into pTwv228 (AmpR transformants were selected on AK1290 (purA::kan)). pKH276 (from Y1089) and pKH278 (from SY798) thus obtained carried the chromosomal hflX-hflK-hflC-purA segments.

Purification of the hflA Mutations—KpnI fragments from the chromosomal DNA of strains Y1089 and SY798 (hflC1) were digested with KpnI and EcoRI and ligated with KpnI-EcoRI-treated pMW119, yielding pH1165 with an insert of hflX-hflK-hflC-hflC1. Finally, we cloned the following fragments into M13 mp18 or M13 mp19: 2.1-kb DraI-SalI fragment of pKH278 (hflC1); 1.2-kb SalI–I fragment (within Mu) fragment of pKH276 (hflC150); and 1.3-kb AaiI–HindIII (within Thn) fragment of pKH165 (hflK:Thn5). Single-stranded DNAs were sequenced (51), using 7-deaza Sequenase kit (U. S. Biochemical Corp.) and the primers described previously (36).

Plasmids—pKH258, carrying hflK’ and hflC’, was constructed by cloning a 2.9-kb EcoRI–HindIII fragment from pKH191 (36) into pTWV229 (a pBR322-derived lac promoter vector obtained from Takara Shuzo). pKH288 (hflK’) was constructed by cloning a 2.1-kb DraI–SalI fragment of pH1A100 (5) into pTWV228 (Takara Shuzo). pKH289 (hflC’) was constructed by cloning a 1.3-kb HpaI–EcoRI fragment of pH1A100 into pTWV229. pKH349, carrying hflK’ and hflC1, was constructed by cloning a 2.9-kb DraI–EcoRI fragment of pH283 into the Smal–EcoRI site of pTWV229.

Media—L medium (52) and M9 medium (53) were used. Ampicillin (50 μg/ml) was used for growing plasmid-bearing strains. Tetracycline (25 μg/ml) and kanamycin (25 μg/ml) were added to L agar, as required, for selection of transductants.

Immunoprecipitation under Denaturing Conditions and Immunoblotting—Immunoprecipitation of once denatured and SDS-solubilized proteins was done as described previously (54). For immunoblotting, proteins were separated by SDS-PAGE and electrophoretically blotted onto nitrocellulose membrane filters (Millipore). The filter was decorated with anti-HflK (4), HflK (4), or FtsY (provided by the late Dr. S. Mizushima), which were then visualized with the ECL detection kit (Amerham Pharmacia Biotech). Anti-HflK sera were preincubated with a cell lysate prepared from AK1316 (ΔhflK-hflC) to reduce backgrounds.

Proteinase K Digestion Assay of HflK and HflC Topology—Cells were grown in an early log phase in M9 medium with indicated supplements, pulse-labeled with [35S]methionine (from American Radiolabeled Chemicals) for indicated periods and chased with unlabeled l-methionine (200 μg/ml) for indicated periods. Samples were mixed with sodium azide (0.02%) and chloramphenicol (100 μg/ml) and chilled on.

FIG. 1. Proteinase K digestion tests of HflK and HflC topography and folding. A, spheroplasts prepared from strain AD202 (wild-type HflKC) were treated with 0 (lanes 1 and 4), 50 (lanes 2 and 3), or 1,000 (lanes 5 and 6) μg/ml of proteinase K (PK) at 0°C for 30 min (lanes 1–3) or 2 h (lanes 4–6). Samples for lanes 3 and 6 received Triton X-100 (Triton) before proteinase K. Proteins were separated by SDS-PAGE, and HflK and HflC were visualized by immunoblotting using anti-HflK antiseraum. P1 and P2 represent the proteinase K resistant fragments of HflK. The dot in lane 3 indicates a nonspecific background. B, cells of AK1316 (hflK-hflC-Δ) carrying PK258 (plac-hflK’-hflC’) (lanes 1–3) or PK288 (plac-hflK-hflC’) (lanes 4–6), pregrown in L medium supplemented with 50 μg/ml ampicillin and 0.2% glucose, were further grown at 37°C in L medium containing 50 μg/ml ampicillin and 0.1 mM cyclic AMP for induction of plasmid-encoded HflK and HflC for 2 h. Spheroplasts were prepared and treated with or without 50 μg/ml proteinase K and 1% Triton X-100 as indicated. After incubation at 0°C for 30 min, proteins were analyzed by SDS-PAGE and immunoblotting using anti-HflK, C, a schematic representation of HflK disposition in the membrane. The coiled region represents the proposed proteinase K-resistant domain.

