ty of the reaction but did allow the acquisition of reproducible data for the rate constant. The reaction followed a second-order dependence on the concentration of **2b**, consistent with a mechanism wherein two discrete catalyst molecules cooperate to activate both the electrophile (epoxide) and the nucleophile (water). Compelling evidence for a similar bimetallic mechanism has been obtained in the asymmetric ring-opening of epoxides by azide nucleophiles catalyzed by related chromium-containing catalysts (*14*). It is noteworthy that bimetallic catalysis is apparently operative with such distinct classes of reactions, and it hints at the possible generality of such a mechanism.

The HKR is an attractive procedure for the preparation of optically enriched terminal epoxides and 1,2-diols. The criteria for evaluating the practicality of chemical processes such as this one have become increasingly stringent. High standards of yield and selectivity in product formation must be met, but additional issues such as reagent cost, volumetric productivity, waste generation, reagent toxicity, and handling risks weigh more heavily than ever before. With these criteria positively met, the HKR appears to hold significant potential for large-scale application (*15*).

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9. Complex **2b** can be generated in situ from **1** (*7*) by treatment with acetic acid (2 equiv) in toluene under air, followed by evaporation of solvent. $^1$H nuclear magnetic resonance (NMR) (400 MHz, dry acetone-$d_6$) δ (ppm) relative to tetramethylsilane: −1.95 (br s, 2 H, $H_2O$), 1.21 (s, 9 H, *t* -Bu), 1.28 (s, 9 H, *t* -Bu), 1.33 (s, 9 H, *t* -Bu), 1.53 (s, 9 H, *t* -Bu), 1.58 [s, 3 H, $CH_3C(O)$], 1.6 (m, 2 H, $CH_2$), 1.9 to 2.0 (m, 2 H, $CH_2$), 2.23 (br q, $J$ = 9.8 Hz, 1 H, C*H*H), 2.79 (d, $J$ = 11.8 Hz, 1 H, C*H*H), 2.88 (br d, 2 H, $CH_2$), 3.27 (brt, 1 H, CHN), 4.35 (brt, 1 H, CHN), 7.17 (d, $J$ = 2.4 Hz, 1 H, ArH), 7.22 (s, 1 H, CH=N), 7.29 (d, $J$ = 2.4 Hz, 1 H, ArH), 7.35 (d, $J$ = 2.4 Hz, 1 H, ArH), 7.46 (d, $J$ = 2.4 Hz, 1 H, ArH), 7.59 (s, 1 H, CH=N). Infrared (KBr) 1719 w, 1638 s, 1611 s, 1545 s, 1540 s, 1526 s, 1461 s, 1436 s, 1408 s, 1390 s, 1361 s, 1339 s, 1323 s, 1270 s, 1255 s, 1235 m, 1202 m, 1169 s, 834 m, 783 m; melting point (open capillary) 108°C (decomposes).
10. The hydrolysis reactions were mildly exothermic on a laboratory scale. For the kinetic resolution of propylene oxide (boiling point, 34°C), the reaction vessel was cooled in an ice bath during the addition of water to limit substrate loss as a result of evaporation.
11. A mixture of (*S*,*S*)-**1** (1.208 g, 2.0 mmol, 0.2 mol %), toluene (10 ml), and acetic acid (0.23 ml, 4.0 mmol, 2 equiv to catalyst) was stirred while open to the air for 1 hour at room temperature. The solvent was removed by rotary evaporation, and the brown residue was dried under vacuum. Propylene oxide (58.7 g, 1.0 mol) was added in one portion, and the stirred mixture was cooled in an ice-water bath. Water (9.9 ml, 0.55 mol, 0.55 equiv) was slowly added until the temperature of the reaction mixture began to rise. The temperature rose to ~25°C before dropping to 15°C, at which point water addition was continued at a rate that maintained the reaction temperature near 20°C. After 1 hour, addition was complete; the ice bath was removed, and the reaction was stirred at room temperature for 11 hours. The flask was then affixed with a distillation head equipped with a receiver cooled to −78°C, and the unreacted epoxide was distilled under $N_2$ until no more material came over with gentle heating. The system was then placed in a mild vacuum to collect any residual epoxide [yield: 26.05 g, >99% pure by gas chromatography (GC), 0.444 mol, 44% yield]. The receiver was changed, and the system was carefully placed in a full vacuum (<65 Pa). The diol was then distilled under vacuum into an ice-cooled receiver and isolated as a colorless, viscous liquid (yield: 38.66 g, >99% pure by GC, 0.503 mol, 50% recovery).
12. It is significant that propylene glycol is isolated in high enantiomeric purity and yield. Even though excellent methods exist for the asymmetric dihydroxylation (AD) of most olefins (*5*), the highest enantioselectivity obtained to date in the AD of propylene is only 49% [K. P. M. Vanhessche and K. B. Sharpless, *Chem. Eur. J.* **3**, 517 (1997)].
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15. The HKR of propylene oxide has been carried out successfully on a 10-kg scale at a pilot plant at Chirex, Inc. (Dudley, UK) (J. Cummins and G. Thorpe, private communication).


