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Co-translocation of a Periplasmic Enzyme Complex by a Hitchhiker Mechanism through the Bacterial Tat Pathway*

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Bacterial periplasmic nickel-containing hydrogenases are composed of a small subunit containing a twin-arginine signal sequence and a large subunit devoid of an export signal. To understand how the large subunit is translocated into the periplasm, we cloned the *hyb* operon encoding the hydrogenase 2 of *Escherichia coli*, constructed a deletion mutant, and studied the mechanism of translocation of hydrogenase 2. The small subunit (HybO) or the large subunit (HybC) accumulated in the cytoplasm as a precursor when either of them was expressed in the absence of the other subunit. Therefore, contrary to most classical secretory proteins, the signal sequence of the small subunit itself is not sufficient for membrane targeting and translocation if the large subunit is missing. On the other hand, the small subunit was required not only for membrane targeting of the large subunit, but also for the acquisition of nickel by the large subunit. Most interestingly, the signal sequence of the small subunit determines whether the large subunit follows the Sec or the twin-arginine translocation pathway. Taken together, these results provide for the first time compelling evidence for a naturally occurring hitchhiker co-translocation mechanism in bacteria.

Proteins destined for secretion, membrane integration, or assembly into organelles are sorted with high fidelity to their respective intracellular sites by virtue of targeting signals encoded within the primary structures of the nascent polypeptides themselves. The principal role of targeting signals is to mediate the engagement of the exported protein with components of the specific translocation machinery (1–3). In these cases, signal sequences are specifically recognized by a cytosolic chaperone or a targeting factor and act as true targeting signals. Alternatively, the function of signal sequences is proposed as being to delay the folding of the mature portion of an exported protein, allowing binding of an export-specific chaperone to its unfolded mature portion and thereby keeping the exported protein in a translocation-competent configuration (4).

Hydrogenases are omnipresent in bacteria and archaea (5). They catalyze the reversible oxidation of hydrogen and allow bacteria to use hydrogen as an energy source for their growth. Hydrogenases can be divided into two major superfamilies: (*a*) nickel-iron hydrogenases (NiFe hydrogenases), and (*b*) iron-only hydrogenases (Fe hydrogenases). They are generally composed of a small subunit of about 30 kDa and a large subunit of 60 kDa. All small subunits of periplasmic or membrane-bound hydrogenases contain an N-terminal signal sequence possessing a conserved twin-arginine motif, which is removed once the hydrogenases are translocated into the periplasm (5, 6). The large subunits of NiFe hydrogenases show no N-terminal processing, but they possess a C-terminal extension sequence composed of one to two dozen residues. The extension sequence seems to keep the precursor of the large subunit in a conformation competent for nickel acquisition, and it is removed by a specific cytoplasmic protease upon nickel incorporation (7). The large subunits, therefore, are devoid of any known signal sequence required for the export of proteins. They are assumed to be co-translocated with the small subunits (5, 6).

The small and large subunits of hydrogenase 2 (HYD2)[1] of *Escherichia coli* are encoded by *hybO* and *hybC* of the *hybOABCDEFG* operon, respectively (8, 9). HYD2 is an extrinsic membranous protein that is attached to the periplasmic side of the cytoplasmic membrane by a 5-kDa fragment of its small subunit (10, 11). An active HYD2 can be released from spheroplasts by limited trypsin proteolysis (11). The acquisition of nickel in the cytoplasm is a prerequisite for HYD2 export (11), which is mediated by the twin-arginine translocation (Tat) pathway (12, 13). In this communication, we show that there is an interdependence between the small and the large subunits for their export and that the signal sequence of the small subunit determines the type of export pathway chosen by the large subunit. We thus provide the first example of a naturally occurring hitchhiker co-translocation of a dimeric enzyme across the bacterial cytoplasmic membrane.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Growth Conditions—Bacterial strains and plasmids used in this study are listed in Table I. To construct the Δ*hybOABC* mutant, the 3421-base pair *Xho*I-*Bgl*II fragment containing *hybOABC* was replaced by the promoterless cassette encoding β-glucuronidase and Kan^R that was obtained by *Sal*I-*Bgl*II digestion of the plasmid pUIDK2 (14). The transcriptional *hybO-uidA* fusion was recombined back to the chromosome of *recD* strain D355 (15) and was further moved into strains MC4100, B834, and HYD720 via P1*cml* transduction (16). Similarly, the *secAts* and *secYts* mutations were introduced into various strains by P1-mediated transduction, selection for tetracycline resistance, and screening for thermosensitivity.

