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Kinetic Analysis of the Translocation of Fluorescent Precursor Proteins into *Escherichia coli* Membrane Vesicles*

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Protein secretion in *Escherichia coli* is mediated by translocase, a multi-subunit membrane protein complex with SecA as ATP-driven motor protein and the SecYEG complex as translocation pore. A fluorescent assay was developed to facilitate kinetic studies of protein translocation. Single cysteine mutants of proOmpA were site-specific labeled with fluorescent dyes, and the SecA and ATP-dependent translocation into inner membrane vesicles and SecYEG proteoliposomes was monitored by means of protease accessibility and *in gel* fluorescent imaging. The translocation of fluorescently labeled proOmpA was largely independent on the position and the size of the fluorescent label (up to a size of 13–16 Å). A fluorophore at the +4 position blocked translocation, but inhibition was completely relieved in the PrlA4 mutant. The kinetics of translocation of the fluorescently labeled proOmpA could be directly monitored by means of fluorescence quenching. Inner membrane vesicles containing wild-type SecYEG were found to translocate proOmpA with a turnover of 4.5 molecules proOmpA/SecYEG complex/min and an apparent K_m of 180 nM, whereas the PrlA4 mutant showed an almost 10-fold increase in turnover rate and a 3-fold increase of the apparent K_m for proOmpA translocation.

Translocase is a membrane-bound enzyme complex that mediates the translocation across and integration of proteins into the cytoplasmic membrane of *Escherichia coli* (for a review see Ref. 1). Secretory proteins are synthesized in the cytosol as precursors with an amino-terminal signal sequence targeted to the membrane and subsequently transported across the membrane in an ATP-dependent manner via a proteinaceous channel formed by a heterotrimeric membrane protein complex composed of SecY, SecE, and SecG (2, 3). SecA is a soluble ATPase that associates with the SecYEG complex (4) where it serves both as a receptor for precursor proteins (5) and as an ATP-driven molecular motor (6, 7). Translocation is a stepwise process that involves cycles of ATP binding and hydrolysis by SecA and concomitant steps of precursor protein SecA association and dissociation. The precursor protein is threaded across the membrane in steps of ~2.5 kDa (7, 8), whereas translocation can be accelerated by the proton motive force (PMF)[1] (7, 9).

During the last decade, the biochemical mechanism of protein translocation has been studied in great detail. This advance has been possible attributed to the availability of a robust *in vitro* translocation assay. This assay is based on a method in which radioactively labeled precursor proteins are added to inner membrane vesicles (IMVs) in the presence of soluble components (SecA, SecB, and so on) and a suitable energy source (ATP). Precursor proteins are subsequently translocated into the lumen of the IMVs whereupon the membranes are treated with externally added proteinase to digest all nontranslocated substrate, whereas the translocated protein is inaccessible. The remaining protein is finally analyzed by SDS-PAGE and autoradiography. Normally, the substrate proteins are synthesized in a (coupled) transcription/translation system resulting in radiochemical amounts of protein. Alternatively, the precursor protein can be isolated from an overproducing strain, purified, and radiolabeled by means of iodination. Although the method is reliable, it is time-consuming, discontinuous, and produces radioactive waste. Because of the use of radiochemical amounts of precursor protein, the exact substrate concentration is often not known; therefore, reactions are frequently performed under non-saturating conditions. Especially for quantitative biochemical and kinetic studies, it is important to obtain exact estimates of initial rates. In the current era of proteomic studies, there is also an increasing demand for simple and rapid assays that can be automated. Such systems are in particular useful for high throughput drug-screening programs.

To overcome the problems associated with the classical protease accessibility assay, we set out to develop a real-time fluorescent protein translocation assay. Here, we show that fluorescently labeled precursor proteins are translocated into IMVs via an authentic mechanism as validated by a fluorescent variant of the classical translocation assay. Translocation of these fluorescent precursor proteins could be directly monitored by means of fluorescence quenching. This allowed a precise determination of the turnover rate and other kinetic parameters of protein translocation.

EXPERIMENTAL PROCEDURES

Materials—IMVs with overexpressed or wild-type levels of SecYEG were derived from *E. coli* strains KM9 or SF100 transformed with pET610 (10). SecA (11), His-tagged SecB (12), and SecYEG (3) were purified as described previously. SecYEG was reconstituted into liposomes of *E. coli* phospholipids (Avanti polar lipids, Alabaster, AL) by detergent dilution (3). Maleimide derivatives of fluorescein, Texas Red, Oregon Green, and Alexa Fluor 633 were from Molecular Probes (Eugene, OR).