16. This work was supported by a grant from the National Institutes of Health (GM-43214) and postdoctoral fellowships to M.T. and F.K. from the Japan Society for the Promotion of Science.

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# Protein Transport by Purified Yeast Sec Complex and Kar2p Without Membranes


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**Posttranslational protein translocation across the endoplasmic reticulum membrane of yeast requires a seven-component transmembrane complex (the Sec complex) in collaboration with the lumenal Kar2 protein (Kar2p). A translocation substrate was initially bound to the cytosolic face of the purified Sec complex in a signal-sequence–dependent but Kar2p- and nucleotide-independent manner. In a subsequent reaction, in which Kar2p interacted with the lumenal face of the Sec complex and hydrolyzed adenosine triphosphate, the substrate moved through a channel formed by the Sec complex and was released at the lumenal end. Movement through the channel occurred in detergent solution in the absence of a lipid bilayer.**


Protein transport across the membrane of the endoplasmic reticulum (ER) occurs through an aqueous channel (*1*) whose major component is the Sec61p complex (*2*–*6*). In posttranslational protein transport in yeast, the Sec61p complex (Sec61p, Sbh1p, and Sss1p) associates with the tetrameric Sec62-63 complex (Sec62p, -63p, -71p, and -72p) to form the Sec complex (*7*, *8*). A lumenal domain of Sec63p (the J domain) interacts with the adenosine triphosphatase (ATPase) Kar2p (BiP) (*9*). Posttranslational protein transport occurs in distinct steps (*2*, *3*, *10*, *11*) and can be reproduced with reconstituted proteoliposomes that contain only the purified Sec complex and Kar2p (*8*). We have now developed a soluble system made up of these components that eliminates many of the problems inherent in reactions that involve membranes and allows in-depth analysis of the molecular mechanism of the translocation process.

To develop a soluble system, we first tested whether an interaction between translocation substrates and the Sec complex would be maintained in detergent


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solution. In vitro synthesized [$^{35}$S]methionine-labeled prepro-α-factor (ppαF) was added to proteoliposomes containing the purified Sec complex, the membranes were solubilized with digitonin, and the complex was immunoprecipitated with antibodies to Sec62p (*12*). A large percentage of ppαF was immunoprecipitated (Fig. 1A). Binding, although less efficient, also was observed with a second translocation substrate, proOmpA. Only background binding was observed with liposomes that lacked protein. Antibodies to Sbh1p and Sss1p also coprecipitated the bound precursor, although less efficiently than antibodies to Sec62p (Fig. 1B). Antibodies that react only poorly with Sec61p in the heptameric complex (*13*) did not recover precursor. Because only a small percentage of the ppαF was translocated in the absence of Kar2p, and proOmpA was not translocated at all (*8*), binding is not the result of translocation. Binding of ppαF to the Sec complex was much reduced with a signal sequence mutant defective in translocation into native microsomes (*3*, *14*) (Fig. 1C).

To test whether the bound precursor could be translocated, ppαF was bound to proteoliposomes containing the Sec complex and lumenal Kar2p. In one-half of the reaction mixture, binding was assessed by coimmunoprecipitation after solubilization; in the other half, adenosine triphosphate (ATP) was added and, after incubation, protease protection was used to determine whether translocation had occurred (Fig. 1D). The large majority of the precursor was both initially bound and later translocated.