The bacteria were routinely grown in LB medium, on LB plates, or in minimal M9 medium as described previously (11, 17, 18).

Preparation of Subcellular Fractions and Enzyme Assays—Periplasm, spheroplasts, membrane, and cytoplasmic fractions were

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[1] The abbreviations used are: HYD2, hydrogenase 2; Tat, twin-arginine translocation.

TABLE I
Bacterial strains, lambda, and plasmids

Strains and plasmids	Description	Source or Ref. no.
Strains		
B1LK0	MC4100 Δ*tatC*	Ref. 12
B834(DE3)	*hsd metB* P_L-T7 gene1	Ref. 28
BH1	B834 (DE3) (Δ*hybO-C*::*uidA* (Kan^R))	P1 transduction: ENF1 × B834
BHA1	BH1 *secAts leu*::Tn*10*	P1 transduction: CK105 × BH1
BHY1	BH1 *secYts*Tn*10*	P1 transduction: CK107 × BH1
CK105	F′ *lac pro [Δlac pro] secAts leu*::Tn*10*	Ref. 29
CK107	F′ *lac pro [Δlac pro] secYts*	Ref. 29
CU164	MC4100 *secY39cs zhd-33*::Tn*10*	Ref. 30
ENF1	MC4100 Δ(*hybO-C*)::*uidA* (Kan^R)	This study
HDJ123	MC4100 Δ*hyc*, Δ*hybBC*::*kan*, Δ*hya*	Ref. 31
HYD720	MC4100 Δ*nikA*	Ref. 16
K38	HfrC *phoA4 pit10 tonA22 ompF627 relA spoT1* λ^+	Ref. 20
MC4100	F^- *araD139* Δ*(argF-lac)* U169 *ptsF25 relA1 flb5301 rpsL150 deoC1 rbsR* λ^-	Laboratory collection
NH1	HYD720 Δ*hybO-C*::*uidA* (Kan^R)	P1 transduction: ENF1 × HYD720
Lambda	2B7,12A8,5C10,6H4,17B2	Ref. 23
Plasmids		
pUC18	Amp^R	Stratagene
pET22b(+)	Amp^R T7 ϕ10	Novagen
pT7–7	Amp^R T7 ϕ10	Ref. 20
pKSM717	Amp^R T7 ϕ10, $P_{lac\text{UV5}}$	Ref. 32
pGP1–2	Kan^R, P_L-T7 gene1, P*lac-c*1857	Ref. 20
pHYB11[a]	*hyb(O-G)*$^+$	This study
pHYB14[b]	*hyb(O-G)*$^+$	This study
pHYB41[c]	*hyb(O-B,D-G)*$^+$, *hybC*$^{\Delta 543bp}$	This study
pHYB84[d]	*hyb(A-G)*$^+$	This study
pHYB30[e]	*hyb(C-G)*$^+$	This study
pHYB411[f]	*hybO*$^+$	This study
pHYB55[g]	*pelB*leader-*hybO*, *hyb(A-G)*$^+$	This study

[a] 15-kb *Bam*HI fragment from the lambda 5C10 cloned in pUC18.
[b] The 10-kb *Xho*I-*Stu*I fragment from pHyb11 cloned in pHyb411.
[c] Derivative of pHyb14, obtained by deletion of 543-bp *Eco*RI fragment of the *hybC* gene that encompasses 181 amino acids of the large subunit.
[d] 7.4-kb *Pst*I-*Bam*HI fragment from pHyb11 cloned in pT7–7, then the 5.4-kb *Hin*dIII-*Stu*I fragment from the resulting plasmid cloned in pKSM717.
[e] Deletion of 2-kb containing *hybAB* of pHyb84.
[f] *Nde*I and *Bam*HI sites were introduced at the ATG codon and immediately after the stop codon of *hybO* by polymerase chain reaction, the resulting 1.1-kb *Nde*I-*Bam*HI fragment was cloned into pT7–7.
[g] 6.5-kb *Nco*I-*Bam*HI fragment from pHyb14 in pET22b(+).

prepared by lysozyme/EDTA/cold osmoshock and ultracentrifugation, as described previously (11, 18). To extract peripherally bound membrane proteins, the membrane was washed with 6 M urea or 100 mM sodium carbonate (pH 10). To further separate membrane proteins from aggregates, the membrane fractions were solubilized by 4% Triton X-100 in 40 mM Tris-HCl (pH 7.6) and centrifuged using an Airfuge at 30 p.s.i. for 10 min. To release HYD2 from the washed spheroplasts, limited trypsin digestion was performed as described previously (11).

