We examined in vitro translocation of pro-OmpA derivatives with a His<sub>6</sub> tag at various positions in their mature proteins and with a c-Myc tag at their C termini across inverted membrane vesicles of *Escherichia coli*. Those with a His<sub>6</sub> tag in the N-terminal region of the mature domain, which corresponds to the "translocation initiation domain" proposed previously (Andersson, H., and von Heijne, G. (1993) Proc. Natl. Acad. Sci. U. S. A. 88, 9751-9754), could not be translocated in the presence of 100 μM Ni<sup>2+</sup>, while OmpA derivatives with a His<sub>6</sub> tag in the middle of or at the C terminus did not show such Ni<sup>2+</sup> sensitivity. The inhibitory action of Ni<sup>2+</sup> on pro-3His-OmpA' (with a His<sub>6</sub> tag after the third amino acid of the mature OmpA-c-Myc region) translocation was exerted only during early events, after which it became ineffective. The inhibition point of Ni<sup>2+</sup> was suggested to lie between membrane targeting and exposure of the signal cleavage site to the periplasm since the unprocessed and membrane-bound form of pro-3His-OmpA' was accumulated by the addition of Ni<sup>2+</sup>. The Ni<sup>2+</sup>-"trapped" precursor was released from its translocation block by 30 mM histidine, which should compete with the His<sub>6</sub> tag on the precursor protein for formation of a Ni<sup>2+</sup> chelating complex. We propose that Ni<sup>2+</sup> confers a reversible positive charge effect on the His<sub>6</sub>-tagged initiation domain of the pro-OmpA derivatives and inhibits an early event(s) of protein translocation, such as presentation of the precursor to the membranous part of the translocase. This system will be useful in dissecting early events of the protein translocation pathway.

Translocation across the cytoplasmic membrane is the first step of protein targeting to the cell surface in bacterial cells. This complex biochemical reaction involving topological change of molecules has been analyzed by combined approaches of genetics and biochemistry in *Escherichia coli* (for reviews, see Refs. 1–4). The biochemical studies, notably purification and reconstitution of protein translocation machinery, have revealed key players of the translocation, translocation ATPase (SecA), a secretory protein-specific chaperone (SecB), and an integral membrane component (SecY-SecE-SecG complex) (5–9). From in vitro analyses using inverted bacterial plasma membrane vesicles, several subprocesses in the protein translocation reaction can be envisaged: 1) recognition of preproteins by chaperones (like SecB) that retain "translocation-competent conformation" of the secretory protein precursors, 2) targeting of the preprotein-SecB complex to SecA bound to the high affinity site of the plasma membrane, 3) ATP binding-dependent partial insertion of the precursors into a translocation channel, and 4) ATP hydrolysis-coupled and Δp-dependent bulk protein translocation (9–13). Translocating secretory proteins are surrounded by SecA and SecY, but not by lipid molecules (14). Recently, Economou and Wickner (15) found that the movement of the secretory protein is coupled with insertion and de-insertion of a 30-kDa segment of SecA. Deep insertion of SecA into the membrane is also detected in vivo (16).

During the course of these analyses, several systems have been developed to trap translocation intermediates during post-translational protein translocation. Except for the cases of kinetic trapping of intermediates by low ATP concentration (12) and formation of a disulfide bond loop of precursor protein in the absence of proton-motive force (11), most of the methods rely on some "tightly folded" structures that block further penetration of the preproteins into the translocase. For instance, translocation of epitope-tagged preprotein was blocked by epitope-specific antibody (13). Covalent attachment of stable structures such as bovine pancreas trypsin inhibitor (12) or methotrexate-binding dihydrofolate reductase (14) moieties to the precursor protein also generates translocation intermediates. But, in all of these cases, the blockades were exerted during the events that occur in the middle of translocation of the bulk of the mature domain.

In the cotranslational translocation system in the eukaryotic endoplasmic reticulum, the ribosome-nascent chain complex offers an ideal experimental tool to define various translocation intermediate states, including those in quite early stages in translocation (17). On the other hand, early biochemical events in the bacterial post-translational system have only insufficiently been investigated due to the lack of convenient methods to accumulate "early intermediates."