We then determined whether one of the two subcomplexes of the Sec complex has independent binding activity. The subcomplexes were reconstituted separately or together, ppαF was added, and binding was examined. Each subcomplex showed weak binding, but together they were as efficient as the intact complex (Fig. 2A). If ppαF was first bound to the intact complex and the subcomplexes were then separated (*8*), no specific coprecipitation occurred with either (Fig. 2B) despite their quantitative recovery (*15*).

Precursor did not bind well to the Sec complex in digitonin (*15*) or deoxyBigCHAP (Calbiochem) (Fig. 3A). However, when phospholipids were added (*16*), efficient binding occurred. To demonstrate that the Sec complex was completely soluble, we examined accessibility of the carbohydrate moiety of Sec71p to added glycosidase (Fig. 3B) (*17*). In detergent solution supplemented with phospholipid, the carbohydrate moiety was as accessible as it was after complete denatur-

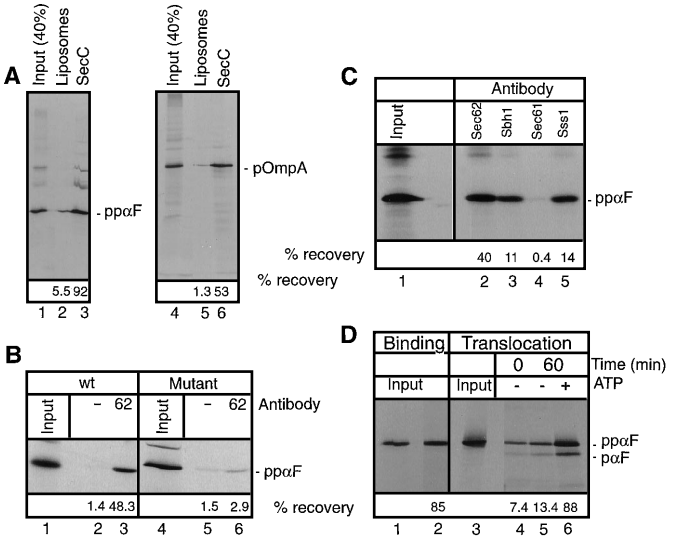


**Fig. 1.** Binding of secretory precursors to reconstituted Sec complex. (**A**) In vitro synthesized [$^{35}$S]methionine-labeled ppαF or proOmpA (pOmpA) was incubated with liposomes containing either no protein (liposomes) or purified Sec complex (SecC), and binding was assessed by coimmunoprecipitation with antibodies to Sec62p after solubilization with digitonin (*12*). Percentage of total input precursor coprecipitated with the Sec complex, determined by analysis with a Phosphorimager, is shown (% recovery); 40% of total input precursor is shown in lanes 1 and 4. (**B**) Coimmunoprecipitation of bound ppαF with antibodies to various components of the Sec complex. (**C**) Binding reactions were done with wild-type ppαF or a signal sequence mutant. (**D**) Bound ppαF can be subsequently translocated. ppαF was bound to proteoliposomes reconstituted with the Sec complex, 10 mM creatine phosphate, creatine phosphokinase (0.1 mg/ml), and ~1.9 μM Kar2p, prepared as described (*8*). Binding was assessed by coimmunoprecipitation with antibodies to Sec62p after solubilization (lane 2). Samples were incubated after the binding reaction with or without 2 mM ATP for 0 or 60 min, as indicated. Translocated protein was determined after treatment with proteinase K (0.5 mg/ml). Pro-α-factor (pαF) produced by contaminating signal peptidase is indicated.