Strains and Plasmids—A plasmid encoding proOmpA (C302S,proOmpA-Cys-290) (pET502) was constructed from pET147 (8)

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[1] The abbreviations used are: PMF proton motive force; IMVs, inner membrane vesicles; OG, Oregon Green; ΔpH transmembrane pH gradient; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate.

by site-directed mutagenesis. Cysteine-less proOmpA-(C290S,C302S) (pET2345) was constructed with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and used to introduce single cysteines at positions +4 and +33 of the mature OmpA, yielding pET2346 (D4C,proOmpA-Cys-4) and pET2348 (N33C,proOmpA-Cys-33). pET502 was used as template to construct the double cysteine mutant proOmpA (C302S,D291C,proOmpA-Cys-290,Cys-291) (pET2349).

Purification and Labeling of proOmpA—ProOmpA was purified as described previously (13). Prior to labeling, the proOmpA (3.5 mg/ml in 8 M urea, 50 mM Tris-HCl, pH 7.0) was reduced with 1 mM tris-(2-carboxyethyl)phosphine and incubated with 2 mM fluorescent maleimide at room temperature for 30 min. The reaction was quenched by the addition of 10 mM dithiothreitol, and the unreacted label was removed by gel filtration on a P6 spin column (Bio-Rad, Hercules, CA). The protein was collected by trichloroacetic acid precipitation and resuspended in 8 M urea, 50 mM Tris-HCl, pH 7.0. The labeling efficiency was determined spectroscopically. With Texas Red, double labeling of proOmpA (C302S,D291C) could be validated by a slower migration on SDS-PAGE of the double-labeled protein relative to the unlabeled or single labeled protein.

In Vitro Translocation and Fluorescent Imaging—Translocation of fluorescently labeled proOmpA into SecYEG proteoliposomes or inner membrane vesicles was assayed by the accessibility to added proteinase K (14). Reactions (final volume 50 μl) were performed in translocation buffer (50 mM Hepes-KOH, pH 7.5, 30 mM KCl, 0.5 mg/ml bovine serum albumin, 10 mM dithiothreitol, 2 mM $Mg(OAc)_2$ with 10 mM phosphocreatine, 50 μg/ml creatine kinase, 20 μg/ml SecA, 32 μg/ml SecB, 5 μg/ml proOmpA) started by the addition of 2 mM ATP, incubated for various time intervals at 37 °C, and stopped by chilling on ice. Translocation reactions were analyzed by proteinase K treatment (8) and SDS-PAGE and directly visualized *in gel* with a Roche Lumi-Imager F1 (Roche Molecular Biochemicals). Exposure was for 500 ms up to 1 s. For the imaging of fluorescein and Oregon Green, Texas Red, and Alexa Fluor 633, high pass filters were used with a cutoff at 520, 600, and 645 nm, respectively.

Direct Fluorescent Monitoring of in Vitro Protein Translocation—Translocation of OG-labeled proOmpA was assayed directly using an Aminco Bowman Series 2 spectrometer (SLM Instruments). Excitation and emission were set to 491 and 515 nm, respectively, with slit-widths of 4 nm. Translocation reaction mixtures in a thermostated microcuvette with a volume of 150 μl were preincubated for 3 min at 37 °C and started by the addition of 2 mM ATP.

Other Techniques—Protein concentrations were determined using the Bio-Rad DC protein assay with bovine serum albumin as a standard. The SecA ATPase activity was assayed as described previously (14). Western blots were developed with a monoclonal antibody against proOmpA (3). Scatchard analysis of the binding of SecA to IMVs was done essentially as described previously (5). The size of various fluorescent dyes was estimated after modeling of the chemicals using the HyperChem package (Hypercube Inc., Gainesville, FL) on a Silicon Graphics workstation.

RESULTS

Translocation of Fluorescently labeled proOmpA into IMVs—Wild-type proOmpA contains two cysteine residues at positions 290 and 302. To obtain a uniform fluorescently labeled proOmpA protein, a single cysteine mutant of proOmpA was created by replacing the cysteine at position 302 by a serine (proOmpA-Cys-290). In addition, cysteine at position 290 was replaced by a serine to obtain a cysteine-less proOmpA that was used to control the degree of nonspecific labeling. Under reducing conditions, Cys-less proOmpA and proOmpA-Cys-290 were translocated with an efficiency equal to wild-type proOmpA as assayed by immunoblotting (data not shown). With both mutant proOmpA proteins, the proOmpA-stimulated SecA translocation ATPase activity was indistinguishable from the wild type (data not shown). Subsequently, the cysteine mutants were labeled with a maleimide derivative of the fluorescent probe OG. Spectroscopic analysis showed that proOmpA-Cys-290 could be labeled with an efficiency of ~70% under conditions that there was only minor labeling of Cys-less proOmpA (Fig. 1*A*). This residual background level is probably because of the presence of some endogenous proOmpA that co-purifies with the overproduced Cys-less mutant. OG-labeled proOmpA-Cys-290 was used next in a translocation reaction with proteoliposomes containing purified SecYEG. Translocation was assayed by means of the protease protection assay, and protease-protected fragments were visualized by direct fluorescent imaging of the SDS-PAGE gel. The presence of protease-protected full-length fluorescent proOmpA was dependent on SecA, ATP (Fig. 1*B*), and the SecYEG complex (data not shown). Similar results were obtained with IMVs bearing wild-type or overexpression levels of SecYEG (Fig. 1*B*). In IMVs, a major fraction of the fluorescent proOmpA was processed by leader peptidase, yielding fluorescent mature OmpA (Fig. 1*B*). Taken together, these data demonstrate that OG-labeled proOmpA is translocated in an authentic Sec-dependent manner.