Hydrogenase activity was measured by following the H_2-linked reduction of benzyl viologen spectrophotometrically at 600 nm or by activity staining as described previously (16, 17).

Immunological Procedures and in Vivo and in Vitro Specific Labeling of hyb Gene Products—Immunoblotting was performed by using the ECL method according to the manufacturer's instructions (Amersham Corp.). To prepare antiserum against HybO, the *hybO* gene was cloned into pET22b+, and 6-His was added at the C terminus of the HybO. The recombinant $HybO^{6His}$ was solubilized by guanidine hydrochloride from inclusion bodies and partially purified on a nickel nitrilotriacetic acid column according to the manufacturer's instructions (Qiagen). Gel slices containing $HybO^{6His}$ were used in standard immunization protocols for rabbits (Eurogentec). The resulting antiserum also contains antibodies that recognize contaminating antigens. Nonspecific antibodies were removed by absorption to an acetone powder prepared with whole cells of ENF1 (Δ*hybOABC*) as described in Ref. 19.

The *hyb* gene products were specifically labeled by [^{35}S]methionine *in vivo* using T-7 RNA polymerase (20) or *in vitro* using a plasmid-directed transcription-translation system (21).

In Vitro Cross-Linking—*In vitro* cross-linking with formaldehyde was performed as described in Ref. 22, with modifications. Soluble S-135 cytoplasmic fractions prepared from the strains ENF1/pHyb84 (Δ*hybOABC*/*hyb(A-G)*$^+$) and ENF1/pHyb411 (Δ*hybOABC*/*hybO*$^+$) were subjected, separately or as a mixture, to treatment with 0.1% formaldehyde. The reaction was incubated at room temperature for 30 min. Aliquots were removed, and the reaction was stopped by the addition of 50 mM Tris-HCl (pH 7.6) and benzonase. To dissociate cross-linked complexes, samples were heated at 100 °C for 15 min, whereas the control was kept at 37 °C. All samples were treated at 90 °C for 5 min before applying them to the gel.

RESULTS

Cloning of the hyb Operon and Construction of the ΔhybO-ABC Mutant—To study the translocation mechanism, we cloned the *hyb* operon by using the Kohara collection (23). Among five cosmids covering the 65 min region of the *E. coli* chromosome, only lambda 5C10 was able to confer HYD2 activity on mutant HDJ123 that is pleiotropically defective in hydrogenase activities (Δ*hya,* Δ*hybBC,* and Δ*hyc*). A 15-kilobase *Bam*HI fragment containing the entire *hyb* operon was obtained from lambda 5C10 and cloned into pUC18, resulting in the plasmid pHyb11. A chromosomal deletion mutant in which the *hybOABC* genes were replaced by a *uidA*-Kan^R cassette was then constructed as described under "Experimental Procedures." As expected, the resulting mutant ENF1 was deficient in HYD2 activity and was devoid of both the small (HybO) and the large (HybC) subunits (data not shown). The wild type phenotype was completely restored to mutant ENF1 by the introduction of plasmid pHyb11 harboring the entire *hyb* operon.

Influence of the Large Subunit on the Targeting of the Small Subunit—In cells expressing the entire *hyb* operon, two forms of HybO, the small subunit of HYD2, were detected. As expected, the larger precursor form was found in the cytoplasm (Fig. 1*A, lane 1*), whereas the mature form, with the signal sequence removed, was recovered from the membrane fraction