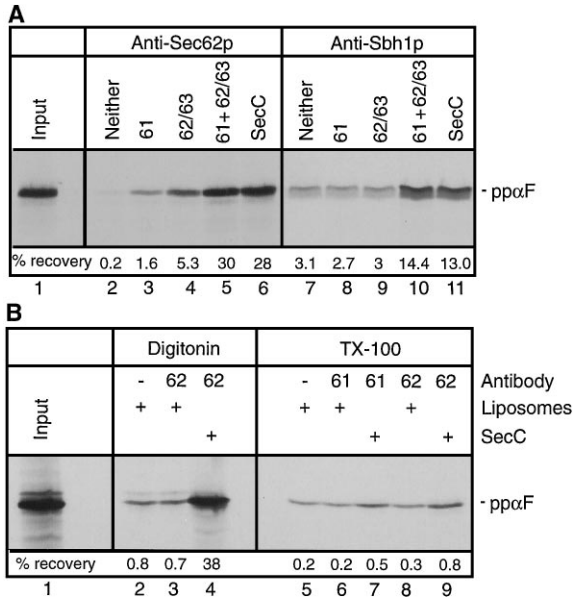


**Fig. 2.** Requirement of an intact Sec complex for precursor binding. (**A**) ppαF was incubated with proteoliposomes containing intact Sec complex (SecC); Sec61p subcomplex (61); Sec62-63 subcomplex (62/63), both prepared as described (*8*); the two subcomplexes together (61 + 62/63); or neither complex. Binding was assessed by immunoprecipitation with antibodies to Sec62p or Sbh1p (anti-Sec62p or anti-Sbh1p) after solubilization in digitonin. (**B**) After binding of ppαF to liposomes containing either intact Sec complex (SecC) or no protein (liposomes), solubilization was done with either digitonin or Triton X-100 (TX-100), which separates the two subcomplexes (*8*). Immunoprecipitation was with antibodies to Sec62p or Sec61p.

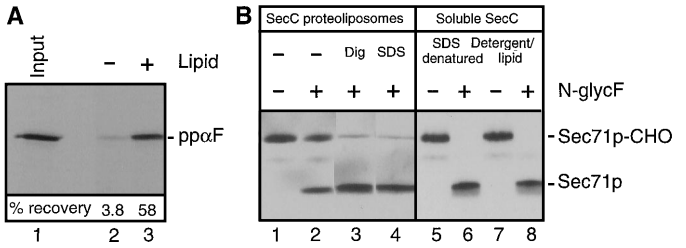


**Fig. 3.** Precursor binding to soluble Sec complex. (**A**) ppαF was incubated with purified Sec complex in detergent with or without phospholipids. Binding was assessed by coimmunoprecipitation with antibodies to Sec62p. (**B**) Purified Sec complex (SecC) was treated with N-glycosidase F (N-glycF) to probe the accessibility of the carbohydrate moiety of glycosylated Sec71p (Sec71p-CHO) under the conditions used in (A) (detergent/lipid; lanes 7 and 8) (*17*). SDS-denatured material (lanes 5 and 6) or proteoliposomes with or without solubilization by digitonin (Dig) or SDS (lanes 1 to 4) were used for controls.

ation of the complex in SDS. With the reconstituted complex, however, only about 50% was accessible unless the membrane barrier was broken by digitonin or SDS. Thus, precursor binding to the Sec complex occurred in solution and required lipids but not an intact bilayer.

