The Rate of Folding Dictates Substrate Secretion by the
Escherichia coli Hemolysin Type 1 Secretion System*§

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Secretion of the Escherichia coli toxin hemolysin A (HlyA) is catalyzed by the membrane protein complex HlyB-HlyD-TolC and requires a secretion sequence located within the last 60 amino acids of HlyA. The Hly translocator complex exports a variety of passenger proteins when fused N-terminal to this secretion sequence. However, not all fusions are secreted efficiently. Here, we demonstrate that the maltose binding protein (MalE) lacking its natural export signal and fused to the HlyA secretion signal is poorly secreted by the Hly system. We anticipated that folding kinetics might be limiting secretion, and we therefore introduced the “folding” mutation Y283D. Indeed this mutant fusion protein was secreted at a much higher level. This level was further enhanced by the introduction of a second MalE folding mutation (V8G or A276G). Secretion did not require the molecular chaperone SecB. Folding analysis revealed that all mutations reduced the refolding rate of the substrate, whereas the unfolding rate was unaffected. Thus, the efficiency of secretion by the Hly system is dictated by the folding rate of the substrate. Moreover, we demonstrate that fusion proteins defective in export can be engineered for secretion while still retaining function.

In Gram-negative bacteria, type 1 secretion systems (T1SS)§ export their cognate substrates in a single step directly from the cytosol to the extracellular medium without the formation of periplasmic substrate intermediates (1, 2). T1SS facilitate the secretion of structurally and functionally distinct proteins, which include pore-forming toxins like hemolysin A (HlyA), hemophores (HasA), adenylate cyclases, lipases, proteases, and surface layer proteins (1).

One of the best-studied T1SS is the hemolysin (Hly) secretion system of Escherichia coli. The Hly translocator consists of the inner membrane protein HlyB, which is an ATP-binding cassette transporter (3), the outer membrane protein TolC (4), and the membrane fusion protein HlyD anchored in the inner membrane (5). The interaction of the substrate HlyA with HlyB and HlyD triggers recruitment of TolC, thereby creating a continuous, but transient channel-tunnel from the cytosol directly into the extracellular medium (6, 7). Whereas the assembly of the Hly complex is nucleotide-independent, HlyA export requires ATP hydrolysis catalyzed by HlyB (7).

HlyA is a member of the RTX (repeats in toxin) protein family as it contains glycine-rich peptide repeats in the C-terminal domain (see Fig. 1A) (8). These repeats have the consensus sequence GGXGXGD (X represents any amino acid) and are important for the binding of Ca2+ ions (see Fig. 1A). This triggers folding of HlyA in the extracellular space, which in turn generates the biologically active form of the toxin. In the cytosol, where the concentration of free Ca2+ is extremely low (~300 nM) (9), folding of HlyA should not be possible. Toxin activity in addition requires the acylation of two specific lysine residues (see Fig. 1A), which is carried out by HlyC in the cytosol (10, 11).

The export of the 107-kDa toxin HlyA by E. coli into the medium is impressive in terms of its size and secreted amounts (12). Consequently, the Hly system has attracted great interest as an alternative secretion system for biotechnological applications (13). Bypassing the periplasm by targeting the synthesized proteins directly to the external medium has major advantages, such as circumventing cellular toxicity, less proteolysis, and the ease of purification of the exported protein (14, 15).

Most of the T1SS substrates of Gram-negative bacteria contain a translocation signal at the carboxyl terminus, and therefore, the process of secretion is assumed to occur post-translationally. The minimal secretion sequence of HlyA is located within the last ~60 C-terminal amino acids and is both necessary and sufficient to direct secretion (16–21). By fusing the C terminus of a given protein to the HlyA secretion sequence, it should, in principle, be possible to engineer proteins that are targeted to and secreted by the Hly system. Indeed, successful secretion of, for example, active forms of β-lactamase and antibodies such as scFv fused to HlyA, has been accomplished (12, 22–24). However, not all passenger proteins fused to the HlyA secretion sequence are efficiently secreted (12), which limits the biotechnological exploitation of this T1SS pathway.