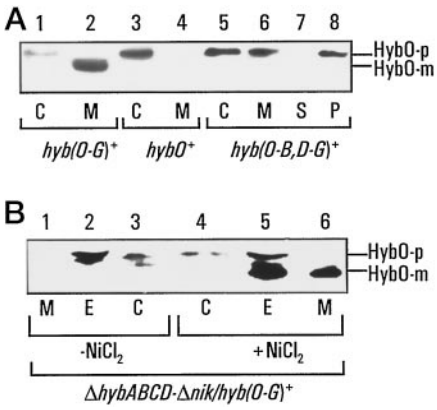

FIG. 1. **Influence of HybC on the membrane targeting of HybO.** Crude extracts (*E*), cytoplasmic fraction (*C*), membrane fraction (*M*), Triton X-100-solubilized membrane (*S*), and the insoluble pellet (*P*) were prepared from ENF1 (Δ*hybOABC*; *panel A*) carrying plasmids pHyb14 (*hyb(O-G)*$^+$), pHyb411 (*hybO*$^+$), or pHyb41 (*hyb(O-B,D-G)*$^+$) or from NH1 (Δ*hybOABC*-Δ*nik*; *panel B*) containing plasmid pHyb14 grown without (−*NiCl*$_2$) or with (+*NiCl*$_2$) 0.3 mM $NiCl_2$ and separated on a 12.5% SDS-gel. Precursor (*HybO-p*) and mature HybO (*HybO-m*) were detected by antiserum against HybO and are indicated on the *right*. Plasmid pHyb41 contains a 543-base pair in-frame deletion in *hybC* and, as verified in an *in vivo* labeling system, directs the biosynthesis of a truncated 42-kDa large subunit.

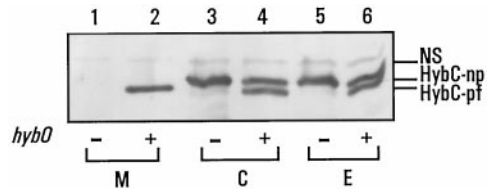

FIG. 2. **Requirement of the small subunit for membrane targeting of the large subunit.** Crude extracts (*E*), cytoplasmic fraction (*C*), and membrane fraction (*M*) were prepared from ENF1/pHyb14 (*hyb(O-G)*$^+$) and ENF1/pHyb84 (*hyb(A-G)*$^+$) and separated on a 7.5% SDS-gel. The presence (+) or absence (−) of the *hybO* gene product in corresponding extracts is indicated. The nonspecific contaminating band (*NS*), non-processed precursor (*HybC-np*), and the processed form of HybC (*HybC-pf*) detected by immunoblot are indicated on the *right*. The increase in mobility of HybC-pf compared with the precursor HybC-np results from the removal of the C-terminal extension sequence of the precursor upon nickel incorporation (11).

(Fig. 1*A, lane 2*). Interestingly, when it was synthesized alone, HybO accumulated as a precursor (HybO-p) in the cytoplasm and was completely absent from the membrane (Fig. 1*A, lanes 3* and *4*). Therefore, unlike most classical secretory proteins, the signal sequence of the small subunit of HYD2 itself is not sufficient for membrane targeting and translocation.

We investigated whether the large subunit (HybC) was necessary for the export of the small subunit. In the presence of a truncated HybC (see the Fig. 1 legend), the small subunit accumulated as a precursor in the cytoplasm (Fig. 1*A, lane 5*). Under these conditions, the precursor was also detected in a *bona fide* membrane pellet (Fig. 1*A, lane 6*). Further analysis, however, revealed that the pelleted precursor of the small subunit reflected aggregated material (Fig. 1*A, lane 8*) because it was absent from the Triton X-100-solubilized membrane fraction (Fig. 1*A, lane 7*). As a consequence, truncation of the large subunit resulted in the formation of aggregates of the small subunit.

We previously showed that in the *nik* mutant, which is deficient in the specific nickel transport system, the large subunit of HYD2 accumulates as a non-processed precursor in the cytoplasm, but the addition of nickel to the growth medium restores processing of the large subunit and its membrane targeting (11, 17). In a double Δ*nik*-Δ*hybOABC* mutant complemented with the entire *hyb* operon ((*hybO-G*)$^+$) and grown in the absence of nickel, the small subunit HybO was detected as HybO-p in crude extracts and the cytoplasm, but it was completely absent from the membrane (Fig. 1*B, lanes 2, 3,* and *1*, respectively). HybO-p synthesized under this condition was very labile, and a slightly smaller breakdown product of HybO was observed in this strain. The addition of nickel to the growth medium resulted in membrane targeting and maturation of the precursor of the small subunit (Fig. 1*B, lanes 5* and *6*), implying a successful translocation of HybO and the removal of its signal sequence. These results indicate that a deficiency in nickel incorporation and in large subunit processing directly or indirectly affects the targeting and translocation of the small subunit of HYD2.