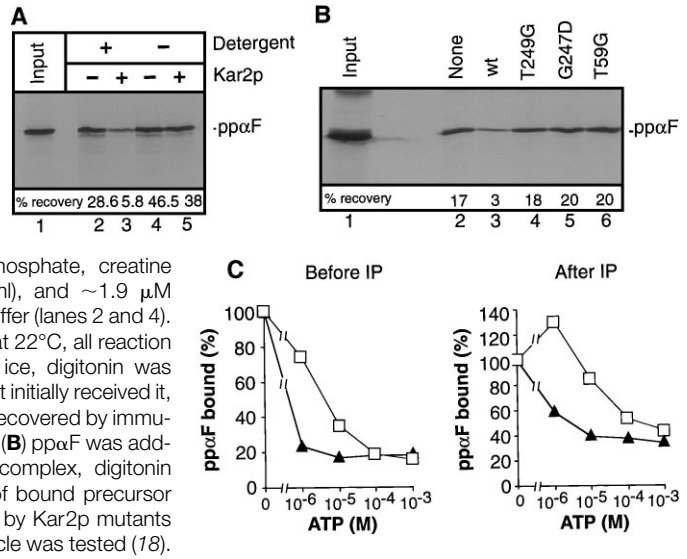


**Fig. 4.** Kar2p uses ATP hydrolysis to break the precursor-Sec complex interaction. (**A**) ppαF was bound to reconstituted Sec complex and one-half of the reaction mixture was treated with digitonin. To both halves was added either 2 mM ATP, 10 mM creatine phosphate, creatine phosphokinase (0.1 mg/ml), and ~1.9 μM Kar2p (lanes 3 and 5) or buffer (lanes 2 and 4). After a 10-min incubation at 22°C, all reaction mixtures were placed on ice, digitonin was added to those that had not initially received it, and Sec complexes were recovered by immunoprecipitation of Sec62p. (**B**) ppαF was added to reconstituted Sec complex, digitonin was added, and release of bound precursor by wild-type (wt) Kar2p or by Kar2p mutants defective in the ATPase cycle was tested (*18*). (**C**) Reaction mixtures were as in (B). On the left [before immunoprecipitation (IP)], release was caused by addition of Kar2p, ATP at the indicated concentrations, and an energy-regenerating system in the absence (filled symbols) or presence (open symbols) of 2 mM AMPPNP. On the right (after IP), release was done after immunoprecipitation. Bound ppαF was determined and normalized to that in the absence of Kar2p.

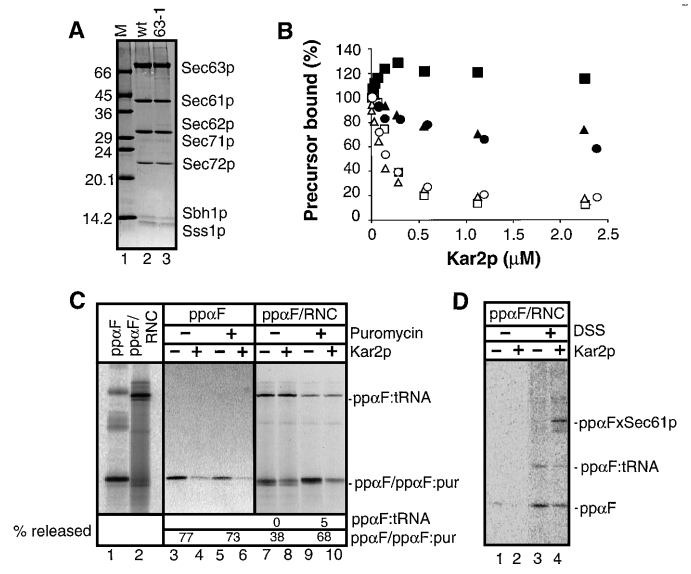


**Fig. 5.** Precursor release on the lumenal face of the Sec complex by Kar2p. (**A**) Sec complex from sec63-1 cells, carrying a mutation in the J domain of Sec63p, was analyzed by SDS-PAGE and staining with Coomassie blue. wt, wild-type complex; M, molecular size standards (kilodaltons). (**B**) ppαF (triangles) and proOmpA (squares) were bound to proteoliposomes containing either wild-type Sec complex (open symbols) or Sec63-1 Sec complex (filled symbols), and release by Kar2p was induced after solubilization with digitonin. The same experiment was done with ppαF bound directly to the soluble wild-type (open circles) or mutant (filled circles) Sec complex. Precursor remaining bound to the Sec complex was normalized to that bound in the absence of Kar2p. (**C**) Ribosome-nascent chain complexes containing ppαF lacking five amino acids from the COOH-terminus (ppαF/RNC) were produced by translation of truncated mRNA and sedimentation of the ribosomes (*3*). Resuspended ppαF/RNC or full-length ppαF was mock treated or treated with 1 mM puromycin for 30 min at 35°C and then brought to 8 M urea to denature the ribosomes. Samples were diluted 1:10 into a binding reaction mixture with reconstituted Sec complex. Digitonin was added and release was induced with Kar2p. Sec62p immunoprecipitates were treated with SDS sample buffer (pH 7.2) at 30°C for 5 min. ppαF:tRNA and ppαF:pur refer to ppαF with an associated tRNA or puromycin molecule, respectively. Release of these species as well as of full-length ppαF was quantitated individually. (**D**) Urea-treated ppαF/RNC was bound to proteoliposomes containing Sec complex, digitonin was added, and reactions were done with or without Kar2p as in (C). One-half of each reaction mixture was treated with 1 mM DSS for 35 min at 0°C; 10 mM ethanolamine was added, followed by 1% SDS; and samples were boiled and diluted to 1% Triton X-100 and 0.1% SDS. After immunoprecipitation with antibodies to Sec61p, the samples were boiled in SDS sample buffer (pH 10.5).

