Figure S1. Control Experiments for the Fluorescence Dequenching Transport Assay. Transport reactions were performed under the conditions of Figure 2D, except that one or more items were missing from the assay. For reference, the red curve ($\tau = 23\pm3\ s$, $N = 3$) in each panel represents the standard transport assay with everything included (proOmpA-HisC-Atto565, SecA, SecB, IMVs, succinate, ATP, and NiCl$_2$). The blue curve ($N = 2$) in each panel represents the assay performed in the absence of: (A) SecB and IMVs; (B) SecA and IMVs; (C) IMVs; (D) SecA; (E) SecA and SecB; and (F) proOmpA-HisC (the mature-length protein, OmpA-HisC, was used in place of the full-length precursor). These results indicate that SecA, SecB, IMVs, and the full-length precursor protein were all required to detect the large and rapid dequenching signal. The curves shown represent data averaged over the indicated number of experiments.
Figure S2. Effect of a Decreased SecYEG Level on the Fluorescence Dequenching Transport Assay.
The blue curve represents transport reactions performed under the conditions of Figure 2D with IMVs made from *E. coli* strain MC4100 (average of two experiments). For reference, the red curve is the same control data shown in Figure S1, where the IMVs were formed from MC4100 overexpressing SecYEG from the pET610 plasmid. These data indicate that the fluorescence dequenching observed under control conditions does not result from an interaction of the SecA/SecB/precursor complex with membranes.
Assumptions for the Dequenching Transport Assay

As outlined in the Dequenching Transport Assay section of the Results, true translocation kinetics are reported by the corrected fluorescence measurements if three conditions hold true. We now discuss the validity of these assumptions in detail.

Assumption 1: The precursor transport rate was identical in the presence and absence of Ni\(^{2+}\)

According to the gel-based assay, 5 \(\mu\)M NiCl\(_2\) decreases the proOmpA-HisC transport rate by \(\sim 27\%\) (Figure S3A). The fluorescence-based transport assay relies on accurate correction for the luminal concentration-dependent quenching effect. Thus, we determined whether the error introduced by the different luminal accumulation rates in the presence and absence of NiCl\(_2\) according to the gel-based assay is significant. The quenching kinetics observed in the absence of NiCl\(_2\) were fit to a two-step first-order kinetic model. The abscissa of the best fit was multiplied by 1.27, to correct for the 27% slower transport rate observed in the presence of 5 \(\mu\)M NiCl\(_2\). This corrected curve was then used to correct the dequenching kinetics observed in the presence of NiCl\(_2\). The shapes of the kinetic curves obtained by this more complicated correction procedure and by the simple subtraction procedure are similar, and both can be fit with a single exponential kinetic model (Figure S3B). The kinetic constants obtained are identical, within error (Figure S3B). Thus, we conclude that any errors introduced by small differences in the kinetics observed in the presence and absence of NiCl\(_2\) do not substantially change the observed shapes or kinetics of the corrected fluorescence curves.

Assumption 2: The dequenching reaction was rapid relative to the transport rate

Important here is the relationship between the rate constants \(k_1\) and \(k_2\) in the following possible reactions:

\[\text{(NiP)}_o \xrightarrow{k_1} \text{(NiP)}_i \xrightarrow{k_2} \text{Ni}^{2+} + \text{P}_i\]  \hspace{1cm} (Eq. S1a)

\[\text{(NiP)}_o \xrightarrow{k_1} \text{(NiP)}^* \xrightarrow{k_2} (\text{P})^* + \text{Ni}^{2+} \xrightarrow{k_3} \text{P}_i\]  \hspace{1cm} (Eq. S1b)