Membrane Targeting and Processing of the Large Subunit of HYD2—Because the large subunits of hydrogenases are devoid of signal sequences, they are assumed to be co-translocated with the small subunits. We therefore analyzed the effect of a complete depletion of *hybO* on the translocation of the large subunit using immunoblot analysis. In the presence of the small subunit, the processed form (HybC-pf) of the large subunit was detected in both the membrane and the cytoplasm (Fig. 2, *lanes 2* and *4*). The non-processed precursor (HybC-np) was present exclusively in the cytoplasm (Fig. 2, *lane 4*). This result indicates that only the processed form of the large subunit is efficiently targeted to the membrane. In addition, the large subunit was successfully translocated into the periplasm because active HYD2 was released from spheroplasts by treatment with trypsin (data not shown). On the other hand, in the absence of the small subunit, the large subunit accumulated exclusively as a non-processed precursor (Fig. 2, *lane 5*), it was absent from the membranes (Fig. 2, *lane 1*), and it was totally localized in the cytoplasm (Fig. 2, *lane 3*). These findings imply a failure of nickel incorporation into the large subunit, which is the normal prerequisite for its processing. Therefore, the small subunit is required not only for membrane targeting, but also for the processing of the large subunit.

Formation of a HybO-HybC Complex—The interdependence between the two subunits for their translocation suggests a complex formation before export. We assessed this possibility using cross-linking and immunoblot analysis. Membrane-free S-135 fractions were prepared from spheroplasts of the mutant ENF1 (Δ*hybOABC)* complemented either by pHyb84 containing the *hyb* operon except *hybO* or by pHyb411 carrying only the *hybO* gene. The two extracts were then treated separately or as a mixture with formaldehyde. A cross-linking product of about 100 kDa detected by antisera against the small or the large subunits was obtained only if both the small and large subunits were present in the reaction medium (Fig. 3, *lanes 4* and *5* compared with *lanes 1, 2, 7,* and *8*). Moreover, as expected for a formaldehyde cross-linking product, this band disappeared when samples were heated to 100 °C (Fig. 3, *lanes 3* and *6*). These results suggest the formation of a HybO-HybC complex under this condition, *e.g.* in the absence of membranes. The low amount of the HybO-HybC complex obtained correlated with the low quantity of small subunit available in the extract (*lane 2*); HybO present in the S-135 fraction seems to be completely converted into the complex (*lane 4 versus lane 2*). However, release of the small subunit from the HybO-HybC complex by heating was not detected, probably because of a degradation or aggregation of HybO due to its labile and poorly soluble nature. The authenticity of HybO was established by comparing the S-135 fraction of ENF1/pHyb411 (*hybO*$^+$; *lane 2*) with that of ENF1/pHyb84 (*hybO*$^-$; lane 1) and was independently confirmed by *in vitro* transcription/translation using various plasmids (data not shown). In contrast, the 92- and 60-kDa bands are contaminating bands that are not related to HybO

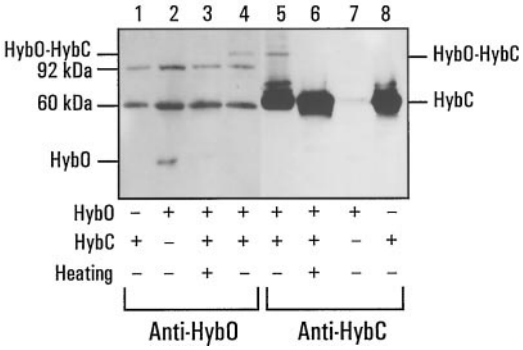

FIG. 3. ***In vitro* cross-linking of HybO and HybC.** Membrane-free S-135 fractions were subjected to treatment with the cross-linker formaldehyde, as indicated under "Experimental Procedures," separated on a 10% denaturing SDS-polyacrylamide gel, and electrotransferred onto a polyvinylidene difluoride membrane. Half of the membrane was incubated with antiserum against HybO (*Anti-HybO*), and the other half was incubated with antiserum against HybC (*Anti-HybC*), and both were developed by the ECL chemiluminescence method (Amersham Corp.). The presence (+) or absence (−) of HybO or HybC in the cross-linking reaction is indicated at the *bottom*. The dissociation of cross-linked complexes was achieved by heating the samples at 100 °C (+), whereas the control was treated at 37 °C (−) before applying the samples to the gel. Polypeptides detected by antisera against HybO and HybC are indicated on the *left* and *right*, respectively.