Mutants affected in the translocation processes provide useful clues about the early events in vivo. Especially the prl mutations in secY and secE loci, which broaden the specificity of signal sequence recognition, suggested a direct interaction between signal sequence and SecY-SecE, the main membranous subunits of the E. coli translocase. Silhavy and co-workers (18, 19) found a striking clustering of prlA mutations in the first periplasmic domain and in the seventh and tenth transmembrane domains, which they proposed are essential for SecY's recognition of signal sequence and SecE. Our isolation of cold-sensitive and dominant sec mutations in secY suggested that the region C-terminal to transmembrane domain 8 is
important for translocation facilitation and that the fourth cytoplasmic region is required for interaction with SecE (20–22). To obtain an integrated picture of translocation in molecular terms, it is essential to analyze the nature of the early interaction between precursors and the SecY-SecE-SecG complex on the membrane in vitro. More specifically, it is highly desired to devise a new method to "trap" translocation intermediates in the early stages in vitro.

In this report, we exploited the technique of hexahistidine tagging to use His$_6$-tagged precursor proteins for easy purification as well as for generation of a new type of translocation intermediates. We found that the pro-OmpA derivatives with a His$_6$ tag in their N-terminal regions of their mature proteins could not be translocated in the presence of a low concentration of Ni$^{2+}$. Ni$^{2+}$ acted only on pro-OmpA derivatives with a His$_6$ tag in the N-terminal region of the mature sequence. This inhibition occurs only at an early stage(s) of the translocation reaction and can be released by adding histidine, which competes for chelating Ni$^{2+}$ with the His$_6$ tag in the preprotein. Ni$^{2+}$ did not inhibit, but rather enhanced, membrane association of His$_6$-tagged OmpA, suggesting that it acts just after the membrane targeting step. This system will be useful for dissecting the early events in bacterial protein translocation.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains—**The following E. coli strains were used in this study: TYE055 (K1279pST30), zdh-33:Tn10, sec24, araD139, rpsE, Δ(argF- lacU169, rpsL10, ΔrA1, flbB301, deC1, pTSF25, rbsF/ [lal-9], lacPL8, lacZ, lacY, lacA), pST30 [cat, plac-syd]: TYE024, araD139, Δ(lacZα-yarF-argU169, rA1, rpsL10, flbB301, deC1, pTSF25, rbsR, ompT:kanF'/ [lacI, lacZ, lacA, lacY, lacB, lacPL8, proAB]; TYE098 (CU148[pKY173], araD139, Δ(lacZα-yarF-argU169, rA1, rpsL10, flbB301, deC1, pTSF25, rbsR, cya238F'/ [lacI, lacZ, lacA, lacB, lacY, lacB, lacPL8, proAB]); TYE050 (M15 proAB ΔrpsL150, pRD87, lacZΔM15, pKY173 [bla, pT7-seCB]). For the construction of plasmids, DH5α/Fα was used. Bacterial strains were cultured according to Ref. 23.

**Materials for In Vitro Translocation Assays—**Inverted inner membrane vesicles (INV) were prepared from TYE024 as described in Ref. 25 with slight modifications. The final membrane pellet was suspended in 50 mM HEPESKOH, pH 7.5, 50 mM KCl, 5 mM MgOAc, 10 mM β-mercaptoethanol.

**Plasmid Constructions—**Reagents for the recombinant DNA technique were purchased from New England Biolabs Inc., Toyobo, Takara Shuzo, Amersham Corp., Bio-Rad, or Perkin-Elmer. Molecular biological experiments were performed according to Ref. 23 or the manufacturers’ instructions.

To construct pTYE005, a His$_6$ fusion vector, the "His$_6$-oligodeoxynucleotides" 5’-GAGAACGAGGCGGCAATCATCATTCCACCATCCACCATCCAGTTGCGA-3’ and 5’-CCATCGATGTTGAGTTGAGTTGACGCTTGCATTGGATTTG-3’ were annealed and ligated between the ClaI and KpnI sites of plasmic blue SK (−). pTYE007, a His$_6$-c-Myc fusion vector, was then constructed by ligating a 1.2-kb ClaI-ClaI fragment of pTYE005 and a 1.84-kb ClaI-ClaI fragment of pTYE006. The His$_6$ and c-Myc oligodeoxynucleotides are designed to encode LEGRHHHHHH (factor Xa site followed by a His$_6$ tag) and EEQKLISEEDLL-RKR-ocher (c-Myc monodonal antibody 9E10 epitope (24)), respectively. When these double-strand oligodeoxynucleotides were cloned into pbBlueScript SK(−) as described above, they did not disrupt the lacZα open reading frame, and the tags were encoded on another reading frame. Therefore, the lacZ assay can be used for cloning an exogenous DNA fragment into these three vectors.