1 On the basis of our sequencing results, we propose the following renaming for the hflA mutations: hflA1 (hfl-1), hflA100, and hflA150, and hflA-Tn5 should be called hflC1, hflC150, and hflK-Tn5, respectively.

2 The abbreviations used are: kb, kilobase pair(s); PAGE, polyacrylamide gel electrophoresis.
The processes of translocation and folding of the HflK and HflC proteins. Wild-type cells were pulse-labeled with [35S]methionine for 30 min and then chased with unlabeled methionine. Spheroplasts were prepared from each sample and treated with 50 μg/ml proteinase K. HflK and HflC were then immunoprecipitated. All the pulse-labeled HflK and HflC proteins were already translocated to the periplasmic side, since they were digested completely with the protease (Fig. 2A, upper panel, lane 2). Without chase, P1 and P2 fragments were only slightly produced, indicating that the majority of HflK was not yet folded into its characteristic conformation. Proportions of the P1 and P2 fragment increased with chase, reaching the maximal level at 2 min (lanes 8 and 9). These results demonstrate that translocation of HflK and HflC occurs within 1 min after their synthesis. This is followed by folding of HflK, taking some 1–2 min.

Sec Factor Dependence of HflK and HflC Translocation—The requirement for SecB was examined using the secB::Tn5 mutant. Pulse-chase experiments showed that translocation of HflK and HflC in this mutant cells (Fig. 2A, lower panel) was as rapid as in the wild-type cells. Thus, targeting/translocation of HflK and HflC does not require the SecB chaperone.

The SecA requirement was examined using sodium azide, a specific inhibitor of this ATPase. When this chemical was added to the culture 1 min before pulse-labeling, both of the pulse-labeled HflK and HflC proteins escaped the proteinase K action (Fig. 2B, lower panel), unless the membrane barrier was destroyed by a detergent (data not shown). Although some fraction of them became proteinase K accessible after chases for a few minutes (a small proportion of fragment P1 was also produced at these time points; lanes 10 and 12), the results shown in Fig. 2B clearly indicate that translocation of HflK and HflC was retarded strikingly when the SecA function was compromised by this inhibitor.

The involvement of the membrane-bound Sec factors in biogenesis of the HflKC complex was examined using cold-sensitive mutants, secY39 (47) and secD1 (48). Cells were exposed to the nonpermissive temperature (20 °C) for 30 min and then subjected to the pulse-chase analysis. Translocation of HflK and HflC in wild-type cells was completed rapidly even at this low temperature (Fig. 2C, upper panel, lanes 1 and 2), although subsequent attainment of the protease-resistant conformation required a longer chase (5–10 min) than at 37 °C (lanes 3–8). In sharp contrast, essentially all the pulse-labeled HflK and HflC proteins were protected from proteinase K digestion in the secY39 and secD1 mutant cells (Figs. 2C, middle and bottom panels, lanes 1 and 2), and they remained so even after a chase for 30 min (lanes 9 and 10). These results show that the HflKC biogenesis strictly depends on the Sec translocase functions.

SRP Dependence of HflK and HflC Membrane Assembly—Translocation of HflK and HflC was studied using the strain in which the ftsY, coding for the E. coli homolog of the SRP receptor α subunit, was placed under the control of the araB promoter (49). Immunoblotting experiments showed that after growth in the absence of arabinose for 5 h, cellular abundance of FtsY decreased to 1⁄33 of that in the wild-type strain (Fig. 3A). Pulse-chase analysis at this stage showed that translocation and folding kinetics of HflK and HflC in the FtsY-depleted cells