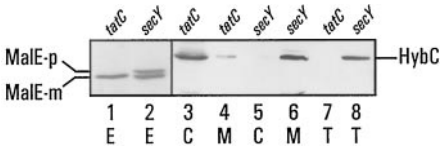

FIG. 4. **Sec-independent translocation of HYD2.** Crude extracts (*E*), cytoplasmic fractions (*C*), membranes (*M*), or trypsin-released protein fractions (*T*) prepared from the *secYcs* (CU164) (*lanes 2, 5, 6,* and *8*) or *tatC* (*lanes 1, 3, 4,* and *7*) mutants were resolved by 13% (*lanes 1* and *2*) or 10% (*lanes 3–8*) SDS-polyacrylamide gel electrophoresis. MalE (*lanes 1* and *2*) and HybC (*lanes 3–8*) were detected by immunoblots. The precursor (*MalE-p*) and the mature form (*MalE-m*) of MalE and the large subunit (*HybC*) are indicated on the *left* and *right*, respectively.

because they were found even in the absence of the *hybO* gene (Fig. 3, *lane 1*).

Sec-independent Translocation of HYD2 through the Tat Pathway—The Tat pathway, which is required for the translocation of proteins carrying a signal sequence with an essential twin-arginine motif, has been recently identified in *E. coli* (12, 13, 24–26). We previously reported that one of the substrates of the Tat pathway, the periplasmic trimethylamine N-oxyde reductase, is exported independently of the Sec machinery (18). The above results indicating a co-translocation of the large and the small subunit of HYD2 are inconsistent with a passage of HYD2 through the Sec machinery, which is believed to accommodate single, unfolded polypeptides. Indeed, the large subunits of HYD2 were found mainly in the membrane fraction of a *secY* mutant (Fig. 4, *lanes 5* and *6*), and HybC was released from spheroplasts by limited trypsin digestion (*lane 8*), indicating a normal translocation of HYD2 across the cytoplasmic membrane of the *secY* mutant. The correct phenotype of the *secYcs* mutant used was confirmed by the accumulation of the precursor of MalE (*lane 2*). On the contrary, HybC accumulated in the cytoplasm of the *tatC* mutant (*lanes 3* and *4*), and it was not accessible from the spheroplasts (*lane 7*), which confirms the previous observation that translocation of Hyd2 is dependent on components of the Tat pathway (12, 13).

The signal sequence of the small subunit determines the translocation pathway to which the large subunit is channeled—The twin-arginine signal sequence of the small subunit is likely to be the determinant for directing HybC to the Tat apparatus. To address this point, we substituted the first 27 N-terminal residues of HybO, including the twin-arginine motif, with the 22 residues of the typical Sec-dependent signal sequence of PelB. The resulting plasmid, pHyb55, directed the biosynthesis of the chimera ^{PelBss}HybO, which accumulated as a 39-kDa precursor and ran between the precursor and mature form of HybO (Fig. 5*A*). Under this condition, the chimera and the large subunit were found mainly in the membrane fractions (Fig. 5*B, lanes 3* and *4*). However, they were absent from either the periplasm or the trypsin-solubilized fraction (data not shown), suggesting that they are targeted to but not translocated across the membrane. Nevertheless, the large subunit was tightly bound to the membrane (see below). It seems likely that the chimera forms a ^{PelBss}HybO-HybC complex that is targeted by virtue of the PelB signal sequence to the translocase SecYEG, and it would then become stuck in the translocation channel.