To construct the OmpA-His$_6$-Myc-expressing plasmid, a 1.23-kb SspI fragment of pRD87 covering the ompA open reading frame was cloned into pTYE007 to obtain pTYE008. A 0.25-kb BglII-EcoRI fragment of pTYE008 was replaced with a 0.15-kb fragment of the 3’-terminal region of the ompA gene amplified by polymerase chain reaction with the primers 5’-AAAGGTATCCCGCGACAG-3’ and 5’-GGGCTACCGTCCCAGGAGTATC-3’ as described above, they did not disrupt the lacZα open reading frame, and the tags were encoded on another reading frame. To insert a His$_6$ and Myc tag at various locations in the OmpA mature domain, pTYE018 was mutagenized with the mutagenic primers described below. 5’-TAGCCCGGCGGCGTCCGAAACAC-CTATCATCCATACATGCAATACACTCCG-3’ was used for the construction of pTYE50, encoding 3His-OmpA, which has His$_6$-Ser after the eighth amino acid of this sequence: 5’-GATAACACGTGACCACACCATACATCACCACGATACTGAATCT-3’ for pTYE086, encoding 8His-OmpA, which has His$_6$-Ser after the 20th amino acid, and 5’-COTTATGGATGTACACCAACCATCACCATCCAAGTCCTGAATCTG-3’ for pTYE112, encoding 11His-OmpA, which has His$_6$-Val after His-114. Mutations were confirmed by sequencing the presence of new restriction sites, underlines in the oligodeoxynucleotides (ClaI, Scal, Scal, and Sall, respectively). To construct SecB-overproducing plasmid pTYE025, a 1.24-kb BamHI-PvuI seCB fragment derived from pAK330 (43) was subcloned into pbBlueScript KSK(−) digested with BamHI and EcoRI (New England Biolabs). Materials for In Vitro Translocation Assays—Inverted inner membrane vesicles (INV) were prepared from TYE024 as described in Ref. 25 with slight modifications. The final membrane pellet was suspended in 50 mM HEPESKOH, pH 7.5, 50 mM KCl, 5 mM MgOAc, 10 mM β-mercaptoethanol.

SecA was prepared from CU148[pKY173], a SecA overproducer, by a combination of Matrix gel red A dye binding column and DEAE-Sepharose Fast Flow chromatography. SecB was overproduced in TYE126 (JM1909DE3[pTYE025]) from the T7 promoter and purified as described (26) up to the Q-Sepharose column step, and the sample was further purified by butyl-Sepharose column chromatography. S-Labeled pro-OmpA derivatives were prepared by in vitro transcription and translation using appropriate plasmids. N. E. coli S130, and [35S]Met (27). Proteins were then precipitated with 5% trichloroacetic acid (final concentration) and dissolved in HU buffer (50 mM HEPESKOH, pH 8.0, 1 mM NaCl, 8 μM urea, 10 mM β-mercaptoethanol).

Purification of Pro-OmpA Derivatives—TYE055 harboring pTYE009 or pTYE50 was cultured until mid-log phase in 2.2 liters of LB medium, 0.04% glucose, isoapropyl-β-D-thiogalactopyranoside was added at a final concentration of 1 mM to induce His$_6$-tagged OmpA derivatives as well as to overproduce Syd, which causes a severe secretion defect (28). After 2–3 h, cells were harvested and lysed by sonication in urea lysis buffer (50 mM sodium phosphate, pH 8.0, 1 mM NaCl, 8 μM urea, 10 mM β-mercaptoethanol) supplemented with 0.5 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 2 μM pepstatin. Cell debris and membranes were removed by two consecutive centrifugations at 4500 × g for 10 min and at 100,000 × g for 60 min at 4 °C. The soluble fraction was loaded onto a Ni$_2$-NTA-agarose column and washed with urea lysis buffer at 4 °C. His$_6$-tagged protein was eluted with a 0–150 mM NaCl gradient, and eluates were examined by Western blotting with anti-cMyc monoclonal antibody. Proteins in the peak fractions were precipitated with 5% trichloroacetic acid (final concentration) and dissolved in 50 mM Tris-HCl, pH 8.0, 8 μM urea, 10 mM β-mercaptoethanol. The sample was loaded onto a Hi-Trap Q column and eluted with a 0–150 mM NaCl gradient at room temperature. Purified pro-OmpA derivatives were again precipitated with 5% trichloroacetic acid, dissolved in HU buffer, dispensed into small aliquots, and then stored at −80 °C.
stored at −80 °C.