RTX toxins like HlyA lack (or have few) cysteine residues and are thought to be largely unstructured in the cytosol (25, 26), with extracellular Ca2+ being important for folding (27). On the other hand, secretion of the hemoprotein HasA of Serratia marcescens, an atypical T1SS substrate because it...
lacks the calcium binding repeats, strictly depends on the molecular chaperone SecB (28, 29). This is the only known T1SS substrate that requires SecB for secretion. Moreover, it was shown that folded HasA in the cytoplasm inhibits its own secretion (28, 29).

Inspired by these findings and indirect evidence from various reports, we suggest that successful secretion of chimeric T1SS substrates may relate to the folding properties of the fusion protein. To investigate this in detail, the maltose binding protein MalE, lacking its own N-terminal signal sequence was chosen as a model substrate. To target MalE to the Hly system, we used a 218-amino acid C-terminal fragment of HlyA that harbors three calcium binding repeats (HlyAc). This HlyAc fragment exhibits high autonomous levels of secretion (see below and Ref. 10), is able to direct the secretion of a large variety of polypeptides (19, 24, 30), and seems to be more effective than shorter C-terminal fragments in directing secretion of large fusion partners (19).

The MalE-HlyAc fusion protein was highly expressed but was secreted at extremely low levels. In striking contrast, introduction of mutations that substantially reduce the rate of folding of the fusion protein yielded chimeric proteins that were secreted in large amounts. Secretion of the slow folding MalE-HlyAc variants did not require SecB. Our studies demonstrate that the folding rate of the substrate determines the amount of secretion and that chimeric proteins defective in export can be engineered for successful secretion. We propose a model in which a slow folding rate for the substrate in addition to the specific C-terminal secretion sequence is essential for secretion of T1SS substrates.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—The *E. coli* strain WM2429 used in this study for expression and secretion analysis is a BW25113 derivative (31) that lacks the *araBAD* genes and is specifically designed for arabinose-dependent induction systems. The *E. coli* strain XL1 (Stratagene) was used for cloning procedures. The expression plasmid pBADHisB was obtained from Invitrogen, whereas the compatible expression plasmid pK184-BspHI is a modified pK184 plasmid containing a tobramycin resistance cassette (supplemental Fig. S1). In this manner, MalE-HlyAc fusion proteins with an N-terminal His$_6$-tag followed by a tobacco etch virus protease cleavage site and C-terminal factor Xa cleavage site were generated. Using the appropriate primer pairs, the V8G, A276G, and Y283D mutations were introduced in MalE-HlyAc.

**Protein Expression and Secretion**—For tryptophan fluorescence studies, HlyAc fusion proteins were expressed in the absence of the secretion apparatus and purified from inclusion bodies. An overnight culture was used to inoculate 2× YT medium at a *A$_{600}$* of 0.05, and cells were grown with agitation at 37 °C. Protein expression was induced at an *A$_{600}$* of 0.8 by adding arabinose to a final concentration of 10 mM. Cells were harvested after a further 5 h of growth. For secretion experiments, cells were grown in LB medium containing isopropyl 1-thio-β-d-galactopyranoside at a final concentration of 1.5 mM for the production of the inner membrane proteins HlyB and HlyD. Cells were grown with agitation at 30 °C to an *A$_{600}$* of 0.8, and production of the HlyAc fusion proteins was induced by the addition of arabinose to a final concentration of 10 mM. Secretion experiments using cells lacking SecB (*E. coli* MC4100 ΔsecB::Cm$^R$) were carried out in an identical fashion. Total cell extracts or culture supernatants were concentrated by precipitation with 10% (v/v) TCA where indicated and analyzed by SDS-PAGE and immunoblotting. Proteins were stained with Coomassie Brilliant Blue (CBB) or probed by immunoblotting using antibodies raised against purified HlyA in rabbit. For immunodetection, the secondary HRP-conjugate antibody was used in conjunction with the ECL advance kit (GE Healthcare).

**Protein Purification**—Harvested cells were suspended in buffer A (50 mM Hepes-KOH, pH 7.6, 150 mM NaCl, and 5 mM urea) and lysed by sonication, and inclusion bodies were dissolved by gently stirring the lysate for 2 h. Undissolved protein was removed by centrifugation (50,000 × g at 4 °C for 10 min) and 1 ml of nickel-nitritriacetic acid resin (Qiagen, Hilden) was added to 10 ml of the cleared cell lysate. For purification, the suspension was mixed with resin and cast in a column. This was washed three times with 5 column volumes of buffer A containing 4 mM urea and 5 mM imidazole to remove nonspecifically bound protein. The fusion proteins were eluted with buffer A containing 4 mM urea and 200 mM imidazole.