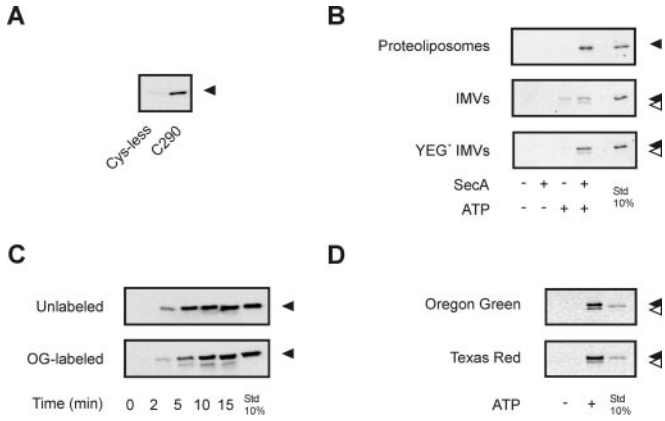

FIG. 1. **Fluorescent labeling and translocation of proOmpA.** *A*, Oregon Green labeling of Cys-less proOmpA and proOmpA-Cys-290. *B*, translocation of OG-labeled proOmpA-Cys-290 into SecYEG proteoliposomes and in urea-treated IMVs derived from wild-type and SecYEG-overproducing cells. Translocation reactions were performed in the presence or absence of SecA (10 μg/ml) and 2 mM ATP, *C*, translocation of unlabeled and OG-labeled proOmpA-Cys-290 into SecYEG-proteoliposomes detected by Western blotting using an anti-body against proOmpA. *D*, translocation of fluorescein, Texas Red, and Alexa Fluor 633-labeled proOmpA-Cys-290 into urea-treated IMVs containing overexpressed SecYEG. The *black* and *open arrows* indicate proOmpA and OmpA, respectively.

Translocation of Fluorescent proOmpA Is Not Impaired by the Size of the Dye—The size of OG (~10–13 Å) could possibly influence the kinetics of translocation. Therefore, the translocation kinetics of the fluorescent proOmpA was compared with the unlabeled proOmpA-Cys-290 by Western blotting. Both proteins were translocated into SecA-SecYEG proteoliposomes with similar kinetics (Fig. 1*C*), demonstrating that Oregon Green labeling does not significantly interfere with the translocation reaction.

To further examine possible size restrictions by the translocation pore, proOmpA-Cys-290 was labeled with fluorescent dyes of different sizes and translocation was assayed in time. Of the maleimide probes used (fluorescein, eosin, Alexa Fluor 488, Alexa Fluor 633, and Texas Red), Texas Red maleimide was the largest fluorescent molecule with a size of ~13–16 Å. However, Texas Red did not interfere with the kinetics of proOmpA translocation into SecYEG-proteoliposomes (data not shown) or SecYEG IMVs (Fig. 1*D*). Even double labeling of proOmpA with Texas Red on two consecutive cysteines (proOmpA-Cys-290,Cys-291) did not impair translocation (data not shown). These data demonstrate that the translocation pore is sufficiently wide or flexible to allow the translocation of proOmpA labeled with bulky fluorescent probes measuring up to at least 16 Å.

A Fluorophore at the +4 Position of proOmpA Interferes with Translocation—The fluorescent probe OG was attached to

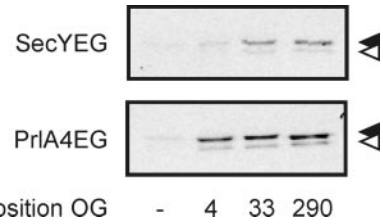

FIG. 2. **Translocation of Oregon Green-labeled proOmpA single Cys mutants into IMVs derived from cells overexpressing the SecYEG and PrlA4 complex.** The *black* and *open arrows* indicate proOmpA and OmpA, respectively. Membranes were treated with urea to inactivate the endogenous SecA.

other unique cysteine positions in proOmpA to determine whether there are positional restrictions. For this purpose, a unique cysteine was introduced at the +4 position from the signal sequence cleavage site (proOmpA-Cys-4) and at position +33 (proOmpA-Cys-33). These positions were chosen to map in loop regions of the OmpA structure to minimize any interference of the labeling in the formation of the β-sheet structure (15). Both cysteine mutants were efficiently translocated into IMVs (data not shown). However, after labeling with OG (Fig. 2*A*) or Texas Red, the presence of the fluorophore at position +4 strongly inhibited proOmpA translocation. On the other hand, OG did not interfere with translocation when the probe was bound to a cysteine at position +33 (Fig. 2*A*) or +66 (proOmpA-S66C).[2] Strikingly, when the translocation of OG-labeled proOmpA-Cys-4 mutant was assayed with IMVs bearing the overexpressed PrlA4 mutant (16), translocation was restored (Fig. 2). This demonstrates that PrlA4 not only suppresses secretion defects caused by a defective or missing signal sequence but that it also restores the translocation of defective precursor proteins with aberrant structural elements that are not part of the signal sequence.