Iodination of Pro-OmpA Derivatives—100 μg of a pro-OmpA derivative in HU buffer was trichloroacetic acid-precipitated and redissolved in 200 μl of 0.1 m sodium phosphate, pH 7.0, 0.15 m NaCl, 8 m urea. Two IODO-BEADS (Pierce), 5 μl of carrier-free Na125I (1.9 MBq), and 2 μl of 1 m unlabeled NaI were added to the solution. After a 10-min incubation at room temperature, the beads were removed, and dithiothreitol was added at a final concentration of 10 mM to add to terminate iodination. Iodinated protein was precipitated with 10% trichloroacetic acid (final concentration) and redissolved in 200 μl of HU buffer, and its radioactivity and protein concentration were determined.

In Vitro Translocation Assay—An in vitro translocation assay was performed in 25 μl of standard assay buffer (50 m Hepes/KOH, pH 8.0, 0.15 m NaCl, 5 m MgCl2, 0.1 mM bovine serum albumin) as described (14) with the following modifications. 1) 1.6 mg/ml INV or 1 mg/ml 6M urea-extracted INV was used; 2) membrane and soluble factors were premixed, and the translocation reaction was started by adding pro-OmpA derivatives; and 3) the translocation reaction was terminated at 15 min unless otherwise mentioned. Reactions without ATP/ATP regenerase system and sodium succinate were used as negative controls. After the translocation reaction, two 10-μl aliquots were withdrawn and subjected to a 10-min incubation in the presence or absence of 0.25 mg/ml TPCK-treated trypsin on ice, followed by a further 10-min incubation on ice with a 2-fold weight of chicken egg trypsin inhibitor. In the reactions with radioactive substrates, samples were subjected to SDS-PAGE, and radioactivities of pro-OmpA and mature OmpA derivatives were measured by the use of a combination of a Fuji BAS2000 analyzer and a PDI image analyzer. In the reactions with nonradioactive substrates, the OmpA species were visualized by Western blotting and quantified by a PDI image analyzer.

Binding of Pro-3His-OmpA and Pro-OmpA-His9 to INV—The binding of pro-OmpA derivatives to INV was examined as described (9). Substrate (1 μg) was mixed with 100 μl of standard reaction mixture containing 1 mg/ml 6M urea-extracted INV with appropriate supplements. After incubation for 15 min at 0 °C, an 80-μl aliquot was loaded on 160 μl of 20% (w/v) sucrose in standard assay buffer and centrifuged at 12,100 × g for 20 min to fractionate into soluble (upper phase) and membrane (pellet) fractions. Relative amounts of total, unbound, and bound pro-OmpA were determined by Western blotting. Distribution of SecA and SecY was examined similarly. In the case of the translocation assay of prebound prepptrotein, binding reactions were performed in 175 μl with 0.35 μg of 125I-pro-3His-OmpA, and 150-μl portions were subjected to centrifugation.

Protein Techniques—For Western blotting, proteins were transferred to Immobilon P polyvinylidene difluoride membrane (Millipore Corp.) and visualized with the appropriate primary antibodies. Signals were visualized by horseradish peroxidase-conjugated secondary antibodies and the ECL Western blotting detection system (Amersham Corp.) or by alkaline phosphatase-conjugated secondary antibody and a nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate system.

SecA ATPase was assayed as described in Ref. 7. Protein concentration was determined by the Bio-Rad protein assay solution with bovine γ-globulin as a standard.