This assumption was confirmed by the following observations. First, the doubling time during the growth of MC4100/pHyb55, which synthesizes the ^{PelBss}HybO-HybC complex, increased by 50% compared with that for MC4100/pHyb14 in which HybO is synthesized with its native twin-arginine signal sequence. Second, the precursor of MalE accumulated in MC4100/pHyb55 (Fig. 5*C, lane 4*) to the same extent as in the *secY* mutant (*lane 1*), but it was absent from MC4100/pHyb14 (*lane 3*). Therefore, the jamming of the Sec translocon by the ^{PelBss}HybO-HybC complex resulted in an inhibitory effect on bacterial growth and led to the accumulation of a precursor of a Sec substrate. Finally, in contrast to the native HybO-HybC complex that is located exclusively in the membrane of the *secY* mutant (Fig. 5, *B1* and *B2*, *lanes 1* and *2*), targeting of the chimeric ^{PelBss}HybO-HybC complex was clearly affected by *sec* mutations. Thus, the large subunit HybC accumulated mainly in the cytoplasms of both *secY* and *secA* (Fig. 5*B2, lanes 5* and *7*), whereas the amount of ^{PelBss}HybO detected in these mutants was strongly reduced (Fig. 5*B1, lanes 5–8*), presumably due to a degradation of misfolded or non-protected material. The remainder of HybC found to co-sediment with membranes (*lanes 6* and *8*) was further analyzed as to the nature of its membrane association. HybC co-expressed with ^{PelBss}HybO was completely removed from *secA* membranes by 6 M urea (Fig. 5*D, lane 3*), whereas no HybC was extracted from the membranes of the wild type strain and the *secY* mutant (Fig. 5*D, lanes 1* and *2*). A second extraction of these membranes with sodium carbonate did not further remove HybC (data not shown). When the extracted membranes were treated with Triton X-100, only HybC in the wild type strain was solubilized (Fig. 5*D, lane 4*), whereas that in the *secY* mutant remained in the insoluble fraction (Fig. 5*D, lane 8*). Thus, in the presence of ^{PelBss}HybO, most HybC looses its membrane association upon inactivation of SecY and SecA, with residual sedimenting material being only loosely attached or aggregated. The combined results therefore indicate that HybC in a ^{PelBss}HybO-HybC complex is targeted to the membrane by the Sec machinery recognizing the PelB signal sequence of the chimeric small subunit. These findings strongly support the idea that HybO and HybC form a complex before translocation and that HybC is targeted to the membrane via a hitchhiker mechanism.

DISCUSSION

Most extracytoplasmic proteins are synthesized with a signal sequence that targets them for export. Removal of the signal sequence or mutations in the signal sequence considerably decrease the efficiency of protein export. However, it has been reported that polypeptides lacking a signal sequence can be effectively imported into the peroxisomal matrix in a piggyback fashion on other polypeptides containing signal sequences (27).