Real-time Monitoring of the Translocation of Fluorescently Labeled proOmpA—To study the kinetics of protein translocation, a real-time spectroscopic assay was developed. Initially, the translocation of OG-labeled proOmpA-Cys-290 into IMVs containing overexpressed SecYEG was assayed in the presence of a membrane-impermeable quencher. For this purpose, *p*-xylene-bis-pyridinium bromide and potassium iodine were tested, because these compounds had no effect on the efficiency of protein translocation when used at a concentration of 40 and 60 mM, respectively[3] (data not shown). Translocation was initiated by the addition of ATP, and the fluorescence level was monitored in time. It was reasoned that if the fluorescence of OG-labeled proOmpA would be quenched at the outside of the vesicles, translocation of the proOmpA into the vesicles would result in an increase in fluorescence. However, after the initiation of translocation, instead of an increase, a progressive decrease in fluorescence was observed to ~15% of its initial value after 5 min. Identical results were obtained when fluorescein or Texas Red-labeled proOmpA was used (data not shown). Strikingly, in the absence of added quencher, a similar decrease in fluorescence was observed (Fig. 3*A*), suggesting that this phenomenon is caused by an intrinsic quenching process. This could either be self-quenching, resulting from the accumulation of precursor protein in the lumen of the IMVs, or be attributed to the presence of an intrinsic quencher that is associated with the IMVs. Therefore, further experiments were performed in the absence of added quencher. The decrease in fluorescence was dependent on SecA (data not shown) and hydrolyzable ATP (Fig. 3*C*) and was inhibited when a 100-fold excess of unlabeled proOmpA was added as a competitor (Fig. 3*B*). In the absence of nucleotide or in the presence of ADP or the non-hydrolyzable ATP analogue AMP-PNP, the fluorescence remained constant (Fig. 3*C*). The presence of an ATP-regenerating system, *i.e.* creatine kinase and creatine phosphate, stimulated protein translocation (Fig. 3, *A* and *C*) (17).

The addition of ATP to IMVs not only energizes SecA but also results in the generation of a PMF via the proton-pumping F_0F_1-ATPase. The transmembrane pH gradient (ΔpH) component of the PMF affects the luminal pH of the IMVs. Because the quantum yield of some fluorophores is pH-dependent, we had to ascertain that the observed quenching is not caused by an acidic pH in the lumen of the IMVs. Therefore, the translocation was assayed in the absence and presence of the ionophore nigericin (1.5 μM) to dissipate the ΔpH. With OG-labeled proOmpA, nigericin had no effect on the fluorescent signal. However, when fluorescein was used, the addition of nigericin resulted in a marked increase in fluorescence (data not shown). Nigericin had no effect on the decrease in fluorescence with fluorescein-labeled proOmpA when SecYEG overexpression IMVs were used derived from an *unc*$^-$ strain NN100 (18) or when urea-treated IMVs were used that are unable to generate a ΔpH by ATP hydrolysis. Therefore, with fluorescein, quenching is partially caused by a pH-dependent change in fluorescence quantum yield. This implies that the fluorescent proOmpA derivatives are indeed translocated into the lumen of the IMVs.

To examine whether the decrease in fluorescence was directly correlated to the amount of translocated proOmpA, samples were drawn from the fluorescence cuvette at different time points and further analyzed in a protease protection assay and *in gel* fluorescent imaging. The kinetics of the fluorescence decrease was paralleled by an increase in the amount of protease-protected proOmpA (Fig. 3*D*) showing that within this range a linear relation exists between the quenching of the fluorescent signal and the amount of translocated proOmpA. Similar results were obtained when instead of IMVs SecYEG proteoliposomes were used (data not shown). These data demonstrate that the fluorescent on-line assay can be used for a direct kinetic analysis.