where \((\text{NiP})_o\) and \((\text{NiP})_i\) are the Ni\(^{2+}\) quenched precursor protein outside and inside the IMVs, respectively, \(\text{Ni}^{2+}_i\) and \(\text{P}_i\) are the free Ni\(^{2+}\) and the precursor in the IMV lumen, respectively, and \(\text{Ni}^{2+}_o\) is the free Ni\(^{2+}\) outside the IMVs. The first rate constant \((k_1)\) describes the translocation process until the point of Ni\(^{2+}\) dissociation, and the second rate constant \((k_2)\) describes the dissociation of Ni\(^{2+}\) from the 6xHis tag resulting in fluorescence dequenching. In Eq. S1a, it is assumed that the Ni\(^{2+}\) dissociates after translocation. For this model, the most likely scenario is that dissociation is governed by the spontaneous Ni\(^{2+}\) dissociation rate. The spontaneous Ni\(^{2+}\) dissociation rate constant was estimated by dilution of the Ni\(^{2+}\)-quenched precursor as \(\sim 0.01\ s^{-1}\) (\(\tau = \sim 100\ s\); Figure S3C). Since the fluorescence-based kinetics were all characterized by a \(\tau\) less than 60 s, the spontaneous Ni\(^{2+}\) dissociation rate of \(\tau = \sim 100\ s\) cannot determine
Figure S3. Effect of Ni$^{2+}$ on the Transport Kinetics and the Spontaneous Dissociation Rate. (A) Translocation kinetics in the presence and absence of NiCl$_2$. The gel-based assay (conditions of Figure 3A) was used to monitor the transport kinetics of proOmpA-HisC-Atto565 (50 nM) in the presence (red, $\tau = 28\pm1$ s, $N = 3$) and absence (blue, $\tau = 22\pm1$, $N = 3$) of 5 $\mu$M NiCl$_2$. The slower kinetics in the presence of NiCl$_2$ is consistent with a slight retardation of transport by a 6xHis-Ni$^{2+}$ chelation structure at the C-terminus of the precursor protein. Reactions were initiated by ATP addition. (B) Effect of differential transport rates in the presence and absence of NiCl$_2$ on the fluorescence-based assay. Using the reference data from Figure S1, the data were corrected as described in the text (Assumption 1). (black) single exponential fits; (red) average kinetics obtained by the normal correction procedure ($\tau = 23\pm3$ s; $N = 3$); (blue) average kinetics obtained when the abscissa of the 0 $\mu$M NiCl$_2$ quenching kinetics was multiplied by 1.27 ($\tau = 27\pm3$ s; $N = 3$). (C) The spontaneous Ni$^{2+}$ dissociation rate measured by dilution of NiCl$_2$. (red) The pre-formed SecA/SecB/precursor complex in 5 $\mu$M NiCl$_2$ (40 $\mu$L) was added to 760 $\mu$L of transport buffer in a stirred cuvette at the 50 s timepoint. The resultant kinetics ($\tau \approx 100$ s) reflect the increase in precursor fluorescence as Ni$^{2+}$ spontaneously dissociated from the precursor protein due to the decrease in NiCl$_2$ concentration resulting from the 20-fold dilution. (blue) The same experiment, except that no NiCl$_2$ was present in the SecA/SecB/precursor solution (unquenched fluorescence control). (green) The same experiment, except that 5 $\mu$M NiCl$_2$ was present in the dilution buffer (no dilution of Ni$^{2+}$ control). (arrowhead) Injection of 5 $\mu$M NiCl$_2$, demonstrating rebinding of NiCl$_2$ after dissociation upon dilution. (arrow) Injection of 10 mM EDTA, demonstrating that Ni$^{2+}$ chelation leads to dequenching. Concentrations after dilution: [proOmpA-HisC] = 2.5 nM; [SecA] = 10 nM; [SecB] = 0.4 $\mu$M. (D) Estimation of the translocation-catalyzed Ni$^{2+}$ dissociation rate. The data from (A) obtained in the presence of NiCl$_2$ were fit by a two-step first-order model, as described in the text. The best fit (shown) results when $k_1 = 0.045$ ($\tau = 22$ s) and $k_2 = 0.18$ ($\tau = 5.5$ s). The Ni$^{2+}$ dissociation rate is estimated by $k_2$. Considering the paucity of data points, these values cannot be considered reliable. However, the data do suggest that $k_1 \leq k_2$. 