**Folding Experiments**—Folding was monitored by changes in intrinsic tryptophan fluorescence as described in Ref. 35 using a Jobin-Ivon Horiba Fluorolog-3 fluorescence spectrophotometer. All measurements were performed at 30 °C. Excitation was performed at 295 nm, and fluorescence emission was monitored at 350 nm with slit widths set to 2 and 5 nm, re-
respectively. To avoid photobleaching at long recording times (>1000 s), the shutter was closed between single data point acquisitions. Native protein (supplemental Fig. S2) was obtained by removal of urea by extensive dialysis against ice cold 20 mM Hepes-KOH, pH 7.6. For unfolding, reactions were initiated by adding native protein to a solution of 20 mM Hepes-KOH, pH 7.6, containing different concentrations of urea in a stirred cuvette. The time between protein addition and the first recording of fluorescence intensity was ~5 s. For refolding, native fusion protein was first unfolded by incubation overnight in 20 mM Hepes-KOH, pH 7.6, containing 5 mM urea. Refolding was initiated by rapidly diluting the unfolded protein into a solution of 20 mM Hepes-KOH, pH 7.6, containing urea at various concentrations. In all cases, the final protein concentration in the cuvette was 60 nM. Folding experiments on the MalE core proteins were carried out in a similar fashion. The relaxation time required to achieve a new equilibrium for the kinetic folding studies was determined by plotting the change of intrinsic tryptophan fluorescence against time and fitting the data according to Equation 1 for folding and Equation 2 for unfolding. All fitting procedures were performed using Prism (GraphPad, Inc). The relaxation time \( \tau \) is defined by Equation 2.

\[
F_{\text{obs}} = F_0 + (F_\infty - F_0) \times (1 - e^{-t/\tau}) \quad (\text{Eq. 1})
\]

\[
F_{\text{obs}} = F_\infty + (F_0 - F_\infty) \times e^{-t/\tau} \quad (\text{Eq. 2})
\]

Here, \( F_{\text{obs}} \) is the fluorescence observed at a certain time point, \( F_0 \) is the fluorescence observed at time 0, \( F_\infty \) is the fluorescence at infinite time, and \( k \) is the rate constant.

\[
k = \frac{1}{\tau} \quad (\text{Eq. 3})
\]

RESULTS

Construction of a Dual Plasmid-based System for Expression and Secretion of HlyA Fusion Proteins—The commonly used laboratory strains of \textit{E. coli} such as K12 lack the hlyCABD genes. Therefore, we designed a suitable set of vectors to study the secretion of substrate proteins via the Hly T1SS. We employed a dual vector setup, where one plasmid, pK184 (36), encodes the inner membrane components HlyB and HlyD under the control of the lacZ promoter, whereas the second plasmid pSOI-HlyAc (a pBAD derivative), encodes a 23-kDa C-terminal fragment of HlyA containing the secretion signal. The pSOI-HlyAc plasmid contains sequences that enable efficient and easy high throughput ligation-independent cloning (33) of fusion partners in frame with the HlyAc fragment (Fig. 1B). The fusion protein is expressed from an arabinose-inducible promoter.

When only the HlyAc fragment was produced, large amounts were secreted into the medium (Figs. 1C, right panel, and 3A), indicating that the HlyBD transport components were functional. Only a minor amount of HlyAc remained associated with the cells (supplemental Fig. S5A, left panels). These results are in agreement with previous data reporting a high autonomous level of secretion for this 218-amino acid C-terminal domain of HlyA (10). In our setup, depending on the expression conditions and the medium composition, yields of secreted HlyAc were as high as ~100 mg protein per liter of culture corresponding to ~7 mg/A600 of cell culture (Fig. 1C).