Kinetics of proOmpA Translocation—To measure true kinetics of protein translocation, it is important that a saturating concentration of precursor protein is used and that initial rates conditions are assessed. Both requirements can be met by the real-time fluorescent assay; therefore, we set out to determine the turnover number of the translocase *in vitro*. The number of translocation sites was determined by Scatchard analysis of the binding of SecA to SecYEG IMVs. For further calculations, it was assumed that a single SecA dimer binds to one translocation site (formed by a SecYEG oligomer). For the IMVs bearing overexpressed SecYEG or PrlA4, the number of SecA binding sites was estimated to be ~80 and 35 pmol/mg IMVs, respectively. The rate of proOmpA translocation was determined over a wide range of proOmpA concentrations (0–0.9 μM) at a fixed IMV concentration and saturating concentrations of the other Sec components (Fig. 4*B*). With IMVs bearing the overexpressed SecYEG complex, the turnover appeared ~4.5 molecules of proOmpA/translocation site/min with an apparent K_m for proOmpA of 180 nM. A marked increase in turnover was observed with the PrlA4 mutant yielding a value of 40 molecules of proOmpA/translocation site/min and an apparent K_m for proOmpA of 420 nM. Remarkably, with PrlA4 IMVs, also a higher level of fluorescence quenching was obtained as compared with the wild-type SecYEG complex (Fig. 4*A*). This finding suggests that the PrlA4 mutant is capable of translocating a larger fraction of the added proOmpA possibly

[2] N. Nouwen, unpublished results.
[3] P. Natale, unpublished results.

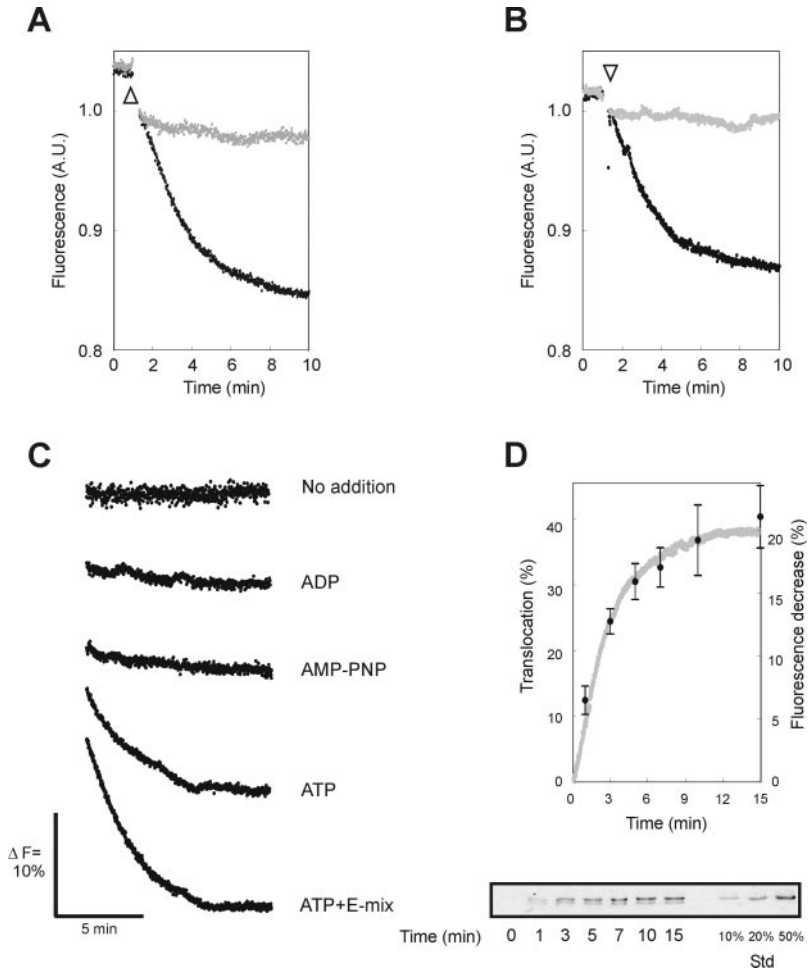

FIG. 3. **Real-time monitoring of proOmpA translocation.** *A*, OG-labeled proOmpA (4 μg/ml) was preincubated for 3 min at 37 °C in the presence of SecA, his-SecB, and IMVs containing overexpressed SecYEG. Translocation was started by the addition of ATP (2 mM) (*black line*) or translocation buffer (*gray line*). The initial drop in the fluorescence is because of dilution of the sample after the addition of ATP (indicated by *open arrow*). No corrections were applied. *B*, translocation of OG-labeled proOmpA (2 μg/ml) into IMVs in the presence (*gray*) or absence (*black*) of a 100-fold excess of unlabeled proOmpA. Translocation was started by the addition of 2 mM ATP (indicated by *open arrow*). *C*, translocation of proOmpA in the presence of ADP, AMP-PNP, ATP, and ATP with an ATP-regenerating system (*ATP+E-mix*) or in the absence of nucleotides. *D*, direct comparison of the translocation of proOmpA-Cys-290-OG as measured spectroscopically and by protease protection assay using immunoblotting for detection. Protease-protected proOmpA was quantified with OptiQuant 3.00 software (Packard Instrument Co.). The values are an average of three independent experiments.

including some proOmpA that is translocation-incompetent with the wild-type SecYEG complex. Taken together, these data demonstrate that the PrlA4 mutation enhances the turnover of the functional translocase.