RESULTS

Purification of Precursors of OmpA Derivatives—As an application of the His6 tag method (29, 30), we constructed His69 and c-Myc-tagged OmpA derivatives as described under “Experimental Procedures.” Among several in vivo conditions we examined to accumulate unprocessed precursor proteins, the tight inhibition of protein export in the secY24 mutant upon overexpression of the secY gene (28) was most effective. In our system, SecA and a precursor protein were overproduced from two compatible plasmids under the regulation of lac promoter. Plasmid-encoded proteins were detected by anti-c-Myc monoclonal antibody (24) during purification and in the in vitro translocation assay. We expressed and purified two types of His69 and c-Myc-tagged OmpA precursors, pro-OmpA-His9 with a His6 tag at the C terminus of the OmpA sequence and pro-3His-OmpA with a His3 tag in the N-terminal region of the mature domain (Fig. 1), as described under “Experimental Procedures” in detail (Fig. 2, A and B). The proteins were kept in buffers containing 8 m urea to maintain their translocation competence in vitro. We used a DEAE-Sepharose or HiTrap Q anion-exchange column after the Ni2+ -NTA-agarose column to remove residual contaminations and small amounts of mature proteins. Typically, 7.5 mg of OmpA precursor with a purity of >95% was obtained from 2.2 liters of culture. Purified precursor was translocated into E. coli wild-type INV in an ATP-dependent manner (Fig. 2C). In the following experiments, translocation of these purified preproteins was assayed either by Western blotting with anti-c-Myc epitope antibody, Lane 1, total lysate; lane 2, supernatant; lane 3, pellet. Similar results were obtained for OmpA-His9, p, precursor; m, mature protein. B, pro-OmpA-His9 and pro-3His-OmpA were purified as described under “Experimental Procedures,” and 0.2 μg each was analyzed on 12.5% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Lane M, molecular mass standards; lane 1, pro-OmpA-His9; lane 2, pro-3His-OmpA. There is a slight contamination of mature OmpA-His9 in lane 1. C, translocation of purified pro-OmpA-His9 was tested using INV prepared from TYE024. Precursor protein (1.32 μg in 8 m urea) was diluted into translocation reaction mixture (40 μg/ml SecA, 20 μg/ml SecB, 1.6 mg/ml INV in standard assay buffer) and incubated at 37 °C for 30 min. Lanes indicated - ATP received an ATP/ATP regenerase system and 5 mM NADH (final concentration). After the incubation, three 7-μl aliquots were withdrawn and subjected to mock treatment, trypsin treatment (0.25 mg/ml TPCK-treated trypsin), or trypsin/Triton X-100 treatment (0.25 mg/ml TPCK-treated trypsin, 0.2% Triton X-100), as indicated, at 0 °C for 10 min. The trypsin-resistant portions of OmpA derivatives were visualized by Western blotting with anti-c-Myc antibody.

Effects of Ni2+ on Translocation of His69-tagged OmpA Precursors—We examined the possible effects of Ni2+ on translo-
In vitro translocation of pro-3His-OmpA, but not of pro-OmpA-His', is sensitive to Ni\(^{2+}\). A, \(^{35}S\)-labeled pro-OmpA, pro-OmpA-His', and pro-3His-OmpA were synthesized in vitro and post-translationally translocated into INV in the presence of various concentrations of NiCl\(_2\) and/or NTA/Na\(_2\)B. In lanes 1–3, 20, 10, and 0\% of the in vitro translated precursors used in the reactions were run, respectively. Each reaction mixture contained the reagents indicated above the panel. Portions of translocation reactions were treated either with trypsin (lanes 4–11) or with buffer (lanes 12–19). Lanes 4 and 12 show negative controls of the reaction without ATP, and lanes 5 and 13 represent positive controls of the standard reaction mixture without NiCl\(_2\) or NTA/Na\(_2\)B. p, precursor; m, mature protein. B, purified pro-3His-OmpA (lanes 1–8) and pro-OmpA-His' (lanes 9–16) were translocated in the absence (lanes 1, 5, 9, and 13) or presence (lanes 2, 6, 10, and 14) of 100 \(\mu\)M NiCl\(_2\). Lanes 3, 7, 11, and 15 are the minus-ATP controls, and lanes 4, 8, 12, and 16 are reactions without INV. Samples were subjected to SDS-PAGE with (lanes 1–4 and 9–12) or without (lanes 5–8 and 13–16) one-fourth of the trypsinized samples were used for trypsinization, and OmpA species were visualized by Western blotting. Asterisks represent unrelated bands.