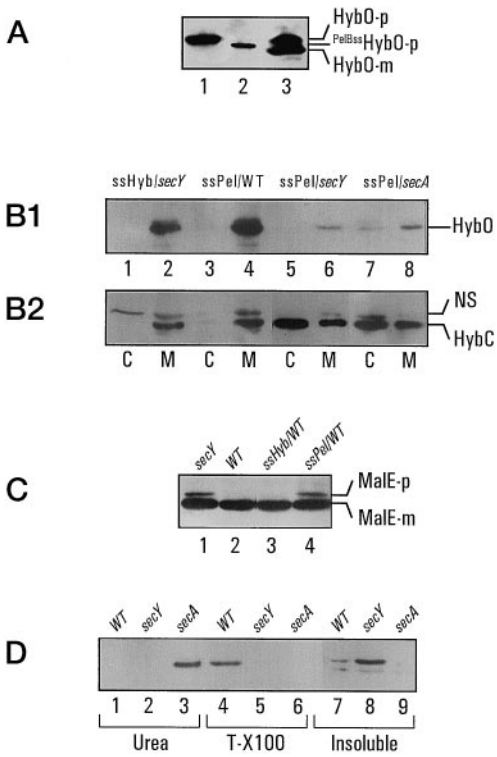

FIG. 5. **Influence of the signal sequence of HybO on the choice of export pathway used by HybC.** *A*, crude extracts of ENF1/pHyb411 (Δ*hybOABC*/*hybO*$^+$; *lane 1*), ENF1/pHyb55 (Δ*hybOABC*/*hyb-*(^{pelBss}*O-G*)$^+$; *lane 2*), and ENF1/pHyb14 (Δ*hybOABC*/*hyb(O-G)*$^+$; *lane 3*) were separated on a 12.5% SDS-gel and analyzed by immunoblot using antiserum against HybO. The precursor (*HybO-p*) and mature form (*HybO-m*) of the small subunit and the precursor of the chimera (^{PelBss}*HybO-p*) are indicated on the *right*. *B*, cells containing pHyb14 (*hyb(O-G)*$^+$; *lanes 1* and *2*) or pHyb55 (*hyb*(^{pelBss}*O-G*)$^+$; *lanes 3–8*) were grown at 30 °C to early exponential phase, shifted to 42 °C, and incubated for an additional 3 h. Thirty μg each of proteins of cytoplasmic fractions (*C*) and membrane fractions (*M*) prepared from Δ*hybOABC* (*lanes 3* and *4*), *secYts*-Δ*hybOABC* (*lanes 1, 2, 5,* and *6*), and *secAts*-Δ*hybOABC* (*lanes 7* and *8*) mutants were separated on a 10% SDS-gel and probed with antiserum against the small (*B1*) or large subunits (*B2*). To analyze both the small and large subunits on the same gel, a compromised concentration (10%) of polyacrylamide was used. This concentration is not appropriated for visualizing the tiny migration difference between HybO-m and ^{PelBss}HybO-p, as observed on a 12.5% gel in *A*. The small (HybO) or large (HybC) subunits and the nonspecific band are indicated on the *right*. *C*, crude extracts prepared from *secYts* (*lane 1*), wild type strain without plasmid (*lane 2*), or complemented by pHyb14 (*lane 3*) or pHyb55 (*lane 4*) grown at 30 °C were separated on 13% SDS-gels and analyzed by immunoblotting using antisera to MalE. The precursor (*MalE-p*) and processed MalE (*MalE-m*) are indicated on the *right*. *D*, membrane fractions were prepared from Δ*hybOABC* (*lanes 1, 4,* and *7*), *secYts*-Δ*hybOABC* (*lanes 2, 5,* and *8*), and *secAts*-Δ*hybO-ABC* (*lanes 3, 6,* and *9*) complemented by pHyb55. Urea-extracted fractions (*lanes 1–3*), Triton X-100-solubilized membranes (*T-X100*; *lanes 4–6*), and insoluble fractions (*lanes 7–9*) were separated on a 7.5% SDS-gel and analyzed by immunoblot using antiserum against the large subunit.

Bacterial hydrogenases are composed of small subunits with the twin-arginine signal sequence and large subunits devoid of any export signal. A naturally occurring co-translocation between the subunits has been proposed (5, 6). In this study, we observe an interdependence between the small and the large subunits for the translocation of HYD2 across the cytoplasmic membrane. Most importantly, we show that the signal sequence of the small subunit determines the export pathway followed by the large subunit.

We considered two models consistent with a hitchhiker co-translocation mechanism. According to the first model, membrane targeting of the two subunits of HYD2 is a sequential event. The precursor of the small subunit is targeted alone to the membrane with the help of its signal sequence. The incorporation of nickel into the large subunit leads to the removal of its C-terminal extension sequence and results in a conformational change, which allow the processed large subunit to specifically interact with the membrane-bound small subunit. This interaction triggers the membrane insertion of the large subunit and the formation of the complex, which then crosses the membrane by an unknown mechanism. According to the second model, the small and the large subunits of HYD2 first form a complex, which is followed by processing of the large subunit and then by membrane targeting of the complex by virtue of the signal sequence of the small subunit. Our findings are more in favor of the second model. When the small subunit was expressed alone, it accumulated in the cytoplasm as a precursor. In addition, membrane targeting of the small subunit required not only the presence of the large subunit but also nickel incorporation into the large subunit and processing of the large subunit. Reciprocally, depletion of the small subunit prevented the large subunit from being targeted to the membrane and affected the incorporation of nickel into the large subunit and its processing. In addition, the substitution of the twin-arginine signal sequence of the small subunit with a Sec-dependent signal sequence inhibited nickel acquisition and processing of the large subunit (data not shown). Because the acquisition of nickel by the large subunit occurs in the cytoplasm and is a prerequisite for HYD2 translocation (11), the interdependence implies a direct contact between the subunits and strongly suggests the formation of a complex before translocation. Consistently, we observed that HybO and HybC can form a complex *in vitro* in the absence of membranes. Taken together, our results show that the bacterial Tat pathway is capable of translocating oligomeric complex across the cytoplasmic membrane in a piggyback fashion, and thus it shares mechanistic similarities with the pathway used in protein import into the peroxisomes, in addition to the characteristics common to the Sec-independent, ΔpH-driven import pathway of plant thylakoids.

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