Protein translocation is stimulated by the PMF. To study the effect of the PMF on the translocation rate, assays were performed in the absence and presence of the ionophores nigericin and valinomycin (both at 1.5 μM). Under these conditions, a complete loss of the PMF is obtained as confirmed by fluorescent ΔpH measurements using the dye 9-amino-6-chloro-2-methoxyacridine (data not shown). Dissipation of the PMF in SecYEG overexpression IMVs resulted in a >3-fold reduction of the turnover rate to 1.2 molecules of proOmpA/translocation site/min with no significant change in apparent K_m for proOmpA (Table I). Translocation in the PrlA4 mutant has been shown to occur largely independent of the PMF (19). Consistently, the turnover of PrlA4 IMVs was hardly affected by the dissipation of the PMF, yielding a value of 35 molecules of proOmpA/translocation site/min and an apparent K_m for proOmpA of ~500 nM (Fig. 4*B* and Table I).

With wild-type SecYEG overexpression IMVs, the apparent K_m values for SecA and ATP were determined for the translocation of OG-labeled proOmpA (Fig. 4, *C* and *D*). IMVs were treated with urea to inactivate SecA and other ATPases. Urea treatment results in a reduction of the turnover of the translocase from 4.5 to 0.5 proOmpA/translocation site/min, which is only partially caused by the loss of a PMF. The kinetic analysis revealed that for proOmpA translocation, the apparent K_m values for SecA and ATP are 50 nM and 45 μM, respectively (Table I).

DISCUSSION

The availability of an efficient *in vitro* protein translocation system has resulted in major advances in our understanding of bacterial protein secretion. Essentially, this reaction monitors the movement of a translocation-competent precursor protein into the lumen of the isolated inner membrane vesicles or proteoliposomes by their protection against treatment with an externally added protease. Protease-protected polypeptide fragments are visualized by Western blotting or autoradiography and, if necessary, quantified by densitometry. This method allows the detection of fully translocated precursor proteins, processed forms, and even of translocation intermediates of which only the translocated amino-terminal fragments become protected against protease treatment. Because the protease accessibility method is discontinuous, *i.e.* time points are taken at various intervals, kinetic studies are rather complex. In addition, the visualization is time-consuming (hours to days) and difficult to implement in high throughput drug-screening protocols or other robotics-driven technologies. Here, we show that when a fluorescently labeled proOmpA is used as substrate, protein translocation can be detected more rapidly and even followed in real time. Instead of radiolabeled fragments, the polypeptide bands can be readily detected by *in gel* fluorescence imaging with a sensitivity that is at least similar to that with radioactivity but requires only a fraction of the time (within a few min). Similar results have been obtained with the fluorescently labeled maltose-binding protein.[2] Therefore, the method appears generally applicable for studies on posttranslational translocation.