We then tested whether Kar2p would release precursor from the Sec complex. In a primary incubation, ppαF was bound to proteoliposomes containing Sec complex; digitonin was then added to solubilize the membranes. In a secondary incubation, Kar2p and an ATP regenerating system were added. Substrate still bound to the Sec complex was detected by coimmunoprecipitation with antibodies to Sec62p. The presence of Kar2p and ATP in the second incubation caused loss of ~80% of the bound substrate (Fig. 4A). Release did not occur if Kar2p and ATP were added to the nonsolubilized proteoliposomes, which suggests that, to effect release, Kar2p must gain access to the face of the Sec complex opposite that on which substrate is presented.

To determine whether ATP hydrolysis is required for release, we used purified mutant Kar2 proteins that were unable to perform various steps of the ATPase cycle. Mutants $Gly^{247} \rightarrow Asp^{247}$ (G247D), $Thr^{59} \rightarrow Gly^{59}$ (T59G), and $Thr^{249} \rightarrow Gly^{249}$ (T249G) are defective in ATP binding, a conformational change after ATP binding, and hydrolysis after the conformational change, respectively (*18, 19*). None of these mutant proteins supported release (Fig. 4B). The nonhydrolyzable ATP analog adenylyl-imidodiphosphate (AMPPNP) did not support release but inhibited the ATP-dependent reaction (Fig. 4C). These results indicate that Kar2p must hydrolyze ATP to release bound substrate from the Sec complex. Release also occurred when Kar2p and ATP were added after immunoprecipitation and extensive washing of the complex with a detergent solution (Fig. 4C).

To determine whether release requires an interaction between Kar2p and Sec63p, we prepared the Sec complex from a yeast strain that carries a mutation in the J domain (sec63-1) (*20*). The mutant complex contained all the components of the wild-type complex in the same apparent stoichiometries (Fig. 5A) and bound ppαF and proOmpA with the same efficiency as the wild-type complex (*15*). However, in the release reaction, the mutant complex was significantly less active, even at very high concentrations of Kar2p (Fig. 5B). A similar observation was made when the precursor was bound directly to the soluble Sec complex (Fig. 5B). We conclude that Kar2p must interact with the J domain of Sec63p to release the substrate from the Sec complex.

To test whether release of the substrate occurred on the cytosolic or the lumenal face of the Sec complex, we used as translocation substrate a fragment of ppαF,

only five amino acids shorter than full length, which contains a tRNA at the COOH-terminus (ppαF:tRNA). If release occurs by passage of ppαF through a translocation channel, the bulky tRNA moiety should cause stalling of the translocating polypeptide chain and prevent its release from the Sec complex. If release occurs on the cytosolic side, it should be unaffected. ppαF:tRNA was synthesized in vitro by translating a truncated mRNA (*3*), released from the ribosomes with 8 M urea, and diluted into a reaction mixture containing proteoliposomes with Sec complex. Detergent was added and binding was assessed by coimmunoprecipitation. The attached tRNA did not prevent binding to the Sec complex (Fig. 5C). Upon addition of Kar2p, little if any release of ppαF:tRNA was detected (Fig. 5C). In the same reactions, the ppαF fragment produced by spontaneous removal of the tRNA by hydrolysis was released by Kar2p. Release was not as efficient as with full-length ppαF, probably because some hydrolysis occurred during immunoprecipitation. When the tRNA at the COOH-terminus of ppαF:tRNA was removed by treatment with puromycin before binding to the Sec complex, release was as efficient as with normal ppαF. Residual ppαF:tRNA remaining after the puromycin reaction was still unaffected by Kar2p. These data indicate that release occurs by Kar2p-dependent transport through the channel. Similar results were obtained when ppαF:tRNA was used with intact proteoliposomes (*21*), which suggests that tRNA-tethered substrates can be used to generate intermediates in posttranslational translocation.

We used crosslinking to verify that Kar2p moves ppαF:tRNA into a stalled position within the solubilized translocation channel. Addition of the lysine-directed bifunctional reagent disuccinimidyl suberate (DSS) to bound ppαF:tRNA produced crosslinks to Sec61p only in the presence of Kar2p (Fig. 5D). These results are consistent with the COOH-terminal region of ppαF, containing all the lysines in the protein, having been moved into the Sec61p channel by the action of Kar2p. Crosslinks were not observed with full-length ppαF or with the ppαF fragment from which tRNA had been removed (*15*).