Mutations That Affect Folding of MalE Facilitate Secretion via Hly System—The Hly T1SS is able to export a large variety of proteins that are C-terminally fused to a fragment containing the HlyA secretion sequence (12, 22–24, 37). However, not all passenger proteins are secreted efficiently (12, 38). To investigate whether folding interferes with secretion, we used the well characterized maltose binding protein (MalE) as a passenger protein. In vivo, MalE is produced as a preprotein containing an N-terminal signal sequence required for targeting to the Sec translocon (39, 40). The signal sequence, in addition, retards folding allowing the molecular chaperone SecB to bind and keep MalE in a conformational state that is competent for export to the periplasm. Interestingly, defects in the signal sequence can be suppressed by intragenic mutations that reduce the folding rate of MalE (41). We introduced the previously described “slow folding” suppressor mutations V8G, A276G, and Y283D in MalE (lacking its natural signal sequence) and investigated the secretion of these MalE variants when fused to the HlyAc fragment (Fig. 2A). Analysis of total cell extracts revealed identical cellular amounts of the fusion proteins (supplemental Fig. S5). As judged by SDS-PAGE and CBB staining (Fig. 2A, left panel) and immunoblotting (Fig. 2A, right panel) the wild-type fusion protein is poorly secreted by the Hly system. The MalE mutants on the
Slow Substrate Folding Facilitates Type 1 Secretion

FIGURE 2. Secretion and folding of the MalE(WT)-HlyAc fusion protein and its variants. A, cells containing pK184-HlyBD and a plasmid encoding MalE-HlyAc fusion protein as indicated were grown in LB medium supplemented with 1.5 mM isopropyl-1-thio-β-d-galactopyranoside to induce hlyBD coexpression. At A600 = 0.8, the production of the MalE-HlyAc fusion proteins was induced by the addition of arabinose to a final concentration of 10 mM arabinose. Five hours after induction, protein in the culture supernatants was TCA-precipitated and analyzed by SDS-PAGE with CBB staining (left panel). B, shown is a chevron plot displaying relaxation times for the refolding and unfolding of the MalE-HlyAc fusion proteins at different urea concentrations. Relaxation times for refolding (closed symbols) and unfolding (open symbols) of wild-type (circles), V8G (triangles), A276G (diamonds), and Y283D (squares) fusion protein are indicated. The intersection of the dashed and solid lines with the y axis indicate the relaxation time for unfolding and refolding at zero denaturant concentration, respectively (see also Table 1).

TABLE 1

| Protein        | Refolding τ (s) | Unfolding τ (s) |
|----------------|-----------------|-----------------|
| MalE(WT)       | 26.3 ± 4.9      | 372 ± 0.7       |
| MalE(V8G)      | 249.4 ± 14.6    | 372 ± 0.7       |
| MalE(A276G)    | 549.8 ± 88.4    | 372 ± 0.7       |
| MalE(Y283D)    | 6206 ± 824      | 372 ± 0.7       |

Note: τ values are extrapolated to a urea concentration of zero. The relaxation time for unfolding of the MalE(WT)-HlyAc fusion protein was 3.72 ± 0.7 s, and the relaxation time for unfolding of MalE(A276G)-HlyAc was 249.4 ± 14.6 s. The relaxation time for unfolding of MalE(Y283D)-HlyAc was 6206 ± 824 s.

other hand were all secreted at a higher level. Notably, the MalE(Y283D)-HlyAc fusion was secreted best, at levels that were readily detected on a CBB-stained polyacrylamide gel (Fig. 2A).

Slow Folding of MalE-HlyAc Fusion Protein Facilitates Secretion—The point mutations V8G, A276G, and Y283D are known to reduce the rate of folding of MalE, whereas the rate of unfolding remains unchanged (35). Here, we investigated whether these mutations affected also the folding of the MalE-HlyAc fusion protein. We used time-resolved intrinsic tryptophan fluorescence spectroscopy to determine the kinetics of both refolding and unfolding of the MalE-HlyAc fusion protein and its variants. First, the kinetics of spontaneous refolding were determined. For this, purified fusion protein was completely unfolded by incubation overnight in 5 M urea. Unfolded protein was then rapidly diluted into buffer containing various concentrations of urea, and the change in tryptophan fluorescence was measured over time. From the kinetic traces obtained, the relaxation time (τ) for refolding was determined (see “Experimental Procedures”) and plotted against urea concentration (Fig. 2B, closed symbols). At all urea concentrations tested, the wild-type fusion protein folded the fastest. For MalE(WT)-HlyAc extrapolation to a urea concentration of zero revealed a relaxation time for refolding of 26.3 ± 4.9 s (Fig. 2B and Table 1), which is nearly identical to the 28 s reported for MalE carrying its natural signal sequence (42). The mutant fusion proteins V8G, A276G, and Y283D on the other hand, folded markedly slower with respective relaxation times of 249.4 ± 14.6 s, 549.8 ± 88.4 s, and 6206 ± 824 s (Fig. 2B and Table 1). Note that MalE(Y283D)-HlyAc, which folds extremely slowly, was secreted the best by the Hly system (Fig. 2A).