The above results suggested that the inhibition of translocation by Ni\(^{2+}\) was dependent on the position of the His\(_6\) tag in the OmpA protein. We constructed a series of OmpA derivatives with a His\(_6\) tag at various positions in the mature domain of the OmpA protein (Fig. 1). The Ni\(^{2+}\) sensitivities of their translocation were compared using in vitro translated preproproteins (Fig. 4). Translocation of pro-3His-OmpA' (Fig. 4A, ▲) and pro-8His-OmpA' (●) with His\(_6\) tags after the third and eighth amino acids of the mature domain, respectively, was completely inhibited by 120 \(\mu\)M Ni\(^{2+}\) (Fig. 4A). The same concentration of Ni\(^{2+}\) did not inhibit translocation of pro-OmpA', pro-OmpA-His', or pro-114His-OmpA'. Pro-20His-OmpA showed an intermediate sensitivity to Ni\(^{2+}\) (Fig. 4A, ×). Although 40 \(\mu\)M Ni\(^{2+}\) somewhat inhibited translocation of various pro-OmpA derivatives, but not of pro-OmpA', we did not pursue this Ni\(^{2+}\) effect further. We conclude that 20–100 \(\mu\)M Ni\(^{2+}\) affects translocation of only precursor proteins with a His\(_6\) tag in the N-terminal region of the mature domain.

The spectrum of the positional effect in the His\(_6\) tag and the Ni\(^{2+}\)-dependent translocation block (Ni\(^{2+}\) inhibition at 120 \(\mu\)M; summarized in Fig. 4B) reminds us of the results of Andersson and von Heijne (31), who found that the first 30 amino acid residues of the mature domain of the secretory precursor are particularly sensitive to introduction of positive charges (6 consecutive lysines). They proposed to call this region the “translocation initiation domain.” The inhibitory effect of Ni\(^{2+}\) on translocation of N-terminally His\(_6\)-tagged pro-OmpA may also result from introduction of positive charges in the translocation initiation domain by chelating Ni\(^{2+}\). Consistent with this notion, protein translocation of pro-3His-OmpA' in the presence of 80 \(\mu\)M NiCl\(_2\) was restored by adding NTA (Fig. 3A, lower panel, compare lanes 6 and 10), which should interact with the His\(_6\) tag with high affinity through chelating Ni\(^{2+}\) (30). Because an NTA molecule has two minus charges at pH 8.0, at which we performed the translocation assay, the antagonistic activity of NTA on Ni\(^{2+}\) inhibition may simply be explained by its charge neutralization effect. Still other explana-
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**Fig. 5.** Ni²⁺ affects only early steps of translocation reaction. Two types of translocation reactions were performed as summarized above the graph. In reaction scheme I, a 150-μl reaction was started at 37°C by adding 0.6 μg (14.6 pmol) of 125I-labeled pro-3His-OmpA. At the indicated times, a 10-μl aliquot was withdrawn and transferred to a new tube containing 1 μl of 1 mM NiCl₂ and further incubated for a total of 25 min. Another 150-μl reaction was carried out as described above, and a 10-μl aliquot was cooled in ice water to measure the amounts of translocated 3His-OmpA at each time point (scheme II). After the reaction, samples were trypsin-treated and subjected to SDS-PAGE. A, translocation efficiencies of the two reaction schemes at each time point are plotted. ●, scheme I; ○, scheme II. Translocation efficiency is expressed by the amount of trypsin-resistant pro-OmpA and mature OmpA derivatives in a 25-μl standard reaction for comparison with other experiments. B, efficiency of Ni²⁺ inhibition after its addition was calculated from the data at each time point in A by the following formula: % inhibition efficiency = ((([scheme I value] − [scheme II value])/([scheme I value at 25 min] − [scheme II value])) × 100.

Ni²⁺ Affects Only Early Event(s) in Translocation—We next investigated the time course of the Ni²⁺ effects on translocation of pro-3His-OmpA. NiCl₂ at a final concentration of 100 μM was added at various time points to the reaction mixture, and translocation of 125I-labeled pro-3His-OmpA was allowed for a total of 25 min (Fig. 5, scheme I). As a control time course (Fig. 5, scheme II), the amounts of 3His-OmpA already translocated at each time point of Ni²⁺ addition were measured by immediately chilling the reaction mixture.