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the observed fluorescence-based kinetics. It is possible, however, that the Ni\(^{2+}\) can dissociate significantly faster from the freshly translocated protein than from the SecA/SecB/precursor protein complex that we used in Figure S3C – however, we consider this possibility remote. In Eq. S1b, it is assumed that the Ni\(^{2+}\) dissociates before complete translocation, and the stars (*) indicate the translocation intermediates before and after Ni\(^{2+}\) dissociation, respectively. Considering the slow spontaneous Ni\(^{2+}\) dissociation rate and the discussion above, we consider the Eq. S1b situation more likely than the Eq. S1a scenario. Three situations are considered:

a) \(k_1 > k_2\):
   This condition implies that the observed kinetics are determined by the Ni\(^{2+}\) dissociation rate, which is much slower than the transport time. The fact that the fluorescence-based transport kinetics were never slower than the gel-based transport kinetics (Figure 3B) suggests that this is not the case.

b) \(k_1 \approx k_2\):
   In this case, the transport kinetics should be sigmoidal, e.g., like the case for \(n = 2\) in Figure 7. Considering all of our data and the fact that sometimes \(n = 2\) fits were reasonable (see Figure S8), this condition could have applied for some experiments (the transport kinetics and dequenching kinetics could both have been variable).

c) \(k_1 < k_2\):
   In this case, the observed kinetics are determined by the precursor transport rate, and hence, this is the ideal situation. If this case is true, the Ni\(^{2+}\) likely dissociated from the precursor protein at or within the time resolution of the experiments (1 s).

The relationship between the rate constants \(k_1\) and \(k_2\) can be estimated from the data provided in Figure S3A. Here, we make the assumption that a single rate constant \((k_1)\) describes the data obtained in the absence of NiCl\(_2\). Further, we assume that the data obtained in the presence of NiCl\(_2\) is described by a two-step first-order process where the first (translocation) step is described by the same rate constant \(k_1\) obtained in the absence of NiCl\(_2\) and the second (Ni\(^{2+}\) dissociation) step is described by \(k_2\). The result is \(k_1 = 0.045\) (\(\tau = 22\) s) and \(k_2 = 0.18\) (\(\tau = 5.5\) s) (Figure S3D). Thus, \(k_1 \leq k_2\), indicating that case (b) or (c) holds true. Our major conclusions remain the same whether (b) or (c) hold true. These data argue strongly that case (a) cannot be true.

Assumption 3: The two quenched states in the IMV lumen were identical in terms of fluorescence emission intensity (i.e., \(F_{Q1} = F_{Q2}\))

This condition must be true since the end of the black and red curves in Figure 2D end with the same fluorescence intensity. The end of the black curve represents the total fluorescence arising from a combination of two fluorescent precursor species: external precursor \((F_D)\), and precursor in the IMV lumen \((F_{Q1})\). The end of the red curve also represents the total fluorescence arising from a combination of two fluorescent precursor species: external precursor from which the Ni\(^{2+}\) has been completely
removed by EDTA ($F_D$), and precursor in the IMV lumen ($F_{Q2}$). The same proportion of each species exists in both cases since the same amount of precursor was transported under the two sets of conditions (Figure 4B). Thus, the total fluorescence intensity at 600 s in Figure 2D is given by $F_{\text{black}} = (1-\alpha)F_D + \alpha F_{Q1}$ and $F_{\text{red}} = (1-\alpha)F_D + \alpha F_{Q2}$ for the black and red curves, respectively, where $\alpha$ is the transport efficiency. By inspection, $F_{Q1} = F_{Q2}$.
Figure S4. Effects of IMV and Precursor Concentrations on Transport Efficiency and Transport Yield. (A) Effect of IMV concentration on transport efficiency. The transport efficiency of proOmpA-HisC-Atto565 (10 nM) was measured via gel-based transport assays (Figure 3A) at different IMV concentrations ($N = 3$). Reactions were initiated by IMV addition. These data indicate that the transport efficiency was largely independent of IMV concentration. (B) Transport yield as a function of precursor concentration. ProOmpA-HisC-Atto565 transport was measured via gel-based transport assays (Figure 3A). Reactions were quenched at an early time point (~20 s) to minimize the possibility of multiple turnover events ($N = 3$). These data indicate that the 10 nM precursor concentration typically used for fluorescence-based transport assays is substantially below the saturating precursor concentration. Higher precursor concentrations were not tested due to the difficulty of obtaining large quantities of fluorescently tagged precursors (see Materials and Methods). Reactions were initiated by ATP addition. [IMV] ($A_{280}$) = 0.5; [succinate] = 5 mM; [ATP] = 1 mM.
Effect of AMP-PNP on the PMF