By direct spectroscopic monitoring, the translocation of Or-

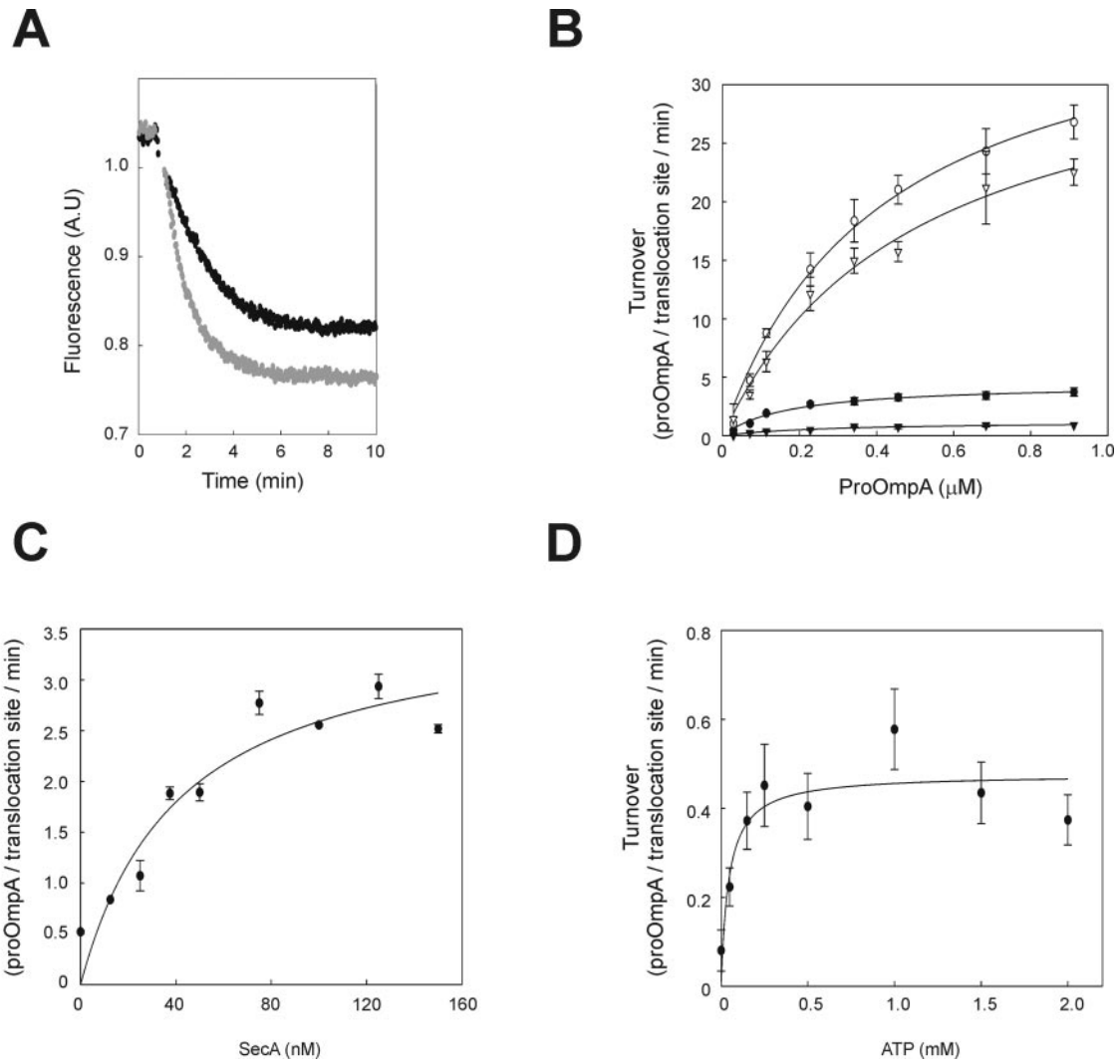

FIG. 4. **Kinetic analysis of proOmpA translocation.** *A*, translocation of OG-labeled proOmpA into IMVs containing overexpressed wild-type SecYEG (*solid*) or PrlA4 (*dashed*). *B*, initial rate of proOmpA in SecYEG (*black*) or PrlA4 (*white*) overexpression IMVs in the presence (○) or absence (△) of a PMF as a function of the proOmpA concentration. Translocation rates were measured at 0–0.9 μM proOmpA-Cys-290-OG, 0.25–1 μM SecB, 0.1 μM SecA, 2 mM ATP, and 0.1 mg/ml IMVs. *C*, initial rate of proOmpA translocation in SecYEG IMVs as a function of the SecA concentration. Rates were measured with 0.2 μM proOmpA-Cys-290-OG, 0.5 μM SecB, 2 mM ATP, and 50 μg/ml urea-treated SecYEG IMVs. *D*, initial rate of proOmpA translocation into SecYEG IMVs as a function of the ATP concentrations. Rates were determined with 0.1 μM proOmpA-Cys-290-OG, 0.25 μM SecB, 0.1 μM SecA, and 50 μg/ml urea-treated SecYEG IMVs.

TABLE I
Kinetics parameters of protein translocation

Parameters were determined under the conditions described in Fig. 4. Values determined in the presence of the ionophores nigericin/valinomycin are given within brackets.

	proOmpA		SecA	ATP
	Turnover[a]	Apparent K_m	Apparent K_m	Apparent K_m
	proOmpA/site/min	*nM*	*nM*	*μM*
SecYEG	4.5 (1.2)	180 (280)	50	45
PrlA4	40 (35)	420 (500)		

[a] Assuming a SecA dimer per translocation site.

egon Green-labeled proOmpA into the IMVs could also be followed in real time. This probe is rather insensitive to pH and ionic composition of the buffer solution. Conditions that specify an authentic SecA and ATP-dependent reaction yield a time-dependent decrease in the fluorescence, which corresponds to the translocation of the fluorescent precursor into the lumen of the vesicles. A similar phenomenon is observed when proOmpA is labeled with fluorescein or Texas Red. The observed quenching is 1) dependent on the presence of SecYEG, SecA, and hydrolyzable ATP; 2) competitively inhibited by an excess unlabeled proOmpA; and 3) the kinetics of the fluorescence decrease correspond to the kinetics of protein translocation as assayed by the classical protease-protection method. In the case of fluorescein-labeled proOmpA-(Cys-290), the fluorescence signal appeared dependent on the ΔpH. This effect, however, is caused by the acidic luminal pH that reduced the fluorescence quantum yield of translocated labeled precursor protein. Interestingly, this implies that the carboxyl-terminal region of the protein must have crossed the membrane. Therefore, we conclude that the decrease in fluorescence represents an authentic translocation reaction.