Although it has not been established to what extent the in vitro translocation reaction using the E. coli INV system is synchronized and how long it takes for a single precursor molecule to complete translocation, the following considerations will be useful for interpretation of the data obtained. If one assumes that a single cycle reaction occurs synchronously and that an inhibitory action is exerted at a specific step of the reaction, addition of the inhibitor prior to the inhibition step in the scheme I reaction completely blocks the final yield of translocation, whereas after the inhibition step, the inhibitor is totally ineffective in lowering the final yield. As shown in Fig. 5A (○), the total amount of translocation (scheme II) increased steadily up to the 25-min incubation period examined. The translocation yields in the scheme I reaction (●) were significantly higher than those in scheme II, except for the 0- and 1-min time points. The effectiveness of the Ni²⁺ inhibition after its addition is shown in Fig. 5B. It was found that the inhibitory action was gradually lost during the course of this in vitro reaction. This indicates that Ni²⁺ does not inhibit the reaction uniformly at every step or, at least, the final step of translocation. Rather, the inhibition point(s) should be located early in the translocation pathway. This interpretation is also supported by the fact that Ni²⁺ blocked the signal cleavage of pro-3His-OmpA, which occurs early in translocation (12). The lack of a clear “cutoff” point in Fig. 5B may be due to asynchrony in the initial process as well as to possible random slowing down during late steps of translocation. Therefore, we conclude that Ni²⁺ acts early in the translocation event(s).

Ni²⁺ Does Not Inhibit Recruitment of Precursor to INV—Several events are assumed to occur early in translocation, including interaction of a soluble translocation precursor with a secretory protein-specific chaperone (SecB) and targeting of the precursor to the inner membrane. We observed that the purified pro-OmpA derivatives were eluted from the gel filtration column as a high molecular mass form with SecA in the presence of SecA and SecB irrespective of the presence of Ni²⁺ (data not shown). This suggested that Ni²⁺ did not affect SecA/SecB recognition of pro-OmpA derivatives. Next, we addressed precursor recruitment to INV. We incubated pro-OmpA derivatives with INV in translocation reaction mixture on ice for 15 min and isolated membranes by centrifugation through a sucrose cushion. SecY, a marker of INV, was quantitatively recovered in the membrane fraction, whereas after the incubation of pro-OmpA-His in the membrane fraction (Fig. 6, lanes under pro-OmpA-His + Ni²⁺). A slight increase in recovery in the membrane fraction, but not as significant as that of pro-3His-OmpA, was observed in the case of pro-OmpA-His (Fig. 6, lanes under pro-OmpA-His' + Ni²⁺). Ni²⁺ may enhance the membrane targeting of...
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An intermediate state(s) on the membrane, such that complex formation with soluble Sec factors and targeting to INV are skipped when released from this trap.

We do not believe, however, that the apparent translocation rates shown in Figs. 5 and 8 represent the kinetics of translocation of individual precursor molecules. They must represent a sum of the heterogeneous population at different stages of the reactions. The fact that the Ni\textsuperscript{2+} trap only shortened the initial lag period but did not enhance the apparent translocation rate upon release may suggest that there are multiple “bottleneck” processes in vitro and that some of them occur after the Ni\textsuperscript{2+}-sensitive step. Although we need a further investigation of the molecular nature of the Ni\textsuperscript{2+}-trapped intermediate, it is a new type of “reversible” inhibition of an early event of translocation in the bacterial system.

**DISCUSSION**

In this study, we made use of the His\textsubscript{6} tag method developed by Bush et al. (29) not only to purify chemical amounts of E. coli pro-OmpA derivatives, but also to dissect their translocation across INV of E. coli in vitro. Our system using a combination of secY24 mutation and overexpression of syd (28) will be useful to accumulate bacterial precursor proteins in E. coli cells. We found that a His\textsubscript{6} tag introduced into the N-terminal region of the OmpA mature domain confers Ni\textsuperscript{2+} sensitivity to its translocation. Ni\textsuperscript{2+} inhibits only the early step(s) of the translocation reaction, which is after the association of precursor with the membrane, but before the signal cleavage. Inhibition can be released by addition of histidine, which breaks the His\textsubscript{6}-Ni\textsuperscript{2+} chelating complex.