To determine the kinetics of unfolding, the native fusion proteins were rapidly diluted into buffer containing various concentrations of urea. The change in tryptophan fluorescence was measured over time, and the relaxation times (τ) for unfolding were calculated (see “Experimental Procedures”). The wild-type and mutant fusion proteins showed nearly identical unfolding behavior (Fig. 2B, open symbols). Extrapolation to a urea concentration of zero revealed a relaxation time for unfolding of 3.72 × 10^4 s for MalE(WT)-HlyAc and its variants (Fig. 2B and Table 1). From these data, it can be concluded that it is the rate of refolding rather than the rate of unfolding that dictates the secretion of the fusion proteins. Moreover, the velocities of refolding for the fusion proteins follow the order: wild-type > V8G > A276G > Y283D, which inversely correlates with their amounts secreted, i.e. a
slower refolding rate relates to higher levels of secreted protein (Table 1 and Fig. 2).

To study whether the HlyAc domain affects the folding of the fusion proteins, folding experiments were also carried out on the MalE core domain. For this, fusion proteins were treated with factor Xa to remove the HlyAc domain (Fig. 1B). Purified wild-type and mutant MalE core domains were then subjected to the same set of folding experiments as described above. Relaxation times were plotted against urea concentration (supplemental Fig. S3) and extrapolated to zero denaturant concentration (Table 1). The MalE(WT) core domain exhibits a relaxation time for refolding of about 19.2 ± 5.5 s, indicating that it folds marginally faster than the MalE(WT)-HlyAc fusion protein. For the mutant MalE core domains, however, a pronounced effect is observed. Compared with the corresponding fusion protein, the V8G and A276G MalE core domain folded ~5-fold faster, whereas the Y283D core domain folded ~10-fold faster (Table 1). The core domains of the wild-type, Y283D, and A276G showed nearly identical unfolding behavior (supplemental Fig. S3). Extrapolation to a urea concentration of zero reveals a relaxation time for unfolding of ~7.2 × 10^5 s, which is 2-fold slower as compared with the corresponding fusion proteins. Thus, the presence of the HlyAc domain seems to have a somewhat destabilizing effect on the MalE domain. The HlyAc domain does not, however, seem to have an effect on the slow folding mutant MalE(Y283D). Taken together, the data suggest that the HlyAc domain reduces the rate of folding of the MalE domain.

Introduction of a Second Slow Folding Mutation in MalE(Y283D)-HlyAc Further Enhances Secretion—Above, it was shown that slow folding variants of MalE-HlyAc fusion protein are secreted at a higher level than the wild-type fusion protein. We therefore argued that the introduction of a second mutation that further reduces the rate of folding may lead to enhanced secretion. To test this hypothesis, MalE-HlyAc fusions with the double mutations V8G/A276G, V8G/Y283D, and A276G/Y283D were generated. Indeed, as compared with the well secreted, slow folding single mutant Y283D, the double mutants V8G/Y283D and A276G/Y283D were secreted at an even higher level (Figs. 2A and 3A). Importantly, under identical experimental conditions, the secreted amount of MalE(V8G/Y283D)-HlyAc was very similar to that of the HlyAc fragment alone (Fig. 3A). Quantification based on comparison with known amounts of BSA indicates that under these conditions up to 15 mg of MalE(V8G/Y283D)-HlyAc were secreted per liter of culture, which corresponds to ~3 mg/A of cell culture (supplemental Fig. S4). As expected, control experiments showed that secretion was strictly dependent on the presence of the membrane components of the translocator, HlyB and HlyD (supplemental Fig. S4). Finally, the level of secretion of the various MalE-HlyAc fusion proteins was ranked according to the amounts of fusion protein detected in the medium: wild-type < V8G < A276G and V8G/A276G < Y283D < A276G/Y283D < V8G/Y283D (Figs. 2A and 3A). We note that refolding experiments with the double mutants were also attempted; however, due to very slow folding, especially in case of the V8G/Y283D and A276G/Y283D mutants, it was not possible to obtain quantitative data.