As an ATP analog, AMP-PNP could potentially interfere with PMF generation by the F$_1$F$_0$ ATPase. This in turn could affect precursor transport rates. We examined this issue as follows. In the presence of an equimolar mixture of ATP and AMP-PNP, no effect on the $\Delta \psi$ was typically observed, compared with ATP alone (Figure S5A). In contrast, a small effect on the $\Delta \mathrm{pH}$ generation rate was typically observed (Figure S5B). To determine whether this difference in $\Delta \mathrm{pH}$ generation rate could explain the slower translocation rate observed in the presence of AMP-PNP, nigericin was added to slow $\Delta \mathrm{pH}$ generation in the presence of ATP alone. At 0.31 nM nigericin, the $\Delta \mathrm{pH}$ generation rate was similar to that observed in the presence of AMP-PNP (Figure S5B), and the magnitude of the $\Delta \psi$ was unaffected (Figure S5A). This nigericin concentration had no effect on proOmpA-HisC transport rate (Figure S5C). Therefore, the difference in transport rates observed in the presence and absence of AMP-PNP cannot be explained by changes in the PMF.

Figure S5. Effect of AMP-PNP on PMF Generation. (A and B) $\Delta \psi$ (A) and $\Delta \mathrm{pH}$ (B) generation in the presence of 0.25 mM ATP (blue), 0.25 mM ATP + 0.25 mM AMP-PNP (black) and 0.25 mM ATP + 0.31 nM nigericin (red). In all cases, gradient formation was initiated by succinate (5 mM) addition to IMVs ($A_{280} = 1.0$) in import buffer (−BSA) at 100 s. ATP/AMP-PNP was added at 130 s. This was the timing typically used for import reactions (Figure 2D). The partial gradient collapse that occurred at $\sim$320-350 s resulted from the consumption of the dissolved oxygen. Gradients were fully collapsed by 5 μM nigericin + 5 μM valinomycin (arrows). (C) The transport time of proOmpA-HisC-Atto565 observed under the conditions described in (A) and (B). ATP alone, $\tau = 31 \pm 4$ s; ATP + nigericin, $\tau = 31 \pm 1$ s; ATP + AMP-PNP, $\tau = 69 \pm 6$ s ($N = 3$).
Figure S6. AMP-PNP Competitively Inhibits ATP Binding During ProOmpA-HisC-Atto565 Transport. Transport reactions were performed with ATP + AMP-PNP (0.25 mM each, red, \( \tau = 70 \pm 1 \) s, \( N = 2 \)) and with ATP alone (0.25 mM, green, \( \tau = 17 \pm 1 \) s, \( N = 2 \)), as in Figure 6B. To test whether AMP-PNP could trap SecA in an "inserted" state, transport reactions were preincubated with 0.25 mM AMP-PNP for 40 s before the addition of 0.25 mM ATP (blue, \( \tau = 86 \pm 1 \) s, \( N = 2 \)). The slight deviation from single exponential kinetics when the transport reaction was preincubated with AMP-PNP suggests that release of AMP-PNP from the inhibited intermediate is slightly slower than ADP/ATP release.
Transport Kinetics in the Absence of a PMF