It is likely that the effect of Ni\textsuperscript{2+} is due to introduction of positive charges to the N-terminal mature region of the precursor protein. The position-specific Ni\textsuperscript{2+} effects are difficult to explain in terms of nonspecific jamming of the translocation machinery, damage to the \( \Delta\mu\text{H}^{+} \) generating system by heavy metal ion, or the molecular size of the His\textsubscript{6}Ni\textsuperscript{2+} chelating complex. The fact that Ni\textsuperscript{2+} inhibition was observed only when the His\textsubscript{6} tag was positioned within the first 20 residues or so of the mature domain of pro-OmpA suggests that the chelating complex affects some specific event(s) where the translocase interacts with this particular N-terminal region of the precursor protein. Toxicity of positively charged amino acids in the N-terminal mature domain has been reported in several secre-
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Tory proteins in vivo and in vitro (32–35). Systematic insertion of Lys6 after the first or second transmembrane domain of leader peptidase indicates that the 30–40 amino acid residues following the signal sequence or the signal anchor sequence form a special domain that cannot tolerate the positive charges (31). Andersson and von Heijne (31) termed this region the translocation initiation domain. The location of the His6 tag that confers Ni2⁺-sensitive translocation on OmpA coincided well with the translocation initiation domain. The antagonistic cation initiation domain directly interacted with some part of the 9

found that pro-3His-OmpA was less sensitive to Ni2⁺ when INV prepared from the prlA3 mutant was used. 2 We suppose that Ni2⁺ affects translocation of N-terminally His6-tagged pro-OmpA through the step of precursor protein recognition by the SecY-SecE-SecG complex. A similar signal sequence recognition event by translocase is also proposed in the mammalian Sec61 system, as revealed in a reaction of the absence of signal recognition particle (40). Actually, translocation across the endoplasmic reticulum is also sensitive to positive charges in the N-terminal portion of the mature protein albeit its lower sensitivity compared with the prokaryotic system (41).

While signal sequence recognition by SecY could be regarded as essentially a proofreading activity that rejects nonfunctional precursor proteins (18), the fact that histidine addition can restore translocation without a short lag may indicate that a translocase-associated precursor can be reactivated on site, i.e. no rejection occurs on the membrane. We detected efficient cross-linking between pro-3His-OmpA on the membrane with SecA irrespective of the existence of Ni2⁺, 3 supporting this notion. Interaction of SecY(-SecE-SecG) with the signal sequence and the N-terminal portion of the mature protein may be required for some intrinsic mechanism of translocation, such as gate opening of the translocation channel (18, 40). It is interesting to point out that the prlA3 and other prl alleles that suppress the translocation defects caused by the basic amino acids (33) or the His6-Ni2⁺ conjugate (this study) reside in the first periplasmic domain of the SecY protein (18, 42). The idea that this region of SecY acts to accept or reject the early mature part (3) is reasonable in terms of topological consideration of SecY and preprotein; this SecY domain may recognize preprotein during or after the insertion of its hairpin loop structure composed of the signal sequence and the translocation initiation domain. But our results imply that the Ni2⁺-inhibited precursor can still remain associated with the membrane. On the other hand, the His6 tag portion of the Ni2⁺-trapped precursor on the membrane should not be completely buried in the translocation machinery. It is accessible to exogenous histidine added from the cytoplasmic surface. Either the intermediate-bearing translocation channel may be open to the cytoplasmic side, or this intermediate is in a fast equilibrium between a membrane-embedded state and a water-accessible state.

Since most translocation intermediate traps developed so far confer a translocation block at its middle or late stages (11–14), the His6-Ni2⁺-tagged precursor system is a novel tool for analyzing initial translocation events. It will be useful for investigating the nature of signal recognition by the SecY-SecE-SecG complex through in vitro analysis of translocon mutants, especially prlA mutants. Also in vivo characterization of the SecY mutants with lowered secretory efficiency (22) with the His6-Ni2⁺-tagged precursor system will be promising in identifying SecY mutants with a deficiency in the early events.

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