Fig. 2. Pulse-chase analysis of HflK and HflC translocation and folding in wild-type and sec mutant cells. A, cells of MC4100 (wild-type, WT; upper panel) and CK1953 (secB::Tn5; lower panel) were grown at 37 °C to an early log phase, pulse-labeled with [35S]methionine for 1 min, and chased with excess unlabeled methionine for 0, 1, 2, and 4 min as indicated. B, cells of AD202 were grown at 37 °C and pulse-labeled for 30 s with [35S]methionine for 0, 0.5, 1, 2, 4, or 8 min, as indicated. C, cells of AD202 (wild-type, WT; top panel), AK1743 (secY39; middle panel), and THE521 (secD1; bottom panel) were grown at 37 °C, shifted to 20 °C for 30 min, and pulse-labeled with [35S]methionine for 1 min, followed by chase for 0.5, 2, 5, 10, or 30 min as indicated. All samples were then processed for the proteinase K (PK, 50 μg/ml) digestion assay of HflK and HflC disposition/folding as described under “Experimental Procedures.” Triton X-100 (Triton, 1%) was added for A, lanes 3, 6, 9, and 12. Radioactive HflK and HflC were immunoprecipitated, separated by SDS-PAGE, and visualized using a PhosphorImager BAS2000 or BAS1800 (Fuji Film).

Fig. 3. Translocation of HflK and HflC under FtsY-depleted conditions. A, cells of AK1745 were grown at 37 °C in M9 medium supplemented with amino acids (20 μg/ml), thiamine (2 μg/ml), 0.4% glycerol, and 0.4% arabinose. Cells were then washed twice with arabinose-free medium and cultured in the medium containing 0.4% glucose instead of arabinose. After 0, 1, 2, 3, 4, and 5 h, as indicated, whole cell proteins were precipitated with 5% trichloroacetic acid and separated by SDS-PAGE. FtsY was visualized by immunoblotting. Comparison with the intensity of FtsY in wild-type cells (AD202; not shown) showed that the FtsY abundance was reduced 33-fold at 5 h. B, the FtsY-depleted (at 5 h; lower panel) and control (upper panel) AK1745 cells were pulse-labeled with [35S]methionine for 30 s and chased for 0, 0.5, 1, 2, 4, or 5 min, as indicated, and HflK and HflC topology was assayed with proteinase K (PK, 50 μg/ml).
Degradation of Unassembled HflK and HflC Subunits—As already discussed, the folding of HflK into the protease-resistant conformation seems to be dependent on the HflK-HflC interaction. In vivo stability of unassembled forms of the HflK and HflC subunits was studied by overproducing these proteins, individually or in combination, in a strain deleted for the chromosomal hflK and hflC genes. Pulse-chase experiments showed that singly expressed HflK and HflC were degraded with a half-life of about 12 min (Fig. 4, lanes 1–6). In this mutant cells (Fig. 4, lanes 7–12), AK1339 (hflC::tet; lane 2), AK1355 (hflC::Tn5; lane 3), AK1327 (hflC::Tn; lane 4), AK1339 (hflC150; lane 5), and AK1327 (hflC150::kan; lane 6) were grown in L medium at 37 °C. Total proteins were separated by SDS-PAGE, and HflK and HflC were visualized by immunoblotting. The asterisk indicates a putative degradation product of HflK.

(Fig. 3B, lower panel) were indistinguishable from those in cells grown continuously in the presence of arabinose (upper panel). Thus, the SRP pathway is not involved in the membrane targeting of HflK.

The periplasmic disposition of HflK and HflC (4) suggests an involvement of a periplasmic protease in their degradation. We examined stability of the HflK and HflC subunits in mutants lacking one of periplasmic proteases, DegP (also called HtrA) (46, 55), Ppr (56), and Prc (also called Tsp) (57). It was noted that degradation of HflK in wild-type cells was accompanied by putative degradation intermediates (I-1, I-2, I-3, I-4, I-6, I-7, I-8, I-9, and I-10 in Fig. 4A). In the degP::kan mutant, degradation of the HflK subunit was significantly retarded (half-life of 20 min; Fig. 4A, lanes 13–18). The preC::neo mutant cells gave significantly altered patterns of degradation intermediates, although the half-life of the full-length HflK was not significantly changed (Fig. 4A, lanes 7–12). In this mutant, I-6, I-7, and I-8 disappeared, whereas the intensity of I-3 increased and a new band, termed I-5, appeared (Fig. 4A, lanes 7–12). Thus, degradation of HflK may be partly initiated by DegP to produce the I-3 intermediate, which is then cleaved by Ppr. HflK degradation was not appreciably affected by the ppr mutation (data not shown). Degradation of HflC was not significantly affected by either ppr (Fig. 4B, middle panel), degP (Fig. 4B, bottom panel), or ptr (data not shown) mutation. No degradation intermediate of HflC was detected by the antibodies used.