Secreted MalE(V8G/Y283D)-HlyAc Retains Its Functionality—In view of the slow folding of the fusion proteins containing the double mutations the question arose whether they are able to fold to the native state following secretion. This was analyzed by determining the ability of the
Secretion of the MalE-HlyAc fusion proteins by cells lacking SecB. E. coli MC4100 ΔsecB::Cm<sup>+</sup> cells containing pK184-HlyBD and a plasmid encoding the MalE-HlyAc fusion protein or the HlyAc fragment were grown in LB medium supplemented with 1.5 mM isopropyl 1-thio-galactopyranoside for hlyBD coexpression. At A<sub>600 </sub> ~ 0.8, the production of HlyAc and the different MalE-HlyAc fusion proteins as indicated was induced by the addition of 10 mM arabinose. Before (−) and after induction, at the time points indicated, protein in the culture supernatants was TCA-precipitated and analyzed by immunoblotting using HlyA-specific polyclonal antibodies.

Secretion of MalE-HlyAc Fusion Proteins Does Not Require SecB—MalE translocation into the periplasm of E. coli is adversely affected when the molecular chaperone SecB is lacking (43, 44). The N-terminal signal sequence of MalE retards folding enabling efficient SecB binding. SecB keeps preproteins in a translocation-competent state and improves the delivery to the Sec translocon (45, 46). MalE devoid of its signal sequence on the other hand still interacts with SecB but is more rapidly released due to faster folding (47). To determine whether SecB plays a role in the secretion of the MalE-HlyAc fusion proteins, the Hly translocator components HlyB and HlyD were coexpressed together with the different fusions in E. coli MC4100 ΔsecB::Cm<sup>+</sup> (32). Notably, both the HlyAc fragment and the slow folding MalE-HlyAc fusion proteins were still secreted by the secB mutant indicating that SecB is not required for their export (Fig. 4). Moreover, compared with the wild-type fusion protein, the fusions containing the Y283D mutation were secreted at a higher level, whereas MalE(V8G/Y283D)-HlyAc was secreted the best. These data further support the view that only slow folding substrates are exported by the Hly system.

**DISCUSSION**

Type 1 secretion systems of Gram-negative bacteria catalyze the secretion of their dedicated substrates in a single step across the inner and outer membrane. As such, these T1SS are attractive candidates for high level expression and secretion of heterologous polypeptides of biotechnological and biomedical interest (12, 22, 23). Currently, the most important application of the Hly system is the presentation of heterologous antigens by attenuated Gram-negative bacteria in live vaccines (48). Another T1SS that has been successfully exploited is the export machinery of the S-layer protein RsaA of *Caulobacter crescentus*, producing >50 mg/l of fusion protein (49).

However, despite our current knowledge of the general architecture and basic molecular principles of T1SS, the secretion process itself is poorly understood. Previous attempts to target fusion proteins for secretion by T1SS were largely based on a “trial and error” approach. Thus, published data indicates that some fusion proteins fail to be secreted or are secreted poorly by the T1SS (38, 50). For instance, chloramphenicol acetyltransferase or dihydrofolate reductase are not secreted when fused to the HlyA C-terminal domain. In contrast, truncated versions of chloramphenicol acetyltransferase (19) or dihydrofolate reductase containing the point mutation I155K (38) are secreted by the Hly system. This indicates that for some passenger proteins, the presence of the HlyA secretion signal is not sufficient to direct export, and other factors may be limiting for efficient secretion. We reasoned that these observations point to a role for folding in the Hly type 1 secretory pathway, where folding of the substrate might inhibit secretion by occluding access to the Hly translocator.

Here, the influence of the velocity of folding of the substrate protein on the level of secretion by the Hly T1SS was analyzed. For this, the maltose binding protein, MalE (47, 51–54) was chosen as a model substrate, and three MalE variants were selected that exhibit a slower folding rate as compared with the wild-type, whereas the rate of unfolding is not affected (35). Based on these MalE variants, a set of MalE-HlyAc fusion proteins was generated and analyzed with regard to the folding properties and “secretability.”