The exact mechanism of quenching of the fluorescence of the

precursor protein during translocation is unclear. The concentrative effect of the translocation reaction that accumulates the precursor protein into a small vesicle lumen could result in self-quenching. However, preliminary rapid mixing experiments suggest that the quenching is observed directly (within the first few seconds) after the onset of translocation, even in the case that the fluorophore is conjugated with a cysteine (Cys-290) in the COOH terminus. Alternatively, the phenomenon may be caused by the presence of intrinsic quenchers in the membrane, although it should be emphasized that the same reaction was observed with SecYEG proteoliposomes formed from isolated *E. coli* phospholipids. Another possibility is that the environment of the fluorescent probe changes when it is translocated, for instance, as a result of folding at the *trans*-side of the membrane after the release from the translocation pore. Because the fluorescence quenching is linearly related to the amount of proOmpA molecules translocated, the assay is quantitative and can be used to determine the kinetic parameters for the translocation. The turnover number for the wild-type SecYEG complex is ~4.5 molecules of proOmpA translocated/site/min, assuming that a translocation site corresponds to a high affinity binding site for the SecA dimer. The apparent K_m for proOmpA was 180 nM. The turnover number was reduced >3-fold upon the dissipation of the PMF (19), without significant change in the apparent K_m value. Strikingly, the PrlA4 mutant was found to be almost 10-fold more active as the wild type when saturated with proOmpA, whereas the apparent K_m for proOmpA was increased to 420 nM. The kinetic studies confirm that translocation by the PrlA4 mutant is barely affected by the dissipation of the PMF (19) and further demonstrate that the PrlA4 mutant is a highly active translocase. Further kinetic studies on proOmpA translocation showed that the apparent K_m for SecA is ~50 nM, which is ~3-fold higher as the K_d value reported for the binding of SecA to the SecYEG complex (13 nM) (20). The apparent K_m values for proOmpA is in the same range as reported for a chimeric protein containing the signal sequence of *E. coli* alkaline phosphatase and the mature portion of staphylococcal nuclease (570 ± 150 nM) as assessed by the precursor protein-stimulated translocation ATPase activity of SecA (21).

The SecYEG complex forms a protein conducting channel across the membrane, but the exact size of the pore is not known. Various structural studies have shed some light on the possible size limits of the pore. Negative stain electron microscopy and mass measurements with the scanning transmission electron microscope suggest that the SecYEG complex tetramerizes under translocation conditions to form a large ring-like structure with a central 4–5-nm wide stain-filled pit (3). In a recent blue native gel study, trapped precursor proteins were found to associate with a SecYEG dimer (22). Cryoelectronmicroscopy and three-dimensional reconstruction of layered two-dimensional crystals of the dimeric SecYEG complex indicate that this structure lacks a central opening (23). Therefore, the SecYEG dimer structure either represents a closed state of the translocase or a building block for the assembly of a tetrameric pore-like structure. The ribosome-attached Sec61p complex also forms a ringlike structure with a central opening of ~2 nm (24). It has been suggested that these rings accommodate a trimer of the Sec61p complex. Studies on the accessibility of a fluorescent probe that is attached to translocating polypeptide chains to quencher of various sizes (25) suggest that the channel is large, possibly 4–6 nm under translocation conditions. The pore of the active *E. coli* translocase may also be significantly larger than required for the translocation of an unfolded polypeptide chain. Although large folded proteins such as bovine pancreas trypsin inhibitor and dihydrofolate reductase block translocation (7, 26), a disulfide-bridged polypeptide loop up to 18 amino acid residues in proOmpA can still be transported across the membrane provided that a PMF is present (27). Our studies with fluorescently labeled proOmpA indicate that a bulky fluorescent dye such as Texas Red (size of 13–16 Å) is readily translocated. Even proOmpA labeled with Texas Red at two consecutive cysteines was tolerated, suggesting that the translocation pore must have a sufficiently wide flexible structure to allow a large variety of chemical groups, even in the absence of a proton motive force.

The kinetic data demonstrate that translocation is a rapid event. With the wild-type SecYEG complex, the turnover per SecA dimer is close to one precursor protein every 10 s. A single *E. coli* cell consists of ~2,350,000 proteins of which 33% is located in the inner membrane, periplasm, or outer membrane (28). In a cell that doubles every 40 min, approximately 20,000 proteins need to be transported across or inserted into the membrane/min (29). Assuming that ~50% of these proteins are indeed translocated (or membrane-inserted) via the SecYEG complex, the experimentally determined turnover number of 4.5 would require ~2,000 translocation sites. The number of SecY molecules in a cell has been only been estimated at ~500 (30), whereas the number of active translocation pores is presumably less as single pore consists of multiple SecYEG complexes. This would mean that the *in vitro* estimated turnover is insufficient to explain *in vivo* translocation. However, this value is certainly an underestimate. For instance, intact metabolizing cells maintain a much higher PMF (up to −200 mV) as compared with IMVs with ATP as energy source (approximately −80 mV), whereas other stimulatory factors such as SecDFyajC complex may further add to the activity. Moreover, the *in vitro* reaction is known to be uncoupled (9, 31) with unproductive translocation initiation events (32).

In conclusion, the use of fluorescently labeled precursor proteins allowed for the first time the real-time monitoring of protein translocation and a direct assessment of kinetic data. This novel method is generally applicable to other protein translocation system and provides new avenues to study protein translocation including single molecule spectroscopy and a high throughput automation of the translocation reaction.

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