Mutational Alteration of the Stability of the HflK Complex—In early studies, several hflA class mutations were described as causing increased frequency of lysogenization of infecting bacteriophage λ. However, they have not been characterized fully in molecular terms. We determined nucleotide sequence changes of the hflA mutations. In addition, we constructed the ΔhflK/hflC mutation deleting almost the entire hflK-hflC genes, and the hflC::tet mutation disrupting HflC at the Ile88 codon (4, 36). The hflA1 (hfl-1) mutant (33) contained two nucleotide substitutions, G3573 → A and G3902 → A, of the hflA operon (numbering, according to Noble et al. (34)), resulting in Gly270 → Asp and Asp285 → His changes in the HflC protein. We propose a renaming, hflC1, for this mutation. The hflA150 Mu phage insertion (50) was found to follow T3450 of the hflA operon resulting in a disruption of the hflC open reading frame at the Val254 codon; the truncated HflC sequence was significantly altered (Fig. 4C). Interestingly, a marked reduction of both HflK and HflC was observed for the hflC1 missense mutant (Fig. 4C, lane 6). A low molecular mass protein observed in the hflC::tet, hflC150, and hflC1 mutant cells (Fig. 4C, lanes 4–6, asterisk) represents a degradation product of HflK, because it reacted with the anti-HflK antibody (data not shown).

Pulse-chase examination of stability of HflK+ and HflC1 was carried out using the ΔhflK-hflC mutant in which these pro-
HflKC Translocation and Stability

The major domains of the HflK and HflC proteins become exposed to the periplasmic side immediately after their synthesis. We did not detect any cytoplasmic form of these proteins by pulse-labeling experiments at 20 or 37 °C. Neither the SecB chaperone system nor the SRP targeting system contributes to this rapid translocation. The N-terminally located transmembrane segments of HflK (68VVTIAAAAIVIWAASGFYT100) and HflC (4VIAIIIIVLVLYSMVFVVK24) contain longer stretches of hydrophobic amino acids than do conventional signal sequences for protein export (for review, see Izard and Kendall (58)). They show high hydrophobic index values, 1.43 and 1.50, respectively for HflK and HflC, according to the TopPred algorithm (59) using the Persson-Argos hydrophobicity scale (60). In acting as a translocation signal, these signal anchor sequences may be rapidly captured by the SecA/SecYEG-SecDF translocation machinery. Although it is not ruled out that some chaperone factors participate as cytosolic anti-folding or targeting factors for HflK and HflC, it is conceivable that these highly hydrophobic signal anchor sequences are directly recognized by the SecA ATPase (61, 62) for immediate translocation. The N-termini of these proteins may be quickly captured by the SecA/SecYEG-SecDF translocase, which may also recognize the signal sequences (63–65). We have shown that folding of HflK to the protease-resistant conformation depends on its association with HflC. Prior to membrane integration, these proteins may not be able to fold into a tight conformation in the cytosol rapidly enough to bias the kinetic competition between folding and translocation. Thus, the need for a chaperone/SRP assistance may be alleviated for this pair of proteins.

Whereas sodium azide markedly retarded the appearance of proteinase K-accessible forms of HflK and HflC, the secY and secD mutations almost completely blocked the translocation. The latter observation is in contrast to the fact that these highly hydrophobic signal anchor sequences are directly recognized by the SecA ATPase (61, 62) for immediate translocation. The N-termini of these proteins may be quickly captured by the SecA/SecYEG-SecDF translocase, which may also recognize the signal sequences (63–65). We have shown that folding of HflK to the protease-resistant conformation depends on its association with HflC. Prior to membrane integration, these proteins may not be able to fold into a tight conformation in the cytosol rapidly enough to bias the kinetic competition between folding and translocation. Thus, the need for a chaperone/SRP assistance may be alleviated for this pair of proteins.