When ppαF:tRNA was bound to the soluble, nonreconstituted Sec complex directly, release by Kar2p was as efficient as with normal ppαF (*15*). It is unclear whether under these conditions the pore size of the channel is increased or its stability is decreased, either possibility allowing passage of tRNA, or whether release occurs toward the cytosolic side.

Our results indicate that posttranslational movement of a protein through the ER membrane can be reproduced with purified components in detergent solution in the absence of a lipid bilayer or even bulk lipids. Blockage of translocation by a bulky group at the COOH-terminus of the substrate provides direct evidence that the Sec complex forms a protein-conducting channel of limited pore size. A functional signal sequence, but neither Kar2p nor nucleotide, is needed to bind the translocation substrate to the Sec complex. Both Sec62-63 and Sec61 subcomplexes must be present. Possibly, signal sequence recognition requires oligomerization of the Sec61p complex by the Sec62-63 complex (*6*). Because initial binding of the translocation substrate required lipids but maintenance of the interaction did not, the signal sequence appears ultimately to interact with the Sec complex through a protein-protein interaction. In the second phase of translocation, Kar2p interacts with the J domain of Sec63p to move the substrate through the channel. The interaction of Kar2p and Sec63p is probably direct, because it occurs in the absence of other proteins. Our results indicate that the recently observed Kar2p-dependent release of ppαF from proximity of the Sec62-63 complex may have been complete translocation rather than aborted transfer into the Sec61 subcomplex (*11*). We found that release through the channel occurred even though the signal sequence was not cleaved, which suggests that Kar2p weakens binding of the Sec complex with the signal sequence. Because translocation occurs in vivo despite a high concentration of lumenal Kar2p, it is possible either that Kar2p can associate with the J domain only after a signal sequence is in contact with the Sec complex or that the nucleotide state of a prebound Kar2p molecule is affected by signal sequence binding. The ensuing dissociation of the signal sequence would be coupled with transfer of Kar2p to the translocation substrate. ATP hydrolysis by Kar2p may occur upon binding of Kar2p to the Sec complex or upon its interaction with the substrate, and it is likely the source of energy for translocation.

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12. Purification of the Sec complex from *Saccharomyces cerevisiae* cells, reconstitution into proteoliposomes, and in vitro translation in the reticulocyte lysate system were as described (*8*). Binding reaction mixture (5 or 10 μl) contained 50 mM Hepes (pH 7.5), 180 mM potassium acetate, 5 mM magnesium acetate, 2 mM dithiothreitol, 10 to 15% (v/v) glycerol, between 40 and 140 nM Sec complex in proteoliposomes, and 20% (v/v) translation mixture desalted on a NAP-5 column. After incubation at 22°C for 10 min, solubilization with 1% digitonin in 150 mM potassium acetate was on ice in 10 or 20 μl. After dilution to 130 μl under the same conditions, the samples were incubated for 40 min with 1 μl of affinity-purified antibodies to the COOH-terminal 13 amino acids of Sec62p (*8*) and for 20 min with 15 μl of protein A-Sepharose. The immunoprecipitates were extensively washed with digitonin-containing buffer before analysis by SDS–polyacrylamide gel electrophoresis (SDS-PAGE).
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16. Purified Sec complex at ~150 nM (6 μl) in 50 mM Hepes (pH 7.5), 183 mM potassium acetate, 12% glycerol, 2 mM dithiothreitol, and 0.3% deoxyBigCHAP (Calbiochem) was incubated on ice with phosphatidylcholine (2.7 mg/ml) and phosphatidylethanolamine (0.7 mg/ml) (Sigma). Buffer (4 μl) was added to introduce potassium acetate to 150 mM, magnesium acetate to 5 mM, and digitonin to 0.5% while the lipid and deoxyBigCHAP were being diluted. Precursor (1 μl) was added, and the mixture was incubated at 22°C for 10 min before dilution and immunoprecipitation with antibodies to Sec62p.
17. Treatment with N-glycosidase F (~24 units/ml; Boehringer) was at 0°C for 2 hours. Hot SDS sample buffer was added and samples were analyzed by immunoblotting with antibodies to Sec71p.
18. Kar2p mutants T249G, G247D, and T59G were generated by in vitro mutagenesis. Wild-type Kar2p and mutant proteins were expressed as histidine-tagged proteins in *Escherichia coli* and purified by immobilized nickel chromatography (*8*). The expected properties of the mutants were confirmed (*19*).
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