The wild-type MalE-HlyAc fusion protein is virtually not secreted by the Hly system, whereas the MalE(Y283D)-HlyAc fusion, which folds ~240-fold slower than the wild-type, is secreted at high level (Fig. 2). Secretion of the fusion protein could be enhanced by the introduction of a second mutation that further reduced the folding rate. As demonstrated for MalE(V8G/Y283D)-HlyAc, secretion of MalE, which normally is localized in the periplasm, can be effectively directed to the extracellular medium using the Hly system, yielding up to 15 mg of secreted protein per liter of culture (supplemental Fig. S4). Because the rate of unfolding of the fusion protein was not affected by the V8G, A276G, or Y283D mutations, it is evidently the rate of folding that directly relates to the efficiency of secretion, i.e. substrates with a slow folding rate were secreted at a higher level (Table 1 and Figs. 2–4). Moreover, we demonstrated that although the introduction of specific point mutations (that reduce the folding rate of the passenger) can permit secretion, the passenger protein is still able to fold to the active form (Fig. 3B).

Because the translocation of pre-MalE in vivo depends on the molecular chaperone SecB (55–57), we considered the...
possibility that SecB may play a role in the secretion of the MalE-HlyAc fusion proteins. However, the slow folding MalE-HlyAc variants, in particular the ones carrying the Y283D mutation, were all secreted by the ΔsecB strain (Fig. 4), indicating that SecB is not required for their secretion. If available, SecB would of course be expected to bind to the MalE domain of the fusions to slow folding and therefore assist secretion. Indeed, we observed that the level of secretion of the MalE-HlyAc fusions was generally reduced when SecB is absent. Nevertheless, for the slow folding mutant secretion is largely independent of SecB.

The small HasA protein (19.3 kDa) secreted by S. marcescens, unlike other T1SS transport substrates, has no RTX repeats but requires SecB for secretion. Interestingly, variants of HasA containing two mutations that reduced the rate of folding, permitted secretion by the Has system essentially independently of SecB (58). This suggests that normally SecB slows folding of HasA sufficiently to allow productive interaction with the T1SS system. Thus, although the Hly and Has systems secrete structurally and functionally very distinct substrates, it appears that they both only engage and translocate slow folding or (largely) unfolded substrates.

Our data indicate that the presence of the C-terminal HlyAc domain in these cases further slows down the folding of the MalE domain. Although, in general, individual protein domains are thought to fold independently, examples of an interdependence of domains during folding have been reported. In the case of the MalE wild-type precursor protein, the presence of the natural N-terminal signal sequence decreases the folding rate of the downstream domain by a factor of ~40 (42). Our observations therefore offer a reasonable explanation as to why certain passenger proteins are secreted better when fused to an HlyA C-terminal fragment that is extended substantially beyond the minimal secretion sequence (19). A larger HlyAc fusion domain may reduce the speed of folding of the fusion partner, thereby facilitating secretion by the Hly system.

Based on our data presented here, we propose a model for T1SS. Following the synthesis of the wild-type MalE-HlyAc fusion protein, the MalE domain is still able to fold rapidly despite the presence of the 23-kDa HlyA domain, and presumably this occurs before the HlyA secretion sequence can engage the Hly transport complex (and cannot be subsequently unfolded). We observed that for a MalE-HlyAc fusion protein carrying the Y283D mutation the overall rate of folding, including the effect of the HlyA domain (which contains three RTX repeats) is sufficiently reduced to permit efficient docking with and subsequent secretion by the Hly complex. Therefore, competition between folding and transport dictates secretion.

Finally, as it appears that the Hly system is incapable of secreting substrates that fold rapidly in the cytoplasm, our data may help explain why dedicated substrates of T1SS are predominantly RTX toxins. The lack of Ca\(^{2+}\) in the cytosol keeps them unfolded or in a state of very slow folding, whereas extracellular binding of Ca\(^{2+}\) greatly accelerates folding to the native biologically active state. This raises the interesting question how the 107-kDa HlyA can exist in the cytoplasm in an unfolded conformation normally expected to be susceptible to proteolytic degradation. In contrast to HasA, secretion of HlyA has been reported to be independent of SecB, whereas a possible protective role by other molecular chaperones was not excluded (1).

In conclusion, our data demonstrate that folding of a T1SS substrate rules secretion, with the secretion signal being necessary but not sufficient for translocation. This principle could be exploited to engineer fusion proteins on a rational level and in addition be tested for other T1SS.

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