DISCUSSION

The major domains of the HflK and HflC proteins become exposed to the periplasmic side immediately after their synthesis. We did not detect any cytoplasmic form of these proteins by pulse-labeling experiments at 20 or 37 °C. Neither the SecB chaperone system nor the SRP targeting system contributes to this rapid translocation. The N-terminally located transmembrane segments of HflK (68VVTIAAAAIVIWAASGFYT100) and HflC (4VIAIIIIVLVLYSMVFVVK24) contain longer stretches of hydrophobic amino acids than do conventional signal sequences for protein export (for review, see Izard and Kendall (58)). They show high hydrophobic index values, 1.43 and 1.50, respectively for HflK and HflC, according to the TopPred algorithm (59) using the Persson-Argos hydrophobicity scale (60). In acting as a translocation signal, these signal anchor sequences may be rapidly captured by the SecA/SecYEG-SecDF translocation machinery. Although it is not ruled out that some chaperone factors participate as cytosolic anti-folding or targeting factors for HflK and HflC, it is conceivable that these highly hydrophobic signal anchor sequences are directly recognized by the SecA ATPase (61, 62) for immediate translocation. The N-termini of these proteins may be quickly captured by the SecA/SecYEG-SecDF translocase, which may also recognize the signal sequences (63–65). We have shown that folding of HflK to the protease-resistant conformation depends on its association with HflC. Prior to membrane integration, these proteins may not be able to fold into a tight conformation in the cytosol rapidly enough to bias the kinetic competition between folding and translocation. Thus, the need for a chaperone/SRP assistance may be alleviated for this pair of proteins.

Whereas sodium azide markedly retarded the appearance of proteinase K-accessible forms of HflK and HflC, the secY and secD mutations almost completely blocked the translocation. The latter observation is in contrast to the fact that these mutations are somewhat leaky with respect to export of periplasmic or outer membrane proteins (47, 48). Thus, HflK and HflC have exceptionally strong dependence on the SecY subunit of protein translocase and the D subunit of the proton ATPase Fp, part is degraded by the FtsH protease (41, 69). FtsH is membrane-bound and having a cytosolic ATPase/protease domain, while its membrane-bound substrates have multiple membrane spanning segments as well as some cytosolic domains. Thus, FtsH comprises a quality control system that attacks malformed/unassembled membrane proteins from the cytosolic side. Since HflK and HflC have short and insignificant cytosolic residues, respectively, they are unlikely to be degraded by FtsH. Our results indeed showed that degradation of HflK or HflC was unaffected by ftsH mutations (data not shown; note that HflKC forms a complex with FtsH) (36).

We have shown that periplasmic proteases are involved in the degradation of HflK. The HflK subunit was stabilized partially in the degP mutant, and a degradation intermediate (I-3) accumulated in the prc mutant. Thus, in a pathway of HflK degradation, DegP (HtrA) initiates the degradation and Prc (Tsp) succeeds it. DegP is a serine protease responsible for degradation of a number of abnormal periplasmic proteins (46). Prc is also a periplasmic protease and was shown to cleave the C terminus of FtsI (PBP-3) (57) as well as proteins that are marked with an ssrA-encoded peptide tag at the C terminus (70). Since stabilization of HflK in the degP null mutant was not complete, some other proteases also might be involved. Also, proteases responsible for the degradation of the HflC subunit are unknown, since HflC was not stabilized in degP, prc, or ptr mutants. Possibly, DegQ (HhoA) and/or DegS (HhoB) are involved, as these proteins are homologs of DegP and their functional overlaps with Prc have been suggested (71, 72). Accumulation of an abnormal protein in the periplasmic space or the outer membrane elicits signal transducing reactions to activate the expression of genes for cell surface folding catalysts and proteases (73–75). It is of interest to ask whether unassembled plasma membrane proteins with large periplasmic domains, such as HflK and HflC, are also recognized as a surface stress by the signal transduction systems.

Acknowledgments—We thank Yoshinori Akiyama for useful comments, Kiyoko Mochizuki for technical support, the late Prof. Shoji Mizushima (Tokyo University of Pharmacy and Life Science) for anti-FtsY serum, and Takayuki Homma and Hiroshi Harasawa (National Institute of Genetics) for E. coli strains.

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