Tomkiewicz and coworkers (JBC 2006 281:15709) observed a length-dependent lag in the transport kinetics of various proOmpA-derived precursor proteins. Unlike the data in Figure 5B, their data was collected in the absence of a PMF, which does not reflect the normal in vivo situation. They used strain NN100, which lacks the FₐF₁ ATPase and therefore cannot generate a PMF from ATP by running this enzyme in reverse. If this is the only enzyme/enzyme system capable of generating a PMF using ATP, the same results should be obtained by using ionophores to collapse the PMF. We used this latter strategy. We found that transport could be ~90% inhibited by 2 μM ionophores (Figure S7A). These data indicate that the absence of a PMF strongly inhibits precursor translocation, and thus, it is unclear how Tomkiewicz and coworkers were able to observe high precursor translocation efficiencies if their IMVs were truly unable to generate and support a PMF, which they did not directly measure. We have recently reported that membrane potentials capable of driving protein translocation can be undetectable via common fluorescence-based methods to detect transmembrane gradients (Bageshwar & Musser JCB 2007 179:87), suggesting that small (potentially undetectable) gradients may be sufficient to assist with Sec translocation. When the transport rate was reduced by ~8-10-fold by decreasing the PMF with 0.5 μM ionophores (decreasing the transport efficiency about 2-fold – see Figure S7A), the kinetics of proOmpA transport were well-fit by a single exponential (Figure S7B). The lag phase observed by Tomkiewicz and coworkers was not observed. We currently cannot explain this discrepancy between the two data sets.

![Figure S7](image-url)

**Figure S7. Effect of the PMF on proOmpA-HisC Transport Efficiency and Transport Kinetics.**

(A) Gel-based transport assays of proOmpA-HisC-Atto565 in the presence of increasing equal concentrations of both valinomycin and nigericin (N = 3). Transport was ~90% inhibited by ≥ 2 μM of both ionophores. Reactions were initiated by IMV addition. [IMV] (A₂₈₀) = 1; [succinate] = 0 mM; [ATP] = 1 mM; [proOmpA] = 50 nM. (B) Transport kinetics when the PMF was reduced with ionophores. Gel-based assays were performed under the conditions of (A), except that the succinate concentration was 5 mM in the absence of ionophores. Reactions were initiated by ATP addition. (red) no ionophores (τ = 38±4 s, N = 3); (blue) 0.5 μM nigericin + 0.5 μM valinomycin (τ = ~300-400 s, N = 3).
Figure S8. Fitting the Transport Kinetics to an \( n \)-step, First-order Model (Equation 1). (A)-(E) Transport kinetics for precursors of different lengths. The data (black) are part of those used to generate Figure 5 (5 \( \mu \)M NiCl\(_2\)). For all of our data (shown and not shown), a single exponential (\( n = 1 \)) yielded a good fit, though it could be argued in some cases that the \( n = 2 \) fit was also reasonable. An \( n = 3 \) fit was always worse (e.g., Figure 7), even for the longest precursor for which a lag phase should have been most obvious (E). Baselines were allowed to float. For plotting purposes, \( y = 0 \) is defined by the \( n = 1 \) fit. (red) \( n = 1 \), (green) \( n = 2 \), (orange) \( n = 3 \) (only shown for (E)). (F) Fit error generated by discarding the first 3 s of data for proOmpA-HisC transport. The first three or four points (3-4 s) in the beginning of the fluorescence-based transport kinetics were typically discarded due to mixing artifacts. To address the possibility that this time period comprised the lag phase that is expected from Eq. 1, we assumed the worst, i.e., that the kinetics were flat during this time period. Shown is the fluorescence data from Figure 7 (black) assuming that the first 3 s of data are flat (\( y = 0 \)). Both \( n = 1 \) and \( n = 2 \) fits are approximately...
equally good, though clearly poorer than if the baseline is allowed to float (Figure 7). Thus, the absence of the early time points does not affect our conclusion that the transport kinetics are controlled by 1-2 rate-limiting step(s). (red) \( n = 1 \); (green) \( n = 2 \); (orange) \( n = 3 \). Berkeley Madonna\textsuperscript{®} was used for all fitting. Residuals and the root-mean-square deviations (RMSDs) for each fit are shown above the main figure.
Figure S9. Transport Time Dependence on Precursor Length. Total precursor length was plotted against the 5 μM NiCl₂ transport time (τ) obtained from Figure 5A. The dashed line is a linear fit to the data, with an x-intercept of 56 amino acids.